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Early life immune and physical stress directly influences anxiety-like behaviour in adolescent rats: examining sex differences

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Neuroscience

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ABSTRACT AND KEYWORDS

This thesis examined the effects of neonatal acute immune activation with the endotoxin, lipopolysaccharide (LPS) on postnatal days 3 and 5 on adolescent anxiety-like behaviour in rats before and after a stress period. Previous research has shown that adult rats exposed to LPS during the neonatal stage show anxiety-like behaviour following a period of stress. This thesis investigated this effect in adolescence. The present results showed significantly higher anxiety-like behaviour in no injection controls, and a potential resilience effect of low dose LPS (15 µg/kg) contrary to what was reported in adult rats. As well, a phase of stressful, aversive conditioning (conditioned disgust) did not elicit anxiety-like behaviour in LPS-treated adolescent rats. This study provides novel findings about the adolescent period, and suggest the use of no injection controls for neonatal research. This thesis presents data that suggests the importance of no injection controls in future neonatal research involving. This thesis also provides support to previous literature investigating sex differences in anxiety-like behaviour; female adolescent rats showed less anxiety-like behaviour compared to male adolescent rats. Overall, endotoxin exposure did not appear to be a significant risk factor for the development of anxiety disorders in adolescence. Physical stress during the early-life period may be of importance when researching risk factors for anxiety disorders.

Keywords: lipopolysaccharide, adolescence, stress, neonatal, endotoxin, sex differences, anxiety, light-dark test, conditioned disgust

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All experimental work was carried out by Jordan Ward. Drs. Martin Kavaliers and Klaus-Peter Ossenkopp contributed to the design of the experiments presented, as well as their assistance in editing the manuscript.

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DEDICATION

I would like to dedicate this thesis to my parents. You have both sacrificed so much to allow me to accomplish my dreams, and I could not be any more thankful. This one's for you, dad.

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CHAPTER 1

GENERAL INRODUCTION

1.1 Introduction

The neonatal period is a time in which the developing brain is going through substantial neurodevelopment. Synaptogenesis, neurogenesis, programmed cell death, and myelination are some key processes that occur during this time (Brunton, 2015). Therefore, disturbances of these processes could have a profound impact on how the brain develops; this is known as early-life programming. There is now substantial evidence in pre-clinical models suggesting that early life stress can influence the appearance of psychiatric disorders later in life, including affective disorders (O'Mahony et al., 2009). Studies from human populations have revealed similar results, such that early-life adverse events increase the risk of later life psychopathologies (Gilmer and McKinney, 2003). The hypothalamic-pituitary-adrenal (HPA) axis is a vulnerable pathway during the perinatal period, and early-life stress directly influences its development (Kehoe et al., 1998; Walker et al., 2006). Pathological changes in the HPA axis putatively result in anxiety disorders later in life by alteration of excitatory and inhibitory inputs (Gunn et al., 2015).

1.1.2 Early-life Immune Stress

One method to induce stress during the early life period is to stimulate the immune system. There is now substantial evidence showing that abnormal activation of immune cells early in life may result in pathological brain changes that persist into adulthood (Harvey and Boksa, 2012; Bennet and Gunn, 2006). Due to the critical nature of early postnatal development, and the underdevelopment of the immune system at that time, it is crucial to understand the effect of postnatal pathogen exposure and its effects on future behaviour (Walker et al., 2006). Another consideration is that the blood brain barrier (BBB) during the early life period is also

underdeveloped (Bilbo and Schwarz, 2012). This underdeveloped BBB is susceptible to the crossing of lipid-insoluble molecules, which includes peripheral proinflammatory cytokines released by the immune system in response to an immunogen (Barichello et al., 2012). It is also well known that there is bidirectional communication between the endocrine system and the immune system (Silverman et al., 2005). Due to bidirectional communication between the immune and endocrine system, these abundant proinflammatory cytokines produced by immune stimulation may alter the developmental pattern of the HPA-axis (Shanks et al., 2000). The proinflammatory cytokines result in the substantial release of glucocorticoids, which in turn results in the alteration of glucocorticoid receptor densities within the hypothalamus and hippocampus that persist into adulthood (Shanks et al., 1995). Due to the alterations in glucocorticoid receptor densities, changes in synaptic transmission occur, leading to the appearance of psychopathology (Shanks et al., 1995). Microglia are also likely involved in the alteration of neurodevelopment due to early-life immune stress (Hoeijmakers et al., 2014). These immune cells (microglia) are heavily involved in neuronal activation and plasticity, and therefore, abnormal activation of microglia could alter homeostasis during the early life period and result in abnormal brain development (Hoeijmakers et al., 2014; Allen and Barres, 2009). The brain changes induced by early life immune stimulation have been theorized to manifest as various psychopathologies including schizophrenia, autism, anxiety and stress disorders (Bilbo and Schwarz, 2009).

To model this pathological development in rodents, an experimental protocol known as the dual-exposure-to-endotoxin (DEE) is often employed. This protocol involves intraperitoneal (i.p.) administration of an immunogen, such as lipopolysaccharide (LPS), on post-natal day (PND) 3 and PND 5 in rats (Walker et al., 2004). Studies investigating the effect of neonatal

DEE on behaviour typically involve doses of 50 µg/kg up to 200 µg/kg. The timing of drug treatment in DEE is based on the nature of endocrine development during the first 2 weeks of life. There is a hyporesponsivity to ACTH during the neonatal period between PND 5 and PND 14 (Sapolsky and Meaney, 1986; Witek-Janusek, 1998). These effects are likely due to reduced adrenal gland size during this post-natal age range and a reduced response by the adrenal cortex (Sapolsky and Meaney, 1986; Witek-Janusek, 1998).

LPS is an immunogen that is found on the cell wall of Gram-negative bacteria that can mimic a bacterial infection by initiating an immune response (Bilbo and Schwarz, 2009). This immunogen (LPS) binds to toll-like receptor 4 (TLR4) on immune cells. A cascade is initiated by the receptor activation, resulting in the synthesis and release of a broad range of cytokines including: IL-1 β , IL-6, and TNF α .

From the perspective of the neuroendocrine system, LPS administration results in an alteration in HPA functioning in terms of measurement of basal corticosterone and basal blood ACTH levels in LPS treated rats when compared to saline controls in adulthood (Shanks et al., 2000). There is growing literature supporting the hypothesis that early postnatal pathogen exposure in rats may be one of several causes of anxiety disorders in adulthood (Tenk, et al., 2013; Walker et al., 2009; Breivik et al., 2002). Anxiety is an affective state, consistent between rats and humans, and is characterized by various physiological and behavioural responses (Gray and McNaughton, 2003). This affective state of anxiety can be elicited by either a perceived or a true threat. The most characteristic stress response is the release of corticoids (cortisol in humans and corticosterone in rats) following the activation of the HPA axis. However, the results of various behavioural assessments are inconsistent. It has been reported that neonatal LPS exposure results in anxiety-like behaviour in adults (Breivik et al., 2002, Walker et al., 2004, and

Walker et al., 2009); however, decreases or no changes in anxiety-like behaviour following neonatal endotoxin exposure have also been determined (Breivik et al., 2002, Tenk et al., 2013, and Walker et al., 2008). In general, previous results are suggestive of the hypothesis that anxiety-like behaviour is increased in rats exposed to an endotoxin via a stressor effect (Walker et al., 2008). This theory states that rats exposed to DEE will show anxiety-like behaviour in adulthood if they are exposed to an acute stressor.

There is currently a gap in the literature investigating the impact of DEE on adolescent behaviour. Behaviourally, it has been found that the DEE (LPS) does not alter anxiety-like behaviour in adolescence (Walker et al., 2004); however, it is important to note that this study did not involve a stressor effect, which has been theorized to induce anxiety-like behaviour. To the best of the author's knowledge, no other studies have been conducted investigating the impact of neonatal LPS exposure on behaviour in the adolescent age range.

1.1.3 Early-life Physical Stress

Numerous models for early-life physical stress have been investigated in the rodent model. The methods used to induce early life stress in rodents include the following: acute maternal deprivation (de Kloet et al., 2005), maternal separation (Sanchez et al., 2001; Huot et al., 2002), handling (Durand et al., 1998), and early weaning of pups (Kikusui and Mori, 2009). Another interesting early-life stressor that has not been extensively researched is the role of neonatal injection and toe clipping on future behaviour. Exposure of neonatal rats to these mentioned procedures can directly influence behavioural and physiological measures later in life, most commonly changes in the hypothalamic-pituitary-adrenal (HPA) axis in response to stress (Walker et al., 2006; Brunton, 2015; Maccari et al., 2014; Wingensfeld and Wolf, 2011).

Research investigating early-life stress on behaviour later in life suggest that the amount of stress and the time period is critical to the development of anxiety-like behaviour in rats. There have been few studies looking at this effect in adolescence; however, the results are consistent for early-life stress inducing anxiety-like behaviour in adulthood. Weininger (1954) found that handling for as little as 10 min daily for 21 days resulted in changes in later life physiology and behaviour that was indicative of fearfulness. This early-life handling elicits an endocrine response in the pups which is indicative of a stress response (Meaney et al., 2000). Adult female C57BL/6 mice, who underwent maternal separation during the early life period, showed reduced anxiety-like behaviour in the elevated-plus maze, and adult males showed an increase in anxiety-like behaviour (Romeo et al., 2003). Adult male and female rats exposed to periodic maternal separation from PND 3-10, showed significant increases in anxiety-like behaviour measured in the elevated plus-maze (Wigger and Neumann, 1999). This was further supported by a study by Kalinichev and colleagues (2002), where it was found that both male and female adult rats, who had been exposed to maternal separation during early life, spent significantly less time in the open arms of the elevated plus maze compared to non-handled controls.

Results of early-life stress on adolescent anxiety-like behaviour are more inconsistent. Maternal separation during the neonatal period, consisting of 15 minute periods every day from post-natal day (PND) 2 to PND 21, results in decreased adolescent anxiety-like behaviour for adolescent females but not adolescent males (McIntosh et al., 1999). Maternal deprivation, consisting of dam separation for 24 hr from PND 9 to PND 10, resulted in no significant changes in anxiety-like behaviour on the elevated plus maze test, unless they were subsequently challenged with saline injection stress. A similar effect was also seen if rats were not exposed to

maternal deprivation, but were exposed to saline injections (Girardi et al., 2014). To the best of the current author's knowledge, there have been no other studies investigating the effect of early life stress on adolescent anxiety-like behaviour.

Research procedures during the early life period (including DEE) frequently employ the use of toe clipping and injections in the neonatal period to assess the effect of neonatal drug treatment (e.g. lipopolysaccharide) on anxiety-related behaviour and physiology. Toe clipping procedures are used for future identification of animals given drug treatments. Both toe clipping and injections can be considered early life stressors. The physical stress associated with intraperitoneal injections and toe clipping in the early post-natal period have not been investigated in terms of their effects on adolescent behaviour changes when compared to untreated rats. It is important to consider the effects of these handling procedures, as they have similarity to maternal separation. The handling procedures involve the removal of pups from the dam for a period of 10 minutes (Tenk et al., 2007). Alterations in behaviour from neonatal injection and toe clipping stress can be assessed in adolescence using paradigms sensitive to anxiety-like behaviour (e.g. time spent in the light chamber of the light-dark test). There is an evident gap in literature regarding the use of untreated controls in neonatal research as a comparison group to a variety of experimental treatments which can affect behaviour later in life.

Research in adolescent NIH-Swiss mice has shown that acute i.p. injection of saline shows a behavioural profile of mice with heightened anxiety-like behaviour in the elevated plus maze (Lapin, 1995). Chronic administration of saline over a two-week period (7 saline injections; 3 sham injections) in adolescent mice has been associated with habituation over the two-week period regarding the physiological release of corticosterone (Ryabinin et al., 1999). Ryabinin and colleagues (1999) also found that a single injection of saline resulted in a

significant release of corticosterone. Therefore, injections show a physiological stress response by the subject that habituates over-time. The lack of an untreated control group may be a factor influencing the inconsistent data in neonatal endotoxin research on future anxiety-like behaviour. Neonatal injections, toe clipping procedures, and the brief maternal separation may be stressful to the pups and induce pathological changes in neuroendocrine communication and functioning. Previous research has suggested no significant differences in HPA activity between saline controls and no injection controls regarding blood measures of HPA activity in adulthood following restraint stress (Meaney et al., 1987; Shanks et al., 1995). However, results on behavioural measures of anxiety-like behaviour have not been previously investigated in either adolescence or adulthood.

1.1.4 Concluding Remarks

The present thesis investigated the impact of DEE on adolescent rat behaviour measured with the light-dark test before (Chapter 2; PND 35) and after chronic injection stress (Chapter 3; PND 47). Novel to the DEE model, the studies within this thesis involved the use of untreated controls that were not toe clipped, maternally separated, or neonatally treated with either LPS or saline. The studies involved dual exposure to one of two doses of LPS (15 µg/kg or 50 µg/kg) on PND 3 and PND 5 to examine a potential dose response of immune stimulation on anxiety-like behaviour in adolescence. Sex differences in anxiety-like behaviour were also examined by including both males and females from each litter into the studies. All behaviour was measured using the light-dark test to measure approach-avoidance behaviour that are indexes of both locomotor activity and anxiety.

The study presented in Chapter 3 examined the effect of chronic injection stress on rats neonatally treated with either LPS or saline on PND 3 and PND 5, and in untreated neonatal

controls (rats that did not receive treatment during the neonatal period). Previous research has suggested that rats exposed to LPS on PND 3 and PND 5 show anxiety-like behaviour in adulthood only following a stressor, typically restraint stress (Walker et al., 2008). Chapter 3 employed a different method to induce stress in rats. The stress procedure used in Chapter 3 involved rats acquiring conditioned disgust. Conditioned disgust is a common method to assess anticipatory nausea in rats, and involves the i.p. injection of LiCl, to induce toxicosis, or a saline control. Following the injection, the rats are placed in a novel context for a period of 30 minutes. Rats receive these injections over 4 conditioning trials with a 72h interval separating each trial. It was hypothesized that this procedure would be sufficiently stressful to alter anxiety-like behaviour in rats treated with LPS on PND 3 and PND 5. Untreated controls were involved to allow for comparison with the saline controls. The saline controls did not have their immune systems stimulated; however, they still underwent early-life stress involving toe clipping (PND 3), saline injections (PND 3 and PND 5), and maternal separation (15 min; PND 3 and PND 5). Therefore, the untreated controls provide a meaningful comparison to examine the effect of early-life stress on anxiety-like behaviour before and after chronic injection stress.

It was hypothesized that untreated controls would be less anxious before and after the stress induced by conditioned disgust compared to all other treatments. It was also hypothesized that a dose of LPS 15 $\mu\text{g}/\text{kg}$ would result in similar anxiety-like behaviour compared to saline controls, and that an LPS dose of 50 $\mu\text{g}/\text{kg}$ would result in significant anxiety-like behaviour across all indexes. Finally, it was hypothesized that anxiety-like behaviour would increase in all LPS treated animals following the stress induced by conditioned disgust.

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CHAPTER 2

**EARLY LIFE IMMUNE AND PHYSICAL STRESS DIRECTLY INFLUENCES
ANXIETY-LIKE BEHAVIOUR IN ADOLESCENT RATS**

2.1 Introduction

Exposure to environmental events during the early-life period of an organism can result in significant changes in neurodevelopment that may ultimately lead to abnormal behaviour later in life (Hida et al, 2013). Anxiety disorders are of a concern within the human population, and have been hypothesized to be a result of early-life environmental events that induce physiological stress in the newborn (Faravelli et al., 2012). Pre-clinical rodent models have investigated this association between early-life environmental stress and psychopathologies later in life. A variety of models have been employed that include physical stress and immune stress to mimic the environments seen in the human population. Physical stress often uses one of the following methods: acute maternal deprivation (de Kloet et al., 2005), maternal separation (Sanchez et al., 2001; Huot et al., 2002), chronic early life stress (Ivy et al., 2008), handling (Durand et al., 1998), and early weaning of pups (Kikusui and Mori, 2009). Immune stress is a method that involves activation of the immune system at an early age using an endotoxin (e.g. lipopolysaccharide; LPS) on PND 3 and PND 5 (Tenk et al., 2007). This chapter focuses on the effect of physical stress and immune stress on PND 3 and 5, and its subsequent effects on adolescent anxiety-like behaviour assessed with the light-dark test.

