

Exposure to elevated prenatal testosterone metabolites induce autism-like behavior in rats: Evidence for the extreme male brain and implications for human health

by

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### ABSTRACT

Neurodevelopmental disorders such as autism spectrum disorder (ASD) originate during early brain development and are the result of complex interactions between genetic and environmental factors. Disruptions in the prenatal hormone environment have been associated with increasing the risk for autism. The extreme male brain theory of autism states that ASD results from abnormally high levels of prenatal testosterone that result in a hypermasculinized (autistic) brain. Prenatal androgen signaling programs behavior through the actions of androgen or estrogen receptors throughout diverse brain regions. The balance of this sex hormone signaling is critical for neuronal organization, and disruptions in normal prenatal hormone levels lead to aberrations in critical behaviors such as juvenile social play, which is important for normal cognitive and social development. This research demonstrates that exposure to excess testosterone metabolites during development induces autism-like behaviors in the rat, including reduced social interactions, abnormal stress response, and enhanced spatial ability, which support the extreme male brain theory.



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## CHAPTER 1: INTRODUCTION

The intimate link between hormones and behavior has been studied since the 1850s. Arnold Adolph Berthold castrated roosters after hatch and observed that mature castrates did not perform typical adult male behavior, such as crowing and male copulatory behavior. This first experiment in behavioral endocrinology showed the importance of early hormone signaling on later behavior (Klein 1968). A century later, Phoenix et al. (1959) manipulated the prenatal hormone environments of Guinea pigs by administering testosterone to pregnant females. Female offspring born after prenatal treatment with testosterone had masculinized genitalia and did not perform lordosis, a female-specific reproductive behavior, and instead performed mounting, a male-specific reproductive behavior (Phoenix et al. 1959). For the first time the organizational effects of hormones on neural tissues that direct sex-specific behaviors, such as reproductive behavior, were connected. Since this pioneering work, the organizational effect of prenatal hormones has been a research area with important implications for many aspects of human health, including neurodevelopment.

The fetal hormonal environment plays a profound role in the organization and programming of the brain, and is sensitive to relatively minor changes in hormone levels (Vandenbergh 2003). Research in mice has shown that nuances such as the position of developing fetuses with respect to their male and female siblings can influence physiology and sex-specific behavior (Vom Saal 1989). Female fetuses that develop in between male fetuses are masculinized in morphology and behavior by androgens produced by their adjacent brothers (Ryan & Vandenbergh 2002). Alternatively, female fetuses that develop between other females do not experience this type of masculinization, and those that develop between one female and one male present an intermediate morphology and behavior profile (Clark & Galef Jr 1998; Ryan

& Vandenberg 2002; Vandenberg 2003, 2004; Vom Saal 1989). Collectively, this body of research illustrates the importance of hormone levels in the prenatal environment and its powerful influence on the development of both the brain and behavior.

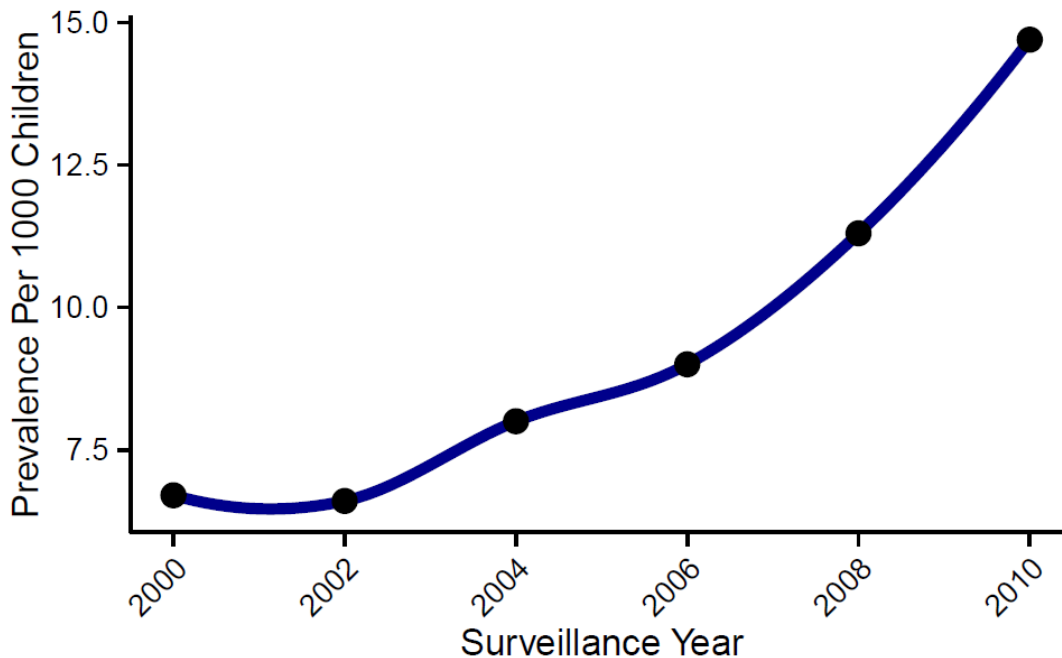
Neurodevelopmental disorders (NDDs) describe a group of human disorders that arise due to altered brain development. A large portion of brain development occurs *in utero*, and many NDDs are thought to originate during prenatal development (Bale et al. 2010; Connors et al. 2008). The specific causes of abnormal brain development that give rise to a NDD remain enigmatic, but NDDs are likely due to complex gene and environment interactions. Among the numerous hypotheses for altered neurodevelopment, the hypothesis that alterations in the prenatal hormone environment result in NDDs has been gaining attention, and is supported by both human epidemiological (Auyeung et al. 2010) and laboratory studies (Xu et al. 2015). Although NDDs encompass a diverse group of human disorders, autism spectrum disorders (ASDs) have remained at the forefront of research efforts due to compelling evidence linking ASD etiology to perturbations in the prenatal environment.

ASDs describe a group of complex neurodevelopmental disorders that are identified by three generalized criteria: 1) atypical reciprocal social interactions (e.g. reduced social play), 2) deficits in social communication, and 3) stereotyped, repetitive, and ritualistic behaviors (APA 2013; Bodfish et al. 2000; Clark & Galef 1998; Dawson et al. 2002; Lord et al. 2000; Volkmar et al. 2004). Individuals that show atypical reciprocal social interactions struggle to engage the social skills necessary to interact with others and form lasting, meaningful friendships (Causton-Theoharis, Ashby, & Cosier 2009; Levy & Perry 2011). However, the degree to which autistic symptoms are manifested varies from mild to severe, thus the characterization of this group of disorders as a broad spectrum.



Like other NDDs, ASD arises from abnormal brain development (Belmonte et al. 2004; Courchesne et al. 2007; Sparks et al. 2002; Walsh, Morrow, & Rubenstein 2008). However, the specific mechanisms underlying the development of autism are not fully understood and myriad factors influence whether or not an individual develops autism. Genetic predisposition, variation in the prenatal environment, and environmental triggers at key stages in development (including after birth) all influence whether ASD develops and its severity (Baron-Cohen, Knickmeyer, & Belmonte 2005; Cook 2001; Hallmayer et al. 2011; State & Levitt 2011). The lack of a clear cause and effect relationship in the development of ASD is likely due to the complex network of interactions between genetic risk factors and environmental variation during key periods in development. In fact, little is known about specific gene-environment interactions that amplify the risk for ASD (Hu 2012). While candidate genes and *de novo* mutations play a role in the etiology of ASD, a recent literature review by Landrigan et al. (2012) reported that only 30-40% of ASD can be attributed to genetic inheritance (Landrigan, Lambertini, & Birnbaum 2012). Importantly, data collected by the CDC show that the prevalence of ASD has increased roughly 200% between 2000 and 2010 despite stable diagnostic criteria (Figure 1, CDC 2015). It is impossible for mutant alleles to increase in frequency in a population at this alarming rate, so it is logical to hypothesize that the increase in ASD can be attributed to environmental influences. While a portion of this increase can be attributed to increased awareness and improved recognition of ASD symptoms (Prior 2003), the diagnostic criteria for ASD have remained relatively stable in editions of the Diagnostic and Statistical Manual of Mental Disorders (DSM) spanning the years illustrated in Figure 1 (Rutter 2005). In fact, one study predicted that changes

in the most recent edition of the DSM (the DSM-V, published in 2013) would result in a nationwide decrease in ASD prevalence (Matson, Hattier, & Williams 2012).



**Figure 1.** Autism spectrum disorder prevalence continues to increase in the US. Adapted from data provided by the CDC, 2015.

However, the broader impacts of the most recent change in ASD diagnostic criteria are currently under debate in the psychological/psychiatric literature and remain unclear (Volkmar & Reichow 2013). This interesting debate is one that will require additional data from future surveys in order to fully assess the impact of changes in recognition and diagnostic criteria, coupled with true changes in the population driven by factors such as environmental exposure.

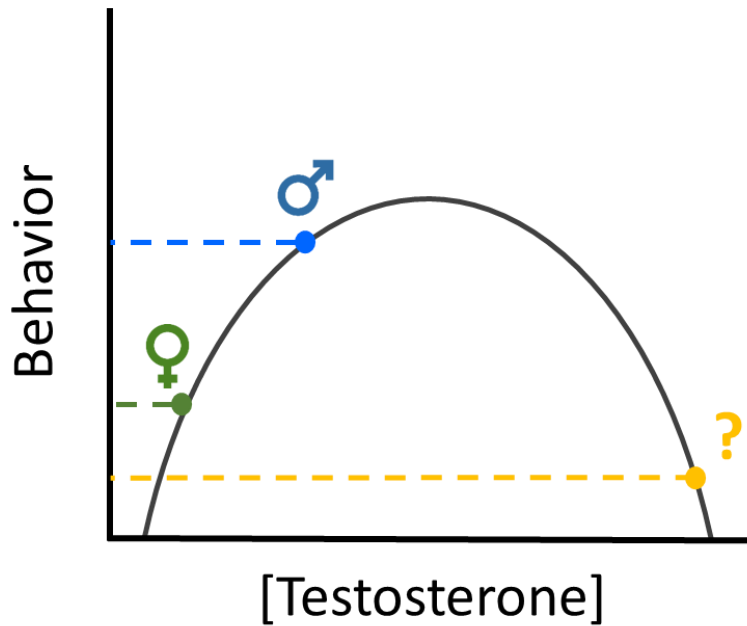
In humans, prenatal exposure to high levels of testosterone influences autistic traits and is associated with an increased risk for ASD (Auyeung et al. 2009; Auyeung et al. 2010; Baron-Cohen et al. 2005; Cohen-Bendahan, van de Beek, & Berenbaum 2005; Knickmeyer et al. 2006; Knickmeyer et al. 2005). For example, Knickmeyer et al. (2005) showed that fetal testosterone

levels for male children, obtained from amniotic fluid, were negatively correlated with the quality of social relationships and positively correlated with restricted interests. These behavioral endpoints are consistent with symptoms used in the diagnosis of autism (APA 2013).

The extreme male brain theory proposes that ASD is a set of behaviors that result from a hyper-masculinized brain (Badcock & Crespi 2008; Baron-Cohen 2002; Crespi & Badcock 2008; Sparks et al. 2002). Normal human brains are sexually dimorphic in many regions including the amygdala and hypothalamus, with male structures having a larger volume than female structures, relative to cerebrum size. Cerebrum sizes also differ between males and females, with male cerebrums being larger, a difference that is not entirely attributed to differences in body size (Giedd et al. 1997). Brain hypermasculinization is thought to result in further enlargement of these structures and cause altered behavior. Furthermore, males are diagnosed with autism four times as often as females. The extreme male brain theory is also supported by research on females with congenital adrenal hyperplasia (CAH), a condition associated with increased levels of prenatal androgens. Females with CAH display more autistic traits than their non-CAH sisters (Knickmeyer et al. 2006). Taken together, these data suggest that organizational effects of excess androgens contribute to the development of autism.

An important distinction in understanding the extreme male brain is that a hypermasculinized brain may result in abnormal behavior that is not necessarily excessively masculine. For example, normal male children engage in rough-and-tumble play behavior more often than female children do. Considering the extreme male brain theory, one might intuitively expect a child with a hypermasculinized brain to engage in even more rough-and-tumble play than an unaffected boy. However, the opposite is true when considering autistic children; children with autism do not engage in extremely high levels of rough-and-tumble play, and often

struggle to engage in successful play interactions with their peers. This is a common symptom of the disorder, and likely due to structural differences in brain regions that control social behavior. Excessively high levels of androgens during development could ultimately reduce androgen signaling through negative feedback and suppress certain behaviors (Figure 2).

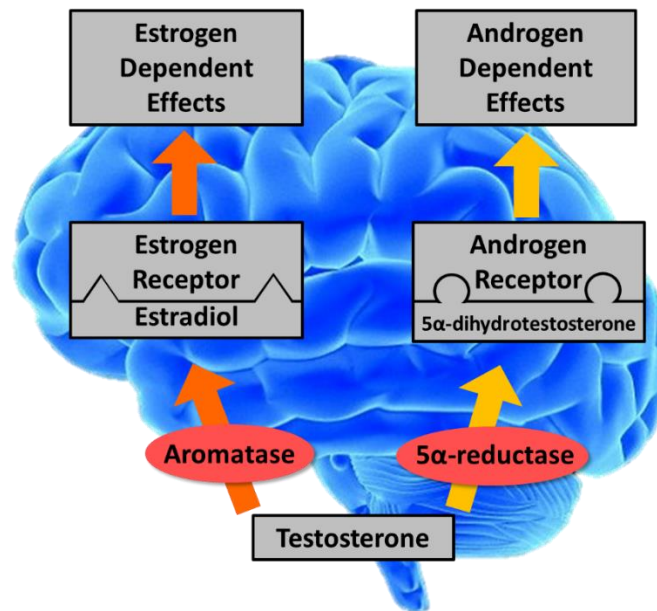


**Figure 2.** A hypothetical dose-response curve showing the relationship between normal male and female concentrations of an androgen and behavioral response in comparison to the behavioral response of an excessive level of androgen.

For example, the endocrine system regulates androgen signaling when androgen concentrations are high by reducing expression or activity of corresponding receptors, reducing overall effects of androgens at high concentrations. Alternatively, excessive levels of androgens can be metabolized into other biologically active hormones (e.g. estradiol) and elicit extreme effects through other signaling pathways.

Although high levels of fetal androgens are proposed to organizationally hypermasculinize regions of the brain that control or direct social behavior, the extreme male

brain does not provide a specific mechanistic explanation for the effects of high levels of prenatal testosterone. Androgens can masculinize the brain through binding to androgen receptor (AR) as testosterone, or as one of several metabolites (e.g. dihydrotestosterone, DHT) which also bind AR, or can be aromatized to estradiol and masculinize the brain through binding to estrogen receptors (ER, Figure 3).



**Figure 3.** Testosterone exerts effects on the developing brain through binding androgen receptor as well as estrogen receptor. Both processes are critical for normal male behavioral programming.

Masculinization of the developing brain is directed by multiple hormones that can have region-specific organizational effects. Understanding the mechanisms through which prenatal sex hormone exposure programs behavior will help to elucidate the pathways influencing neurodevelopmental disorders like ASD. My research investigates the role of prenatal exposure to testosterone metabolites in programming ASD-like behaviors, using the rat as a model and aims to delineate the roles of estrogen- and androgen-dependent signaling.

### *Additional Related Hypotheses*

While the extreme male brain is supported by compelling evidence for the organizational role of prenatal androgens and later behavior, it is not the only hypothesis that attempts to explain an endocrine-based etiology of ASD. For example, rather than ASD being associated with hypermasculinization, a female protective effect has been proposed as another route through which prenatal hormones play an integral role (Skuse et al. 1997; Skuse 2000). Unlike the extreme male brain, the female protective effect suggests a strong genetic component. The female protective effect proposes that females tend to have a higher threshold for developing autistic behavior because they require a greater familial etiologic load. In other words, females present less autistic behavior for a given set of genetic risk factors. An elevated threshold in females would account for both the sex difference in the incidence of ASD and the observation that females with ASD generally have severe symptoms (Robinson et al. 2013). This protective effect has been investigated with regard to the interaction between sex hormones and gene expression. Sarachana et al. (2011) found that androgens and estrogens differentially regulate an important gene, retinoic orphan receptor alpha (*RORA*), in a neuronal cell line, SH-SY5Y (Sarachana et al. 2011). *RORA* is a novel candidate gene for ASD; its product transcriptionally regulates aromatase, the enzyme that converts testosterone to estrogen. This research suggests that the sex difference in ASD may also be implicated by the reciprocal regulatory properties of androgens and estrogens. According to these findings, estrogens are able to up-regulate *RORA* and thus up-regulate the expression of aromatase, increasing the conversion of testosterone to estrogen and creating a positive feedback loop. Alternatively, testosterone down-regulates *RORA*, which down-regulates the expression of aromatase, thus decreasing the conversion of testosterone to estrogen. Estrogen is hypothesized to have neuroprotective effects through

activating microglial cells (Bruce-Keller et al. 2000), but these effects could be non-linear. The activation of microglial cells in regions of neurodevelopmental damage, such as the dorsolateral prefrontal cortex, has been found in autistic brains (Morgan et al. 2012; Morgan et al. 2010). It is possible that elements of the female protective effect and the extreme male brain occur simultaneously to explain the roles of both androgens and estrogens in the pathology of complex disorders like ASD.

Another prominent theory that addresses many of the complicated aspects of ASD is the genetic imprinting theory (Badcock & Crespi 2006). The genetic imprinting theory describes the “battle of the sexes” between expression of paternal and maternal genes. According to this theory, individuals with increasing levels of paternal gene expression experience increasing severity of ASD-related symptoms while individuals with increasing levels of maternal gene expression experience decreasing amounts of ASD-related symptoms. Crespi and Badcock (2008) argue that the autistic brain is not necessarily the result of an extreme male brain, but is instead the result of a brain that has developed under conditions of extreme paternal imprinting (Crespi & Badcock 2008). It is difficult to understand why aberrant genetic imprinting would be increasing in human populations, leading to the exponential increase in autism spectrum disorders that have been recorded over the last decade. However, elements of paternal versus maternal genetic imprinting might affect or be influenced by prenatal hormone signaling. In addition, it is known that estrogens can alter methylation and chromatin remodeling (Prins 2008). Thus, elements of the genetic imprinting theory might overlap with the body of evidence suggesting a role for prenatal hormone signaling in the development of ASDs and should be investigated further.

### *Endocrine Disrupting Chemicals and ASD*

While exposure to endogenous hormones is known to have a significant impact on brain development, questions regarding the influence of exogenous chemicals have begun to emerge in concert with conservation research (Weiss 2002, 2012; Zala & Penn 2004). Endocrine disrupting chemicals (EDCs) describe a class of chemicals that can interfere with normal endocrine signaling through mimicking or antagonizing the actions of endogenous hormones (Colborn, vom Saal, & Soto 1993). EDCs can over-activate hormone receptor actions through agonistic effects and can suppress hormone receptor actions through antagonistic effects. Importantly, some EDCs can act agonistically or antagonistically depending on dose (see Chapter 2) (Liet al. 2012; Vandenberg et al. 2012; Wonget al. 1995).

EDCs can influence the prenatal hormone environment and have been implicated in abnormal behavior in children exposed *in utero*. Indeed, prenatal exposure to EDCs impacts behavior in myriad ways, including increasing the risk for ASD-related behavior (Bouchard et al. 2011; Braun 2012; Braun et al. 2014; De Cock, Maas, & van de Bor 2012; Eskenazi et al. 2007; Hertz-Picciotto et al. 2006; Landrigan et al. 2012; Xu et al. 2015). For example, Miodonovik et al. (2011) found that atypical neonatal and early childhood behavior, such as social impairment, were associated with prenatal exposure to phthalates (Miodovnik et al. 2011). Stewart et al. (2000) found a dose-response relationship between prenatal exposure to highly chlorinated polychlorinated bisphenols (PCBs) through the mother's diet and increases in their children's reflexive and autonomic deviations from the norm as well as a reduced ability to habituate under various conditions (Stewart et al. 2000). These behavioral abnormalities are similar to autistic-like behavioral symptoms. In a recent literature review, de Cock et al. (2012) found positive associations between ASD and exposure to a wide array of endocrine disrupting chemicals



including bisphenol A (BPA), PCBs, and low molecular weight phthalates (De Cock et al. 2012). Similarly, Windham et al. (2006) found an association between exposure to air pollutants (e.g. chlorinated solvents and heavy metals) and the incidence of ASD (Windham et al. 2006). Indeed, these findings collectively show that offspring of mothers exposed to EDCs have an increased risk of developing behaviors deviant from normal childhood social behaviors. However, we do not have a clear understanding of the mechanisms through which EDCs can induce ASD. For example, this body of research does not directly measure how EDC exposure alters prenatal hormone signaling and how those alterations are linked to ASD, and is, therefore, unable to eliminate other factors that may influence ASD-like behavior, such as maternal care, other hormones (i.e. thyroid hormones), etc. My research will investigate how prenatal estrogens and androgens affect behavior and will inform our understanding of how altering the developing endocrine system can scale across time to affect behavior later in life.

#### *Autism Intervention through Play*

Children with ASD struggle to engage in normal social play with peers (Lord et al. 2000). The absence of typical social play interactions in children is one of the hallmarks of ASD and can be identified early, which can lead to further assessment as well as diagnosis (Chakrabarti & Fombonne 2001). There is no cure for ASDs; however early behavioral intervention can substantially facilitate the development of behavior that improves socialization outcomes in those with ASD (Dawson 2008; Sallows & Graupner 2005). In both humans and social mammals, play is important for developing appropriate and necessary social behaviors for adulthood (Yang et al. 2011), as well as stimulating cognitive development (Fone & Porkess 2008; Harlow, Dodsworth, & Harlow 1965; Heim, Plotsky, & Nemeroff 2004), which has direct implications for the development of ASD. A lack of social experience during critical

neurodevelopmental periods may exacerbate ASD symptoms, further hindering the development of normal social behaviors (Dawson 2008).

Research in primates and rodents suggests early social isolation with no opportunity for social play has a profound effect on the development of adult behavior, including social withdrawal (Fone & Porkess 2008), which is related to ASD behavior (APA 1994). Schrijver et al. (2002 and 2004) showed that rats unable to play due to social isolation had increased levels of motor hyperactivity and increased levels of activity in response to environmental novelty in comparison to socially reared rats (Schrijver et al. 2002; Schrijver et al. 2004). These findings reflect symptoms of ASD such as inflexibility towards change (hyperactivity in response to novelty), general motor hyperactivity, and social withdrawal (Howlin 2006; Lord et al. 2010). Early social isolation produces long-term changes including; neophobia, cognitive rigidity and decreased cortical and hippocampal synaptic plasticity (Fone & Porkess 2008). These structural changes reflect those that have been identified as structural abnormalities commonly found in the brains of autistic humans (McAlonan et al. 2005; Sparks et al. 2002).

Yang et al. 2011 showed that autism-like symptoms were rescued in an inbred strain of autistic-like mice through social play intervention with a highly social strain of mice (Yang et al. 2011). Thus, interventions that facilitate social play behavior can alleviate some autism-like symptoms. Similarly, Schneider, Turczak and Przewlocki (2006) found that rats prenatally exposed to valproic acid displayed autistic-like behavioral phenotypes that were reversed by environmental enrichment, which included opportunity for social play as well as access to toys (i.e. tunnels, ladders, swings, etc.). In humans, play-related early behavioral interactions such as symbolic play, sociodramatic play training, and peer mediated social initiations promote appropriate reciprocal social interactions between autistic children and their peers (Koegel,

Koegel, & Brookman 2003; Odom & Strain 1986; Rogers 2000; Strain, Kerr, & Ragland 1979; Thorp, Stahmer, & Schreibman 1995). Indeed, play behavior intervention has been shown to improve autistic symptoms in affected humans (Koegel et al. 2003; Odom & Strain 1986; Rogers 2000; Strain et al. 1979; Thorp et al. 1995). This evidence suggests that social interactions such as play can reduce the severity of ASD presentation.

Indeed, the relationship between altered social behavior and development of ASD is complex. In order to improve the understanding of neurodevelopmental disorders such as ASD, it is important that a potential etiological mechanism is identified. The extreme male brain theory explains key components of ASDs through the presence of elevated levels of androgens (or potentially neuronal estrogens) during fetal development. It does not, however, offer a mechanistic explanation for how ASD-related behaviors are programmed. My research tests the hypothesis that prenatal hormone exposure induces autism-like behavior in a rodent model and informs the fields of autism research and behavioral endocrinology.

CHAPTER 2: HORMONAL PROGRAMMING OF RAT PLAY BEHAVIOR:  
STANDARDIZED TECHNIQUES WILL AID SYNTHESIS AND TRANSLATION TO  
HUMAN HEALTH

*Introduction*

Early social behaviors like juvenile play are important for normal cognitive and social development. Deficits in these behaviors are associated with neurodevelopmental disorders, such as autism. Rat juvenile rough-and-tumble play is a useful behavioral biomarker of neurodevelopment, and is sensitive to chemical factors such as pre and neonatal hormones. Despite a rich body of literature characterizing hormonal programming of rodent juvenile play, the physiological mechanisms that regulate the organization of play behavior are not well characterized. Synthesizing results to understand the role of endocrine signaling in the development of play behavior remains difficult due to methodological inconsistency across studies. In this review, we synthesize what is known about hormonal mechanisms programming play, advocate standardized protocols for investigating rat play, and identify key areas where future research is needed. An integrative understanding of the relationship between endocrine signaling and behavioral programming will improve our ability to understand the development and onset of neurodevelopmental disorders in humans and ultimately will help prevent these devastating conditions.

Fifteen percent of US children have been diagnosed with neurodevelopmental disorders (NDDs), and incidences are dramatically increasing (CDC 2014). Affected children, such as those with autism spectrum disorders (ASDs), often present atypical reciprocal social interactions that impair many important aspects of childhood development including their ability to play normally. Jean Piaget, the founder of the theory of cognitive development, identified

childhood play as a critical stage in successful cognitive development (for a review, see Nicolopoulou 1993). Furthermore, childhood social play behavior is important for developing what psychologists refer to as Theory of Mind, which describes the ability to understand that others have beliefs, desires, and intents that differ from one's own. People with autism are often deficient in Theory of Mind capabilities (Miodovnik et al. 2011) (for a review, see Baron-Cohen 2000). Furthermore, one of the three main diagnostic criteria for autism is aberrant reciprocal social interactions. Therefore, altered social behavior can be indicative of ASDs or other NDDs.

