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Dose-related sex differences in the establishment of conditioned disgust (anticipatory nausea), and the effect of peripubertal and adult immune system stimulation with the endotoxin lipopolysaccharide (LPS) on learning and memory in the rat

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Abstract

In the human population, many immune- and nausea-related illnesses are more prevalent in women, relative to men.However, this apparent difference in not currently considered in animal research. The major question of this thesis was whether this sex difference could be reflected in a rodent model. Furthermore, how does immune system stimulation with LPS affect learning and memory in females? This thesis examined sex differences in the establishment of lithium chloride (LiCl) – induced conditioned disgust behavior (anticipatory nausea) to a distinct context, as well as, the establishment of conditioned place avoidance (CPA) using rodent models. Also examined were potential sex differences in response to treatment with the bacterial endotoxin, lipopolysaccharide (LPS), and its effect on learning and memory. In Chapter 2, male and female naïve Long-Evans rats were injected (intraperitoneally; i.p.) with either 200 µg/kg LPS or 0.9% (NaCl), 90 minutes prior to i.p. injections of either 128 mg/kg LiCl or 0.9% NaCl, and immediately placed into a distinctive context for 30 minutes (repeated over 4 conditioning days, spaced 72 h apart). 72 h following the final conditioning day, each subject was re-exposed to the context on a drug-free test day where orofacial and somatic behaviors were recorded. Results showed that LiCl-treated females conditioned stronger disgust reactions, relative to LiCl-treated males, as evidenced by significantly higher frequencies of conditioned “gaping” behavior and forelimb flailing in females. Pre-treatment with LPS during conditioning led to strong inhibition of conditioned disgust behavior, to levels that did not significantly differ from controls. Although there was no apparent sex difference in the degree of inhibition produced by LPS in this context-based rodent disgust model, males did exhibit significantly greater 24 h body weight losses

following LPS injections on the first two conditioning days, relative to females. In Chapter 3, the sex difference in conditioned gaping behavior found in Chapter 2 was explored further by examining potential dose-related effects. Once again, females displayed significantly higher frequencies of conditioned "gaping" behavior relative to males, in a dose-dependent manner. The results from Chapters 2 and 3 provide strong support for a sex difference in the onset and severity of nausea-related symptoms which is also observed among the human population. This provides a preclinical tool for testing the efficacy of anti-nausea treatments and noxious drug side-effects.

In Chapter 4, the effects of LPS pretreatment on the establishment of conditioned place avoidance (CPA) were examined. Female rats were also injected with LPS or saline during the peripubertal phase of development (6 weeks of age) and later pretreated with LPS again or saline in the classic two-chamber CPA paradigm. Results showed that while peripubertal LPS had no long-term effect on establishing CPA, it did interfere with the ability of a second LPS challenge in adulthood to block CPA, as was shown in subjects pretreated with LPS in adulthood only. The results of this study demonstrated a significant sex difference in the acquisition of conditioned disgust (i.e., anticipatory nausea), and the importance of considering the peripubertal stage of development when evaluating the effects of environmental challenges on adult behavior.

Keywords

Lithium Chloride (LiCl); Lipopolysaccharide (LPS); Anticipatory Nausea (AN); Conditioned Disgust; Rat; Sex Differences; Conditoned Place Avoidance (CPA); Conditioned Taste Avoidance; Conditioned Taste Aversion (CTA): Learning; Memory; Inhibition; Peripubertal; Homotypic; Sickness Behavior; Oncology; Model

Co-Authorship Statement

This thesis was written with the help and guidance of Dr. Klaus-Peter Ossenkopp and Dr. Martin Kavaliers, my doctoral supervisors. They contributed to this work by helping to develop the experimental design, suggestions for data analyses, and revisions help with the writing.

A version of Chapter 2 is in the final stages of revisions for a special issue of Pharmacology, Biochemistry and Behavior; and, Chapter 3 has been submitted to Psychoneuroendocrinology.

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1 General Introduction

In the human population, sex differences in the incidence and severity of certain mental and physical health disorders are not uncommon, especially in conditions characterized by nausea and/or inflammation-associated pathophysiology (Klein, 2012). For example, women report more frequent and severe experiences with nausea, relative to men, which include increased susceptibility to motion sickness (MSS; Paillard et al., 2013), increased nausea in relation to the onset of chronic migraine (CM; Özge et al., 2014), severe post-operative nausea and vomiting (PONV; Fujii, 2009), and, higher incidences of anticipatory nausea and vomiting (AN/V) in oncology patients receiving chemotherapy treatment (Fetting et al., 1983; Hilarius et al., 2012; LeBaron et al., 1988; Williams et al., 1980). Women also comprise 60% and 80% of the populations suffering from asthma (Tam et al., 2011) and autoimmune disease (Voskuhl, 2011), respectively; and, are 2-6 x more likely to die from H5N1 Avian influenza, relative to men (Klein, Pekosz, Passaretti, Anker, & Olukoya, 2010). Furthermore, a greater incidence of anxiety/depression-related disorders (for review, see Altemus, Sarvaiya & Epperson, 2014), as well as, neurodegenerative disease (For review, see Rocca, Mielke, Vemuri & Miller, 2014), are observed in women- all of which have been associated with pro-inflammatory pathology (Abbott et al., 2015; Raz, Knoefel & Bhaskar, 2016; Vivekanantham et al., 2015).

Since there are clear sex differences in nausea- and inflammation-related disorders observed in the human population, it is crucial that animal researchers employ models that reflect these important differences when examining disease etiology and/or treatment. However, the vast majority of research is carried out in male subjects only, thus reducing the potential validity of the model itself, and increasing the likelihood that putative treatments are less efficacious within a population comprised of both males and females. Therefore, the examination of nausea- and inflammation-related behaviors in female animal subjects is a critical factor for elucidating the mechanisms of these phenomena.

1.1 Nausea and learning

Nausea is a feeling of importance in human and animal medicine, which is poorly understood. Feelings of nausea are subjective and described as an unpleasant internal state (Kenward et al., 2015), consisting of queasiness or feeling sick to the stomach (Koch, 1995). Severe nausea and/or vomiting occurs in 40 – 70% of patients in palliative care, which greatly reduces patient quality of life (Keeley, 2009). Nausea also commonly presents following the intake of toxic or intolerable substances (Bischoff and Renzer, 2006), as an adverse post-operative side effect (Fujii, 2009), as a core symptom of motion sickness (Golding, 2006; Matchock et al., 2008; Paillard et al., 2013), or, as a side-effect to pharmacotherapy treatment, including chemotherapy (Morrow et al., 2002; Molassiotis, 2005).

Feelings of nausea play a large role in the development of conditioned disgust-related behavior. Conditioned taste aversion/avoidance is a behaviorally adaptive form of learning that enables animals to successfully reject or avoid consumption of potentially harmful food agents (Garcia, Lasiter, Bermudez-Rattoni, & Deems, 1985). Gustatory conditioning to solutions paired with (Eckel & Ossenkopp, 1996; Kent, Cross-Mellor, Kavaliers, & Ossenkopp, 2000; Ossenkopp & Eckel, 1995; Spector, Breslin, & Grill, 1988) or foods infused with (Cross-Mellor, Clarke, & Ossenkopp, 2004; Loy & Hall, 2002; Ossenkopp & Eckel, 1995; Ossenkopp, Ladowsky, & Eckel, 1997) an emetic toxin is acquired rapidly and can be very robust. It is important to note the distinction between taste aversion and taste avoidance. A conditioned taste *aversion* has been established when animals exhibit active aversive rejection responses (i.e., gapes, forelimb flails, head shakes, passive drip, and chin rubs) to an intraoral infused taste that was previously paired with a nausea-inducing US, such as LiCl (see Parker, 2003). The taste reactivity test (TRT) is commonly employed to test for conditioned taste aversion. This test involves the involuntary infusion (via intraoral cannula) of a salient taste that was previously paired with feelings of nausea during the conditioning phase (Berridge, Grill, & Norgren, 1981; Grill & Norgren, 1978 a,b). Upon infusion of the salient taste, animals will display aversion-related rejection responses to the taste, in the absence of any actual noxious treatment. A conditioned taste *avoidance* has been established when an animal

refuses to *voluntarily* consume a salient taste that was previously paired with a nausea-inducing US (e.g., LiCl). In the classic two-bottle preference test for conditioned taste avoidance, animals previously infused with a palatable taste in conjunction with feelings of nausea will, in a drug-free state, prefer to drink a safe fluid, such as water, and avoid consumption of the taste originally associated with nausea during the conditioning phase (e.g., Rana & Parker, 2008).

Although rats are incapable of vomiting (Borison, 1989; Hatcher, 1924, Horn et al., 2013), they do produce a gaping response that is similar to the orofacial topography of the retch response exhibited by the shrew, Suncus murinus, just prior to an emetic response (see Parker et al., 2008; Parker & Limebeer, 2006). Gaping is defined as the repeated opening and closing of the lower mandible in rapid succession approximately 5-7 times, and it involves the same musculature as the shrew retch (Travers & Norgren, 1986). Conditioned disgust was initially demonstrated in rats by pairing a novel taste with the effects of a nausea-inducing emetic drug, such as lithium chloride (LiCl). Garcia et al. (1974) argued that the rat disgust behaviors of gaping and chin rubbing, observed in response to the conditioned taste cue following such conditioning, were established by an association between the taste and activation of emetic neural circuitry, such as the brainstem emetic chemoreceptor trigger zone (area postrema; Borison, 1989).

By quantifying the rat orofacial and somatic behaviors when challenged with an intraoral infusion of the conditioned taste in a drug free test, Grill and Norgren (1978a) demonstrated that the disgust behaviors of gaping, chin rubbing and paw treading, were a strong index of conditioned taste aversions. Other studies have found that only emetic drugs condition gaping responses (Parker et al., 2006), and that the taste conditioned disgust response of gaping can be reduced or eliminated by lesioning the area postrema in rats (Eckel & Ossenkopp, 1996; Ossenkopp & Eckel, 1994, 1995), or, by treating the rats with anti-emetic drugs, such as ondansetron and 8-OH-DPAT (Limebeer & Parker, 2000; Parker et al., 2008).

In addition to the establishment of taste + illness associations, rodents have also been shown to associate a distinct context with "feelings" of nausea, induced by the toxin

LiCl, which models anticipatory nausea responses in human oncology patients receiving chemotherapy treatment. Anticipatory nausea (AN) is commonly observed among individuals receiving chemotherapy treatment for cancer, who report anticipatory nausea and/or vomiting to be among the most aversive side-effects from chemotherapy treatment (Morrow et al., 2002), oftentimes causing them to reduce or forego potentially life-saving treatment. Produced by classical conditioning, AN is acquired following exposure to a novel context-based conditioned stimulus (CS) (e.g., exposure to a hospital environment) that was paired with a nausea/emesis-inducing unconditioned stimulus (US) (e.g., chemotherapy treatment) (Morrow et al., 2002; Molassiotis, 2005). Anticipatory nausea is acquired when, upon subsequent re-exposure(s) to the contextual environment (CS), nauseous/emetic conditioned responses (CR) are exhibited prior to actual drug treatment.

Anticipatory nausea conditioning can be modeled in the rodent by means of a "conditioned gaping" learning and memory paradigm. As stated, "conditioned gaping" behavior has been shown to be an index of nausea in the rat, evidenced by the prevention of LiCl-induced conditioned gaping when rats are administered an anti-emetic treatment, such as, ondansetron or the 5-HT_{1A} agonist 8-OH-DPAT (Limebeer et al., 2006; Limebeer et al., 2008; Parker and Limebeer, 2006). Furthermore, it has been shown that a distinct context, previously paired with LiCl-induced nausea, has the potential to establish a conditioned taste aversion when this context is paired with a novel saccharin flavor (i.e., second-order conditioning), in the absence of any direct pairing with LiCl (Sticht et al., 2015). This evidence demonstrates that the conditioned disgust responses (i.e., gaping) to the context CS are indicative of feelings of nausea, as nausea is specifically required for the formation of true conditioned taste aversions (see Parker, 2003; Chambers, 2015).

Exposure to a context previously associated with feelings of nausea elicits a "conditioned gaping" response in the rat, providing an animal model that can serve as a valuable preclinical tool for examining anticipatory nausea treatments in chemotherapy patients (Limebeer et al., 2006; Limebeer et al., 2008; Molassiotis, 2005). Thus, rats can learn and remember associations between distinctive environments and experienced

nausea, and subsequently retrieve these associations to show aversion-related behaviors, such as gaping, upon re-exposure to the environment (Limebeer et al., 2006, 2008).

Similar to AN, in the 2-chamber test for Conditioned Place Avoidance (CPA; (see Frisch et al., 1995, Miller et al., 2000, Parker, 1992, Turenne et al., 1996 and White and Carr, 1985), rodents learn to associate one of two distinct chambers with the effects of drug treatment (e.g., LiCl-induced nausea), while the other "safe" chamber is paired with a saline control. These context + illness/saline pairings are alternated over the course of the Conditioning Phase. On a drug-free Test Day, each animal is re-exposed to the previously drug-paired chamber, and allowed to escape into the previously saline-paired chamber through a new opening between the two contexts. Male rats display significant LiCl-induced CPA behavior, spending less time in the previously drug-paired chamber relative to saline controls, and displaying dose-dependent increases in vertical rearing behavior (Tenk et al., 2005).

