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Characterizing putative sexually dimorphic effects of a propionic acid induced autism spectrum disorder phenotype in adult male and female rats

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Abstract

Research suggests that certain gut and dietary factors may worsen symptoms in autism spectrum disorder (ASD). Studies have shown that treatment with the bacterial product, propionic acid (PPA), elicits neuroinflammatory and behavioral responses in rats that are characteristic of ASD in humans. A consistent male bias in ASD prevalence has been observed, and several sex-differential genetic and hormonal factors have been suggested to contribute. However, most studies of ASD, including those involving PPA, focus on males. The present study explored putative sex differences in the effects of PPA (500mg/kg) on a rat behavioral ASD phenotype and the influence of the estrous cycle and fluctuations in estradiol and progesterone. This was accompanied by examinations of the effects of ovariectomy and hormone replacement therapy with estradiol and progesterone. PPA produced no sex-differential effects, and elevated hormonal levels did not seem to play a protective role against the adverse effects of PPA.

Keywords

Gut Microbiome; Short-chain fatty acid; Estradiol; Progesterone; Estrous Cycle; Rat; Sex Differences; Social Behavior; Anxiety

Summary for Lay Audience

Autism spectrum disorder (ASD) refers to a broad range of conditions affecting brain development. Males are diagnosed four times more often than females, but the factors that explain this majority of males are generally unknown (Amaral, Schumann, & Nordahl, 2008; Beggiato et al., 2017; Zablotsky et al., 2015). One's environment and genetics have been suggested to contribute to the expression of autism symptoms, however there is no single known cause for ASD (Zablotsky et al., 2015). Results from scientific studies have suggested that female hormones may provide protection against the expression of several symptoms of ASD. Autism has been said to be primarily a disorder of brain function, but issues with other bodily systems have become of interest. Of particular interest to the present study are issues with the gastrointestinal system, which appear to be very common in children diagnosed with ASD (Quigley & Hurley, 2000). Elevated levels of a bacteria, termed *Clostridia*, have been detected in the gastrointestinal tract of children with ASD. *Clostridia* produces short-chain fatty acids, one of which is known as Propionic Acid (PPA), which at high enough levels has been found to worsen ASD-like behavior in rodents. In this thesis, a PPA rat model for autism was used to study the influence of sex and female sex hormones on social behavior and anxiety. Research studies seeking to explore the effects of PPA in relation to ASD have explored these effects in males, and results from these studies are then generalized to both sexes. It is imperative that research continues to extend these studies to female populations. Findings from the current thesis do not suggest that higher levels of female sex hormones protect against the ASD-like behavioral effects of PPA.

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Chapter 1

1 General Introduction

There are many clinical and behavioral disorders that have been reported to manifest differently in males and females (Manton, 1999; Regitz-Zagrosek & Seeland, 2013). In many cases, the reasons for these sex differences are unclear. In some instances, sex differences can be attributed to differences in the brain, genetics, and hormones, and often a combination of all three. While sex differences have been well documented in the literature, these differences are rarely addressed in healthcare; sex and gender of the patient are not always taken into consideration when making decisions regarding prevention and treatment (Regitz-Zagrosek, 2012). As researchers and physicians are becoming increasingly aware of the variances between males and females in terms of their health, whether it be greater rates of anxiety and dementia in females (Gliatto, 2000; Qizilbash et al., 2015) or a greater chance of autism and Parkinson's disease in males (Baldereschi et al., 2000; Baron-Cohen et al., 2011), there is a lack of research being conducted to determine the mechanisms that underlie these differences.

One area of research that still remains unsolved is the skewed sex ratio toward males in autism spectrum disorder (ASD) prevalence that has been consistently observed (Baron-Cohen et al., 2011), with only one female being diagnosed with ASD for every four males. Although the reason for this sex ratio has been attributed to genetic, hormonal, and other biological differences between the sexes (Hallmayer et al., 2011; Sarachana et al., 2011), the reasons for this bias are still uncertain. It is consistently found that, throughout the autism spectrum, that mean age diagnosis for females is later than males, and that females are more often diagnosed with ASD when they present with comorbid cognitive impairments (Lord, Schopler & Revicki, 1982; Wing, 1981). Scientific findings suggest that this difference could in part be due to an artifact in diagnoses, where physicians expect females to present with the same behavior as males with ASD, creating a male bias. However, females have been found to be significantly better at what is commonly referred to as “camouflaging”, using compensatory behaviors to mask some of their social challenges (Dworzynski et al., 2012; Tierney et al., 2016). This emphasizes the need for

broadening gender and sex-based assessments, considering the variable behavior among males and females in social environments.

The vast majority of ASD research has focused on male subjects. Interpretations are drawn from these studies and are then generalized to both males and females, which ignores the involvement of female physiology and biology. The fact that this ratio is observed across variable times and among disparate populations indicates that perhaps sex-specific factors are at play (Werling & Geschwind, 2013). The present thesis addressed putative sex differences in an animal model of autism spectrum disorder.

1.1 Autism Spectrum Disorder (ASD)

The 5th edition of the Diagnostic Statistical Manual (DSM-5) defines autism, in part, as the presence of continued difficulty with social communication and reciprocity, difficulties adjusting behavior to suit various social contexts, and restricted/repetitive behaviors and interests that are present in early development (American Psychiatric Association, 2013). Diagnostic criteria indicated in the DSM-5 states that individuals with autism exhibit challenges with social approach, as well as failure to initiate and respond to social interactions across multiple contexts. While there are underlying neurological commonalities among individuals with ASD, they vary greatly from one another and symptoms manifest differently in each case (Pellicano, 2013). Most individuals with ASD show difficulty engaging in everyday human interaction. This difficulty is demonstrated even as early as infancy (Ozonoff et al., 2014), the stage in which most babies tend to want to touch and explore other human beings. Outcomes from research studies suggest that feelings of anxiety are common in individuals with ASD, indicating an additional morbidity (Kerns & Kendall, 2012; White, Oswald, Ollendick & Scahill, 2009).

The idea of autism as a spectrum disorder supports the diversity in expression among affected individuals (Carl & Hardan, 2011). Many theories exist about how or why a child develops autism and there is evidence for both genetic and environmental influences. Inconclusive evidence points to the idea that it is most likely that environmental factors influence ASD by interacting with pre-existing genetic vulnerabilities (Benvenuto et al., 2009). Estimates of prevalence have increased dramatically since the 1960's, where diagnoses were around 4 per

10,000 and are now currently near 22 per 1,000 in children ages 3 through 17 (Zablotsky et al., 2015). It is unclear if this change reflects an increase in ASD or a change in the diagnostic criteria.

1.2 The Gut Microbiome

ASD has usually been said to affect the brain primarily (Belmonte et al., 2004), however issues with other systems have recently become more apparent. Of particular interest is the gastrointestinal system. In particular, gastrointestinal issues seem to occur significantly more often in individuals with autism than the general population (Parracho, Bingham, Gibson & McCartney, 2005). While the precise etiology of ASD is not clear, ASD as a disorder of the microbiota-gut-brain axis has been evolving as a probable factor in the expression of ASD behaviors (Li & Zhou, 2016). The human digestive tract is host to many bacteria that produce various metabolic products. Some of the more important products that have been implicated in the etiology of ASD are short-chain fatty acids (MacFabe et al., 2007). The three main short-chain fatty acids include acetate, butyrate and propionate. Propionate (PPA) in particular has been implicated in the etiology of ASD. A family of gram-positive bacteria known as *Clostridia* have been of particular interest, as they are major gut colonizers and are producers of propionate. At physiological levels, PPA is critical for immune related functioning. But at higher levels, it may augment immune function by stimulating the release of proinflammatory cytokines (Li et al., 2018). Increased levels of PPA have also been shown to exacerbate ASD-like symptoms such as reduced social interaction, repetitive behaviors, and deficits in sensorimotor gating in rodents (MacFabe et al., 2007). The effects of PPA have been found to be expressed through a variety of mechanisms, including neurotransmitter release and synthesis, mitochondrial dysfunction, and through epigenetic actions such as inhibition of histone deacetylase. All of these mechanisms have been associated with neurodevelopmental disorders, including ASD (Frye et al., 2013; Inoue et al., 2012; MacFabe, 2012, 2013).

PPA has been implicated in not only ASD, but in several other disorders including Propionic Acidemia (PA). PA is an inherited disorder characterized by the accumulation of propionic acid and its toxic derivatives, caused by the deficiency of a particular enzyme in the body that is unable to properly process certain proteins and fats (Cotrina, Ferreiras & Schneider, 2019).

Children with PA are at a higher risk for psychological and cognitive deficiencies, in addition to intellectual disabilities and impaired language (Cotrina, Ferreiras & Schneider, 2019; Schreiber et al., 2012). Several studies have shown an increased frequency of ASD in children with PA (de la Bâtie et al., 2018; Witters et al., 2016), and abnormal neurodevelopment patterns have been commonly detected in children with PA (Witters et al., 2016). Different from ASD, scientific reports indicate that comorbid PA and ASD is gender balanced and has a greater mean age of diagnosis (Cotrina, Ferreiras & Schneider, 2019).

1.3 Animal Models

Several mouse and rat models of autism spectrum disorder are well-established in the literature. A comprehensive review conducted by Möhrle et al. (2020) outlines how the complex presentation of ASD symptoms can be explored by using animal models and the importance of contextualizing genetic abnormalities within these behavioral models. Assessments that examine social behavior and interaction are often used to investigate reduced social approach and communication, which are considered core symptoms in ASD. Rodents are very social making them appropriate for the modelling of social impairments that are observed in ASD (Kaidanovich-Beilin et al., 2011). This review also mentions the significance of considering other features that resemble emotional and behavioral disorders that often emerge as a comorbidity in individuals with ASD, such as anxiety (Leyfer et al., 2006; Möhrle et al., 2020). Various rodent paradigms, including the elevated plus maze test and the light dark anxiety procedure, are commonly used to assess changes in anxiety-like behavior (Bailey & Crawley, 2009). In addition to providing insight into the mechanisms by which ASD symptoms are expressed in humans, results of studies with animal models can give rise to advances in treatments that can eventually lead to the alleviation of several symptoms that are associated with ASD (Tania, Khan, & Xia, 2014).

As indicated, elevated levels of bacteria flora, including *Clostridia*, have been detected in the GI tract of children with ASD (Martirosian et al., 2011; Tomova et al., 2015). Using animal models, it has been found that central or peripheral administration of PPA to male rats produces both brain and behavioral changes that are consistent with ASD such as increased anxiety, decreased social interaction, deficits in sensorimotor gating, and increased neuroinflammation (Kamen et

al., 2019; MacFabe et al., 2007; Shultz et al., 2008). MacFabe et al. (2007) investigated the effects of central PPA administration at the behavioral, electrophysiological, neuropathological, and biochemical levels of analysis. Results of this investigation suggest that PPA has the ability to produce neuronal and behavioral deficits that resemble human presentations of ASD. These results led to their proposal that forms of autism may be an acquired disorder that involves altered levels of PPA and its metabolism. Shultz et al. (2008) examined social behavior and brain tissue of adult male rats given intraventricular injections of PPA or a control compound and found both brain and behavioral impairments that resemble ASD. PPA treated rats demonstrated less playful interactions, altered responses to playful initiations, and less time spent in close proximity to a naïve stranger. Brain tissue analyses revealed an increase in the number of astrocytes along with the destruction of nearby neurons and neuroinflammation. Additionally, findings from a study conducted by Shams et al. (2019) showed that intraperitoneal treatment of rats with PPA reduced playful initiations and increased anxiety-like behavior. These findings provide compelling evidence that PPA can produce behavioral and neuropathological changes in rodents that align closely with those observed in clinical manifestations of autism.

Animal models of ASD have focused almost exclusively on male samples, and findings from these studies are then generalized to females. Although there is a prominent sex difference in the disorder, ASD also affects the female population. Future examinations exploring the possible mechanisms that are contributing to this sex bias are paramount.

1.4 Sex Differences in ASD

There is a dramatic sex difference in the risk of ASD, with a diagnosis rate of 1 in 42 boys in comparison to 1 in 189 girls (Christensen et al., 2018). Since there is a lack of agreement about clinical signs and assenting laboratory tests specific to ASD, it is difficult to definitively diagnose ASD (Johnson, 2008.) With the vast range of phenotype manifestation in ASD, it becomes less suitable to approach diagnosis with a one-size-fits all method.

While the exact reasons accounting for this bias are unclear, some have speculated that the reason for this difference may be rooted in biological differences between the sexes, or for reasons relating to differences in genetics, hormones and immune function (Klein, 1998; Klein,

Marriott, & Fish, 2015; Werling & Geschwind, 2013). Alternatively, this sex difference may be related to a bias in diagnosis, or perhaps to a lessened tendency to diagnose females who are presenting with ASD criteria. Compared to males, females have been reported to present fewer externalizing symptoms along with less restrictive and repetitive patterns of behavior (Mandy et al., 2012). These differences in symptom presentation suggest that this bias may be due in part to disparate phenotypic presentations of ASD between the sexes. In general, females that have been clinically diagnosed with ASD have also been reported to exhibit more profound presentations of some of the characteristics of this disorder, due to the fact that the vast majority of milder cases in females are left undiagnosed (Kreiser & White, 2014).

Findings from a large body of research supports the expression and prevalence of co-occurring anxiety disorders in autism. Nearly 80% of children, both male and female, diagnosed with autism display clinically significant anxiety (Leyfer et al., 2006; Simonoff et al., 2008). Elevated levels of sex hormones, particularly estrogen and progesterone, in female rats have been found to have anxiolytic properties. Heightened levels of estradiol observed during rodent proestrus, or after exogenous hormone replacement to ovariectomized (OVX) females, exert anxiolytic actions in the elevated plus maze anxiety paradigm (Leret, Molina-Holgado & Gonzalez, 1994). Nomikos & Spyraiki, 1988). Additionally, when endogenous estrogens are removed, via ovariectomy, behavioral indices of anxiety increase (Díaz-Véliz et al., 1997; Mora, Dussaubat & Díaz-Véliz, 1996). Rats in behavioral estrus, which show naturally high levels of progesterone, exhibit reduced anxiety behavior in several behavioral tasks (Bitran et al., 1991; Brot et al., 1997; Farr et al., 1995; Fernandez-Guasti and Picazo 1992). These findings lend support to the idea that heightened levels of estrogen and progesterone in females can provide protection against the manifestation of anxiety in individuals with ASD.

1.5 Objectives of the Current Thesis

The current thesis, which addressed putative sex differences in the effects of PPA in male and female rats, was comprised of three distinct phases. Given the strong male bias in ASD prevalence, the objective of the first phase was to characterize putative sex differences in a propionic acid (PPA) induced ASD behavioral phenotype. The second phase was conducted to examine whether or not the effects of PPA changed during different stages of the estrous cycle,

where there are varying levels of estradiol and progesterone. The two stages of interest of the rat estrous cycle were diestrus (lowest observed levels of estradiol and progesterone) and proestrus (highest observed levels of estradiol and progesterone; Butcher, Collins & Fugo, 1974). The third and final phase involved using ovariectomized (OVX) female rats treated with a cyclic hormone replacement regimen. This regimen was used to determine whether an exogenous source of estradiol and progesterone would produce differential effects on a PPA-induced ASD behavioral phenotype. The same behavioral tests, measuring anxiety and social behavior, were conducted in all three phases of the present thesis.

While the mechanisms that are responsible for the sex difference in the expression of ASD phenotypes have yet to be established, there is some evidence that implicates estradiol and progesterone (Singer et al., 1996). It was hypothesized that there are sex differences in the expression of ASD with males showing greater reductions in social behavior and higher levels of anxiety, and that hormonal fluctuations across the rat estrous cycle are a contributing factor to females showing less pronounced symptoms.

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Chapter 2

2 Characterizing putative sexually dimorphic effects of a propionic acid induced autism spectrum disorder phenotype in adult male and female Long-Evans rats

2.1 Introduction

Neurodevelopmental disorders, including autism, disproportionately affect the male population. The reasons why males are more vulnerable and/or females are protected from ASD are unknown. Defining the mechanisms that play a role here is imperative to elucidating the etiology and furthering treatment of neurodevelopmental disorders like ASD. Because of this pronounced sex difference, research on ASD focuses almost entirely on male samples. Although there is an abundance of studies exploring ASD with males, relatively little research has been conducted using females.

Findings from clinical investigations suggest the potential involvement of gut and dietary factors in the expression of autism spectrum disorder (ASD). Greater levels of the *Clostridia* bacteria have been detected in the gastrointestinal tract of children with autism (Song, Liu & Finegold, 2004). Via the breakdown of various carbohydrates and amino acids, *Clostridia* produces short-chain fatty acids (e.g. butyrate, propionate, acetate). Elevated levels of short chain fatty acids, which have been found to exert effects at the gut, neuronal, and behavioral levels (MacFabe et al., 2007), have been associated with neurodevelopmental disorders such as autism (El-Ansary & Al-Ayadhi, 2014). Studies have reported social, cognitive, motor, and EEG irregularities, as well as neuroinflammation, heightened oxidative stress, and altered lipid metabolism, all of which are consistent with symptoms in ASD (MacFabe et al., 2007; Shultz et al., 2008; Song et al., 2004).

Findings from previous investigations have suggested that the short chain fatty acid, propionate (PPA), may produce brain and behavior alterations in rodents that have been commonly observed in human cases of ASD, such as elevated anxiety, gastrointestinal disturbance, and social impairments (Foley et al., 2014; MacFabe et al., 2007; Ossenkopp et al., 2012). Foley et al. (2014) examined the effects of prenatal PPA and lipopolysaccharide (LPS) treatment on rat social

behavior. Although their results did not point to a strong sex difference in the effects of PPA when administered during the prenatal period, there have been no examinations of possible sex differences in the effects at later points of development. Systemic treatment with PPA has produced reductions in social and play behavior in adolescent and adult male rats, such that PPA-treated males display fewer approach behaviors and reduced time spent in close proximity to a stranger (Shams et al., 2019). Wah et al. (2019) detected anxiogenic effects of PPA in the light-dark box test in adolescence and adult male rats, as indicated by less time spent in the light chamber and fewer transitions into the light chamber (Wah et al., 2019). These patterns of results suggest that elevated PPA can have discernible effects on anxiety and social behavior in adolescent and adults. With nearly all of these subjects having been male, it is impossible to generalize these findings to both sexes. The possible sexually dimorphic effects of PPA, and at different points across the lifespan, need to be examined.

2.1.1 The Potential Role of Gonadal Hormones

Results from investigations exploring the sex bias in ASD have suggested that this difference may be rooted in biological differences between the sexes, however the exact reasons for this bias remain unknown (Jeon et al., 2018). Findings from several studies have suggested that prominent female gonadal hormones, specifically estradiol and progesterone, have neuroprotective effects against the development and pathogenesis of ASD symptoms (Barth, Villringer, & Sacher, 2015).

Two critical neurotransmission systems, glutamate and GABA, are involved in ASD pathophysiology, and these neurotransmitters have been found to display sex differences in their expression and regulation (Al-Suwailem, Abdi & El-Ansary, 2018). Glutamate release reaching abnormal levels is one of the pathological events that moderates the interaction between different causative risk factors for autism (Essa et al., 2013). Al-Suwailem et al. (2018) explored gonadal hormones as a potential mechanism underlying the sex bias in neurodevelopmental disorders. Their findings suggest that the male bias observed in autism can potentially be explained by female's reduced susceptibility to altered glutamate levels, due to proposed neuroprotection by sex hormones such as estrogens. Excessive glutamate release is associated with adverse alterations in intracellular calcium levels, often producing cell cycle arrest and apoptosis

(Ankarcrona et al., 1995), and treatment with estradiol has been found to protect against glutamate associated cell death (Perrella & Bhavnani, 2005). Additionally, progesterone has been found to enhance GABAergic inhibitory transmission through its interaction with GABA_A receptors in the mature brain, helping keep glutamate within its normal functioning range (Essa et al., 2013).

Environmental factors, including gut health and gut-microbiome alignment, influence immune development differently in males and females (Klein & Flanagan, 2016). These differences can ultimately result in variable susceptibilities to different diseases and disorders involving immune functioning, such as ASD, with males exhibiting greater vulnerabilities (Klein, 1998; Klein, Marriott, & Fish, 2015). Sex hormones have been implicated in immune response and the development of the immune system, with testosterone leading to increased risk of infection and susceptibility to disease, while estrogens produce the opposite effect (Furman et al., 2014; McCrudden & Stimson, 1991). Findings from several reports also indicate that estrogens prevent the occurrence of neuroinflammatory processes (Villa, Vegeto, Poletti & Maggi, 2016), which play a crucial role in the pathogenesis and development of these diseases (Hanamsagar & Bilbo, 2016).

2.1.2 Hormones and the Estrous Cycle

The female rat reproductive cycle, or estrous cycle, typically lasts four days and is comprised of four distinct phases. The four phases include: estrus, metestrus, diestrus, and proestrus (Long & Evans, 1922). The estrous cycle can be followed by collecting vaginal samples and discerning cell type and quantity under a phase contrast microscope. The impact of estradiol and progesterone fluctuations throughout the estrous cycle on certain behaviors can be investigated by tracking the estrous cycle and quantifying behaviors on specific days of the cycle.

Depending on what phase of the estrous cycle the rodent is in, there are different levels of the hormones estradiol and progesterone (see Figure 2.1). During proestrus, estradiol and progesterone levels are observed to be highest while during diestrus, they are at their lowest (Lebron-Milad & Milad, 2012). Estrous stage seems to be a major determinant of anxiety, with diestrus females behaving more anxiously than males or estrus, metestrus, and proestrus females.

Frye et al. (2000) explored hormonal fluctuations throughout the estrous cycle in combination with hippocampal progesterone concentrations. Proestrus females were found to have the highest concentrations of progesterone and displayed more social behaviors and reduced anxiety-like behaviors compared to diestrus females and males (Frye et al., 2000). These results suggest that differences in anxiety-like and social behavior displayed by females during different phases of the estrous cycle coincide with differential hormonal concentrations. In a study conducted by Frye et al. (2008), sex differences and estrous cycle variations in anxiety-like behaviors in the open-field task and a social behavior task were examined. It was found that proestrus females exhibited less anxiety-like behavior, as measured in the elevated plus maze and social interaction tests, than animals in all other phases. Findings from these studies indicate that estrous phase influences the expression of anxiety like behaviors.

2.1.3 Phase One

In phase one, male and female rats were treated with PPA to explore the potential sex differences in the expression of ASD-like behaviors. A test of social interaction was conducted to observe whether or not systemic PPA-treatment produces deficits in social behavior. The variables measured in the present study (i.e. social initiations and probability of defense) have been measured in prior clinical investigations exploring analogous measures of social behavior in human ASD (Yuill et al., 2007) and have been shown to be affected by PPA in male rats (Shultz et al., 2008). The light-dark test was used to examine levels of anxiety-like behavior. A number of common behaviors seen in children with ASD overlap with symptoms seen in varying anxiety disorders (Kerns & Kendall, 2012). In addition, PPA was previously shown to elicit anxiety-like behavior in male rats (Wah et al., 2019). The light-dark test is useful in predicting anxiety-like and anxiogenic-like behavior in rodents as it is based on their intrinsic aversion to novel and brightly lit environments (Bourin & Hascoët, 2003). It was hypothesized that there would be sex differences in the effects of PPA, with males exhibiting significantly greater levels of anxiety-like behavior and reductions in social and play behavior compared to female rats.

2.1.4 Phase Two

Phase two of the present study explored the potential differential effects of administration of

PPA in young-adult female rats at two different phases of their estrous cycle: proestrus and diestrus. The estrous cycle of the female rat was followed, and they were tested when estradiol/progesterone levels were highest, during proestrus, and when they were lowest, during diestrus (Butcher, Collins & Fugo, 1974; See Figure 2.1). It was predicted that the effects of PPA in female rats would differ depending on what stage of the estrous cycle they were in. When estradiol and progesterone levels were high (proestrus), females were expected to display less severe ASD-like impairments (i.e. lower anxiety and increased social interactions/play behavior) relative to when estradiol and progesterone levels were low (diestrus).