Physical stress has been extensively used during the early-life period in animal models to understand various mechanisms that may induce a predisposition to anxiety-like behaviour later in life. The use of early-life handling as a natural environmental stressor has been used since 1954 (Weininger, 1954). Weininger (1954) found that handling for as little as 10 min daily for 21 days resulted in changes in later life physiology and behaviour that was indicative of fearfulness. This early-life handling elicits an endocrine response in the pups which is indicative of a stress response (Meaney et al., 2000). Early-life handling, however, is a physical stressor

that is multifactorial and elicits behavioural changes (Macrì and Würbel, 2006). This period of early handling results in an increase in maternal care (Liu et al., 1997). This increase in maternal care is linked to a general reduction in HPA-responses in adulthood, suggesting that early-life handling reduces the endocrine response to stress in adulthood (Macrì and Würbel, 2006).

However, if this early-life stress begins to increase, the resilience effects produced by an increase in maternal care become negligible (Macrì and Würbel, 2006). Maternal separation is a method that is more stressful to the pups, when compared to early-life handling, which entails separation of pups from the dam for 180 minutes (Plotsky and Meaney, 1993). When exposed to a stressor later in life, maternal separation results in a significant increase in HPA-axis response compared to both no handling controls and early-life handling (Plotsky and Meaney, 1993). Overall, this suggests that the adulthood response to acute stress is dependent upon the severity of early-life stress experienced (Macrì and Würbel, 2006).

The immune system is known to have a vital role in host defense as it prevents substantial damage by foreign pathogens and objects. Current research on the immune system has shown its critical involvement in homeostasis. There is now substantial evidence that abnormal activation of the immune system during the early life period may result in pathological brain changes (Harvey and Boksa, 2012). There are currently several methods employed to induce an immune response within a rodent that can mimic bacterial infection; however, the primary method used is the dual exposure to endotoxin (DEE) method. The DEE method involves the systemic administration of lipopolysaccharide (LPS) on PND 3 and PND 5 to induce an immune response.

LPS is a component of the cell wall of Gram-negative bacteria. This immunogen induces an immune response without the damage often associated with live infection, allowing for

examination of immune activation specifically. LPS binds to TLR-4 receptors found on immune cells, where it causes the release of proinflammatory cytokines into the extracellular space.

Behaviourally the impact of neonatal exposure to an endotoxin is relatively inconsistent. However, findings suggest the potential involvement of immune activation on anxiety-like behaviour into adulthood (Tenk et al., 2012; Walker et al., 2009; Breivik et al., 2002). Several studies have investigated the effect of a neonatal administration of 50 µg/kg of LPS on adulthood behaviour, and it has been found to induce anxiety-like behaviours (Breivik et al., 2002, Walker et al., 2004, and Walker et al., 2009). On the other hand, several studies using this same protocol have obtained nonsignificant results regarding anxiety-like behaviour differences between neonatal saline treatments and peripheral injection of 50 µg/kg LPS (Breivik et al., 2002, Tenk et al., 2013, and Walker et al., 2008). It is also important to note that very few studies have investigated the effect of neonatal administration of endotoxins on adolescent anxiety-like behaviour. Adolescence is a key time period that should be explored as changes in microglial colonization occur at this age (Rebuli et al., 2016).

A potential explanation for the inconsistent effects seen in the DEE method is that previous studies did not employ the use of untreated controls during the early post-natal period. The lack of untreated controls may be a factor influencing the inconsistent data in neonatal endotoxin research on future anxiety-like behaviour. The neonatal injections, coupled with toe clipping procedures and the brief maternal separation, may reach a stress threshold that induces pathological changes in neuroendocrine communication and functioning. Previous research has found no significant differences in restraint stress induced HPA activity between saline treated controls and no injection controls regarding blood measures of HPA activity in adulthood (Meaney et al., 1987; Shanks et al., 1995). However, results on behavioural measures of

anxiety-like behaviour have not been previously investigated in either adolescence or adulthood. To date, there have been no studies investigating the impact of untreated neonatal controls on anxiety-like behaviour. The handling associated with the DEE method is unlikely to induce long-lasting changes in behaviour and HPA-axis functioning, as previous research has shown that this may induce resilience; however, in combination with the stressful saline injections on PND 3 and 5 and toe clipping on PND 3, this stress may not be recoverable by maternal care. The lack of information regarding this combination of stressors signifies the need to explore its effect on future behaviour.

Therefore, the aim of the present study was to further examine the effect of early life stress on anxiety-like behaviour in adolescence, with an emphasis placed on the use of untreated controls. It was hypothesized that the handling, injections, and toe clipping combination during the early neonatal period would be sufficient to induce long lasting behavioural changes in neonatal saline controls that could be measured in adolescence using the light-dark test. Sex differences were also investigated as there is relatively little data on the effect of DEE in both males and females.

2.2 Methods

2.2.1 Animals

Fifteen sexually naïve Long-Evans females (226-250) were paired with sexually experienced male Long-Evans males (300-400g). Breeders were obtained from Charles River, Canada. Two weeks following pairing, the male rat was removed from the cage leaving the female single-housed in a standard polypropylene cage (45 cm x 22 cm x 20 cm). Rooms were temperature-controlled ($20 \pm 1^\circ \text{C}$) maintained on a 12:12 h light-dark cycle. *Ad libitum* access to food (Prolab RMH3000 lab chow) and tap water. Cages were checked twice daily for birth of

litters. Day of birth was designated as postnatal day 1 (PND 1). Litters were culled to 12 rats per litter on PND 5. On PND 22 rats were weaned and group-housed with same-sex littermate (3-5 rats per cage). Same-treatment was preferred; however, due to sample sizes this did not always occur. Forty-eight no injection controls were ordered from Charles River, Canada at a weight of 50-75g and were same-sex group-housed. Rats were ordered at this weight range, as this was the earliest possible age following weaning at Charles River and allowed habituation to the research environment prior to testing. Furthermore, the age of the No Injection controls was estimated to be PND 35 when their average weight was within the weight range chart provided by Charles River. All experimental manipulations were conducted during the light phase of the light:dark cycle. Vaginal smears were collected at 14:00 h following the behavioural test and the day following at the same time to assess estrous cycles. It is important to consider that the vaginal smear collection procedure was stressful to the female rats. Analysis revealed that females were proportionally tested across all estrous stages minimizing estrous cycle effects. 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.

2.2.2 Neonatal drug administration

Each litter was assigned either LPS 15 µg/kg or LPS 50 µg/kg, with 0.9% isotonic saline controls in each litter. LPS (derived from *E. coli* serotype 0111:B4, no. L2630, Sigma Chemical, St. Louis, MO, USA) was dissolved in 0.9% isotonic saline. The low dose of LPS 15 µg/kg was chosen as it has not been previously used in this field of research, and falls above the effective dose, as a low dose of 5 µg/kg still results in slight alterations in cytokine release (Lenczowski et al., 1997). The dose of 50 µg/kg was chosen as it has been previously used in our research group

with significant results, and is one of the most common doses used in neonatal immune stimulation on anxiety-like behaviour (Tenk et al., 2007; Tenk et al., 2008; Tenk et al., 2013). On PND 3, rats were randomly assigned to the respective LPS dose for that litter, or saline as a control. All injections were made intraperitoneally using a Hamilton syringe with a 30-gauge needle tip at a volume of 1 ml/kg. Final group numbers are shown in Appendix A. An attempt was made to balance drug treatment group sizes per litter, however, due to mortality rates and birth defects, group sizes per litter varied. No more than three pups of each sex were assigned to a treatment from a single litter. Following the injection on PND 3, toe clipping was performed to assist with future identification of the rat's treatment. Prior to toe clipping, the respective feet for the toe clipping were cooled with an ice pack to minimize pain during the procedure. On PND 5, rats were again injected with their respective treatment. All injections were administered between 10:00 and 12:00 h. During the injection and toe clipping procedures, the entire litter was removed from the home cage and placed under a heat-lamp for the entire duration. Each litter took no more than 10 minutes for injections and toe clippings. Following PND 5, rats were left untouched in their home cage other than for weekly cage changes until PND 22 for weaning. Rats were group housed following weaning with same-sex rats, and preference was also placed on same-treatment groups.

2.2.3 Light-dark apparatus

Eight modified VersaMax Animal Activity Monitors (Accuscan Model RXYCZM-16, Columbus, OH) were used to conduct light-dark testing. Each monitor consisted of a clear Plexiglas open-field (40 cm x 40 cm x 30.5 cm) with a clear Plexiglas lid with air holes. Infrared photo beams were located 2.54 cm apart and 5 cm above the floor around the perimeter of the

open field. Two additional banks of 16 photo beams were located on opposite sides of the open-field, 15.6 cm above the floor.

An opaque black Plexiglas box (40 cm x 20 cm x 30 cm) was inserted into each Animal Activity Monitor which modified it in such a way that divided the open-field into two equal sized chambers. This opaque black box contained a 10 cm x 8.5 cm entry, which allowed unrestricted access between both chambers. The side containing the black box was considered the “dark” chamber, and the other side the “light” chamber. Above the apparatus was three fluorescent bulbs providing a light source of approximately 900 lux at the floor of the light chamber. The black Plexiglas box contained holes around each side to allow the infrared beams to pass through the box.

The light-dark test was used in this thesis as our research group has previously used the same apparatus with significant results (Tenk et al., 2007; Tenk et al., 2008; Tenk et al., 2013).

2.2.4 Variables quantified by the Versamax System during light-dark testing

The Versamax system automatically quantified the variables during behavioural testing.

Anxiety-like Behaviour Variables

Anxiety-like behaviour variables recorded by Versamax include: Duration of time spent in seconds in the light and dark chamber, number of Nose Pokes into the light chamber, and Chamber Transitions into the light chamber (animal crossed entire body into the opposite chamber).

Locomotor Activity Variables

Locomotor activity variables were assessed to validate anxiety-like behaviour findings, and to explore potential changes in locomotor activity due to LPS treatment. Locomotor activity variables recorded by Versamax include: Total Distance in each chamber (corrected for the unequal amount of time spent in each of the chambers; cm/s), Vertical Time (time spent moving in the vertical plane as a proportion of time spent in each chamber) and Number of Vertical Movements (number of movements in the vertical plane per second spent in the respective chamber).

2.3 Procedures

2.3.1 Behavioural testing procedure

On PND 35, adolescent rats were tested in the light-dark apparatus. Male and female rats were tested separately, with males being tested prior to females. Before testing, rats were habituated to the Versamax monitoring room for a duration of 45 minutes. Immediately following the 45 min habituation period, they were placed facing the Plexiglas wall on the opposite end of the “dark” chamber entrance. Rats were allowed unrestricted access to both chambers while behavioural data was collected for a duration of 30 minutes; however, only the first 10 minutes of data were analyzed.

2.4 Statistical analyses

All analysis was conducted using a 2x4 ANOVA accounting for the random effect of litter (16 litters, where no injection controls were considered as an additional litter). Between subject factors consisted of sex (at 2 levels: male; female) and neonatal treatment (at 4 levels: no injection; saline; LPS 15 µg/kg; LPS 50 µg/kg). Post-hoc pair-wise comparisons were made using least significant difference (LSD) analysis to investigate group comparisons following a

significant main effect and interaction effect. LSD post-hoc analysis was chosen for this study due to the exploratory nature of the study being conducted. All hypothesis tests used an alpha of .05 criterion, and analyzed using SPSS 24.0 for Windows.

2.5 Results

2.5.1 Chamber Transitions

A significant main effect of neonatal treatment on total chamber transitions was obtained, $F(2, 13.149) = 4.104, p = .041$. Post-hoc analysis revealed that no injection controls ($M = 35.292, SE = 1.629$) had significantly more chamber transitions compared to neonatal saline ($M = 28.256, SE = 1.463, p = .000$), LPS 15 $\mu\text{g}/\text{kg}$ ($M = 31.994, SE = 1.861, p = .028$), and LPS 50 $\mu\text{g}/\text{kg}$ ($M = 26.157, SE = 2.007, p = .000$), as seen in Figure 2.1. Rats treated with LPS 15 $\mu\text{g}/\text{kg}$ also made significantly more transitions compared to LPS 50 $\mu\text{g}/\text{kg}$ ($p = .018$). No main effect of sex was found and no significant interaction was revealed.

2.5.2 Time Spent in the Light Chamber

A significant main effect of neonatal treatment on time spent in the light chamber was found, $F(2, 14.565) = 14.478, p = .000$. Post-hoc analysis revealed multiple significant comparisons. No neonatal injection controls ($M = 160.723, SE = 8.973$) spent significantly more time in the light chamber compared to neonatal saline ($M = 67.463, SE = 8.059, p < .001$), LPS 15 $\mu\text{g}/\text{kg}$ ($M = 115.977 \text{ s}, SE = 10.251, p = .000$), and LPS 50 $\mu\text{g}/\text{kg}$ ($M = 74.931, SE = 11.057, p = .000$), as seen in Figure 2.2. Furthermore, rats receiving LPS 15 $\mu\text{g}/\text{kg}$ also spent significantly more time in the light chamber compared to neonatal saline ($p = .003$) and neonatal LPS 50 $\mu\text{g}/\text{kg}$ ($p = .020$). There was no significant main effect of litter. No significant main

effect of sex was found. $F(1, 3.621) = 5.855, p = .079$, and no significant interaction effects was obtained.

2.5.3 Nose Pokes into the Light Chamber

Analysis revealed no significant main effect of neonatal treatment on nose pokes into light chamber, $F(2, 12.717) = 3.022, p = .084$. A significant main effect of sex was obtained, $F(1, 11.310) = 7.918, p = .016$, with females ($M = 11.899, SE = .585$) making significantly more nose pokes compared to males ($M = 8.636, SE = .556, p = .000$), as seen in Figure 2.3. No other significant interactions were found.

2.5.4 Total Distance in Light Chamber (Corrected)

ANOVA analysis found no significant main effect for sex or neonatal treatment and no significant interaction effects.

2.5.5 Vertical Movement Number in the Light Chamber (Corrected)

A significant main effect of neonatal treatment was found, $F(2, 14.005) = 4.485, p = .031$. Post-hoc analysis revealed that neonatal saline treated rats ($M = .050, SE = .006$) made significantly fewer vertical movements per second in the light chamber compared to no injection controls ($M = .080, SE = .007, p = .001$) and LPS 15 $\mu\text{g}/\text{kg}$ treated rats ($M = .071, SE = .007, p = .025$). No significant main effect of sex was found. A significant interaction between sex and neonatal treatment was found, $F(2, 12.162) = 7.488, p = .008$. Pairwise comparisons revealed that male rats treated with saline ($M = .037, SE = .008$) had significantly less vertical movements per second in the light chamber compared to no injection control ($M = .078, SE = .009, p = .001$),

LPS 15 µg/kg ($M = .083$, $SE = .010$, $p = .001$), and LPS 50 µg/kg ($M = .068$, $SE = .011$, $p = .026$), as seen in Figure 2.4. Furthermore, males treated with saline had significantly fewer vertical movements per second compared to females treated with saline ($M = .061$, $SE = .009$, $p = .038$). A significant interaction between sex and litter was also revealed, $F(12, 8.744) = 3.204$, $p = .046$.

2.5.6 Vertical Movement Time in the Light Chamber (Corrected)

No significant main effect for sex or neonatal treatment was revealed, and no interaction effect was found.

2.5.7 Distance in the Dark Chamber (Corrected)

A significant main effect of sex was found following the ANOVA analysis, $F(1, 9.529) = 7.307$, $p = .023$. Females exhibited significantly more total distance (cm) per second spent in the chamber ($M = 1.615$, $SE = .060$) compared to males ($M = 1.333$, $SE = .057$, $p = .001$), as seen in Figure 2.5. No significant main effect of neonatal treatment was found, and no significant interactions were revealed.

2.5.8 Vertical Movement Number in the Dark Chamber (Corrected)

No significant main effect was found for sex or neonatal treatment, and no significant interaction effects were revealed.