To date, potential sex differences in the conditioning of nausea/disgust responses in rodents have not been closely examined. Previous studies indicate that human females condition anticipatory nausea more often and in greater strength, relative to males; however, this sex difference has not yet been examined in the rodent models of anticipatory nausea (i.e., context aversion) and conditioned place avoidance.

1.2 Immune system stimulation

Accumulating evidence suggests that both bacterial and viral infections can have diverse behavioral and physiological effects. These effects include the production of an adaptive profile of behaviors termed "sickness behavior", as well as, reported deleterious effects on learning and memory in some, but not all, paradigms. Lipopolysaccharide (LPS), the active component of Gram-negative bacteria outer cell wall (Dinarello, 1984), is used to mimic bacterial infections. Administration of LPS stimulates the immune system, thus activating phagocytes and resulting in the release of pro-inflammatory cytokines. Cytokines, such as, interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TFN-α), and interleukin-6 (IL-6), produce a specific set of behaviors collectively known

as “sickness behavior”. The sickness behavior profile often includes, fever (Hart, 1988; O’Reilly et al., 1988; Roth et al., 1997), decreased locomotor activity (Hart, 1988; Engeland et al., 2003 a; Franklin et al., 2007; Yirmiya et al., 1994), hypersomnia and decreased grooming (Hart, 1988), adipsia, and anorexia (Cross-Mellor et al., 2000; Gayle et al., 1998; Langhans, 2000; Langhans et al., 1990), all of which are considered to be behaviorally adaptive and serve to help the organism counter the bacterial infection.

Bacteria-related immunogens have been shown to affect learning and memory in a variety of learning paradigms. However, the results from previous studies, which have examined the effects of acute versus chronic LPS administration on learning and memory, have been somewhat inconsistent. It has been reported that marked learning deficits are observed following LPS-treatment in the Morris water maze (MWM) (Arai et al., 2001; Shaw et al., 2001; Sparkman et al., 2005 b), yet others have reported slower swimming speeds that may account for longer search latencies to the hidden platform (Sparkman et al., 2005b). However, performance deficits in the MWM have been shown to be associated with disruptions in NMDA-dependent, and NMDA-independent long-term potentiation, following chronic intracerebroventricular administration of LPS (Min et al., 2009). Similarly, it has been suggested that LPS may impair the ability to form representations of distinct contexts in contextual fear conditioning paradigms, as demonstrated by a reduction in freezing responses upon re-exposure to a context previously paired with an aversive foot shock in LPS-treated rats and mice (Kranjac et al., 2012; Pugh et al., 1998).

Learning paradigms that require the animal to produce significant motor output present potential confounds to the interpretation of task performance. As LPS is known to produce reductions in locomotor behavior (Franklin et al., 2007; Hart, 1988; Yirmiya et al., 1994) during the acute-phase response to endotoxin treatment, it can be difficult to determine whether learning decrements are a product of disruptions in cognitive processes or simply due to reductions in locomotor behavior (Cunningham & Sanderson, 2008). Indeed, it has been suggested that the effects of LPS on learning and memory must be tested in different paradigms that do not present such potential confounding factors

(Cunningham & Sanderson, 2008). Thus, the investigation of LPS effects on learning and memory in rodent conditioned disgust paradigms is of interest.

The rodent models of taste aversion/avoidance, anticipatory nausea, and conditioned place avoidance utilize the rat's ability to associate the aversive feelings of toxin-associated nausea with distinct tastes or contexts. LPS pre-treatment during the conditioning phase of the AN and CTA paradigms has been shown to significantly attenuate the establishment of conditioned disgust responses (e.g., gaping, paw treading, chin rubbing). Treatment with LPS has been shown to produce an initial drop in voluntary saccharin (Yirmiya, 1996; Langhans, 1996) or sucrose (Cross-Mellor et al., 1999) consumption. The act of feeding involves both appetitive and consummatory factors (see Parker, 2003 for review). In a taste avoidance paradigm, intake is voluntary, and thus both appetitive and consummatory factors are involved. Since LPS produces anorexia and adipsia during the acute-phase response, there is often little motivation to consume liquids or food (i.e., appetite). Thus, it would follow that without appetite, voluntary consumption would be low as well. In the conditioned taste aversion paradigm, which uses the involuntary TRT, appetite is not a factor in terms of intrinsic motivation to drink, therefore only consummatory factors are being evaluated (Parker, 2003).

The results of prior studies demonstrate that LPS by itself does not produce conditioned taste aversion, instead, it has been shown to block robust conditioned taste aversion that is typically produced through the pairing of an emetic treatment (i.e., LiCl) and a palatable sucrose solution (Cross-Mellor et al., 2009). Male rats pre-treated with LPS during LiCl conditioning also fail to acquire robust conditioned "gaping" responses in the rodent model of AN, displaying similar behavioral frequencies to saline controls (Chan et al., 2009; Chan et al., 2013; Cloutier et al., 2011; Cloutier et al., 2012b).

The effects of LPS treatment on CPA learning have not yet been evaluated in male or female subjects. Likewise, the effects of LPS pre-treatment on the establishment of AN in have not yet been examined in female subjects. Furthermore, since LPS developmental models are now being used to elucidate potential links between early-life (i.e., prenatal or neonatal) infection and mental illness, it is of interest to examine the

consequence of immune challenges during other sensitive periods of development, such as, puberty, on learning and memory in adulthood. Female mice treated with LPS at 6 weeks of age have been shown to develop long-term alterations in behavior, displaying increased plasma concentrations of estradiol, and surprisingly, increased anxiety-like behavior (Blaustein et al., 2011). Neonatal male rat pups treated with LPS displayed exacerbated sickness behavior responses to homotypic challenge in adulthood, while females did not (Tenk et al., 2008). Thus, it is clear that the long-term effects of LPS treatment on behavior may be dependent, in part, on biological sex. However, the effects of a peripubertal immune challenge on learning and memory in adulthood have not yet been examined.

1.3 Objectives of the current thesis

Conditioned disgust and other aversion-related behaviors that become associated with salient conditioning stimuli have been studied for decades, though data from female subjects is scarce. Given the evident human sex differences in the incidences of nausea- and inflammation-related ailments, the need for female subjects as animal models is therefore fundamental to the understanding of disease etiology and treatment.

The first objective of this thesis was to examine the ability of female rodents to process and to associate an external mode of stimulus presentation (i.e., a distinct context) with an internal sickness cue (i.e., LiCl-induced nausea) in the rodent model of Anticipatory Nausea (i.e, conditioned place aversion), and to test whether a significant sex difference was present. The second part of this study examined the effects of pre-treatment with lipopolysaccharide, during the 4-day AN Conditioning Phase, on the expression of learning and memory deficits on the Drug-Free Test Day, in order to elucidate any further sex differences in the response to LPS effects on learning and memory.

The second objective of this thesis was to further investigate the sex difference observed in the first study, where it was shown that female rats conditioned significantly

higher frequencies of conditioned disgust-related behavior, relative to males. In this second experiment, sex differences in the dose response to LiCl-induced AN were examined. Adult male and female rats were treated with one of three doses of LiCl (64 mg/kg, 96 mg/kg, or 128 mg/kg), or, a saline control, prior to immediate exposure to a distinct context over a 4-day Conditioning Phase. Dose- and sex-dependent increases in conditioned disgust behavior (e.g., "conditioned gaping") were examined.

The third objective of this thesis was to examine, for the first time, Conditioned Place Avoidance behavior in female rats using a 2-chamber CPA apparatus, wherein one distinct chamber is paired with LiCl-induced nausea, and the other distinct chamber is paired with saline. Acute LPS pre-treatment, during the 8-day CPA Conditioning Phase, and its effects on establishing CPA behavior were also examined in females. To the best of our knowledge, this is the first time the effects of LPS on CPA learning have been examined in any rodent. LPS challenge during sensitive developmental phases, such as, the neonatal and peripubertal stages, has been shown to produce long-lasting alterations in behavior. Therefore, the effect of a single immune system challenge with LPS on CPA learning in adulthood was examined. Futhermore, long-term changes in the response to a second homotypic drug challenge in adulthood were also evaluated.

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Chapter 2

2 Rodent sex differences in disgust behaviors (anticipatory nausea) conditioned to a context associated with the effects of the toxin LiCl: Inhibition of conditioning following immune stimulation with Lipopolysaccharide

Chemotherapy treatment plays a major role in cancer survival rates; however, its cytotoxicity produces undesirable side effects, including severe acute and delayed nausea. This aversive nausea experience serves as an unconditioned stimulus in the establishment of a phenomenon termed Anticipatory Nausea and/or Vomiting (AN/V). Anticipatory nausea presents as a classically conditioned response, produced by the pairing of the hospital treatment room or context with the nausea produced by the chemotherapy, resulting in the establishment of a robust conditioned nausea/vomiting response upon re-entry into the hospital context on subsequent visits, prior to actual drug treatment (Hickok et al., 2003). It is estimated that 30% of patients suffer from conditioned anticipatory nausea, and report it as the most aversive component of chemotherapy treatment (Rodriguez, 2013). Due to the severity of this conditioned response, patient non-compliance increases as many choose to forego further treatment that could be life-saving (Molassiotis, 2005).

Anticipatory nausea conditioning can be modeled in rodents by means of "conditioned disgust" learning and memory paradigms. Although rats are incapable of vomiting (Borison, 1989; Hatcher, 1924, Horn et al., 2013), they do produce a gaping response which is similar to the orofacial topography of the retch response exhibited by the shrew, Suncus murinus, just prior to an emetic response (see Parker et al., 2008; Parker & Limebeer, 2006), and involves the same musculature (Travers & Norgren, 1986). Conditioned disgust was initially demonstrated in rats by pairing a novel taste with the effects of a nausea-inducing emetic drug, such as lithium chloride (LiCl). Garcia et al.

(1974) argued that the rat disgust behaviors of gaping and chin rubbing, observed in response to the conditioned taste cue following such conditioning, were established by an association between the taste and activation of emetic neural circuitry, such as the brainstem emetic chemoreceptor trigger zone (area postrema; Borison, 1989). By quantifying the rat orofacial and somatic behaviors when challenged with an intraoral infusion of the conditioned taste in a drug free test, Grill and Norgren (1978a) demonstrated that the disgust behaviors of gaping, chin rubbing and paw treading, were a strong index of conditioned taste aversions. Other studies have found that only emetic drugs condition gaping responses (Parker et al., 2006), and that the taste conditioned disgust response of gaping can be reduced or eliminated by lesioning the area postrema in rats (Eckel & Ossenkopp, 1996; Ossenkopp & Eckel, 1994, 1995), or, by treating the rats with anti-emetic drugs, such as ondansetron and 8-OH-DPAT (Limebeer & Parker, 2000; Parker et al., 2008).

Exposure to a context previously associated with feelings of nausea elicits not only a robust conditioned context place avoidance (e.g., Tenk et al., 2005), but also a conditioned disgust response (gaping) and this paradigm has been offered as an animal model of anticipatory nausea (see Limebeer et al., 2006; Parker et al., 2008). Dose related increases in rat gaping behavior have been observed in a drug free test trial following multiple pairings of a novel context with the effects of LiCl (Ossenkopp et al., 2011). Thus, rats can learn and remember associations between distinctive environments and experienced nausea, and subsequently retrieve these associations to show aversion-related behaviors, such as gaping, upon re-exposure to this environment (Limebeer et al., 2008). This model can serve as a valuable preclinical tool for examining anticipatory nausea in chemotherapy patients (Limebeer et al., 2008; Molassiotis 2005; Parker et al., 2008). As this population comprises one third of all patients undergoing chemotherapy treatment, individual differences, such as sex differences, in AN/V should be examined in order to determine the possible reasons why some patients establish strong anticipatory nausea, while others do not.

In addition to examining sex differences in the establishment of conditioned disgust, it is also important to evaluate sex differences in the efficacy of drug treatments

that have been shown to disrupt learning and memory in these paradigms. Lipopolysaccharide (LPS) treatment is an experimental procedure that has been shown to reduce or abolish the conditioning of disgust responses to novel taste or context cues in rats (Chan et al., 2009, 2013; Cloutier et al., 2012a,b; Cross-Mellor et al., 2009). LPS is the active component of the cell wall of Gram negative bacteria, and when injected into an animal results in the production of pro-inflammatory cytokines (Dinarello, 1984), chiefly interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). LPS treatment also initiates a specific set of pathophysiological and behavioral changes collectively known as "sickness behaviors". These "sickness behaviors" include induction of fever (Hart, 1988; O'Reilly et al., 1988; Roth et al., 1997), anorexia and adipsia (Cross-Mellor et al., 2000; Gayle et al., 1998; Langhans, 2000; Langhans et al., 1990), reductions in locomotor activity (Hart, 1988; Engeland et al., 2003 a; Franklin et al., 2007; Yirmiya et al., 1994), hypersomnia and reduction in grooming (Hart, 1988), helping the organism counter the bacterial infection.