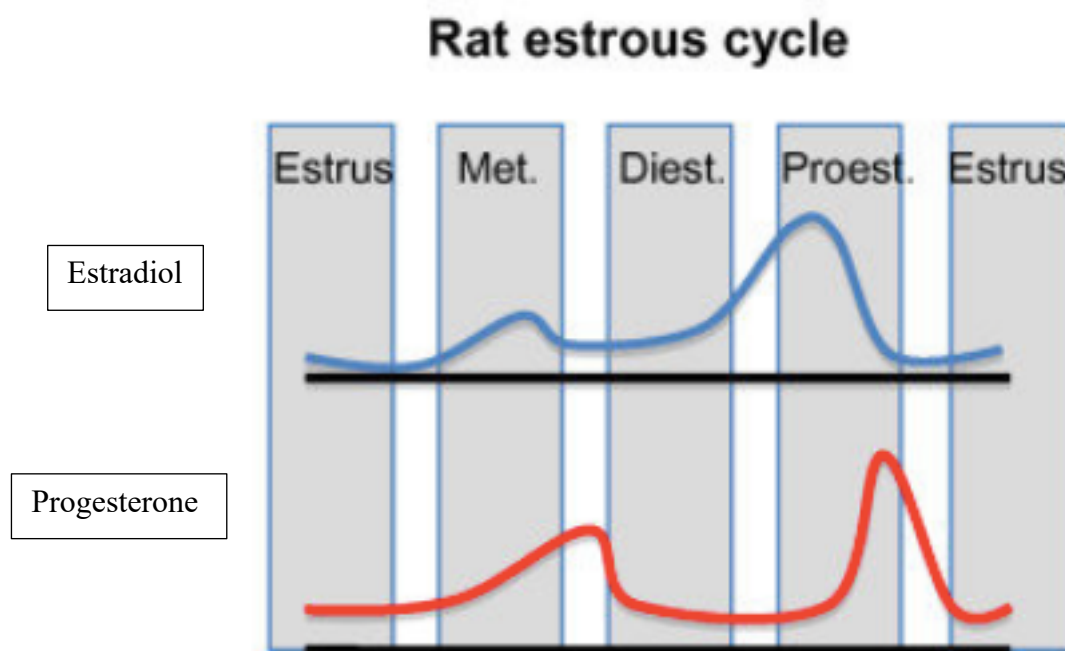


Figure 2.1. Hormonal Fluctuations Across the Estrous Cycle. Estradiol and progesterone levels measured throughout the rodent estrous cycle. Estradiol levels are indicated by the blue line and progesterone levels are indicated by the red line. Modified image from Lebron & Milad, 2012.

2.2 Method

2.2.1 Animals

The subjects for phase one consisted of 26 young adult male (Post-Natal Day; PND 50) and 25

young- adult female Long Evans rats and subjects in phase two were 32 female Long-Evans rats (Charles River, Quebec); all animals weighed between 201-225 g. Rats were housed in same-sex and same-treatment pairs in polypropylene cages (45 cm x 22 cm x 20 cm), in a colony room at 21 ± 1 °C and under a 12:12 light to dark cycle, with lights on at 0700h. Each cage was provided with ProLab (RMH3000) rat chow and water ad libitum. All testing took place during the early light phase (0900-1200h) of the light-dark cycle. All procedures were approved by the University of Western Ontario Animal Care Committee and were in accordance with the Canadian Council of Animal Care (CCAC) Guidelines. See Figure 2.2 for Group Designation.

2.2.1.2. Stranger Animals

Age and weight matched “stranger” animals for the social behavior task were utilized for each phase. Stranger animals were not treated with any drug and were handled according to the same schedule as experimental rats. The sample of stranger rats consisted of 20 Long Evans rats for phase one (10 male and 10 female) and 16 (all female) for phase two. Stranger animals were housed in same-sex pairs.

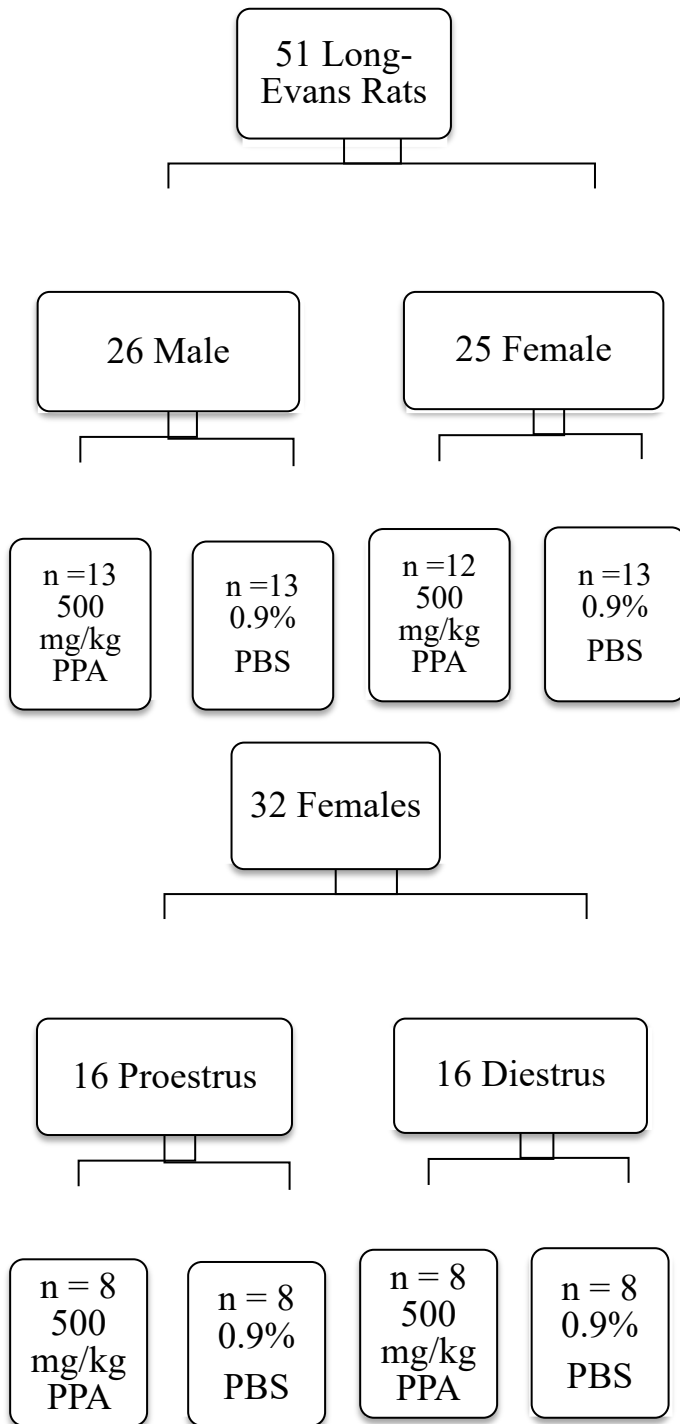


Figure 2.2. Group Designation. Phase One (Top); Phase Two (Bottom)

2.2.2 Drugs

Sodium propionate (PPA, P1880, Sigma Chemical, St. Louis, MO, USA) was dissolved in 0.1 M Phosphate Buffered Saline (PBS) and administered intraperitoneally (i.p.) at a dose of 500mg/kg (250 mg/mL, 0.26 M, pH corrected to 7.4 with concentrated HCl) once a day for four days. This dose was chosen based on prior investigations (Ossenkopp et al., 2012) and selected to model states of metabolic dysfunction. Due to its short half-life (30 min, Brusque et al., 1999), several injections of PPA were administered. PBS was injected i.p. as a vehicle control (2 mL/kg) to yield a control group. In both phase one and two, male and female samples were randomly divided into two groups to be treated with either PPA (500mg/kg) or the vehicle control drug (0.1mol/L PBS; see Figure 2.2 for Group Designation).

2.2.3 Experimental Timeline

The experimental procedure for phase one is summarized in Figure 2.3A. When animals arrived, they were first acclimated to their colony rooms for a week. After three days of handling, estrous samples were taken from female cohorts for three consecutive estrous cycles (12 days) to determine projected estrous phases throughout the duration of the study. Male and female experiments were run at different times. As shown in Figure 2.2, the four experimental groups were: Male+PPA, Male+PBS, Female+PPA, and Female+PBS.

The experimental procedure for phase two is summarized in Figure 2.3B. When animals arrived, they were first acclimated to their colony rooms for a week. After three days of handling, estrous samples were taken for three consecutive estrous cycles to determine when each animal should be injected so that half were tested during diestrus and half were tested during proestrus. A previous study found that the behavioral deficits produced by PPA return to baseline after a 7-day recovery period, and one injection with high dose PPA (500mg/kg) has been found to produce impairments (Mepham et al., 2019). Phase two had a delay of eight days between social behavior and anxiety testing to confirm an accurate determination of estrous phase before each test. As shown in Figure 2.2, the four experimental groups were: Proestrus+PPA, Proestrus+PBS, Diestrus+PPA, and Diestrus+PBS.

Phase 1: Injection Days (13, 14, 15, 16, 20)

Phase 2: Injection Days (13, 14, 15, 16, 24)

- Injected i.p. 5x (1x/Day) with either 500mg/kg PPA or 0.9% PBS
- Placed in respective behavioral apparatus 10-min post-injection

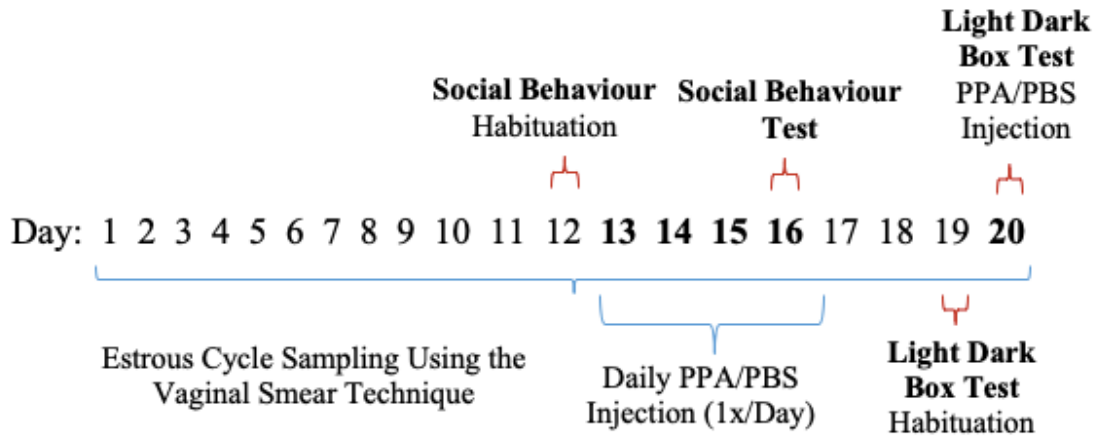


Figure 2.3A. Experimental Overview for Phase One.

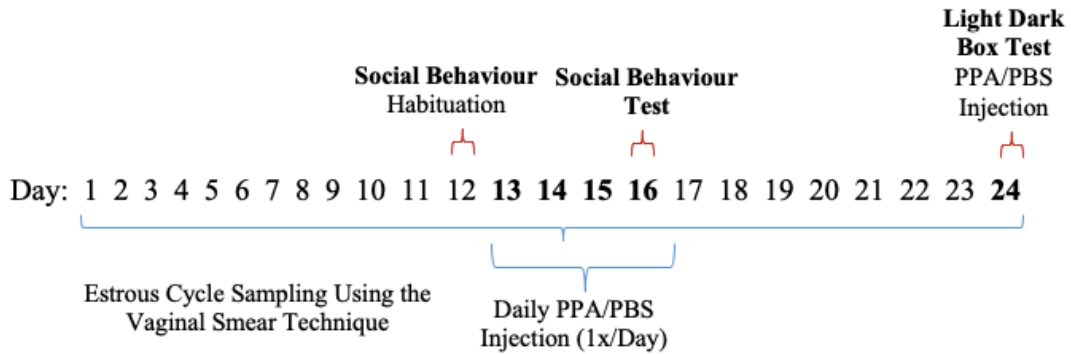


Figure 2.3B. Experimental Overview for Phase Two.

2.2.4 Materials and Apparatus

2.2.4.1 *Social Behavior Test*

Social behaviors were recorded in a circular open-field arena (90 cm diameter, 40 cm high) with a video camera placed above the center. The camera was connected to a computer, allowing behavior to be recorded using the EthoVision 3.0.15 Behavioral Monitoring and Analysis System. The camera was also connected to a VCR, allowing behavior to be recorded for later analysis. On the day of testing, the dorsal surface of the treated rat from each pair was colored black using an unscented black marker so that the EthoVision Tracking System could distinguish between the experimental rat and stranger rat (Lazar et al., 2008; Shultz et al., 2008). The social behaviors described in Table 2.1 were manually scored based on criteria previously defined by Field et al. (2006) and Shultz et al. (2008).

Twenty-four hours before injections began, experimental animals (excluding stranger animals) were individually habituated to the social behavior apparatus for ten minutes. On the test day, rats received a single PPA (n = 41) or PBS (n = 42) injection and were placed in the arena 10 minutes after the injection. Once the injected rat was placed into the center of the arena, a non-treated same-sex stranger rat was placed into the center of the arena and their social behavior was recorded for 20 minutes. Following this session, rats were returned to their home cages.

Table 2.1*Behavioral Measures Recorded for Social Behavior (Shultz et al., 2008).*

<i>Behavioral Measure</i>	<i>Definition</i>
Frequency of Social Initiations	Number of nape to snout contacts displayed by injected rat.
Probability of Defense	Number of defenses elicited (defense defined as withdrawal of the nape from the stranger's snout) divided by the total number of social initiations by the stranger $\times 100$. This measure was recorded only if at least one social initiation occurred.
Type of Defense	
(i) Facing Defense	(i) The number of facing defenses (defined as withdrawal of the nape from the stranger's snout by turning to face the stranger), divided by the total number of defenses by the injected rat $\times 100$. These measures were recorded only if a rat displayed at least one defensive response.
(ii) Evasive Defense	(ii) The number of evasive defenses (defined as withdrawal of the nape from the stranger's snout by either leaping, running, or turning away from the stranger), divided by the total number of defenses by the injected rat $\times 100$.

2.2.4.2 Light Dark Test

Anxiety-related behavioral variables from the light-dark apparatus were collected using eight VersaMax Animal Activity Monitors (Accuscan Model RXYZCM-16, Columbus, OH).

Monitors were made of clear Plexiglas (42 \times 42 x 30 cm) enclosed by a Plexiglas lid that contained holes to allow air to pass through. The box contained 16 infrared sensors positioned every 2.54 cm along the perimeter and 7 cm above the floor. An additional 16 sensors were located 18 cm above the floor on opposite sides. This apparatus was divided into a light or dark chamber using a black opaque Plexiglas box insert (40 \times 20 x 23 cm). The light chamber was illuminated by 3 linear fluorescent cool white light sources (approximately 900 lux at the floor of the light chamber) located above the monitors. The dark chamber had small holes located on the sides to permit infrared beams to pass through. A gap (10 x 8 cm) in the partition between the light and dark chambers allowed the rat to freely pass between chambers. Data were collected

and analyzed by a VersaMax Analyzer (Accuscan Model CDA-8, Columbus, OH) and sent to a computer where it was recorded and later analyzed. Procedures were adapted from Banasikowski et al. (2015) and Ossenkopp et al. (2005).

As seen in Table 2.2, the VersaMax Analyzer created two types of variables: chamber-choice and activity variables. The chamber-choice variables included the number of nosepokes into the light chamber, the number of transitions into the light chamber, and duration of time spent in the light chamber. These reflect the rat's willingness to explore, their degree of risk assessment and anxiety levels (Banasikowski et al., 2015; Ossenkopp et al., 2005). The other type of behavioral variables collected by the VersaMax Analyzer were activity variables in both the light and dark chambers (i.e. total distance travelled and vertical time).

Table 2.2*Behavioral Measures Recorded for the Light Dark Box Test.*

Behavioral Measures	Definition
<i>Chamber Choice Variables</i>	
Nosepokes	Number of investigative postures extending into the light chamber.
Transitions	Number of transitions between the light and dark chambers.
Duration	Time spent in the light/dark chamber.
<i>Activity Variables</i>	
Total Distance Travelled	Cumulative horizontal distance travelled in the light/dark chamber.
Vertical Time	Time spent in rearing position. This measure does not consider the time when the animal goes below the vertical sensor.

Twenty-four hours prior to conducting the light dark anxiety test in phase one, animals were habituated to the testing room for thirty minutes and then placed in the center of the light chamber of the apparatus to measure baseline levels of anxiety. Animals were given the fifth and final injection with either PPA or PBS three days following the social behavior test (see Figure 2.3 for Experimental Timeline). The light-dark test was conducted approximately 10min following the final injection.

In phase two, females were given the fifth and final injection with either PPA or PBS on their respective estrous phase day: diestrus or proestrus. At this time, animals had not been given an injection of PPA/PBS for two consecutive estrous cycles (eight days). Before the injection was given, animals were habituated to the light-dark testing room for thirty minutes. After this habituation period, animals were weighed and injected. The light-dark test was conducted

approximately 10min following the injection.

2.2.5 Estrous Cycle Determination

Images illustrating different phases of the estrous cycle are shown in Figure 2.4. The estrous cycles were tracked using the vaginal smear technique. This involved sampling the cells of the vaginal canal with distilled water using a cotton swab. The recovered material on the cotton swab was then placed on individual microscope slides to later determine respective estrous phases of each female. This was completed using a phase-contrast microscope under 10X magnification. Estrous samples were taken in phase one to ensure regular cycling in all females but were not used in the analyses. In phase two, proestrus was determined by the dispersed scattering of predominantly cornified cells and the presence of nucleated cells. Diestrus was identified by the reduction in the number of cells and the presence of leukocytes (Marcondes, Bianchi, & Tanno, 2002). Following determination of respective estrous phases for each rat sample, it was confirmed that each rat assigned to the diestrus or proestrus group was in fact tested during diestrus or proestrus, respectively, on both the social behavior day of testing and during the light-dark box test. No disruptions to estrous cycle phases were observed in the rats following drug treatment. All rats used in this study exhibited a regular 4-day cycle.

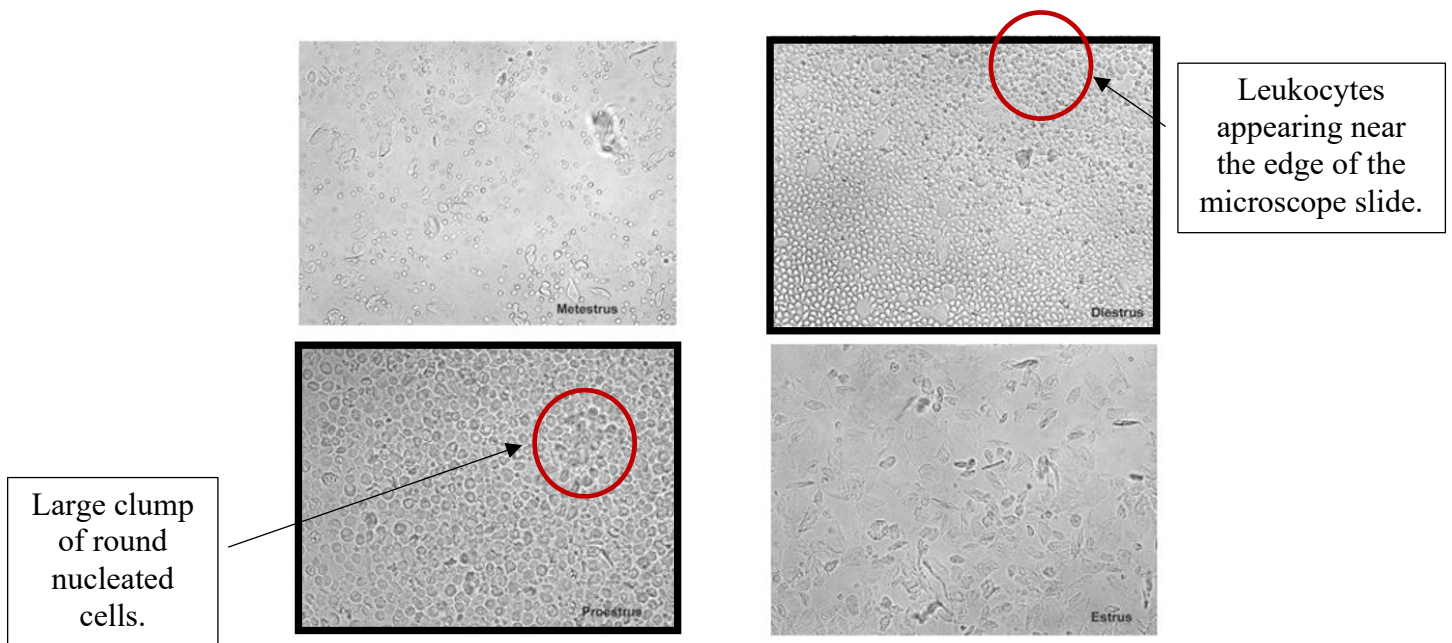


Figure 2.4. Estrous Cycle Phases. Reference images for all four phases of the rodent estrous cycle. Metestrus contains equal proportions of cell types (i.e. leukocytes, cornified cells, nucleated cells). Diestrus contains many small leukocytes which often branch near the edges of microscope slides. Proestrus contains big clusters of round, nucleated cells. Estrus contains sheets of cornified cells and few to no nucleated cells. Phases utilized in this study (Proestrus and Diestrus) are indicated in boxes.

2.2.6 Data Analysis

The statistical analyses conducted were the same in all three phases of the present study.

2.2.6.1 Social Behavior

A randomly selected subset of videos were manually scored by another blinded experimenter in order to calculate inter-rater reliability via Pearson correlations. All statistical tests used $\alpha = 0.05$ as a significance criterion. A 2x2 Univariate Analysis of Variance (ANOVA) was conducted to compare group means. Statistical analyses were performed using IBM SPSS Statistics 23 for Windows.

2.2.6.2 Light Dark Box Test

All activity variables were corrected for the total time spent in the light and dark chamber respectively (e.g. corrected total distance travelled in the light chamber = total distance travelled per second spent in the light chamber). A Univariate Analysis of Variance (ANOVA) was conducted to compare PPA sample means to PBS sample means in both males and females in phase one as well as proestrus and diestrus sample means in phase two. Additionally, a 3-way Mixed Model ANOVA was performed to compare baseline levels of anxiety to anxiety levels 24hr after habituation when PPA or PBS was injected in phase one. All statistical tests used $\alpha = 0.05$ as a significance criterion. Statistical analyses were performed using IBM SPSS Statistics 23 for Windows.

2.3 Results

2.3.1 Phase One

2.3.1.1 Social Behavior

Frequency of Social Initiations. As shown in Figure 2.5, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 47) = 83.57, p < .001$). A main effect of sex was found ($F(1, 47) = 4.85, p < .05$). No significant Drug X Sex interaction was found ($F(1, 47) = 1.53, p = .223$). Rats treated with PBS, regardless of whether they were male or female, displayed significantly more social initiations than rats treated with PPA. Males displayed significantly more social initiations than females, regardless of the drug injected.

Probability of Defense. As shown in Figure 2.6, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 47) = 30.60, p < .001$). A main effect of sex was not found ($F(1, 47) = .921, p = .342$). No significant Drug X Sex interaction was found ($F(1, 47) = .179, p = .674$). Rats treated with PBS, regardless of whether they were male or female, were significantly more likely to elicit a defense in response to a social initiation than rats treated with PPA.

Type of Defense. Two types of defenses were observed, facing and evasion. As shown in Figure 2.7A, the 2 x 2 between-subjects ANOVA did not reveal a main effect of drug ($F(1, 47) = 3.69, p = .061$) or sex ($F(1, 47) = .017, p = .897$) for facing defense. A significant Drug X Sex interaction for facing defense was not detected ($F(1, 47) = .094, p = .761$). As shown in Figure 2.7B, no significant main effect of drug ($F(1, 47) = .960, p = .332$) or sex ($F(1, 47) = .391, p = .535$) was detected for evasion. No significant Drug X Sex interaction was found ($F(1, 47) = .080, p = .778$) for evasive defense. There were no statistically significant differences between PPA and PBS treated animals, regardless of sex, with regards to type of defense (facing or evasive) elicited.

Inter-rater reliability for the manually scored variables was high: Frequency of Social Initiations ($r(16) = .94, p < .001$), Facing Defense ($r(16) = .82, p < .001$), and Evasive Defense ($r(16) = .97, p < .001$).