2.5.9 Vertical Time in Dark Chamber (Corrected)

Analysis revealed no significant main effect for sex or neonatal treatment, and no significant interaction effects

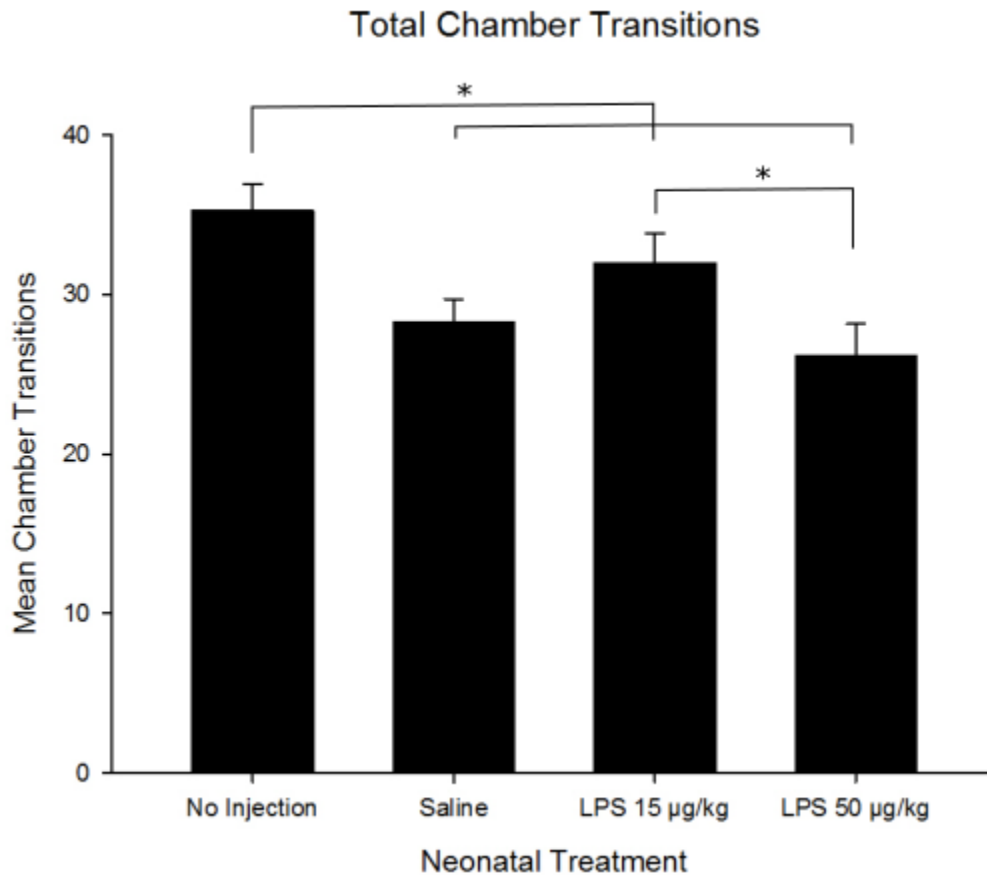


Figure 2.1: Group mean (\pm S.E.M.) activity behaviour of total chamber transitions during adolescence (PND 35). * $p < .05$ indicates a significant Neonatal Drug Treatment effect, where no injection controls made significantly more chamber transitions compared to all other neonatal treatments. Rats neonatally treated with LPS 15 $\mu\text{g}/\text{kg}$ also made more chamber transitions compared to rats receiving 50 $\mu\text{g}/\text{kg}$ LPS (No Injection; $n = 48$, Saline; $n = 72$, LPS 15 $\mu\text{g}/\text{kg}$; $n = 42$, LPS 50 $\mu\text{g}/\text{kg}$; $n = 39$).

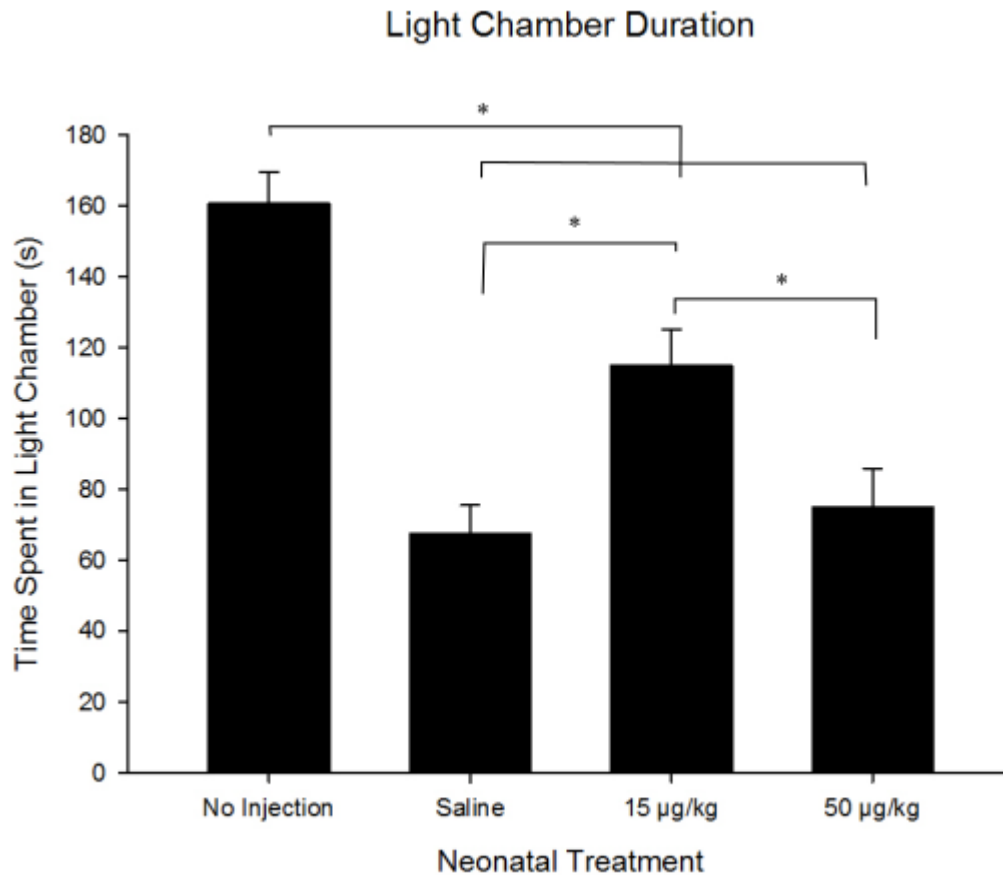


Figure 2.2: Group mean (\pm S.E.M.) activity behaviour of time spent in the light chamber during adolescence (PND 35). * $p < .05$ indicates a significant Neonatal Drug Treatment effect, where no injection controls spent significantly more time in the light chamber compared to all other neonatal treatments. Rats neonatally treated with LPS 15 $\mu\text{g}/\text{kg}$ also spent significantly more time in the light chamber compared to saline controls and rats receiving 50 $\mu\text{g}/\text{kg}$ LPS (No Injection; $n = 48$, Saline; $n = 72$, LPS 15 $\mu\text{g}/\text{kg}$; $n = 42$, LPS 50 $\mu\text{g}/\text{kg}$; $n = 39$).

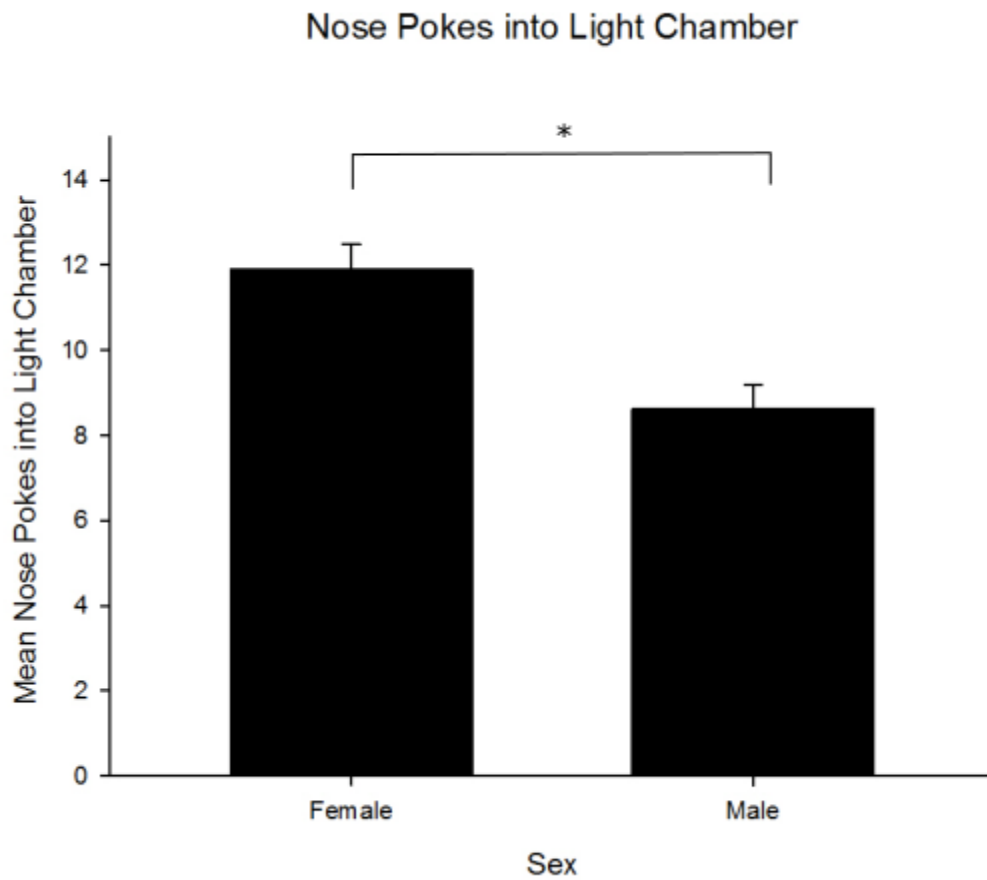


Figure 2.3: Group mean (\pm S.E.M.) activity behaviour of nose pokes into the light chamber during adolescence (PND 35). * $p < .05$ indicates a significant Sex effect, where females made significantly more nose pokes into the light chamber compared to males. (Female; $n = 99$, Male; $n = 102$).

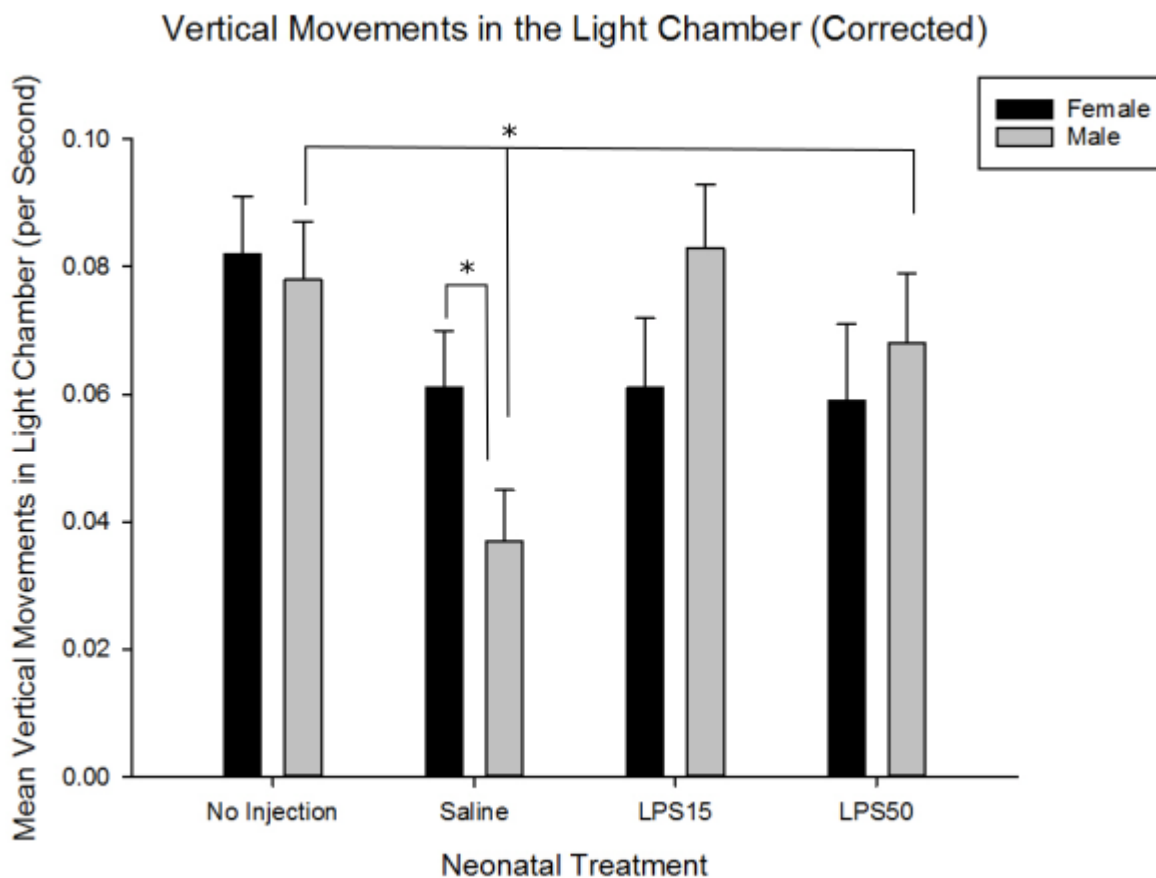


Figure 2.4: Group mean (\pm S.E.M.) activity behaviour of vertical movements in the light chamber, per second spent in the chamber, during adolescence (PND 35). * $p < .05$ indicates a significant Neonatal Drug Treatment by Sex interaction effect. Male rats receiving saline injections during the neonatal period made significantly less vertical movements per second spent in the light chamber compared to all other neonatal treatments. Females receiving neonatal saline also made significantly more vertical movements per second spent in the light chamber compared to their male counterparts (male-No Injection; $n = 24$, male-Saline; $n = 36$, male-LPS 15 $\mu\text{g}/\text{kg}$; $n = 21$, male-LPS 50 $\mu\text{g}/\text{kg}$; $n = 21$, female-No Injection; $n = 24$, female-Saline; $n = 36$, female-LPS 15 $\mu\text{g}/\text{kg}$; $n = 21$, female-LPS 50 $\mu\text{g}/\text{kg}$; $n = 18$).

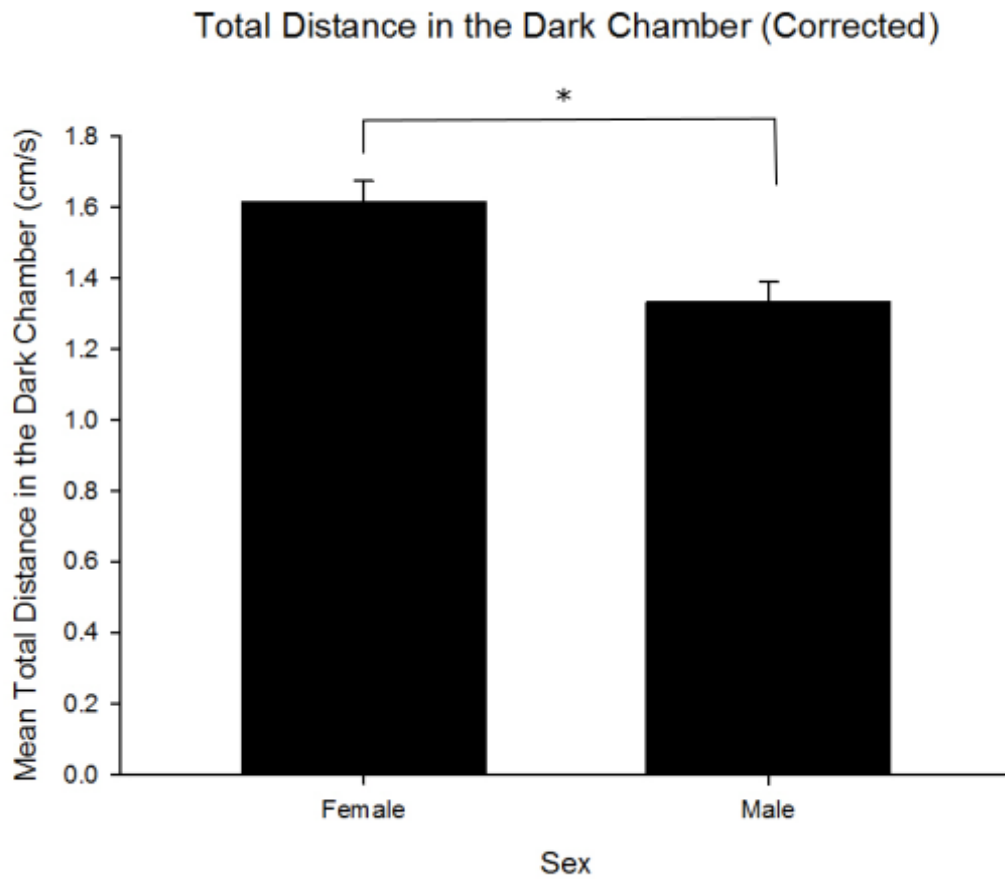


Figure 2.5: Group mean (\pm S.E.M.) activity behaviour of total distance in the dark per second spent in the dark chamber during adolescence (PND 35). * $p < .05$ indicates a significant Sex effect, where females moved significantly more in the dark chamber compared to males.