LPS treatment has been found to have deleterious effects in some learning and memory paradigms. Studies have found effects on the consolidation phase in contextual fear conditioning, context discrimination, and two-way active avoidance learning (e.g., Pugh et al., 1998; Kranjac et al., 2012; Sparkman et al., 2005a). However, the nature of the sickness behaviors produced by LPS treatment can make it difficult to separate cognitive effects from changes in locomotor output. For example, some studies have shown learning and memory impairments in spatial reference learning in the Morris water maze (Arai et al., 2001; Shaw et al., 2001; Sparkman et al., 2005b) and in the radial arm maze (Song et al., 2004). However, other studies have considered the influence of LPS-induced reductions in locomotor output on spatial performance and have concluded that latencies to reach the hidden platform in LPS-treated rats are due to decreased swimming speeds, as opposed to increased distances travelled to reach the platform (Sparkman et al., 2005b). The conditioned disgust (gaping) paradigm is not influenced by locomotor reductions (Grill and Norgren, 1978b).

The conditioned disgust rodent model of anticipatory nausea has to date only used male subjects. In the human population, anticipatory nausea occurs more frequently in

female patients (Fetting et al., 1983; Hilarius et al., 2012; LeBaron et al., 1988). Thus, it was of interest to examine the conditioning of nausea related disgust responding in female rats. Females also exhibit enhanced immune functioning, relative to males (e.g., Bilbo & Nelson, 2001; Engeland et al., 2003a,b; Gaillard & Spinedi, 1998; Lahita, 2000), and such effects on learning and memory require further examination. The current study investigated the effects of treatment with the bacterial endotoxin LPS on the establishment of LiCl conditioned disgust reactions in both male and female rats. As women exhibit anticipatory nausea more frequently than men, it was predicted that a sex difference in the frequency of conditioned disgust responses would be observed, evidenced by a higher frequency of conditioned disgust reactions in the female rats, relative to the males. Given this specific objective of the present study, a priori pairwise comparisons for sex differences in conditioned disgust behaviors were applied. Secondly, as females show enhanced immune functioning, relative to males, it was predicted that a sex difference in response to LPS treatment would be observed in terms of acute phase sickness behaviors (less weight loss) during conditioning and in its effects on learning (less reduction in conditioned disgust responses).

2.1 Materials and Methods

2.1.1 Subjects

Subjects were 29 male and 31 female adult naive Long-Evans rats (Charles River, Quebec, Canada) weighing between 175-250 g at the start of the experiment. The rats were pair-housed in standard polypropylene cages (45 x 22 x 20 cm) in a colony room with a temperature of 21 ± 1 °C and maintained on a 12-h light: 12-h dark cycle with the lights on from 07:00 to 19:00 h. All rats had free access to food (ProLab RMH 3000 rat chow) and tap water throughout the experiment. 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.

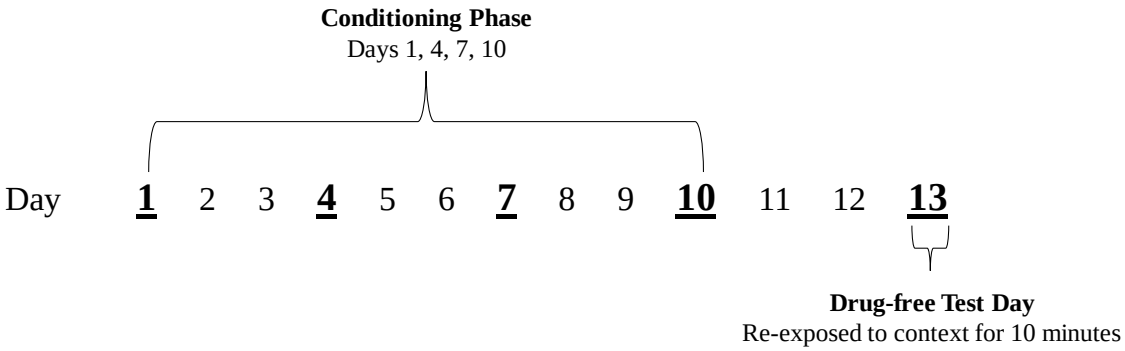
2.1.2 Apparatus

The apparatus (used on all conditioning days and the test day) consisted of a white Plexiglas box (29 cm × 25 cm × 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 (see Figure 1B). Lighting cues were kept consistent with previous studies employing this rodent model of anticipatory nausea (e.g., Chan et al., 2009). Behavioral responses exhibited on the Drug-Free Test Day were videotaped with a video camera (Sony DCR-DVD201; London, Ontario) positioned approximately 1 m from the mirror.

2.1.3 Experimental procedure

An illustration of the testing injection schedule and group designation is provided in Figure 1A-B and Table 1. All conditioning and testing was performed during the light phase of the LD cycle. The experiment consisted of two phases, included a Conditioning Phase (4 days, spaced 72 h apart), and one Drug-free Test Day, 72 h following the final conditioning day. There were four experimental groups for each sex resulting in 8 groups (n = 7-8/group) in total.

A



B

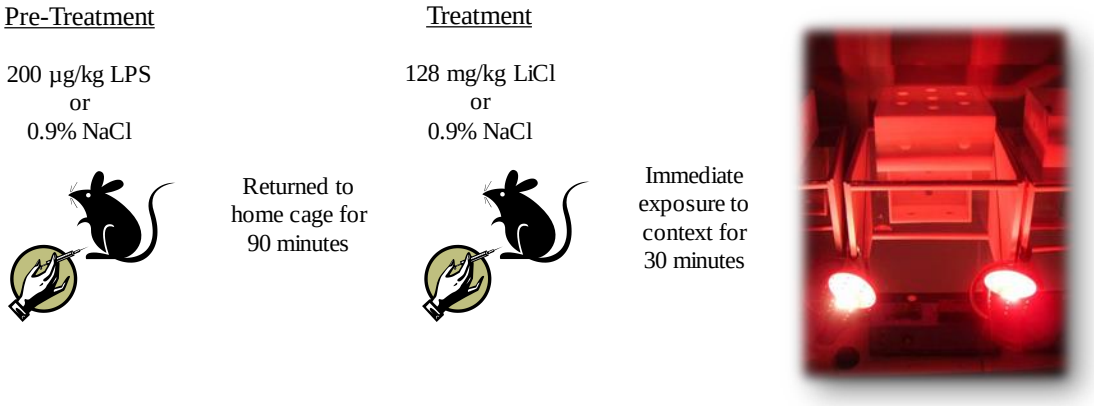


Figure 1 A-B. Procedure. Rats were pretreated with LPS or saline followed 90 minutes later by an injection of either LiCl or saline, and quickly placed into a distinctive context for 30 minutes, on each conditioning day. On the drug-free test day, each rat was reexposed to the distinctive context for 10 minutes.

Pre-Treatment	Treatment	Group Name	Male n =	Female n =
0.9% NaCl	0.9% NaCl	**NaCl-NaCl**	7	8
0.9% NaCl	128 mg/kg LiCl	**NaCl-LiCl**	8	8
200 µg/kg LPS	0.9% NaCl	**LPS-NaCl**	7	8
200 µg/kg LPS	128 mg/kg LiCl	**LPS-LiCl**	7	7

Table 1. Group designation.

2.1.3.1 Conditioning phase drug treatment

On each day of the Conditioning Phase (4 days, 72 h apart), animals were pre-treated with an intraperitoneal injection of either 200 µg/kg LPS or an equal volume of 0.9% isotonic saline (NaCl; 1 ml/kg), 90 minutes prior to a second intraperitoneal injection of 128 mg/kg lithium chloride (LiCl; 20 ml/kg; 0.15 M), or 0.9% isotonic saline (NaCl; 20 ml/kg). Each rat was then immediately placed into the distinctive context for 30 minutes. Following each exposure to the distinctive context, animals were immediately returned to the home cage. The eight groups consisted of: LPS-LiCl, LPS-NaCl, NaCl-LiCl, and NaCl-NaCl, for both males and females. An illustration of the group composition is shown in Figure 1C. Doses of LiCl and LPS were selected based on previous findings that demonstrated robust conditioned gaping behavior following treatment with 128 mg/kg LiCl, and significant impairment of conditioned gaping responses using 200 µg/kg LPS (Chan et al., 2009; Cloutier et al., 2012; Chan et al., 2013).

2.1.3.2 Change in Body Weight

Body weight was measured prior to conditioning and again 24 h later on each of the four conditioning days. LPS induces an acute-phase response, wherein the initial immune system stimulation produces a specific set of sickness behaviors that includes anorexia and adipsia. The anorectic and adipsic effects lead to significant weight loss following LPS treatment (Hart, 1988; Li et al., 2000) - a reliable measure of LPS influence.

2.1.3.3 Drug-free test day

72 h following the final Conditioning Day, each animal was again placed in the distinctive context for 10 minutes in a drug-free state, and behavioral responses were video recorded for later scoring. Dependent behavioral variables analyzed consisted of the frequency of the active aversive disgust-related behaviors; “gaping”, forelimb flailing, chin rubbing, head shaking, and paw treading (see Grill & Norgren, 1978a). Gaping was

defined as lowering of the mandible and the pushing or thrusting out of the lower teeth (see Parker & Limebeer, 2006).

2.1.3.4 Vaginal lavage collection

After arriving from the supplier, the animals were allowed one week to acclimatize to the colony room before being handled. Following handling over 3 days, the estrous cycle was tracked daily, at the same time each day throughout the experiment. Vaginal lavages were collected with a pipette filled with distilled warm water, and samples were placed onto microscope slides for later analysis in order to determine the cycle phase of each rat on the drug-free test day.

2.1.4 Data analysis

2.1.4.1 Changes in body weight

Following LPS treatment, significant reductions in body weight are typically observed 24 h post-treatment, relative to controls (Li et al., 2000). Percent of body weight change was calculated by subtracting the Conditioning Day body weight from the 24 h post-treatment weight, then dividing by the Conditioning Day body weight and multiplying by 100.

Percent change in body weight was analyzed with a repeated-measures split-plot Analysis of Variance (ANOVA), with 3 between-subjects factors: Sex (at 2 levels: Male or Female), Drug 1 (at 2 levels: LPS or NaCl), and Drug 2 (at 2 levels: LiCl or NaCl); and, one within-subjects factor: Conditioning Day (at 4 levels: C1-C4).

Post-hoc pairwise comparisons were performed using Tukey's Honestly Significant Difference (HSD) test. All statistical tests used a significance criterion of $\alpha = 0.05$.

2.1.4.2 Drug-free test day

On the 10-minute drug-free Test Day frequency of gaping responses, paw treading, forelimb flailing, and head shaking were each analyzed using a between-

subjects ANOVA with 3 between-subjects factors: Sex (at 2 levels: Male or Female), Drug 1 (at 2 levels: LPS or NaCl), and Drug 2 (at 2 levels: LiCl or NaCl).

As this part of the study was exploratory, Fisher's least significant difference (LSD) test was used for a priori and post-hoc pairwise comparisons. All statistical tests used a significance criterion of $\alpha = 0.05$.

2.2 Results

Results showed that all rats treated with LiCl (i.e., males and females in Group NaCl-LiCl) established robust conditioned gaping behavior, relative to controls. Furthermore, females acquired stronger condtioned disgust responses relative to males. LPS blocked the formation of conditioned gaping behavior in both sexes, with no significant differences observed between sexes.

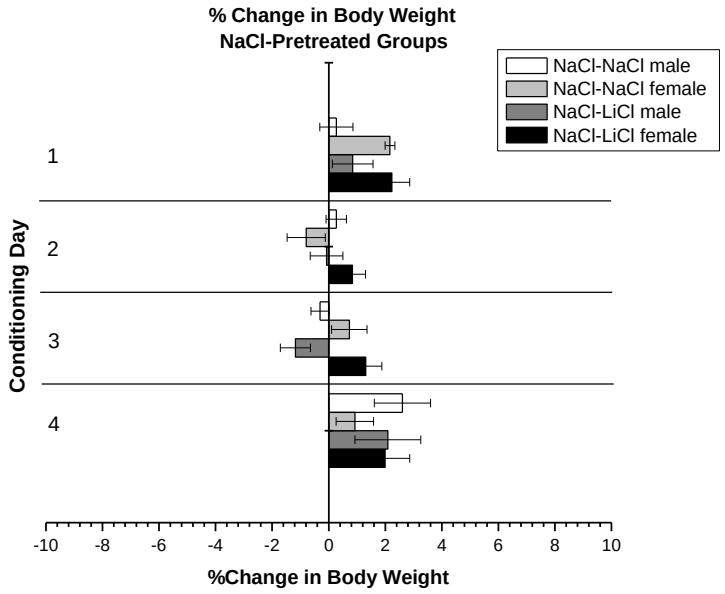
2.2.1 Body weight

The ANOVA revealed significant main effects of Conditioning Day, $F(3, 119) = 37.693$, $p < 0.001$; Sex, $F(1, 52) = 19.375$, $p < 0.001$; and, Drug 1 (LPS or NaCl), $F(1, 52) = 64.562$, $p < 0.001$. Significant interactions were revealed for Sex x Drug 1, $F(1, 52) = 4.240$, $p < 0.05$; Conditioning Day x Sex, $F(3, 119) = 3.387$, $p < 0.05$; Conditioning Day x Drug 1, $F(3, 119) = 51.019$, $p < 0.001$; and, Conditioning Day x Sex x Drug 1, $F(3, 119) = 4.326$, $p < 0.02$.