Frequency of Social Initiations

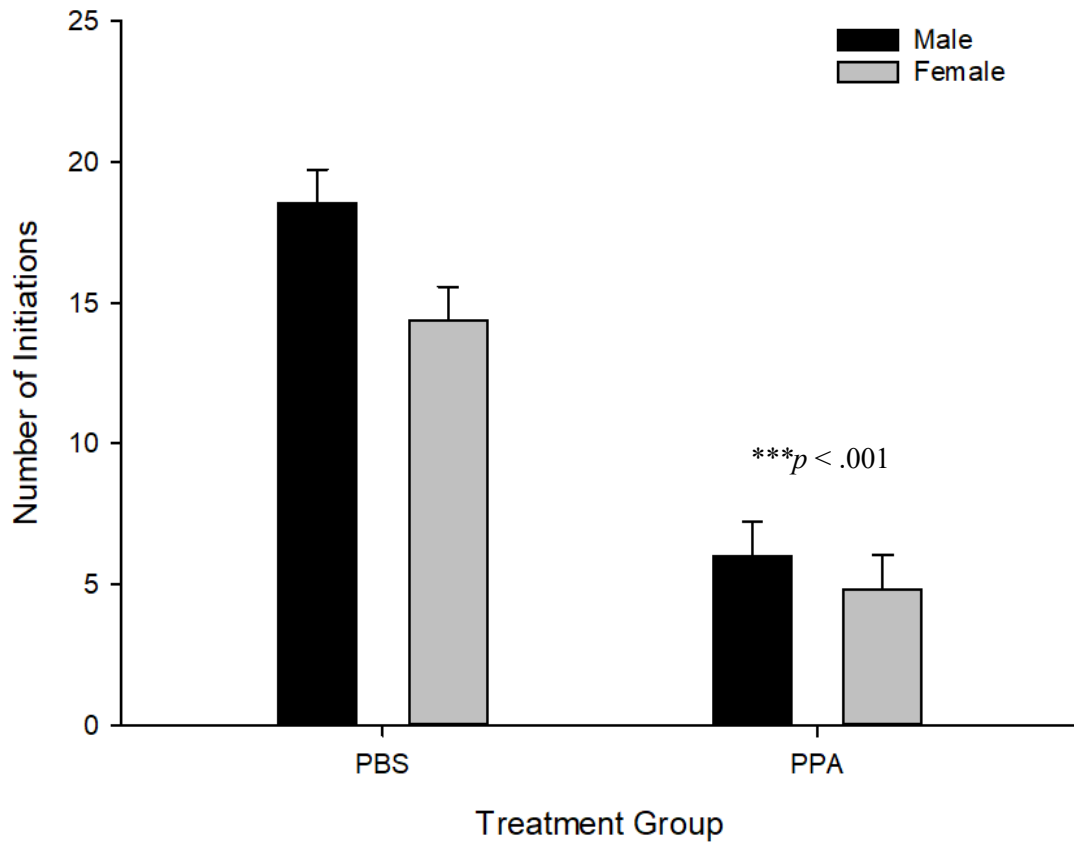


Figure 2.5. Frequency of Social Initiations Displayed by PPA or PBS Treated Rats. Mean (\pm S.E.M) frequency of social initiations expressed by the 4 groups (Male+PPA ($n = 13$), Male+PBS ($n = 13$), Female+PPA ($n = 12$), & Female+PBS ($n = 13$)), during the 20-minute social behavior test. Only the first 10 minutes were scored. Both PBS groups displayed significantly greater social initiations than PPA groups ($***p < .001$). No significant Drug X Sex interaction was detected ($p = .223$).

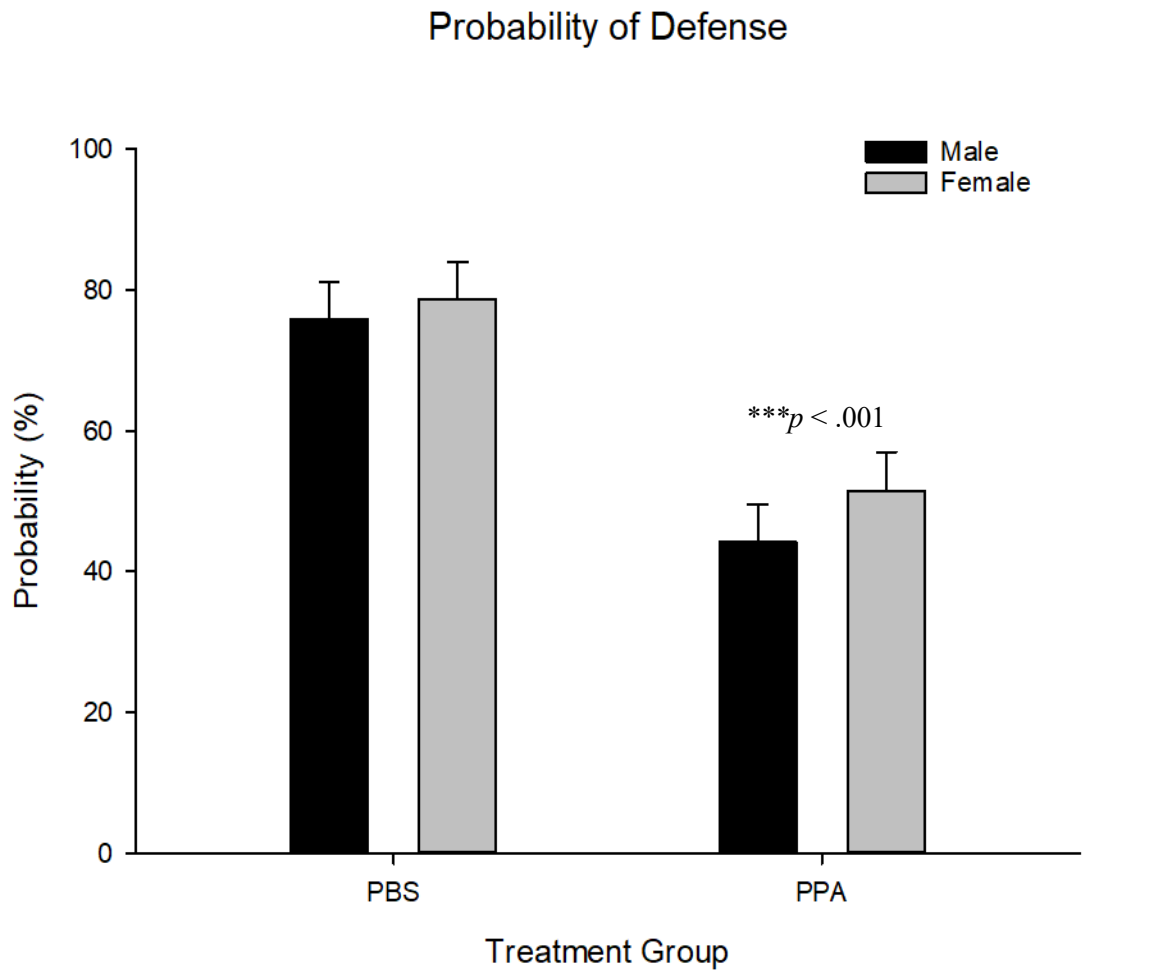


Figure 2.6. Probability of Defense by PPA or PBS Treated Rats (defined as a withdraw of the nape from the stranger’s snout divided by the total number of social initiations by the stranger x 100). Mean (\pm S.E.M) probability of defense shown by the 4 groups (Male+PPA (n = 13), Male+PBS (n = 13), Female+PPA (n = 12), & Female+PBS (n = 13)), during the 20-minute social behavior test. Only the first 10 minutes were scored. Both PBS groups were significantly more likely to elicit a defense in response to a social initiation than PPA groups ($***p < .001$). No significant Drug X Sex interaction was detected ($p = .674$).

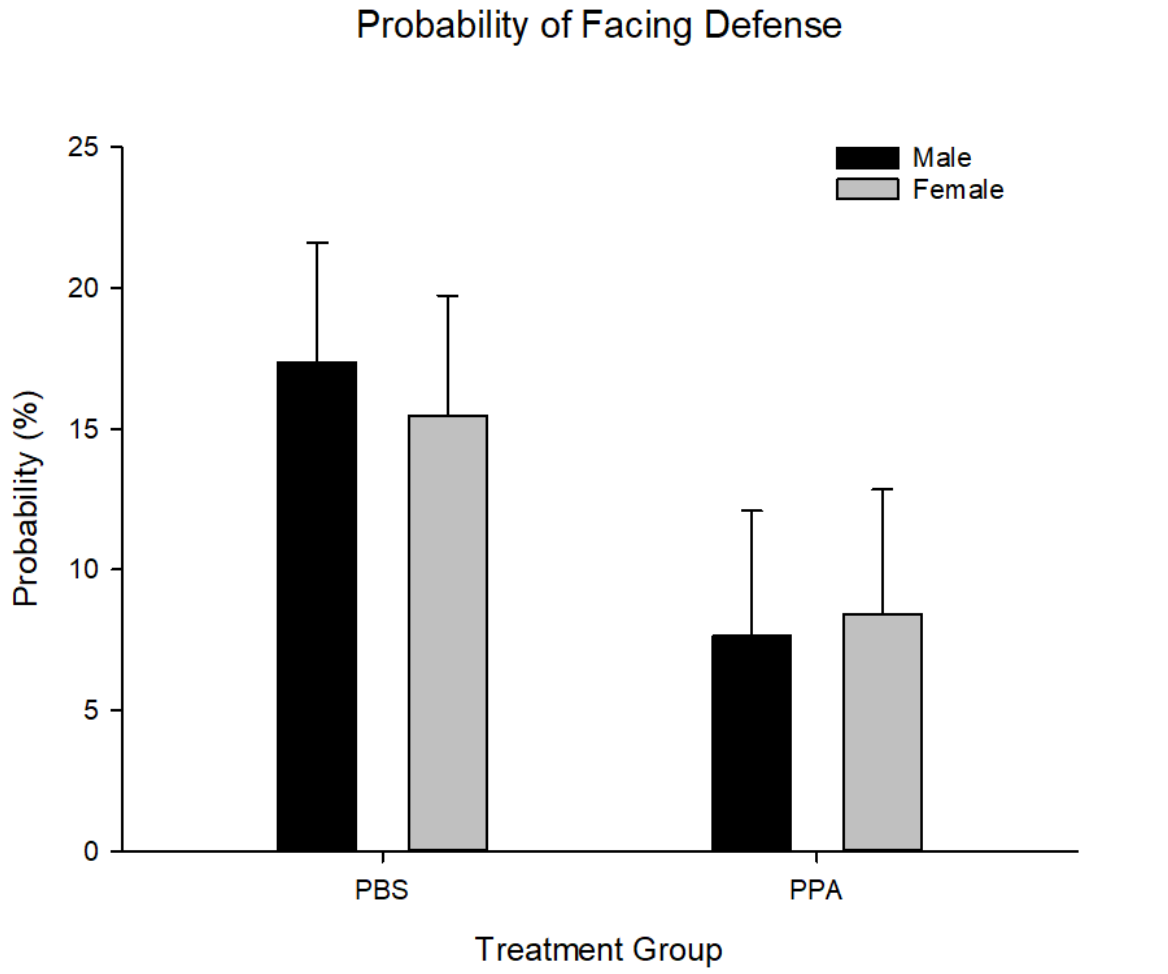


Figure 2.7A. Probability of Facing Defense (defined as a withdraw of the nape from the stranger's snout by facing the stranger divided by the total number of defenses x 100). Mean (\pm S.E.M) probability of facing defense shown by the 4 groups (Male+PPA ($n = 13$), Male+PBS ($n = 13$), Female+PPA ($n = 12$), & Female+PBS ($n = 13$)), during the 20-minute social behavior test. Only the first 10 minutes were scored. PPA and PBS treated rats, regardless of sex, did not display significantly different probabilities of facing defense ($p = .061$). No significant Drug X Sex interaction was detected ($p = .761$).

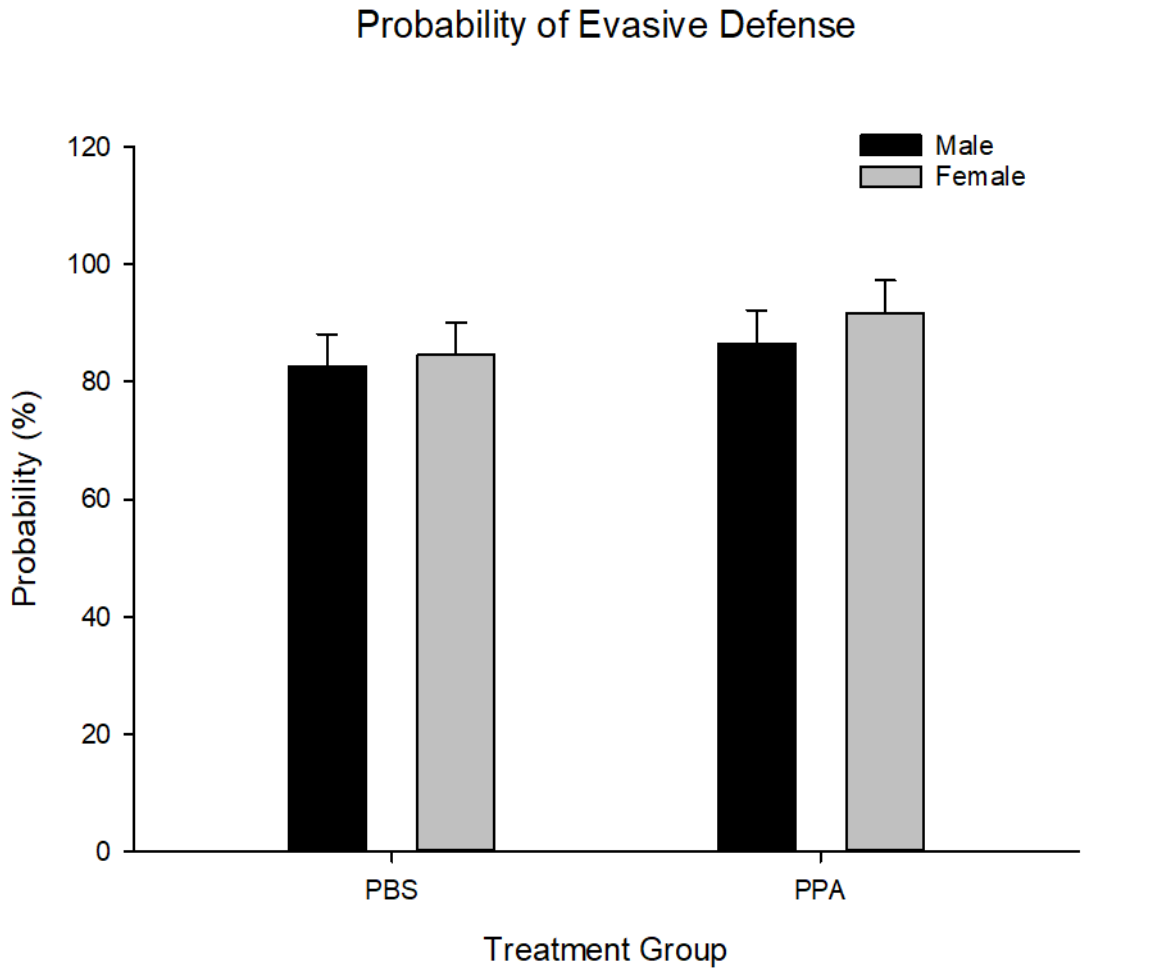


Figure 2.7B. Probability of Evasive Defense (defined as a withdraw of the nape from the stranger’s snout by either leaping, running, or turning away from the stranger, divided by the total number of defenses x 100). Mean (\pm S.E.M) probability of evasive defense shown by the 4 groups (Male+PPA (n = 13), Male+PBS (n = 13), Female+PPA (n = 12), & Female+PBS (n = 13)), during the 20-minute social behavior test. Only the first 10 minutes were scored. PPA and PBS treated rats, regardless of sex, did not display significantly different probabilities of evasive defense ($p = .332$). No significant Drug X Sex interaction was detected ($p = .778$).

2.3.1.2 Light Dark Test

2.3.1.2.1 Habituation Day (Session 1; No injection) compared to Testing Day (Session 2; PPA/PBS Injection)

Nosepokes into the Light Chamber.

As shown in Figure 2.8, the Mixed Model ANOVA revealed a significant main effect of time ($F(1, 47) = 67.16, p < .001$) and drug ($F(1, 47) = 5.73, p < .05$). A significant main effect of sex was found ($F(1, 47) = 4.05, p < .05$). A significant Time X Drug interaction was found ($F(1, 47) = 11.35, p < .001$). A significant Time X Sex interaction was found, ($F(1, 47) = 19.28, p < .001$). A significant Time X Group X Sex interaction was not detected ($F(1, 47) = 2.65, p = .110$). Animals, regardless of sex and drug injected, displayed significantly ($p < .001$) more nosepokes into the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2) when the respective drug (PPA or PBS) was injected. PBS treated animals, regardless of time and sex, displayed more nosepokes ($p < .05$) in the light chamber compared to PPA treated animals. Males, regardless of drug and time, displayed more nosepokes ($p < .05$) compared to females. PPA treated animals displayed significantly less nosepokes ($p < .001$) than PBS in Session 2, but not in Session 1. Males displayed more nosepokes ($p < .001$) into the light chamber compared to females in Session 1 but not during Session 2.

Duration of Time Spent in the Light Chamber.

As shown in Figure 2.9, the Mixed Model ANOVA revealed a significant main effect of time ($F(1, 47) = 40.52, p < .001$) and drug ($F(1, 47) = 6.15, p < .05$). A significant main effect of sex was found ($F(1, 47) = 12.38, p < .001$). A significant Time X Drug interaction was found ($F(1, 47) = 7.14, p < .01$). A significant Time X Sex interaction was not found, ($F(1, 47) = .441, p = .510$). A significant Time X Group X Sex interaction was not found ($F(1, 47) = 1.10, p = .301$). Animals, regardless of sex and drug injected, spent significantly ($p < .001$) more time in the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2) when the respective drug (PPA or PBS) was injected. PBS treated animals, regardless of time and sex,

spent more time ($p < .05$) in the light chamber compared to PPA treated animals. Males, regardless of time and drug, spent less time ($p < .001$) in the light chamber compared to females. PPA treated animals displayed significantly less time ($p < .01$) than PBS treated animals in Session 2 but not in Session 1.

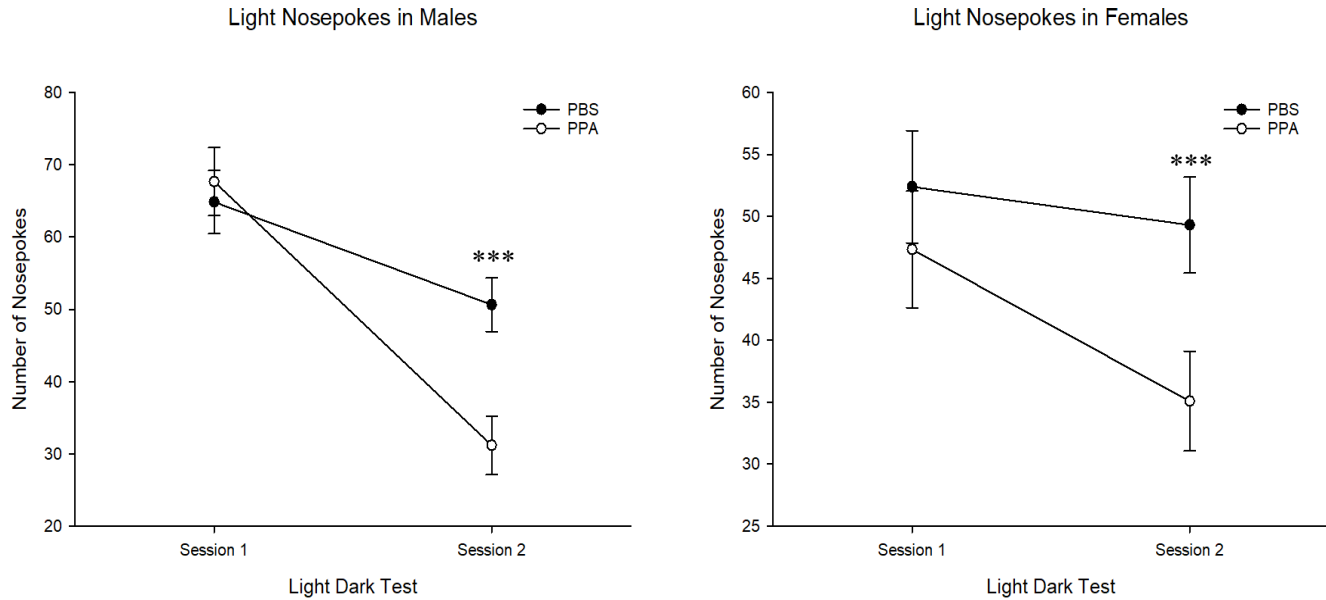


Figure 2.8. Light Nosepokes. Mean (\pm S.E.M) light nosepokes shown by the 4 groups (Male+PPA ($n = 13$), Male+PBS ($n = 13$), Female+PPA ($n = 12$), & Female+PBS ($n = 13$)), during the two 20-minute light-dark tests. Animals, regardless of sex and drug injected, displayed significantly more nosepokes into the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2) when the respective drug (PPA or PBS) was injected ($***p < .001$). PBS treated animals, regardless of time and sex, displayed more nosepokes in the light chamber compared to PPA treated animals ($p < .05$). Males, regardless of drug and time, displayed more nosepokes compared to females ($p < .05$). PPA treated animals displayed significantly less nosepokes than PBS in Session 2, but not in Session 1 ($p < .001$). Males displayed more nosepokes into the light chamber compared to females in Session 1 but not during Session 2 ($p < .001$).

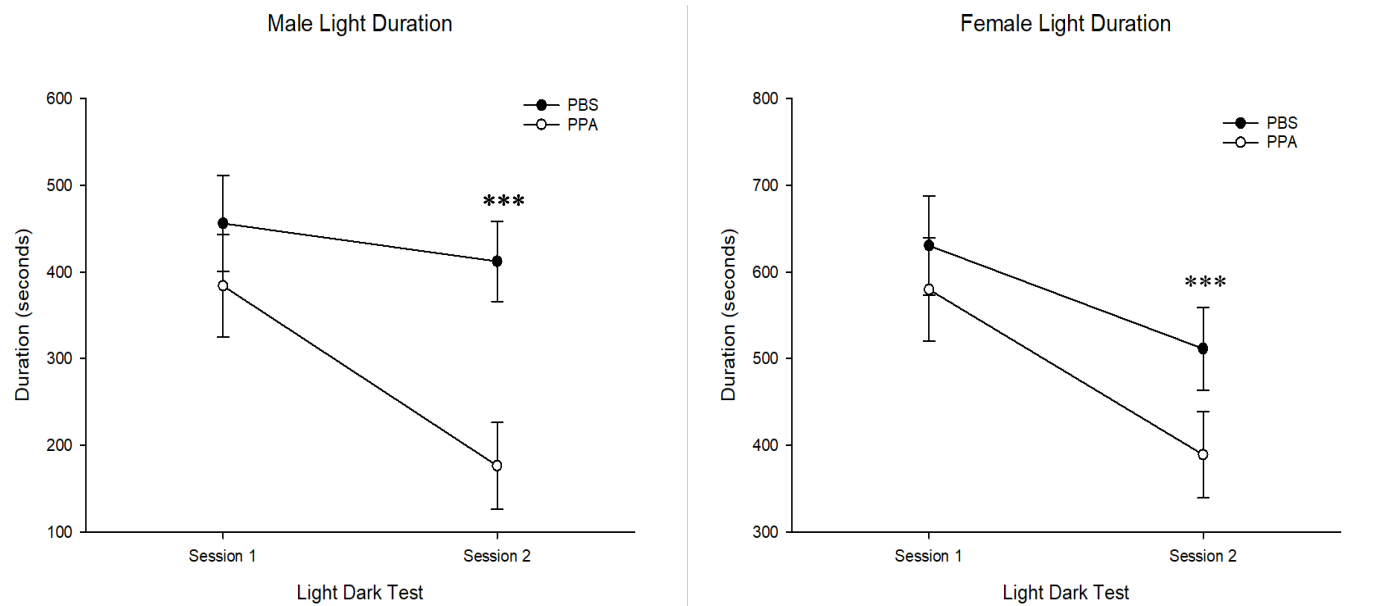


Figure 2.9. Light Duration. Mean (\pm S.E.M) light duration shown by the 4 groups (Male+PPA ($n = 13$), Male+PBS ($n = 13$), Female+PPA ($n = 12$), & Female+PBS ($n = 13$)), during the two 20-minute light-dark tests. Animals, regardless of sex and drug injected, displayed a significantly greater duration in the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2) when the respective drug was injected ($***p < .001$). PBS treated animals, regardless of time and sex, spent more time in the light chamber compared to PPA treated animals ($p < .05$). Males, regardless of time and drug, spent less time in the light chamber compared to females ($p < .001$). PPA treated animals displayed significantly less time than PBS treated animals in Session 2 but not in Session 1 ($p < .01$).

2.3.1.2.2 Testing Day

Nosepokes into the Light Chamber. As shown in Figure 2.10, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 47) = 18.54, p < .001$), but not for sex ($F(1, 47) = .290, p = .743$). No significant interaction was detected ($F(1, 47) = .321, p = .506$). PPA treated animals, regardless of sex, displayed significantly fewer nosepokes into the light-chamber in comparison to PBS treated animals.

Transitions into the Light Chamber. As shown in Figure 2.11, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 47) = 43.20, p < .001$) and sex ($F(1, 47) = 8.05, p < .01$). No significant Drug X Sex interaction was found ($F(1, 47) = .040, p = .312$). PPA treated animals, regardless of sex, displayed significantly less transitions into the light-chamber in comparison to PBS treated animals. Females exhibited a significantly greater number of transitions into the light chamber, regardless of the drug administered.

Duration of Time Spent in the Light Chamber. As shown in Figure 2.12, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 47) = 13.65, p < .001$) and sex ($F(1, 47) = 10.36, p < .01$). No significant Drug X Sex interaction was found ($F(1, 47) = .446, p = .248$). PPA treated animals, regardless of sex, displayed significantly less time in the light-chamber in comparison to PBS treated animals. Females spent significantly more time in the light chamber, regardless of the drug administered.