(Female; $n = 99$, Male; $n = 102$).

2.6 Discussion

The purpose of the current investigation was to examine the potential long-term programming of adolescent behaviour following the exposure to physical and/or immune stress during the neonatal period. Sex differences were also explored in light of the inherent differences in microglial colonization during the neonatal period, and hormonal differences during the adolescent age period (Schwarz et al., 2013). Inconsistent results in neonatal endotoxin effects using the DEE model on anxiety-like behaviour suggested the need for further investigation. Furthermore, these previous studies did not employ untreated neonates to control for the physical stress of handling, injection, and toe clipping on future behaviour.

The results of the present study suggest that both LPS and saline injections during the early post-natal life result in changes in anxiety-like behaviour, as measured by time spent in the light chamber, chamber transitions, and vertical movements in the light chamber. However, these results were obtained only when comparisons were made against the untreated controls. Interestingly, it was also found that an LPS dose of 15 $\mu\text{g}/\text{kg}$ resulted in resilience, such that the rats receiving this dose were significantly less anxious when compared to the LPS dose of 50 $\mu\text{g}/\text{kg}$ and saline controls, as measured by time spent in the light chamber and total chamber transitions. Rats receiving LPS at a dose of 15 $\mu\text{g}/\text{kg}$ also showed significantly less anxiety-like behaviour compared to the saline controls, as measured by time spent in the light chamber. The present results also revealed that males receiving neonatal saline showed significantly less anxiety-like behaviour compared to females receiving neonatal saline, as measured by vertical movements in the light chamber. Other sex differences consisted of differences in locomotor activity, as measured by total distance in the dark chamber, and significantly less anxiety-like behaviour in females, as measured by nose pokes into the light chamber. Collectively, the results

of this study suggest that the inconsistent effects seen in the DEE literature may be due to a lack of control for the early life physical stress associated with the methods being used for early life drug treatment.

The present results add to the current literature on the effect of DEE on behaviour later in life, specifically in adolescence. Currently, there has been only one study investigating the effect of a 50 µg/kg dose of LPS on behaviour in adolescence, where it was shown to not induce anxiety-like behaviour on the elevated plus maze (Walker et al., 2004). The results of the present study are consistent with this literature, the 50 µg/kg dose of LPS group was not significantly different from saline controls on time spent in the light chamber in the light dark test. It is important to note that the study conducted by Walker and colleagues (2004) did not include untreated controls. The present study also found that rats treated with a 15 µg/kg dose of LPS spent significantly less time in the light chamber compared to both the saline controls and the 50 µg/kg dose of LPS group.

From a physical stress viewpoint, the three groups (neonatal saline, LPS 15 µg/kg and LPS 50 µg/kg) experienced exactly the same procedures, and all showed significantly less time spent in the light chamber compared to the untreated controls. The physical stress involved in the DEE method involves handling and separation from the dam for a period of 10 minutes, intraperitoneal injections, and toe clipping on PND 3. Thus, the physical stress involved in the procedures during neonatal drug manipulation predisposed the rats to anxiety-like behaviour in adolescence, as seen by time spent in the light chamber.

Previous research has shown that maternal separation in rats during the first 2 weeks of life results in neurodevelopmental changes in HPA-axis functioning, such that there is corticotrophin-releasing hormone (CRH) promoter hypomethylation and an enhancement of

CRH transcriptional responses in response to stress in adulthood (Chen et al., 2012). Neonatal mice and their respective dams showed enhanced excitatory glutamatergic drive to the CRH neurons in the paraventricular nucleus when they were exposed to early life stress involving reduced environmental enrichment (Gunn et al., 2013). Inhibitory inputs in the HPA-axis also appear to be altered in models of early life stress, and they are likely dependent on the type of early life stress. Plotsky and Meaney (1993) investigated the differences between no handling, handling, and maternal separation. The difference between handling and maternal separation was dependent on the amount of time the pups spent away from the dam: handling involved a separation of 15 min daily for 2 weeks, and maternal separation consisted of separation from the dam for 180 min daily for 2 weeks. The results of the present study, and previous studies, suggest that rats subjected to no handling during early-life show enhanced HPA-axis response to stress when compared to handled neonates (Plotsky et al., 1993; Meaney et al., 1989; Viau et al., 1993). This suggests that the baseline plasma corticosterone levels are better at inhibiting HPA axis activity in rats exposed to handling at an early age (Plotsky et al., 1993). In other words, it appears that handling is protective rather than an inducer of pathological HPA-axis activity in later life. The hippocampus appears to be a primary target for these differences, as rats exposed to early life handling show greater glucocorticoid receptor density in the hippocampus compared to the non-handled controls (Plotsky et al., 1993). Early life stress in the Japanese quail, which involved the removal of food for 25% of the day (which has been previously shown to be stressful in birds; Buchanan et al., 2003), resulted in a decrease in glucocorticoid receptor density within the hippocampus (Zimmer and Spencer, 2014). This trend also appeared in the human population where a reduction in glucocorticoid receptor mRNA was found in the hippocampus of post-mortem brains of individuals with a history of childhood abuse who committed suicide

(McGowan et al., 2010). Overall, it is suggested that the effects of early life stress on hippocampal glucocorticoid receptor density is dependent on the severity of the stress during the early life period. It is also concluded that the 10 minutes of handling in the DEE method in the present study is unlikely to induce the anxiety-response in the neonatal treatment groups.

During the neonatal procedure of the current study, rat pups were removed from their dam for 10 minutes, placed under a heat lamp, exposed to an ice pack for local anesthetic for the toe clipping procedure, and injected intraperitoneally. It is possible that the toe clipping procedure may have induced the effect seen in the present study, such that untreated controls were less anxious overall compared to all neonatal treatments. Previous research has shown that toe clipping on PND 7 resulted in significantly-less time spent in the open arms of the elevated plus maze compared to sham toe clipping on PND 17 (Paluch et al., 2014); the authors attributed this effect to individual variation. A separate study conducted by Castelhana-Carlos and colleagues (2010) found no significant difference between toe clipping on PND 5 and controls on elevated plus maze activity in adulthood. Therefore, it is plausible that the results seen in the present study may have been due to the effect of toe clipping, as previous literature is inconsistent. However, it is difficult to make a definitive conclusion as there has been limited research conducted on the effect of toe clipping in rats, and subsequent behavioural assessments in adolescence.

Lastly, the other difference between the untreated controls and the neonatal treatments was the use of intraperitoneal injections. There is very little research conducted on this effect, and only a few studies have investigated the differences between neonatal saline and untreated controls during the neonatal period. It has, however, been found that neonatal saline injections do not alter biological markers of stress (Meaney et al., 1987). A single study in mice was found

where untreated controls were used to investigate the effect of neonatal morphine on time spent in the open arms of the elevated plus maze (Boasen et al., 2009). It was found that there were no significant differences in anxiety-like behaviour in adulthood between neonatal saline injections and untreated controls; however, it is important to note that this study was conducted in mice and behavioural tests occurred in adulthood. The current study involved the use of rats and behavioural testing in adolescence. It is plausible that the neonatal injections, whether saline or LPS, may have induced changes in the brain resulting in the anxiety-like behaviour in adolescence. More investigation is needed to explore this effect of neonatal injections on adolescent behaviour in rats.

Combining these factors (handling, toe clipping, injection), it is possible that toe clipping or a combination of all three factors may have produced the increased anxiety-like behaviour in the current study, as measured by time spent in the light chamber and total chamber transitions. As stated earlier, the more stressful the test situation (e.g. handling versus maternal separation) the greater the impact on glucocorticoid receptor densities within the hippocampus. Individually, these factors may not be stressful enough to induce pathological brain changes; however, in combination it may be sufficient. Future studies should explore these neonatal factors to validate neonatal research methods.

The unexpected result of the current study was the anxiolytic effect induced by the low 15 µg/kg dose of LPS. Previous research has shown that LPS acutely induces an increase in plasma ACTH, plasma CORT, and plasma IL-6; however, a low 5 µg/kg dose of LPS does not induce an increase in plasma IL-1 β when compared to a high 100 µg/kg dose of LPS (Lenczowski et al., 1997). When combining this result by Lenczowski and colleagues (1997), the results can be seen to be inconsistent with previous literature, as IL-1 treatment has been

previously shown to reduce corticosterone levels in adult Wistar rats following acute stress (Plagemann et al., 1998). Blunted corticosterone responses are also seen in adult male rats following a stress protocol, when exposed to the DEE method with a 50 µg/kg dose of LPS (Walker et al., 2009). A 50 µg/kg dose of LPS in the present study revealed a significant reduction in chamber transitions, indicating greater anxiety-like behaviour (i.e. less risk taking).

Sex differences were also revealed, such that females showed greater nose pokes and locomotor activity within the dark chamber. It is quite possible that the result associated with nose pokes is a result of estrogen effects, as it has been previously shown to regulate HPA-axis activity and normalize glucocorticoid receptor densities within the hippocampus (Ferrini et al., 1999). Vertical movements in the light chamber also reveal a sex by drug interaction, such that females did not show any overall significant differences between treatments on vertical movements. Males, however, did show a significant difference among all treatment groups when compared to saline controls. This again suggests a potential role that estrogen played in the female groups, especially given the fact that the rats were tested in adolescence where there is a sudden rise of estrogen during puberty in females. There are also significant sex differences in microglial functioning during the neonatal period in which LPS and physical stress are induced, which may be relevant to the results seen in the present study (Schwartz et al., 2013).

It is unclear what links can be made, especially given the fact that saline controls had similar anxiety-like behavior measures compared to the 50 µg/kg dose of LPS in the light-dark test of the present study. Quite possibly, the effects being seen in the present study may be more strongly related to the effect of physical stress rather than immune stress. Untreated controls were significantly less anxious overall compared to neonatal saline controls and LPS treatments, as measured by time spent in the light chamber.

2.7 References

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CHAPTER 3

**NEONATAL EXPOSURE TO IMMUNE AND PHYSICAL STRESS DOES NOT
PREDISPOSE RATS TO EXAGGERATED ANXIETY-LIKE BEHAVIOUR DURING
ADOLESCENCE FOLLOWING STRESSOR EXPOSURE**

3.1 Introduction

The early life period is pivotal for the development of the organism. During the first few weeks of life, the organism is highly vulnerable and responsive to external and internal threats. These threats can have a profound impact on the developmental outcome of the organism. It is widely hypothesized that early life stress, including both physical and immune, can be linked to psychopathologies later in life within the human population (Heim et al., 2001; Bale et al., 2010). Specifically, this physical and immune stress elicits changes in the functioning of the organism through alteration of the normal developmental pattern. This is known as early life programming. Early life programming has been linked to the development of anxiety-related disorders due to the alterations in endocrine and nervous system functioning during the early life period (Bale et al., 2010). Both early life physical and immune stress are common scenarios in both the animal and human population, and is therefore important to investigate their changes on behaviour later in life.

Animal literature has placed a heavy emphasis on the role of early life physical stress on the development of anxiety-related disorders. Common methods used in the animal population to induce physical stress are the following: acute maternal deprivation (de Kloet et al., 2005), maternal separation (Sanchez et al., 2001; Huot et al., 2002), chronic early life stress (Ivy et al., 2008), handling (Durand et al., 1998), and early weaning of pups (Kikusui and Mori, 2009). Maternal separation is the most common method used to induce early life physical stress. This method involves removing pups from the dam for an extended amount of time, which limits passive care and feeding to the pup. When pups exposed to maternal separation reach adulthood, changes in endocrine functioning are often seen in response to stress (Plotsky et al., 1993; Meaney et al., 1989; Viau et al., 1993). More specifically, these rats show an overall general

increase in stress hormones throughout the body when they are exposed to a secondary stressor in adulthood, and is therefore suggestive of early life programming by maternal separation. Adult female C57BL/6 mice, who underwent maternal separation during the early life period, showed reduced anxiety-like behaviour in the elevated-plus maze, and adult males showed an increase in anxiety-like behaviour (Romeo et al., 2003). Adult male and female rats, who were exposed to periodic maternal separation from PND 3-10, both showed significant increases in anxiety-like behaviour measured in the elevated plus-maze (Wigger and Neumann, 1999). This was further supported by a study conducted by Kalinichev and colleagues (2002), where it was found that both male and female adult rats who underwent maternal separation during the early life spent significantly less time in the open arms of the elevated plus maze compared to non-handled controls during the neonatal period.

Anxiety disorders are multifactorial. There are several factors that can be implicated in its development. Early life physical stress in animal models can mimic the human scenarios, however, maternal separation is still considered to be vastly different than early life stress in humans. From an evolutionary standpoint, early life immune stress has greater cross species relevance as both animals and humans can be infected at an early age. Activation of the immune system during the early life period elicits an endocrine response that is quite like the effect seen in physical stress models, such that there is an acute increase in systemic corticosterone (Walker et al., 2004). A method commonly employed in the animal models is the injection of lipopolysaccharide (LPS) on post-natal day (PND) 3 and 5 to induce an immune response. LPS is an immunogen found on the cell walls of Gram-negative bacteria that induces an immune response in the host. The use of LPS in early life immune stress models is ideal due to its controlled and reliable effects, as it mimics an infection without the subsequent damage caused

by live bacteria. If rats are exposed to LPS on PND 3 and 5, and reach adulthood, changes in neuroendocrine functioning are seen. The PVN is an important structure within the endocrine system and is associated with the feedback loop of the HPA axis. Adult rats neonatally exposed to LPS show significant increases in CRH mRNA within the PVN and are therefore indicative of an alteration in the feedback loop, which results in increased HPA-axis activity (Shanks et al., 1995). Furthermore, we can also see changes in glucocorticoid receptor binding within the hypothalamus, frontal cortex, and hippocampus in adult rats that were neonatally exposed to LPS (Shanks et al., 1995). In response to acute stress, the activation of glucocorticoid receptors results in negative feedback; however, with the lack of binding, we see a decrease in effective termination of the stress response with an overall increase in corticosterone response (Shanks et al., 2000; Hodgson et al., 2001; Walker et al., 2008). Overall, it has been reported that neonatal LPS exposure results in anxiety-like behaviour in adults (Breivik et al., 2002, Walker et al., 2004, and Walker et al., 2009); however, decreases or no changes in anxiety-like behaviour following neonatal endotoxin exposure have also been reported (Breivik et al., 2002, Tenk et al., 2013, and Walker et al., 2008). In general, previous results are suggestive of the hypothesis that anxiety-like behaviour is increased in rats exposed to an endotoxin via a stressor effect (Walker et al., 2008).

The current study employed the use of neonatal endotoxin exposure; however, it also contains several different physical stressors that may elicit similar changes as maternal separation. Administering LPS on PND 3 and 5 involves pup handling, injection stress, and toe clipping on PND 3. All of which may be physically stressful. This effect can be seen in Chapter 2, where it was revealed that saline controls showed significantly more anxiety-like behaviour compared to no injections controls.

Much of the research on the effect of early life stress on behaviour has focused on adulthood, with very little focus placed on adolescence. Adolescence is an important period of life to consider, as the average age of onset for all anxiety disorders combined is 11 years (Kessler et al., 2005). For early-life physical stress in rodents, only one study has been conducted on the effect of maternal separation on adolescent behaviour. Maternal separation during the neonatal period - 15 minutes every day from post-natal day (PND) 2 to PND 21 - resulted in decreased adolescent anxiety-like behaviour for adolescent females but not for adolescent males (McIntosh et al., 1999). Only one study is known to have investigated the effect of early life immune stress on anxiety-like behaviour in adolescence, and it was found that no behavioural changes were seen on PND 43 on the elevated plus maze (Walker et al., 2004). However, neither of these studies employed the use of an acute stress, as previous research in adults has suggested the need of a stressor to elicit endocrine and behavioural changes.