Post hoc analyses (Tukey's HSD) revealed that, 24 h following Conditioning Day 1, all LPS-treated male and female groups lost significantly more body weight relative to all saline control groups (p's< 0.01). Furthermore, both LPS-treated groups of males lost significantly more body weight relative to females in Group LPS-NaCl (p's< 0.05), but not relative to females in Group LPS-LiCl. Twenty-four hours following Conditioning Day 2, males in Group LPS-LiCl lost significantly more weight relative to female Groups LPS-LiCl and NaCl-LiCl only (p's< 0.05). Whereas LPS-treated males continued to show small body weight decreases, LPS-treated females exhibited body weight increases following Conditioning Day 2. No other significant differences in weight loss were found

in LPS-treated subjects, relative to controls, following Conditioning Days 3 and 4. Mean percent change in body weight and S.E.M. are depicted in Figure 2.

A


% Change in Body Weight
NaCl-Pretreated Groups
NaCl-NaCl male
NaCl-NaCl female
NaCl-LiCl male
NaCl-LiCl female
Conditioning Day
1
2
3
4
-10
-8
-6
-4
-2
0
2
4
6
8
10
%Change in Body Weight

B

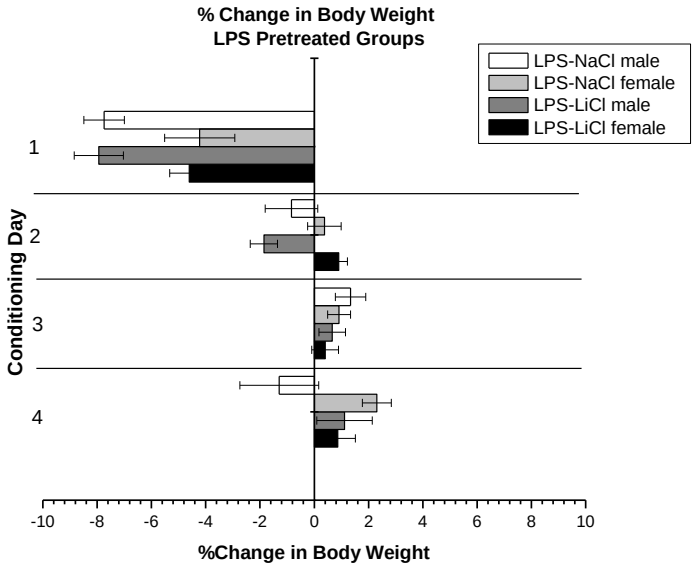

% Change in Body Weight
LPS Pretreated Groups
LPS-NaCl male
LPS-NaCl female
LPS-LiCl male
LPS-LiCl female
Conditioning Day
1
2
3
4
-10
-8
-6
-4
-2
0
2
4
6
8
10
%Change in Body Weight

Figure 2 A-B. Mean (+ S.E.M.) percent change in body weight over four conditioning days for groups NaCl-NaCl, NaCl-LiCl, LPS-NaCl, and LPS-LiCl (males and females; n = 7-8/group). 24 h following Conditioning Day 1 (Fig. 2B), all LPS-pretreated groups lost significantly more body weight relative to NaCl controls (p's< 0.05). LPS-pretreated males also lost significantly more weight than females in Group LPS-NaCl ($p< 0.05$).

2.2.2 Drug-free test day

All Test Day data presented reflect conditioned responding only, while the animal was in a completely drug-free state.

2.2.2.1 Conditioned gaping behavior

The between-subjects ANOVA revealed significant main effects of Drug 1 (LPS or NaCl), $F(1, 52) = 19.26$, $p < 0.001$; and, Drug 2 (LiCl or NaCl), $F(1, 52) = 25.74$, $p < 0.001$, on the frequency of conditioned the disgust reaction of gaping. In addition, a significant Drug 1 x Drug 2 interaction was found, $F(1, 52) = 19.05$, $p < 0.001$. No other significant main effects or interactions were found.

A priori analyses (Fisher's LSD) for sex differences revealed that both males and females in Group NaCl-LiCl established conditioned disgust, evidenced by gaping frequencies that were significantly higher than those displayed by males and females in Groups NaCl-NaCl, LPS-NaCl, and LPS-LiCl (p's < 0.02). Thus, LPS pre-treatment prior to LiCl treatment blocked the establishment of conditioned gaping behavior in both males and females. Most notably, females in Group NaCl-LiCl displayed higher frequencies of conditioned gaping relative to males in Group NaCl-LiCl ($p < 0.02$), evidence for a significant sex difference in the establishment of conditioned gaping responses. Mean gaping frequencies and S.E.M. are depicted in Figure 3.

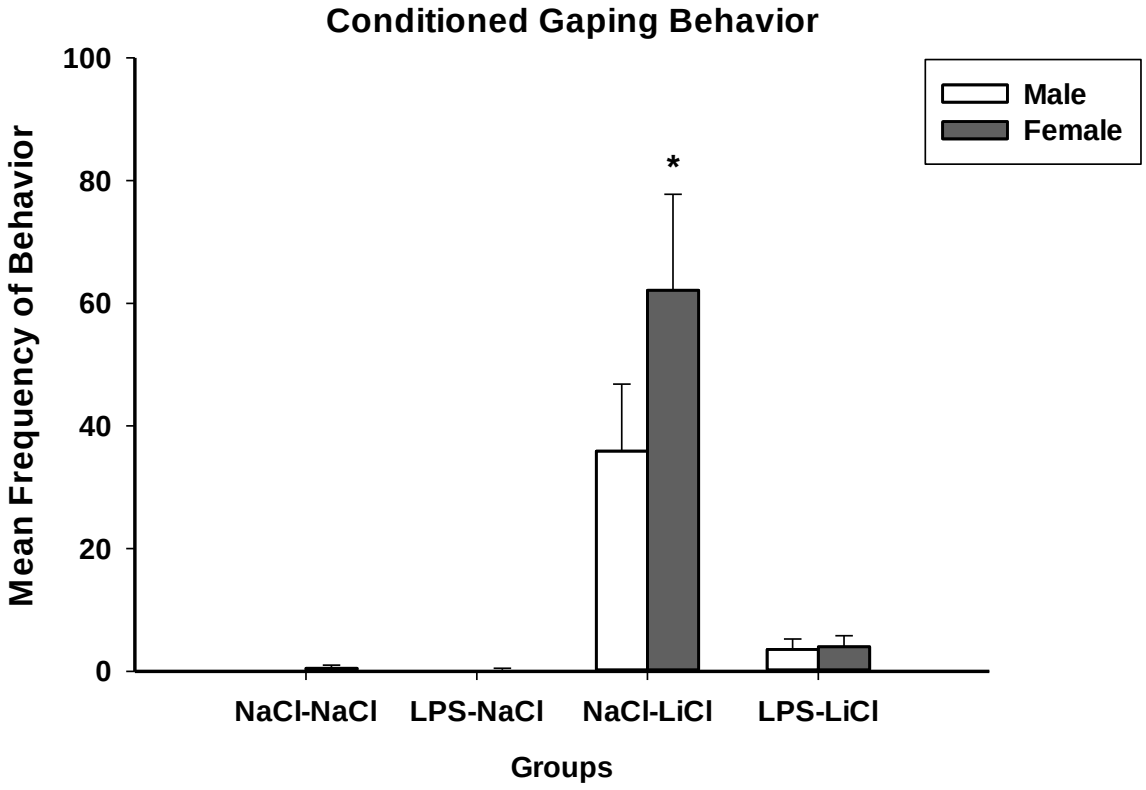


Figure 3. Mean (+ S.E.M.) frequency of conditioned "gaping" behavior expressed by Groups NaCl-NaCl, NaCl-LiCl, LPS-NaCl, and LPS-LiCl (males and females; n = 7-8/group) during the 10-minute test in the distinctive context in the absence of drug treatment. Both males and females in Group NaCl-LiCl displayed significantly higher "gaping" frequencies relative to controls. * denotes that females in Group NaCl-LiCl also displayed significantly higher "gaping" frequencies relative to males in Group NaCl-LiCl ($p < 0.05$).

2.2.2.2 Conditioned forelimb flails

The between-subjects ANOVA revealed significant main effects of Sex, $F(1, 52) = 7.91$, $p < 0.01$; Drug 1 (LPS or NaCl), $F(1, 52) = 6.24$, $p < 0.02$; and, Drug 2 (LiCl or NaCl), $F(1, 52) = 13.07$, $p = 0.001$, on the frequency of conditioned forelimb flailing behavior. Significant interactions were also found for Sex x Drug 1, $F(1, 52) = 4.32$, $p < 0.05$; Sex x Drug 2, $F(1, 52) = 4.32$, $p < 0.05$; Drug 1 x Drug 2, $F(1, 52) = 10.44$, $p < 0.01$; and, Sex x Drug 1 x Drug 2, $F(1, 52) = 7.907$, $p < 0.01$.

A priori analyses (Fisher's LSD) for sex differences revealed that females in Group NaCl-LiCl displayed significantly more forelimb flails relative to all other groups, including a sex difference when compared to males in Group NaCl-LiCl (p's< 0.001), which did not differ from controls. Mean frequencies of forelimb flailing and S.E.M. are depicted in Figure 4.

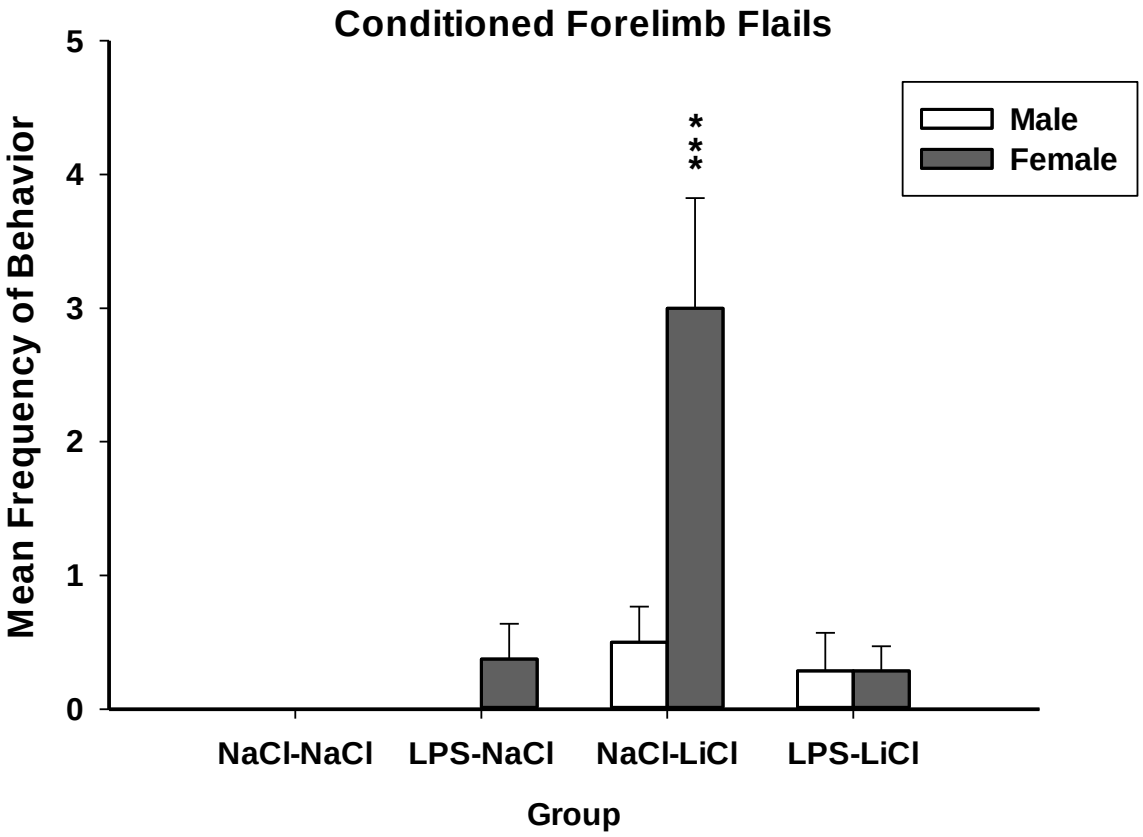


Figure 4. Mean (+ S.E.M.) frequency of conditioned forelimb flailing behavior expressed by Groups NaCl-NaCl, NaCl-LiCl, LPS-NaCl, and LPS-LiCl (males and females; n = 7-8/group) during the 10-minute test in the distinctive context in the absence of drug treatment. Females in Group NaCl-LiCl displayed significantly higher forelimb flailing frequencies relative to controls. *** denotes that females in Group NaCl-LiCl also displayed significantly higher "gaping" frequencies relative to males in Group NaCl-LiCl ($p < 0.001$).