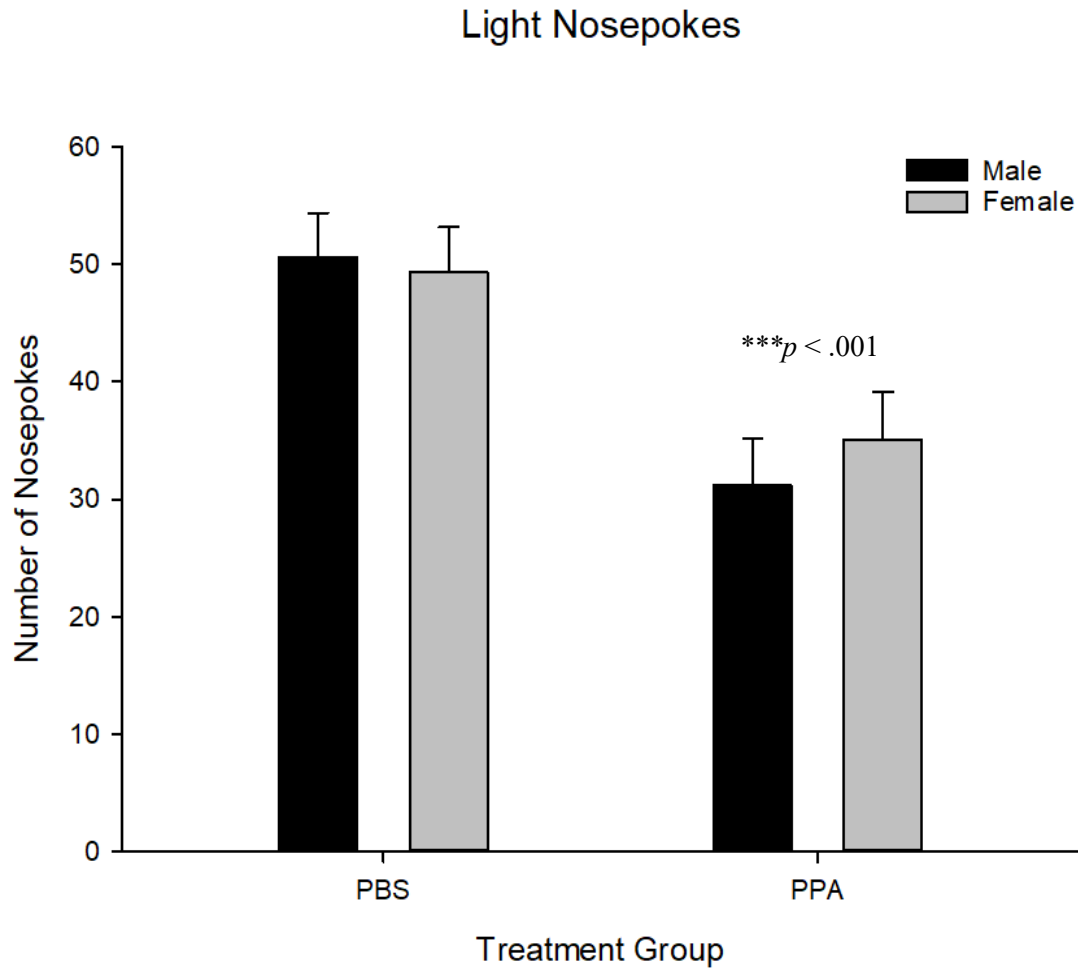


Figure 2.10. Light Nosepokes. Mean (\pm S.E.M) number of nosepokes into the light chamber of the light-dark apparatus expressed by the 4 groups (Male+PPA ($n = 13$), Male+PBS ($n = 13$), Female+PPA ($n = 12$), & Female+PBS ($n = 13$)), during the 20-minute light-dark box test. PPA treated animals, regardless of sex, displayed significantly fewer nose pokes into the light-chamber in comparison to PBS treated animals ($***p < .001$). No significant Drug X Sex interaction was detected ($p = .506$).

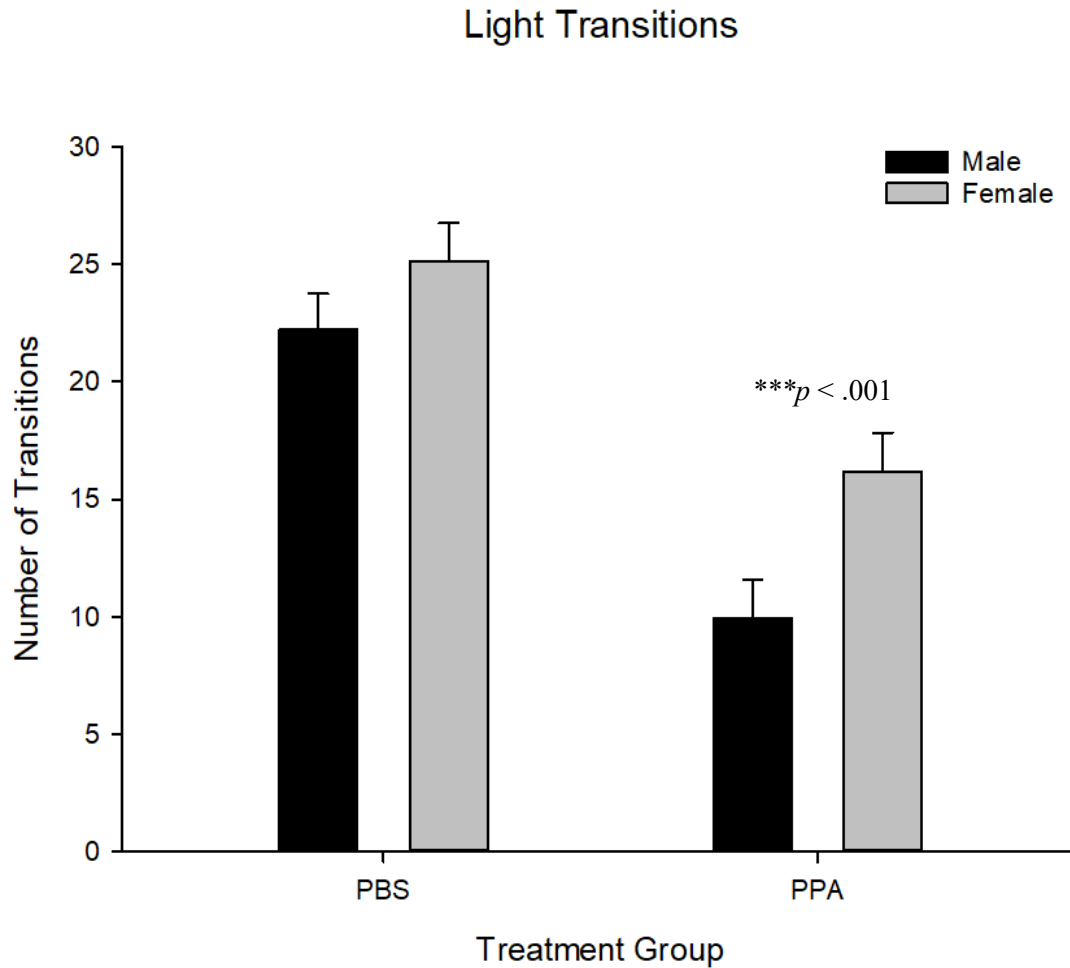


Figure 2.11. Light Transitions. Mean (\pm S.E.M) number of transitions into the light chamber of the light-dark apparatus expressed by the 4 groups (Male+PPA ($n = 13$), Male+PBS ($n = 13$), Female+PPA ($n = 12$), & Female+PBS ($n = 13$)), during the 20-minute light-dark box test. PPA treated animals displayed significantly less transitions into the light-chamber in comparison to PBS treated animals ($***p < .001$). Females exhibited a significantly greater number of transitions into the light chamber compared to males, regardless of the drug administered ($p < .01$). No significant Drug X Sex interaction was detected ($p = .312$).

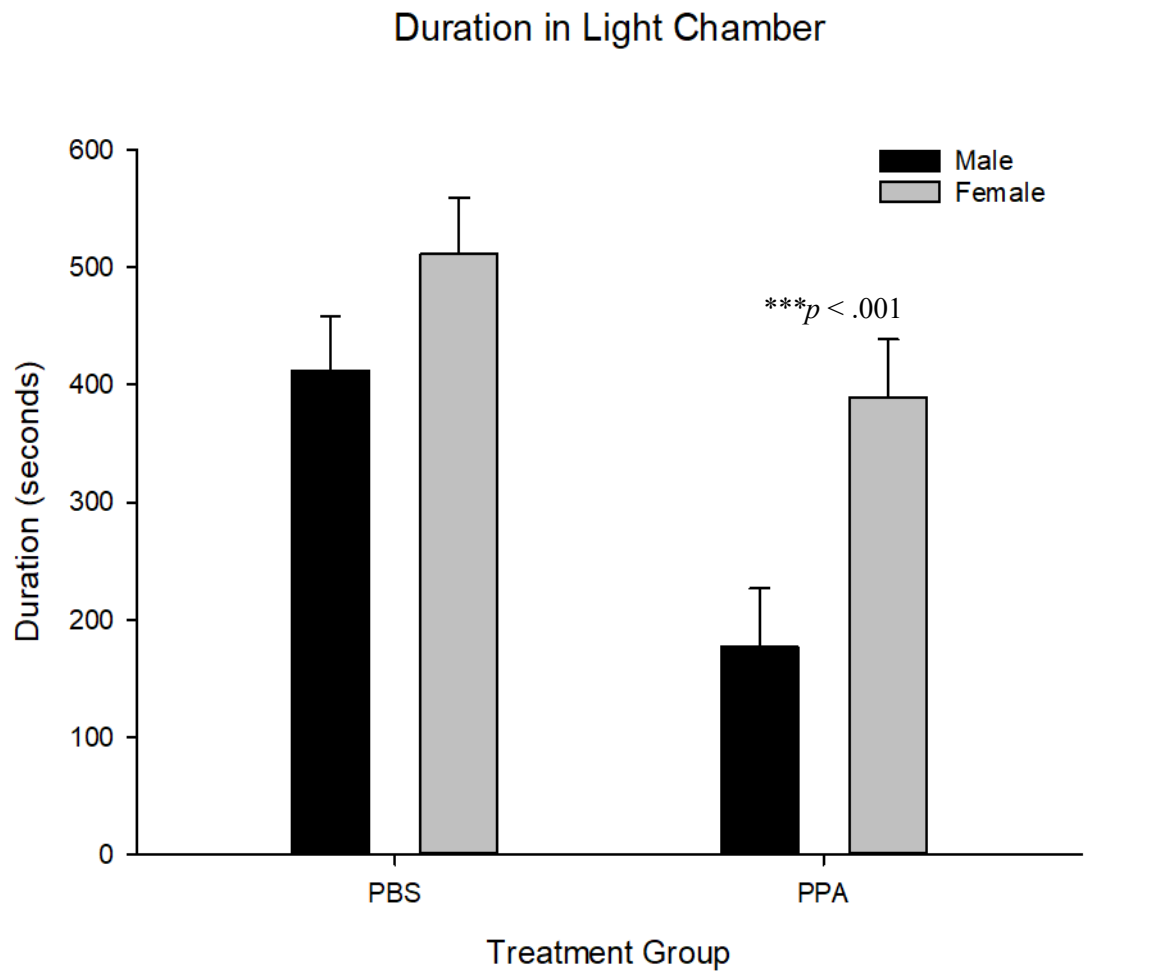


Figure 2.12. Duration in Light Chamber. Mean (\pm S.E.M) duration of time spent in the light chamber of the light-dark apparatus expressed by the 4 groups (Male+PPA ($n = 13$), Male+PBS ($n = 13$), Female+PPA ($n = 12$), & Female+PBS ($n = 13$)), during the 20-minute light-dark box test. PPA treated animals displayed significantly less time in the light-chamber in comparison to PBS treated animals ($***p < .001$). Females spent significantly more time in the light chamber compared to males, regardless of the drug administered ($p < .01$). No significant Drug X Sex interaction was detected ($p = .248$).

2.3.2 Phase Two

2.3.2.1 Social Behavior

Frequency of Social Initiations. As shown in Figure 2.13, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 28) = 13.299, p < .001$). No main effect of estrous phase was detected ($F(1, 28) = 1.894, p = .180$). No significant Drug X Estrous Phase interaction was found ($F(1, 28) = .473, p = .497$). Rats treated with PBS, regardless of whether they were tested during proestrus or diestrus, displayed significantly more social initiations than rats treated with PPA.

Probability of Defense. As shown in Figure 2.14, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 28) = 15.821, p < .001$). No main effect of estrous phase was found ($F(1, 28) = 3.034, p = .093$). No significant Drug X Estrous Phase interaction was found ($F(1, 28) = .083, p = .775$). Rats treated with PBS, regardless of whether they were tested during proestrus or diestrus, were significantly more likely to elicit a defense in response to a social initiation than rats treated with PPA.

Type of Defense. Two types of defenses were observed, facing and evasion. As shown in Figure 2.15A, the 2 x 2 between-subjects ANOVA did not reveal a significant main effect of drug ($F(1, 28) = .849, p = .365$) or estrous phase ($F(1, 28) = .115, p = .737$) for facing defense. A significant Drug X Estrous Phase interaction was not detected ($F(1, 28) = .075, p = .786$) for probability of facing defense. As shown in Figure 2.15B, no main effect of drug ($F(1, 28) = .864, p = .361$) or estrous phase ($F(1, 28) = .121, p = .730$) was found for evasion. A significant Drug X Estrous Phase interaction was not found ($F(1, 28) = .071, p = .792$) for probability of evasive defense. There were no statistically significant differences between PPA and PBS treated animals, regardless of estrous phase, with regards to type of defense (facing or evasive) elicited.

Frequency of Social Initiations

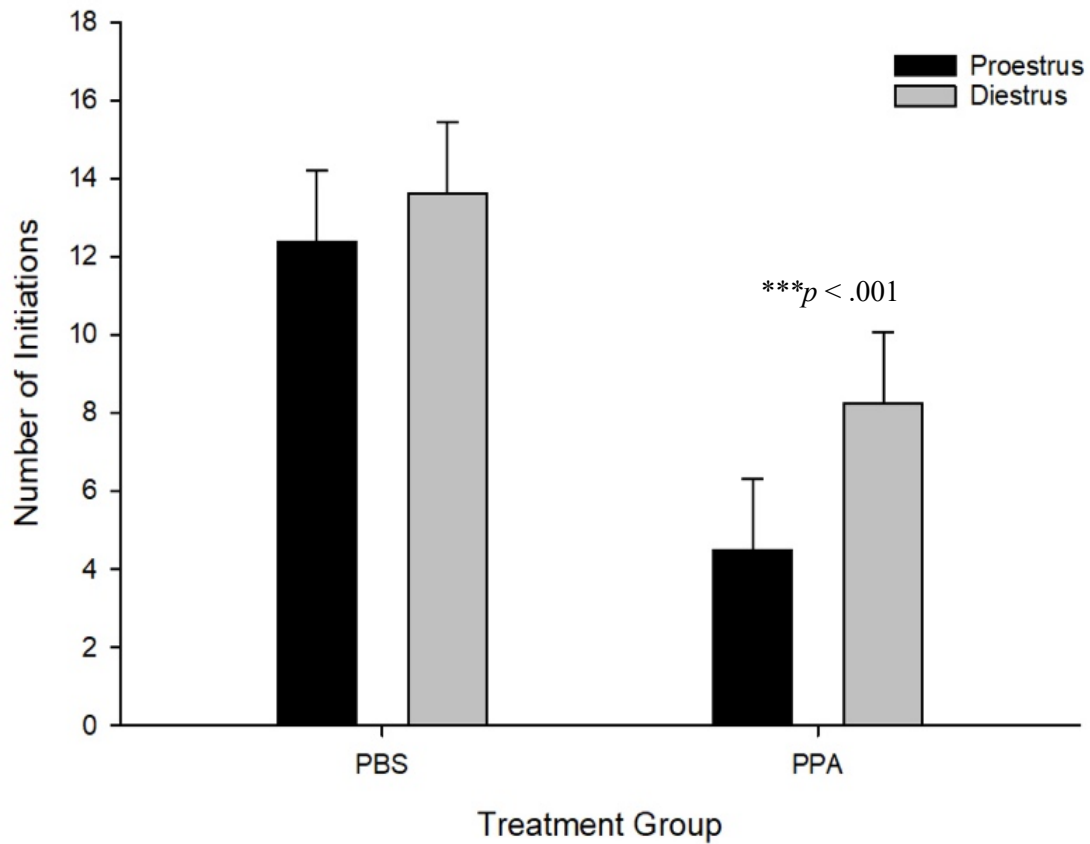


Figure 2.13. Frequency of Social Initiations Displayed by PPA or PBS Treated Rats. Mean (\pm S.E.M) frequency of social initiations expressed by the 4 groups (Proestrus+PPA ($n = 8$), Proestrus+PBS ($n = 8$), Diestrus+PPA ($n = 8$), & Diestrus+PBS ($n = 8$)), during the 20-minute social behavior test. Only the first 10 minutes were scored. Rats treated with PBS, regardless of estrous phase, displayed significantly more social initiations than rats treated with PPA ($***p < .001$). A significant Drug X Estrous Phase interaction was not detected ($p = .497$)

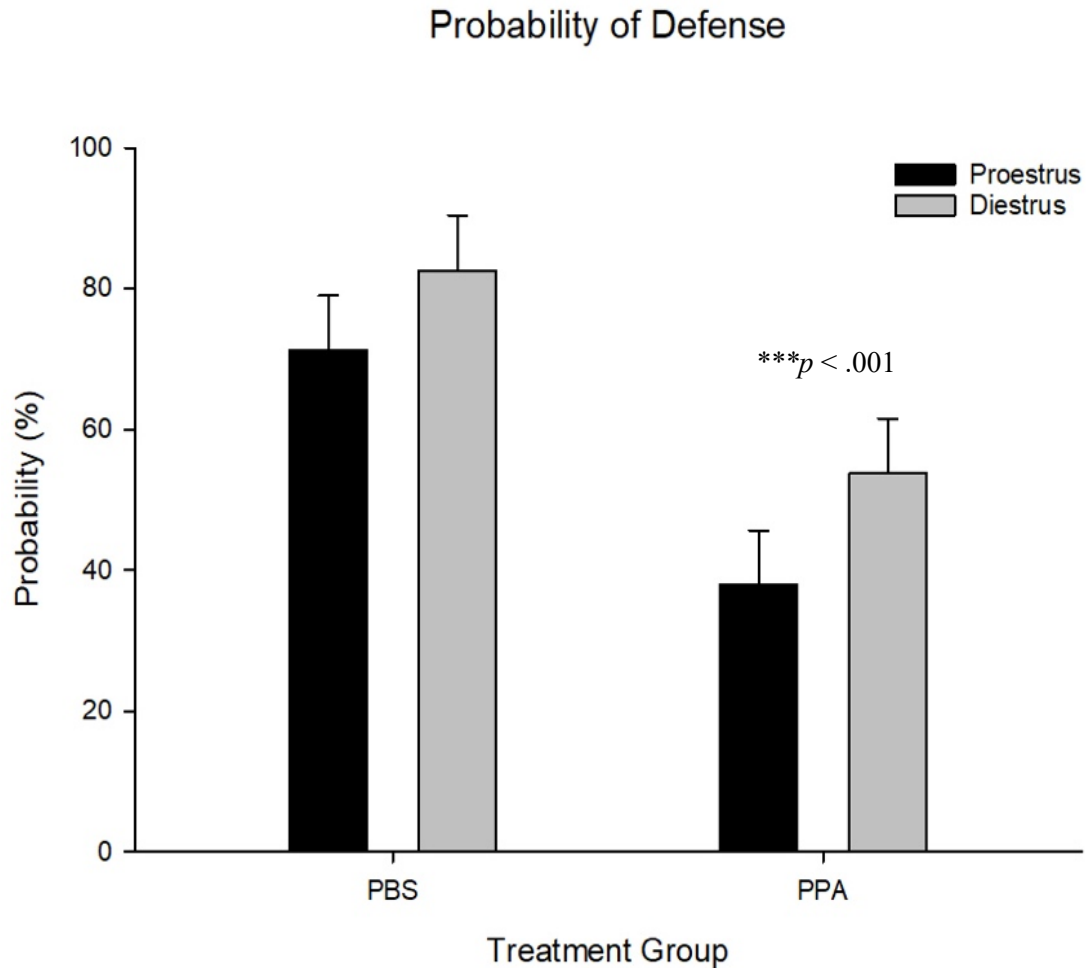


Figure 2.14. Probability of Defense by PPA or PBS Treated Rats (defined as a withdraw of the nape from the stranger's snout divided by the total number of social initiations by the stranger x 100). Mean (\pm S.E.M) probability of defense shown by the 4 groups (Proestrus+PPA (n = 8), Proestrus+PBS (n = 8), Diestrus+PPA (n = 8), & Diestrus+PBS (n = 8)), during the 20-minute social behavior test. Only the first 10 minutes were scored. Both PBS groups were significantly more likely to elicit a defense in response to a social initiation than PPA groups (** $p < .001$). No significant Drug X Estrous Phase interaction was detected ($p = .775$)

Probability of Facing Defense

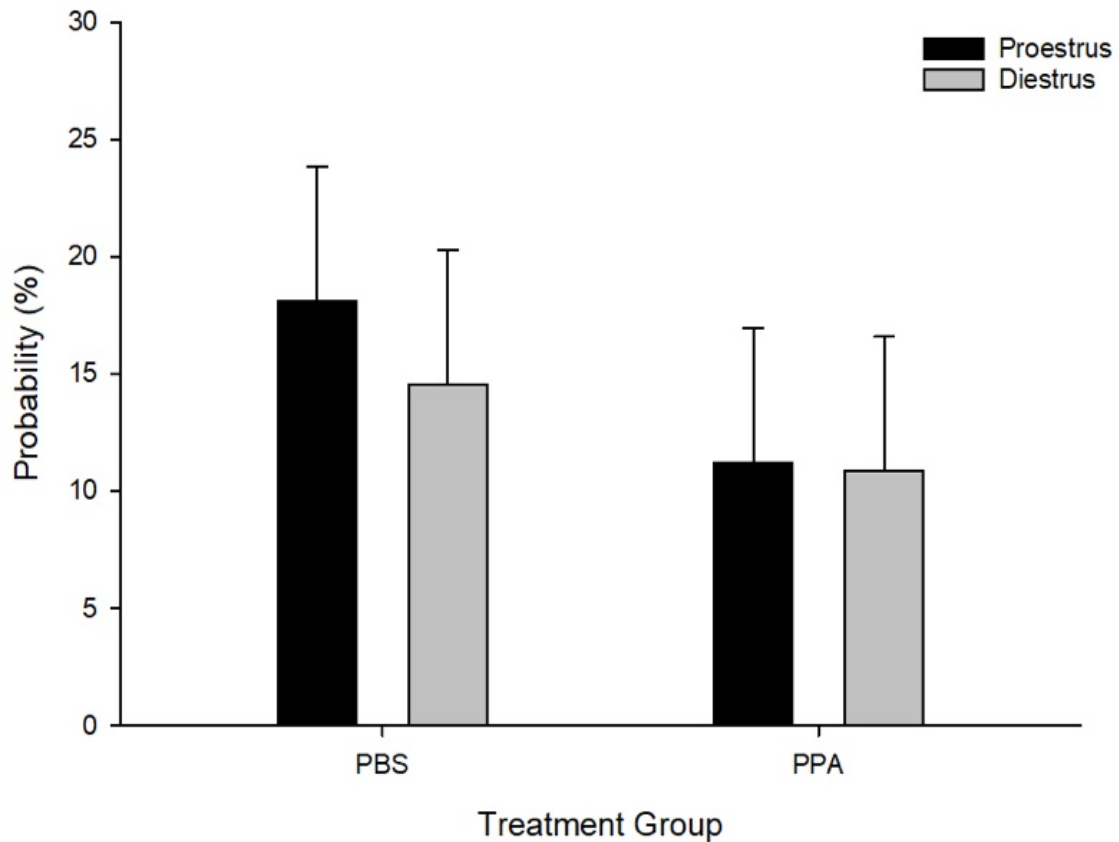


Figure 2.15A. Probability of Facing Defense (defined as a withdraw of the nape from the stranger's snout by facing the stranger divided by the total number of defenses x 100). Mean (\pm S.E.M) probability of facing defense shown by the 4 groups (Proestrus+PPA ($n = 8$), Proestrus+PBS ($n = 8$), Diestrus+PPA ($n = 8$), & Diestrus+PBS ($n = 8$)), during the 20-minute social behavior test. Only the first 10 minutes were scored. PPA and PBS treated rats, regardless of estrous phase, did not display significantly different probabilities of facing defense ($p = .365$). No significant Drug X Estrous Phase interaction was detected ($p = .786$).