The current study made use of an aversive conditioning procedure to elicit a stress response in rats previously exposed to early life stress. Conditioning stress in this study involved the injection of either saline or lithium chloride (LiCl) intraperitoneally on 4 conditioning trials with 72 h separating each trial. It was hypothesized that the stress from the procedure to induce conditioned disgust would be strong enough to elicit anxiety-like behaviour differences in the light-dark test 48 h following the final conditioning trial. As such, the current study examined the effect of neonatal endotoxin exposure, with both saline and untreated controls, on anxiety-like behaviour in the light-dark test following conditioning stress. Furthermore, the data from Chapter 2 were used to compare anxiety-like behaviour before and after conditioning stress.

3.2 Materials and Methods

3.2.1 Animals

All animals used in the previous experiment (Chapter 2) were used. Animals remained group-housed (3-4 rats per cage, same-sex littermates, and same conditioned disgust treatment) in standard polypropylene cages (45 cm x 22 cm x 20 cm). They remained in the same temperature-controlled colony room ($20 \pm 1^\circ \text{C}$) that was maintained on a 12:12 h light-dark cycle. *Ad libitum* access to food (Prolab RMH3000 lab chow) and tap water was provided. Vaginal smears were collected for female adolescent rats throughout the procedures. Analysis revealed that females were proportionally tested across all estrous stages minimizing estrous cycle effects. The experimental methodology was carried out according to the Canadian Council on Animal Care guidelines and was approved by the University of Western Ontario Animal Care Sub-Committee.

3.2.2 Light-dark apparatus

The same light-dark apparatus used in Chapter 2 was used for this experiment. Results from Chapter 2 were also used for repeated measures analysis to compare data before and after conditioned disgust.

3.2.3 Variables quantified by the Versamax System during light-dark testing

The same variables quantified in Chapter 2 were used for this experiment.

3.2.4 Conditioned disgust apparatus

The apparatus (used on all conditioning days and the test day) consisted of a distinctive context that was a white Plexiglas box (29 cm x 25 cm x 29 cm) set atop a clear glass plate. A

mirror was mounted at a 45° angle beneath the glass plate in order to view the rat's ventral surface. Two 40 W red lights were placed below the glass plate. Lighting cues were kept consistent with previous studies employing the rodent model of anticipatory nausea (e.g. Chan et al., 2009). Behavioural responses exhibited on the Drug-Free Test Day were videotaped with a video camera (SONY DCR-DVD202; London, Ontario) positioned approximately 1 m from the mirror.

3.2.5 Experimental procedure

On PND 36, one day following the light-dark testing in Chapter 2, rats underwent conditioned disgust. The light-dark data from Chapter 2 will be used to assess changes in anxiety-like behaviour following the conditioned disgust procedure. All conditioning and testing was performed during the light phase of the light:dark cycle. The conditioned disgust experiment consisted of two phases, including a Conditioning Phase (4 days spaced 72 h apart) and one Drug-free test Day, 72 h following the final conditioning day. The procedure can be visualized in Appendix B. On PND 47, one day before the Drug-free test day, rats were again exposed to the light-dark apparatus used in Chapter 1. Like Chapter 2, rats were placed in the light-dark apparatus for a period of 30 minutes, with the first 10 minutes being used for data analysis. Conditioned disgust results on Drug-free test day were used for a separate study.

3.2.6 Conditioning phase drug treatment

On each day of the Conditioning Phase (4 days, 72 h apart) rats received an intraperitoneal injection of 96 mg/kg lithium chloride (LiCl; 15 ml/kg), or 0.9% isotonic saline (NaCl; 15 ml/kg). Each rat was immediately placed into the distinctive context for a period of 30

minutes. Following the exposure to the distinctive context, rats were immediately returned to their home cage. There were a total of sixteen treatment groups consisting of (neonatal treatment:conditioning treatment): No Injection:NaCl, No Injection:LiCl, NaCl:NaCl, NaCl:LiCl, LPS 15 µg/kg:NaCl, LPS 15 µg/kg:LiCl, LPS 50 µg/kg:NaCl, and LPS 50 µg/kg:LiCl, for both males and females. Sample sizes for each group can be seen in Appendix B.

3.2.7 Statistical analysis

All analysis was conducted using a 2x4x2 ANOVA accounting for the random effect of litter (16 litters, where no injection controls were considered as an additional litter). Between subject factors consisted of sex (at 2 levels: male; female), neonatal treatment (at 4 levels: no injection; saline; LPS 15 µg/kg; LPS 50 µg/kg) and conditioning treatment (at 2 levels: NaCl; LiCl). A repeated measures test was employed to measure differences in light-dark test results on PND 35 and light-dark test results on PND 47. Post-hoc pair-wise comparisons were made using least significant difference (LSD) analysis to investigate group comparisons following a significant main effect and interaction effect. LSD post-hoc analysis was chosen for this study due to the exploratory nature of the study being conducted. All hypothesis tests used an alpha of .05 and analyzed using SPSS 24.0 for Windows.

3.3 Results

Light-Dark Test Repeated Measures: Before and After Conditioned Disgust Conditioning

3.3.1 Chamber Transitions

A 2x4x2 repeated measures ANOVA was conducted to compare the effect of Sex, Neonatal Treatment, and Conditioning Treatment on Chamber Transitions before and after conditioned disgust. There was a significant main effect of Neonatal Treatment on chamber transitions, as seen in Figure 3.1. Rats receiving no neonatal injection made significantly less chamber transitions after conditioning ($M = 10.458, SD = 5.979$) compared to before ($M = 14.771, SD = 5.470$), Wilks' Lambda = .910, $F(3,184) = 6.048, p = .001$.

3.3.2 Time Spent in the Light Chamber

A 2x4x2 repeated measures ANOVA was conducted to compare the effect of Sex, Neonatal Treatment, and Conditioning Treatment on Time Spent in the Light Chamber before and after conditioned disgust. There was a significant effect of Neonatal Treatment, Wilks' Lambda = .955, $F(3, 184) = 2.918, p = .036$. Post-hoc LSD comparisons were made for Neonatal Treatment and revealed that Neonatal Saline treatment spent significantly more time in the light chamber after conditioned disgust ($M = 122.430, SE = 8.566$) compared to before conditioned disgust ($M = 73.775, SE = 7.726$), $p = .000$, as seen in Figure 3.2. A significant two-way interaction effect was found for Conditioning Treatment and Sex, Wilks' Lambda = .981, $F(3, 184) = 6.063, p = .015$. Post-hoc LSD comparisons revealed that male rats who received NaCl during conditioning spent significantly more time in the light chamber after conditioning ($M = 110.416, SE = 10.584$) compared to before conditioning ($M = 85.570, SE = 85.570, SE = 9.546$), $p = .032$. Furthermore, female rats who received LiCl during conditioning spent significantly more time in the light chamber after conditioning ($M = 163.250, SE = 10.308$) compared to before conditioning with LiCl treatment ($M = 111.892, SE = 9.297$), $p = .000$, as

seen in Figure 3.3. No other significant main effects or interaction effects were found following repeated measures analysis.

A main effect of sex was found on PND 47 alone, $F(1, 10.950) = 6.584, p = .026$, such that females ($M = 148.781, SE = 6.620$) spent significantly more time in the light chamber compared to males ($M = 96.389, SE = 6.561, p = .000$).

3.3.3 Nose Pokes into the Light Chamber

A 2x4x2 repeated measures ANOVA was conducted to compare the effect of Sex, Neonatal Treatment, and Conditioning Treatment on Nose Pokes into the Light Chamber before and after conditioned disgust. There was a significant main effect of Neonatal Treatment on Nose Pokes into the Light Chamber, Wilks' Lambda = .910, $F(1, 184) = 6.048, p = .001$. Pairwise comparisons revealed that No Injection neonatal controls made significantly less Nose Pokes into the Light Chamber following conditioning stress ($M = 10.587, SE = .772$) compared to before conditioning ($M = 14.781, SE = .811$), $p = .000$, as seen in Figure 3.4. Furthermore, a significant interaction between Neonatal Treatment and Conditioning treatment was also found, Wilks' Lambda = .953, $F(3, 184) = 3.023, p = .031$, as seen in Figure 3.5. Pairwise comparisons revealed that rats who received No Injection neonatally and NaCl during conditioning made significantly fewer nose pokes into the light chamber after conditioning ($M = 10.296, SE = 1.068$) compared to before conditioning ($M = 14.219, SE = 1.122$), $p = .003$. Similarly, if No Injection neonatal controls received LiCl during conditioning, they made significantly fewer nose pokes into the light chamber following conditioning ($M = 10.879, SE = 1.068$) compared to before conditioning ($M = 15.344, SE = 1.122$), $p = .001$. Rats who received NaCl during the neonatal treatment and LiCl during conditioning treatment made significantly more nose pokes

into the light chamber after conditioning ($M = 10.483$, $SE = .836$) compared to before conditioning (7.957 , $SE = .878$). No other significant main effects or interactions were found in the repeated measures analysis.

Analysis revealed a significant main effect of sex on nose pokes into the light chamber on PND 47 alone, $F(1, 9.63) = 8.548$, $p = .016$. Females ($M = 12.051$, $SE = .587$) made significantly more nose pokes into the light chamber compared the males ($M = 8.208$, $SE = .582$, $p = .000$).

3.3.4 Total Distance in Light Chamber (Corrected)

Repeated measures analysis revealed a significant main effect of sex on total distance spent in the light chamber (corrected for time spent in the chamber), $F(1, 184) = 3.924$, $p = .049$, as seen in Figure 3.6. Females moved significantly more per second in the chamber before conditioning ($M = 3.306$, $SE = .276$) compared to females after conditioning ($M = 2.607$, $SE = .116$), $p = .020$. Males showed a similar effect, such that they moved significantly more in the dark chamber before conditioning ($M = 3.701$, $SE = .269$) compared to their results after the conditioning stress ($M = 2.180$, $SE = .113$), $p = .000$. No other significant main effects or interactions were found in the repeated measures analysis.

A significant main effect of sex was found on PND 47 alone, $F(1, 2.760) = 14.954$, $p = .035$. Females ($M = 2.691$, $SE = .136$) had significantly more distance moved per second in the light chamber compared to males ($M = 2.241$, $SE = .135$), $p = .020$.

3.3.5 Vertical Movement Number in the Light Chamber (Corrected)

A significant main effect of vertical movement in the light chamber was found, $F(1, 184) = 10.780$, $p = .001$. There were significantly more vertical movement movements per second

spent in the light chamber after conditioning ($M = .090$, $SE = .003$) compared to before conditioning ($M = .067$, $SE = .004$).

A significant interaction effect was also found between Vertical Movement Number (corrected), Sex, and Neonatal Treatment, $F(1, 184) = 3.750$, $p = .012$, as seen in Figure 3.7. Post-hoc LSD analysis revealed that female rats who received No Injection during the Neonatal Treatment made significantly more Vertical Movements per second of time spent in the light chamber after conditioning ($M = .105$, $SE = .010$) compared to before conditioning ($M = .077$, $SE = .010$), $p = .012$. Similar results were found for the No Injection controls for male rats, where the No Injection control male rats made significantly more corrected Vertical Movements in the light chamber after conditioning ($M = .102$, $SE = .010$) compared to before conditioning ($M = .072$, $SE = .010$), $p = .012$. Female rats who received neonatal saline made significantly more Vertical Movements per second in the chamber after conditioning ($M = .091$, $SE = .008$) compared to before conditioning ($M = .070$, $SE = .008$), $p = .025$. Male rats treated with saline during the Neonatal Treatment also saw a similar effect, where they made significantly more vertical movements per second in the light chamber after conditioning ($M = .086$, $SE = .008$) compared to before conditioning ($M = .036$, $SE = .010$), $p = .000$. Female rats who received LPS 15 $\mu\text{g}/\text{kg}$ for Neonatal treatment also made significantly more Vertical Movements following the conditioning stress ($M = .092$, $SE = .010$) compared to before conditioning ($M = .052$, $SE = .011$), $p = .002$.

A significant interaction between Vertical Movement Number in the light chamber (corrected), Sex, and Condition was also revealed, $F(1, 184) = 3.924$, $p = .049$, as seen in Figure 3.8. Post-hoc LSD analysis revealed that females who received NaCl during the conditioning period made significantly more Vertical Movements in the light chamber per second spent in the

chamber after conditioning ($M = .099, SE = .007$) compared to before conditioning, $p = .013$. Similar results were found for females who received LiCl during the conditioning period (After: $M = .093, SE = .007$; Before: $M = .061, SE = .007$), $p = .000$. Males who received NaCl during the conditioning period also saw a similar effect, where they made significantly more Vertical Movements in the light chamber per second spent in the chamber after conditioning ($M = .093, SE = .007$) compared to before conditioning ($M = .064, SE = .007$), $p = .000$. The males who received LiCl during conditioning saw no significant effect when comparing before and after conditioning.

No other significant main effects or interactions were found within the repeated measures analysis for Vertical Movements in the Light Chamber (corrected).

3.3.6 Vertical Movement Time in the Light Chamber (Corrected)

Repeated measures analysis revealed a significant main effect of Vertical Time in the Light Chamber corrected for time spent in the light chamber, $F(1, 184) = 52.463, p = .000$. Rats spent significantly more time in Vertical Movements after conditioning ($M = .132, SE = .006$) compared to before conditioning ($M = .070, SE = .004$), $p = .000$. A significant interaction between Vertical Time in the Light Chamber (corrected) and Litter was also found, $F(1, 184) = 4.756, p = .030$. No other significant effects were found.

3.3.7 Distance in the Dark Chamber (Corrected)

Analysis revealed a significant main effect of total distance in the dark chamber corrected to account for the time spent in the chamber (cm/s), $F(1, 184) = 7.720, p = .006$. Total distance

corrected in the dark chamber was significant lower in the light dark test following conditioning stress ($M = 1.330$, $SE = .049$) compared to before conditioning ($M = 1.543$, $SE = .038$), $p = .000$.

A main effect of sex was found, $F(1, 12.010) = 6.370$, $p < .027$. Females ($M = 1.604$, $SE = .048$) moved significantly more per second compared to males ($M = 1.140$, $SE = .047$), $p = .000$.

3.3.8 Vertical Movement Number in the Dark Chamber (Corrected)

The repeated measures analysis found that there was a significant main effect on the number of vertical movements made in the dark chamber per second, $F(1, 184) = 4.811$, $p = .030$. A significant interaction between the repeated measure dependent variable and litter was also found, $F(1, 184) = 4.811$, $p = .030$. No other significant effects or interactions were found.

3.3.9 Vertical Time in Dark Chamber (Corrected)

A significant main effect on the vertical time in the dark chamber, $F(1, 184) = 5.626$, $p = .019$. A significant interaction between the repeated measure dependent variable and litter was also found, $F(1, 184) = 4.706$, $p = .031$. No other significant effects or interactions were found.