2.2.2.3 Conditioned chin rubs

The ANOVA indicated significant main effects for Drug 1, $F(1,52) = 12.34$, $p = 0.001$, and Drug 2, $F(1,52) = 12.78$, $p = 0.001$, as well as a significant Drug 1 x Drug 2 interaction $F(1,52) = 12.34$, $p = 0.001$. Post-hoc comparisons indicated that both the male and female groups NaCl-LiCl exhibited significantly higher levels of chin rubbing relative to all other groups ($ps < .02$) but there was no sex difference in these two groups or any other comparisons (see Figure 5).

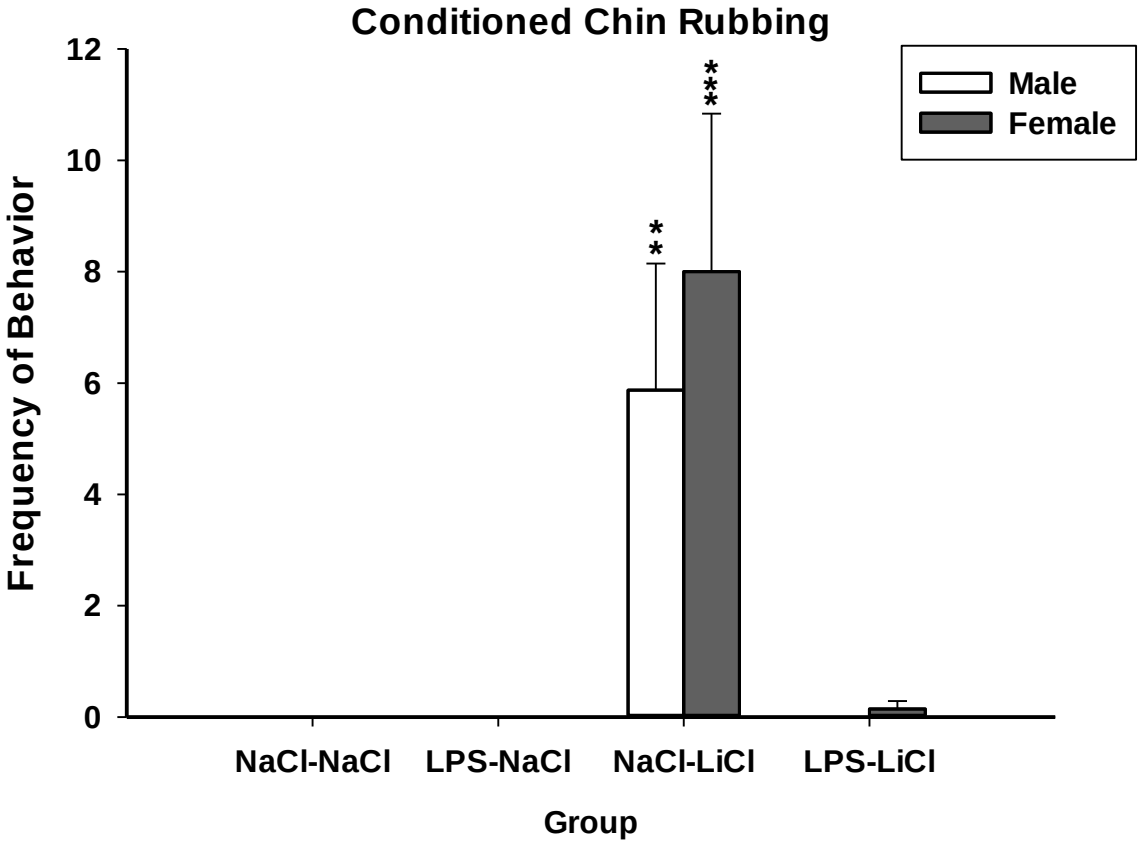


Figure 5. Mean (+ S.E.M.) frequency of conditioned chin rubbing behavior expressed by Groups NaCl-NaCl, NaCl-LiCl, LPS-NaCl, and LPS-LiCl (males and females; n = 7-8/group) during the 10-minute test in the distinctive context in the absence of drug treatment. ** and *** indicate that NaCl-LiCl males and females displayed significantly higher frequencies of conditioned chin rubbing relative to all other groups (*p*'s< 0.01 and 0.001, respectively).

2.2.2.4 Conditioned paw treads

The ANOVA showed significant main effects for Drug 1, $F(1,52) = 7.08$, $p = 0.01$ and Drug 2, $F(1,52) = 10.94$, $p < .01$, as well as a significant Drug 1 x Drug 2 interaction, $F(1,52) = 7.81$, $p < .01$. Again, post-hoc comparisons indicated that both the male and female groups NaCl-LiCl exhibited significantly higher levels of paw treading relative to all other groups ($ps < .02$) except the female LPS-LiCl group. As well, there was no sex difference in any comparisons (see Figure 6).

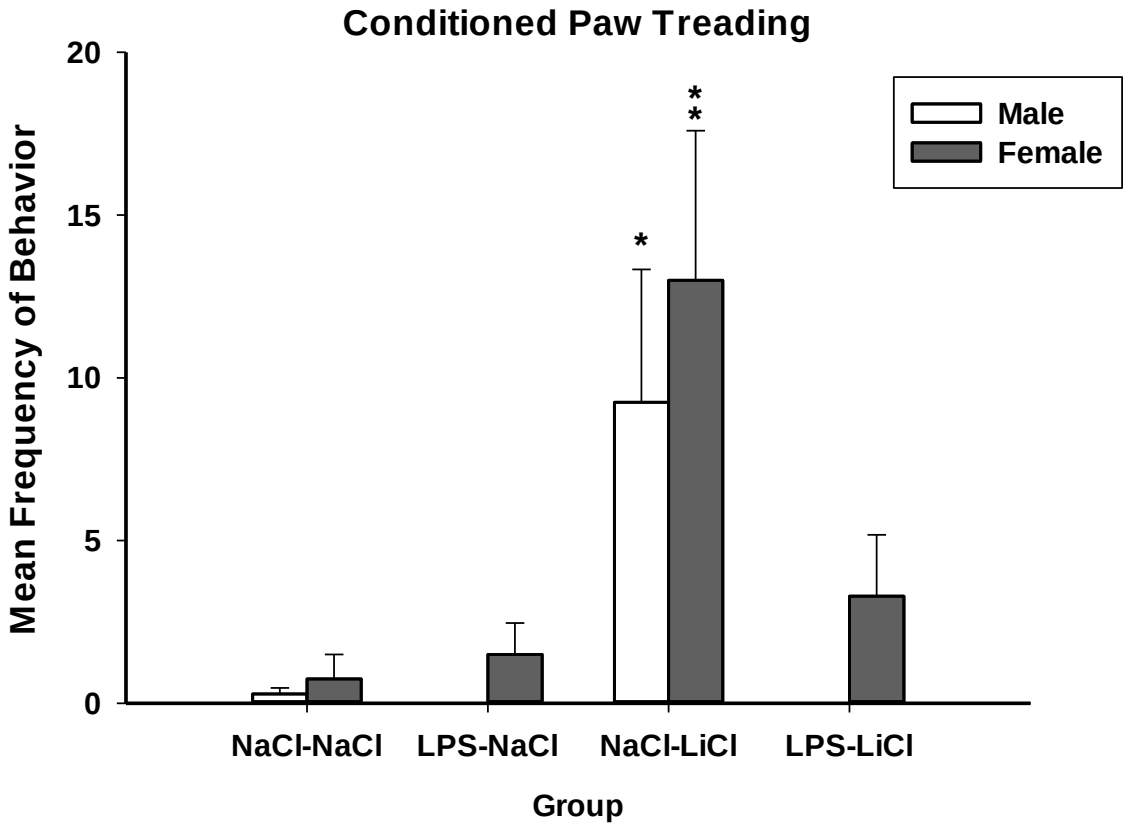


Figure 6. Mean (+ S.E.M.) frequency of conditioned paw treading behavior expressed by Groups NaCl-NaCl, NaCl-LiCl, LPS-NaCl, and LPS-LiCl (males and females; n = 7-8/group) during the 10-minute test in the distinctive context in the absence of drug treatment. * and ** indicate that NaCl-LiCl males and females displayed significantly higher frequencies of conditioned paw treading relative to all other groups (*p*'s< 0.05 and 0.01, respectively), except Female Group LPS-LiCl.

2.2.2.5 Conditioned head-shakes

No significant main effects or interactions were obtained in the ANOVA for the head-shake data (data not shown). Post-hoc comparisons also failed to reveal any significant group differences.

2.2.3 Vaginal Lavage

Following coding of the lavage samples under the microscope (magnification 10x), it was noted that, in each group, there was a relatively even distribution of cycle phases across group members Furthermore, since conditioning occurred every 72 h, each animal experienced one conditioning day on each day of the estrous cycle. Therefore, the samples in each group were pseudo-randomized, and not selected for, based on cycle phase. In addition, no disruptions to cycle phase were observed in any females following any drug treatment.

2.3 Discussion

Results of the present study showed that female rats (Group NaCl-LiCl) conditioned stronger disgust reactions (i.e., higher frequencies of "gaping" and forelimb flailing) to a distinct context, relative to male rats (Group NaCl-LiCl). This finding is consistent with previously observed sex differences in the human population (Fetting et al., 1983; Hilarius et al., 2012; LeBaron et al., 1988). Pre-treatment with LPS during the Conditioning Phase led to significant reductions in 24 h body weight following LPS treatment, in both male and female groups (Groups LPS-LiCl and LPS-NaCl). However, LPS-treated males lost significantly more body weight on the first two Conditioning Days relative to LPS-treated females. This finding is consistent with previous studies showing that, during the light phase, locomotor decrements (i.e., acute-phase response sickness behavior) are less pronounced in female rats, relative to males (Engeland et al., 2003 b; Franklin et al., 2006). Despite a sex difference in the acute-phase response following systemic LPS treatment, there was no apparent behavioral sex difference in the ability of LPS to disrupt the establishment of conditioned disgust reactions in this model. Both

LPS-treated males and females failed to establish significant frequencies of conditioned disgust behavior (Group LPS-LiCl), relative to saline pre-treated controls (Groups NaCl-LiCl and NaCl-NaCl). These results provide strong evidence of a sex difference in the establishment of conditioned disgust behavior in the rat, as well as, differences in response to immune system stimulation with LPS. The results of the current study are discussed further in the following sections.

2.3.1 Body weight change

Consistent with prior reports (Chan et al., 2009; Engeland et al., 2003a,b) treatment with LPS produced significant decreases in body weight, relative to saline pre-treated groups, 24 hours following Conditioning Days 1 and 2. Although some significant differences between groups were observed following Conditioning Days 3 and 4, the data show that LPS pre-exposed animals did, in fact, gain weight, though not at the same rate as saline pre-treated animals. This index of systemic LPS tolerance development (significantly decreased physiological effects of LPS with repeated treatment) is consistent with tolerance effects to LPS observed in previous studies (e.g., Cross-Mellor et al., 1999; Dantzer, 2004; Engeland et al., 2001; Engeland et al., 2003 a,b).

Male rats pre-treated with LPS (Groups LPS-NaCl and LPS-LiCl) lost significantly more body weight 24 h following treatment on Conditioning Days 1 and 2, relative to LPS-treated females. These findings add further evidence of a sex difference in the behavioral response to LPS treatment. Female rats develop tolerance to LPS treatment, but this tolerance does not develop as strongly if the two initial treatments are each administered during the proestrus phase of the estrous cycle (Engeland et al., 2006). A previous study has also shown that, during the light phase of the light-dark (LD) cycle, males showed greater locomotor decrements relative to females (Franklin et al., 2003). In the current study, conditioning episodes were carried out during the light cycle every 3 days which ensured a different estrous cycle day for females on each of the 4 conditioning days. Furthermore, estrous cycle phases for each rat in each group were pseudo-randomized (i.e., a relatively even number of rats began conditioning in each phase of the estrous cycle) across the estrous cycles.