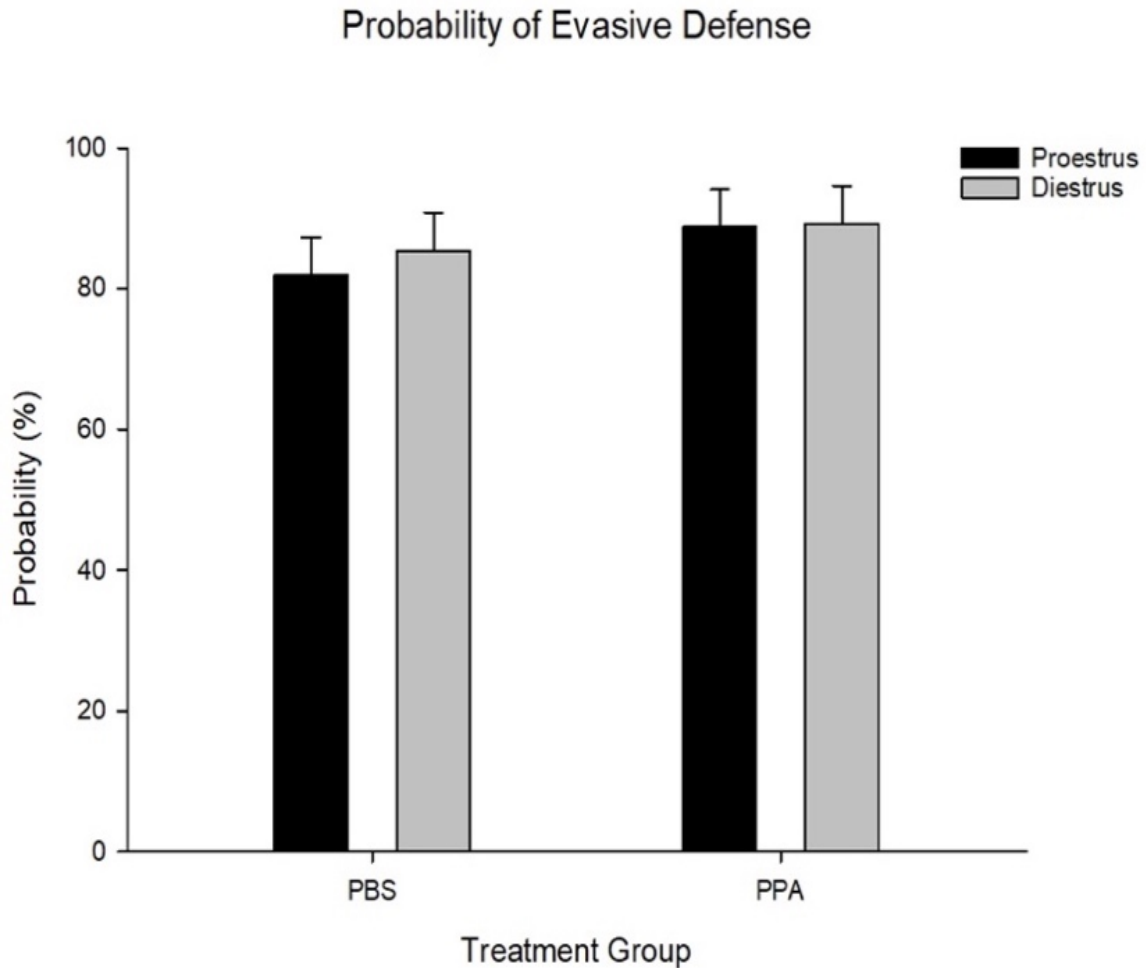


Figure 2.15B. Probability of Evasive Defense (defined as a withdraw of the nape from the stranger's snout by either leaping, running, or turning away from the stranger, divided by the total number of defenses x 100). Mean (\pm S.E.M) probability of evasive defense shown by the 4 groups (Proestrus+PPA ($n = 8$), Proestrus+PBS ($n = 8$), Diestrus+PPA ($n = 8$), & Diestrus+PBS ($n = 8$)), during the 20-minute social behavior test. Only the first 10 minutes were scored. PPA and PBS treated rats, regardless of estrous phase, did not display significantly different probabilities of evasive defense ($p = .361$). No significant Drug X Estrous Phase interaction was detected ($p = .792$).

2.3.2.2 Light Dark Box Test

Nosepokes into the Light Chamber. As shown in Figure 2.16, the 2 x 2 between-subjects ANOVA did not reveal a significant main effect of drug ($F(1, 28) = .504, p = .484$) or estrous phase ($F(1, 28) = .224, p = .640$). No significant Drug X Estrous Phase interaction was detected ($F(1, 28) = .418, p = .523$). There was no significant difference in the number of nosepokes into the light chamber between PPA and PBS-treated rats regardless of estrous phase.

Transitions into the Light Chamber. As shown in Figure 2.17, the 2 x 2 between-subjects ANOVA revealed a main effect of drug ($F(1, 28) = 8.629, p < .01$) but not estrous phase ($F(1, 28) = 1.177, p = .287$). No significant Drug X Estrous Phase interaction was detected ($F(1, 28) = .001, p = .979$). PPA-treated females, regardless of estrous phase, displayed significantly less transitions into the light chamber compared to PBS treated females.

Duration of Time Spent in the Light Chamber. As shown in Figure 2.18, the 2 x 2 between-subjects ANOVA did not reveal a main effect of drug ($F(1, 28) = 1.715, p = .201$) or estrous phase ($F(1, 28) = .483, p = .493$). A significant Drug X Estrous Phase interaction was detected ($F(1, 28) = 5.967, p < .05$). The duration of time in the light chamber displayed by PPA-treated females was dependant on the estrous phase they were in. PPA treated females during diestrus displayed significantly more time in the light chamber than PPA treated females during proestrus.

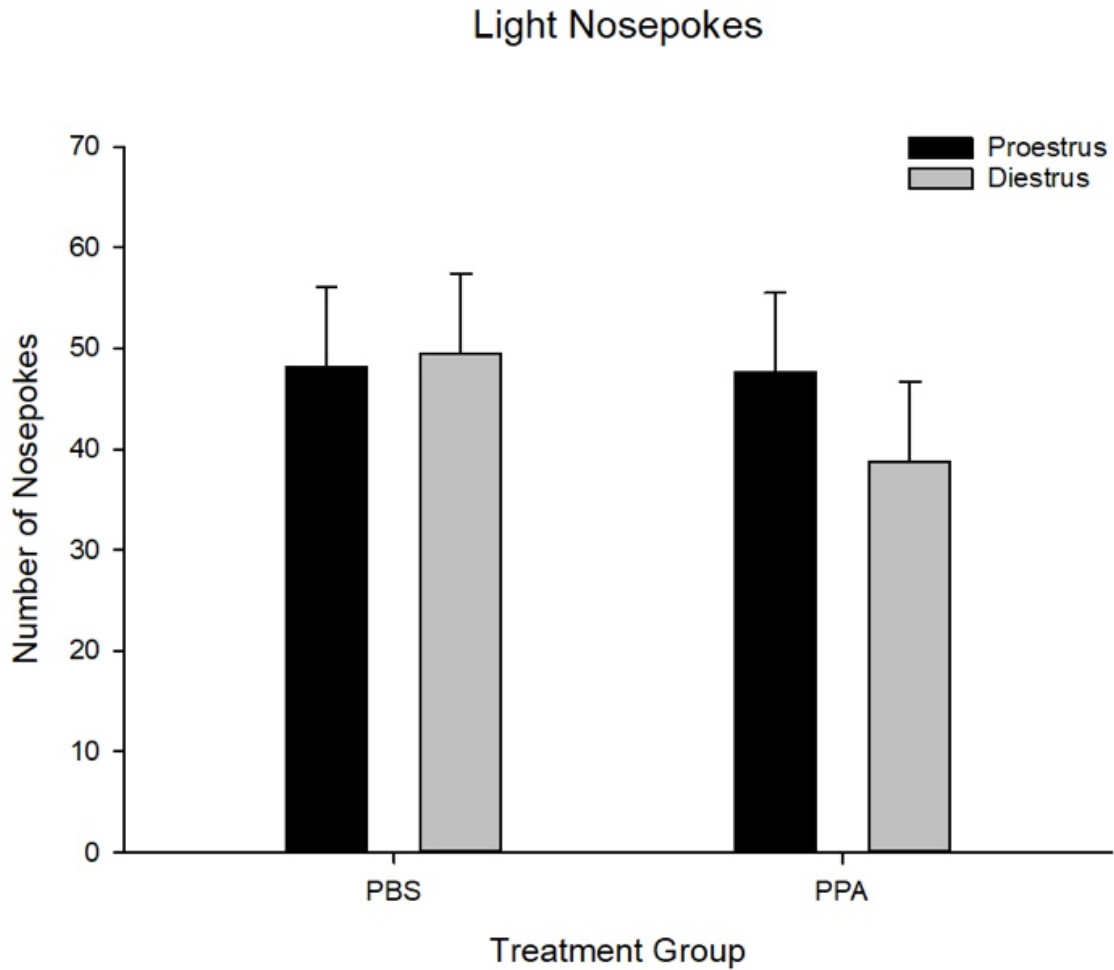


Figure 2.16. Light Nosepokes. Mean (\pm S.E.M) number of nosepokes into the light chamber of the light-dark apparatus expressed by the 4 groups (Proestrus+PPA ($n = 8$), Proestrus+PBS ($n = 8$), Diestrus+PPA ($n = 8$), & Diestrus+PBS ($n = 8$)), during the 20-minute light-dark box test. PPA and PBS treated animals did not display a significantly different number of nosepokes into the light chamber ($p = .484$). No significant Drug X Estrous Phase interaction was detected ($p = .523$).

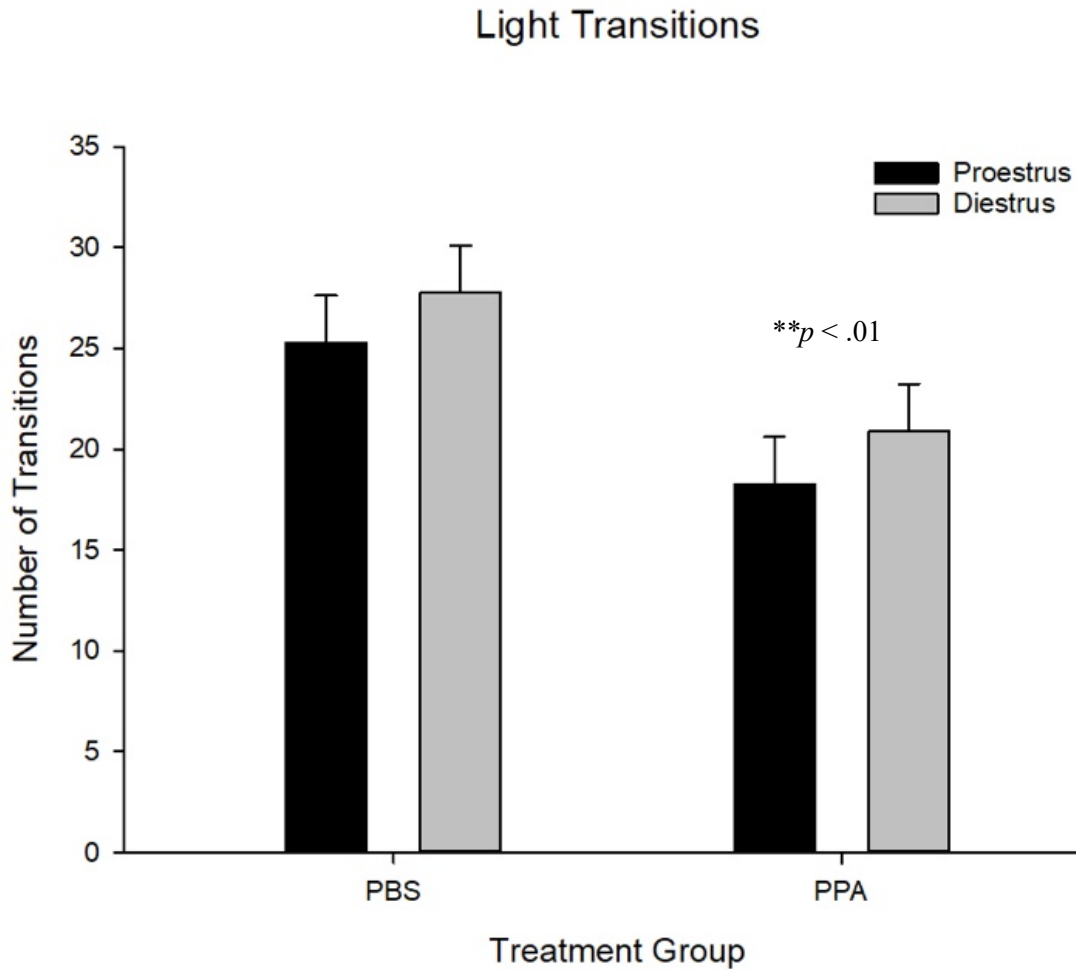


Figure 2.17. Light Transitions. Mean (\pm S.E.M) number of transitions into the light chamber of the light-dark apparatus expressed by the 4 groups (Proestrus+PPA (n = 8), Proestrus+PBS (n = 8), Diestrus+PPA (n = 8), & Diestrus+PBS (n = 8)), during the 20-minute light-dark box test. PPA-treated females, regardless of estrous phase, displayed significantly less transitions into the light chamber compared to PBS treated females (** $p < .01$). No significant Drug X Estrous Phase interaction was detected ($p = .979$).

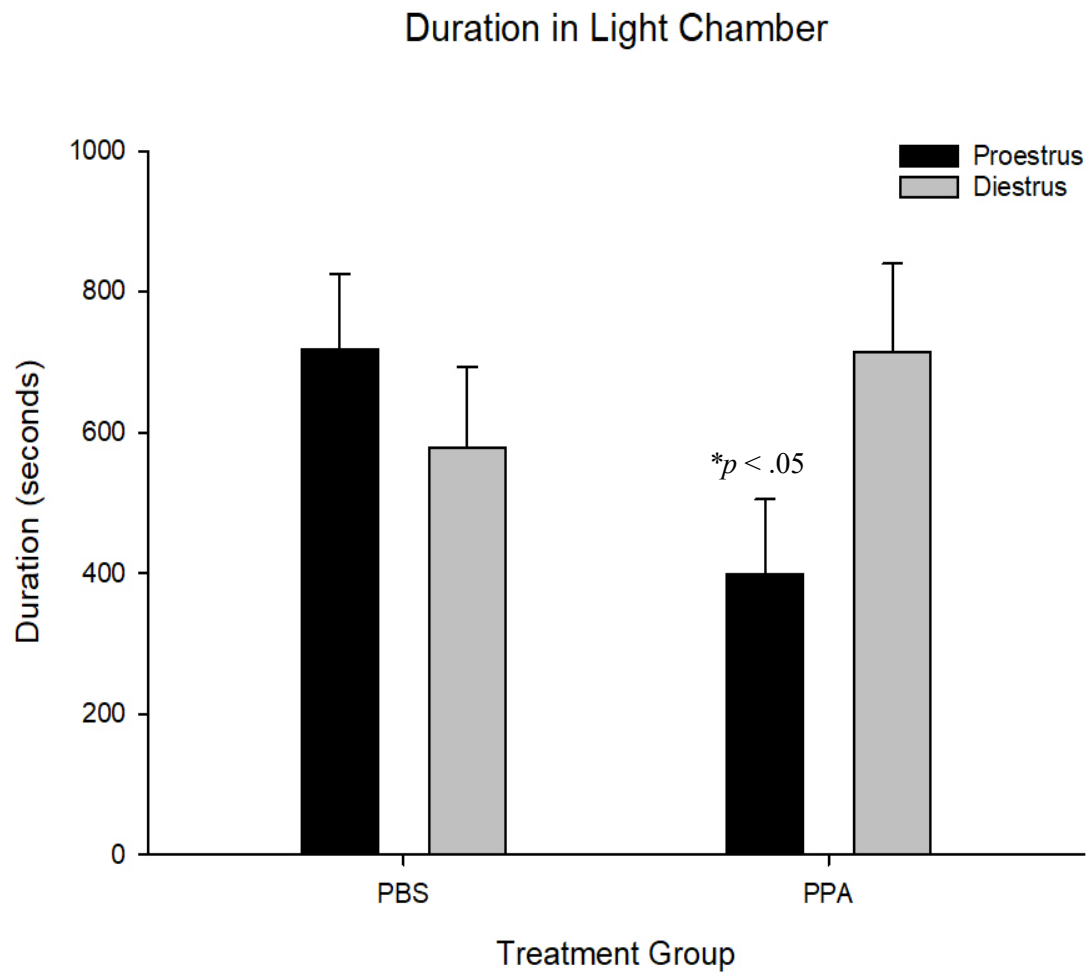


Figure 2.18. Duration in Light Chamber. Mean (\pm S.E.M) duration of time spent in the light chamber of the light-dark apparatus expressed by the 4 groups (Proestrus+PPA (n = 8), Proestrus+PBS (n = 8), Diestrus+PPA (n = 8), & Diestrus+PBS (n = 8)), during the 20-minute light-dark box test. PPA treated diestrus females spent significantly more time in the light chamber in comparison to PPA treated proestrus females ($*p < .05$).

2.4 Discussion

A persistent male bias in ASD prevalence is observed in the literature, and research investigations seeking to explain this bias are inconclusive. Recent reviews have suggested that predominantly female hormones, estradiol and progesterone, may provide some mode of protection in females against the development of several etiological markers for ASD (Crider & Pillai, 2017). The present study explored possible sex differences when PPA is systemically injected and its relation to social behavior and anxiety. The objective of the first phase was to investigate putative sex differences in the effects of intraperitoneal (i.p.) PPA administration in male and female rats. It was predicted that females would display less severe ASD-like impairments in their behavior in the social behavior test (i.e. greater social initiations and increased probability of eliciting a defense) and light dark anxiety paradigm (i.e. increased time spent in the light chamber and greater number of transitions into the light chamber). These results were not obtained, as PPA at 500mg/kg produced statistically similar deficits in male and female rats. The deficits that were obtained were similar to those previously reported in adult males receiving PPA (Shultz et al., 2008; Wah et al., 2019).

The absence of sex differences could be due to the variable modes of action by which PPA induces its effects (MacFabe et al. 2007, Wyse et al., 1998), not all of which may be sexually dimorphic. Due to their ability to cross lipid bilayers, PPA and other short-chain fatty acids are capable of inducing neuroinflammation via the activation of G-Protein Coupled Receptors (GPCR's; Venegas et al., 2019). PPA has been reported to adversely affect brain structures that have been implicated in social and play behavior (i.e. the hippocampus and cingulate cortex), by inducing a neuroinflammatory response (MacFabe et al., 2007; Shultz et al., 2008). PPA has also been found capable of producing significant alterations in dopamine and serotonin transmission, in addition to acting via its own receptor mechanisms, GPCR's (Cannizzaro et al., 2003; Mitsui et al., 2005). The present study explored two variables relating to ASD: reduced social behavior and anxiety. It is possible that the mechanisms by which PPA impacts social behavior and anxiety are the same in males and females, although this has not been investigated. Consistent with the results of rodent PPA studies, findings from structural imaging reports looking at human ASD brains seem to suggest brain abnormalities in regions such as the amygdala and cingulate cortex that are associated with emotion and social behavior (Bauman & Kemper, 1985; Cody,

Pelphrey, & Piven, 2002; Haznedar et al., 2000). However, female representation in these studies is lacking. Larger samples including females are required to characterize brain abnormalities associated with the manifestation of ASD in both sexes as well as the specificity of the actions of PPA in these brain areas.

PPA significantly reduced frequency of social initiations and probability of defense in males and females. Both social initiations and defences are considered a form of pro-social response (Field et al., 2006), demonstrating that PPA decreases social interaction in rodents. Male rats have been found to exhibit greater levels of social and play behavior than female rats. A study by Stack et al. (2010) explored the impact that estrous phase has on social interaction by comparing the behavior of male and female rats. It was observed that male rats exhibited greater levels of social behavior than female rats, regardless of which estrous phase the females were tested in (Stack et al., 2010). These findings lend support to a pattern of results demonstrated in phase one of the present study, where males were significantly more likely to initiate play with the non-treated stranger rat than females, regardless of whether they were treated with PPA or the vehicle.

In line with previous literature (Scholl et al., 2019), baseline levels of anxiety were not consistently different between males and females in phase one. The Mixed Model ANOVA found that males displayed more nose pokes into the light chamber in comparison to females during the habituation day (Session 1), but not on testing day (Session 2) after receiving the injection. However, females spent significantly more time in the light chamber than males during habituation day. Prior investigations have not been able to make well-founded conclusions concerning sex differences in levels of anxiety. Often several anxiety assays are performed, and their results vary in their patterns of male and female differences (Scholl et al., 2019).

The objective of the second phase was to explore whether PPA elicited different behavioral effects at high versus low estradiol/progesterone levels during the rat estrous cycle. The two phases of interest were proestrus and diestrus, as these are the phases when estradiol and progesterone have been measured to be at their highest and lowest levels, respectively (Lebron-Milad & Milad, 2012). It was predicted that the effects of PPA in female rats would be different depending on what stage of the estrous cycle they are in. When estradiol and progesterone levels are high (proestrus), it was expected that females would display less severe ASD-like

impairments as demonstrated through behavior in the social interaction test and light dark anxiety paradigm. The prediction that elevated levels of estradiol and progesterone during proestrus would protect against the adverse effects of PPA was not supported. PPA-treated females tested during proestrus did not display increased social interaction or reduced anxiety-like behavior compared to PPA-treated diestrus females.

PPA-treated females, regardless of estrous phase, were equally likely to engage in social behavior with an untreated female conspecific. This finding suggests that elevated levels of estradiol and progesterone, as measured by vaginal smear estrous tracking, do not provide observable protection against ASD-like social behavior deficits produced by PPA. PPA-treated females were significantly less likely to display social initiations toward the naïve conspecific in comparison to PBS-treated animals, regardless of whether they were tested during the proestrus phase or the diestrus phase. PPA-treated females were also significantly less likely to elicit a defense in comparison to PBS treated females, regardless of estrous phase. Previous literature has demonstrated a greater likelihood of evasive defenses compared to facing defense among PPA-treated animals (Shultz et al., 2008). However, PPA and PBS animals did not exhibit significantly different probabilities in the type of defense elicited. This could be attributed to the different modes of analyses conducted, or perhaps the disparate modes of administration used compared with past studies.

A statistically significant main effect of drug was not consistently observed in the light-dark variables quantified in phase two. The light dark procedure is often utilized in investigations exploring anxiety-like behavior due to rodent's innate tendency to avoid brightly lit and novel environments (Bourin & Hascoët, 2003). Thus, it was expected that PPA-treated animals would display an observable aversion to the light chamber. PPA treated females were not significantly different from PBS treated females in terms of duration of time spent in the light chamber or nosepokes in the light chamber. Because PPA was found to produce anxiogenic behavior in the light dark test in the previous phase, these disparate findings could be attributed to the difference in experimental schedules between phase one and phase two and the duration of PPA's effects in the rodent. The length of time between the social behavior and the light dark test was twice as long in phase two than it was in phase one to ensure that estrous tracking was accurate and that animals were tested during their correct respective estrous phase. It is possible that the

anxiogenic effects of PPA wore off by the time the light dark test was conducted and that the final dose given before the test was not sufficient to produce the effects that were observed in phase one. This has been supported by previous studies that have found that behavioral effects produced by PPA have returned to baseline after eight days of a drug-free recovery period (Mephram et al., 2019).

A significant Drug X Estrous Phase interaction was detected for duration of time spent in the light chamber in phase two, however this interaction was observed in the opposite direction that was predicted. Diestrus females treated with PPA displayed greater durations of time in the light chamber compared to proestrus females treated with PPA. This interaction does not suggest protective effects of estradiol and progesterone against the anxiogenic actions of PPA given that during diestrus, these hormones are at their lowest (Butcher, Collins & Fugo, 1974). Moreover, this was only one significant interaction detected among the many variables quantified. Light-dark findings for phase two were not as expected, perhaps due to the variable lengths of time between the behavioral tests in phase one and phase two. This finding, while not in line with the present hypotheses, could provide direction with regards to where effects could be looked for in future studies.

Vaginal smears of all animals were taken at the same time each morning. It is possible that the determination of estrous phases was accurate (that animals were in-fact in proestrus/diestrus when tested), but that animals were not observed in the social behavior and anxiety paradigms at the exact time when estradiol/progesterone levels were highest and lowest. According to several studies, progesterone levels increase several hours prior to ovulation, which, in the estrous cycle, is termed proestrus (Baum, 2002). Maximum estradiol release from the ovary has been detected 18 h before ovulation with serum estradiol levels reaching their highest approximately 6–12 h before ovulation (Freeman, Smith & Neil, 1974; Freeman, 1994). Additionally, a significant increase in progesterone occurs 4 – 6 h after the rise in estradiol, during the afternoon of proestrus (Becker et al., 2005). When using the vaginal smear technique to determine estrous phase, future studies studying hormonal influences should consider measuring serum hormonal levels before observing behavior for more accurate results.

Collecting estrous samples to track rodent reproductive cycles is a suitable method to explore the reputed effects of elevated hormonal levels on various behaviors such as anxiety and social interaction. However, the obvious caveat is that looking to the phases of the estrous cycle to measure the effects of estradiol and progesterone is only a relative measure of hormonal influence and this method is not precise. Thus, although it was predicted that estradiol and progesterone may have moderated the effects of treatment with PPA, phase two of the present study does not conclusively prove this. Phase three addresses this caveat and serves as a follow up study to explore this further by means of ovariectomy and estradiol and progesterone replacement.