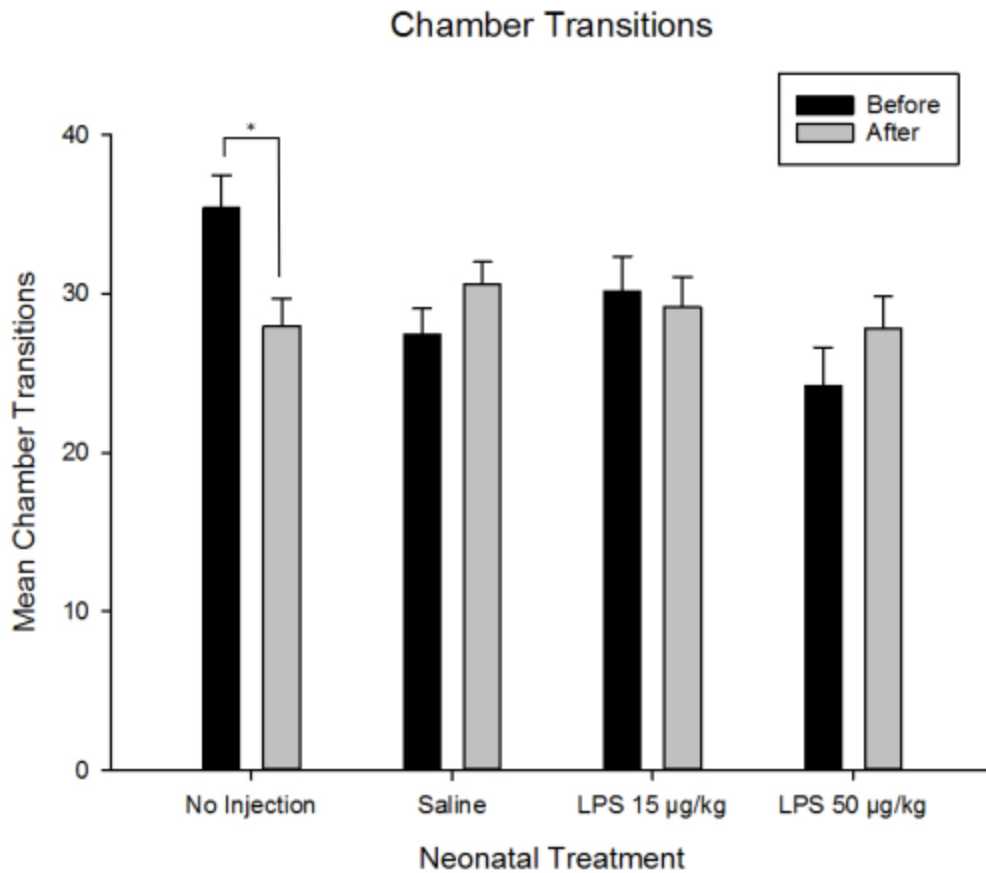


Figure 3.1: Group mean (\pm S.E.M.) activity behaviour of chamber transitions before (PND 35) and after (PND 47) conditioned disgust. * $p < .05$ indicates a significant Neonatal Drug Treatment by Time effect, where no injection controls made significantly more chamber transitions before conditioning compared to after conditioning (No Injection; $n = 48$, saline; $n = 72$, LPS 15 $\mu\text{g}/\text{kg}$; $n = 42$, LPS 50 $\mu\text{g}/\text{kg}$; $n = 39$).

Time Spent in Light Chamber Before and After Conditioned Disgust

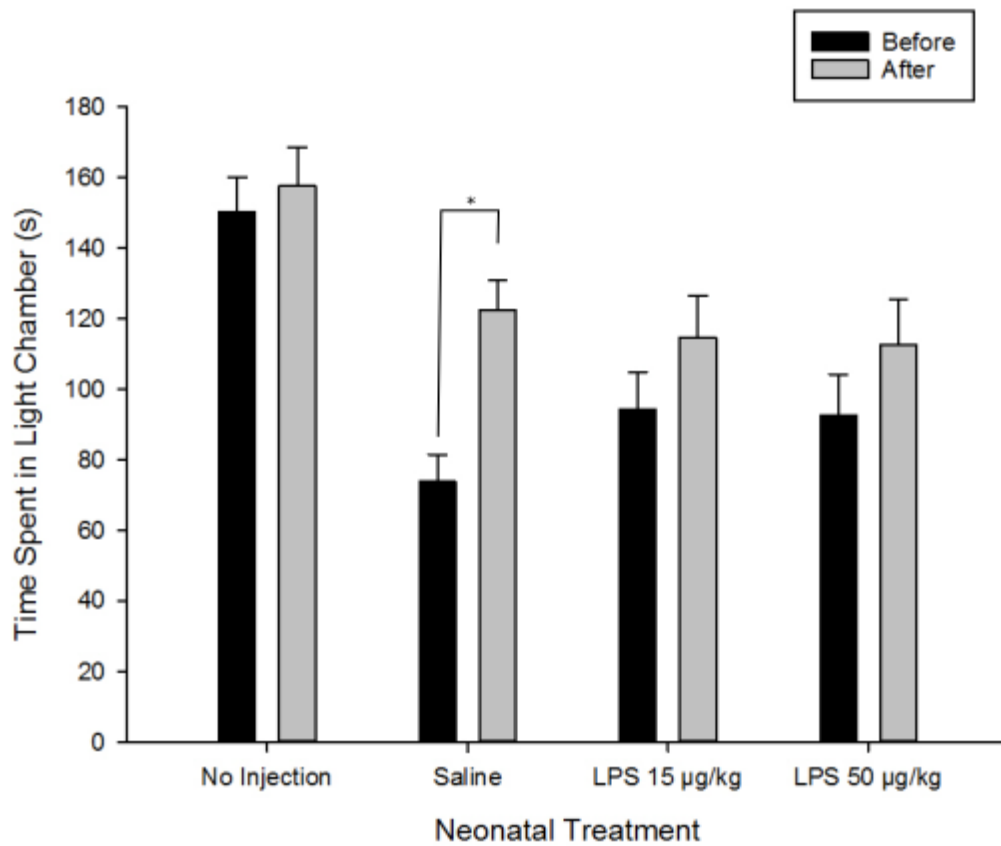


Figure 3.2: Group mean (\pm S.E.M.) activity behaviour of time spent in the light chamber during adolescence before (PND 35) and after (PND 47) conditioned disgust. * $p < .05$ indicates a significant Neonatal Drug Treatment by Time effect, where saline controls spent significantly more time in the light chamber after conditioning compared to before (No Injection; $n = 48$, saline; $n = 72$, LPS 15 $\mu\text{g}/\text{kg}$; $n = 42$, LPS 50 $\mu\text{g}/\text{kg}$; $n = 39$).

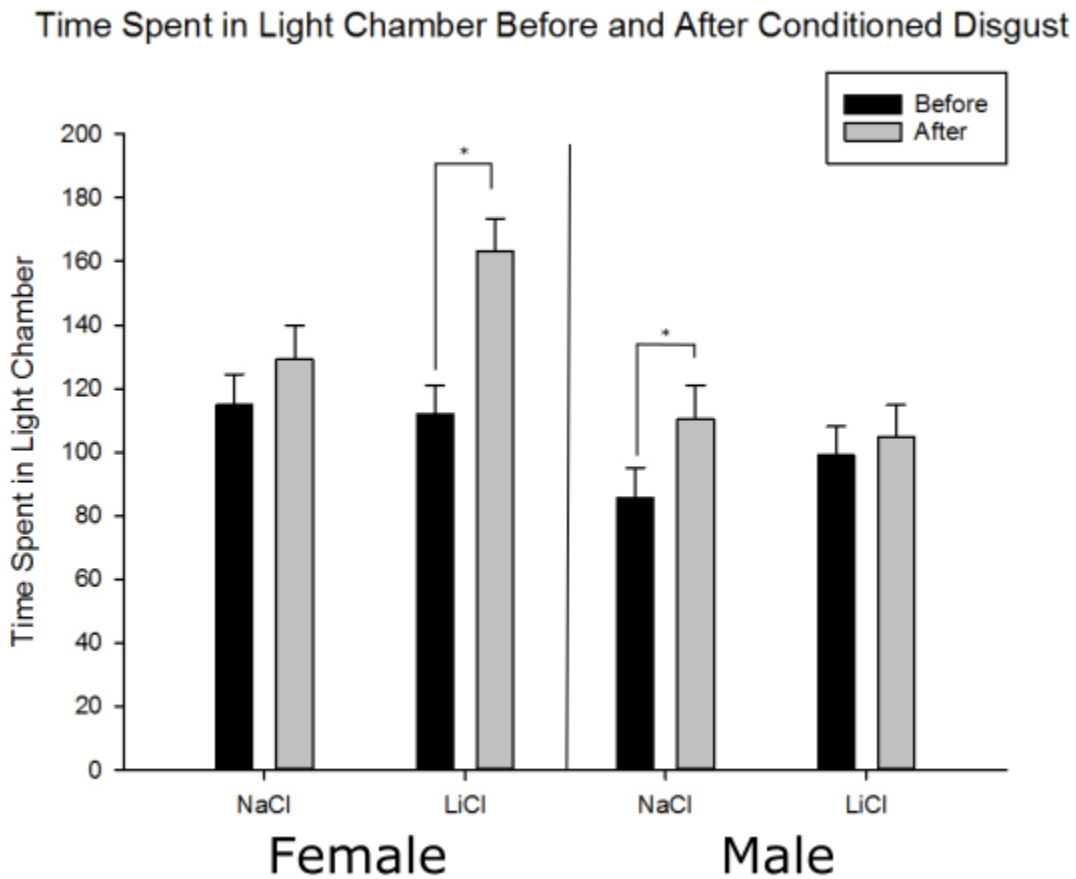


Figure 3.3: Group mean (\pm S.E.M.) activity behaviour of time spent in the light chamber during adolescence before (PND 35) and after (PND 47) conditioned disgust. * $p < .05$ indicates a significant Conditioning Treatment by Sex by Time effect, where females receiving LiCl during conditioning spent significantly more time in the light chamber after conditioning compared to before conditioning. Male rats who received saline during the conditioning period spent significantly more time in the light chamber after conditioning compared to before (Female-NaCl; $n = 48$, Female-LiCl; $n = 51$, Male-NaCl; $n = 48$, Male-LiCl; $n = 54$).

Nose Pokes into Light Chamber Before and After Conditioned Disgust

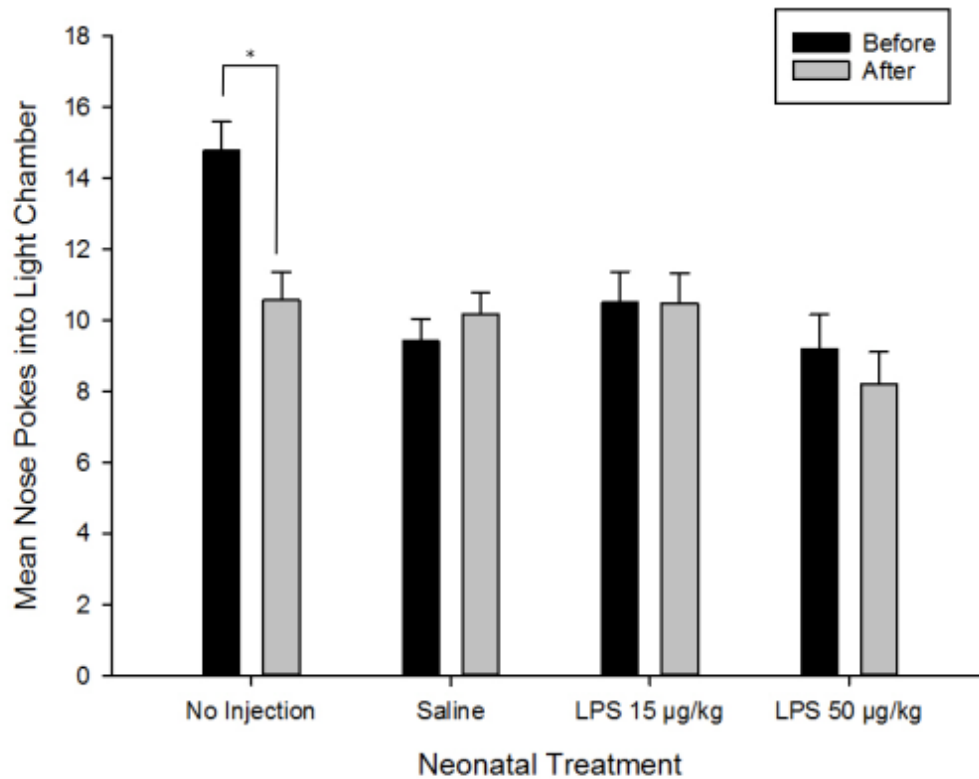


Figure 3.4: Group mean (\pm S.E.M.) activity behaviour of nose pokes into the light chamber during adolescence before (PND 35) and after (PND 47) conditioned disgust. * $p < .05$ indicates a significant Neonatal Drug Treatment by Time effect, where no injections controls made significantly more nose pokes into the light chamber before conditioning compared to after conditioning (No Injection; $n = 48$, saline; $n = 72$, LPS 15 $\mu\text{g}/\text{kg}$; $n = 42$, LPS 50 $\mu\text{g}/\text{kg}$; $n = 39$).

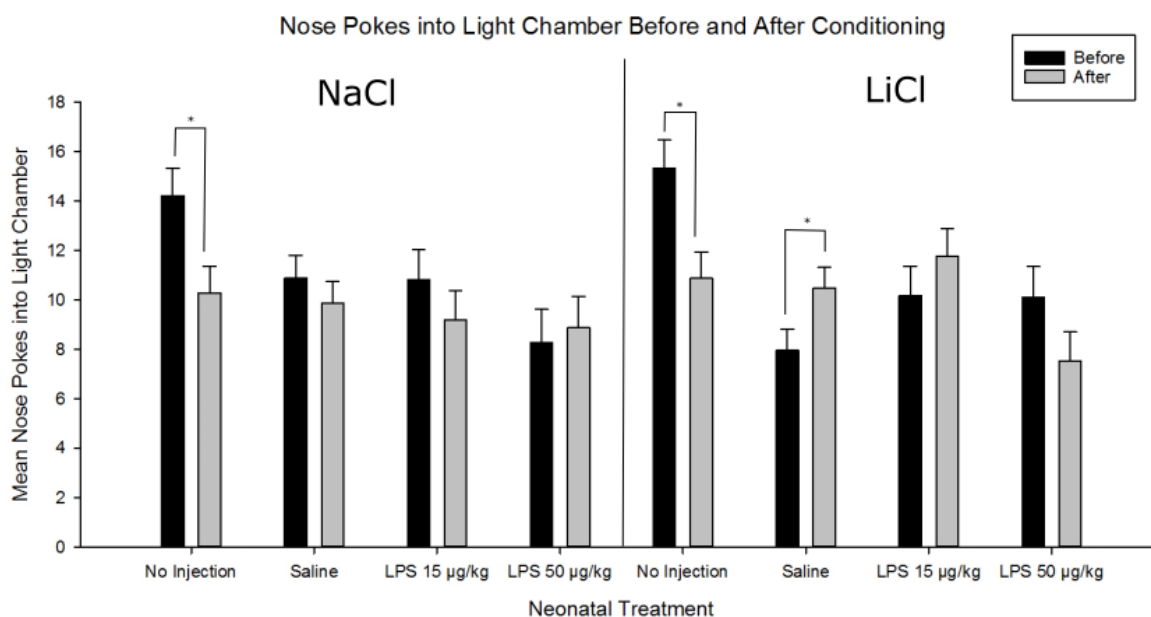


Figure 3.5: Group mean (\pm S.E.M.) activity behaviour of nose pokes into the light chamber during adolescence before (PND 35) and after (PND 47) conditioned disgust. * $p < .05$ indicates a significant Neonatal Drug Treatment by Conditioning Treatment by Time effect, where no injections controls receiving saline and LiCl during conditioning made significantly more nose pokes into the light chamber before conditioning compared to after conditioning. Rats receiving neonatal saline treatment made significantly more nose pokes after conditioning compared to before if they received LiCl during conditioned disgust (No Injection-Saline; $n = 24$, No Injection-LiCl; $n = 24$, Saline-Saline; $n = 34$, Saline-LiCl; $n = 38$, LPS 15 $\mu\text{g}/\text{kg}$ -NaCl; $n = 20$, LPS 15 $\mu\text{g}/\text{kg}$ -LiCl; $n = 22$, LPS 50 $\mu\text{g}/\text{kg}$ -Saline; $n = 18$, LPS 50 $\mu\text{g}/\text{kg}$ -LiCl; $n = 21$).