NDDs arise from complex gene X environment interactions, and exposure to the correct hormonal milieu during specific developmental windows is critical for appropriate neuronal organization and later life activation of the social behavioral repertoire (Arnold & Breedlove 1985). Recent work indicates that several NDDs, including ASDs and hyperactivity, are associated with altered hormone concentrations during development (Auyeung et al. 2009; Auyeung et al. 2010; Baron-Cohen 2002; Baron-Cohen et al. 2011; Gore et al. 2014; Knickmeyer et al. 2006; Knickmeyer et al. 2005). The endocrine system is integral to normal neurodevelopment, which ultimately manifests as an individual's behavioral phenotype, and the function of the endocrine system, especially during development, is sensitive to external factors such as environmental contaminants.

Endocrine disrupting chemicals (EDCs) are ubiquitous environmental pollutants and through their antagonistic and agonistic actions can disrupt hormone dependent behavioral programming (Bergman et al. 2013). Recent research has linked EDC exposure during pre and neonatal development to the incidence and severity of NDDs in humans (Betts 2014; Bouchard et al. 2011; Braun 2012; Braun et al. 2014; de Cock, Maas, & van de Bor 2012; Eskenazi et al. 2007; Hertz-Picciotto et al. 2006; Miodovnik et al. 2011; Roberts et al. 2007; Stewart et al. 2000;

Weiss 2012; Windham et al. 2006). In fact, the extreme male brain theory states that subsets of ASD result from abnormally high levels of prenatal testosterone that result in a hypermasculinized brain (Baron-Cohen 2002). It is known that maternal exposure to EDCs can alter the prenatal hormone environment, which can disrupt the balance of the endocrine system's intricate network of feedback loops (Schönfelder et al. 2002). The effects of EDCs are further complicated by non-monotonic dose responses, for example high levels of response at very low concentrations (Vandenberg et al. 2012).

In order to understand how endocrine disrupting chemicals are linked to NDDs, we must first understand how endocrine signaling organizes behaviors used to diagnose these disorders, such as social play. Investigating how aberrations in the prenatal hormone milieu in model species influence the behavioral repertoire will advance our understanding of the relationship between EDC exposure and the incidence and severity of NDDs in humans. Presently, however, our understanding of the link between perturbations in endocrine signaling and altered rodent social play behavior is limited due to an inability to synthesize the existing literature. Unfortunately, investigations focused on identifying physiological mechanisms have nearly excluded studies of prenatal programming, and there are no widely accepted standardized protocols for defining and quantifying play behavior, thus procedural differences limit our ability to synthesize across studies.

Like many behaviors, juvenile play behavior is organized by physiological factors (i.e. hormones) during early development (Beatty et al. 1981; Hines & Kaufman 1994; Meaney 1981, 1983). However, the physiological mechanisms that regulate programming of play behavior are not well characterized—especially across species—because 1) investigations focused on identifying physiological mechanisms have taken different approaches, 2) most research has excluded

prenatal programming and focused on postnatal organization, and 3) standardized protocols for defining and quantifying play behavior are not utilized.

In this review we aim to provide a foundation upon which our understanding of the implications of play behavior on later life outcomes can be built. We focus on studies using the laboratory rat (*Rattus norvegicus*) because they are a highly social species that has a rich repertoire of juvenile play behavior and are a model system for studying NDDs (Belzung et al. 2005). In this review, we synthesize what is known about the hormonal mechanisms that organize play behavior and throughout the manuscript identify key areas where future research is needed. While many factors, such as nonsteroidal hormones, maternal care, and elevated stress during critical developmental periods, can have profound effects on the development of social behavior, this review focuses on the role of sex hormones and hormone disruptors. We attempt to unravel what is known about the various hormonal mechanisms underlying the organization of play fighting in the rat and suggest the use of standardized techniques to aid future synthesis and translation to human health.

### *Hormonal Mechanisms Programming Play*

Play behavior has captivated the scientific community for over a century (Burghardt 2005; Groos & Baldwin 1898; Smith 1982), and in rats it is critical for the development of appropriate social interactions (Pellis & Pellis 1998), improved cognition (Humphreys & Einon 1981), and reproductive success (Meaney & Stewart 1981; Pellis 1993; Thor & Holloway 1984). In addition, research on human juvenile play behavior shows that it is necessary for the development of normal social behavior in adults (Smith 1982; Von Frijtag et al. 2002). In fact, play behavior therapy is used in young children to alleviate social deficits common in NDDs, such as ASD (Dawson 2008; Kasari, Freeman, & Paparella 2006; Stahmer 1995; Strain, Kerr, &

Ragland 1979; Thorp, Stahmer, & Schreibman 1995). Before we can understand the link between play and social development, we must determine the mechanisms through which play behavior is programmed.

Rat rough-and-tumble play behavior (play fighting) is sexually dimorphic; males engage in play fighting more frequently than females (Olioff & Stewart 1978; Pellis 2002). Importantly, the sexual dimorphism in juvenile play fighting inspired research that has largely focused on the role of hormones, such as androgens, in the development of play behavior (Meaney & Stewart 1981; Meaney et al. 1983). Indeed, juvenile play fighting in rats is organized by the actions of sex hormones during a critical period of development that begins prior to and beyond birth (Casto, Ward, & Bartke 2003; Meaney 1988; Meaney et al. 1983).

However, it has not been fully resolved whether play fighting is organized through the actions of androgen receptor (AR) or estrogen receptor (ER), or if it results from the interaction of sex hormones and other biochemical factors (e.g., dopamine, a neurotransmitter, has been shown to interact with ER). Differentiating between the roles of androgens and estrogens in programming play behavior is further complicated by the conversion of testosterone into metabolites that can bind AR or ER. Additionally, there is evidence that other hormones, such as progesterone, are involved in the organization of play fighting behavior (Birke & Sadler 1983, 1984, 1988).

Sexual differentiation, a process that leads to the development of distinct sexes, is generally initiated by sex steroids. Each sexually dimorphic behavior arises due to specific mechanisms of sexual differentiation that occur during distinct, sensitive periods of development. In both rodents and primates, for instance, male specific behaviors are organized by appreciable increases in circulating androgens during the embryonic period, perinatal period,



and during sexual maturation (Arnold & Breedlove 1985; Berenbaum & Beltz 2011; McCarthy 2008; Meaney 1988; Whalen & Edwards 1967; Winter et al. 1975; Zuloaga et al. 2008). Juvenile rough-and-tumble play is interesting in that both sexes engage in play fighting, but males typically engage in the behavior at a higher frequency than females (Olioff & Stewart 1978; Pellis et al. 1997; Pellis & Pellis 1990; Pellis, Pellis, & McKenna 1994; Whalen & Edwards 1967). The underlying mechanisms driving the propensity for each sex to exhibit rough-and-tumble play behavior are not fully understood but many studies report that testosterone and its metabolites, which include estradiol, play an important role (Meaney & McEwen 1986; Meaney & Stewart 1981; Meaney et al. 1983; Olioff & Stewart 1978).

However, a bias exists in the literature; in rats, high levels of rough-and-tumble play are considered normal male behavior, while lower levels of rough-and-tumble play constitute normal female behavior. The possibility that female rats engage in a different set of juvenile social play behavior(s) more often than males has not yet been characterized or experimentally quantified in this species. Ethograms of juvenile female behavior should be attempted so that we can begin to understand the mechanisms driving female play behavior.

### *Neonatal Hormone Exposure*

#### *Neonatal Testosterone*

In the male rat, testosterone surges before and after birth and is important for both masculinization and defeminization of the male brain (Habert & Picon 1984; Konkle & McCarthy 2011; Weisz & Ward 1980). Testosterone masculinizes some regions of the brain through AR dependent gene expression (Clemens, Gladue, & Coniglio 1978; Whalen & Edwards 1967; Zuloaga et al. 2008), or it can be aromatized into estradiol and defeminize or masculinize

the brain through ER dependent mechanisms (McCarthy 2008; Whalen & Edwards 1967). The importance of the neonatal testosterone surge in the development of male typical behaviors has been recognized for several decades (Meaney & Stewart 1981). Consequently, the majority of studies investigating the effects of developmental exposure to hormones and subsequent changes in play behavior focus on this neonatal period (Table 1). We now know that the neonatal period in rats is sensitive to alterations in hormone signaling and can have long lasting effects on behavior, including on juvenile play. Importantly, this suggests that the neonatal period in humans is likely an important time of neuronal development and that disruptions to the neonatal hormonal milieu could induce NDDs.

What if hormonal signaling responsible for behavioral programming is absent? Androgen insensitivity occurs when tissue fails to respond to androgens. Androgen insensitive male rats, have a mutation in the androgen receptor gene that renders it dysfunctional. They are referred to as testicular feminized mutants (*tfm*), and develop as phenotypic females (e.g. they have perforate vaginas, a nipple line, and smaller body sizes compared to wild types males) but have abdominal testes that produce testosterone (Yarbrough et al. 1990). Male *tfm* rats do not experience AR directed neuronal programming during the pre or neonatal testosterone surges,

**Table 1.** Dosing regimens across studies.

Authors	Strain	Treatment(s)	MOA <sup>a</sup>	Method of exposure	Dose <sup>b</sup>	Behavior(s) recorded	Direction of effect
Beatty et al. 1981	Holtzman albino	Castration on PND 1, 6, 10 or 20	No production of T	Castration on PND 1, 6, 10, or 20	N/A	Play initiation, total play	PND 1 castrates had reduced play initiation and total play
Birke and Sadler 1983	Wistar	MPA	PR agonist/anti-androgen	Subcutaneous injection into the dam on PND 1	5 µg/kg	Wrestling, pinning, pouncing, chasing, boxing	MPA treated animals showed less play than control animals for both sexes. MPA treated animals were less likely to initiate play behavior and less likely to pounce than controls and were more likely to end a play bout than controls.
Birke and Sadler 1984	Wistar	Progesterone	PR agonist	Subcutaneous injection into the pup on PND 2-4	2.5 µg	Play, social sniffing, exploring, rearing, self-grooming	MPA and progesterone treated animals of both sexes played less than controls. Anti-P treated males played more than matched controls while anti-P treated females played less than matched controls.
		MPA	PR agonist/anti-androgen	Subcutaneous injection into the pup on PND 2	0.12 mg		
		Anti-P	Antiserum to progesterone	Subcutaneous injection into the pup on PND 2-4	0.025 mg		
Birke and Sadler 1988	Wistar	Anti-T	T inhibitor	Injection into pup PND 2-4	0.025 mg	Play fighting, pouncing, exploration	Progesterone treated animals of both sexes showed increased play behavior. Males given anti-T+anti-P had reduced play and pouncing. Males given anti-T+MPA did not differ from control males. Females given MPA+T had reduced play relative to females treated with T alone. Females treated with T alone had increased play compared to controls.
		Testosterone	AR agonist	Injection into pup PND 2-4	2.5 µg		
		Progesterone	PR agonist	Injection into pup PND 4-7	2.5 µg		
		Anti-T+anti-P	T inhibitor + P inhibitor	Injection into pup PND 2-4	0.025 mg + 0.025 mg		
		MPA+anti-T	PR/AR agonist + T inhibitor	Injection into pup PND 2-4	0.12 mg + 0.025 mg		
		MPA+testosterone	PR/AR agonist + AR agonist	Injection into pup PND 2-4	0.12 mg + 2.5 µg		
		Anti-P+T	P inhibitor + AR	Injection into pup PND			

			agonist	2-4	0.025 mg + 2.5 µg		
Casto et al. 2003	Sprague Dawley	Flutamide	AR antagonist	Subcutaneous injection into the dam GD 11 – 21	5 mg	Total play, wrestling	Flutamide treated males had reduced total play and reduced wrestling.
Colbert et al. 2005	Long Evans	Vinclozolin	AR antagonist	Dams gavaged GD 14 – PND 3	1.5 mg/kg 3 mg/kg 6 mg/kg 12 mg/kg	Total play, nape attack, pounce	6 and 12 mg/kg Vinclozolin treated males had increased total play. 12 mg/kg Vinclozolin treated males had increased nape attack and pounce.
Dessi-Fulgheri et al. 2002	Sprague Dawley	Bisphenol A	ER agonist	Oral administration via pipette of 0.04 mg/kg beginning 10 days prior to pairing through PND 21 Oral administration via pipette of 0.4 mg/kg on GD 14 – PND 6	40 µg/kg 400 µg/kg	Nape attack, pounce, chase, and withdraw (female directed), nape attack, pounce, crawl over, riding (male directed)*	Play behavior directed towards females was increased by BPA exposure, the low dose was more effective in females and the high dose was more effective in males.
Field et al. 2006	Male <i>tfm</i> and wild type Sprague Dawley	<i>tfm</i>	Androgen insensitivity	N/A	N/A	Playful attack, playful defense (complete and partial rotation)	<i>tfm</i> males did not differ from control males. Control females played more than control and <i>tfm</i> males.
Flynn et al. 2000	Sprague Dawley	Genistein	ER agonist	Dams fed chow with genistein GD 7 – PND 22	2 mg/kg 20 mg/kg 100 mg/kg	Pin	None
Flynn et al. 2001	Sprague Dawley	Vinclozolin	AR antagonist	Dams fed chow with vinclozolin GD 7 – PND 22	0.8 mg/kg 10 mg/kg 60 mg/kg	Pin	None
Flynn et al. 2005	Sprague Dawley	Methoxychlor	ER agonist	Dams fed chow with methoxychlor GD 7 – PND 22	0.8 mg/kg 8 mg/kg 80 mg/kg	Pin	None
Hotchkiss et al. 2003	Sprague Dawley	Flutamide	AR antagonist	Subcutaneous injection into the pup	50 mg/kg	Play bout, rostral sniff, caudal sniff, dorsal contact, chase	Flutamide and vinclozolin treated males had reduced chasing and play bouts. Testosterone propionate treated females had increased play bouts.
		Vinclozolin	AR antagonist	PND 2 – 3	200 mg/kg		
			AR agonist		250 µg/kg		

		Testosterone Propionate					
Meaney et al. 1983	Long Evans hooded	Flutamide	AR antagonist	Subcutaneous injection into pup on PND 1, implant on PND 2-11	300 µg + implant	Pouncing, wrestling, boxing, on-top/on-the-back postures	Flutamide treated males played less than control males, and did not differ from control females.
		<i>tfm</i>	Androgen insensitivity	N/A	N/A		
Meaney and McEwen 1986	Long Evans hooded	Testosterone	AR agonist	Implants PND 1-6	~10 µg ~1.0 µg ~0.1 µg	Play initiation, play fighting	~10 µg treated females had increased play initiation and play fighting.
Olesen et al. 2005	Sprague Dawley	Estradiol benzoate	ER agonist	PND 0-2	100 µg	Wrestling/boxing, biting, pouncing, pinning	EB treated females played more than control males, while females given tamoxifen + EB did not differ from control females. SKF 38393 treated animals played more than control animals while tamoxifen + SKF 38393 treated animals did not differ from controls.
		Tamoxifen	ER antagonist		100 µg		
		SKF 38393	D1 receptor agonist		100 µg		
		Tamoxifen + EB			100 µg + 100 µg		
		Tamoxifen + SKF 38393			100 µg + 100 µg		
Porrini et al. 2005	Sprague Dawley	Bisphenol A	ER agonist	Dams consumed dosage suspended in oil fed from a micropipette tip GD 0 – PND 21	40 µg/kg	Pounce, chase, crawl over	Females exposed to BPA showed a decrease in pounce, chase, crawl over
Tonjes et al. 1987	Wistar	Testosterone propionate	AR agonist	Amygdalar implants on PND 3	2µg in a 25% solution	Total play, play initiation	Testosterone and DHT treated females had increased play and did not differ from control males. Oestradiol benzoate females did not differ from control females.
		5α-DHT	AR agonist		2µg in a 25% solution		
		Oestradiol benzoate	ER agonist		1µg in a 10% solution		

<sup>a</sup> MOA = Mechanism of action

<sup>b</sup> Some studies adjusted dosing volumes for body weight while other studies gave standard doses regardless of animal weight.

although *tfm* males have elevated levels of testosterone compared to wild type males (Field et al. 2006; Zuloaga et al. 2008). The lack of AR dependent behavioral programming suggests that *tfm* males would not present male typical behavior, but would instead behave more similar to wild type females. However, inconsistencies among the findings of play behavior studies using *tfm* rats indicate the role of gonadal hormones in programming play behavior is not thoroughly understood. While testosterone and *one* of its main metabolites, 5 $\alpha$  dihydrotestosterone (DHT), cannot bind to the mutated AR, another important metabolite of testosterone—estradiol—is still able to bind ER. The biochemical conversion of testosterone to estradiol may be a key component to discrepancies across studies (McCarthy 2008; Whalen & Edwards 1967), discussed in further detail below.

*Tfm* male rats and wild type male rats exposed to the anti-androgenic pharmaceutical agent flutamide display reduced play which supports the hypothesis that androgen signaling through AR is responsible for organizing play behavior (Meaney et al. 1983). If play is programmed through neonatal androgen signaling then females exposed to androgens are expected to exhibit masculinized play behavior, and they do (Meaney & McEwen 1986). For both male and female rats, these results suggest a role for androgens as previously reported. Importantly, however, these studies do not rule out the hypothesis that increased testosterone could be converted to estrogen and signal through the estrogen receptor (ER) (McCarthy 2008; Whalen & Edwards 1967).

However, in a different study, *tfm* male rats did not find a reduction in rough-and-tumble play relative to wild type female and male rats (Field et al. 2006). Despite using the same mutant line of rat, no differences in *tfm* male play behavior were observed. However, these results could be anomalous because wild type females displayed higher levels of play than wild type males

which is likely indicative of stressful cage environments, or differences in how play behaviors were defined and measured (Table 2), or differences in play environments (i.e. pairs vs groups; see Table 3). This work highlights the need for use of standardized techniques when evaluating play behavior.

In wild type male rats, testosterone surges on the day of birth and programs male-typical behavior in the developing brain. The role of the neonatal testosterone surge and its influence on the development of rough-and-tumble juvenile play was tested by castrating male neonates on postnatal day (PND) 1, 6, 10 and 20 (Beatty et al. 1981). Males castrated the day after birth, PND 1, showed a reduction in both play initiation and total play compared to control males, and did not significantly differ from control females. PND 6 castrates tended to have reduced play, while PND 10 and 20 castrates played normally. The neonatal testosterone surge is, therefore, important for the organization of male typical play behavior, but is not necessary for normal masculinized play after organization has occurred (during the first week of life). Furthermore, play is reduced in neonatal males given an antibody against testosterone while females given testosterone over the same period had elevated levels of play relative to control females (Birke & Sadler 1988; see Table 3). Anti-testosterone antibody treatment should limit the ligand available for aromatization, so this work suggests organization of play behavior during the neonatal period is driven by AR-mediated signaling. Furthermore, female rats neonatally exposed to DHT, a nonaromatizable androgen that binds AR exhibit increased play (Tonjes, Docke, & Dorner 1987), which strongly suggests that AR directed programming induces play behavior. Collectively, this body of evidence illustrates the importance of AR-mediated signaling during the neonatal period. However, more work should be done to determine the extent to which neonatal ER mediated signaling organizes play behavior

**Table 2.** Behaviors defined across studies.

Authors	Behaviors defined
Beatty et al. 1981	<p>Pouncing—one rat lunges at another with its forepaws extended</p> <p>Wrestling—rats roll and tumble about</p> <p>Boxing—rats stand erect pawing at one another with their forepaws</p> <p>Pinning—one rat stands on another while the rat on the bottom struggles to escape</p> <p>Play-biting—one rat bites another usually on the tail or leg</p>
Birke and Sadler 1983	<p>Social sniffing—sniffing at any part of the body of one of the other animals.</p> <p>Exploring—active movement around the cage, sniffing at the walls or floor.</p> <p>Rearing—the animal rears up on its hind legs</p> <p>Self grooming—any part of the body is cleaned</p> <p>Play—cites definitions by Meaney and Stewart (1981), lists the following behaviors: pouncing, chasing, wrestling, and boxing</p> <p>Wrestling—the animals roll over each other</p> <p>Boxing—both animals adopt the upright “boxing” posture.</p>
Birke and Sadler 1984	<p>Play—includes several components that were not separated out in this experiment. Wrestling—the animals roll over each other</p> <p>Boxing—both animals adopt the upright “boxing” posture.</p> <p>Social sniffing—sniffing at any part of the body of one of the other animals.</p> <p>Exploring—active movement around the cage, sniffing at the walls or floor.</p> <p>Rearing—the animal rears up on its hind legs</p> <p>Self grooming—any part of the body is cleaned</p>
Birke and Sadler 1988	<p>Play—includes several components that were not separated out in this experiment. Wrestling—the animals roll over each other</p> <p>Boxing—both animals adopt the upright “boxing” posture.</p> <p>Social sniffing—sniffing at any part of the body of one of the other animals.</p> <p>Exploring—active movement around the cage, sniffing at the walls or floor.</p> <p>Rearing—the animal rears up on its hind legs</p> <p>Self grooming—any part of the body is cleaned</p>
Casto et al. 2003	<p>Pouncing—one animal lunges at another animal with its forepaws extended</p> <p>Wrestling—two animals tumble and roll over one another in a seeming attempt to gain a dominant position</p> <p>Boxing—both animals rear up on their hind legs and make jabbing movements at each other with their forepaws</p> <p>Pinning—the animal that is pinning stands over the opponent with its forepaws on the ventral surface of the opposing animal</p>



On-the-back—this is the reciprocal behavior of pinning, when an animal is in this position the entire ventral surface is exposed

Colbert et al. 2005

Nape attack—the snout of the target animal makes contact with the nape area of the partner animal

Pounce—the target animal lunges forward with its forepaws extended and makes contact with the partner animal

Pin—the target animal is positioned on top of the partner animal with its forepaws placed on the partner, the partner animal lies on its back, fully exposing its ventral surface to the target animal

Wrestle—the target and partner animal roll and tumble with each other

Mount—a component of the male copulatory pattern where the target animal approaches the partner animal from the rear, clasps its flanks, and mounts

Dessi-Fulgheri et al. 2002

Play—any activity involving exaggerated movements and inhibited attacks; it appears to achieve no obvious goal.

Social behaviors—approaching (moving toward another), crawl-over (moving over another), crawl-under (moving under another), social investigation (sniffing another's body except anogenital area), anogenital sniffing, allogrooming (gentle grooming of another's fur), aggressive grooming (vigorous grooming of another), pouncing (bouncing over another), charging (rushing toward another with vigorous bouncing gait), chasing, riding (forepaws over the back of a moving partner), sideways posture (the animal orientates itself broadside on to another), aggressive posture (the animal orientates itself at right angle to and over another), submissive posture (lying on the back with belly exposed to another), biting, withdrawing (all movements away from another), upright posture (with erect posture the rat exposes its belly to another), boxing (both rats stand up facing each other and boxing with forepaws), jumping (animal leaps vigorously into the air).

Field et al. 2006

Attack—Snout contact with a body part initiated a playful defensive maneuver by the partner, or it the partner failed to respond defensively when one pair mate brought the tip of the snout either into contact with or within 1-4 cm from the partner's nape.

Defense—withdrawal of the nape area by the recipient of an attack.

Complete rotation—upon nape contact the defending animal, in order to break the contact of the snout of the attacking animal to its nape, rolls over cephalocaudally from prone to supine.

Partial rotation—similar to a complete rotation except that the rotation around the longitudinal axis stops at the pelvis and at least one hindpaw maintains contact with the substrate.

Upright—this occurs when the animal rotates and faces the attacking animal but does so in an upright position rather than rolling over to supine.

Evasion—this occurs when the recipient of an attack leaps or serves away from the attacking animal to prevent nape contact.