2.4.1 Conclusions

It was predicted that PPA would have sex differential effects, producing more profound ASD-like behavioral impairments in males than in females. It was also predicted that proestrus rats treated with PPA would demonstrate less profound ASD-type behavioral impairments in the social behavior task and the light dark test. In the statistical analyses conducted, no Drug X Sex interactions were detected in phase one. With the exception of light duration, however in the opposite direction as predicted, no Drug X Estrous Phase interactions were found in phase two. PPA produced the same deficits in males and in females, and PPA treated females displayed impairments regardless of whether they were tested during proestrus or diestrus. These findings suggest that hormonal levels, as indicated by determining estrous phase using the vaginal smear technique, is either not an accurate way of indicating hormonal levels, or that estradiol and progesterone levels do not influence social and anxiety-like behavior when a relatively high dose (500mg/kg) of PPA is administered.

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Chapter 3

3 Exploring the effects of propionic acid in cyclic hormone-replacement treated ovariectomized rats

3.1 Introduction

3.1.1 Gonadal Hormones and ASD

Recent studies have shown that hormones play critical roles in several brain functions, including cognition, learning, as well as neurodevelopment (Crider & Pillai, 2017; Ferri, Abel & Brodtkin, 2018). Distinct sex differences in clinical symptoms and incidence are apparent in neurodevelopmental disorders such as schizophrenia and autism (Hill et al., 2004; Kokras and Dalla, 2014; Mottron et al., 2015). This suggests that gonadal hormones are involved in the underlying vulnerability for behavioral abnormalities or are ameliorating or exacerbating the severity of symptoms (Romano, Cosentino, Laviola & De Filippis, 2016). In view of the marked male predominance in ASD, there is reason to believe that estradiol and progesterone are potentially involved in the manifestation and/or pathogenesis of ASD. Studies using human subjects are lacking as it is impossible to ethically manipulate fetal hormonal levels in humans. One investigation, however, looking at a small sample of post-mortem adolescents with ASD brains uncovered a trend of reduced estrogen receptor subtypes in addition to reduced aromatase, the enzyme responsible for the conversion of androgens to estrogens (Crider & Pillai, 2017). Rodent models exploring the mechanisms that underlie ASD-like social and neuronal abnormalities in mice have reported that neonatal administration of estradiol rescues these phenotypes (Macri et al., 2010), pointing to the developmental effects of these hormones during a critical period of neurodevelopment. The present study explores the acute effects of estradiol and progesterone when administered in young adulthood, which can provide insight into the potential variable manifestation of ASD-relevant behaviors in males and females. The involvement of gonadal hormones in the context of ASD as a potential factor that differentially confers risk or protection to males and females requires further investigation.

3.1.2 Ovariectomized (OVX) Females

Phase two did not find that propionic acid (PPA) produced differential effects in behavior according to estrous phase (with the exception of light duration, see Figure 2.18). It was postulated that these findings could be due to the method used to track hormonal levels. The advantage of using the vaginal smear technique is that it is non-invasive and does not put the animal through a stressful procedure. However, the clear drawback is that using this technique to determine estrous phase is only a relative measure of hormonal levels and is not precise. In fact, even when researchers are perfectly consistent in terms of taking vaginal smears at the same time each day, physiological timing is often not perfect. There is individual variability within rats in terms of at what time exactly hormonal peaks occur as well as variability in the duration of each phase (Becker et al., 2005). The use of OVX female rats ensures that the endogenous source of estradiol and progesterone is removed, making it possible to administer these hormones externally and thus be more accurate in making observations about the effects of elevated hormonal levels.

A variety of studies have examined the effects of ovariectomy and the acute effects of exogenous hormone replacement. Ovariectomy in rodents has been reported to produce deficiencies in social interaction and play behavior. Hlíňáček (1993) reported that ovariectomized females, compared to those treated with estradiol, displayed significantly reduced social recognition and investigatory behavior. Ovariectomized females have also been found to display reduced likelihood of social exploration, and that long-term treatment with estradiol rescues this phenotype (Tang et al., 2005). Pandaranandaka et al. (2009) compared the effects of endogenous and exogenous estradiol on anxiety. In the elevated T-Maze test, estradiol treated OVX females displayed reduced inhibitory avoidance compared to other groups, including females in proestrus, suggesting that exogenous estradiol has anxiolytic properties. Locomotor activity was not affected by treatments, suggesting that increased locomotor activity often produced by heightened estradiol (Ogawa et al., 2003) was not accounting for this effect. Additionally, Frye and Walf (2004) found that combined subcutaneous injections of estradiol and progesterone to OVX females had anxiolytic effects as measured by behavior in several anxiety tasks.

The female rat and human share neuroanatomical and physiological similarities, but there are

notable differences that further support the use of ovariectomized female rats as opposed to relying on the rodent estrous cycle (Gorski, Mennin, & Kubo, 1975). There is a significant difference in the length of the reproductive cycle of the female rat and human. In the rat, the reproductive cycle lasts four days whereas the reproductive cycle of the human lasts 28 days (Staley & Scharfman, 2005). Throughout the reproductive cycle, there are also clear differences between both the cycling pattern and amplitude of the fluctuations of estradiol and progesterone. Peak levels of estradiol and progesterone occur during the same phase of the rodent estrous cycle whereas in humans they reach peak levels at two distinct phases (Staley & Scharfman, 2005). Since ASD is a human condition, these differences give merit to using ovariectomized females and administering hormones externally when exploring the possible influence of heightened estradiol and progesterone in females as a mechanism behind the sex bias observed in ASD.

3.1.3 Phase Three

With the consistently observed male predominance in autism spectrum disorder, researchers are exploring various mechanisms that can possibly account for such a pronounced sex difference (Schaafsma & Pfaff, 2014). Phase three of the present study explored the potential differential effects of intraperitoneal (i.p.) administration of PPA in young adult ovariectomized (OVX) female rats that were randomly assigned to be treated with a cyclic four-day hormone replacement therapy (HRT) with estradiol and progesterone or a vehicle control (sesame oil; Clarke & Ossenkopp, 1998). Treatment with PPA or PBS vehicle followed the first round of hormone replacement. This was accompanied by the behavioral testing schedule that was used in the first and second phases of the study. It was hypothesized that the effects of PPA in OVX female rats would be significantly different depending on whether or not OVX females received the HRT. When estradiol and progesterone levels are administered exogenously (HRT group), OVX females were expected to display less severe ASD-like impairments (i.e. reduced anxiety and greater social interactions/play behavior) relative to animals who were given the vehicle control.

3.2 Method

3.2.1 Animals

The subjects consisted of 30 young- adult female Long Evans rats (Charles River, Quebec) that weighed between 201-225 g and were ovariectomized (OVX) on Post-Natal Day (PND) 30 before arrival. Rats were housed in same-treatment pairs in polypropylene cages (45 cm x 22 cm x 20 cm) in a colony room that was 21 ± 1 °C and in a 12:12 light to dark cycle, with lights on at 0700h. Each cage was provided with ProLab (RMH3000) rat chow and water ad libitum. All testing took place during the light phase (0900-1200h) of the light-dark cycle. All procedures were approved by the University of Western Ontario Animal Care Committee and were in accordance with the Canadian Council of Animal Care (CCAC) Guidelines.

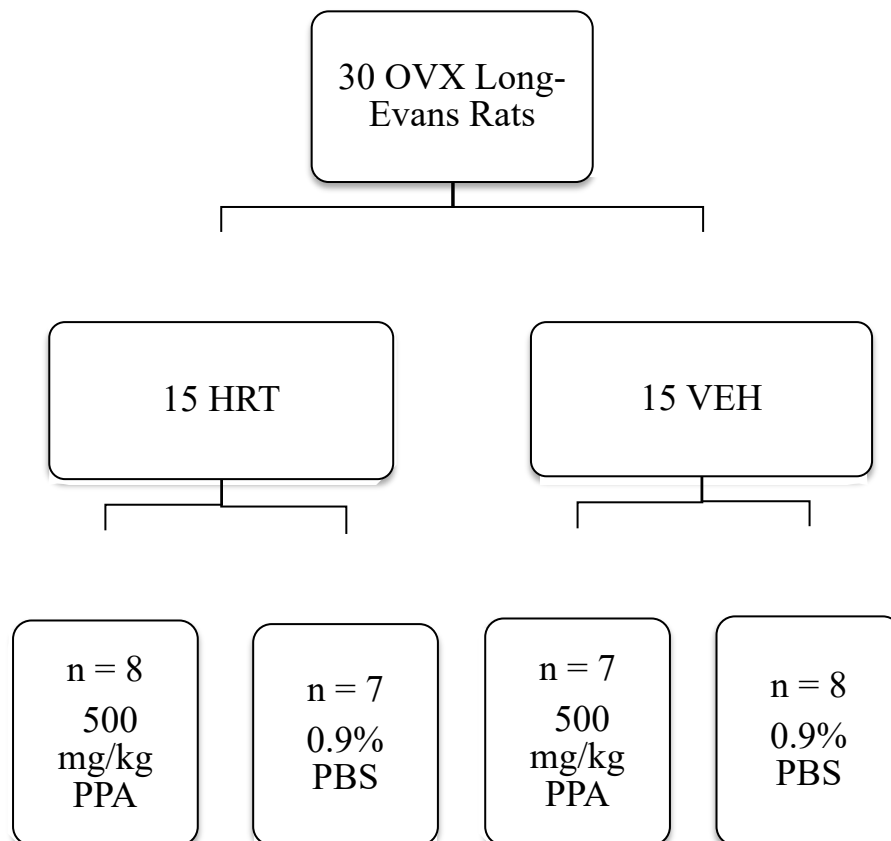


Figure 3.1. Group Designation. HRT = Hormone Replacement Therapy; VEH = Vehicle (Sesame Oil).

3.2.2 Drugs

Propionic acid (PPA) and the vehicle control, Phosphate Buffered Saline (PBS), were prepared the same way in all three phases of the present study. See Section 2.2.2 for drug preparation processes. OVX females were randomly divided into four groups (see Figure 3.1 for Group Designation). Rats were randomly assigned to be injected with PPA (500mg/kg), or a vehicle control compound, PBS (0.1mol/L), and treatment with either a cyclic Hormone Replacement Therapy (HRT) or sesame oil (VEH).

3.2.3 Hormone Replacement

Cyclic hormonal replacement began 20 days after ovariectomy (PND 50). Rats were divided into two groups (refer to Figure 3.1 for Group Designation): OVX-HRT (n = 15), and OVX-VEH (n = 15). These groups received subcutaneous (s.c.) injections (0.1 ml) of 10 μ g 17- β -estradiol (Sigma Diagnostics Canada, Ontario, Canada) and 500 μ g progesterone (Sigma) in sesame oil, or the sesame oil vehicle alone, starting at 0900 h (refer to Table 3.1). This regimen of hormonal replacement produces behavioral estrus in rats tested in the afternoon of the 4th day of the replacement cycle (i.e., the day of progesterone administration; Schumacher et al., 1990, 1991). Behavioral tests (social behavior and light-dark box) were both conducted on the 4th day of the replacement cycle.

3.2.4 Experimental Timeline

The experimental procedure for phase three is summarized in Figure 3.2. When animals arrived, they first acclimated to their colony rooms for a week. Hormone replacement therapy (HRT) began after anestrus state confirmation (see Section 3.2.6). Females were randomly assigned to be given intraperitoneal injections of 500mg/kg of PPA or vehicle injections of the PBS control, as well as random assignment to either the HRT or VEH treatment groups. On the final day of PPA/PBS treatment, social behavior was recorded for 20 min in a circular open field arena. The next behavioral assay was the light-dark box (LDB) test three days following social behavior. As shown in Figure 3.1, the four experimental groups were: HRT+PPA, HRT+PBS, VEH+PPA, and VEH+PBS.

Injection Days (13, 14, 15, 16, 20)

- Hormones injected s.c. in 0.1 ml sesame oil starting at 0900 h. Cycle was repeated 3x.
- Injected i.p. 5x (1x/Day) with either 500 mg/kg PPA or 0.9% PBS
- Placed in respective behavioral apparatus 10-min post-injection

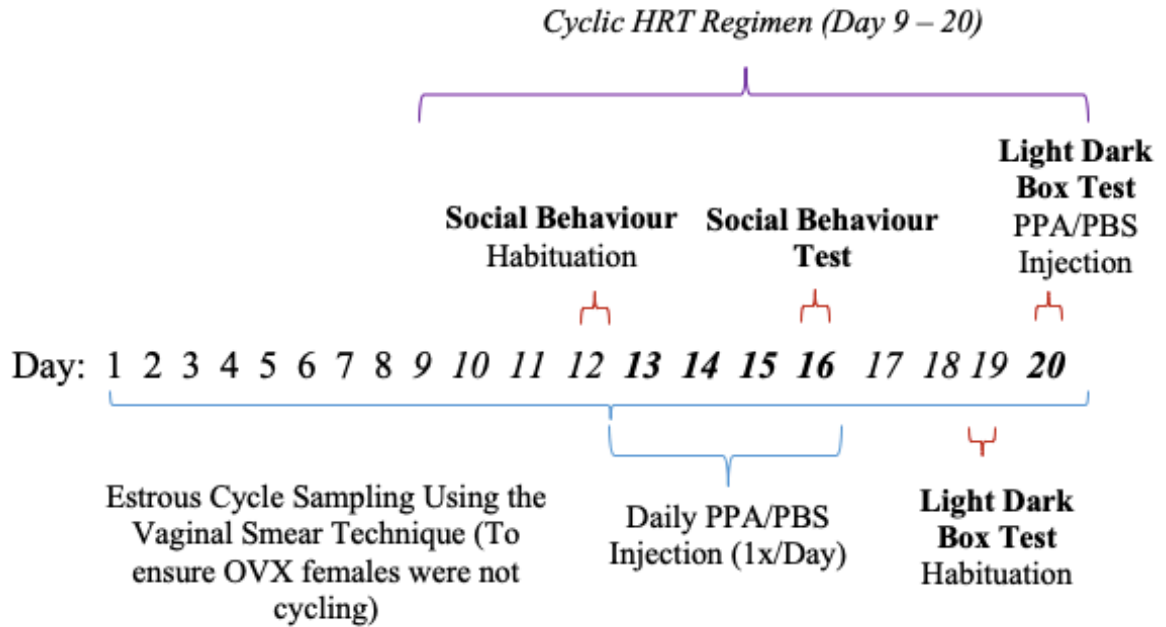


Figure 3.2. Experimental Timeline for Phase Three.

Table 1.1*Cyclic Regimen of Hormone Replacement.*

Group	Day 1	Day 2	Day 3	Day 4
OVX + VEH	V	V	—	V
OVX + HRT	E	E	—	P

Hormones were injected s.c. in 0.1 ml sesame oil starting at 0900 h. E = 10 μ g 17- β -estradiol; P = 500 μ g progesterone; V = sesame oil vehicle alone. Cycle was repeated 3x.

3.2.5 Materials and Apparatus

3.2.5.1 Social Behavior Test

The social behavior test utilized in phase three was conducted and analyzed in an identical manner to phase one and two.

Twenty-four hours before injections began, animals were habituated to the social behavior apparatus for ten minutes one at a time. 10 minutes before being placed in the apparatus, the rat being tested received a single PPA (n = 15) or PBS (n = 15) injection. Once the injected rat was placed into the arena, a non-treated female stranger rat was placed into the open field and behavioral data was recorded for 20 minutes. Following this session, rats were returned to their home cages. Variables quantified in phase three were the same as those quantified in phase one and two.

3.2.5.2 Light Dark Test

The light dark anxiety test utilized in phase three was conducted and analyzed in an identical manner to phase one and two. Anxiety variables quantified in phase three were the same as those quantified in phase one and two.

Twenty-four hours before conducting the light dark anxiety test, animals were habituated to the

testing room for thirty minutes and placed in the test to measure baseline levels of anxiety. Animals were given the fifth and final injection with either PPA or PBS three days following the social behavior test. At this time, animals had not been given an injection of PPA/PBS for three days, however they continued to receive their respective HRT/VEH injections. The light-dark test was conducted approximately 10min following the injection.

3.2.6 Anestrous State Confirmation

Reference images for determination of estrous cycle phase can be seen in Figure 2.4. Rats were acclimatized to their home cages for one week and then handled for three days. Following handling, vaginal smears were tracked daily at the same time each day for eight days (two rodent estrous cycles) before the cyclic hormone replacement regimen began in order to confirm that the ovariectomy was successful and that all females were anestrous. The estrous cycles were tracked by the vaginal smear technique. All rats used in this study were anestrous.

3.2.7 Data Analysis

3.2.7.1 *Social Behavior*

Variables quantified for the social behavior test were identical in all three phases of the present study (See Section 2.2.4). A randomly selected subset of data was manually scored by another blinded experimenter in order to calculate inter-rater reliability via Pearson correlations. A Univariate Analysis of Variance was conducted to compare OVX-VEH and OVX-HRT sample means, as well as PPA and PBS group sample means. All statistical tests used $\alpha = 0.05$ as a significance criterion. Statistical analyses were performed using IBM SPSS Statistics 23 for Windows.

3.2.7.2 *Light Dark Test*

Variables quantified for the light-dark test were identical in all three phases of the present study (See Section 2.2.4). A Univariate Analysis of Variance (ANOVA) was conducted to compare OVX-VEH and OVX-HRT sample means, as well as PPA and PBS group sample means. In addition, a Mixed Model ANOVA was conducted to compare behavior in the light-dark box test

without PPA/PBS injections (Session 1) and with PPA/PBS injections (Session 2). All statistical tests used $\alpha = 0.05$ as a significance criterion. Statistical analyses were performed using IBM SPSS Statistics 23 for Windows.

3.3 Results

3.3.1 Social Behavior

Frequency of Social Initiations. As shown in Figure 3.3, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 26) = 51.621, p < .001$). No main effect of hormone replacement therapy (HRT) was found ($F(1, 26) = .677, p = .418$). No significant Drug X HRT was found ($F(1, 26) = .494, p = .488$). Rats treated with PBS, regardless of whether they were given the HRT, displayed significantly more social initiations than rats treated with PPA.

Probability of Defense. As shown in Figure 3.4, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 26) = 41.866, p < .001$). A main effect of HRT was found ($F(1, 26) = 4.646, p < .05$). No significant Drug X HRT was found ($F(1, 26) = 2.683, p = .113$). Rats treated with PBS, regardless of whether they were given the HRT, exhibited significantly greater probabilities of defense compared to PPA-treated females. HRT-treated females were significantly less likely to elicit a defense compared to VEH-treated females.

Type of Defense. Two types of defenses were observed, facing and evasion. As shown in Figure 3.5A, the 2 x 2 between-subjects ANOVA did not reveal a significant main effect of drug ($F(1, 26) = .106, p = .748$) or HRT ($F(1, 26) = 1.587, p = .219$) for facing defense. No significant Drug X HRT was detected ($F(1, 26) = .686, p = .415$). As shown in Figure 3.5B, no main effect of either drug ($F(1, 26) = .106, p = .747$) or HRT ($F(1, 26) = 1.588, p = .219$) was found for evasion. There was no significant Drug X HRT interaction found ($F(1, 26) = .685, p = .415$) for probability of evasive defense. There were no statistically significant differences between PPA and PBS treated animals, regardless of whether they were given the HRT, with regards to type of defense (facing or evasive) elicited.

Frequency of Social Initiations

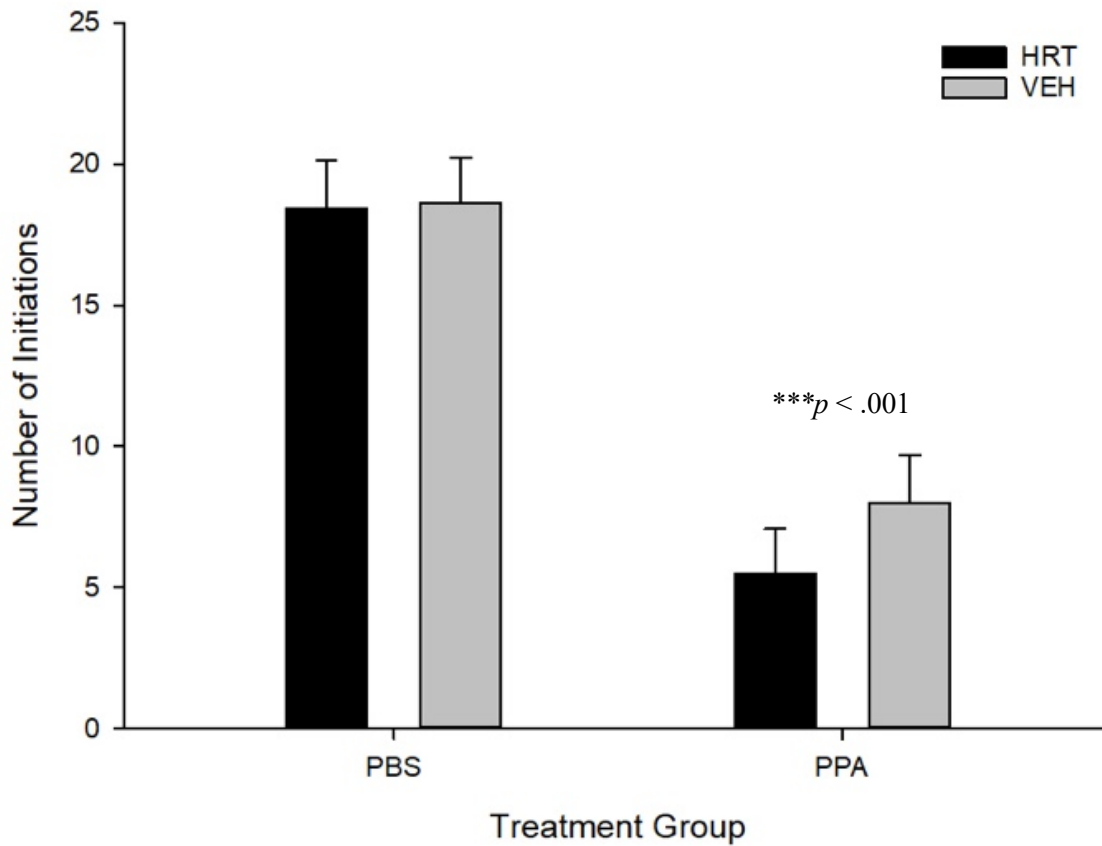


Figure 3.3. Frequency of Social initiations Displayed by PPA or PBS Treated Rats. Mean (\pm S.E.M) frequency of social initiations as shown by the 4 groups (HRT+PPA (n = 8), HRT+PBS (n = 7), VEH+PPA (n = 7), & VEH+PBS (n = 8)), during the 20-minute social behavior test. Only the first 10 minutes were scored. Rats treated with PBS displayed significantly more social initiations than rats treated with PPA ($***p < .001$). No significant Drug X HRT was found ($p = .488$).

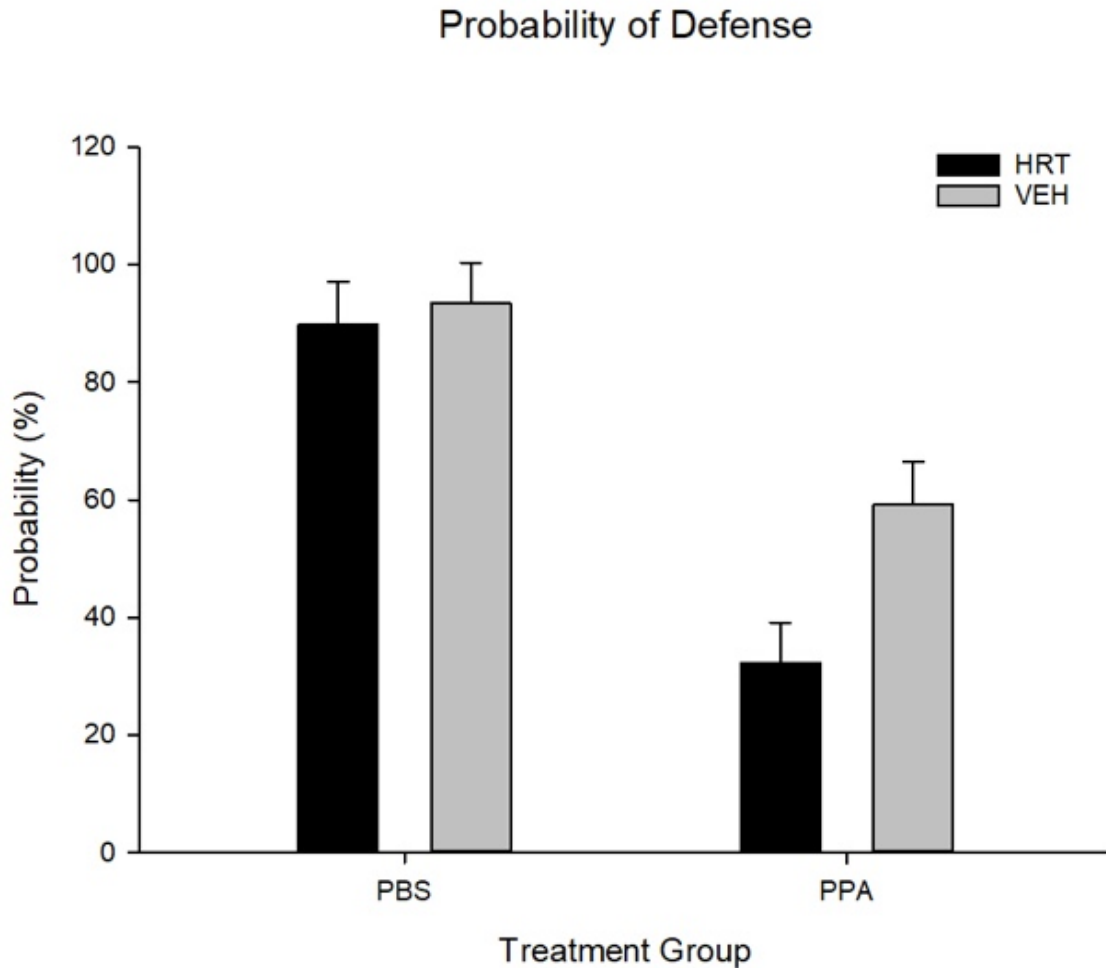


Figure 3.4. Probability of Defense by PPA or PBS Treated Rats (defined as a withdraw of the nape from the stranger's snout divided by the total number of social initiations by the stranger x 100). Mean (\pm S.E.M) probability of defense as shown by the 4 groups (HRT+PPA ($n = 8$), HRT+PBS ($n = 7$), VEH+PPA ($n = 7$), & VEH+PBS ($n = 8$)), during the 20-minute social behavior test. Only the first 10 minutes were scored. Rats treated with PBS exhibited significantly greater probabilities of defense compared to PPA-treated females ($***p < .001$). No significant Drug X HRT was found ($p = .113$).