2.3.2 Context-conditioned disgust behaviors

Pre-treatment with saline (NaCl) followed by LiCl treatment (male and female Groups NaCl-LiCl) during the conditioning phase resulted in the robust conditioned "gaping" behavior, relative to all other groups, when re-exposed to the conditioning context on a drug-free test day. This finding is consistent with prior reports demonstrating that rats can associate "feelings" of nausea with a specific context and display anticipatory nausea (conditioned "gaping" behavior) when placed in the context in a drug-free state (Chan et al., 2009; Cloutier et al., 2012a,b; Limebeer et al., 2006; Ossenkopp et al., 2011). Furthermore, the present study expands on these previous findings by showing a significant sex difference in the degree of conditioned disgust responses, evidenced by higher frequencies of conditioned "gaping" behavior and forelimb flailing in female rats, relative to males. Rats lack an emetic reflex (e.g., Horn et al., 2011), but display a conditioned disgust reaction when exposed to tastes or contexts previously associated with nausea (Limebeer et al., 2006). Forelimb flailing is an active aversive behavior most often displayed during conditioned taste aversion testing with the Taste Reactivity Test (TRT; see Grill & Norgren, 1978a; Parker, 2003). Although this behavior is most commonly observed during the TRT with aversive tastes, it also has been observed (along with paw treading, chin rubbing and head shaking) during the 10-minute drug-free test for context-based conditioned disgust (i.e., anticipatory nausea) (Limebeer et al., 2006). In the present study only gaping, forelimb flailing, chin rubbing and paw treading behaviors were conditioned to the distinct context with LiCl treatment, and only gaping and forelimb flailing exhibited significant sex differences.

2.3.3 Effects of immune system stimulation with LPS

Pre-treatment with LPS in both male and female LiCl-treated groups led to significant inhibition of all conditioned disgust behaviors. The current study used a relatively large dose of LPS (200 µg/kg) which may have resulted in a ceiling effect in its ability to inhibit learning and memory. LPS-treated females displayed significantly less body weight loss on conditioning Days 1 and 2, relative to males, but there was no sex difference in the degree of inhibition of the conditioned disgust responses. Future studies

should investigate the effects of lower doses of LPS on the establishment of conditioned disgust, in order to explore possible sex differences in the degree of inhibition of learning and memory.

2.3.4 Putative mechanisms

The acquisition of anticipatory nausea relies on the association of feelings of nausea with a specific context, thus, it is likely that this task is largely hippocampus-dependent (Kranjac et al., 2012; Limebeer et al., 2006). It is the consequential innate immune response, not the LPS itself, that leads to behavioral changes. While LPS does not readily cross the blood-brain-barrier, toll-like receptors (e.g., TLR-4) are found to be expressed in brain endothelium cells, thus increasing BBB permeability, and triggers a series of events ultimately leading to a central inflammatory response (see Singh & Jiang, 2004). Peripheral and central routes of administration could bring about different outcomes, but one is not wholly distinct from the other, and thus, both should be considered. In addition, the link between endotoxin administration and LiCl treatment on vagal afferent activity should be addressed. Firstly, although LiCl stimulates various vagal afferents, the vagus nerve is not necessary for the establishment of conditioned disgust responses (Niijima & Yamamoto, 1994). In fact, sympathetic nerve activity is more important for relaying LiCl sensory information relative to vagal afferents (Niijima & Yamamoto, 1994), and vagotomy does not disrupt the establishment of CTA (Martin et al., 1978). The evidence for the role of the vagus nerve in peripheral and central cytokine relationships is in debate. Conflicting evidence suggests that while vagotomy blocks sleep enhancement induced by systemic TNF-α and LPS (Zielinski et al., 2012), other studies suggest that vagotomy fails to block LPS-induced fever (Hansen et al., 2000), nor is the vagus nerve the major pathway by which abdominal IL-1β and LPS affect behavioral, hypothalamic-pituitary-adrenal (HPA) axis, and brain catecholamine responses (Wieczorek et al., 2005).

Here, we discuss the putative mechanisms of LPS-induced memory disruptions in the form of inhibited consolidation processes in the hippocampus. Indeed, there is evidence suggesting that LPS treatment inhibits aversion conditioning by disrupting

memory consolidation processes. Examination of LPS-induced chronic neuroinflammation on the induction of NMDA-dependent, and NMDA-independent, long-term potentiation (LTP) shows that intracerebroventricular administration of LPS produces significant spatial memory impairment in the Morris water maze (Min et al., 2009). LPS treatment also impaired the ability to form representations of distinct contexts in a contextual fear conditioning paradigm, as demonstrated by a reduction in freezing responses upon re-exposure to a context previously paired with an aversive foot shock in LPS-treated rats (Pugh et al., 1998). In both studies, it was suggested that LPS could affect the functioning of the hippocampus. Recordings of postsynaptic potentials showed that the induction of NMDA-dependent and NMDA-independent LTP were impaired in the Schaffer collateral-CA1 synapse of the hippocampus (Min et al., 2009).

Contextual fear conditioning has been shown to be, at least in part, a hippocampus-dependent learning paradigm, as demonstrated by the elimination of contextual fear conditioned responses after hippocampal lesions one day following conditioning (Kim & Fanselow, 1992). Tanaka et al. (2006) reported that LPS administration to the CA1 region of the hippocampus activated microglial cells and resulted in an increased production of IL-1β and TNF-α in this region. After 5 days of injections, it was found that long-term activation of microglia, induced by LPS, resulted in a decrease of glutamatergic transmission and learning and memory impairments without neuronal cell death (Tanaka et al., 2006).

Kranjac et al. (2012) showed that a single LPS injection, following contextual fear conditioning training, not only impaired memory consolidation, but could also disrupt memory reconsolidation processes in mice. This was evidenced by a decrease in freezing responses in LPS-treated mice, along with heightened peripheral and central cytokine and chemokine levels, and significantly decreased brain-derived neurotrophic factor (BDNF) mRNA expression in the hippocampus and cortex (Kranjac et al., 2012).

It has been shown that peripheral inflammation by LPS causes a reduction of trophic supply in the brain (Schnydrig et al., 2007). Neurotrophins, such as BDNF and nerve growth factor (NGF), are known to play an important role in synaptic plasticity and

long-term potentiation (Schnydrig et al., 2007). An experiment by Hennigan, Trotter, & Kelly (2007) demonstrated that synaptic plasticity in the dentate gyrus of the hippocampal complex is related to neurotrophin signaling changes, and that the disruption of these changes in plasticity by LPS may be partially due to a strong effect on these signaling cascades. Guan and Fang (2006) found that LPS treatment decreased BDNF expression in not only the hippocampus, but also the frontal, parietal, temporal, and occipital cortices. LPS also exerts a depressive effect on the expression of other neurotrophins, such as, NGF and neurotrophic factor 3 (NT-3), where expression was significantly reduced in cortical regions, as well as, the hippocampus (Guan & Fang, 2006).

The results of the studies discussed above strongly suggest that treatment with LPS, or specific cytokines, such as IL-1β, disrupts memory consolidation processes that are vital for associative learning in paradigms, such as those for anticipatory nausea and taste avoidance. Furthermore, these neurotrophin data also suggest that, although tolerance develops to the systemic effects of LPS treatment (i.e., reduction in behavioral sickness behaviors), it seems to have longer lasting central effects. Indeed, it has previously been shown that systemically LPS-tolerant animals failed to acquire LiCl-induced context-conditioned disgust (anticipatory nausea) when treated with LPS during conditioning (Chan et al., 2013).

There is evidence for a sex difference in hippocampus-dependent cognition and neurogenesis (Duarte-Guterman et al., 2015), where sex steroids, such as, estradiol and progesterone, have been implicated in learning and memory processes. Ovariectomy has been shown to impair cognitive function across different tasks; and, depending on the experimental parameters (e.g., age, strain, task), estradiol replacement has been shown to reverse overiectomy-induced deficits (for review, see Gibbs, 2010; Hogervorst et al., 2000). Estrogens have been shown to influence hippocampus-dependent memory in tasks that typically demonstrate a sex difference in performance, such as, spatial and context learning paradigms (Duarte-Guterman et al., 2015). Increases in the proliferation of new neurons in the hippocampus, and facilitation of hippocampal-dependent learning and memory, have been observed following acute estradiol treatment (Barha et al., 2010;

Mahmoud et al., 2016). Some evidence suggests that the effects of estrogen on neurogenesis are decreased by a single dose of progesterone administered 24 hours following estradiol treatment (Tanapat et al., 2005). While acute administration of estradiol into ovariectomized rats has been shown to improve context-dependent memory, chronic treatment with estradiol has been shown to have no effect on the acquisition of such tasks, such as, contextual fear conditioning (Barker & Galea, 2010). Importantly, when evaluating the influence of sex steroids on learning and memory, many factors need to be considered, such as, age, species, dosing schedule, type of task, and type of estrogen employed (Duarte-Guterman et al., 2015). In the current study, hormone concentrations were not measured, thus, the influence of gonadal hormones on the acquisition of conditioned disgust cannot be determined. However, in the present experiment, female subjects were conditioned during different phases of the estrous cycle with no apparent influence of cycle phase on the establishment of conditioned disgust behavior (i.e., "gaping" and forelimb flailing). Future research should investigate the influence of cycle phase and differing hormone concentrations on the acquisition of toxin-induced context-based conditioned disgust reactions.

2.3.5 Conclusions

It was hypothesized that female rats would condition stronger conditioned disgust responses relative to males, in the rodent model of anticipatory nausea (AN), and that pre-treatment with the immune system stimulant, lipopolysaccharide , would differentially inhibit this conditioning in male and female rats. It was found that female rats exhibited significantly stronger conditioned disgust (gaping and forelimb flailing) to a context paired with LiC treatments than did males. Although LPS significantly inhibited LiCl-induced context conditioned disgust behaviors in both males and females, there was no evidence of a significant sex difference in the degree of this inhibition. Further investigation is required to replicate and expand on the finding of this novel sex difference in anticipatory nausea, and to elucidate whether LPS has significantly different effects on the male and female brain during learning and memory for aversive internal states. The present findings are consistent with sex differences observed within the human oncology population that conditions AN, providing further validation of this

rodent model and its preclinical use in the evaluation sex differences in response to anti-emetic/anti-nausea drug treatments.

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Chapter 3

3 A sex difference in the expression of conditioned disgust behaviors (anticipatory nausea) to a context previously paired with the toxic effects of LiCl in the rat: A toxin dose-response examination

Nausea is a feeling of importance in human and animal medicine, which is poorly understood. Feelings of nausea are subjective and described as an unpleasant internal state (Kenward et al., 2015), consisting of queasiness or feeling sick to the stomach (Koch, 1995). Severe nausea and/or vomiting occurs in 40 – 70% of patients in palliative care, which greatly reduces patient quality of life (Keeley, 2009). Nausea also commonly presents following the intake of toxic or intolerable substances (Bischoff and Renzer, 2006), as an adverse post-operative side effect (Fujii, 2009), as a core symptom of motion sickness (Golding, 2006; Matchock et al., 2008; Paillard et al., 2013), or, as a side-effect to pharmacotherapy treatment, including chemotherapy (Morrow et al., 2002; Molassiotis, 2005). The study of nausea, and the need for valid animal models, is therefore crucial, given the problems nausea presents in health care and medicine.

Individual differences in sex, age, family environment, and personality traits have also been associated with the incidence of nausea. Sex differences in incidence and severity of nausea have been reported in studies examining Motion Sickness Susceptibility, Chronic Migraine, and Post-Operative Nausea and Vomiting. It has been shown that females have higher Motion Sickness Susceptibility relative to males (Paillard et al., 2013). Furthermore, it has been observed that fluctuating estrogen levels over the menstrual cycle may influence the severity or likelihood of motion sickness-related nausea. Peaks in nausea occur during the peri-menses phase, relative to the peri-ovulatory phase, but this effect is not observed among women using oral contraceptives (Matchock et al., 2008). Nausea is also reported as a main contributing factor to Chronic Migraine Syndrome more often in females, relative to males (Özge et al., 2014); and, female sex is

one of the main predictors of Post-Operative Nausea and Vomiting (Fujii, 2009). Taken together, these findings suggest that there are sex differences in the experience of nausea. Thus, sex is an important factor to consider when modeling nausea in animals in order to examine the efficacy of anti-nausea drug therapy, nausea-related side-effects of therapeutic drug treatment, and, the mechanisms underlying the conditioning of nausea responses. One particular example is that of anticipatory nausea and vomiting in oncology patients receiving cytotoxic chemotherapy treatment, who report nausea to be the most aversive side effect of the treatment (Morrow et al., 2002).

Chemotherapy treatment plays a major role in cancer survival rates, however its cytotoxicity produces undesirable side-effects, including severe acute and delayed nausea. Such nausea can lead to the establishment of Anticipatory Nausea and/or Vomiting (AN/V). AN/V presents as a classically conditioned phenomenon (Stockhorst et al., 2006; Bovbjerg, 2006), where the pairing of a conditioned stimulus (e.g., a novel hospital context) with the unconditioned stimulus (nausea produced by chemotherapy) leads to the establishment of a robust conditioned nausea/vomiting response upon re-entry into the hospital context on subsequent visits, prior to actual drug treatment (Morrow et al., 2002; Molassiotis, 2005). Due to the severity of this conditioned response, patient non-compliance with treatment increases and many choose to forego further treatment that could be life-saving (Morrow et al., 2002). Reports estimate that 30% of patients suffer from conditioned anticipatory nausea (Rodriguez, 2013).