Flynn et al. 2000	Pin—one animal having its dorsal surface to the ground while the other animal was on top
Flynn et al. 2001	See Flynn et al. 2000
Hotchkiss et al. 2003	Chasing—one animal actively pursuing another Dorsal contact—contact with the dorsal surface of the partner anywhere from the nape of the neck to the rump area Play bout—an individual is positioned with its back on the floor with another individual on top
Meaney et al. 1983	Pouncing—one animal lunging at another
Meaney and McEwen 1986	Pouncing—one animal lunging at another Wrestling Boxing On-the-top/On-the-back postures or pinning
Olesen et al. 2005	Wrestling/boxing—two animals engaged in rolling and tumbling over each other or making jabbing movements at each other with the forepaws. Biting—one rat biting another Pouncing—one rat pounces or lunges at another. Pinning—one rat standing over another, with its forepaws on the ventral surface of the opposing rat.
Porrini et al. 2005	See Dessi-Fulgheri et al. 2002
Tonjes et al. 1987	N/A

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**Table 3. Methodological approaches across studies.**

Authors	Strain	Age	Social Isolation	Habituation	Groups or pairs	Same sex	Time of testing	Data collection method	Scoring	Behaviors scored
Beatty et al. 1981	Holtzman albino	PND 31-40	None	None	Groups of 6-7	Both sexes	Onset of dark phase of L:D cycle	105, 20 second long intervals each day for 10 days.	Score of "1" for category Social Play if individual participated in play during interval. Score of "1" for category Play Initiation if individual initiated play during interval.	Social play: Pouncing, wrestling, boxing, pinning, play-biting  Play initiation: Pouncing, tail and leg pulling
Birke and Sadler 1983	Wistar	PND 22-24, 28-30, 35-37, and 41-43	None	None	Groups of 3	Same sex	Dark phase of L:D cycle	Behavior of each animal was noted every 30 seconds	Scores were generated by summing the number of times an individual engaged in each behavior and by calculating the mean time spent in play per cage member	Social sniffing, exploring, rearing, self grooming, play (wrestling, pinning, pouncing, chasing, boxing)
Birke and Sadler 1984	Wistar	PND 26-33	None	None	Pairs	Same sex	--	Behavior was sampled every 15 seconds for a period of 10 minutes	Scores were generated by summing the number of times an individual engaged in each behavior	Play, social sniffing, exploring, rearing, pouncing, self-grooming
Birke and Sadler 1988	Wistar	PND 26-33	None	None	Pairs	Same sex	--	Behavior was sampled every 10 seconds for a period of 10 minutes	Scores were generated by summing the number of times an individual engaged in each behavior	Play, social sniffing, exploring, rearing, pouncing, self-grooming
Casto et al. 2003	Sprague-Dawley	PND 27, 31, and 35	None	None	Groups of 6	Both sexes	First 4 hours of the dark cycle	15, 30 second observation periods per day	Recorded frequency of behavior per individual	Pouncing, pinning, wrestling, boxing, on-the-back

Colbert et al. 2005	Long Evans hooded	PND 22 and 34	24 hours isolation	None	Pairs	Same sex	Middle of dark phase in L:D cycle	10 minute testing period	Frequency of behavior recorded per individual	Nape attack, pounce, pin, wrestle, mount
Dessi-Fulgheri et al. 2002	Sprague Dawley	PND 35, 45, and 55	None	None	Groups of 6	Both sexes	Light phase of L:D cycle	6 minutes of observation, behaviors recorded during minute 2 and 3 of each observation	Frequency of behavior recorded per individual	Crawl over, crawl under, pouncing, charging, chasing, riding, boxing
Field et al. 2006	Male <i>t/m</i> and wild type Sprague Dawley	PND 33 and PND 90	24 hours isolation	15 minutes on PND 30, 31, and 32	Pairs	Same sex	--	10 minutes observation	Each 10 minute test period was scored for both the attack and defense components of play (Pellis & Pellis 1987). Attacks scored as number of attacks/animal/10 minutes. The rate of defense by each rat was scored as the number of defenses divided by the total number of attacks received, then multiplied by 100 to create a percentage of use of a particular defense maneuver for each pair mate.	Attack, defense (complete rotation, partial rotation, upright, evasion)
Flynn et al. 2000	Sprague Dawley	PND 35	24 hours isolation	None	Pairs	Same sex	--	5 minute testing period	Number of pins was counted per individual	Pinning
Flynn et al. 2001	Sprague Dawley	PND 35	24 hours isolation	None	Pairs	Same sex	--	5 minute testing period	Number of pins was counted per individual	Pinning
Hotchkiss et al. 2003	Sprague Dawley	PND 36 or 37	24 hours isolation	None	Pairs	Same sex	1-4 hours after lights	10 minute observation period	Frequency of behaviors recorded per individual	Rostral sniffing, caudal sniffing,

							out during L:D cycle			chasing, dorsal contact, play bout
Meaney et al. 1983	Long Evans hooded	PND 26-40	None	None	Groups of 6	Both sexes	One hour after lights out during dark phase of D:L cycle	70, 20 second observation periods per day	Score of "1" given to individuals that engaged in play, score of "0" given for those that did not during the observation periods.	Pouncing, wrestling, boxing, on-top/on-the-back postures
Meaney and McEwen 1986	Long Evans hooded	PND 26-40	None	None	Groups of 6	Both sexes	N/A	50, 20 second observation periods per day	Score of "1" given for participation in play, score of "0" given for no participation in play during 20 second periods.	Pouncing, wrestling, boxing, pinning
Olesen et al. 2005	Sprague Dawley	PND 25-29	None	None	Groups of 6	Both sexes	2 trials 3 hours after lights off and 2 trials 6 hours after lights off	4, 2 minute observation periods per day	Scores were generated by summing the number of times an individual engaged in each behavior	Wrestling/boxing, biting, pinning, pouncing
Porrini et al. 2005	Sprague Dawley	PND 35, 45, and 55	None	None	Groups of 6	Both sexes	Dark phase of L:D cycle	6 minutes of observation, only female behaviors recorded	Frequency of behavior recorded per individual	See Dessi-Fulgheri <i>et al.</i> 2002
Tonjes et al. 1987	Wistar	PND 26-40	None	None	Groups of 6	Both sexes	--	70, 20 second observation periods per day	A score of "1" was given to an individual if engaged in play, or a score of "0" if not engaged in play per each observation. A score of "1" was given if the individual initiated play.	Total play, play initiation

### *Neonatal Progestins and AR Response*

Progestins comprise another class of hormones that influence the mechanisms of sexual differentiation (Quadros et al. 2002) and have been implicated in the development of sexually dimorphic play behavior. Progestins can bind to and activate progesterin receptor (PR), but there is evidence that progestins can antagonize or weakly agonize AR, depending on the concentrations of both progestins and endogenous androgens (Bardin et al. 1983). In fact, the relationship between androgen receptor activation and the competitive binding between similar ligands (i.e. progestins and androgens) does not follow a classic allosteric model but is instead described by a steric allosteric model. In other words, the relative concentrations of progestins:androgens greatly influences the degree to which AR responds to ligand binding. It is important to note that progestins are not strong androgens, but can interact with AR and induce some level of response, especially in the absence of androgens (a high progestins:androgens ratio). The effects of progestins on play behavior have almost exclusively been viewed through the lens of their anti-androgenic effects and not within the context of the function of the PR as a transcription factor for PR responsive genes.

Medroxyprogesterone acetate (MPA), a synthetic progesterone, is a strong AR antagonist with a binding affinity equivalent to the testosterone metabolite DHT (Kemppainen et al. 1999). Indeed, MPA has been used as an anti-androgen in clinical practice in humans (e.g., anti-androgenic cancer treatment, see Bentel et al. 1999). Neonatal rat pups exposed to MPA directly or via the dam's milk exhibited reduced play and reduced play initiation (Birke & Sadler 1983). The mechanism by which MPA altered play is not understood, but at the dosage studied (Table 1), MPA likely antagonized endogenous AR (Birke & Sadler 1983, 1984).

In order to better understand the role of endogenous progestins, the hypothesis that native progesterone modulates organization of play behavior was tested by treating neonatal male pups with either progesterone or to anti-P, an antibody against progesterone. Neonatal male and female pups exposed to progesterone exhibited reduced play, however, an interesting sex difference was observed in pups with depressed levels of progesterone. Anti-P treated males played more than controls (as expected) but treated females played less than controls. Surprisingly, rat pups of both sexes exposed to progesterone during a longer exposure window exhibited increased play behavior (Table 1, Birke & Sadler 1988). These findings support the widely accepted idea that both timing and dose of hormone exposure during organizational events is alters the direction and magnitude of observed effects.

In addition to timing and dose, the interaction between hormones is critically important for regulating behavioral programming. For example, in males with depressed levels of testosterone, MPA treatment “rescued” male typical levels of play fighting (Birke & Sadler 1988). This finding suggests that when androgen levels are low, weak AR signaling via MPA binding may suffice to elicit downstream effects needed for the organization of play behavior. However, females given MPA + testosterone had reduced play relative to those treated with testosterone alone, which suggests that in females the presence of testosterone interacts to make MPA anti-androgenic. An alternative explanation could be that the observed effects resulted from an interaction between MPA and estrogen receptor signaling (Pazol, Wilson, & Wallen 2004), but this relationship remains uncharacterized in the context of juvenile play. Females could be more sensitive to changes in AR antagonism via progesterone because they have lower levels of androgens compared to males, thus large quantities of progesterone would competitively bind to AR. Another explanation for this result could involve positive feedback

(termed a “rebound effect”), which would lead to increased progesterone levels following anti P treatment, resulting in high levels of progesterone instead of depressed levels. This type of feedback should be more likely to occur in females relative to males given progesterone’s diverse roles in female physiology and known feedback mechanisms. These two explanations are not mutually exclusive.

However, it is important to note that differences in the dose concentrations and methods of administration (via the dam’s milk or directly injected into the pup) could also influence the strength of responses reported in these experiments (Table 1). Furthermore, it is possible that MPA and progesterone interact with AR differently and elicit diverse responses. While this series of experiments serves as an important first step toward understanding the role of progestins, many questions have yet to be addressed. For example, what role does PR play?

#### *Neonatal Estrogen*

Estrogen is a critical steroid hormone involved in the activation of female typical behavior in adults (i.e. activation of precopulatory/proceptive behavior), yet counterintuitively it plays a role in organizing the developing male brain so that normal male reproductive behavior can occur (McCarthy 2008; Schwarz & McCarthy 2008; Wersinger et al. 1997). In developing fetuses, testosterone within specific regions of the brain is converted by the enzyme into estradiol. Estradiol binds estrogen receptor and drives estrogen dependent transcription, causing changes in brain development and organization. The conversion of testosterone to estradiol is critical in males for proper male typical neurodevelopment and behavioral programming (Juntti et al. 2010; McCarthy 2008; Whalen & Edwards 1967). The role of estrogens in female behavioral programming is not as well understood, and the role of neonatal estrogen in the development of play behavior in both sexes is largely understudied.



In one of the few studies investigating the role of early neonatal estrogens in organizing play behavior programming, oestradiol benzoate (EB) an estradiol analog that binds to ER $\alpha$  in both humans and murine rodents did not affect play behavior of female rats (Table 1). This suggests that early postnatal estrogen signaling through ER $\alpha$  does not alter the organization of female typical play behavior (Tonjes et al. 1987).

In contrast, female rat pups exposed to higher concentrations of EB that paralleled male typical levels of estradiol engaged in play more often than control females, and as often as control males (Table 1, Table 3). Female rat pups treated with both EB and tamoxifen, a pharmaceutical agent that antagonizes ER, did not display elevated levels of play (Olesen et al. 2005). These findings implicate a role for ER in the neonatal masculinization of play behavior in female rats.

Indeed, steroid hormone receptors can be activated by nonsteroid compounds, such as neurochemicals. For example, ER activation by the neurochemical dopamine increases the expression of ER responsive genes and has been linked to changes in social play behavior (Olesen et al. 2005). Specifically, dopamine D1 receptor agonism has been linked to masculinized rough-and-tumble play and masculinized sexual behavior in female rats (Gotz et al. 1991; Olesen et al. 2005; Tonjes et al. 1989). Female rats dosed with a dopaminergic chemical exhibited an increase in total play (Olesen et al. 2005). The organization of complex social behaviors like play likely results from the interaction of multiple molecular pathways and linkages between physiological systems. Additional research is required to address the role of ER signaling in the development of play behavior in male rats.

Synthesizing the literature regarding ER mediated programming of play behavior is difficult due to the surprisingly sparse number of studies and their lack of concordance. This

could be due to study differences in experimental design (i.e. differences in scoring methods; Table 3), in dose and timing of exposure (Table 1), and in how play behaviors were defined (no definitions of play behaviors were provided by Tönjes et al. 1987; Table 2). These differences in methodology further highlight the need for standardized behavioral scoring techniques.

*En masse*, experiments focusing on neonatal hormone exposure indicate that play behavior is likely not programmed by a single steroid. It is, therefore, important to understand how the hormonal milieu during critical windows of development exerts its influences on the organization of complex social behaviors.

#### *Prenatal Hormone Exposure*

Studies from the 1980s provided evidence that play behavior is organized neonatally (Beatty et al. 1981; Birke & Sadler 1983, 1984; Meaney & McEwen 1986; Meaney & Stewart 1981; Meaney et al. 1983; Tonjes et al. 1987). As a result, few studies have attempted to assess whether alterations in the prenatal environment influence the development of play behavior. Therefore, our ability to estimate how the prenatal hormonal environment influences development of human social behavior is extremely limited. Prenatal testosterone peaks in male rodents at gestational days 15-19 (Habert & Picon 1984; Konkle & McCarthy 2011; Weisz & Ward 1980) and thus could organize play behavior prior to birth via AR or ER directed processes.

In fact, male pups exposed prenatally to flutamide, an anti-androgenic pharmaceutical agent displayed reduced and female typical levels of play (Casto et al. 2003). This study was the first to demonstrate that juvenile rough-and-tumble play in male rats is demasculinized when androgen function is disrupted during a prenatal period (Table 3). The implications of this work

are compelling, as many industrial and agricultural chemicals consumed during pregnancy disrupt normal endocrine function by acting as AR antagonists (Vandenberg et al. 2012).

### *Endocrine Disrupting Chemicals*

Many chemicals including those used in industry and agriculture, pharmaceuticals, and personal care products are classified as endocrine disrupting chemicals (EDCs) because they interfere with normal endocrine system function and can alter behavior (Zala & Penn 2004). Many EDCs are able to bind to specific hormone receptors and can be either agonistic or antagonistic. Certain EDCs can have duality in their mechanism of action and can act agonistically or antagonistically depending on their concentration, similar to the example with the compound MPA above (Li et al. 2012; Wong et al. 1995). EDCs exert their effects through a variety of mechanisms. For example, metabolites (M1 and M2) of the pesticide vinclozolin binds AR and reduces AR-ligand complex DNA binding, which decreases androgen dependent gene expression (Lambright et al. 2000) whereas atrazine, an herbicide, increases expression of aromatase and conversion of testosterone to estradiol (Holloway et al. 2008).

EDCs are now ubiquitous in the environment and have been found in regions far from industrial and agricultural use (Sonne et al. 2012). The effects of EDCs impact all vertebrate classes, including humans (Bergman et al. 2013; Hayes et al. 2011). Our endocrine system naturally responds to very low concentrations of endogenous hormones, thus very low concentrations of EDCs can disrupt its function and can be more dangerous than high doses due to the involvement of negative feedback loops and detoxification mechanisms (Vandenberg et al. 2012). In addition to the complexity of EDCs and their mechanisms of action, the body of literature regarding the influence of endocrine disruptors on the development of play behavior in

the rat is difficult to synthesize partly due to lack of consistency in methodology across studies (Table 3, Table 2).

### *Prenatal AR Antagonism*

Vinclozolin (metabolites act as AR antagonists) exposure from gestation through weaning had no significant treatment effects on play behavior (Flynn et al. 2001). This lack of effect might have been related to the dose of vinclozolin (Table 1). However, control rats did not display the typical level of sexual dimorphism, as the mean number of times one play partner pinned down the other play partner was not different between control male and females. It is difficult to evaluate the sex-specific effects of an anti-androgen in a system where there are no sexual dimorphisms. However, because only one behavior was measured for a short time period (average number of pins in 5 minutes, see Table 3), it might be that other play behaviors were affected but not evaluated.

In another study, developmental exposure to intermediate and high doses of vinclozolin did affect juvenile play behavior. Males exposed to vinclozolin from mid-gestation to birth exhibited increased levels of pinning (Colbert et al. 2005). These findings are surprising relative to prior evidence from neonatal studies suggesting the development of male typical play behavior is directed through AR (Meaney et al. 1983). If the selected doses did indeed competitively bind to AR and block normal activation by testosterone, then a dose dependent decrease in play behavior for the vinclozolin treatments would be expected, assuming a linear or monotonic dose response (see Table 3). This suggests that other mechanisms independent of AR likely are involved in masculinizing play behavior during the prenatal period. Alternatively, it has been suggested that in some contexts androgen antagonists can act as agonists (Nguyen, Yao, & Pike 2007; Wong et al. 1995). This duality of mechanism highlights the complexity of effects

that can be induced by EDCs, and sheds new light on data from Colbert et al. (2005), which suggest that vinclozolin might have a variety of prenatal effects depending on which mechanism is activated. However, AR antagonists can increase plasma levels of testosterone, ultimately increasing T metabolites (Hellman et al. 1977; Sardanons et al. 1989). A substantial increase in the concentration of T and its metabolites could in fact outcompete antagonistic binding of AR. The increase of T and its metabolites in response to an initial reduction in AR signaling due to antagonism could very well be the mechanism through which the observed agonistic effects occur. Importantly, the methodology employed by Colbert et al. (2005) were more powerful than those of previous studies as they examined multiple behaviors (see Table 1). The increased variety of observed behaviors and increased behavioral data points per individual recorded by Colbert et al. (2005) likely increased the ability to detect treatment effects.

#### *Neonatal AR Antagonism*

To determine the role of AR directed behavioral programming of male typical juvenile play during the neonatal period male neonates were exposed to either flutamide or vinclozolin, and female neonates to testosterone propionate (TP) (Hotchkiss et al. 2003). As expected, androgenized (TP) females displayed an increase in play while flutamide and vinclozolin treated males exhibited a decrease in play. These results suggest AR activation during an early neonatal period is important for the development of masculinized play behavior. This study differed from other studies in that the vinclozolin dosage was substantially higher than those used by Flynn et al. (2001) and Colbert et al. (2005) (see Table 1) and was administered neonatally rather than prenatally or across both developmental windows. Although the dose deviates from an ecologically relevant range, the findings illustrate that this anti androgenic EDC is able to affect

neonatal organization of male typical juvenile play and suggests that neonatal androgen signaling is important for development of play behavior.

### *ER Agonists*

Estrogenic EDCs typically function by binding ER and increasing ER signaling. Bisphenol A (BPA) is an estrogenic plasticizer widely used in the food industry (Brotons et al. 1995; Steinmetz et al. 1997). Dessi Fulgheri et al. (2002) compared the play behavior of offspring from pregnant dams exposed to a high dose of BPA (400ug/kg) from mid-gestation through weaning) or to a low dose of BPA (40ug/kg) administered prior to pregnancy through weaning (Table 3 and Table 1). Males and female offspring in both the high and low dose groups exhibited increased play behavior directed toward female playmates.

In another study, however, offspring were exposed to BPA (40ug/kg) from conception through weaning, play behavior was not increased. BPA treated females exhibited reduced play behavior directed towards males, but the level of play behavior directed towards females did not change (Porrini et al. 2005). The disparity between these findings and those of Dessi Fulgheri et al. (2002) may be explained by recent evidence showing that the estrogenic actions of BPA are dose dependent, and that BPA could act as an ER agonist at high doses (Dessi-Fulgheri et al. 2002) and as an ER antagonist at low doses (Li et al. 2012; Porrini et al. 2005).

The phytoestrogen, genistein, has multiple physiological mechanisms but exerts its estrogenic effects on the endocrine system by binding ER, with a stronger affinity for ER $\beta$  than ER $\alpha$ . Offspring exposed to genistein during gestation and through weaning did not have any significant changes in play behavior (Flynn et al. 2000). The authors attributed the lack of effect of genistein to the organizational role of early androgens, which were not influenced by the treatments used (Flynn et al. 2000). Alternatively, play behavior might depend on estrogen

receptor type, with signaling through ER $\alpha$  being more important. However, exposure to methoxychlor, a synthetic pesticide with metabolites that are agonists for ER $\alpha$  and antagonists for ER $\beta$  and AR, also found no significant effects on the frequency of pinning (Flynn et al. 2005), further supporting the hypothesis that rough and tumble play is organized via AR. It is possible that the dose used in this study was not high enough or was too high to elicit anti androgenic effects on play behavior. In addition, these studies focused only on pinning behavior (Table 3), which might not be the most sensitive measure of play behavior. The effects on other aspects of rough-and-tumble play are unknown. Standardized techniques for measuring play behavior will allow us to synthesize data across studies so that we can make general conclusions about effects induced by different hormones or EDCs.

#### *Summary of Effects*

Play is a critically important juvenile behavior that facilitates normal social development, yet we still have only a cursory understanding of the mechanisms that organize and regulate sexually dimorphic expression of play. It is evident from the majority of studies reviewed here that androgens and AR have important effects on the development of play behavior. However, several studies suggest a potential role for ER signaling (Dessi-Fulgheri et al. 2002; Olesen et al. 2005; Porrini et al. 2005), and the roles of AR and ER might be dependent on prenatal or neonatal developmental timing. Researchers have largely focused on the role of androgens, but we have not disentangled the pathway through which testosterone exerts its effects on organizing play behavior, as testosterone can bind to and activate AR but can also be metabolized to estradiol and activate ER. Moreover, many of the studies reviewed here attempt to address the role of AR signaling by using chemicals that are not specific to the androgen receptor (Birke & Sadler 1983, 1984). Finally, most studies investigate the effects of hormones and hormone

disruptors that are known to have non-linear dose responses using only one or a few concentrations. Conclusions and inference drawn from a single dose in this context is limited and the use of dose response experiments will strengthen our ability to truly understand the effects elicited by the chemical(s) being studied. Until we achieve a clear understanding of how endogenous hormone signaling influences the development of critical social behaviors such as play, our ability to predict and understand the impacts of endocrine disrupting compounds on hormone receptor dependent pathways will remain restricted.

While this body of literature allows us to address many questions regarding how changes in endocrine signaling during critical windows of development influence juvenile play behavior in the rat, it is difficult to identify generalities due to differences in study designs, treatment delivery, and experimental endpoints. Progress towards understanding the relationship between endocrine signaling and the development of play behavior is hindered by: 1) use of idiosyncratic methods across experimental designs (Table 3) 2) a primary focus on studying the postnatal period, leaving the critical prenatal window conspicuously understudied, (Table 1), 3) a lack of standardized protocols for assessing play behavior (Table 3 and Table 2), and the absence of dose response experiments. This deficient methodological consistency across studies introduces important confounding factors. For example, it is well established in the rat literature that a brief period of social isolation can increase motivation for play in juvenile rats. In the studies reviewed here, some individuals were socially isolated for 24 hours prior to play behavior data collection while others experienced no social isolation. For example, it will be difficult to detect treatment effects in animals that are not motivated to play relative to those that are motivated, because motivated animals will behave while unmotivated ones might not. Comparing results from



studies that use different approaches such as this limits our ability to draw general conclusions about specific chemicals.

It is evident from the existing body of literature that developmental exposure to EDCs can disrupt the development of juvenile play behavior in the rat. The hypothesis that disruptions in endocrine signaling can influence this critically important juvenile behavior has broad implications for both conservation biology and human health, but without a standardized methodology that allows for comparisons across studies, scientific progress in understanding the relationship between alterations in the embryonic and neonatal environment and subsequent changes in behavior will remain limited.

### *Suggested Standardized Protocol*

#### *Standardizing Methodology*

Standardized methodology will allow for making comparisons across studies, which is critical in order to synthesize results from experiments that evaluate the effects of endogenous hormones or endocrine disruptors. Play behavior is studied from a multitude of perspectives, from the basic science of how play develops in natural settings to the effects of environmental exposures on the development of play. In order to be able to synthesize findings across diverse research perspectives, it is crucial that play behavior is studied in a way that can be meaningfully compared (e.g. quantitative meta-analysis). To achieve this, the methodology must be standardized to a reasonable extent—one that allows for meaningful comparison without limiting the potential for unique research questions. Here we make recommendations for a methodology that aims to achieve this goal.

There has been a lack of consistency with respect to testing conditions before and during data collection—for example, some studies have included a habituation period whereas others

have not. Data collected from animals that did not undergo a habituation period would likely record behaviors exhibited when placed in a novel, unfamiliar environment. Lack of habituation likely induces a stress response (and accompanying stress-related behaviors), which would give a false impression of the animals' motivation to engage in the target behavior, play. Alternatively, animals had been habituated to the play arena would not experience the same stress and would quickly engage in play behaviors. Synthesizing across studies to evaluate general effects of a treatment will be limited when studies use different approaches. The characterization and quantification of the components of play behavior also differ across studies—for example, some studies have counted the total number of pins over a five minute play session while others characterized each behavior as part of a complex ethogram consisting of groupings of specific behaviors (see Table 3 and Table 2). The studies that record more types of play have a higher probability of detecting effects and can identify specific play behaviors that are affected by treatment versus those that are not. Detailed information such as this will help identify mechanisms through which different types of play are organized. In order to allow synthesis researchers and clinicians to begin synthesizing the play literature in a meaningful way, we propose a standardized methodology adapted from Himmler et al. 2013, which is accompanied by a freely available and detailed instructional video supplement (Himmler, Pellis, & Pellis, 2013).

First, data collection should, at a minimum, include observations taken on PND 35 when juvenile rough-and-tumble play behavior peaks in the rat (Thor & Holloway 1984). Data collection should be preceded by a habituation period of 30 minutes per day for three days (at age 32, 33, and 34 days) prior to the behavioral trial (Himmler et al. 2013). Behavioral observations on day 35 should then be taken on two same sex, same treatment play partners

while in the neutral “play arena” within which the animals were habituated, but decisions regarding animal group sizes and sex combinations can be modified depending on the experimental question being addressed. Both the habituation periods and play trials should occur in the play arena under red light during the first few hours of the dark cycle when rats are most active (Himmler et al. 2013). This prevents introducing variation from recording behavior at different times during the dark cycle. The play arena should always be sanitized, completely dried, and clean bedding added prior to habitation or trials to eliminate odors from previous animals and cleaning materials (Himmler et al. 2013). Because brief periods of social isolation have been shown to increase motivation for engaging in play behavior (Panksepp & Beatty 1980), the habituation and data collection process should be done in three stages. In stage 1 play partners are placed together in play arena (for 30 minutes, under red light, just after dark cycle begins), and then returned to their home cages. This is conducted for two consecutive nights, thus stage 1 includes both the first and second days of habituation. Stage 2 begins on the third day of habituation when play partners are again placed together in play arena (for 30 minutes, under red light, just after dark cycle begins), and then are placed in separate cages for a 24 hour period of social isolation (Himmler et al. 2013). This will standardize motivation on the focal analysis day. Stage 3 begins on the trial day, a video camera is placed in front of the arena and titled down at an angle where all four corners of the play arena are clearly visible. Play behavior of newly reunited play partners should be recorded for 10-15 minutes (Himmler et al. 2013). We recommend using software designed for quantifying behavior, such as J watcher (produced by Dr. Daniel Blumstein's Lab University of California Los Angeles & The Animal Behaviour Lab, Macquarie University, Sydney).

Previous studies have measured play behavior with various groupings of juvenile rats (i.e. mixed treatment and mixed sex groups, same sex different treatment pairs, different sex same treatment pairs, etc). Decisions regarding the playgroup design are largely influenced by the experimental questions that the study aims to address. For questions that aim to address how a specific developmental treatment influences play behavior, using same sex, same treatment pairs allows for efficient quantification of treatment effects on play behavior and play dynamics. Placing treated and untreated individuals together will allow for tests of dominance. Each individual in the pair should be separately observed as the focal animal so that individuals can be independently scored as the attacker, defender, or counterattacker. Indeed, small mixed sex groups will allow for assessment of how specific treatments influence play directed at one sex relative to another.

*Standardizing how play behavior is characterized*

Juvenile rough-and-tumble play behavior is comprised of a complex suite of movements including: pouncing, pinning, wrestling, boxing, and chasing. Pellis and Pellis (1987 and 1990) have characterized specific forms of attack and defensive tactics employed by juvenile rats (Pellis & Pellis 1987; Pellis & Pellis 1990). Importantly, Pellis and Pellis (1990) highlighted that juvenile play is generally comprised of three types of tactics: playful attack (any approach by one animal that brings its snout into contact or near contact with the nape of the partner), playful defense (withdrawal of the nape area from the snout of an approaching partner), and playful counterattack (an attack launched immediately at the play partner following a defense).