Probability of Facing Defense

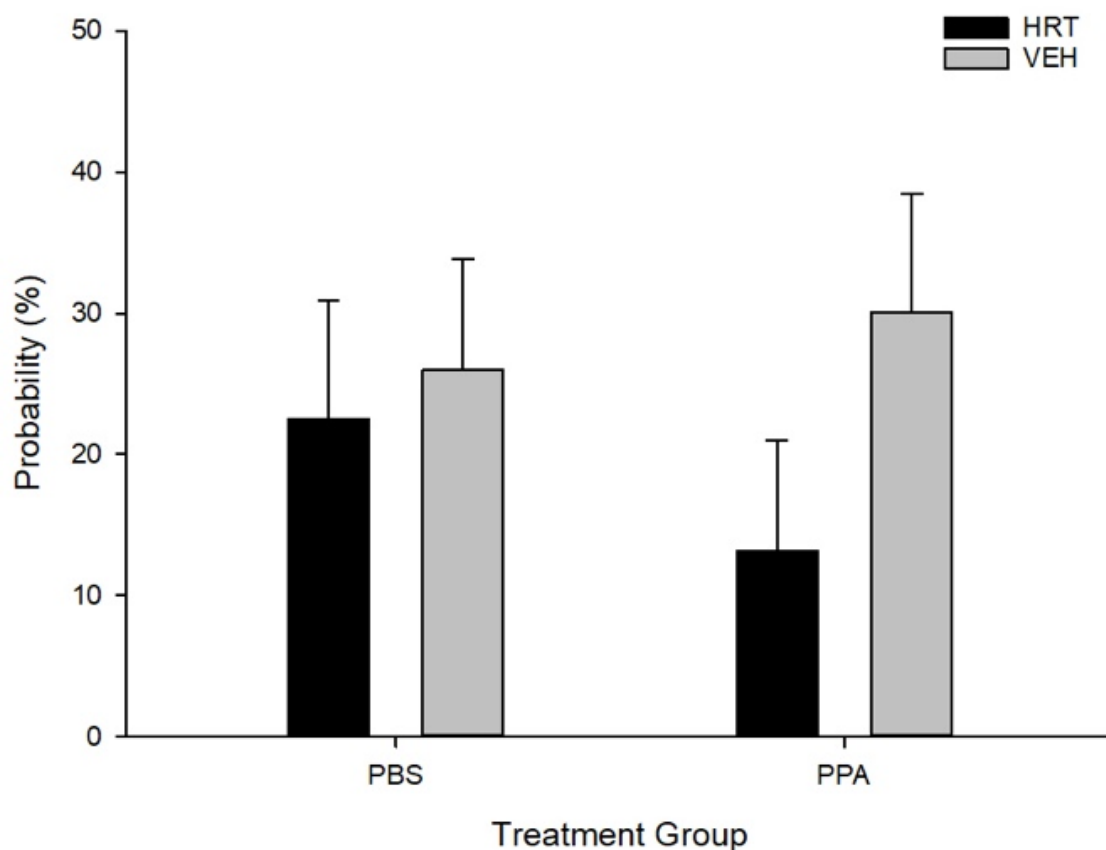


Figure 3.5A. Probability of Facing Defense (defined as a withdraw of the nape from the stranger's snout by facing the stranger divided by the total number of defenses x 100). Mean (\pm S.E.M) probability of facing defense as shown by the 4 groups (HRT+PPA (n = 8), HRT+PBS (n = 7), VEH+PPA (n = 7), & VEH+PBS (n = 8)), during the 20-minute social behavior test. Only the first 10 minutes were scored. PPA and PBS treated rats, irrespective of HRT, did not display significantly different probabilities of facing defense ($p = .748$). No significant Drug X HRT was found ($p = .415$).

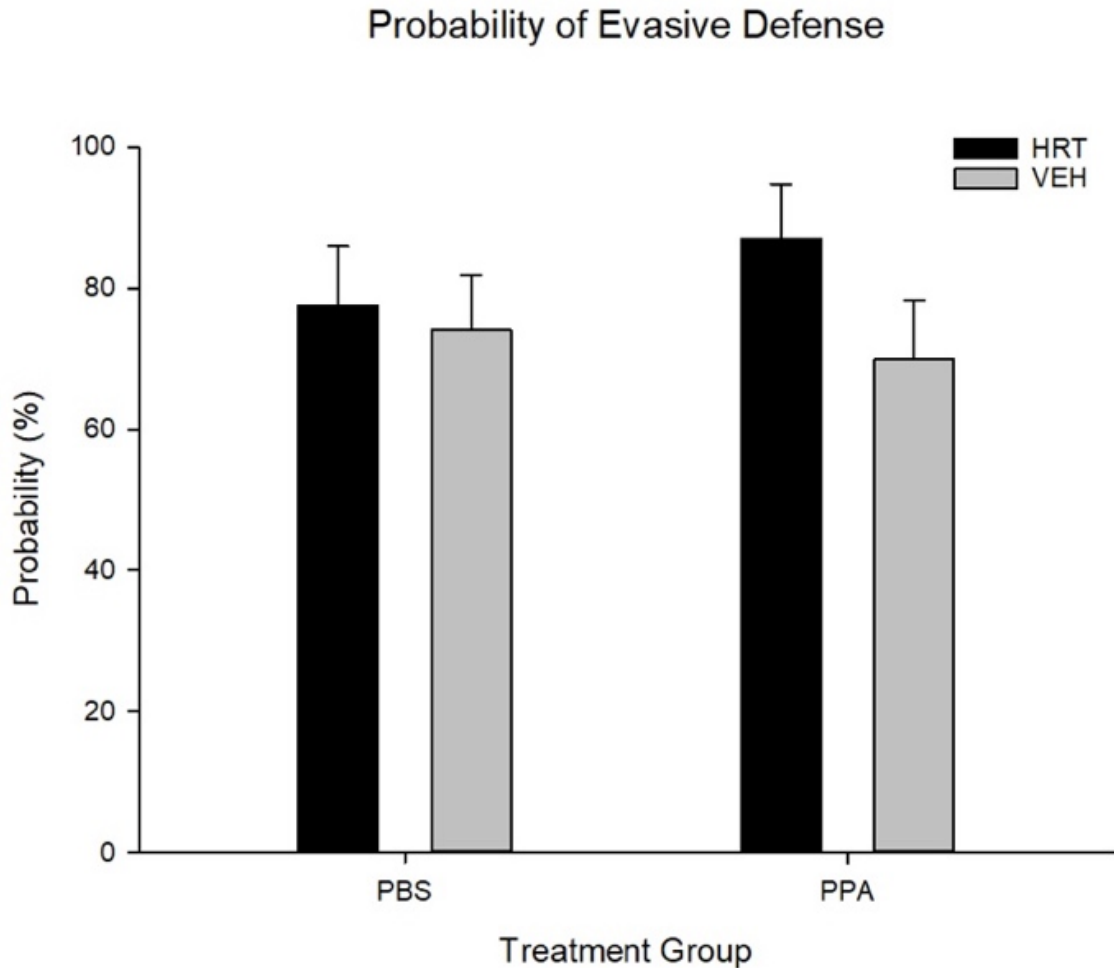


Figure 3.5B. Probability of Evasive Defense (defined as a withdraw of the nape from the stranger's snout by either leaping, running, or turning away from the stranger, divided by the total number of defenses x 100). Mean (\pm S.E.M) probability of evasive defense as shown by the 4 groups (HRT+PPA ($n = 8$), HRT+PBS ($n = 7$), VEH+PPA ($n = 7$), & VEH+PBS ($n = 8$)), during the 20-minute social behavior test. Only the first 10 minutes were scored. PPA and PBS treated rats, irrespective of HRT, did not display significantly different probabilities of evasive defense ($p = .747$). No significant Drug X HRT was found ($p = .415$).

3.3.2 Light Dark Box

3.3.2.1 Habituation Day (Session 1; No injection) compared to Testing Day (Session 2; PPA/PBS Injection)

Nosepokes into the Light Chamber. As shown in Figure 3.6, the Mixed Model ANOVA revealed a significant main effect of time ($F(1, 26) = 72.567, p < .001$). No significant main effect of drug was found ($F(1, 26) = 2.651, p = .116$). No significant main effect of Hormone Replacement Therapy (HRT) was found ($F(1, 26) = .347, p = .561$). A significant Time X Drug interaction was found ($F(1, 26) = 32.572, p < .001$). A significant Time X HRT interaction was not found, ($F(1, 26) = .305, p = .585$). No significant Drug X HRT interaction was found ($F(1, 26) = .492, p = .489$). A significant Time X Drug X HRT interaction was not found ($F(1, 26) = 1.113, p = .301$). Animals, regardless of HRT and drug injected, displayed significantly ($p < .001$) more nose pokes into the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2) when the respective drug (PPA or PBS) was injected. PPA treated females, regardless of time and HRT, displayed less nose pokes ($p < .001$) into the light chamber compared to PBS treated females during Session 2 but not Session 1.

Duration of Time Spent in the Light Chamber. As shown in Figure 3.7, the Mixed Model ANOVA revealed a significant main effect of time ($F(1, 26) = 24.774, p < .001$). No significant main effect of drug was found ($F(1, 26) = .937, p = .342$). No significant main effect of Hormone Replacement Therapy (HRT) was found ($F(1, 26) = .000, p = .989$). A significant Time X Drug interaction was not found ($F(1, 26) = 3.158, p = .087$). A significant Time X HRT interaction was not found, ($F(1, 26) = .057, p = .813$). No significant Drug X HRT interaction was found ($F(1, 26) = .427, p = .519$). A significant Time X Drug X HRT interaction was not found ($F(1, 26) = .028, p = .869$). Animals, regardless of HRT and drug injected, spent significantly ($p < .001$) more time in the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2).

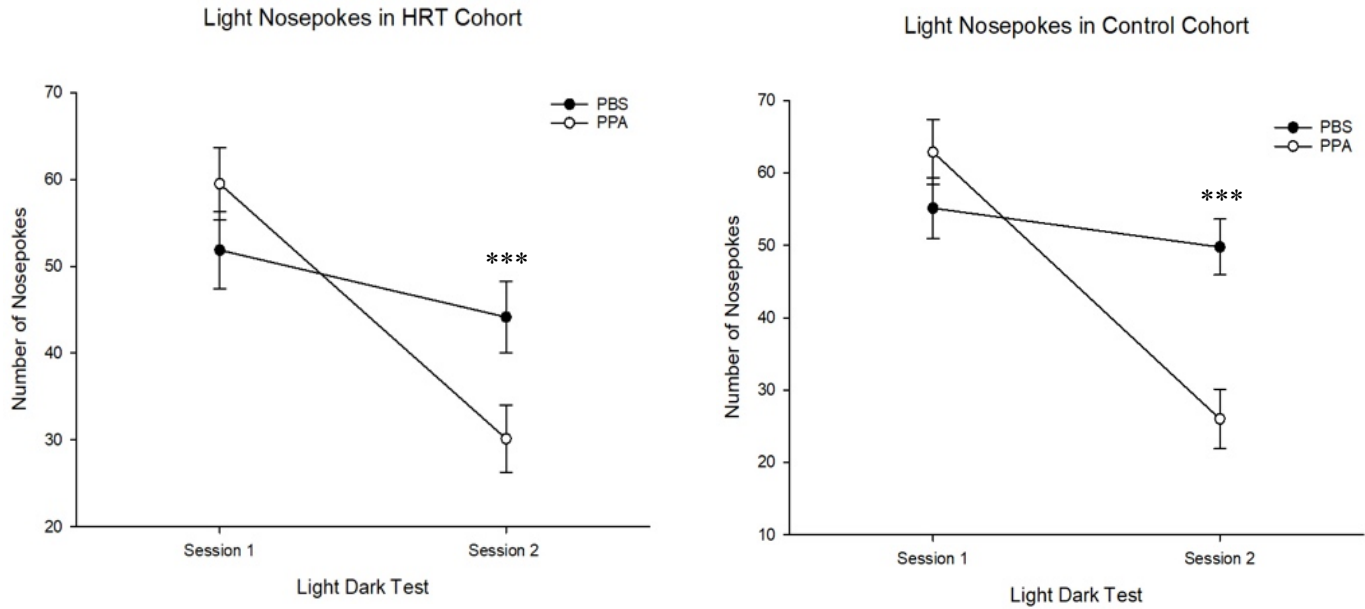


Figure 3.6. Light Nosepokes. Mean (\pm S.E.M) light nosepokes shown by the 4 groups (HRT+PPA ($n = 8$), HRT+PBS ($n = 7$), VEH+PPA ($n = 7$), & VEH+PBS ($n = 8$)), during the two 20-minute light-dark tests. Animals, regardless of HRT and drug injected, displayed significantly more nosepokes into the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2) when the respective drug was injected ($***p < .001$). PPA treated females displayed less nosepokes into the light chamber compared to PBS treated females during Session 2 but not Session 1 ($p < .001$).

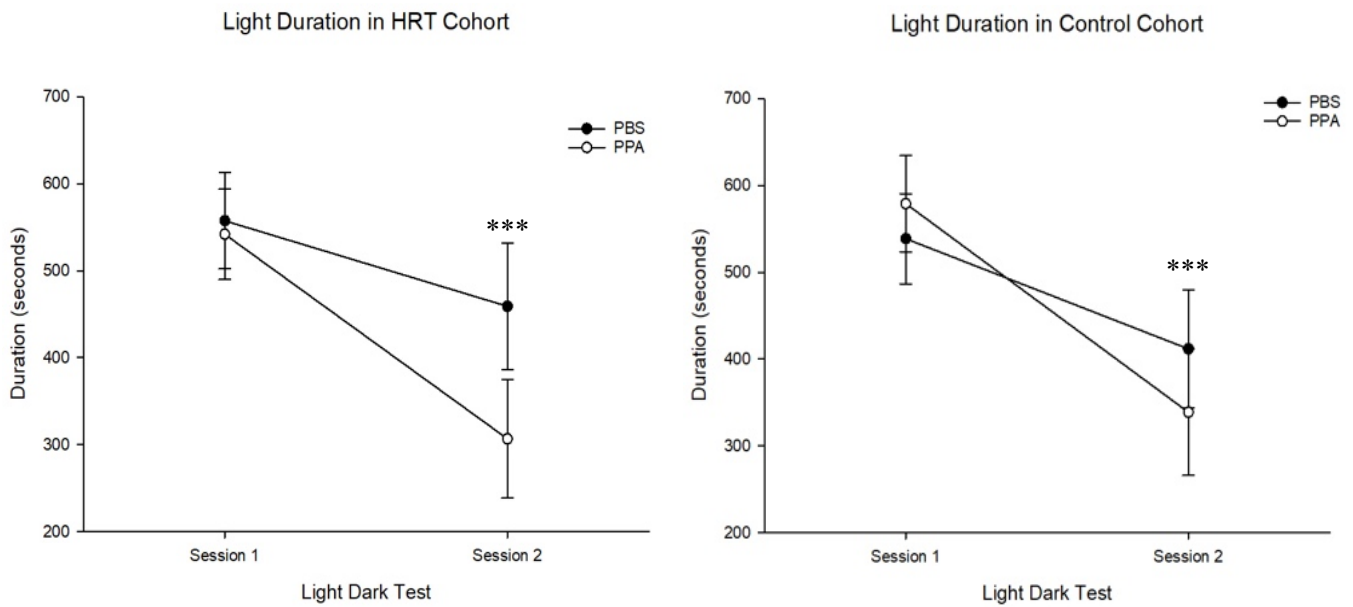


Figure 3.7. Light Duration. Mean (\pm S.E.M) light duration shown by the 4 groups (HRT+PPA (n = 8), HRT+PBS (n = 7), VEH+PPA (n = 7), & VEH+PBS (n = 8)), during the two 20-minute light-dark tests. Animals, regardless of HRT and drug injected, displayed significantly greater durations in the light chamber on the habituation day (Session 1) in comparison to the testing day (Session 2) when the respective drug was injected (***) $p < .001$.

3.3.2.2 Testing Day

Nosepokes into the Light Chamber. As shown in Figure 3.8, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 26) = 22.482, p < .001$) but not of HRT ($F(1, 26) = .035, p = .854$). No significant interaction was detected ($F(1, 26) = 1.493, p = .233$). PPA treated animals, regardless of HRT, displayed significantly less nosepokes into the light-chamber in comparison to PBS treated animals.

Transitions into the Light Chamber. As shown in Figure 3.9, the 2 x 2 between-subjects ANOVA revealed a significant main effect of drug ($F(1, 26) = 26.963, p < .001$) but not of HRT ($F(1, 26) = .069, p = .795$). No significant Drug X HRT interaction was found ($F(1, 26) = .014, p = .905$). PPA treated animals, regardless of HRT, displayed significantly less transitions into the light-chamber in comparison to PBS treated animals.

Duration of Time Spent in the Light Chamber. As shown in Figure 3.10, the 2 x 2 between-subjects ANOVA did not reveal a significant main effect of drug ($F(1, 26) = 2.556, p = .122$) or of HRT ($F(1, 26) = .012, p = .914$). No significant Drug X HRT interaction was found ($F(1, 26) = .318, p = .578$). PPA treated animals, regardless of HRT, did not exhibit significantly different durations of time in the light-chamber in comparison to PBS treated animals.

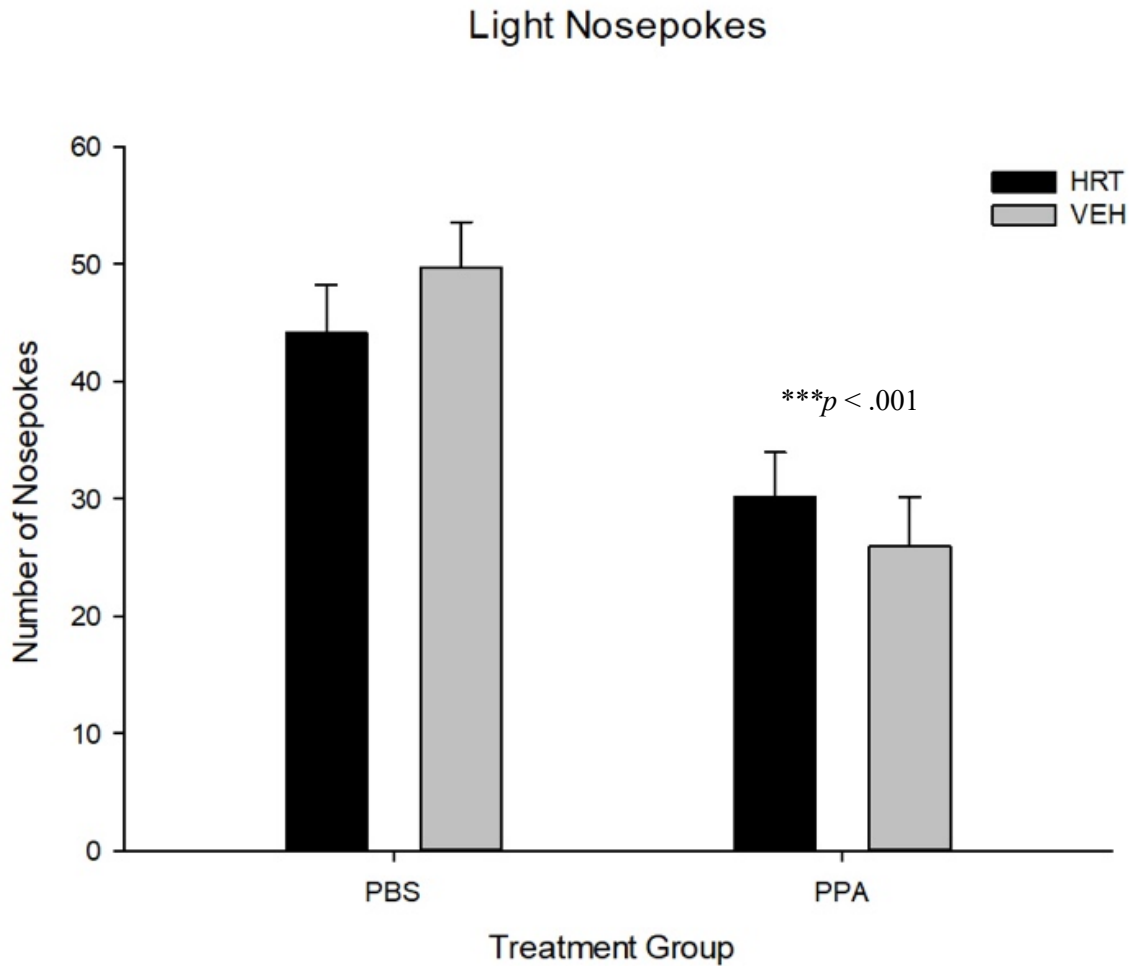


Figure 3.8. Light Nosepokes. Mean (\pm S.E.M) number of nosepokes into the light chamber of the light-dark apparatus expressed by the 4 groups (HRT+PPA (n = 8), HRT+PBS (n = 7), VEH+PPA (n = 7), & VEH+PBS (n = 8)), during the 20-minute light-dark box test. PPA treated animals displayed significantly less nosepokes into the light chamber compared to PBS treated animals ($***p < .001$). No significant Drug X HRT interaction was detected ($p = .233$).