Within the subset of individuals who condition AN/V, case reports and cohort studies suggest a higher incidence in females relative to males, including both adolescents and adults (Williams et al., 1980; Fetting et al., 1983; LeBaron et al., 1988; Hilarius et al., 2012). It is possible that anxiety related to such factors as alopecia and amenorrhea (in adolescent females), or the greater willingness in females to express feelings, may contribute to this apparent sex difference (LeBaron et al., 1988). However, other evidence suggests that sex differential experiences with nausea may also have an innate component.

The subjectivity of the feeling of nausea presents a challenge when developing animal models for examination of nausea-related responses. Rodent models of conditioned nausea and disgust have been successfully developed by using "conditioned gaping" learning and memory paradigms (Limebeer et al., 2006, 2008). "Conditioned gaping" behavior has been shown to be a useful index of nausea in the rat, evidenced by the prevention of LiCl-induced conditioned gaping when rats are administered anti-emetic treatments, such as, ondansetron or the 5-HT_{1A} agonist 8-OH-DPAT (Limebeer et al., 2006; Limebeer et al., 2008; Parker and Limebeer, 2006). As well, the orofacial component of the "retch" reflex that precedes vomiting in shrews is topographically similar to the rat "gape" response (Parker and Limebeer, 2006; Parker et al., 2008), requiring the same musculature as the rat gape (Travers and Norgren, 1986).

Re-exposure to a salient sucrose or saccharin taste that was previously paired with nausea elicits conditioned aversion-related disgust responses, such as gaping, in the taste reactivity test (TRT) for conditioned taste aversion (Grill and Norgren, 1978). Exposure to a specific context that has previously become associated with feelings of toxin-induced nausea can also elicit robust conditioned disgust (gaping) responses in the rat, providing a rodent model that can serve as a valuable preclinical tool for examining anticipatory nausea treatments in chemotherapy patients (Limebeer et al., 2006; Limebeer et al., 2008; Molassiotis, 2005). Thus, rats can learn and remember associations, not only between distinctive tastes and experienced nausea, but also between distinctive contexts that were previously paired with nausea, and they can subsequently retrieve these associations to show aversion-related disgust behaviors, such as gaping, upon re-exposure to the context in a drug-free state. Furthermore, it has been shown that a distinct context, previously paired with LiCl-induced nausea, has the potential to establish a conditioned taste aversion when this context is paired with a novel saccharin flavor (i.e., second-order conditioning), in the absence of any direct pairing with LiCl (Sticht et al., 2015a). This evidence demonstrates that the conditioned disgust responses (i.e., gaping) to the context CS are indicative of feelings of nausea, as nausea is specifically required for the formation of true conditioned taste aversions (see Parker, 2003; Chambers, 2015).

Female sex is a predictor of not only nausea-related phenomena, such as, motion sickness susceptibility, chronic migraine, and post-operative nausea (Fujii, 2009; Matchock et al., 2008; Özge et al., 2014, Paillard et al., 2013) but also appears to be a predictive factor in the development of anticipatory nausea and vomiting following chemotherapy treatment (Williams et al., 1980; Fetting et al., 1983; LeBaron et al., 1988; Hilarius et al., 2012). It is thus of interest to examine potential sex differences in the establishment of anticipatory nausea in the rodent model. Such information would allow for further improvements in anti-emetic and anti- nausea treatments, drug side-effects, and possible elucidation of neural mechanisms of nausea responses and how these are influenced by individual differences. The rodent model of anticipatory nausea has to date mainly been evaluated in male subjects, and sex differences in conditioned disgust responses to salient contexts have not yet been evaluated extensively.

The present study examined potential dose-related sex differences in conditioned disgust reactions, using four different doses of the nausea-inducing toxin, LiCl: 0 mg/kg (NaCl control), 64 mg/kg, 96 mg/kg, and 128 mg/kg. Based on reported sex differences in nausea responses in the human population it was hypothesized that adult female rats would exhibit stronger conditioned nausea responses (higher frequencies of conditioned disgust behaviors), relative to adult males.

3.1 Material and methods

3.1.1 Subjects

Subjects were 37 male and 39 female adult naive Long-Evans rats (Charles River, Quebec, Canada) weighing between 175-250 g at the start of the experiment. The rats were pair-housed in standard polypropylene cages (45 x 22 x 20 cm) in a colony room with a temperature of 21 ± 1 °C. The colony room was maintained on a 12-h light: 12-h dark cycle with the lights on from 07:00 to 19:00 h. All rats had free access to food (ProLab rat chow RMH 3000) and tap water throughout the experiment. Rats were acclimatized to the colony room for one week and were then handled on three separate days. The experimental methodology was carried out according to the Canadian Council

on Animal Care guidelines and was approved by the University of Western Ontario Institutional Animal Care Committee.

3.1.2 Apparatus

The apparatus (used on all conditioning days and the test day) consisted of a white Plexiglas box (29 cm × 25 cm × 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 those used in previous studies employing this rodent model of anticipatory nausea (e.g., Chan et al., 2009; Cloutier et al., 2011). Behavioral on the Drug-Free Test Day were videotaped with a video camera (Sony DCR-DVD201; London, Ontario) positioned approximately 1 m from the mirror.

3.1.3 Vaginal lavage collection

Animals were allowed one week to acclimatize before being handled. Following handling, the estrous cycle was tracked daily in female subjects, at the same time each day. Vaginal lavages were collected with a pipette filled with distilled warm water, and samples were placed onto microscope slides for later scoring of the estrous cycle.

3.1.4 Experimental procedure

An illustration of the drug injection and behavioral testing schedule is provided in Figure 1 A-B. All conditioning and testing was performed during the afternoon light phase (between 1200 and 1600 h) of the light-dark cycle, with females being conditioned and tested between 1400-1600 h. The two phases of the experiment consisted of a Conditioning Phase (4 days, spaced 72 h apart), and one Drug-free Test Day 72 h following the final conditioning day. There were 4 groups of male and 4 groups of female rats (n = 9 -10/group). Rats of each sex were randomly assigned to 1 of 4 groups (64 mg/kg LiCl, 96 mg/kg LiCl, 128 mg/kg LiCl, or NaCl control). Since conditioning

occurred every 72 h, each female experienced each conditioning day in a different phase of the estrous cycle.

A

Treatment

(intraperitoneal; 0.15M)

64 mg/kg LiCl

or

96 mg/kg LiCl

or

128 mg/kg LiCl

or

0.9% NaCl

Immediate exposure to distinct context for 30 minutes

B

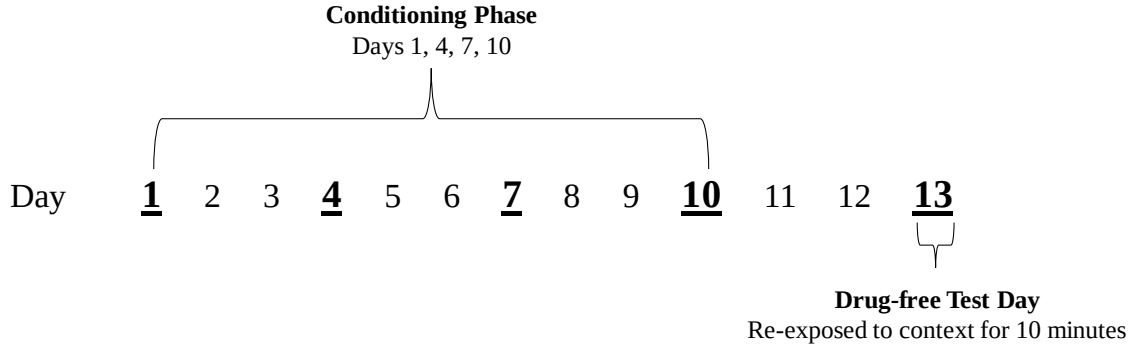

Figure 1 A. Conditioning Procedure. Subjects were treated with either 64, 96, or 128 mg/kg LiCl or NaCl (intraperitoneal) and immediately placed into a distinctive context alone for 30 minutes. **B. Experimental Timeline**. There were 4 Conditioning Days, spaced 72 h apart. 72 h following the final Conditioning Day, each animal was re-exposed to the distinct context in a drug-free state, and conditioned aversion-related disgust behaviors (gaping, paw treading, forelimb flails, head shakes, chin rubs) were recorded and analyzed.

3.1.4.1 Conditioning phase

On each day of the Conditioning Phase (4 days, 72 h apart), animals were treated with an intraperitoneal injection of either 64, 96 or 128 mg/kg lithium chloride (LiCl; 10, 15, or 20 ml/kg; 0.15M), or with 0.9% isotonic saline (NaCl; 20 ml/kg) as the control (0 mg LiCl). Each rat was immediately placed into the distinctive context for 30 minutes. Following each exposure to the distinctive context, animals were quickly returned to the home cage. There were 8 groups in total: NaCl male, 64 mg/kg LiCl male, 96 mg/kg LiCl male, 128 mg/kg LiCl male, NaCl female, 64 mg/kg LiCl female, 96 mg/kg female, and 128 mg/kg LiCl female. An illustration of the group composition is shown in Table 1. Prior studies have demonstrated conditioned gaping behavior to be a robust response to a distinctive context following a conditioning phase that employed 128 mg/kg LiCl in male rats (Chan et al., 2009; Chan et al., 2013; Cloutier et al., 2012b). More variable, but significant, conditioned gaping has also been shown following treatment with 32 and 64 mg/kg LiCl in male rats (Cloutier et al., 2011; Cloutier et al., 2012a; Ossenkopp et al., 2011). Conditioned gaping following 96 mg/kg LiCl treatment in the anticipatory nausea rodent model has not yet been evaluated in males or females. However, a LiCl dose of 96 mg/kg has been shown to produce a significant conditioned place avoidance in male rats, evidenced by significantly less time spent in a previously drug-paired chamber, relative to a safe, saline-paired chamber (Tenk et al., 2005).

LiCl Dose (0.15M)	Male *n*	Female *n*
0 mg/kg (0.9%NaCl)	10	10
64 mg/kg	9	9
96 mg/kg	9	10
128 mg/kg	9	10

Table 1. Group Designation. Numbers of subjects per treatment group.

3.1.4.2 Drug-free test day

72 h following the final Conditioning Day, each animal was re-exposed to the distinctive context for 10 minutes in a drug-free state, and behavioral responses were video recorded for later scoring.

3.1.5 Data analysis

On the 10 minute drug-free Test Day, dependent variables included conditioned gaping behavior, and other aversion-related disgust behaviors, such as, paw treading, chin rubs, forelimb flails, and head shakes, as well as, a composite score (cf. Ossenkopp and Mazmanian, 1985) of these non-gaping aversion-related behaviors (see Grill and Norgren, 1978). Gaping was defined as lowering of the mandible and the pushing or thrusting out of the lower teeth (see Parker and Limebeer, 2006). All dependent variables were scored blind.

A 2 x 4 between-subjects analysis of variance (ANOVA) was used to analyze each dependent variable. The between-subjects factors were Sex (at 2 levels: male or female), and Drug (at 4 levels: 0, 64, 96, or 128 mg/kg LiCl). Linear contrasts were performed for each dependent variable in order to analyze sex- and drug-dose response effects. As this study was exploratory, all post hoc pairwise comparisons were performed using Fisher's Least Significant Difference (LSD). All statistical tests used a significance criterion of $\alpha = 0.05$. All data presented reflects conditioned responding, while the animal was in a completely drug-free state (no injections). Statistical analyses were performed using IBM SPSS Statistics 23 for Windows.

3.2 Results

Results demonstrated that all LiCl-treated animals treated with either 128 mg/kg or 96 mg/kg LiCl developed significantly stronger conditioned gaping behavior, relative to controls. At each of these doses, females further displayed significant gaping frequencies relative to LiCl-treated males, indicative of dose- and sex-related effects.

3.2.1 Conditioned gaping behavior

The between-subjects ANOVA revealed a significant main effects of Sex $F(1, 68) = 11.862$, $p < 0.001$, and Drug $F(3, 68) = 34.997$, $p < 0.0001$, on the expression of gaping behavior on the drug-free test day. Furthermore, a significant Sex x Drug interaction was obtained, $F(3, 68) = 3.107$, $p < 0.032$, as well as, significant sex- and dose-dependent increases in gaping behavior, evidenced by significant linear contrasts (p's< 0.001). Means and their Standard Errors (S.E.M.) are depicted in Figure 2.

Post hoc pairwise comparisons revealed that females treated with 96 or 128 mg/kg LiCl displayed significantly higher frequencies of conditioned gaping behavior, relative to males treated with the same doses (p's< 0.002).