Delineating the specific roles of each play partner is critical for understanding the dynamics of social play—one play partner may attempt to elicit playful behavior from the other partner with a higher frequency, which would result in a high number of attacks by that

individual whereas the play partner may not be motivated to play, and therefore would result in a low rate of defense tactics for this individual. One could imagine a situation where the total number of playful interactions is the same, yet the rate of attacks, defenses, and counter attacks varies immensely. Classifying these three main components of playful interactions for both play partners will allow us to compare the dynamics of play across treatment groups.

While attack, defense, and counterattack account for the three major components of playful interactions, there is a suite of specific behaviors that occur during the course of play bouts. These behaviors include: chasing, pouncing, pinning, wrestling, and boxing. Table 2 highlights the inconsistencies across studies in how these behaviors have been defined and characterized and Table 3 highlights the numerous scoring designs that have been implemented to quantify juvenile play behavior data. Latency, frequency (events), and duration (states) of behavior are three of the most important parameters to consider when quantifying behavior and should therefore be incorporated in the play scoring design. We suggest that the total time the play partners spend engaged in play should be quantified for the 10-15 minute play behavior trial. Playful attack, counterattack, pouncing, wrestling, pinning, boxing, and chasing should be scored individually for each play partner. Classifying each individual's play behavior will help provide insight into the composition of the play dynamics between each set of play partners and what aspects of play are modified by experimental manipulations.

### *Conclusions*

Beginning to understand how the early hormone environment shapes critical juvenile behaviors like play will help us gain insight into what types of environmental insults may be contributing to the increase in childhood neurodevelopmental disorders—many of which impair a child's ability to successfully engage in social play with peers. For example, it has been shown

that children prenatally exposed to higher levels of polychlorinated biphenyls and dioxins had altered levels of sex-typical play behavior (Vreugdenhil, Slijper, Mulder, & Weisglas-Kuperus, 2002). Social behaviors, such as juvenile play behavior, are important for normal cognitive and social development and have been utilized as behavioral biomarkers for altered development in humans and rodent models. Indeed, rat social play behavior has been used as a behavioral biomarker in rat models of autism (Schneider et al. 2005). Despite a rich body of literature characterizing pre and neonatal hormonal programming of rodent juvenile play, synthesizing the results to understand the role of endocrine signaling in the development of this early social behavior remains exceedingly difficult due to methodological inconsistency across studies. Developing standardized techniques for measuring rat juvenile play behavior is critical because play behavior is an important behavioral biomarker for endocrine-based insults during sensitive periods of development. Standardization of collection and analysis of play behavior will enhance our ability to make comparisons across future studies and will facilitate our ability to make translations to human health.

## CHAPTER 3: ELEVATED PRENATAL ANDROGEN METABOLITES INDUCE AUTISM-LIKE BEHAVIOR IN THE RAT

### *Introduction*

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is broadly characterized by atypical social behavior, repetitive behaviors, and deficits in communication, but can also present with other symptoms such as hyperactivity and anxiety (APA 2013). Although the etiology of ASD remains enigmatic, evidence suggests it is multifactorial and likely occurs due to gene-environment interactions (Bill & Geschwind 2009; Braun 2012; Courchesne et al. 2007; Dawson et al. 2002; Hallmayer et al. 2011).

Normal fetal neurodevelopment is dependent on sex-steroid signaling, and the dysregulation of hormone signaling has been implicated in ASD (Auyeung et al. 2010; Baron-Cohen 2002; Baron-Cohen et al. 2005; Braun 2012; Gore et al. 2014; Ingudomnukul et al. 2007; Sarachana et al. 2011). Autism is male biased, with an incidence rate four times greater in males compared to females (Baron-Cohen et al. 2011). One explanation for this preponderance is the extreme male brain theory, which states that a hyperandrogenic environment programs the developing brain in an aberrant manner (Baron-Cohen 2002), resulting in an autistic behavioral phenotype. Human studies have shown that children born from mothers with elevated androgens have an increased risk for developing ASD (Knickmeyer et al. 2006).

Androgens impact brain development through two main endocrine signaling pathways (McCarthy 2008). Testosterone (T) can bind to androgen receptor (AR) directly or as an androgen metabolite (e.g. 5 $\alpha$ -dihydrotestosterone, DHT) and elicit AR-dependent responses, T can also be converted to estradiol (E) through the enzyme aromatase, which binds to estrogen receptors (ER) and elicits ER-dependent responses (McCarthy 2008; Schwarz & McCarthy

2008). Both AR- and ER-mediated pathways are important for early brain development. However, the relative contribution of excessive AR- versus ER-mediated signaling has not been explored in the context of understanding the link between fetal endocrine disruption and later behavioral abnormalities, such as ASD.

Here we test the extreme male brain theory that prenatal exposure to elevated metabolites of testosterone are related to ASD-like behavior in the rat, and we delineate the contributions of elevated prenatal androgen- and estrogen-dependent signaling to later presentation of ASD-like behaviors in the rat. Our findings show that prenatal exposure to elevated androgens and estrogens differentially impact behaviors associated with autism, and consistent with ASD presentation in humans, these effects are more common in males.

### *Methods*

#### *Study System*

The rodent is an appropriate model organism for studying ASD-like behavior (Moy et al. 2004; Pletnikov et al. 2001; Silverman et al. 2010). Rats are especially useful for studying social behavior and have recently emerged as an important model system for ASD (Wöhr & Scattoni 2013). Rats induced to have autism-like phenotypes have abnormal behavioral responses in a variety of assays that parallel the three main diagnostic criteria of autism. Atypical reciprocal social interactions have been observed by measuring juvenile play behavior (Bobee et al. 2000; Pletnikov, Moran, & Carbone 2002; Schneider & Przewłocki 2005; Shultz et al. 2008; Wolterink et al. 2001) and interest in social novelty (Bambini-Junior et al. 2011; MacFabe et al. 2011; McFarlane et al. 2008). Anxiety and hyperactivity have been modeled using tests that measure behavioral responses in a novel environment, such as the open field, (Narita et al. 2010; Schneider & Przewłocki 2005). Coincident with predictions of the extreme male brain theory,



enhanced spatial learning has been observed in ASD-like rats (Edalatmanesh et al. 2013). Importantly, the influence of early hormone signaling on juvenile play behavior (Beatty et al. 1981; Birke & Sadler 1983, 1984; Blake & McCoy 2015; Meaney et al. 1983), and spatial learning ability (Imwalle et al. 2006; Isgor & Sengelaub 2003; Williams & Meck 1991) have been extensively studied, providing an important link between autistic behavioral phenotypes and the endocrine system.

### *Hormones*

To test the extreme male brain theory and delineate the relative contributions of prenatal exposure to elevated prenatal androgen- and estrogen-dependent signaling to later presentation of ASD-like behavior in the rat, we exposed pregnant rat dams to either 5 $\alpha$ -dihydrotestosterone propionate (DHTP, Advance Scientific & Chemical Inc.), estradiol benzoate (EB, Sigma-Aldrich Co.), or a corn oil control. DHTP is esterified to DHT and binds to AR, and EB is esterified to estradiol, which binds to ERs.

### *Animals and Hormone Treatments*

Male and female Sprague Dawley rats were obtained from Charles River Laboratories, NC. Animals were housed undisturbed for 5 days for adjustment and all animal handling and housing adhered to East Carolina University IACUC regulations (AUP #D300). All animals were housed in the same colony room with 12 hours of light and dark (L:D cycle), and standard rodent chow and water were available *ad libitum*. Female rats were paired with stud males overnight and checked for copulatory plugs the following morning. If a copulatory plug was not visible, a vaginal smear was obtained and examined under a light microscope for sperm. The presence of sperm in the vaginal smear or the presence of a copulatory plug was used to confirm pregnancy, and at this point pups were considered to be at embryonic day (E) 0.5. Pregnant rats

were randomly assigned to a treatment and co-housed until treatment began on E 15.5. From E 15.5-17.5, pregnant rats received subcutaneous injections of the assigned treatment suspended in corn oil, either 8 mg/kg dihydrotestosterone propionate (DHTP, N=5), 50 µg/kg estradiol benzoate (EB, N=4), or corn oil alone (vehicle, N=5, Table 4). Injection volume was standardized for all treatments by animal weight as 0.5 ml/kg. Injections were administered between 4 to 5 hours after the start of the light phase of the L:D cycle. After the dosing period ended, pregnant rats were left undisturbed until parturition.

#### *Tissue Collection and Physiological Measures*

On the day of birth (E 21-22), weight and body length were measured and pups received paw pad tattoos for identification (AIMS Neonate Rodent Tattoo System, Braintree Scientific Inc.). Litters were culled to eight pups consisting of four males and four females based on anogenital distance when possible, and returned to the dam. At weaning, two of each sex were randomly selected for behavioral testing and the remaining two were donated for a different study. One litter of control animals yielded only three males, so five females were kept in the cage to maintain a litter size of eight pups. One litter of EB animals yielded only five pups (three female and two male), so this litter was not culled, but was still used in behavioral assays.

On PND 35 and PND 55-60, after behavioral assays, animals were weighed and anogenital distance, a sensitive measure of genitalia masculinization, was measured using electronic calipers. At the end of the project, approximately PND 120-140, one male and one female were randomly selected from each litter and humanely euthanized using standard euthanasia mix, and brains were perfused using 4% paraformaldehyde, extracted, weighed, and then stored in 70% EtOH at -4 C.

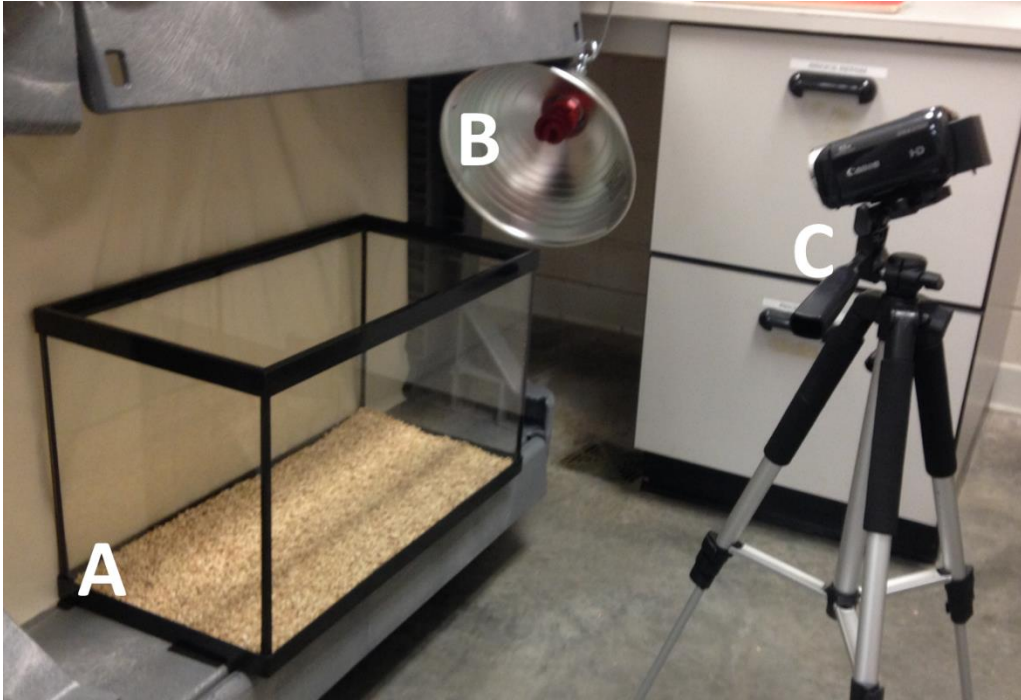
## *Behavioral Testing and Data Collection*

### *Juvenile Play Behavior*

Previous work on rat models of autism has shown that rats exhibit reduced juvenile play (Pletnikov et al. 2002; Schneider & Przewłocki 2005; Wolterink et al. 2001). Play behavior testing in the current study followed methods described by Blake and McCoy (2015). Same treatment, same sex siblings (play partners) were habituated to the play arena for 30 minutes during the first two hours of the dark phase of the L:D cycle under red light on PND 32-34. The play arena was a 50.8 x 27.9 x 33.0 cm glass aquarium with neutral corn cob bedding. Play partners were socially isolated for 24 hours beginning on PND 34. On PND 35, play partners were dorsally marked using a non-toxic marker and reunited in the play arena. Play behavior was recorded using a Sony Vixia Handycam Camcorder for 15 minutes under red light during the first two hours of the dark phase (Figure 4). Play partners were returned to their home cages, and the play arena was thoroughly sanitized using Cavicide (Metrex).

Play behavior was quantified using J Watcher software (produced by Dr. Daniel Blumstein's Lab University of California Los Angeles & The Animal Behaviour Lab, Macquarie University, Sydney). Characterization and quantification of behavior followed previously described methods (Blake and McCoy 2015). Videos were randomized and coded by a trained researcher blind to treatment using a play behavior ethogram (Table 5). Video coding began when animals were placed in the arena and coding ended 10 minutes after this time point.

Play behavior data obtained from video coding were counted as events (e.g. total wrestling bouts). For each play pair, behaviors were divided into two categories: single animal behaviors (pins, playful attacks, counterattacks) and pair behaviors (chasing, wrestling, boxing). To calculate the total number of play behavior events that occurred during each trial, single



**Figure 4.** Set up of play arena. A) Standard 10-gal aquarium with neutral corn cob bedding in the bottom, B) Red light for filming in the dark, C) Video camera positioned so that all four corners of the play arena were in the frame.

animal behaviors performed by each playmate were counted and added to the number of pair behaviors. All pairs of animals engaged in play during the behavioral assay except for one pair of control females. The data points generated by these females were determined to be over 2.5 standard deviations from the mean using the package *LMERConvenienceFuctions*, and thus were deemed outliers. In order to avoid having extreme values inflate differences among groups, these non-behaving control females were dropped from play behavior analyses. These females were not found to be outliers in any other behavioral assay, and were included in subsequent behavioral analyses.

**Table 4.** Dosing concentrations and dam sample sizes across treatments.

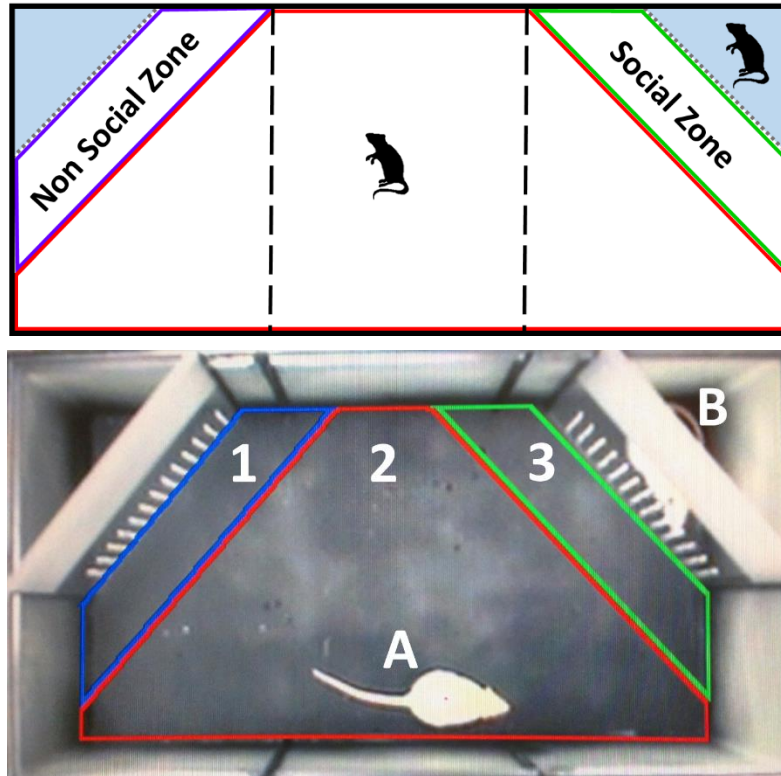
TREATMENT	DOSE (E 15.5-17.5)	N
ESTRADIOL BENZOATE	50 µg/kg	5
DIHYDROTESTOSTERONE PROPIONATE	8 mg/kg	5
CORN OIL	--	4

### *Social Approach Test*

Social approach behavior tests are established assays to determine whether ASD-like atypical social behavior is displayed (Moy et al. 2004; Silverman et al. 2010). Rat models of autism exhibit aberrant social approach to a stranger rat and a reduced preference for social novelty (Bambini-Junior et al. 2011; MacFabe et al. 2011).

On PND 55-60, each individual was tested in a three-chambered social approach apparatus during the first four hours of the light phase. The social approach apparatus consisted of a 91.4 x 45.7 x 43.2 cm glass aquarium with two triangular side chambers located in the top corners where one stranger rat was placed by random assignment (Figure 5). On the day of testing, a stranger rat of the same sex as the test animal was randomly assigned to and placed in one of the triangular side chambers. The test animal was placed in the center chamber for a 5 minute acclimation period. Then, the dividers were removed and the test animal was given 10 minutes to explore the apparatus. SMART tracking software (SMART, PanLab, Cornellá, Spain) was used to record the test animal's movement and specific zones were defined in order to determine the amount of time spent in as well as entries into the social zone versus the non-social zone (Figure 5). After completion of the trial, test animals were returned to their home cages and the apparatus was thoroughly sanitized using Cavicide (Metrex). Social approach behavior was

analyzed as the proportion of entries into the Social Zone relative to total entries into both the Social Zone and Nonsocial Zone (Figure 5).



**Figure 5.** Top, cartoon depiction of social approach arena. Triangular side chambers were separated from the main test area by clear, perforated plexiglass walls so that test animals could see and smell the stranger rat. The apparatus was further divided into three equal chambers, separated by removable, opaque plexiglass walls. Bottom, screen shot of social approach trial. A) Test animal, B) Same sex stranger. Numbered sections represent specified zones: 1) Non social zone, 2) Center area, 3) Social zone.

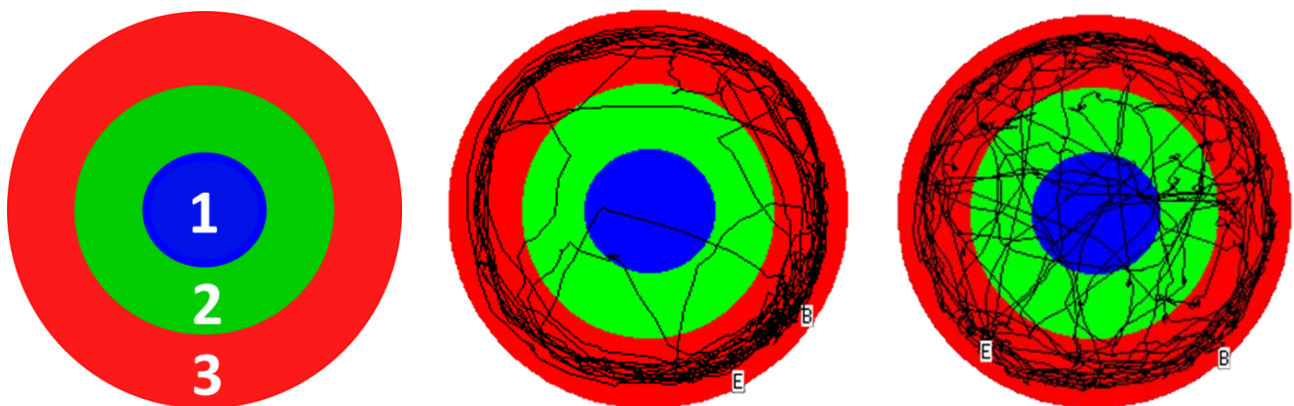
### *The Open Field*

Previous work has used the open field to assess behavior in rat models of autism, and showed ASD-like rats are hyperactive in the open field (Narita et al. 2010; Schneider & Przewłocki 2005).

Forty-eight hours after the social approach test on PND 57-62, animals were tested in the open field during the first four hours of the light phase of the L:D cycle. The open field was a

circular arena measuring 63.5 cm in diameter. Zones were defined with three concentric circles in order to determine whether the animal crossed the center of the open field or remained near the edges, and to quantify overall movement among zones (Figure 6). On the day of testing, the animal was placed inside the arena near the edge and movement was tracked using SMART tracking software for 10 minutes (SMART, PanLab, Cornellá, Spain). Animals were returned to home cages after the open field test and left undisturbed until PND 80-90.

Behavior in the open field was analyzed in two ways: 1) as an estimate of abnormal anxious behavior, the total number of times the animal crossed the center circle of the open field was counted (Bailey & Crawley 2009), and 2) as a measure hyperactivity, the total number of times the animal crossed from one zone to another was counted (Anderson 1941, Figure 7A).

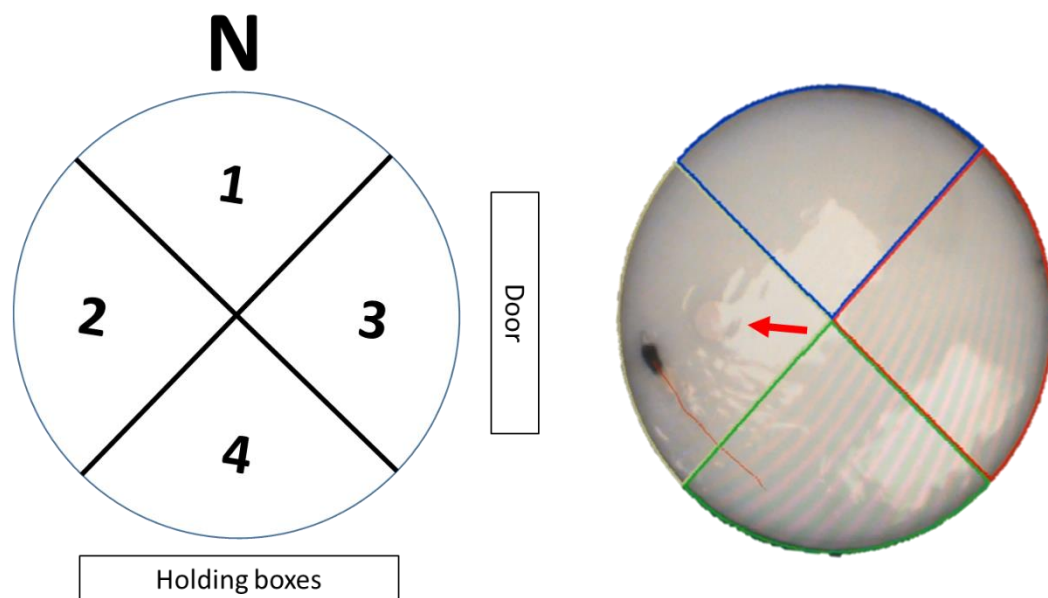


**Figure 6.** The open field. A) Zone 1: Center, Zone 2: Mid-region, Zone 3: Outer circle, B) Example of normal behavioral response, C) Example of abnormal behavioral response.

### *Morris Water Maze*

Rats induced to have an ASD-like phenotype show enhanced spatial learning, particularly in males (Edalatmanesh et al. 2013).

On PND 80-90, animals were tested using the “place” version of the Morris water maze (MWM) using standard methods adapted from Vorhees and Williams (2006). The acquisition phase occurred over three consecutive days in the first five hours of the light phase. Each day of the acquisition phase, test animals completed four trials of the water maze and were recorded using SMART software (SMART, PanLab, Cornellá, Spain). Acquisition trials lasted a maximum of 60 seconds. If an animal was unable to locate the escape platform in 60 seconds, then it was gently placed on the platform. All animals were given 30 seconds to observe the room and external visual cues before being returned to the holding boxes (Figure 7). Animals had at least 15 minutes of rest in between the four trials. After the fourth trial on acquisition days 1-3, animals were returned to their home cages. On the fourth day of testing (the probe day), the hidden platform was removed from the water maze, and the test animal was given 2 minutes to swim to the platform. After the probe day trial, animals were returned to their home cages.



**Figure 7.** The water maze set up. Left, cartoon schematic of water maze zones and visual landmarks in testing room. “N” represents cardinal North, displayed as a large letter N printed on paper in the testing room. Right, screen shot of the test in progress, the red arrow indicates where the hidden platform is (Quadrant 2).

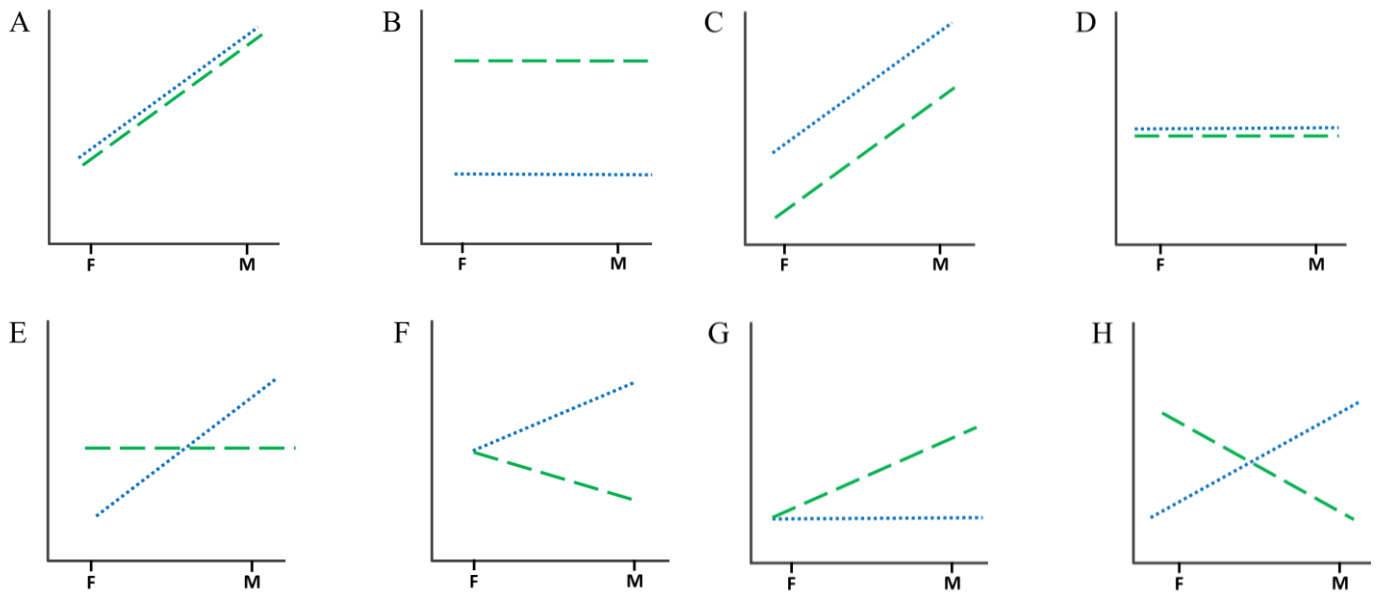


Latency and distance to the escape platform during the acquisition trials were analyzed to detect differences in learning between experimental groups. Both time and distance were analyzed to account for differences in swimming speed. Probe day performance, indicative of memory, was measured by comparing the distance swam and time spent in the quadrant previously containing the escape platform (the target) and the quadrant directly opposite of the target quadrant (Figure 7).