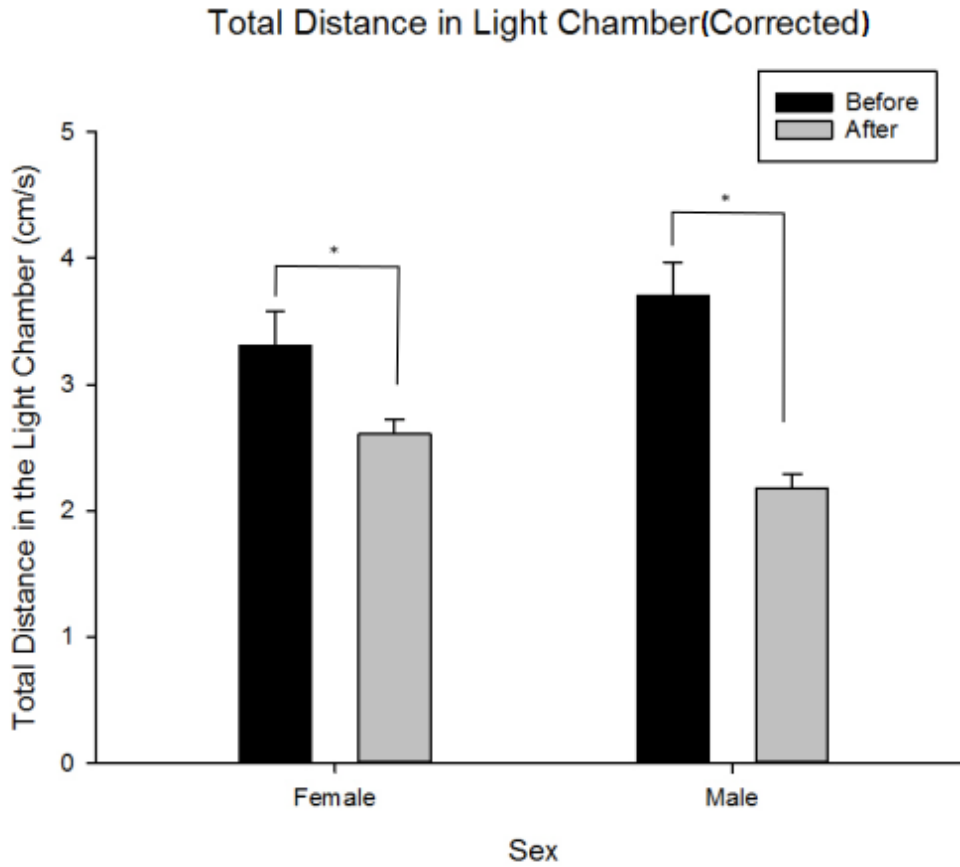


Figure 3.6: Group mean (\pm S.E.M.) activity behaviour of total distance in the light chamber per second spent in the light chamber during adolescence before and after conditioned disgust (PND 47). * $p < .05$ indicates a significant Sex effect, where both females and males moved significantly more in the light chamber before conditioning compared to after. (Female; $n = 99$, Male; $n = 102$).

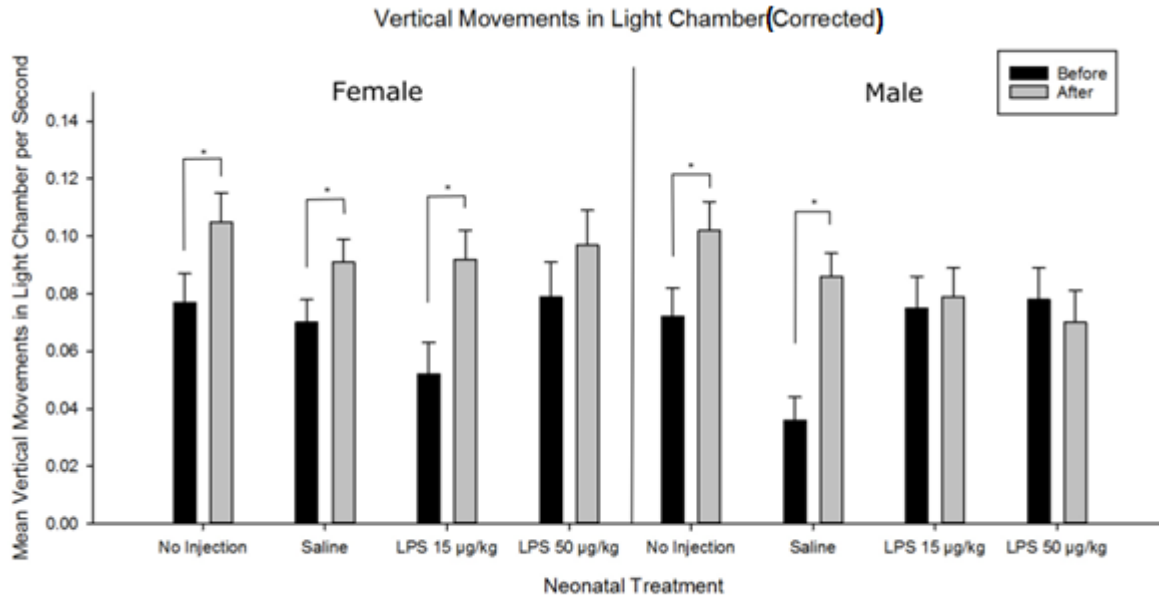


Figure 3.7: Group mean (\pm S.E.M.) activity behaviour of vertical movements in the light chamber per second spent in the light chamber during adolescence before (PND 35) and after conditioned disgust (PND 47). * $p < .05$ indicates a significant Neonatal Treatment by Sex effect, where females showed significantly more vertical movements in the light chamber after conditioning compared to before for all neonatal treatments except an LPS dose of 50 $\mu\text{g}/\text{kg}$. Males saw a similar effect for no injection and saline neonatal treatments, where vertical movements per second were significantly higher after conditioning compared to before (male-No Injection; $n = 24$, male-Saline; $n = 36$, male-LPS 15 $\mu\text{g}/\text{kg}$; $n = 21$, male-LPS 50 $\mu\text{g}/\text{kg}$; $n = 21$, female-No Injection; $n = 24$, female-Saline; $n = 36$, female-LPS 15 $\mu\text{g}/\text{kg}$; $n = 21$, female-LPS 50 $\mu\text{g}/\text{kg}$; $n = 18$).

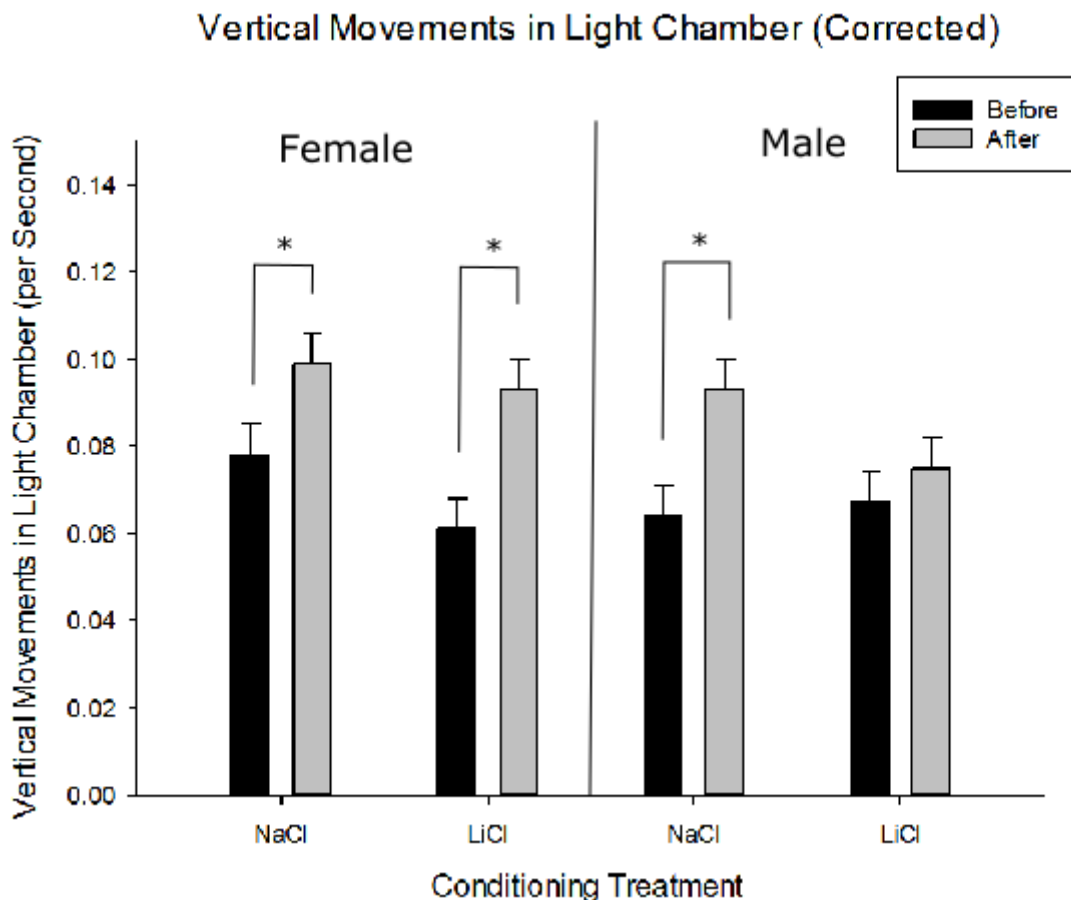


Figure 3.8: Group mean (\pm S.E.M.) activity behaviour of vertical movements in the light chamber per second spent in the light chamber before (PND 35) and after (PND 47) conditioned disgust. * $p < .05$ indicates a significant Conditioning Treatment by Sex by Time effect, where females receiving NaCl or LiCl during conditioning made significantly more vertical movements per second after conditioning compared to before conditioning. Male rats who received saline during the conditioning made significantly more vertical movements in the light chamber after conditioning compared to before (Female-NaCl; $n = 478$ Female-LiCl; $n = 51$, Male-NaCl; $n = 48$, Male-LiCl; $n = 54$).

3.4 Discussion

The purpose of this study was to examine the effect of a conditioned disgust stress protocol on rats that had been exposed to both physical and immune stress on PND 3 and PND 5. Sex differences were also of interest to explore the potential influence of microglial colonization differences during the neonatal period into adolescent (Schwartz et al., 2013). Prior research has not investigated the role of early life physical stress associated with the DEE model; therefore, the comparisons against the no injection control in the present study were of interest.

The results of the present study suggest that a background of stress does not elicit anxiety-like behaviour in adolescent rats that underwent DEE. Neonatal treatment, regardless of sex and conditioned disgust treatment, showed no changes in anxiety-like behaviour for no injection controls and both LPS doses; however, an increase in time spent in the light chamber was seen for the saline controls. This suggests that the background of stress influenced the neonatal saline treated rats in such a way that they were less anxious following conditioned disgust compared to before. It is unclear why the effect was seen, but may be due to a habituation effect that was not seen in LPS treated rats. Interestingly, we also saw this effect in females who received LiCl during conditioned disgust, and males who received saline during conditioned disgust, regardless of neonatal treatment. Both groups spent significantly more time in the light chamber compared to the dark, indicative of anxiety-like behaviour. These results run contrary to previous research in adult Fischer 344 rats, which suggests that repeated saline injections twice a day for 14 days results in reduced open field activity (Izumi et al., 1997).

Another measure often investigated in the light-dark test for anxiety-like behaviour is vertical movements, which is indicative of risk taking behaviour (Arrant et al., 2013). The results of the present study suggested that all treatments, other than the 50 µg/kg dose of LPS

during the neonatal period, resulted in significantly more vertical movements in the light chamber compared to before conditioned disgust. This is suggestive of less risk taking, and potentially reduced anxiety. A similar result was seen in both the control groups for male rats (no injection and saline controls), such that there was an increase in vertical movements in the light chamber. By collapsing the groups, regardless of neonatal treatment, it was shown that an increase in vertical movements was also seen in females receiving either NaCl or LiCl, and males who received NaCl. Overall, these results suggest that the adolescent rats showed reduced anxiety-like behaviour in the light-dark test following conditioned disgust stress, when compared to the light-dark test results prior to conditioned disgust.

On the contrary, the present study did suggest some increases in anxiety-like behaviour following conditioned disgust for both male and female rats. The primary variables for these findings were nose pokes into the light chamber and total chamber transitions. Interestingly, the present study found that the no injection controls, who received either NaCl or LiCl during conditioned disgust, had significantly less nose pokes into the light chamber. This suggests that the no injection controls were showing more anxiety-like behaviour following conditioned disgust compared to before. Comparable results were seen in the chamber transition variable, such that no injection controls made significantly less chamber transitions following conditioned disgust compared to before.

There have been very limited studies investigating the hypothesis that the influence of DEE on anxiety-like behaviour later in life is dependent on a background of stress (Walker et al., 2008; Walker et al., 2009). Compared to the present study, the previous studies employed different methods for inducing a background of stress, as well as for testing anxiety-like behaviour. Walker and colleagues (2008) used a stress protocol consisting of 30 minutes of

restraint stress for days 1 and 2, social isolation on day 3, with the test day occurring on day 4. This study also used adult Wistar rats instead of the adolescent age range used in the present study. The study by the Walker group (2008) showed a significant increase in anxiety-like behaviour in LPS treated adult rats, which was evident by the startle amplitude in the acoustic startle response. Walker and colleagues (2009) used the same stress protocol as the one used in 2008, however, they also used the elevated-plus maze and a hide box/open field to assess anxiety-like behaviour. The results of this study by the Walker group (2009) suggested an increase in anxiety-like behaviour across all behavioural tests for LPS treated adult rats, when compared against the DEE saline controls. The results of both of these studies brought forth the 'double-hit' hypothesis, such that neonatal immune stress coupled with psychological stress leads to an increase in anxiety-like behaviour. These results are contrary to the present study results, as the present study found that there were no significant increases in anxiety-like behaviour in the light-dark test following conditioned disgust (used as the stress background).

There are several reasons why the results of the present study are not like those found in previous research. One main reason is that a different strain of rat was used. Both previous studies investigating the 'double-hit' hypothesis employed the use of Wistar rats, while the present study used the Long-Evans rat strain. Rat strains have been previously shown to have inherent behavioural differences in response to stimuli, as well as learning and memory (Shepard and Myers, 2008; Turner and Burne, 2014; Nosek et al., 2008); however, to the best of the author's knowledge, there have been no previous studies investigating differences in anxiety-like behaviour between Long-Evans rats and Wistar rats. The second reason why there may be significant differences in results between the previous studies by the Walker groups and the present study, may be due to the age group being tested. Previous studies investigating a

background of stress on the DEE have tested rat strains in adulthood, while the present study tested the rats in adolescence. Previous research has shown that adolescent rats exposed to the DEE do not show changes in anxiety-like behaviour in the elevated-plus maze on PND 40, which is close to the age tested in the present study (PND 47; Walker et al., 2004). Quite possibly, the current study found no differences in anxiety-like behaviour due to the inherent biological and behavioural differences between adolescent and adulthood. Compared to adulthood, adolescent male rats tend to show less anxiety-like behaviour in the elevated plus-maze (Schramm-Sapyta et al., 2007; Andrade et al., 2003). Female rats also show similar results, such that adolescent ages exhibit less anxiety-like behaviour on the elevated-plus maze compared to adults (Genn et al., 2003; Imhof et al., 1993).

The present study also showed that females had significantly less anxiety-like behaviour compared to the males following conditioned disgust. This result is supportive of previous studies that show that female adult rats are less anxious compared to males in the open field test (Masur et al., 1980) and the elevated-plus maze (Genn et al., 2003; Imhof et al., 1993). It is clear that the biological differences between males and females results in differing responses in anxiety assessing apparatuses. It is, however, unclear why this effect occurred. The results do suggest some level of involvement of the LiCl injections on behaviour. For example, female rats who were given LiCl during the conditioning period exhibited significantly less anxiety-like behaviour, as measured by time spent in the light chamber. LiCl has been previously shown to reduce anxiety-like behaviour in the elevated-plus maze (Wu et al., 2014). More research would be needed to explore why males who received NaCl during conditioned disgust had reduced anxiety-like behaviour.

Overall, the present study suggests that the ‘double-hit’ hypothesis does not hold true in adolescent Long-Evans rats that have been exposed to DEE and then exposed to conditioned disgust. The present study showed significant increases in anxiety-like behaviour following conditioned disgust (when compared to light-dark data prior to conditioned disgust) for no injection controls. In other words, no injection control rats reacted to the conditioned disgust protocol, while all rats that underwent the DEE protocol during the neonatal period did not show any significant increases in anxiety-like behaviour. In fact, the opposite was true. The neonatal protocol involving any form of injection, resulted in a decrease in overall anxiety following conditioned disgust.

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CHAPTER 4

GENERAL DISCUSSION

4.1 General Discussion

The present thesis examined the effects of neonatal physical and immune stress on anxiety-related behaviour in male and female adolescents, as well as potential sex difference with respect to these effects, with the inclusion of neonatally untreated controls. Studies similar to those found in the present thesis help us further understand the potential risks of both physical and immune stress during infancy, and how they may be linked to the development of anxiety disorders that are prevalent at the onset of adolescence.