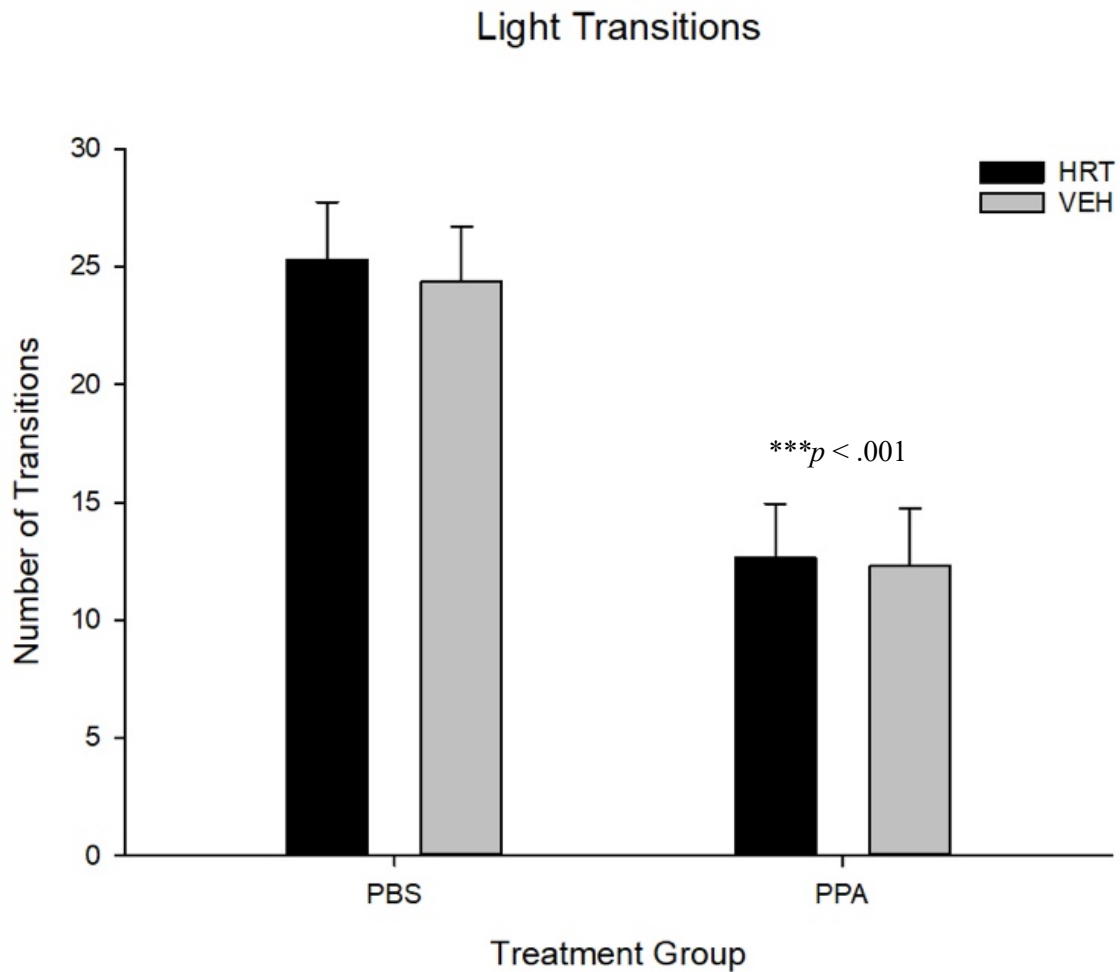


Figure 3.9. Light Transitions. Mean (\pm S.E.M) number of transitions into the light chamber of the light-dark apparatus expressed by the 4 groups (HRT+PPA ($n = 8$), HRT+PBS ($n = 7$), VEH+PPA ($n = 7$), & VEH+PBS ($n = 8$)), during the 20-minute light-dark box test. PPA treated females displayed significantly less transitions into the light chamber in comparison to PBS treated females ($***p < .001$). No significant Drug X HRT interaction was detected ($p = .905$).

Duration in Light Chamber

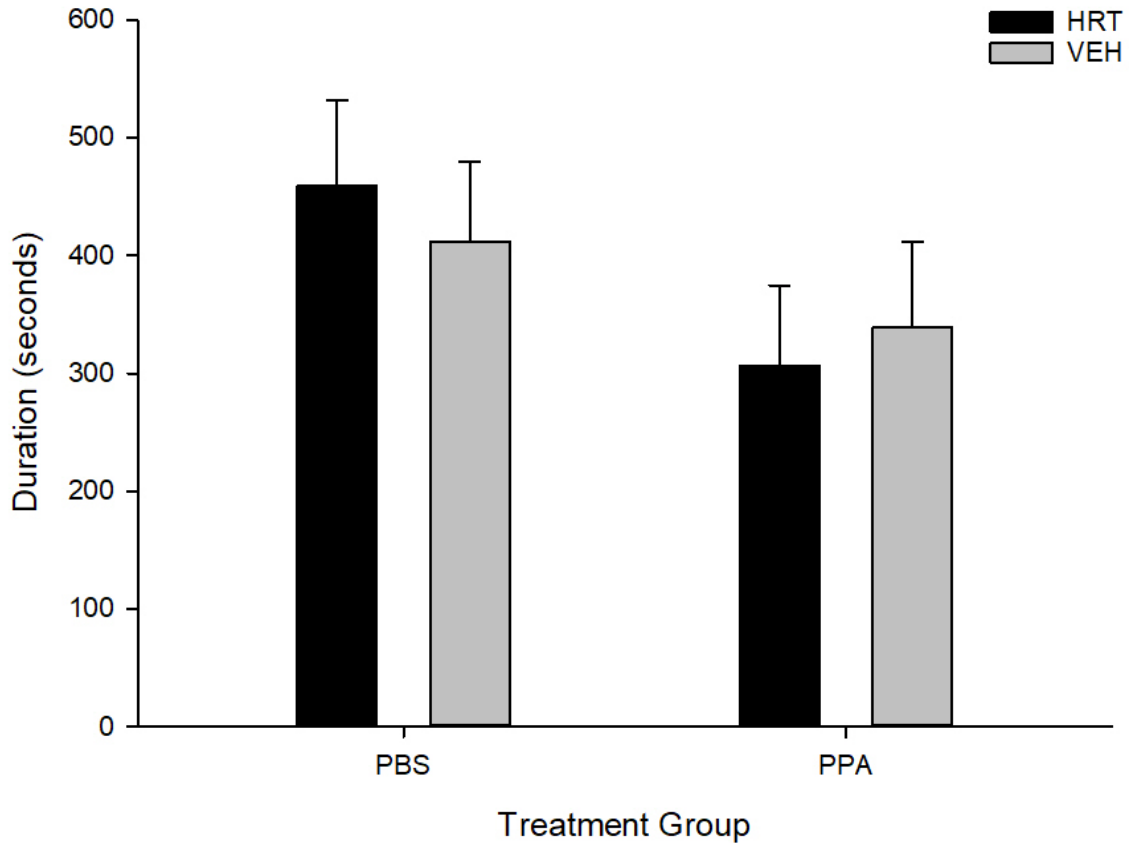


Figure 3.10. Duration in Light Chamber. Mean (\pm S.E.M) duration of time spent in the light chamber of the light-dark apparatus expressed by the 4 groups (HRT+PPA (n = 8), HRT+PBS (n = 7), VEH+PPA (n = 7), & VEH+PBS (n = 8)), during the 20-minute light-dark box test. PPA and PBS treated females did not show significantly different durations of time spent in the light chamber ($p = .122$). No significant Drug X HRT interaction was detected ($p = .578$).

3.4 Discussion

Results of studies exploring the sex difference in ASD have pointed to possible protection by prominent female hormones, estradiol and progesterone, against ASD symptomology (Werling & Geschwind, 2013). To further investigate the possible involvement of gonadal hormones, phase three used ovariectomized (OVX) female rats to explore the acute effects of exogenous estradiol and progesterone administration. It was proposed that PPA treated HRT females would display less severe ASD-like impairments than vehicle PPA treated rats. These results were not obtained, as PPA produced equivalent reductions in social behavior and elevations of anxiety in OVX-VEH and OVX-HRT females. The present study injected OVX females with PPA for four consecutive days at the same time each day, followed by three days “drug-free” before a final injection. A test of social behavior was conducted on the final day of the four-day injection period, and animals were placed in a light-dark anxiety procedure after the final injection. While PPA produced significant deficits compared to the control drug, findings from phase three suggest that PPA’s effects are not sex differential or that perhaps hormones do not play a role in this context.

These data suggest that PPA's ability to impact social behavior and anxiety in rats is not related to sex. This pattern of findings is consistent with previous reports that suggest that PPA impairs social behavior, including abnormal play behavior and other forms of social contact (MacFabe et al., 2011; Shultz et al., 2008). Taken together, these results support PPA’s ability to produce social abnormalities in the rodent that are consistent with social abnormalities seen in ASD. These data, however, do not support the role of PPA in the mechanisms that contribute to the persistent sex bias in ASD, and it is likely that other risk factors such as immune stimulation and genomic mechanisms are contributing (Careaga et al., 2017; Mendelsohn & Schaefer, 2008; Schaefer & Mendelsohn, 2008; Stone et al., 2004). Results from prior investigations are in line with the current results that support PPA’s role in anxiety (Wah et al., 2019) throughout different points of development, but do not support a sex-differential role of PPA in males and females in the light-dark anxiety task. The effects of PPA have been observed during prenatal development and throughout later points of development such as adolescence and early/late adulthood. Continued exposure to PPA during adolescence/early adulthood, as per the present thesis, has been found to produce ASD-like behaviors (Shams et al., 2019; Meeking et al., 2020), while

earlier exposure can delay or alter typical developmental process (Foley et al., 2014, 2015), which is perhaps more closely linked to the pathogenesis and manifestation of ASD. Early gut-microbiome development is one amongst several proposed causative risk factors in ASD (Careaga et al., 2017; Mendelsohn & Schaefer, 2008; Schaefer & Mendelsohn, 2008; Stone et al., 2004), and further research is required in order to support the hypothesis that gut-derived factors are plausible environmental factors that can trigger ASD differently in males and females.

An alternative explanation is conceivably the length of exposure to the PPA. This period of exposure was relatively brief, being just four days. It is likely the case that in order for any hormonal effects to influence PPA-related behaviors (i.e. social behavior, anxiety, sensorimotor gating; MacFabe et al., 2007), that a much longer period of exposure is required. Based on the PPA model of ASD, elevated PPA levels in children with autism are on a consistent basis (Frye, Rose, Slattery & MacFabe, 2015), and the sex difference may be related to longer term developmental aspects and likely chronic exposure to PPA. Additionally, while the present hormone replacement regimen has been used successfully in previous studies (Clarke & Ossenkopp, 1998; Schumacher et al., 1990, 1991), it is possible that in order for estradiol and progesterone to exert protective effects against PPA, that PPA must be administered for a greater length of time, or perhaps multiple times a day. Data collected by Frye et al. (2015) indicate that the effects of PPA are highly dependent on not only dose and concentration, but also length of exposure, and that these effects are intensified in children with ASD. Future investigations should explore a longer exposure period to PPA and possibly during an earlier phase of development, which may be necessary for sex or hormonal influences to appear.

The length of time between the ovariectomy procedure and time of testing should also be taken into consideration. Female rats were ovariectomized (OVX) at post-natal day 30 (post-weaning; Sengupta, 2013), and did not begin testing until post-natal day 50. With a greater time delay, estrogen receptors are possibly slightly down-regulated (Liu & Shi, 2015) and thus the present study may not have been accurately measuring the effects of estradiol. The present study could have measured serum estradiol and progesterone levels to detect whether or not they were comparable to peak levels observed during the proestrus phase of the estrous cycle. A study conducted by Marcondes et al. (2001) examined behavior in females during proestrus and diestrus. Blood was taken from all rats to determine precise estradiol and progesterone levels.

Treating diestrus rats with enough estradiol to match proestrus-estradiol levels abolished the difference in behavior that was initially observed between proestrus and diestrus females in an anxiety task (Marcondes et al., 2001). It is also a possibility that the amount of estradiol administered was not great enough to observe these effects, and that the present study was exploring the effects of progesterone exclusively.

In addition, to ensure that animals were in fact in estrous, animals could have been tested for sexual receptiveness. Female animals could have been presented with a male conspecific to observe whether or not they exhibit lordosis (a unique arched back position), indicative that the female is in estrous and is sexually receptive (Rodriguez-Sierra et al., 1975). Future investigations should consider administering a hormone replacement earlier after the OVX procedure, assessing sexual receptiveness after hormone administration, and measuring serum hormonal levels.

A limitation of the present phase was the small sample size that was used. The sample of 30 rats yielded experimental groups that were no greater than eight per division and should be re-examined with a larger sample size in future studies. The objective of this phase was to explore a more accurate way of investigating the effects of elevated levels of estradiol and progesterone, however, firm conclusions cannot be drawn until additional studies are conducted with a larger sample, with thus a larger power. Increasing the sample size may reveal an interaction between hormone replacement and drug administered, such that differential effects of PPA are observed in OVX females depending on whether the animal is given hormones. Future research looking to further explore prominent female hormones as a potential mechanism behind the sex bias observed in ASD should conduct further OVX studies with greater sample sizes.

A main effect of hormone replacement was detected for probability of defense, such that OVX females treated with the HRT, irrespective of PPA/PBS treatment, were significantly less likely to elicit a defense compared to OVX females treated with the vehicle control. This finding suggests that exogenous hormone replacement can reduce a form of social response in ovariectomized females, although it is likely that this finding is due to the small sample size that was used. This finding is contrary to what has been previously found, such that exogenous administration with estradiol and progesterone did not produce increased social interaction in

phase three OVX females. Previous investigations support the idea that ovariectomy reduces social behavior and that these reductions can be modulated by hormone, particularly estradiol, replacement (Hlíňáček, 1993; Tang et al., 2005). However, this idea is underexplored, and outcomes are highly dependent on the specific hormone replacement regimen that is used and the specific doses that are selected.

Hormones secreted by the ovaries, such as estradiol, have been found to have profound effects on female's physiological and psychological functioning. During the habituation day (no PPA/PBS injection) to the light dark box test, statistically significant differences between OVX females given the vehicle drug and OVX females given the HRT were not detected. These findings suggest that subcutaneous administration of estradiol and progesterone do not have anxiolytic effects in the light dark box. This is inconsistent with previous findings that have demonstrated that estradiol replacement to OVX female rats reduced anxiety-like behavior in the mirror maze, light-dark transition and open field tasks (Nomikos and Spyraiki, 1988; Frye & Walf, 2004; Walf and Frye, 2005). Reports have also suggested that the likelihood of positive responses is closely related to the timing of estradiol replacement (Garcia et al., 2018). As previously indicated, this can perhaps be explained by the dose of estradiol administered, which may have been too low, or the length of time between the OVX procedure and beginning of testing.

Less explored are the effects of progesterone treatment on these behaviors. Studies have reported anxiolytic effects of estradiol and progesterone replacement but have also found discordant findings between these ovarian hormones. Mora and colleagues (1996) looked at the effects of estradiol and progesterone on behavioral markers of anxiety in the elevated plus maze test in OVX female rats. Their results revealed that progesterone increased open-arm exploration of the plus-maze, whereas estradiol antagonized this effect. Additionally, Koss, Gehlert, and Shekhar (2004) tested the effects of estradiol when administered to OVX female rats utilizing the elevated plus maze test to test anxiety and a test of social interaction. Their results suggested anxiolytic effects of the hormone as OVX rats treated with estradiol entered the open arms more and spent more time in the open arms than control OVX rats. However, estradiol-treated OVX females interacted less with a novel stranger suggesting anxiogenic actions of estradiol. Treatment with progesterone did not reverse these effects, which suggests that the absence of progesterone was

likely not responsible for the anxiogenic behavior observed in the social interaction procedure (Koss, Gehlert, & Shekhar, 2004). In order to resolve these inconsistent findings, future investigations should replicate phase three using a greater sample size of ovariectomized females, and perhaps with an additional experimental group to administer estradiol and progesterone separately.

The role of ovarian derived hormones, estradiol and progesterone, in the context of a PPA model of autism spectrum disorder is underexplored. The male susceptibility to autism has been linked to male hormones in the early stages of brain development (Auyeung, Lombardo & Baron-Cohen, 2013), though there is insufficient support for this link and results are inconsistent. Exploring the influence of gonadal hormones in males by means of castration, as well as testosterone replacement in females is another avenue of exploring hormonal influence in the context of ASD that future investigations should pursue. Further studies should continue to seek different ways of exploring gonadal hormones as a potential mechanism in explaining the differential manifestation and/or expression of autism spectrum disorders in males and females.

3.4.1 Conclusions

It was hypothesized that PPA-treated OVX female rats that were administered the cyclic hormone replacement therapy (HRT) would show less pronounced anxiety like behavior in the light dark box test and greater social and play behavior in comparison to PPA-treated OVX females that were given the vehicle control (VEH). It was found that PPA did not produce differential effects in HRT and VEH animals. PPA produced reduced social and play behavior as well as elevated levels of anxiety-like behavior in both groups, regardless of estradiol and progesterone treatment. Along with the results from the first and second phase, these findings do not support the proposal/hypothesis that raised estradiol and progesterone modulate the detrimental effects of elevated PPA in females.

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Chapter 4

4 General Discussion

Autism spectrum disorder (ASD) is a neurobehavioral condition that is characterized by a range of impairments in social skills, a tendency to engage in repetitive behaviors, and difficulties with communication (Powers, 2000). There is a high degree of variability in the manifestation of these disorders, making for a broad range of phenotypic presentations (Santangelo & Tsatsanis, 2005). Currently our understanding of the causation of ASD is incomplete. While affecting the brain primarily, gastrointestinal disturbances in children with ASD have become more prominent (Belmonte et al., 2004; Parracho, Bingham, Gibson & McCartney, 2005). Abnormal levels of a propionic acid (PPA) producing bacteria, *Clostridia*, have been detected in the GI tract of children with ASD, and animal models of ASD have found that both systemic and central administration of PPA has produced brain and behavioral changes that are observed in ASD.

ASDs affect females less frequently than males, with males being diagnosed nearly four times more often than females (Werling & Geschwind, 2013). Although ASD prevalence is higher in males than females, few studies have considered sex differences in the developmental trajectory or clinical manifestations of ASD. The present thesis examined whether there are sexually dimorphic effects of a PPA induced behavioral phenotype in young adult male and female rats and explored the possible involvement of prominent female hormones, estradiol and progesterone.

Phase one revealed that PPA does not produce different effects on social behavior and anxiety in males and females. Both male and female rats treated with PPA exhibited deficits in social interactions and increased anxious-like behavior in the light-dark box. PPA-treated animals, irrespective of sex, were significantly less likely to initiate play with a stranger conspecific and demonstrated less willingness to explore the light chamber of the light dark box.

In phase two, it was found that PPA decreased social and play behavior in females tested during the diestrus and proestrus phase of the estrous cycle. However, minimal effects of PPA were observed in the light-dark anxiety test in both groups of females tested during diestrus and proestrus. This was suggested to be due to a delayed administration of PPA.

In phase three, the effects of ovariectomy and exogenous hormone replacement on the behavioral effects of PPA were examined. OVX females were randomly assigned to be treated with a cyclic hormonal replacement regimen or a vehicle compound, sesame oil, that followed the same cycle. In addition, as per phase one and two, phase three animals were randomly assigned to be treated with PPA or the PBS vehicle control. PPA-treated OVX females showed a reduced willingness to engage in social and play behavior with a stranger female rat and increased anxiety-like behavior in the light dark test, regardless of whether they received the hormone replacement therapy. Taken together, these findings suggest that there are no sex differences in the effects of PPA (500mg/kg), and that elevated estradiol and progesterone in females do not protect against these effects.

Future studies should measure serum progesterone and estradiol levels. This would permit a more accurate indication of hormonal increases and decreases throughout the estrous cycle and allow for a shorter delay between behavioral tests. In a study conducted by Marcondes et al. (2001), anxiety-like behavior of female rats in the elevated plus was examined at all phases of the estrous cycle and this was accompanied by determinations of estradiol and progesterone levels. No significant differences in progesterone levels were observed. However, they found that proestrus females, with the highest levels of serum estradiol, exhibited the least anxiety-like behavior. Further, they found that when diestrus rats were treated with estradiol to produce concentrations that are similar to proestrus levels, the difference in time spent in the open arms between proestrus and diestrus females was no longer observed. When progesterone concentrations were quantified throughout the estrous cycle in a study by Frye et al. (2000), proestrus females exhibited greater social interaction and reduced anxiety-like behavior, accompanied by the highest levels of circulating progesterone. This suggests that variations in behavior observed among females tested during different phases of the estrous cycle correspond with fluctuating hormonal concentrations. The current study indirectly quantified hormonal influences by means of tracking the estrous cycles using the vaginal smear technique only. Direct measurements of hormonal levels are needed before any clear conclusions regarding hormonal effects can be drawn.

The absence of sex differences could also be attributed to the relatively high dose of PPA that was chosen, such that a ceiling effect occurred and sexually dimorphic effects of PPA were

obscured. The dose of PPA used in this exploratory study was 500mg/kg, which is higher than some of the doses that have been used in previous investigations that have found significant effects (Kamen et al., 2019; MacFabe et al., 2007). Compared to the physiological levels at which PPA and other short-chain fatty acids act as critical signaling molecules in microbiota-host communication, the present dose is high (Venegas et al., 2019).

Ultimately, the present study did not yield results that support the proposal that PPA produces behavioral effects in a sexually dimorphic manner due to heightened estradiol and progesterone in females. It is conceivable that females need to be exposed earlier on during what might be considered a more critical or sensitive period of neurodevelopment in order for this sex difference to appear. If this is the case, it is possible that the current findings are a more accurate reflection of purely the neurotoxic effects of PPA which more closely resembles phenotypic presentations of propionic acidemia (PA). Several symptoms of PA overlap with those of autism spectrum disorders (Feliz et al., 2003) and several case reports have revealed multi-morbidities of the two clinical conditions (Al-Owain et al., 2012; de la Bâtie et al., 2018; Witters et al., 2016). Findings from the Propionic Acidemia International Patient Registry have shown that PA is gender balanced and is characterized by symptoms that are similar to those observed in ASD (Baio et al., 2014; Cotrina, Ferreiras & Schneider, 2019). Studies looking to elucidate the mechanisms by which PPA accurately acts as a reliable animal model for ASD should explore and compare high and low doses (250mg/kg; Kamen et al., 2019) of PPA in young adult males and females.

When PPA is administered during development, it has the ability to alter developmental processes in the rodent and produce behaviors relevant to ASD (Foley et al., 2014, 2015). During young adulthood the PPA model of ASD is based on the premise that continuous high levels of PPA will result in the appearance of an ASD-like phenotype (Shams et al., 2019; Meeking et al., 2020). Considering PPA's vast applicability in several other disorders and broad interest as a gut metabolite, it is possible that administration with propionic acid, especially during adolescence and adulthood, is not a perfect model for autism spectrum disorder. Additionally, gastrointestinal environment and gut-microbiome development are only one amongst many of the causative risk factors that have been associated with ASD. Other potential causes must also be taken into account, such as genetic and environmental factors and prenatal environment (Devlin & Scherer,

2012; Ornoy, Weinstein-Fudim & Ergaz, 2015). While PPA is able to produce social behavior impairments in rodents, as indicated by the present and prior investigations, social issues are not exclusive to ASD and present themselves as problems in several other developmental disorders such as schizophrenia and attention deficit hyperactivity disorder (ADHD), which have also been related to gut health and propionic acid (Cenit et al., 2017; McConaughy et al., 2011; Mueser, 1998; Sandgren & Brummer, 2018; Shaw, 2010). Propionic acid's wide-ranging involvement in various diseases makes it challenging to conclude that this gut metabolite is specific to ASD.

The initially stated aim of this research was to explore whether elevated hormonal levels in female rats influences phenotypic presentations of ASD-relevant behavioral impairments. Findings from the aforementioned three phases do not support the prediction that PPA produces sex-differential effects in male and female rats, or that elevated estradiol and progesterone levels influence the effects of a relatively high dose of PPA.

4.1 Conclusions

While the increased risk for males to develop ASD suggests a potential role of sex hormones in the pathophysiology of ASD, the current findings do not support either the presence of sex differences or the hypothesis that elevated progesterone and estradiol influence the expression of social and anxiety impairments in the PPA rodent model of ASD that was presently used. Further, a model using different doses, a longer duration of exposure to PPA, or at a different developmental period may produce different outcomes. The short-chain fatty acid, propionic acid (PPA) was used as an animal model of ASD as it has been found to produce both brain and behavioral related changes that are consistent with ASD (MacFabe et al., 2011, 2012). Further research, predominantly at the neurodevelopmental and clinical levels, is still needed to have a clearer understanding of the possible role of PPA and associated fatty acids in autism.

These results provide a basis for future studies to explore both endogenous hormone manipulation in the animal model, as well as a cyclic hormone replacement regimen. Findings from the present study suggest that the effects of propionic acid are not sexually dimorphic and lend support to the idea that PPA is not exclusive to ASD. Further research is required to understand the mechanisms that underly the sex bias consistently observed in ASD. Uncovering

this mechanism will bring research closer to a more fluid understanding of the sex biases observed not only in ASD, but several other neurodevelopmental disorders (Bale et al., 2010).

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Curriculum Vitae

Education

Master of Science: Neuroscience, 2020 **Western University** - London, ON

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Research Experience

WESTERN UNIVERSITY, London, ON

(2018-2020) Master's Level Thesis: *Supervisors: Dr. Klaus-Peter Ossenkopp and Dr. Martin Kavaliers*

Project: Exploring the potential sexually dimorphic effects of propionic acid on autism spectrum disorder phenotypes in adult rats

Roles: Complete research project responsibility including study design, animal care and handling, thesis preparation, presenting results in seminar course. Involves intraperitoneal injections, subcutaneous injections, vaginal smear technique, scoring behavioral responses, and various analyses using SPSS software.

WESTERN UNIVERSITY, London, ON

(2018) Community Experiential Learning

Project: Literature Review assessing treatment outcomes in clients with substance use disorders at Westover Treatment Centre

Roles: Complete research project responsibility including study design, literature review preparation, presenting results in the course and at a public poster presentation. Comprehensive evaluation of Stage-Two program at Westover Treatment Centre.

WESTERN UNIVERSITY, London, ON

(2018) Undergraduate Psychology Honors Thesis: *Supervisors: Dr. Klaus-Peter Ossenkopp and Dr. Martin Kavaliers*

Project: Investigating the effects of propionic acid on social odour in adult male rats: Implications for an animal model of autism spectrum disorder

Roles: Complete project responsibility including study design, animal care and handling, thesis writing and preparation, presenting results at thesis poster presentation. Involves intraperitoneal injections and various data analyses using SPSS software.

Publications

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(2019-2020) Teaching Assistant- Course Instructor: Dr. Graeme Haynes

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