### *Statistical Analyses*

All data were analyzed using the statistical software program R v. 3.1.2 (R Core Team 2015). Data were analyzed using linear or generalized linear mixed models (LMM and GLMM, respectively) using the *lme4* package (Bates et al. 2013). Statistical parameters were generated using the *lme4* (Bates et al. 2013) and *lmerTest* packages (Kuznetsova, Brockhoff, & Christensen 2015). We used likelihood ratio tests to choose the best model. After determining the best model, Wald's z test statistics provided by the *lmer4* output or t statistics provided by the *lmerTest* output were used to distinguish significant effects of specific interactions or main effects. Models included the response variable, predictors of interest, and their interaction. All models contained a random effect to account for variation in maternal effects that pups experienced throughout development and dosing. Count data were analyzed using a generalized linear mixed effect regression with a Poisson distribution, all other data were analyzed using linear mixed effect regressions.

Play behavior, open field, social approach, and Morris water maze probe data were visualized as interaction plots to compare differences between both sex and treatment. To illustrate all possible interaction outcomes, hypothetical examples are described in Figure 8. Figure of the data display means and standard error of both the fixed and random effects.



**Figure 8.** Examples of the hypothetical interaction plot outcomes where line types represent two treatments and F and M represent male and female sexes. A) Main effect of sex, No main effect of treatment, No interaction, B) Main effect of treatment, No main effect of sex, No interaction, C) Main effect of treatment, Main effect of sex, No interaction, D) No effect of sex, No effect of treatment, No interaction, E) Main effect of sex, No main effect of treatment, Interaction between treatment and sex, F) Main effect of treatment, No main effect of sex, Interaction between treatment and sex, G) Main effect of sex, Main effect of treatment, Interaction between treatment and sex, H) No main effect of treatment, No main effect of sex, Interaction between treatment and sex.

## Results

### *Juvenile Play Behavior*

*Total play behavior events.* There was a significant interaction between treatment and sex for total play,  $\chi^2(2) = 26.102$ ,  $p < 0.0001$  (Figure 9). Control males and females did not differ in total play behavior events ( $z = 0.271$ ,  $p > 0.05$ ). Play behavior in EB males and EB females did not differ drastically, but EB reduced play behavior in males by 22% compared to control males and reduced play behavior in females by 33% compared to control females. DHTP also reduced play behavior in both sexes, but effects were much stronger in females. DHTP males played 27% less than control males, while DHTP females played 55% less than control females ( $z = -2.935$ ,  $p = 0.00334$ ).

*Wrestling.* A significant interaction between treatment and sex was identified for wrestling,  $\chi^2(2) = 11.587$ ,  $p = 0.003$  (Figure 10). DHTP females wrestled 55% less than control females ( $z = -2.644$ ,  $p = 0.008$ ).

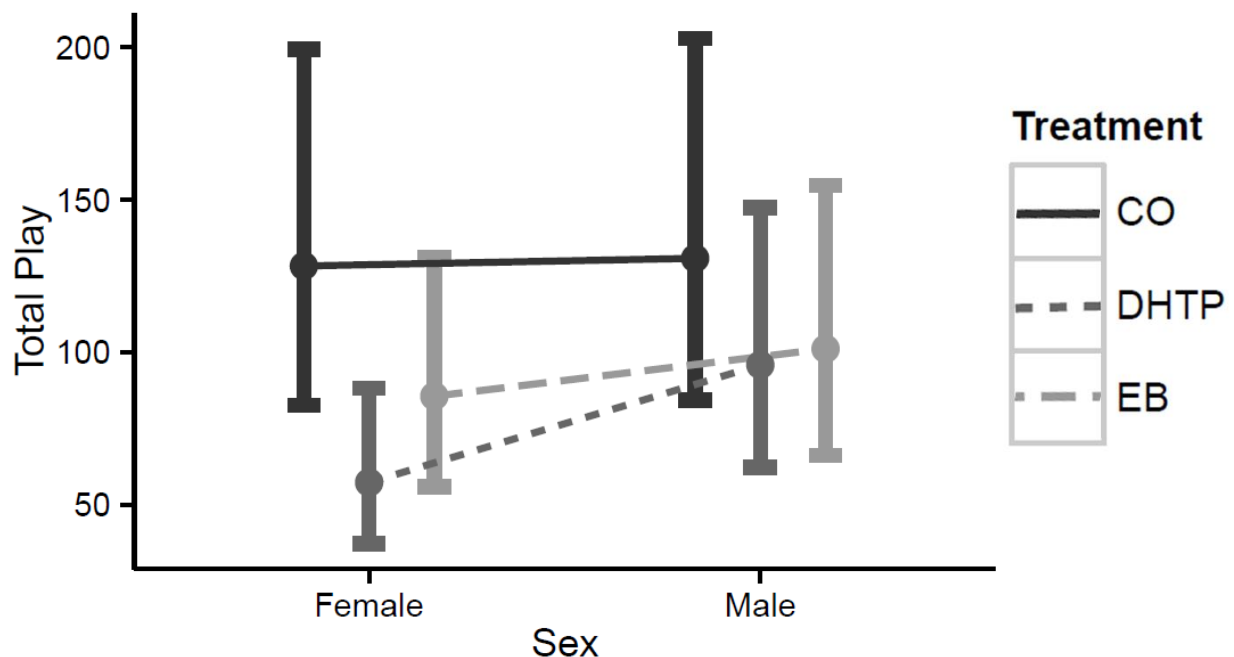
*Chasing.* Significant main effects of sex,  $\chi^2(1) = 5.1839$ ,  $p = 0.0228$ , and treatment,  $\chi^2(1) = 6.7973$ ,  $p = 0.03342$ , were detected for chasing (Figure 11). Overall males chased more than females ( $z = 2.281$ ,  $p = 0.023$ ), and both treatment groups exhibited reduced levels of chasing relative to controls. EB animals chased 49% less than control animals ( $z = -2.370$ ,  $p = 0.0178$ ) and DHTP animals chased 56% less than control animals ( $z = -2.768$ ,  $p = 0.0056$ ).

*Boxing.* Boxing events did not significantly differ between treatment groups or between sexes (data not shown).

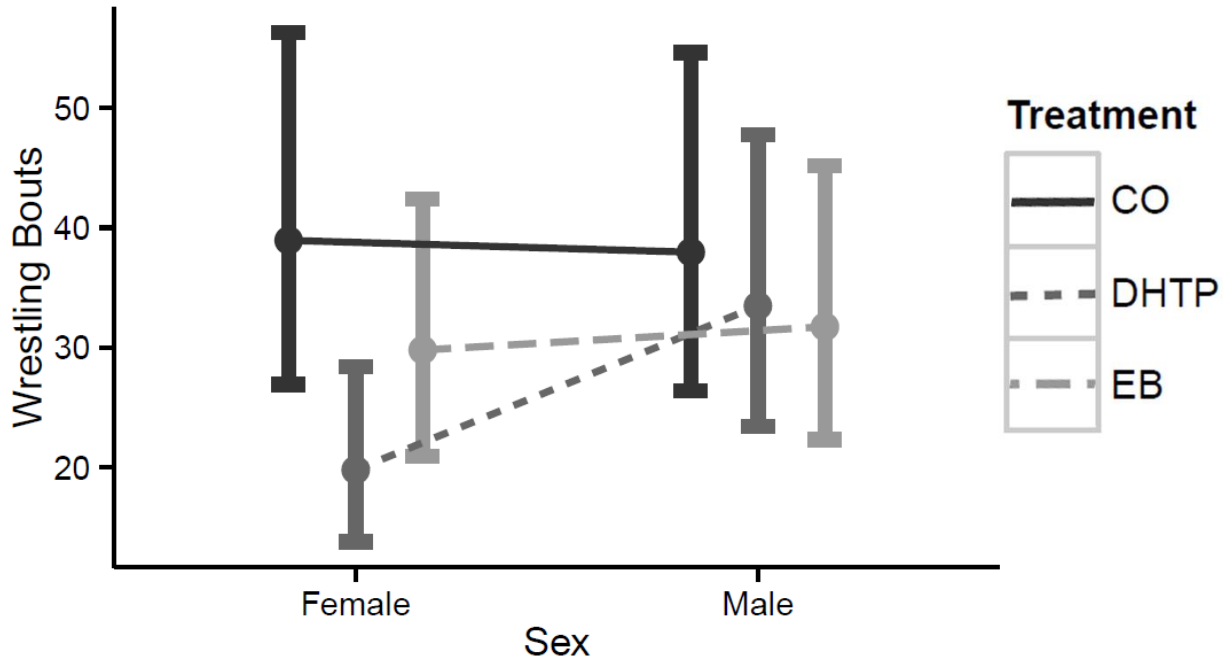
*Playful Attack.* There was a significant effect of treatment on playful attacks,  $\chi^2(1) = 12.3401$ ,  $p = 0.0004433$  (Figure 12). DHTP animals attacked 43% less than controls ( $z = -2.146$ ,  $p = 0.0319$ ).

*Pinning.* A significant interaction between treatment and sex existed in pinning behavior,  $\chi^2(2) = 12.303$ ,  $p = 0.00213$  (Figure 13). DHTP females pinned 57% less than control females ( $z = -2.196$ ,  $p = 0.0281$ ).

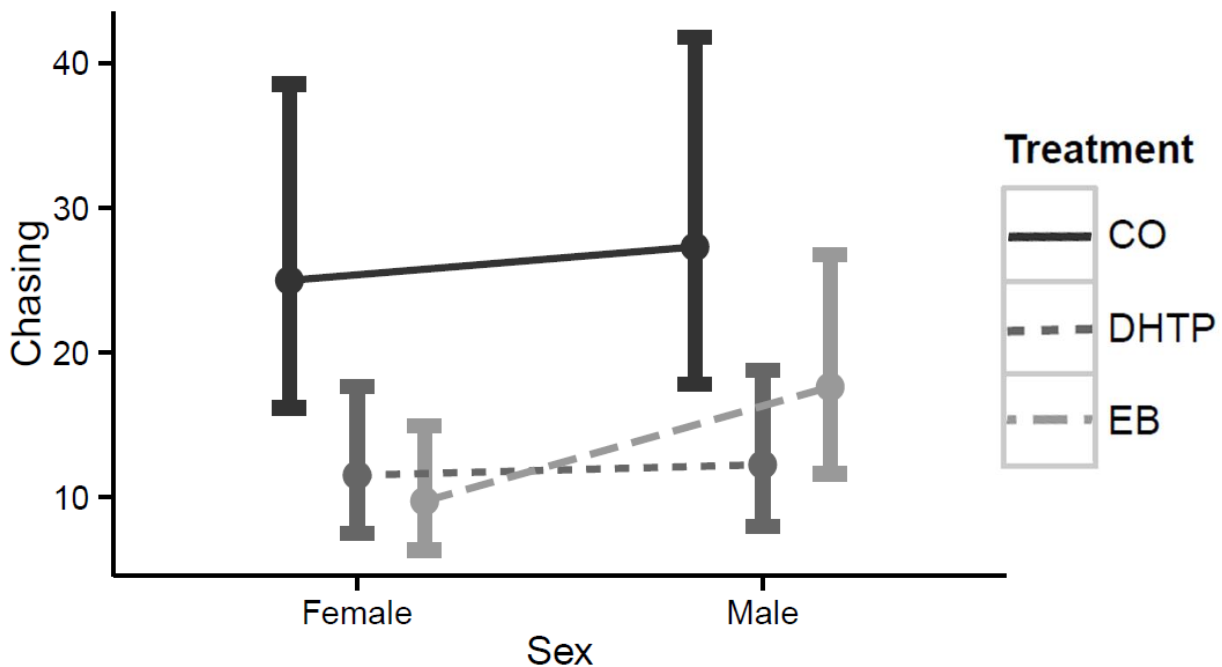
*Counterattack.* There was a significant interaction between treatment and sex in counterattacks,  $\chi^2(2) = 14.75$ ,  $p = 0.0006$  (Figure 14). DHTP females exhibited 85% less counterattacks than control females ( $z = -2.513$ ,  $p = 0.01198$ ).



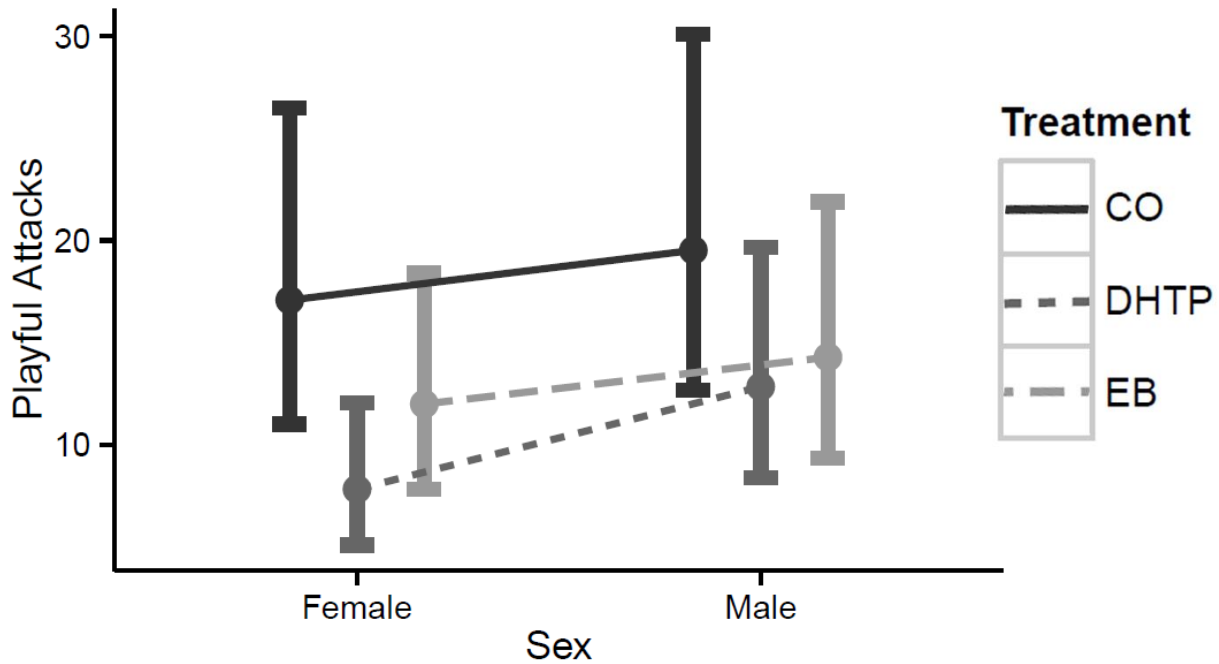
**Figure 9.** Total play behavior (attack, chase, wrestle, pin, counterattack, and boxing) was significantly affected by an interaction between treatment and sex (likelihood ratio test,  $\chi^2(2) = 26.102$ ,  $p < 0.0001$ ). Control males and control females did not differ in total play behavior events ( $z = 0.271$ ,  $p > 0.05$ ). DHTP reduced play behavior in both sexes, but effects were much stronger in females. DHTP males played 27% less than control males, while DHTP females played 55% less than control females ( $z = -2.935$ ,  $p = 0.00334$ ).



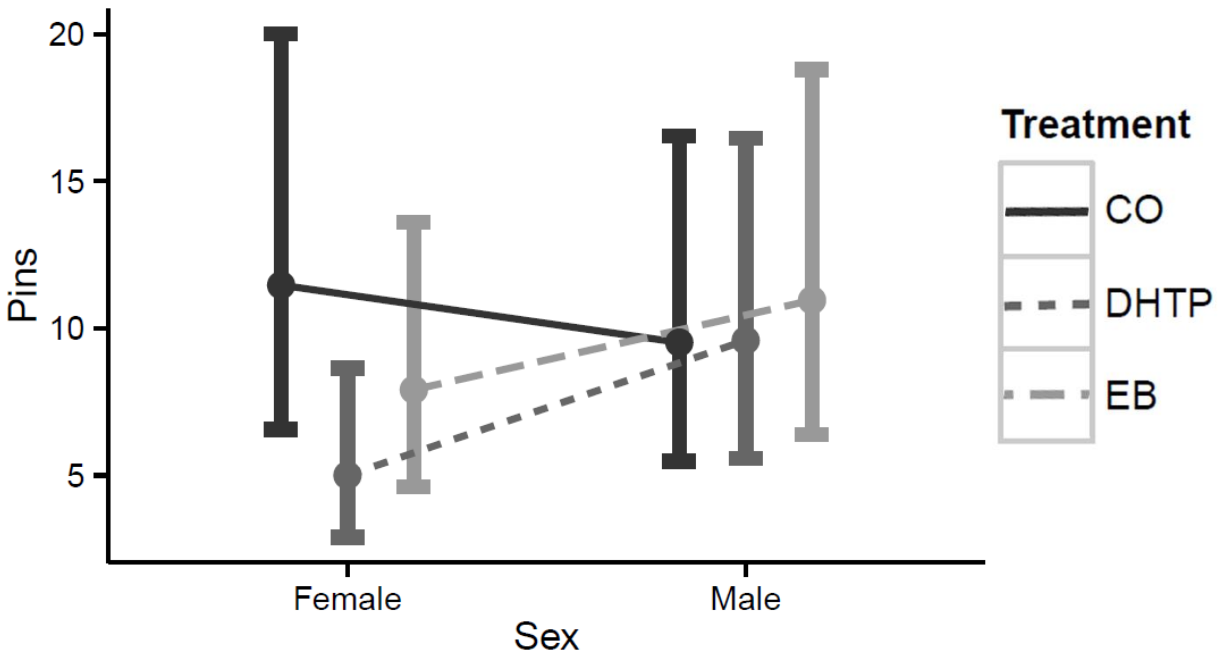
**Figure 10.** Wrestling events showed a significant interaction between treatment and sex (likelihood ratio test,  $\chi^2(2) = 11.587$ ,  $p = 0.003$ ). DHTP females wrestled 55% less than control females ( $z = -2.644$ ,  $p = 0.008$ ).



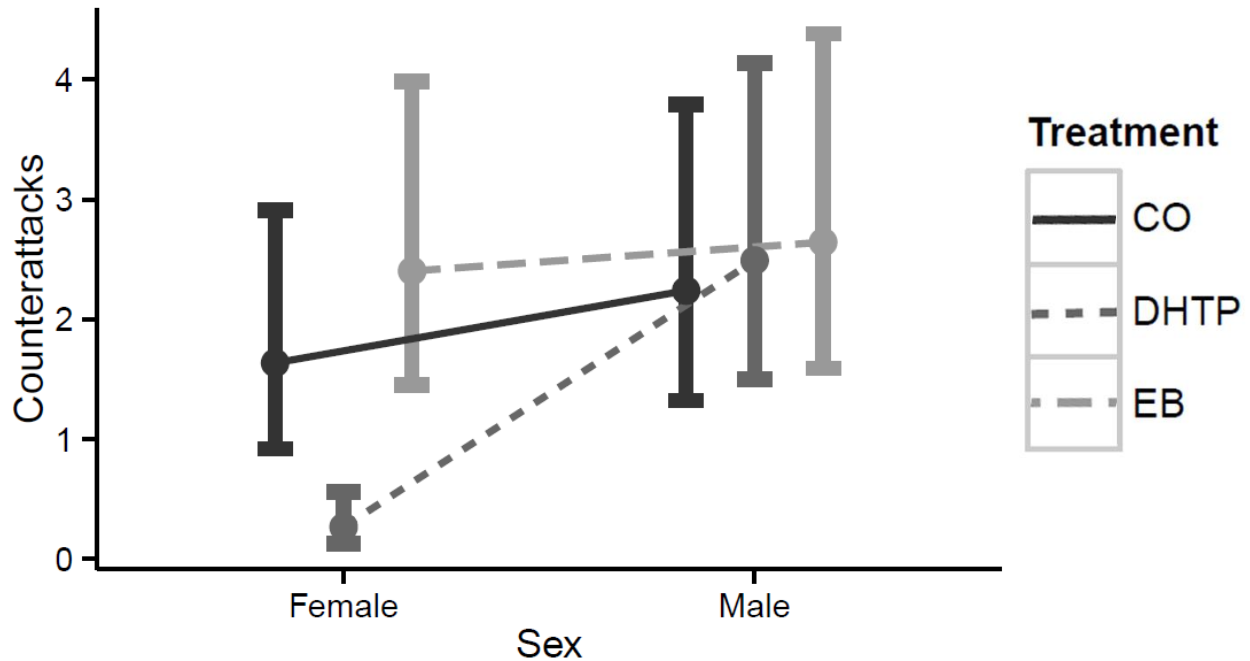
**Figure 11.** A significant interaction between treatment and sex was not detectable (likelihood ratio test,  $\chi^2(2) = 5.528$ ,  $p = 0.063$ ). Significant main effects of sex and treatment were detected. Overall males chased more than females ( $z = 2.281$ ,  $p = 0.023$ ), and both treatment groups exhibited reduced levels of chasing relative to controls. EB animals chased 49% less than control animals ( $z = -2.370$ ,  $p = 0.0178$ ) and DHTP animals chased 56% less than control animals ( $z = -2.768$ ,  $p = 0.0056$ ).



**Figure 12.** There was a significant effect of treatment on playful attacks, but no significant interaction (likelihood ratio test,  $\chi^2(1) = 12.3401$ ,  $p = 0.0004433$ ). DHTP animals attacked 43% less than controls ( $z = -2.146$ ,  $p = 0.0319$ ).



**Figure 13.** A significant interaction between treatment and sex existed in pinning behavior (likelihood ratio test,  $\chi^2(2) = 12.303$ ,  $p = 0.00213$ ). DHTP females pinned 57% less than control females ( $z = -2.196$ ,  $p = 0.0281$ ).



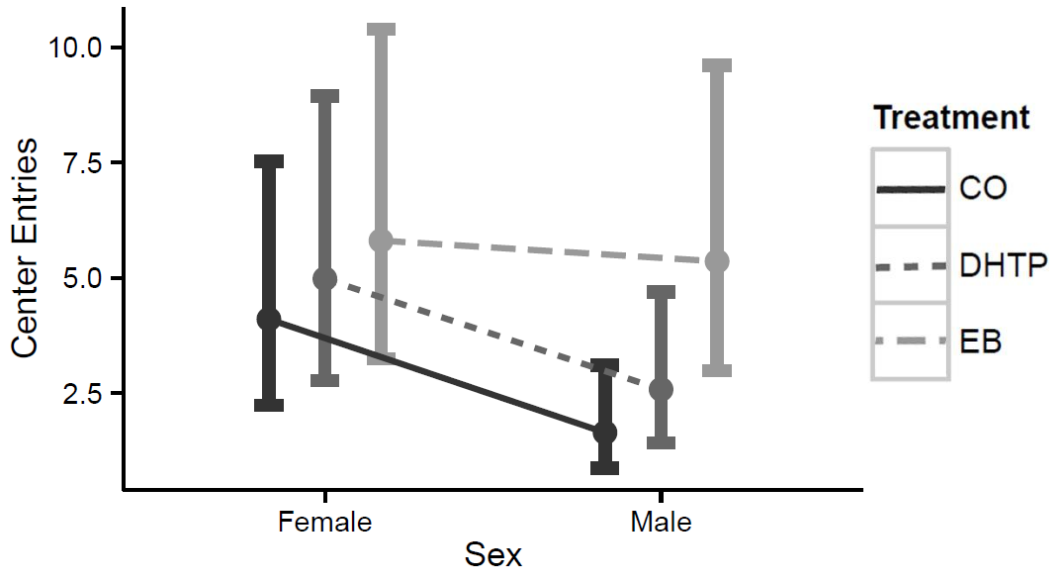
**Figure 14.** There was a significant interaction between DHTP treatment and sex in counterattacks (likelihood ratio test,  $\chi^2(2) = 14.75$ ,  $p = 0.0006$ ). DHTP females exhibited 85% less counterattacks than control females ( $z = -2.513$ ,  $p = 0.01198$ ).

#### *Open Field Behavior*

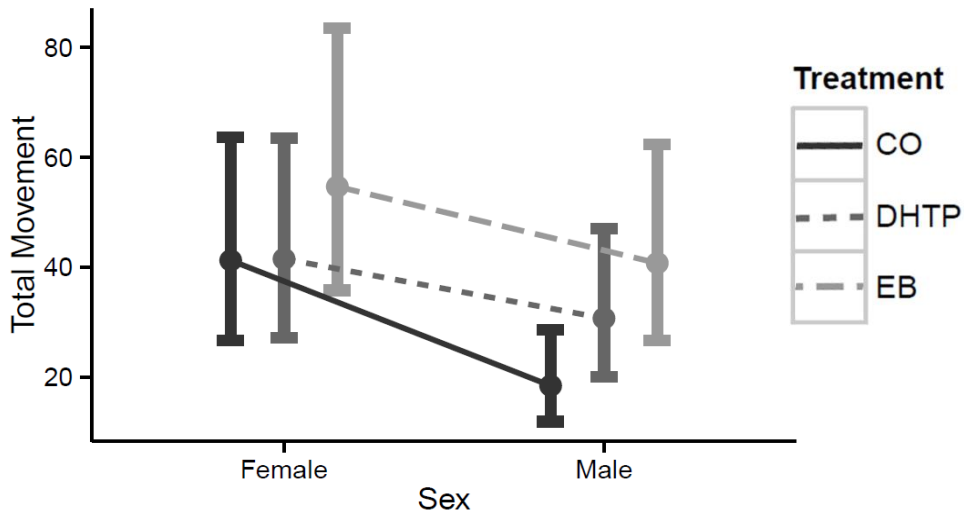
*Center crosses (anxiolytic behavior).* A significant interaction between treatment and sex was detected,  $\chi^2(2) = 7.5146$ ,  $p = 0.02335$  (Figure 15). Control males crossed the center of the open field less often than control females ( $z = -3.129$ ,  $p = 0.00716$ ), and EB males crossed the center of the open field 227% more than control males ( $z = 5.36$ ,  $p = 0.01528$ ), suggesting an abnormal stress response.