The results of this study suggest that the inconsistent results in current literature on the effects of neonatal manipulations on future anxiety-like behaviour may be due to a lack of untreated controls. Overall, it was found that untreated neonatal controls were significantly less-anxious on measures of time spent in the light chamber, chamber transitions and vertical movements in the light chamber compared to all other neonatal treatments; these effects were seen prior to a period of injection stress from conditioned disgust. This novel result has not been investigated in previous literature and shows the magnitude of its importance in future neonatal studies. It was also found that a low dose of LPS (15 $\mu\text{g}/\text{kg}$) induced resilience during the neonatal period, resulting in less anxiety-like behaviour compared to saline controls and the 50 $\mu\text{g}/\text{kg}$ dose of LPS on the measure of time spent in the light chamber prior to conditioning stress. No previous literature has investigated a dose this small on anxiety-like behaviour in either adolescence or adulthood. Quite possibly, this effect may be linked to the cytokine release profile associated with a smaller dose. Previous research has shown that a dose of 5 $\mu\text{g}/\text{kg}$ of LPS does not cause an increase in IL-1 β blood serum levels that is often seen with a dose of 50 $\mu\text{g}/\text{kg}$ of LPS (Lenczowski et al., 1997). IL-1 β has been associated with the activation of the HPA axis and results in profound neuroactive steroid release (Silverman et al., 2005). Nose pokes into the

light chamber also revealed a sex difference, such that females were less anxious than males during the adolescent period. This is consistent with previous research in adolescence and this effect has been shown to continue into adulthood (Tenk, 2007; Hughes, Desmond and Fisher, 2004).

As suggested earlier, it was also hypothesized that a period of injection stress from conditioned disgust would elicit significant differences in neonatal immune treatment. This hypothesis was not supported by the results. No significant differences in anxiety-like behaviour were found in the measures used for the light-dark chamber. However, like the results prior to conditioning stress (Chapter 2), females were shown to be less-anxious overall compared to male adolescent rats. Locomotor activity was also investigated and it was shown that neonatal saline rats moved significantly more per second in the light chamber compared to rats who received an LPS dose of 15 µg/kg and LiCl during the conditioning period. No previous research has investigated this interaction effect, especially at such a low dose.

Repeated measures analyses were also conducted to determine if there were any changes within groups over the conditioned disgust period. Rats that were neonatally treated with saline showed a significant increase in time spent in the light chamber following conditioning stress. This is not consistent with previous research in rats where chronic mild stress showed a relative decrease in time spent in the light chamber in adult rats (Farhan and Haleem, 2016). When the data was analyzed regarding conditioning treatment and sex, females who received LiCl during the conditioning period showed a significant increase in time spent in the light chamber following conditioning compared to before conditioning stress. Lithium chloride is often used in the general population as a mood stabilizer (Ihne et al., 2012). A study conducted by Ihne and colleagues (2012) showed that adult male mice fed a 4 g/kg dose of drug-containing pellets had

rescued effects of restraint stress on light-dark exploration. In the present study, there may be a sex dependent effect where the LiCl they receive during conditioning may be treating the anxiogenic effects elicited by conditioning stress in the female rats. A similar effect was seen in the male rats of the current study; however, this was found in the rats who received saline during the conditioning period. This is not consistent with previous research showing that chronic saline injections in adult rats were associated with a decrease in time spent in the light chamber (Souza-Pinto et al., 2007). A decrease in nose pokes was also seen in the no injection control rats after the conditioning stress, which may be associated with habituation to the light-dark test. Both males and females also saw a significant drop in total distance per second in the light chamber, however, there was a profound increase in vertical movements per second in the light chamber after conditioning for all neonatal treatment groups in females. In males, an increase in vertical movements per second was only seen for the no injection controls and saline controls. A reduction in chamber transitions was also seen in the no injection controls. Previous research regarding chronic stress on behaviour in the light-dark tests occur during adulthood. The results seen in the current study may be due to differences in adolescent and adulthood behaviours. Adolescent rats typically show more risk-taking behaviour and less anxiety-related behaviour than adults (Stansfield and Kirstein, 2006). Risk-taking behaviour in adolescence is theoretically believed to be associated with appropriate development and maturation into adulthood (Spear, 2000).

Overall, it is reasonable to conclude that no injection controls appear to be critical for comparisons between neonatal manipulations. The neonatal procedure for saline controls involves separation from the dam for a period of 15 minutes, an injection, and toe clipping on PND 3. These three variables may have a profound impact on the development of the brain in the

first week of life. We also observed several effects of immune stress prior to a conditioning stress, such that LPS 15 µg/kg showed resilience effects (less anxiety-like behaviour) compared to the saline controls. A period of chronic stress, using conditioned disgust, did not appear to elicit anxiety-like behaviour in the rats that would be consistent with previous literature. Repeated measures also revealed less anxiety-like behaviour over several parameters in the light-dark test following conditioned disgust.

Previous studies investigating the impact of early life stress and immune stimulation have not used untreated controls. There is a large amount of research that has concluded that neonatal LPS exposure does not elicit anxiety-like behaviour unless the rats are exposed to a stressor later in life. However, there may be a ceiling effect where the saline controls have heightened anxiety like the neonatal treatments, as the controls were exposed to early life stress during the neonatal manipulations. Therefore, the present thesis paves the way for future research calling for the use of untreated controls in neonatal drug manipulation research associated with psychopathologies involving anxiety.

4.2 References

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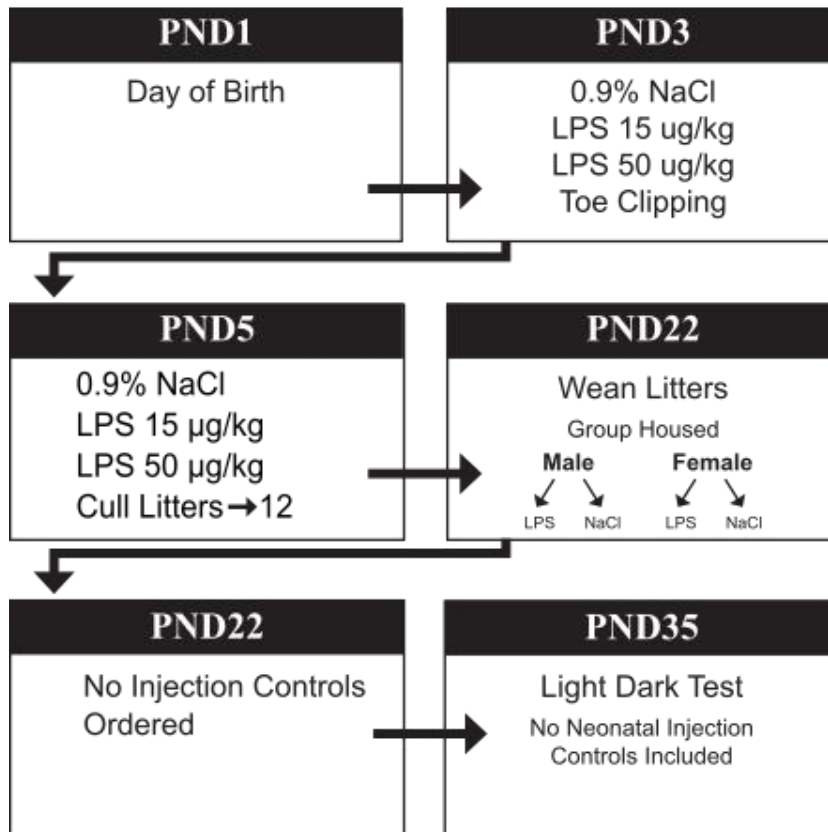
Tenk, C. M., Foley, K. A., Kavaliers, M., & Ossenkopp, K. P. (2007). Neonatal immune system

activation with lipopolysaccharide enhances behavioural sensitization to the dopamine

agonist, quinpirole, in adult female but not male rats. *Brain, behavior, and immunity*,

21(7), 935-945.

Appendix A



Sample Sizes

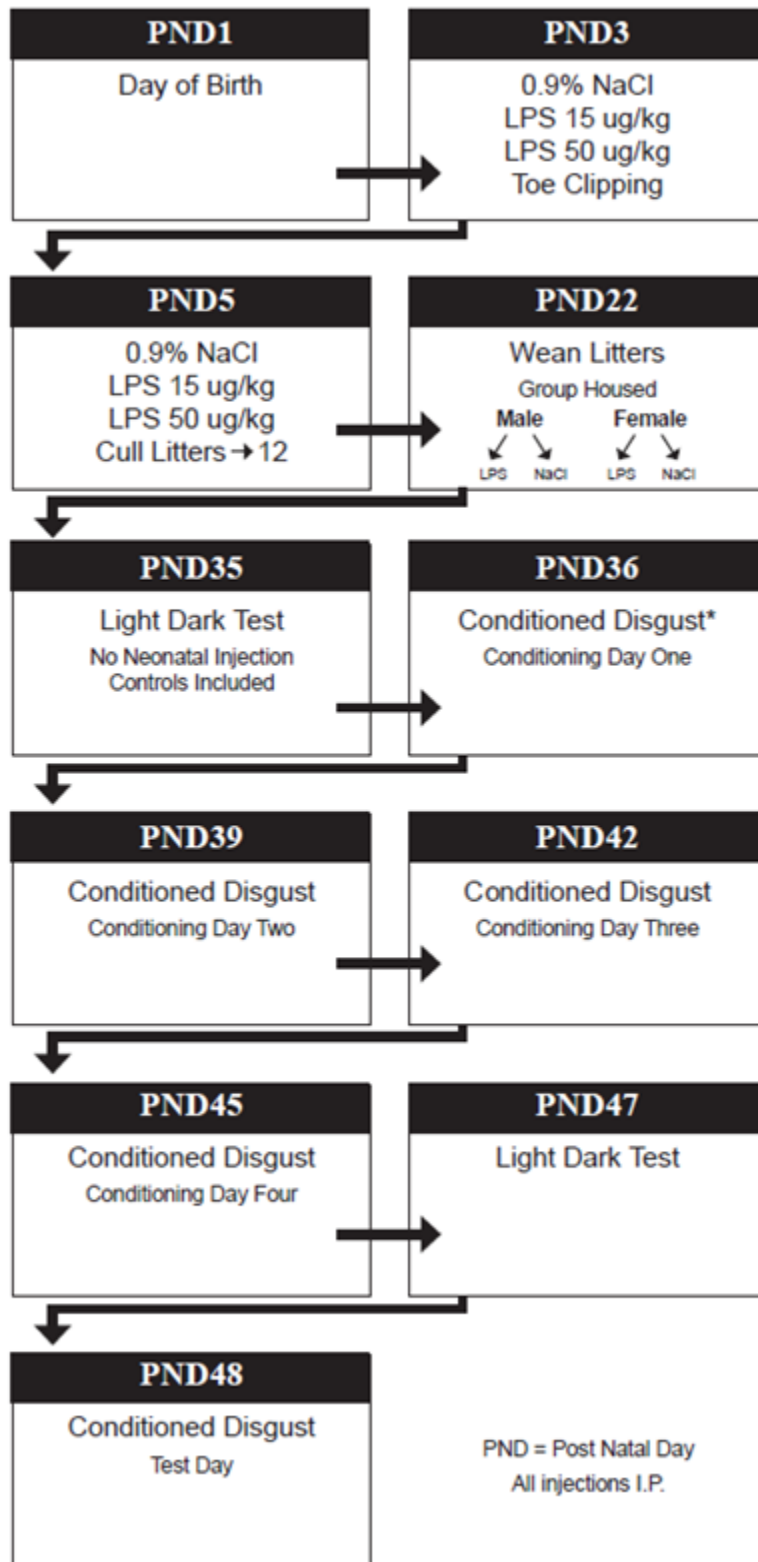
Neonatal Treatment	Male	Female
No Injection	n = 24	n = 24
0.9% NaCl	n = 36	n = 36
LPS15 µg/kg	n = 21	n = 21
LPS 50 µg/kg	n = 21	n = 18

SUPPLEMENTARY IMAGES



Light Dark Test Apparatus

Appendix B

* **CONDITIONED DISGUST**

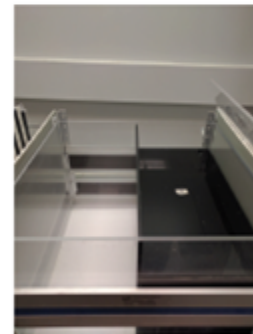
Male

Neonatal Treatment	Conditioned Disgust Treatment	n
No Injection	NaCl	12
	LiCl	12
NaCl	NaCl	16
	LiCl	20
LPS 15 ug/kg	NaCl	10
	LiCl	11
LPS 50 ug/kg	NaCl	10
	LiCl	11

Female

Neonatal Treatment	Conditioned Disgust Treatment	n
No Injection	NaCl	12
	LiCl	12
NaCl	NaCl	18
	LiCl	18
LPS 15 ug/kg	NaCl	10
	LiCl	11
LPS 50 ug/kg	NaCl	8
	LiCl	10

SUPPLEMENTARY IMAGES



Light Dark Test Apparatus



Conditioned Disgust Apparatus

CURRICULUM VITAE

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Post-Secondary Education and Degrees:

Western University
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2016 – Present
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Western University
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Honours and Awards:

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Western Graduate Research Scholarship
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Academic and Teaching Experience

Teaching Assistant

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- Psychology 1000 – Introduction to Psychology
- Psychology 3209 – Neuroscience of Motivation and Emotion

Undergraduate Honours Thesis Student Co-Supervisor

Western University
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- Provided guidance and assistance with experimental procedures, data collection, analysis, and writing of thesis

Clinical Research Experience

Clinical Research Intern

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- Developed Standard Operating Procedures for regulatory training to streamline clinical research
- Helped with the development of research budgets for a variety of Phase I-Phase IV clinical trials
- Assisted with the acquisition of informed consent from various patients outlined by GCP and approved protocol
- Reviewed adverse events for various clinical trials to determine reporting eligibility
- Attended various site selection visits, site initiation visits, and trial screening committee meetings

Peer Reviewed Published Articles:

Ward, J., James, F. & Monteith, G. (2016). The effect of topical lidocaine on muscle artefacts in awake canine electroencephalogram recordings. *The Veterinary Journal*, 213: 6-8.

Parker, L.A., Limebeer, C.L., Rock, E.M. Sticht, M.A., **Ward, J.**, Turvey, G...Makriyannis, A. (2016). A comparison of novel, selective fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL) or dual FAAH/MAGL inhibitors to suppress acute and anticipatory nausea in rat models. *Psychopharmacology*, 233(12): 2265-2275.

Rock, E.M., Limebeer, C.L., **Ward, J.M.**, Cohen, A., Grove, K., Niphakis, M.J., Cravatt, B.F. & Parker, L.A. (2015). Interference with acute nausea and anticipatory nausea in rats by fatty acid amide hydrolase (FAAH) inhibition through a PPAR α and CB1 receptor mechanism, respectively: a double dissociation. *Psychopharmacology*, 232(20): 3841-3848.

Conference Presentations:

Ward, J.M., Tenk, C., Kavaliers, M. & Ossenkopp, K-P. (2015). Effects of neonatal infection on adolescent anxiety in male and female rats and the development of 'anticipatory nausea'. Poster session presented at the *Society for Neuroscience* conference, Chicago, IL.

Professional Certifications:

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- Collaborative Institutional Training Initiative (CITI Program)

Health Canada Division 5- Drugs for Clinical Trials Involving Human Subjects

May 2017

- Collaborative Institutional Training Initiative (CITI Program)

Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans

May 2017

Transportation of Dangerous Goods TDG/IATA Training

May 2017

- Certified to Transport Class 6.2 & 9 Materials
 - Category B
 - Exempt Human/Animal Specimens
 - Genetically Modified Organisms
 - Limited Excepted Quantities
 - Dry Ice

Western University Animal Care and Veterinary Services

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- Approved by Canadian Council on Animal Care
 - WebCT Animal Care and Use Course
 - Basic Rat and Basic Mouse Training
 - Injections and Blood Collection Techniques Training

Western Basic WHMIS Training

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Western University General Laboratory Safety and Hazardous Waste Management Training

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