*Total movement (hyperactivity).* A significant interaction between treatment and sex was detected,  $\chi^2(2) = 23.995$ ,  $p < 0.0001$  (Figure 16). Control males had lower levels of total movement in the open field compared to control females ( $z = -8.590$ ,  $p < 0.0001$ ). Treated males had elevated levels of total movement; DHTP males moved 67% more than control males ( $z =$

4.229,  $p < 0.001$ ) and EB males moved 112% more than control males ( $z = 4.512$ ,  $p < 0.001$ ), indicating hyperactivity.



**Figure 15.** Treatment and sex significantly interacted in the number of center crosses in the open field test (likelihood ratio test,  $\chi^2(2) = 7.5146$ ,  $p = 0.02335$ ). Control males crossed the center of the open field less than control females ( $z = -3.129$ ,  $p = 0.00716$ ), and EB males crossed the center of the open field 227% more than control males ( $z = 5.36$ ,  $p = 0.01528$ ).

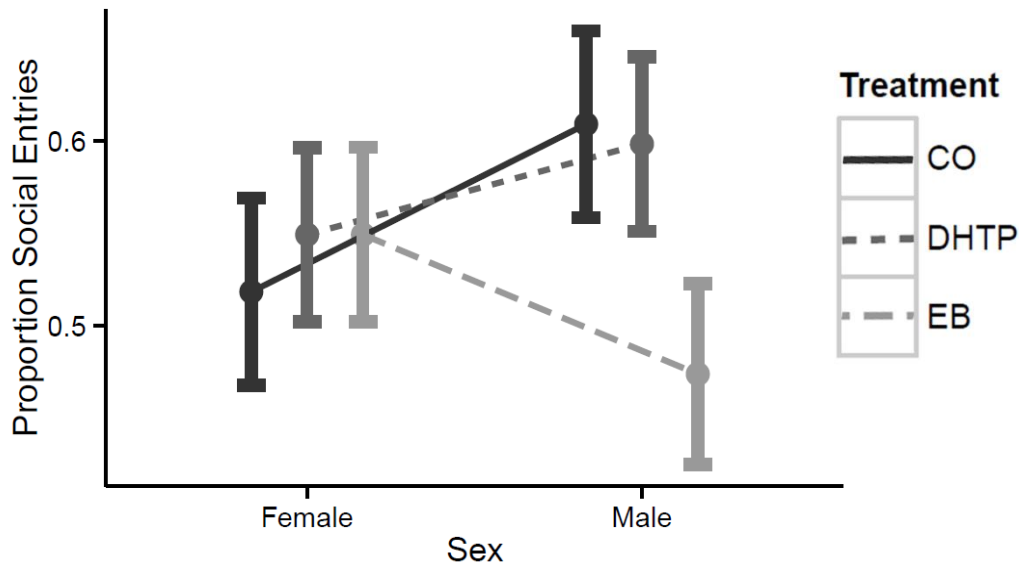


**Figure 16.** Treatment and sex significantly interacted in total movement between zones during the open field test (likelihood ratio test,  $\chi^2(2) = 23.995$ ,  $p < 0.0001$ ). Control males had lower levels of total movement in the open field compared to control females ( $z = -8.590$ ,  $p < 0.0001$ ). Treated males had elevated levels of total movement; DHTP males moved 67% more than control males ( $z = 4.229$ ,  $p < 0.001$ ) and EB males moved 112% more than control males ( $z = 4.512$ ,  $p < 0.001$ ).



### *Social Approach*

There was a significant interaction between treatment and sex,  $\chi^2(2) = 6.0043$ ,  $p = 0.04968$  (Figure 17). Comparing entries into the two zones (social and non-social), control males made entries into the social zone 61% of the time while EB males made entries into the social zone only 47% of the time, indicating a reduced interest in social novelty ( $t = -2.237$ ,  $p = 0.0308$ ).



**Figure 17.** The proportion of social entries interacted between treatment and sex (likelihood ratio test,  $\chi^2(2) = 6.0043$ ,  $p = 0.04968$ ). Comparing entries into the two zones (social and non-social), control males made entries into the social zone 61% of the time while EB males made entries into the social zone only 47% of the time, indicating a reduced interest in social novelty ( $t = -2.237$ ,  $p = 0.0308$ ).

### *Morris Water Maze*

Latency to the escape platform was significantly different between treatments,  $\chi^2(1) = 11.7105$ ,  $p = 0.002865$  (data not shown), DHTP animals located the escape platform more quickly than EB or control animals ( $t = -3.59$ ,  $p = 0.00473$ ). Distance to the escape platform was significantly different between the sexes,  $\chi^2(1) = 10.6339$ ,  $p = 0.00111$  (data not shown), males swam a shorter distance to the platform than females ( $t = -3.306$ ,  $p = 0.0012$ ).

On the probe day, treated males swam further in the target zone relative to the opposite zone, DHTP  $\chi^2(1) = 5.037$ ,  $p = 0.0006873$ , EB  $\chi^2(1) = 5.4909$ ,  $p = 0.01912$ , (Table 6), indicating enhanced spatial memory. The time spent in the target zone and opposite zone reflected the same pattern (data not shown). However, interpretation of these observations is limited due to a lack of expected differences in control male and control female performance, which may be the result of high variation and low sample sizes.

**Table 6.** Distance swam in the target zone compared to the opposite zone (OPP). Likelihood ratio tests were performed to determine if distance swam in the target zone or opposite zone significantly differed. DHTP and EB males swam further in the target relative to the opposite zone on the probe day.

	<b>Target vs OPP</b>
<b>Control Females</b>	Target = OPP $\chi^2(1) = 3.0869$ , $p = 0.07892$
<b>EB Females</b>	Target = OPP $\chi^2(1) = 0.3493$ , $p = 0.5545$
<b>DHTP Females</b>	Target = OPP $\chi^2(1) = 0.1222$ , $p = 0.2853$
<b>Control Males</b>	Target = OPP $\chi^2(1) = 1.9216$ , $p = 0.1657$
<b>EB Males</b>	Target > OPP $\chi^2(1) = 5.4909$ , $p = 0.01912^*$
<b>DHTP Males</b>	Target > OPP $\chi^2(1) = 5.037$ , $p = 0.0006873^*$

## *Discussion*

This study provides evidence that supports the extreme male brain theory (Baron-Cohen 2002) by showing that testosterone metabolites, especially estradiol, alter behavior to phenocopy behavioral deficits observed in rat models of autism, and are consistent with behaviors common in humans with autism. We found that prenatal exposure to elevated testosterone metabolites induce autism-like reductions in typical social behavior, increases in abnormal stress response and hyperactivity, and enhancements in spatial learning. The developing male and female rat brain were found to be differentially susceptible to extreme levels of androgen and estrogen-dependent signaling, providing a potential explanation for the observed sex bias of autism in our human population.

Males exposed to high levels of estrogen exhibited an abnormal stress response, increased hyperactivity, reduced interest in social novelty, and enhanced spatial memory, suggesting that the embryonic male brain is more broadly affected by exposure to estrogens than the developing female brain. ASD is more common in males and may present symptoms more often than females because developmental exposure to estrogens can modulate more areas of the male brain. Indeed, females are thought to have evolved mechanisms that protect their brains from the masculinizing effects of estrogens (Bakker et al. 2006). Taken together, these results provide compelling evidence in support of the extreme male brain theory. Developmental exposure to androgen metabolites, especially estrogen, contributes to the development of autism-like behaviors.

Endocrine disrupting chemicals (EDCs) are ubiquitous environmental contaminants (Sonne et al. 2012) that structurally and functionally mimic endogenous hormones, and can exert agonistic or antagonistic effects. Ninety-seven percent of mothers tested for contaminants in

urine tested positive for biphenyl A (BPA), a common estrogenic chemical in plastics (Braun et al. 2011). Like BPA, a large portion of EDCs that humans are exposed to are estrogenic. Thus, daily exposure to common estrogenic compounds could alter the maternal-fetal endocrine system and result in aberrant estrogen signaling. Indeed, maternal exposure to EDCs like pesticides (Eskenazi et al. 2007), air pollution (Windham et al. 2006), and plastics (Braun et al. 2011) has been associated with an increased risk for ASD in their children (for reviews, see Braun, 2012; Braun et al. 2014; De Cock et al. 2012; Gore et al. 2014). Our results provide evidence for the extreme male brain theory of autism and suggests that exposure to environmental endocrine disruptors, especially estrogenic compounds, contribute to the development of autism. Future work must explicitly evaluate the link between maternal exposure to EDCs and ASD-like behavior in offspring using environmentally relevant concentrations and mixtures.

Human children with autism often have difficulty engaging in successful reciprocal playful social interactions with peers (Atlas & Lapidus 1987). Consistent with previous research in rat models of autism (Pletnikov et al. 2002; Schneider & Przewłocki 2005; Wolterink et al. 2001), we observed decreased play behavior in animals prenatally exposed to elevated testosterone metabolites. Females embryonically exposed to DHTP experienced consistent reductions in play behaviors, which suggests specific brain regions controlling play (e.g. the amygdala) were altered. Consistent with our findings, a recent related study reported that female offspring of pregnant rats exposed to letrozole, an aromatase inhibitor, throughout gestation had reduced social interactions and impaired ultrasonic vocalizations, another behavioral endpoint indicating ASD-like symptoms (Xu et al. 2015). The mechanism of inducing a hyperandrogenic prenatal environment differed between this study and ours, which shows that female rats are consistently behaviorally impaired by elevated developmental androgens in an ASD-like manner.

Taken together, these concordant results provide compelling evidence for the extreme male brain theory of autism and suggest that females are particularly sensitive to the testosterone metabolite, DHT.

Developing females are known to be sensitive to embryonic androgens, as developing between two male siblings can masculinize reproductive organs and behavior (Clark & Galef 1998; Ryan & Vandenberg 2002). Normally during development females have low levels of circulating androgens but must sequester excess estradiol via alpha-fetoprotein, which has a low binding affinity for androgens (Bakker et al. 2006). It is conceivable that DHTP affected organization of females more because females have not evolved strong mechanisms to control high quantities of androgens. DHTP females did not exhibit behavioral abnormalities in other behavioral assays in this experiment, which suggests that the effects of androgens are relatively specific in females or that females have stronger compensatory mechanisms that correct abnormal programming.

Humans with autism prefer repetitive routines and familiar people and places, and can have autism-related episodes of abnormal behavioral responses to novelty (Frith & Happé 2005; Kanner 1943), and atypical reciprocal social interactions are common (APA, 2013). Consistent with these characteristics of autism, males prenatally overexposed to estrogen, a testosterone metabolite, exhibited abnormal behavior and hyperactivity in the novel open field activity and displayed an ASD-like reduced interest in social novelty. Because estrogen plays an integral role in sexual differentiation of the male brain (McCarthy 2008), males likely lack a protective mechanism to prevent estrogens from reaching the brain (Bakker & Baum 2008). Indeed, extreme levels of estrogens could program a hypermasculinized brain and autistic phenotype through estrogen-mediated regulation of developing neurons, promoting aberrant growth in

certain regions while inducing widespread cell death due to uncontrolled excitotoxicity in others (McCarthy 2008).

There is evidence to suggest that humans with autism have enhanced spatial abilities (Caron et al. 2004), and this is also true for rats induced to have an ASD-like phenotype (Edalatmanesh et al. 2013). Over repeated learning trials, DHTP treated animals located the escape platform more quickly, suggesting prenatal DHTP improves spatial learning. When spatial memory was tested on the probe day, EB and DHTP males spent more time in and swam a further distance within the target zone than the opposite zone, suggesting enhanced spatial memory, which is indicative of a hypermasculinized brain (Gurzu et al. 2008). Importantly, enhanced spatial memory was observed in EB treated males that exhibited other key aspects of ASD-like behaviors, including abnormal stress response and asocial behavior. Although humans with autism suffer from behavioral impairments, it is common for individuals to be gifted in certain abilities (Assouline, Nicpon, & Doobay 2009; Neihart 2000). Our findings demonstrate how aberrant estrogen signaling could damage certain functions of the brain while enhancing others.

In this study, we demonstrate in rats that elevated prenatal levels of two testosterone metabolites impacted social behaviors, stress response, and spatial ability consistent with rat models of autism and in ways that parallel aspects of human autism spectrum disorders. Importantly, our findings are consistent with predictions of the extreme male brain theory of autism, and thus provide further evidence that high levels of prenatal testosterone metabolites are likely involved in the etiology of autism spectrum disorder. This has important implications for human health, as increasing evidence suggests ASDs can be modulated by environmental factors that affect the maternal-fetal endocrine system.

## CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The fetal environment plays a profound role in the organization of the brain and is sensitive to relatively minor changes in hormone levels (Vandenbergh 2003). In fact, a large portion of brain development occurs *in utero* and it is thought that many NDDs including ASDs originate during early brain development (Bale et al. 2010; Connors et al. 2008). There is mounting evidence from human and animal studies suggesting that aberrant neonatal hormone signaling is involved in the etiology of neurodevelopmental disorders, including autism. The extreme male brain theory suggests that high levels of androgens program a hypermasculinized brain in developing fetuses, which later presents as a suite of abnormal behaviors characteristic of autism (Baron-Cohen 2002).

My research supports this hypermasculinized brain hypothesis and shows that prenatal exposure to two metabolites of testosterone (dihydrotestosterone and estradiol) can induce a range of behaviors that are consistent with ASDs. Estradiol induced the highest number of effects, which were more common in males. Indeed, male humans are diagnosed with ASDs more often. Here we report that alterations in androgen and estrogen concentrations lead to behavioral alterations consistent with those seen in rat models of autism and humans with ASD, including reduced juvenile play (Pletnikov et al. 2002; Schneider & Przewlocki 2005; Wolterink et al. 2001), abnormal stress response (Narita et al. 2010; Schneider & Przewlocki 2005), reduced interest in social novelty (Bambini-Junior et al. 2011; MacFabe et al. 2011), and enhanced spatial ability (Edalatmanesh et al. 2013). Juvenile play behavior was reduced in females treated prenatally with DHTP, while abnormal stress responses and reduced interest in

social novelty presented in males treated prenatally with EB. Both EB males and DHTP males exhibited enhanced spatial ability.

### *Prenatal Exposure to EDCs and Implications for Autism*

The finding that fetal hormone exposure induces autism-like behavior in the rat has important implications for human health because there are many environmental chemicals that induce endocrine disruption. Many of these endocrine disrupting chemicals (EDCs) mimic estrogens and are common in plastics (Le et al. 2008), paints, flame retardants (Gosavi et al. 2013), and products used in food processing such as pesticides (Soto, Chung, & Sonnenschein 1994). Estrogenic chemicals are so common that they can be detected in nearly all people tested. For example, in 2005 the CDC found that 90% of the 2400 individuals studied had mixtures of pesticides in their systems (CDC 2005), and in the same year another study found that 95% of people studied had detectable levels of bisphenol A (BPA), a known estrogenic chemical in many plastics (Calafat et al. 2005). More importantly, however, these chemicals are detected in amniotic fluid (Foster et al. 2000), umbilical cord blood (Barr et al. 2010), and breast milk (Stefanidou, Maravelias, & Spiliopoulou 2009), meaning that fetuses likely are exposed to estrogenic chemicals during critically important times of neuronal development.

Another important and common class of EDCs are classified as anti-androgens because they block the normal function of testosterone or dihydrotestosterone. Anti-androgens can block the androgen receptor (Sohoni & Sumpter 1998). I have shown that increased androgens can decrease female rat play behavior, so it may seem counter-intuitive that environmental anti-androgens could influence or induce autism. However, blocking the androgen receptor can lead to increases in androgen concentration due to reduced negative feedback. If elevated androgens can influence molecular signaling independent of androgen receptor binding, then it is possible



that consequences of extreme concentrations of androgens could be induced by exposure to anti-androgens.

Here we demonstrate that overexposure to testosterone metabolites during development results in autism-like behaviors, especially in male rats. These results are consistent with the observation in humans that autism is male-biased, and support the extreme male brain theory for subsets of patients with of autism. Humans are exposed to endocrine disrupting chemicals on a daily basis, many of which are estrogenic. Thus, maternal exposure to commonly occurring estrogenic EDCs may in fact be contributing to the increasing prevalence of autism in our population.

Indeed, evidence from human epidemiological studies has implicated EDCs as risk factors for autism. Maternal exposure to air pollution (Windham et al. 2006), pesticides (Bouchard et al. 2011; Braun et al. 2014; Eskenazi et al. 2007; Roberts et al. 2007), and chemicals found in plastics such as biphenyl A (BPA) (Braun et al. 2011), polychlorinated bipheyls (PCBs) (Braun et al. 2014; Darvill et al. 2000; Jacobson & Jacobson 1996; Koopman- Esseboom et al. 1996; Lai et al. 2001) and phthalates (Kim et al. 2011; Miodovnik et al. 2011) have all been associated with increased risk for autism or autistic behaviors in their children. Importantly, many of these chemicals are known to be estrogenic, including PCBs (Bitman & Cecil 1970), phthalates (Harris et al. 1997), and BPA (Krishnan et al. 1993).

The research presented here extends our knowledge of the adverse neurodevelopmental effects of estrogens and implicates estrogenic endocrine disruption in the development of disorders like autism. Given the substantial evidence from human epidemiological studies, research efforts should be directed towards characterizing the neurodevelopmental consequences of prenatal overexposure to estrogenic compounds.

### *Future Work*

Our findings underline the complex interactions between sex and hormones, and suggest that the fetal neuroendocrine environment can have brain region specific effects. For example, DHTP treated females exhibited depressed levels of juvenile social play behavior, but were indistinguishable from control females in the open field test and social approach test. This suggests that the brain regions controlling juvenile play behavior are sensitive in female rats to high levels of androgen-dependent signaling during the developmental window of exposure used in our study. During the experiment, neonatal brains of culled littermates were preserved and can be used for making comparisons in structures important for play behavior. Future work can test the hypothesis that changes to brain regions important for play behavior, like the amygdala, sexually dimorphic nucleus of the pre-optic area, and cortex, among others, were affected in DHTP treated females.

Additionally, EB treated males displayed typical levels of play behavior, but had abnormal stress responses in the open field and reduced interest in social novelty during the social approach test. This suggests that brain regions controlling stress response and sociability are sensitive in males to elevated levels of estrogen-dependent signaling. The hypothalamus-pituitary-adrenal (HPA) axis is activated during stress, such as exposure to a novel environment. In this study, we observed movement in the open field test to measure abnormal stress responses. In addition to measuring behavior, stress responses can be measured at the physiological level. For example, the amount of corticosterone, a stress hormone released by the adrenals, can be measured in the blood or feces of test animals. Rat feces were collected and preserved after both the open field test and the social approach test, and corticosterone levels in these samples will be measured as a physiological marker of stress. This will allow for testing the hypothesis that

ASD-like individuals have elevated corticosterone levels due to exposure to a novel environment and exposure to social novelty.

Spatial learning is a hippocampal dependent learning ability, and previous work suggests that spatial ability is enhanced in people with autism (Caron et al. 2004), a finding that has been supported in the rodent literature as well (Edalatmanesh et al. 2013). Here we showed that EB and DHTP males exhibited improved spatial memory. EB males exhibited ASD-like behaviors in other behavioral assays, further supporting the hypothesis that exposure to estrogens during fetal development induced ASD-like behavior. This finding also supports the hypothesis that enhanced spatial abilities of these males is due to structural changes in the hippocampus induced by estrogen during development. The hypothesis that prenatal estrogen exposure results in morphological changes in the hippocampus associated with improved spatial abilities (e.g. increased cell density of CA1 and CA3 pyramidal cells, Isgor & Sengelaub 1998) can be tested by comparing adult rat brains obtained from animals used in this study. Additionally, hippocampus size and cell density can be compared in the brains of neonates; unlike the amygdala, which may not maintain size differences over time, the hippocampus remains the same relative size with age in autistic humans (Schumann et al. 2004). We can verify that these brain structure patterns are consistent in rodents by comparing regions like the amygdala and hippocampus in neonatal and adult brains.

#### *Final Remarks*

The work presented in this thesis explicitly tests the extreme male brain theory of autism in a well-accepted model organism that is known to exhibit ASD-like behavior. This work makes important contributions to understanding the mechanisms through which elevated prenatal testosterone affect the developing brain. For the first time, we present data suggesting that

testosterone metabolites that operate through distinctly different signaling pathways (androgen dependent and estrogen dependent) impact the developing male and female brain in unique ways, resulting in altered behavior consistent with predictions of the extreme male brain theory. This work takes an important first step towards making important translations from rodent models to human health. Elevated prenatal androgens are associated with autism, and our work suggests that males are particularly sensitive to estrogen, an important metabolite of testosterone. The results of this experiment highlight the effects of perturbations in prenatal estrogen signaling, which has important implications for maternal exposure to estrogenic endocrine disrupting chemicals. The relationship between prenatal estrogen signaling and later behavioral outcomes was shown to be important in autism-like behaviors, and should be the focus of future research.

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APPENDIX A: IACUC APPROVAL



Animal Care and  
Use Committee

212 Ed Warren Life  
Sciences Building

East Carolina University  
Greenville, NC 27834

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

February 19, 2014

Krista McCoy, Ph.D.  
Department of Biology  
Howell Science Complex  
East Carolina University

Dear Dr. McCoy:

Your Animal Use Protocol entitled, "Do Prenatal Sex Hormones Influence Development of Autism-Like Behavior?" (AUP #D300) was reviewed by this institution's Animal Care and Use Committee on 2/19/14. The following action was taken by the Committee:

"Approved as submitted"

**\*Please contact Dale Aycock at 744-2997 prior to hazard use\***

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

Sincerely yours,

A handwritten signature in black ink that reads 'S. B. McRae'.

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

SM/jd

Enclosure

**EAST CAROLINA UNIVERSITY  
ANIMAL USE PROTOCOL (AUP) FORM  
LATEST REVISION NOVEMBER, 2013**

**Project Title:**

Do prenatal sex hormones influence development of autism-like behavior?

	Principal Investigator	Secondary Contact
<b>Name</b>	Krista A. McCoy	Bevin Blake
<b>Dept.</b>	Biology	Biology
<b>Office Ph #</b>	252-737-2730	Click here to enter text.
<b>Cell Ph #</b>	571-315-2884	440-465-7246
<b>Pager #</b>	Click here to enter text.	Click here to enter text.
<b>Home Ph #</b>	252-565-5780	440-465-7246
<b>Email</b>	mccoyk@ecu.edu	blakeb13@students.ecu.edu

**For IACUC Use Only**

AUP #	D300			
New/Renewal	New 11/31/14			
Full Review/Date		DR/Date		
Approval Date	2/19/14			
Study Type	hormones			
Pain/Distress Category	D			
Surgery		Survival	Multiple	
Prolonged Restraint				
Food/Fluid Regulation				
Other				
Hazard Approval/Dates		Rad	IBC	EHS 2/19/14
OHP Enrollment				progesterone,
Mandatory Training				testosterone,
Amendments Approved				estradiol

*Regulate +  
Howell Science*

*single housing - temporary*



**I. Personnel**

**A. Principal Investigator(s):**

Krista McCoy

**B. Department(s):**

Biology

**C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:**

Name/Degree/Certification	Position/Role(s)/Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Dr. Krista McCoy, Ph.D.	PI	Yes	~20 years of experience working with fresh and saltwater fish, amphibians, reptiles, birds and rodents. I have taken the AALAS learning module for <i>Xenopus laevis</i> as well as Introduction to Mice and Working With the Laboratory Mouse. I have also attended a rodent handling course through University of Florida and been trained by their vet staff to conduct cervical dislocations. In addition to my animal training, I also have formal hazardous waste training.
Bevin Blake	Graduate student	Yes	Bevin is new to animal research, but has taken ECU's rodent handling class and the AALAS learning modules: Introduction to Mice and Working With the Laboratory Mouse. She will

			be trained on additional specific procedures by K. McCoy
Ciro Amato	Graduate student	Yes	Two years of research experience working with marine wildlife. One year of experience handling dogs, cats, birds and mice in a veterinary setting. Ciro has taken ECU's rodent handling class and survival surgery class. In addition, he has taken the AALAS learning modules: Introduction to Mice and Working With the Laboratory mouse.
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.
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## **II. Regulatory Compliance**

### **A. Non-Technical Summary**

**Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)**

***Do not cut and paste the grant abstract.***

Prenatal hormone signaling has powerful and long lasting effects on brain organization. There is evidence that excessive androgen signaling during critical periods of fetal development might contribute to the development of neurodevelopmental disorders such as autism spectrum disorder (ASD). Increased levels of prenatal testosterone (T) are associated with autism and autism-related traits. The extreme male brain (EMB) theory of autism states that autism is the result of abnormal brain development due to high levels of T, which results in exceedingly male-directed brain development.

Juvenile play behavior is important for normal cognitive and social development. Children with autism are unable to successfully engage in social play with peers. In fact, this disruption in normal play patterns can feed back on autism and exacerbate the symptoms. Like ASD, prenatal exposure to endocrine disrupting chemicals (EDCs) can disrupt normal social play in both rodents and humans. Play behaviors are also known to be programmed pre- and neonatally in rodents by the actions of hormone receptors. Thus, the prenatal programming for ASD and play behaviors may be linked.

We propose an integrative approach to test the EMB theory through investigating the link between prenatal hormone receptor signaling and its effects on juvenile play behavior, autistic-like behavior, and masculinized behavior (spatial learning and reproductive behavior). This work will improve our understanding of the mechanisms underlying the effects of prenatal hormone receptor signaling on brain development and subsequent changes in social behavior that are indicative of increased risk for ASD.

Rats are a useful model for understanding how the prenatal environment influences behavior at different periods in life. They are a particularly relevant model organism for understanding autism; the rich repertoire of social behaviors makes it possible to use rat models to study neurodevelopmental disorders characterized by social deficits. Furthermore, behaviors we wish to assess (including social play, spatial learning, and reproductive behaviors) have been well studied in this model organism.

Pregnant dams will be given subcutaneous injections of estradiol,  $5\alpha$  dihydrotestosterone, or a control on gestational days 14-19. After birth, litters will be culled to two males and two females. On post natal day (PND) 21, pups will be weaned and play behavior observations will begin. Play behavior testing will be conducted in order to assess whether or not the treatment conditions had an effect on juvenile play. This behavioral test will consist of pairing same sex littermates together in a neutral play box and recording their behaviors. Pups will be returned to their home cage and cared for until the next behavioral test (~35 days later).

On PND 55, one female and one male pup will be randomly assigned to the sociability and social approach test and the remaining female and male will be assigned to the open field test. The sociability and social approach test will assess whether or not the treatment condition altered the animal's social behavior. The sociability and social approach test will consist of placing the test animal in the testing apparatus (for which is has been habituated), which will contain a stranger animal in a smaller cage and an empty cage. The test animal's behavior and time spent investigating the stranger animal versus the empty cage will be recorded. Normal individuals are expected to be curious and inspect the new animal. The open field test will be used to assess the animals' reaction to a sudden change in environment. The open field test will consist of placing the test animal in the open field arena and recording their behavior. Rats will be returned to their home cage and cared for until the next behavioral test (~50 days later).

On PND 110, reproductive behavior and water maze tests will begin. The reproductive behavior test will assess whether embryonic hormone treatment altered reproductive behavior. The reproductive behavior test will consist of independently pairing male and female test individuals with a sexually primed female and observing behavior. The water maze test will assess whether spatial learning was altered by the treatment condition. The water maze test will consist of placing the test animal in a pool of water and recording their trajectory and swimming time as the animal navigates towards the hidden platform. Animals will be trained to learn where the platform is by using visual cues on prior to testing.

## **B. Ethics and Animal Use**

### **B.1. Duplication**

**Does this study duplicate existing research?** No

**If yes, why is it necessary? (note: teaching by definition is duplicative)**

[Click here to enter text.](#)

### **B.2. Alternatives to the Use of Live Animals**

**Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here?** No

**If yes, please explain why you cannot use these alternatives.**

[Click here to enter text.](#)

### **B.3. Consideration of Alternatives to Painful/Distressful Procedures**

**a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.**

**1. Please list all of the potentially painful or distressful procedures in the protocol:**

Dams will be injected subcutaneously once per day for five days (on embryonic days 14.5-19.5). Subcutaneous injections are rarely painful (Wolfensohn and Lloyd, 1994). However, after injections, females will be presented with wet (basic) chow in an effort to reduce stress.

The hormone treatments are expected to alter the dams' sex hormone concentrations but should not make the females ill.

Offspring will complete the Morris Water Maze test. Our animals might experience temporary distress during the test, which will require swimming in a pool of warmed water to navigate towards a platform. If an animal takes longer than 90 seconds to complete the task, they will be guided by an observer to the platform. Animals will have sufficient rest time in between swimming trials. Animals completing the water maze will be carefully monitored by an observer and removed from the water if any signs of distress or potential drowning occurs. Animals will be gently dried with a towel after each trial.

**2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):**

<b>Date Search was performed:</b>	1/16/13	Click here to enter text.
<b>Database(s) searched:</b>	Google Scholar	Click here to enter text.
<b>Time period covered by the search (i.e. 1975-2013):</b>	All years	Click here to enter text.
<b>Search strategy (including scientifically relevant terminology):</b>	Alternatives to subcutaneous injections	Click here to enter text.
<b>Other sources consulted:</b>	Pubmed.gov	Click here to enter text.

**3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers**

to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

We have read the following sources to help us address the three R's (Replacement, Reduction, and Refinement): <http://www.nc3rs.org.uk/page.asp?id=7>, <http://www.nal.usda.gov/awic/pubs/enrich/rodents.htm>, <http://or.ucsf.edu/larc/10666-DSY.html>

Refinement: Subcutaneous injection is the least stressful method that we are aware of to provide our treatments in a controlled manner. Although protocols to train animals to eat dosed food items are available we cannot guarantee that our rats will eat the entire food item and if it is not completely consumed that dam would have to be sacrificed. We will also provide animals with enrichment items to help alleviate boredom. Refinement is further detailed below in the section on refinement techniques (III 3 b).

Replacement: Live animal research is necessary for this type of research because it includes evaluating whole animals affects and behavioral assays. These behavioral assays are integral to the study design and must be completed in order to test our research questions.

Reduction: We are confident that we will not need as many samples as our power analyses suggest. We will, therefore, conduct our experiment in two time blocks where the second block will be run only if needed to increase sample size. In addition, as another way to reduce future use of animals, when dams and offspring are euthanized, several organs of interest will be collected and utilized. Other organs may be affected by the treatment (brain, genitals, kidney, and liver) and will be collected, properly fixed and stored in an -80 freezer. They will be held for future examination and potential collaboration.

## C. Hazardous Agents

### 1. Protocol related hazards (chemical, biological, or radiological):

Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	Radiation	Click here to enter text.	Choose an item.
Ionizing radiation	Radiation	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	IBC	Click here to enter text.	Choose an item.

Toxins of biological origins (venoms, plant toxins, etc.)	IBC	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	IBC	Click here to enter text.	Choose an item.
Human tissues, cells, body fluids, cell lines	IBC	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	IBC	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	EH&S	Click here to enter text.	Choose an item.
Nanoparticles	EH&S	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	DCM	Click here to enter text.	Choose an item.
Other agents	EH&S	Estradiol, 5-alpha dihydrotestosterone estradiol benzoate, progesterone	Choose an item.

## 2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

No

## III. Animals and Housing

### A. Species and strains:

*Rattus norvegicus*, Sprague-Dawley rat

### B. Weight, sex and/or age:

Adult male and female rats, pregnant dams, and offspring. Offspring will be reared through sexual maturity.

### C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project
30 females 300 pups	5 male breeders 10 incidental females (see below)	345 animals

**2. Justify the species and number (use statistical justification when possible) of animals requested:**

Because our work is attempting to model effects of prenatal chemical signaling on human neurological development and behavior, rodents are among the “lowest” animals that we can use. Most fish, amphibians, birds, and reptiles do not display quantifiable play and social behaviors, so they do not function as suitable models for our research questions. Of the mammals, rodents are especially appropriate because previous research has phenotyped behaviors in rodents that are relevant to ASD (i.e. repetitive grooming, reduced sociability).

**Statistical justification:** We will replicate each treatment in each experiment at least 5 times and up to 10 times. Our experiment will be conducted in two time blocks with a sample size of five in each block. A sample size of ten will provide at least a statistical power of 0.85, anticipated effect size of 1.25, with a probability level of 0.05.

Further details of the animal numbers being requested are detailed below in section IV A.

**3. Justify the number and use of any additional animals needed for this study:**

[Click here to enter text.](#)

**a. For unforeseen outcomes/complications:**

There is a small probability that certain females could have small litters or litters that are sexually biased. If this happens our design will require that we treat another pregnant dam so that we have the appropriate sex ratio for our study. Additional females have been included in the table above (Ill 1) to account for this issue.

**b. For refining techniques:**

**Refinement:** Because rats are social animals females will be housed together until the day of first injection (14.5). After treatment begins females will be housed with a female of the same treatment or singly in anticipation of the birth of her pups. Females will not be housed singly for more than 7 days. To reduce injection stress, moist basic chow will be given to the rats as a “reward” to help alleviate any mental or physical stress. In the event that any rat is showing signs of distress that cannot be ameliorated with moistened chow diet we will ask for a veterinarian consultation. If an animal requires euthanasia it will be euthanized with isoflurane and decapitation will ensure death.



**c. For breeding situations, briefly justify breeding configurations and offspring expected:**

Two sexually mature females and one male will be housed together, individuals will not exceed 400g in weight. If a male exceeds 400g, only one female will be housed with him. Vaginal plugs will be checked every morning. When a plug is encountered the female will be considered pregnant, and the embryos will be given the designation of embryonic day (E) 0.5. Pregnant females will be removed, and a new breeder female will be added to the male's cage. This breeding configuration will allow us to obtain the correct number of pregnant dams per treatment.

Females will be housed singly with their young. It is expected that each female yields a litter with at least four males and four females. However, some of the genitalia and anogenital distances will be ambiguous due to the prenatal hormone treatment. Therefore, pups will be ear punched to obtain tissue samples in order to assign genetic sex to each individual. Once genetic sex is determined, and at weaning, litters will be culled to two females and two males. Tissues from culled offspring will be used for physiological/mechanistic work. This design will allow us to study both the behavior and physiology of animals of the same treatment, thus reducing the number of dams required and increasing our productivity from this experiment. The remaining four offspring will be weaned and housed together based on sex.

**d. Indicate if following IACUC tail snip guidelines: N/A  
(if no, describe and justify)**

[Click here to enter text.](#)

**4. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? No (if yes, describe)**

[Click here to enter text.](#)

**5. Are there any deviations from standard husbandry practices?**

**YES If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**

- 1) No animals may receive any food other than the standard rat diet. Rationale: it is unknown how supplemental foods may impact developmental and behavioral parameters.
- 2) Personnel from K. McCoy's laboratory will be responsible for all injection procedures. Rationale: this requires more time and effort than we feel is reasonable to ask of animal care personnel.
- 3) Personnel from K. McCoy's laboratory will pair breeder animals and monitor females for mucous plugs and signs of pregnancy

**6. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study?**

**Yes (If yes, describe housing and justify the need to singly house social species):**

Stud males will be housed in groups of two three according to weight. Cages will be monitored for aggression and animals will be separated if necessary. Pregnant females will always be paired with other pregnant females until the first injection (on embryonic day 14.5). We cannot co-house females from different treatments because feces and urine from one treatment might contaminate the cage and therefore the animal of the other treatment (e.g., DHT to control). In most cases, females will be able to be housed with another co-treated female until just prior to birth. Just before birth, females will be housed singly to give birth.

As a standard protocol, pups will be housed individually for a brief period of social isolation (24 hours) prior to play behavior observations in order to increase the likelihood of play behaviors and other relevant social interactions. This is a standard method employed when studying play behavior in rodents and is not known to have significant adverse effects.

**7. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? Yes**

**(If yes, describe and justify the need to withhold enrichment)**

We cannot use plastic enrichment items because they can be made of endocrine active materials, but alternate enrichment will be acceptable. Rats will be given enrichment activities including enviro-dri and wooden chew blocks to improve living conditions and reduce stress. To provide stimulation several dry food items will be scattered in the bedding.

**8. If wild animals will be captured or used, provide permissions (collection permit # or other required information):**

N/A

**9. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.**

Ragsdale Annex and S112A Howell Science Center

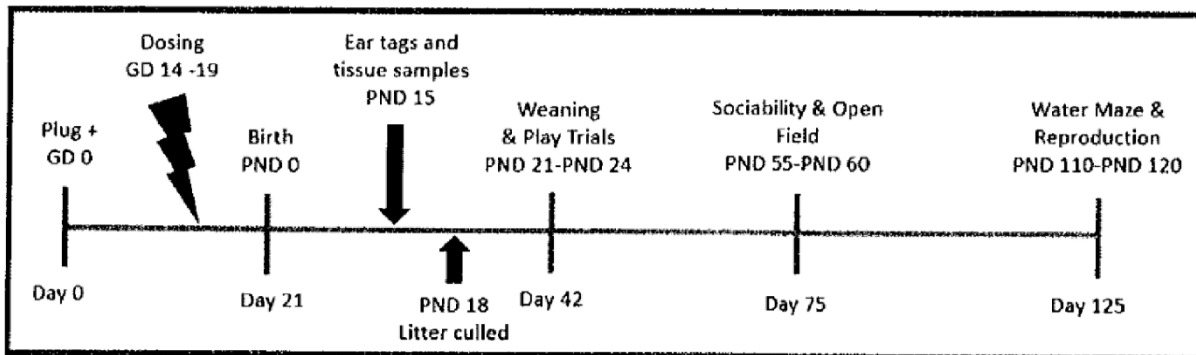
#### **IV. Animal Procedures**

**A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.**

We will test the extreme male brain theory of autism in order to achieve a better understanding of how the prenatal hormone environment can contribute neurodevelopmental disorders.

There is one experiment planned that will follow three treatment groups from conception to adulthood and measure behavior at three different points in time. Females will be time mated, and the observation of vaginal plugs will be recorded as gestational day (GD) 0.5. Pregnant dams will be randomly assigned to three treatment groups (max n=10/treatment) and subcutaneously injected with a vehicle control, DHT, or estradiol. Injections will be given on GD 14-19 (Figure 1).

Prior to the day of birth, females will be housed singly. Between days 10 and 15 Pups will be ear punched for identification purposes and the tissue samples will be collected for genotyping. The tissue sample is necessary in order to determine the genetic sex of each individual via PCR. At weaning, litters will be culled to two females and two males. Culled pups will be deeply anesthetized and blood will be collected via cardiac puncture. After blood collection they will be humanely euthanized (see below), perfused with fixative, and tissues of interest will be preserved.



**Figure 1.** Experiment timeline

On PND 21, pups will be weaned and all pups will be housed in same sex groups. Details of behavioral studies are outlined in the appropriate sections below. Briefly, same sex pairs will be habituated to a readily sanitizable (glass) play arena/box over a three-day period and then will be housed singly for 24 hours. After this 24-hour separation play-pairs will be reunited in the play arena and behavior will be recorded in order to assess social play.

On PND 55-60, sociability and open field behavioral tests will be run (detailed below). After these trials, rats will be returned to their same sex sibling cages until the next behavioral tests. On PND 110-120, the Morris water maze and reproductive behavior testing will be conducted. After these behaviors are recorded individuals will be deeply anesthetized and blood will be collected via cardiac puncture. Then animals will be euthanized and perfused with fixative (see below).

***In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.***

***Please refer to DCM and IACUC websites for relevant guidelines and SOPs.***

**B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:**

***Adequate records describing anesthetic monitoring and recovery must be maintained for all species.***

**If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:**

[Click here to enter text.](#)

**1. Describe the pre-procedural preparation of the animals: N/A**

**a. Food restricted for** [Click here to enter text.](#) **hours**

**b. Food restriction is not recommended for rodents and rabbits and must be justified:**

[Click here to enter text.](#)

**c. Water restricted for** [Click here to enter text.](#) **hours**

**d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:**

[Click here to enter text.](#)

**2. Anesthesia/Analgesia for Procedures Other than Surgery**

	Agent	Concentration	Dose (mg/kg)	Max Volume	Route	Frequency	Number of days administered

<b>Pre-procedure analgesic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Pre-anesthetic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Anesthetic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Post procedure analgesic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Other</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

**3. Reason for administering agent(s):**

Click here to enter text.

**4. For which procedure(s):**

Click here to enter text.

**5. Methods for monitoring anesthetic depth:**

Click here to enter text.

**6. Methods of physiologic support during anesthesia and recovery:**

Click here to enter text.

**7. Duration of recovery:**

Click here to enter text.

**8. Frequency of recovering monitoring:**

Click here to enter text.

**9. Specifically what will be monitored?**

Click here to enter text.

**10. When will animals be returned to their home environment?**

Click here to enter text.

**11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:**

Click here to enter text.

**C. Use of Paralytics**

**1. Will paralyzing drugs be used?** Choose an item

**2. For what purpose:**

Click here to enter text.

**3. Please provide scientific justification for paralytic use:**

Click here to enter text.

**4. Paralytic drug:**

Click here to enter text.

**5. Dose:**

Click here to enter text.

**6. Method of ensuring appropriate analgesia during paralysis:**

Click here to enter text.

#### **D. Blood or Body Fluid Collection**

**1. Please fill out appropriate sections of the chart below:**

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
<b>Blood Collection</b>	cardiac	21gauge	Max 15mL	one	Terminal at sacing
<b>Body Fluid Collection</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Other</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

#### **E. Injections, Gavage, & Other Substance Administration**

**1. Please fill out appropriate sections of the chart below:**

	Compound	Location & Route of admin	Needle/catheter /gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
<b>Injection/ Infusion</b>	Estradiol, dihydrotestosterone progesterone	Subcutaneous injection into the scruff (the loose skin over the interscapular area)	22 G X ½ in.	1.0 ml	Once per day	Max of 5 days	500 ug/kg, 5mg/kg, 200 ug/kg
<b>Gavage</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
-------	---------------------------	---------------------------	---------------------------	---------------------------	---------------------------	---------------------------	---------------------------

- 3. Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:**
- a. Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.
  - b. Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).

All hormones injected will be pharmaceutical grade.

#### **F. Prolonged restraint with mechanical devices**

**Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.**

**1. For what procedure(s):**

Click here to enter text.

**2. Explain why non-restraint alternatives cannot be utilized:**

Click here to enter text.

**3. Restraint device(s):**

Click here to enter text.

**4. Duration of restraint:**

Click here to enter text.

**5. Frequency of observations during restraint/person responsible:**

Click here to enter text.

**6. Frequency and total number of restraints:**

Click here to enter text.

**7. Conditioning procedures:**

Click here to enter text.

**8. Steps to assure comfort and well-being:**

Click here to enter text.

**9. Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

**G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.**

**1. Describe methodology:**

Subcutaneous administrations are made into the loose skin over the interscapular or inguinal area. Subcutaneous injections will be delivered by doing the following:

1. The animal will be weighed and the appropriate dosing volume will be determined.
2. The animal will be restrained. To restrain the rat we will: scruff the rat, grasping the skin over the shoulders with the thumb and forefinger. The animal will then be placed on a clean towel or sterile surface.
3. The needle will be inserted under the skin of the interscapular area tented by the thumb and forefinger and the injection will be delivered.
4. Return the animal to the cage and provide moist chow.

**2. Expected model and/or clinical/pathological manifestations:**

Offspring of our treated dams are expected to develop some genital abnormalities (females in the DHT treatment will have masculinized genitalia). Offspring of treated dams are



expected to develop some behavior abnormalities (reduced sociability, reduced spatial learning, altered reproductive behavior). The exposed dams are not expected to show any adverse effects.

**3. Signs of pain/discomfort:**

The signs of pain and discomfort include: depression, anorexia, labored respiration, increased aggression, periorbital/nasal discharge, abnormal posture and immobility. If dams are injected correctly there should not be any signs of pain and discomfort from any of the exposure treatments. All animals, will be provided moist chow after each injection.

**4. Frequency of observations:**

Daily

**5. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

If any of the above signs of distress are seen in individuals a veterinarian will be consulted. If the animal needs to be euthanized, isoflurane will be used with decapitation to insure death.

## **H. Treadmills/Swimming/Forced Exercise**

**1. Describe aversive stimulus (if used):**

[Click here to enter text.](#)

**2. Conditioning:**

Animals will be trained to learn where the hidden platform is for the Morris water maze so that spatial learning can be assessed. No aversive stimuli will be used.

**3. Safeguards to protect animal:**

Animals will be gently guided to the platform by the observer if unable to find it. Animals will be removed from the water if swimming for more than 90 seconds.

**4. Duration:**

Animals will not be required to swim for more than 90 seconds.

**5. Frequency:**

Each animal will complete the Morris water maze one time. One water maze trial consists of 15 sub-trials. Including, three training trials (60 second each), and three experimental sub-trials (max 90 seconds) from each of the four cardinal directions (north, east, south, and west = 12).

**6. Total number of sessions:**

For the Morris water maze each animal will undergo 15 sub-trials with sufficient rest time in between each trial.

**7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

The animals may experience temporary stress due to the forced swimming task, but adverse effects are typically not expected during a water maze test. If an animal shows signs of excessive distress, they will be immediately removed from the water by the observer and will not be required to complete the test.

## **I. Projects Involving Food and Water Regulation or Dietary Manipulation**

*(Routine pre-surgical fasting not relevant for this section)*

### **1. Food Regulation**

**a. Amount regulated and rationale:**

[Click here to enter text.](#)

**b. Frequency and duration of regulation (hours for short term/weeks or months for long term):**

[Click here to enter text.](#)

**c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):**

[Click here to enter text.](#)

**d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

[Click here to enter text.](#)

### **2. Fluid Regulation**

**a. Amount regulated and rationale:**

[Click here to enter text.](#)

**b. Frequency and duration of regulation (hours for short term/weeks or months for long term):**

[Click here to enter text.](#)

**c. Frequency of observation/parameters documented (body weight, hydration status, etc.):**

[Click here to enter text.](#)

**d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

[Click here to enter text.](#)

### **3. Dietary Manipulations**

**a. Compound supplemented/deleted and amount:**

Click here to enter text.

**b. Frequency and duration (hours for short term/week or month for long term):**

Click here to enter text.

**c. Frequency of observation/parameters documented:**

Click here to enter text.

**d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

### **J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging**

**1. Describe animal methodology:**

Click here to enter text.

**2. Duration of procedure:**

Click here to enter text.

**3. Frequency of observations during procedure:**

Click here to enter text.

**4. Frequency/total number of procedures:**

Click here to enter text.

**5. Method of transport to/from procedure area:**

Click here to enter text.

**6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

**7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:**

Click here to enter text.

### **K. Polyclonal Antibody Production**

**1. Antigen/adjuvant used and justification for adjuvant choice:**

Click here to enter text.

**2. Needle size:**

Click here to enter text.

**3. Route of injection:**

Click here to enter text.

**4. Site of injection:**

Click here to enter text.

**5. Volume of injection:**

Click here to enter text.

**6. Total number of injection sites:**

Click here to enter text.

**7. Frequency and total number of boosts:**

Click here to enter text.

**8. What will be done to minimize pain/distress:**

Click here to enter text.

**9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

**L. Monoclonal Antibody Production**

**1. Describe methodology:**

Click here to enter text.

**2. Is pristane used:** Choose an item.

**Volume of pristane:**

Click here to enter text.

**3. Will ascites be generated:** Choose an item.

**i. Criteria/signs that will dictate ascites harvest:**

Click here to enter text.

**ii. Size of needle for taps:**

Click here to enter text.

**iii. Total number of taps:**

Click here to enter text.

**iv. How will animals be monitored/cared for following taps:**

Click here to enter text.

**4. What will be done to minimize pain/distress:**

Click here to enter text.

**5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

## **M. Temperature/Light/Environmental Manipulations**

### **1. Describe manipulation(s):**

Click here to enter text.

### **2. Duration:**

Click here to enter text.

### **3. Intensity:**

Click here to enter text.

### **4. Frequency:**

Click here to enter text.

### **5. Frequency of observations/parameters documented:**

Click here to enter text.

### **6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

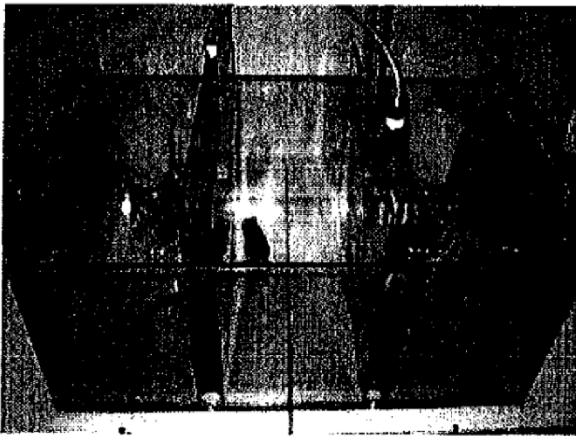
## **N. Behavioral Studies**

### **1. Describe methodology/test(s) used:**

**Social Play.** Social play will be tested using methods described by Himmler et. al (2013). A controlled environment that is designed to maximize the occurrence of play behavior will be used. Play behavior will be recorded in a “play box”—a large arena (36"L x 18"W x 16"H) with neutral bedding which will create a natural setting for playing. Pups will be weaned on PND 21 and separated into sex-specific cages with their sibling. Starting on PND 21, habituation to the play box will begin. On PND 21, play pairs will be placed in the play box for 30 minutes to allow habituation to occur. After 30 minutes of habituation, play pairs will be returned to their home cages. The play box will be emptied and sanitized using Quatricide PV-15. The next play pair will be placed inside and their first 30 minute habituation will begin. This process will be repeated again on PND 22 and PND 23. After the final habituation session on PND 23, play pairs will be housed singly for 24 hours. This isolation is a standard procedure, does not induce excessive stress, and is known to increase motivation for play behavior upon reuniting the play pair. On PND 24, each play partner will be marked on their dorsal side with a “+” or a “0” so that the trained observer can distinguish between individuals when reviewing the recording. Play pairs will be reunited in the play box on PND 24 and their behavior will be recorded for 30 minutes under red light. After the 30 minutes of recording are finished, play pairs will be returned together to a new cage and housed together until the next behavioral trial. The observer will use Jwatcher behavioral analysis program to code behaviors performed by each focal animal including non-aggressive play fighting, approach, chasing, sniffing and pouncing.

**Open field test-**The open field test will measure anxiety and hyperactivity, which will provide information regarding whether or not autism-like behavioral phenotypes are expressed. Open field behavior will be tested on PND 55-60. Each rat will be placed in the periphery of the open field and allowed to explore the apparatus for 20 minutes, with the experimenter out of the rat's sight. The behavior will be video recorded and analyzed using Jwatcher software. The distance travelled, the number of rears, and time spent in the central and peripheral regions will be recorded and measured per test session. The number of entries into the center, and resting time in each zone of the arena will also be recorded.

**Sociability and preference for social novelty-**At the same time (PND 55-60) but using different animals than used above in the open field test, sociability and preference for social novelty tests will be conducted to assess the presence or absence of autism-like behavioral phenotypes in the animals. A social behavior apparatus (36"L x 18"W x 16"H) with three sections will be used (illustrated by Figure 3; Moy et al. 2007). Procedures for the sociability and preference for social novelty will follow those described by Moy et al. (2007). The test rat will be placed in the middle chamber and allowed 10 minutes for exploration and habituation. The doors to the neighboring chambers will then be opened and each chamber will contain an empty wire cage. After 10 minutes, the test rat will be enclosed in the center compartment and an unfamiliar rat of the same sex (stranger 1) will be placed in the sectioned off side compartment of the testing apparatus. The location of the unfamiliar rat will be randomized to the left or right chamber per testing session. Once the unfamiliar rat is placed, the doors to the side compartments will be opened and the test rat will be



**Figure 4.** Social approach and sociability apparatus (Moy et al. 2007)

observed for another 10 minutes. All observations will be video recorded and analyzed to assess the amount of time the test rat spends with the unfamiliar rat or the empty chamber, as well as the number of entries into each side chamber. After the first 10 minutes pass, the test rat will again be temporarily isolated to the center chamber while a second unfamiliar of the same sex (stranger 2) rat is placed in the empty wire cage. The test rat will again be permitted to explore all areas of the testing apparatus. The test rat will be video recorded for another 10 minutes to assess how much time is spent investigating the now

familiar rat (stranger 1) versus the novel unfamiliar rat (stranger 2). The apparatus will be sanitized with Quatricide PV-15 after every trial.

**Home cage behavior**-In order to measure the extent to which autism-like behavioral phenotypes are expressed in the animals, home cage behavior will be monitored. Home cage behavior observations will be modeled after the methods described by Moy et al. (2007). Observations will take place at PND 61-64. Observations of same-treatment, same-sex siblings in their home cages will be made for 20 minutes. Nest shredding, nest building, sleeping in huddles, activity, non-aggressive play fighting, social approach and any aberrant behaviors will be measured. Female mounting behavior will be recorded as evidence for masculinized reproductive behavior.

**Water maze test**-The Morris water maze will be used to assess spatial learning abilities in the rat (Beatty 1984). Tests will be conducted between PND 110-120. A standard Morris water maze protocol will be followed to test the rats (Nunez 2008). The Morris water maze is a warmed pool of water that has a hidden, slightly submerged platform that the animals must navigate to using four visual cues that are located at each cardinal direction (north,



**Figure 5.** The Morris water maze (Moy et al 2007)

south, east and west). The animals will be pre-trained to learn where the hidden platform in the pool of water is. Each animal will undergo three 60 second training trials. If the animal is unable to find the platform, the observer will gently guide the animal to the platform using their hand. This will ensure that the animal learns where the platform is. After the learning trials are conducted for each animal, the testing trials will begin. Twelve testing trials will be completed per animal. This is to provide three data sets per starting point per individual. Each trial will consist of no longer than 90 seconds of swimming while the animal's trajectory is recorded by the video software. Animals will have opportunity to rest and dry off in between the 12 trials inside individual test boxes. Once the 12 trials are completed, animals will be dried off and returned to their cage.

The water in the maze is maintained at 25-27°C via a temperature-regulated pond heater. The water will be changed and the pool will be sanitized according to the number of days it

is used. If behavioral tests are run for 5 days, the pool will be cleaned after day 3. If behavioral tests are run for 10 days, the pool will be cleaned after day 5. After the pool is drained on the day of cleaning, it will be dried and sanitized with Quatricide PV-15, allowed to air dry again, rinsed with tap water then re-filled with tap water.

The test boxes in which the rats will be housed in between water maze trials will be sanitized after each rat completes all trials. The test boxes will be emptied of feces and sanitized with Quatricide PV-15 and then dried.

**Sexual reproduction**-Masculine sexual reproduction behaviors are programmed prenatally by the actions of estrogens and ER (K. McCoy, unpublished data). Measuring masculine reproductive behavior will indicate the extent to which ER programmed the brain prenatally. Reproductive behavior will be assessed between PND 110-120. Male and female rats will be paired with a sexually receptive (hormonally primed) female in his home cage. Behavior will be recorded for 20 minutes and a trained observer will score and quantify sexual behavior (e.g., mount latency, number of mounts, total time spend mounting).

Hormonal priming of non-exposed females will involve three injections. Two days before the female is paired with the male she will be injected with estradiol benzoate (10ug/kg). The following day she will be injected with another dose of estradiol benzoate (5ug/kg). The day of reproductive trials (four hours before the female is paired with male) she will be injected with progesterone (~10ug/kg).

## **2. Will conditioning occur? If so, describe:**

Conditioning will occur for the Morris water maze prior to testing trials. The animals will be pre-trained to learn where the hidden platform in the pool of water is by being introduced to the hidden platform by the observer. Each animal will undergo three 60 second training trials. If the animal is unable to find the platform, the observer will gently guide the animal to the platform using their hand. This will ensure that the animal learns where the platform is.

## **3. If aversive stimulus used, frequency, intensity and duration:**

N/A

## **4. Length of time in test apparatus/test situation: (i.e., each test is ~10 mins)**

Play trials: 30 minutes

Open field: 20 minutes

Water maze: 15 trials lasting no longer than 90 seconds each

Reproductive trials: 20 minutes

## **5. Frequency of testing and duration of study: (i.e., 5 tests/week for 6 months)**

Play trials: One test over one day

Open field: One test over one day

Water maze: One test consisting of fifteen trials over one day

Reproductive trials: One test over one day

## **6. Frequency of observation/monitoring during test:**



Play trials: Continuous monitoring  
Open field: Continuous monitoring  
Water maze: Continuous monitoring  
Reproductive trials: Continuous monitoring

**7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Play trials: There is no evidence that juveniles should be aggressive, however if aggression is observed the animals will be immediately separated by the observer.

Open field: Animals may experience mild stress associated with being exposed in the open field, but this stress should not have adverse effects.

Water maze: Animals will be monitored during all trials and removed from the water by the observer if they show signs of distress or struggling. There is a risk for hypothermia, but this risk is low as animals will not be placed in the water maze unless the water is within the range of 25-27°C .

Reproductive trials: Potential for aggressive interactions. If aggression is observed, animals will be separated by the observer. Aggression has never been observed by K. McCoy during similar mating trials.

**O. Capture with Mechanical Devices/Traps/Nets**

**1. Description of capture device/method:**

Click here to enter text.

**2. Maximum time animal will be in capture device:**

Click here to enter text.

**3. Frequency of checking capture device:**

Click here to enter text.

**4. Methods to ensure well-being of animals in capture device:**

Click here to enter text.

**5. Methods to avoid non-target species capture:**

Click here to enter text.

**6. Method of transport to laboratory/field station/processing site and duration of transport:**

Click here to enter text.

**7. Methods to ensure animal well-being during transport:**

Click here to enter text.

**8. Expected mortality rates:**

Click here to enter text.

**9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

[Click here to enter text.](#)

## **P. Manipulation of Wild-Caught Animals in the Field or Laboratory**

**1. Parameters to be measured/collected:**

[Click here to enter text.](#)

**2. Approximate time required for data collection per animal:**

[Click here to enter text.](#)

**3. Method of restraint for data collection:**

[Click here to enter text.](#)

**4. Methods to ensure animal well-being during processing:**

[Click here to enter text.](#)

**5. Disposition of animals post-processing:**

[Click here to enter text.](#)

**6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

[Click here to enter text.](#)

## **Q. Wildlife Telemetry/Other Marking Methods**

**1. Describe methodology (including description of device):**

[Click here to enter text.](#)

**2. Will telemetry device/tags/etc. be removed? Choose an item. If so, describe:**

[Click here to enter text.](#)

**3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

[Click here to enter text.](#)

## **R. Other Animal Manipulations**

**1. Describe methodology:**

[Click here to enter text.](#)

**2. Describe methods to ensure animal comfort and well-being:**

Click here to enter text.

**3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

## **S. Surgical Procedures**

***All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.***

**1. Location of Surgery (Building & Room #):**

Click here to enter text.

**2. Type of Surgery (check all that are appropriate):**

Click here to enter text.

**Non-survival surgery (animals euthanized without regaining consciousness)**

**Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**

**Minor survival surgery**

**Multiple survival surgery**

**If yes, provide scientific justification for multiple survival surgical procedures:**

Click here to enter text.

**3. Describe the pre-op preparation of the animals:**

**a. Food restricted for** Click here to enter text. **hours**

**b. Food restricted is not recommended for rodents and rabbits and must be justified:**

Click here to enter text.

**c. Water restricted for** Click here to enter text. **hours**

**d. Water restriction is not recommended in any species for routine pre-op prep and be justified:**

[Click here to enter text.](#)

**4. Minimal sterile techniques will include (check all that apply):**

*Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).*

**Sterile instruments**

**How will instruments be sterilized?**

[Click here to enter text.](#)

**If serial surgeries are done, how will instruments be sterilized between surgeries:**

[Click here to enter text.](#)

**Sterile gloves**

**Mask**

**Cap**

**Sterile gown**

**Sanitized operating area**

**Clipping or plucking of hair or feathers**

**Skin preparation with a sterilant such as betadine**

**Practices to maintain sterility of instruments during surgery**

**Non-survival (clean gloves, clean instruments, etc.)**

**5. Describe all surgical procedures:**

**a. Skin incision size and site on the animal:**

[Click here to enter text.](#)

**b. Describe surgery in detail (include size of implant if applicable):**

[Click here to enter text.](#)

**c. Method of wound closure:**

[Click here to enter text.](#)

**i. Number of layers**

[Click here to enter text.](#)

**ii. Type of wound closure and suture pattern:**

[Click here to enter text.](#)

**iii. Suture type/size/wound clips/tissue glue:**

Click here to enter text.

**iv. Plan for removing of skin sutures/wound clip/etc:**

Click here to enter text.

**6. Anesthetic Protocol:**

**a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:**

Click here to enter text.

**b. Anesthesia/Analgesia For Surgical Procedures**

	<b>Agent</b>	<b>Dose (mg/kg or %)</b>	<b>Volume</b>	<b>Route</b>	<b>Frequency</b>	<b>Number of days administered</b>
<b>Pre-operative analgesic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Pre-anesthetic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Anesthetic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Post-operative Analgesic</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
<b>Other</b>	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

**c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):**

Click here to enter text.

**d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):**

Click here to enter text.

**e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:**

Click here to enter text.

**f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):**

Click here to enter text.

**g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:**

Click here to enter text.

**7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)**

Click here to enter text.

**a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:**

Click here to enter text.

**b. How frequently (times per day) will animals be monitored:**

Click here to enter text.

**c. How long post-operatively (days) will animals be monitored:**

Click here to enter text.

**8. Surgical Manipulations Affecting Animals**

**a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:**

Click here to enter text.

**b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):**

Click here to enter text.

**c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

**V. Euthanasia**

***Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.***

***A. Euthanasia Procedure. All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.***

**1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO<sub>2</sub> or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:**

[Click here to enter text.](#)

**2. Inhalant Method**

**(if other, describe the agent and delivery method)**

[Click here to enter text.](#)

**3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)-**

**Please provide the following:**

**a. Agent:**

Rodent euthanasia mix or pentobarbital overdose solution

**b. Dose or concentration:**

390mg/ml, 100mg/kg IP

**c. Route:**

Rats will receive a left cardiac ventricular perfusion under a fume hood at Ragsdale Hall Room 9 (maintained by EH&S) within 10 days after water maze and sexual behavior assays. They will be euthanized with rodent euthanasia mix or pentobarbital overdose solution (390mg/ml, 100mg/kg IP) obtained from DCM.

**B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):**

Death (absence of reflexes, including corneal and palpebral reflexes, and glazing of eyes) will be confirmed. Bilateral incisions will be made to reveal the chest cavity and a left ventricular blood collection will be performed. Remaining blood will be flushed initially with saline (normally 0.9%), followed by brain tissue fixation with 4% paraformaldehyde.

**C. Describe disposition of carcass following euthanasia:**

Carcasses will be dissected, tissues preserved, and carcasses will be frozen and stored at -20C until they can be properly discarded.





<b>APPENDIX 1-HAZARDOUS AGENTS</b>			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730	Home Phone: 252-565-5780 Cell: 571-315-2884	
IACUC Protocol Number: PENDING	Department: Biology	E-Mail: mccoys@ecu.edu	
Secondary Contact: Bevin Blake Department: Biology	Campus Phone: 440-465- 7246	Home Phone:	E-Mail: Blakeb13@students.ecu.edu
Chemical Agents used: Estradiol, 5-alpha dihydrotestosterone		Radioisotopes used: None	
Biohazardous Agents used: None		Animal Biosafety Level: N/A	Infectious to humans? No
<b>PERSONAL PROTECTIVE EQUIPMENT REQUIRED:</b>			
Route of Excretion: Urine and feces			
Precautions for Handling Live or Dead Animals: Nitrile gloves			
Animal Disposal: Nitrile gloves			
Bedding/Waste Disposal: Nitrile gloves, lab coat, eye protection			
Cage Decontamination: Nitrile gloves, lab coat, eye protection			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment:			
<b>Initial Approval</b> Safety/Subject Matter Expert Signature & Date			
_____			

## Laboratory Safety Plan for Progesterone injections

*Alana Bagley*  
2-19-14

<b>*Process</b>	<u>Progesterone injections</u>
<b>*Hazardous Chemical/ Chemical Class</b>	Progesterone Hazards: Reproductive hazard, suspected carcinogen Target Organs: Reproductive (Male and Female), Liver, Nervous system
<b>*Hazardous Equipment</b>	Injection: Needle/Syringe
<b>*Potential Hazards</b>	Harmful by ingestion, inhalation and skin absorption. Considered a suspected carcinogen. May cause respiratory tract irritation. Possible reproductive hazard for men and women. Consult physician if pregnant or possibility of pregnancy. Chronic disorders may cause reproductive disorders.
<b>*Personal Protective Equipment</b>	Safety glasses/goggles; closed front or back lab coat, compatible gloves (nitrile), long pants and closed toed shoes.
<b>*Engineering and Ventilation Controls</b>	All handling and preparation should be completed in a certified chemical fume hood (Howell S112). Administration of injections will be in the biosafety cabinet in Ragsdale Annex. All extraneous equipment should be removed from the hood before work begins. All equipment required for dilutions will be placed in the hood prior to beginning work. All chemical containers can only be removed from fume hood if tightly capped and the exterior wet wiped. Before relocating to Ragsdale Annex ensure the chemical containers are securely closed and placed in an absorbent lined secondary container, preferably with a lid. When transporting to Ragsdale take the most direct route without interruptions. All sharps should be placed in a red biohazard sharps container. Do not recap sharps.
<b>Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins</b>	Prepare the stock solutions in the laboratory and store away from other chemical storage, preferably in a locked cabinet.
<b>Special Use Procedures</b>	Hands will be washed with a disinfectant soap after handling any chemical containers.
<b>Special Handling and Storage Requirements</b>	Keep container tightly closed in a dry, well-ventilated space. Store stock solutions separately from other chemicals, in a secure area. Store away from oxidizers. Keep away from ignition sources. Substance should be stored away from light. Avoid creating and breathing dust/aerosols from substance.
<b>*Spill and Accident Procedures</b>	Clean spills only if proper materials are available and if researcher is properly trained to do so. All other spills should be reported to EH&S for clean-up. Needed: absorbent materials, plastic bags, sealable container to hold contaminated cleanup materials, protective clothing, gloves, and safety glasses. For minor spills ventilate area. If spill occurs outside hood, cover liquid with absorbent material; and place in plastic bag. Spill materials should be picked up by EH&S Hazardous Waste Management.
<b>*Waste Minimization Plan</b>	Order only the amount needed.
<b>*Hazardous Waste Disposal</b>	Maintain all hazardous waste in closed containers with hazardous waste tag and contact EH&S for disposal. All sharps should be placed in a closed red sharps disposal container. Contact Prospective Health for pickups of biohazards and sharps.
<b>Decontamination Procedures</b>	Hood will be decontaminated, if necessary, by removing the paper liner and wiping the hood interior surfaces from top to bottom then back to front. (PPE as listed above must be worn during decontamination.)
<b>Animal Care Precautions</b>	Animal care workers should wear PPE including double gloves, lab coats and closed -toed shoes animal and waste removal.
<b>*Chemical Procurement</b>	Inventories kept by PI or laboratory manager. Minimum quantities necessary for the procedures confirmed by current funding.
<b>*Revision Date</b>	2-13-2014



APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730		Home Phone: 252-565-5780
IACUC Protocol Number: D300	Department: Biology		E-Mail: mccoyk@ecu.edu
Secondary Contact: Bevin Blake Department: Biology	Campus Phone:	Home Phone: 440-465-7246	E-Mail: blakeb13@students.ecu.edu
Chemical Agents Used: Progesterone		Radioisotopes Used: N/A	
Biohazardous Agents Used:	Animal Biosafety Level:	Infectious to humans?	
<b>PERSONAL PROTECTIVE EQUIPMENT REQUIRED: STANDARD PERSONAL PROTECTIVE EQUIPMENT FOR DCM ANIMAL LAB TECHNICIANS.</b>			
Route of Excretion: Urine			
Precautions for Handling Live or Dead Animals: Use standard precautions when handling alive or dead animals. Always don personal protective equipment when handling animals (dead or alive) and bedding.			
Animal Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Bedding / Waste Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Cage Decontamination: Normal cage washing.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: Always don personal protective equipment when handling animals (dead or alive) and bedding. Use provided biosafety cabinet in Ragsdale Annex when handling alive or dead animals, bedding and waste.			
<b>Initial Approval</b> Safety/Subject Matter Expert Signature & Date			
<u>Alana Bagley 2-19-14</u>			



**Laboratory Safety Plan for Testosterone injections (Controlled Substance)**

*Alana Bagley  
2-19-14*

<b>*Process</b>	Testosterone injections
<b>*Hazardous Chemical/ Chemical Class</b>	Testosterone – Controlled Substance Hazards: Reproductive hazard, suspected carcinogen Target Organs: Reproductive (Male and Female), Liver, Nervous system
<b>*Hazardous Equipment</b>	Injection: Needle/Syringe
<b>*Potential Hazards</b>	Considered a carcinogen. Harmful by ingestion, inhalation and skin absorption. May cause respiratory tract irritation. Possible reproductive hazard for men and women. Consult physician if pregnant or possibility of pregnancy. Chronic disorders may cause reproductive disorders.
<b>*Personal Protective Equipment</b>	Safety glasses/goggles; closed front or back lab coat, compatible gloves (nitrile), long pants and closed toed shoes.
<b>*Engineering and Ventilation Controls</b>	All handling and preparation should be completed in a certified chemical fume hood (Howell S112). Administration of injections will be in the biosafety cabinet in Ragsdale Annex. All extraneous equipment should be removed from the hood before work begins. All equipment required for dilutions will be placed in the hood prior to beginning work. All chemical containers can only be removed from fume hood if tightly capped and the exterior wet wiped. Before relocating to Ragsdale Annex ensure the chemical containers are securely closed and placed in an absorbent lined secondary container, preferably with a lid. When transporting to Ragsdale take the most direct route without interruptions. All sharps should be placed in a red biohazard sharps container. Do not recap sharps.
<b>Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins</b>	Prepare the stock solutions in the laboratory and store away from other chemical storage, in a locked cabinet according to federal regulations for controlled substances.
<b>Special Use Procedures</b>	Hands will be washed with a disinfectant soap after handling any chemical containers.
<b>Special Handling and Storage Requirements</b>	Keep container tightly closed in a dry, well-ventilated space. Store stock solutions separately from other chemicals, in a locked and secure area according to federal regulations for controlled substances. Store away from oxidizers. Keep away from ignition sources. Substance should be stored away from light. Avoid creating and breathing dust/aerosols from substance.
<b>*Spill and Accident Procedures</b>	Clean spills only if proper materials are available and if researcher is properly trained to do so. All other spills should be reported to EH&S for clean-up. Needed: absorbent materials, plastic bags, sealable container to hold contaminated cleanup materials, protective clothing, gloves, and safety glasses. For minor spills ventilate area. If spill occurs outside hood, cover liquid with absorbent material; and place in plastic bag. Spill materials should be picked up by EH&S Hazardous Waste Management.
<b>*Waste Minimization Plan</b>	Order only the amount needed.
<b>*Hazardous Waste Disposal</b>	Maintain all hazardous waste in closed containers with hazardous waste tag and contact EH&S for disposal. All sharps should be placed in a closed red sharps disposal container. Contact Prospective Health for pickups of biohazards and sharps.
<b>Decontamination Procedures</b>	Hood will be decontaminated, if necessary, by removing the paper liner and wiping the hood interior surfaces from top to bottom then back to front. (PPE as listed above must be worn during decontamination.)
<b>Animal Care Precautions</b>	Animal care workers should wear PPE including double gloves, lab coats and closed -toed shoes animal and waste removal.
<b>*Chemical Procurement</b>	Inventories kept by PI or laboratory manager. Minimum quantities necessary for the procedures confirmed by current funding.
<b>*Revision Date</b>	2-13-2014



APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730		Home Phone: 252-565-5780
IACUC Protocol Number: D300	Department: Biology		E-Mail: mccoynk@ecu.edu
Secondary Contact: Bevin Blake Department: Biology	Campus Phone:	Home Phone: 440-465-7246	E-Mail: blakeb13@students.ecu.edu
Chemical Agents Used: Testosterone		Radioisotopes Used: N/A	
Biohazardous Agents Used:		Animal Biosafety Level:	Infectious to humans?
<b>PERSONAL PROTECTIVE EQUIPMENT REQUIRED: STANDARD PERSONAL PROTECTIVE EQUIPMENT FOR DCM ANIMAL LAB TECHNICIANS.</b>			
Route of Excretion: Testosterone metabolites are excreted in feces and urine			
Precautions for Handling Live or Dead Animals: Use standard precautions when handling alive or dead animals. Always don personal protective equipment when handling animals (dead or alive) and bedding.			
Animal Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Bedding / Waste Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Cage Decontamination: Normal cage washing.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: Always don personal protective equipment when handling animals (dead or alive) and bedding. Use provided biosafety cabinet in Ragsdale Annex when handling alive or dead animals, bedding and waste.			
<b>Initial Approval</b>			
Safety/Subject Matter Expert Signature & Date			
<u>Glenn Bagley 2-19-14</u>			



## Laboratory Safety Plan for Estradiol injections

*Alanna Bagley*  
2-19-14

<b>*Process</b>	Estradiol injections
<b>*Hazardous Chemical/ Chemical Class</b>	Estradiol Hazards: Reproductive hazard, suspected carcinogen Target Organs: Reproductive (Male and Female), Liver, Nervous system
<b>*Hazardous Equipment</b>	Injection: Needle/Syringe
<b>*Potential Hazards</b>	Harmful by ingestion, inhalation and skin absorption. Considered a suspected carcinogen. May cause respiratory tract irritation. Possible reproductive hazard for men and women. Consult physician if pregnant or possibility of pregnancy. Chronic disorders may cause reproductive disorders.
<b>*Personal Protective Equipment</b>	Safety glasses/goggles; closed front or back lab coat, compatible gloves (nitrile), long pants and closed toed shoes.
<b>*Engineering and Ventilation Controls</b>	All handling and preparation should be completed in a certified chemical fume hood (Howell S112). Administration of injections will be in the biosafety cabinet in Ragsdale Annex. All extraneous equipment should be removed from the hood before work begins. All equipment required for dilutions will be placed in the hood prior to beginning work. All chemical containers can only be removed from fume hood if tightly capped and the exterior wet wiped. Before relocating to Ragsdale Annex ensure the chemical containers are securely closed and placed in an absorbent lined secondary container, preferably with a lid. When transporting to Ragsdale take the most direct route without interruptions. All sharps should be placed in a red biohazard sharps container. Do not recap sharps.
<b>Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins</b>	Prepare the stock solutions in the laboratory and store away from other chemical storage, in a secure area.
<b>Special Use Procedures</b>	Hands will be washed with a disinfectant soap after handling any chemical containers.
<b>Special Handling and Storage Requirements</b>	Keep container tightly closed in a dry, well-ventilated space. Store stock solutions separately from other chemicals, in a secure area. Store away from oxidizers. Keep away from ignition sources. Substance should be stored away from light. Avoid creating and breathing dust/aerosols from substance.
<b>*Spill and Accident Procedures</b>	Clean spills only if proper materials are available and if researcher is properly trained to do so. All other spills should be reported to EH&S for clean-up. Needed: absorbent materials, plastic bags, sealable container to hold contaminated cleanup materials, protective clothing, gloves, and safety glasses. For minor spills ventilate area. If spill occurs outside hood, cover liquid with absorbent material; and place in plastic bag. Spill materials should be picked up by EH&S Hazardous Waste Management.
<b>*Waste Minimization Plan</b>	Order only the amount needed.
<b>*Hazardous Waste Disposal</b>	Maintain all hazardous waste in closed containers with hazardous waste tag and contact EH&S for disposal. All sharps should be placed in a closed red sharps disposal container. Contact Prospective Health for pickups of biohazards and sharps.
<b>Decontamination Procedures</b>	Hood will be decontaminated, if necessary, by removing the paper liner and wiping the hood interior surfaces from top to bottom then back to front. (PPE as listed above must be worn during decontamination.)
<b>Animal Care Precautions</b>	Animal care workers should wear PPE including double gloves, lab coats and closed -toed shoes animal and waste removal.
<b>*Chemical Procurement</b>	Inventories kept by PI or laboratory manager. Minimum quantities necessary for the procedures confirmed by current funding.
<b>*Revision Date</b>	2-13-2014



APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator: Krista McCoy	Campus Phone: 737-2730		Home Phone: 252-565-5780
IACUC Protocol Number: D300	Department: Biology		E-Mail: mccoyk@ecu.edu
Secondary Contact: Bevin Blake Department: Biology	Campus Phone:	Home Phone: 440-465-7246	E-Mail: blakeb13@students.ecu.edu
Chemical Agents Used: Estradiol		Radioisotopes Used: N/A	
Biohazardous Agents Used:		Animal Biosafety Level:	Infectious to humans?
<b>PERSONAL PROTECTIVE EQUIPMENT REQUIRED: STANDARD PERSONAL PROTECTIVE EQUIPMENT FOR DCM ANIMAL LAB TECHNICIANS.</b>			
Route of Excretion: Urine			
Precautions for Handling Live or Dead Animals: Use standard precautions when handling alive or dead animals. Always don personal protective equipment when handling animals (dead or alive) and bedding.			
Animal Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Bedding / Waste Disposal: All materials will be discarded in biohazard bags and incinerated through ECU hazardous waste management.			
Cage Decontamination: Normal cage washing.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: Always don personal protective equipment when handling animals (dead or alive) and bedding. Use provided biosafety cabinet in Ragsdale Annex when handling alive or dead animals, bedding and waste.			
<b>Initial Approval</b>			
Safety/Subject Matter Expert Signature & Date			
<u>Alana Bagley 2-19-14</u>			