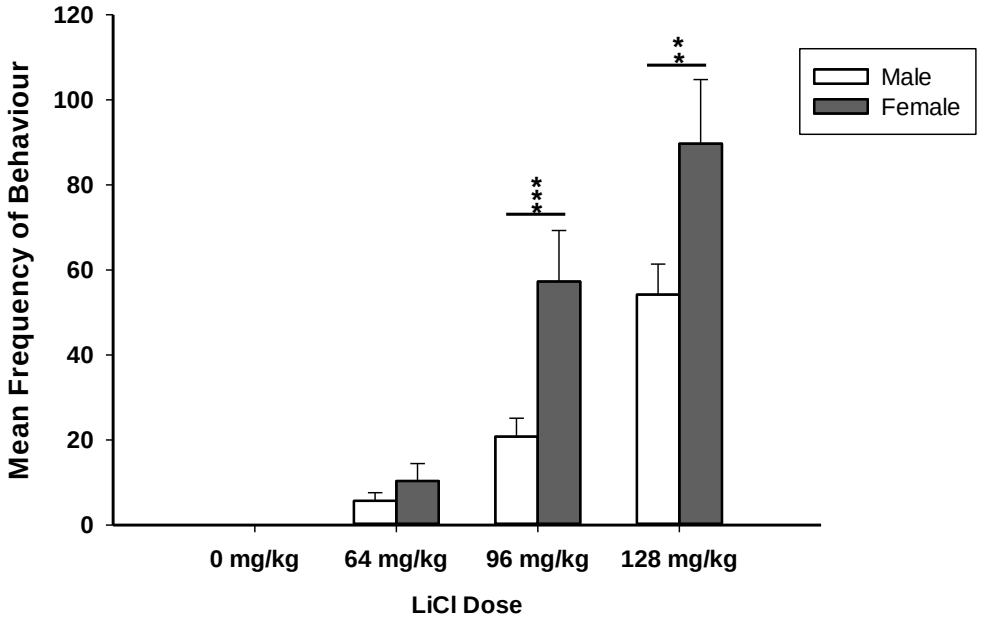

Figure 2. Conditioned Gaping Behavior. Mean (+ S.E.M.) frequency of conditioned "gaping" behavior expressed by Groups NaCl, 64 mg/kg LiCl, 96 mg/kg LiCl, and 128 mg/kg LiCl (males and females; n = 9-10/group) during the 10-minute re-exposure to the distinctive context on the Drug-Free Test Day. LiCl-treated males and females in Groups 96 mg/kg LiCl and 128 mg/kg LiCl displayed significantly higher gaping frequencies relative to controls (p's < 0.05). A dose-related sex difference was also observed with females displaying significantly higher gaping frequencies at 96 mg/kg and 128 mg/kg LiCl relative to males ($p < 0.001$ and $p < 0.01$, respectively).

3.2.2 Other conditioned aversion-related disgust behaviors (gaping excluded)

Conditioned aversion-related behaviors that did not include gaping (i.e., chin rubs, paw treads, forelimb flails, and head shakes) were analyzed separately and as a composite score. The ANOVA revealed a significant main effect of Drug Treatment for Chin Rubs, $F(3, 68) = 14.481$, $p < 0.0001$; Forelimb Flails, $F(3, 68) = 3.906$, $p < 0.012$; Paw Treads, $F(3, 68) = 10.145$, $p < 0.0001$; and, the aggregate score, $F(3, 68) = 17.878$), $p < 0.0001$. No significant main effect of, or interaction with, Sex was found. Significant dose-dependent increases in the frequencies of Chin Rubs, Forelimb Flails, Paw Treads, and the composite score of aversion-related behavior was obtained, evidenced by significant linear contrasts (p's< 0.02). Means and S.E.M are depicted in Figure 3.

No significant sex differences were revealed in the post hoc analyses (LSD) for conditioned aversion-related behaviors that did not include gaping behavior.

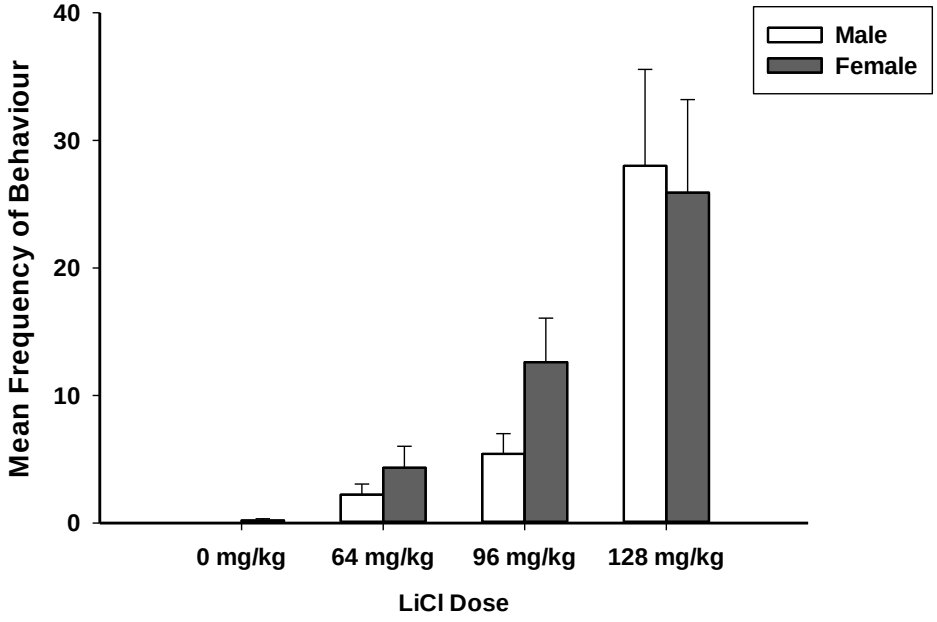

Figure 3. Composite Score of Disgust Behavior (gaping excluded). Mean (+ S.E.M.) frequency of a total aggregate of chin rubs, forelimb flails, paw treads, and chin rubs expressed by Groups NaCl, 64 mg/kg LiCl, 96 mg/kg LiCl, and 128 mg/kg LiCl (males and females; n = 9-10/group) during the 10-minute re-exposure to the distinctive context on the Drug-Free Test Day. LiCl-treated males and females displayed dose-related increases in non-gaping disgust behaviors (p's < 0.05). No significant sex differences were found.

3.2.3 Cycle tracking

Vaginal lavage samples were collected daily and examined under the microscope at 10x magnification for cycle phase classification. Results are based on normally cycling females, where cycle day was observed to be equally distributed across and within groups on the first conditioning day.

3.3 Discussion

The current study examined putative sex differences in the formation of conditioned disgust behaviors, most notably gaping behavior, in the rodent model of anticipatory nausea, using three different doses of the nausea-inducing toxin LiCl (64, 96, or 128 mg/kg), and a saline control (0 mg LiCl). It was hypothesized that female rats would show greater levels conditioned disgust responses relative to males in a toxin dose-dependent manner. Our hypothesis was largely supported. Results showed larger dose-related increases in conditioned gaping behavior in females, relative to males, on the drug-free test day. Although LiCl-treated subjects displayed significantly higher frequencies of other aversion-related behaviors (i.e., chin rubs, paw treads, and forelimb fails), relative to saline-treated subjects, no significant sex effect was observed at any drug dose for these behaviors. The current study thus replicated previous evidence of a LiCl dose relationship to the level of conditioned gaping (Ossenkopp et al., 2011) .

Nausea-producing effects can be produced by chemicals acting on the area postrema (a chemoreceptor trigger zone for emesis), by activating abdominal vagal and glossopharyngeal afferents, as well as with provocative vestibular stimulation (Borison, 1988; Sanger and Andrews, 2006; Kenwood et al., 2015). Nausea is necessary for the establishment of both conditioned taste aversions as well as context conditioned disgust response (anticipatory nausea ; see Parker, 2003; Parker et al., 2006; Parker and Limebeer, 2006). Typically, nausea-inducing toxins, such as LiCl, are employed in rodent conditioned disgust paradigms, but rats have also been shown to condition disgust responses with other nausea-inducing treatments, such as provocative vestibular

stimulation (Cordick et al., 1999; Ossenkopp et al., 2003), a non-pharmacological treatment, and even estradiol injections (Ossenkopp et al., 1996).

Although the neural mechanisms underlying conditioned nausea learning require further examination, it does appear that an intact area postrema is critical for successful taste avoidance/aversion learning with LiCl (Eckel and Ossenkopp, 1996; Ossenkopp and Eckel, 1994,1995). The chemosensitive area postrema is a circumventricular medullary structure implicated in the detection of blood-borne toxins, such as LiCl (Borison, 1989). Animals with area postrema lesions will fail to acquire conditioned taste avoidances/aversions conditioned with LiCl and other toxins (Eckel and Ossenkopp, 1996; Ossenkopp and Eckel, 1994,1995; Ossenkopp et al., 1997). The area postrema has also been associated with estradiol-induced hypophagia, which is salient to taste-illness associations. Markers for neuronal activity, such as c-fos like immunoreactivity (c-FLI), are activated in this structure following exogenous administration of estradiol benzoate, consistent with the typical time period for estradiol-induced hypophagia (Chambers and Hintiryan, 2009). Furthermore, area postrema lesions have been shown to eliminate hypophagia expressed by male rats exposed to chronic estradiol treatment (Bernstein et al., 1986). The role of area postrema in forming associations between feelings of nausea and specific contexts or environments, such as those in anticipatory nausea or conditioned place avoidance paradigms, or, how area postrema may be influenced by sex hormones in this paradigm, have not been yet examined.

The continued development of anti-emetic and anti-nausea pharmacotherapies is critical, and preclinical animal models can provide a valuable method of testing drugs that may attenuate or eliminate nausea in both males and females. The anti-emetic effects of 5-hydroxytryptamine3 (5-HT3) and neurokinin1 receptor (NK_1) antagonists are well-documented and help to alleviate nausea and vomiting to some degree, but do not attenuate nausea in general, leaving it difficult to treat (Sanger and Andrews, 2006). 5-HT3 is effective during the acute-phase following cytotoxic drug treatment (Miner and Sanger, 1986; Costall et al., 1986), and NK_1 helps to attenuate vomiting in the delayed-phase following chemotherapy treatment, but does not significantly reduce feelings of nausea (see Andrews and Rudd, 2004; Sanger and Andrews, 2006). Growing evidence

suggests that treatments targeting the endocannabinoid system may help to regulate nausea. However, the efficacy of these substances, such as, delta-9-tetrahydrocannabinol (THC), canabidiolic acid (CBDA), and fatty acid amide hydrolase (FAAH) inhibitors that increase anandamide (an endogenous cannabinoid), has only been evaluated in male subjects (see Cross-Mellor et al., 2007; Rock et al., 2015; Sticht et al., 2015b), and future research should test these effects in females, given that they show greater conditioned nausea responses, relative to males.

Women report higher incidences of nausea relative to men in anticipatory nausea, but apparent sex differences also exist across a wide variety of other nausea-related health issues. Gender differences in Motion Sickness Susceptibility (MSS) were assessed by self-report questionnaires that measured both MSS (MSSQ) and trait anxiety (STAI-B) (Paillard et al., 2013). In healthy participants who did not have a history of vestibular abnormalities, women were found to have greater motion sickness susceptibility relative to men. Trait anxiety in healthy women yielded a small positive correlation with MSS, however this was not sufficient to explain the sex difference. Motion sickness also has been shown to fluctuate over the menstrual cycle (Golding et al., 2005). However, sex steroids do not fully explain sex differences in MSS, and it has been suggested that perhaps females have "hard-wired" differences that leave them more susceptible to nausea, relative to their male counterparts (Golding et al., 2005). Female sex is also the most relevant predictor of post-operative nausea/vomiting (PONV) in young adulthood (Fujii, 2009) and this sex difference in PONV has been shown to persist into late adulthood (mean age 73), with elderly women reporting higher incidence and severity of PONV relative to men (Conti et al., 2014).

Sex differences in classically conditioned behaviors have been shown to be dependent, in part, on plasma concentrations of sex hormones, such as, estrogen. For example, female rodents learn faster in the classical eyeblink conditioning paradigm, relative to males, when training begins during proestrus, but this sex difference is abolished by ovariectomy (for review see Dalla and Shors, 2009). However, since this sex difference is also apparent when training begins in diestrus, a cycle phase associated with low estrogen, it suggests a role for progesterone and luteinizing hormone as well

(Dalla et al., 2009). Other studies have reported situations in which males consistently outperform females, as in contextual fear conditioning (Dalla and Shors, 2009; Pryce et al., 1999). Ovariectomy, but not castration, abolished this sex difference, with ovariectomized females showing similar levels of freezing responses relative to males (e.g., Leuner et al., 2004).

Future studies should examine a putative role of female sex steroids, such as, estrogen and progesterone, in the acquisition of conditioned disgust behavior. By considering the phases of the estrous cycle, or performing ovariectomy with or without replacement of hormones, the strength and severity of conditioned disgust can be directly examined in female rodents. The continued development of anti-emetic and anti-nausea pharmacotherapies is of importance and preclinical animal models provide a valuable method of testing drugs with the potential to alleviate nausea in both males and females.

3.3.1 Conclusions

It was hypothesized that female rats would condition higher levels of disgust responses relative to males, in the rodent model of anticipatory nausea, in a toxin dose related manner. It was found that female rats exhibited significantly stronger conditioned disgust responding (i.e., gaping) to a context paired with the effects of LiCl treatment at the two largest doses, 96 mg/kg and 128 mg/kg. This sex difference was particular to conditioned “gaping” behavior, as no significant sex difference was found for any other aversion-related disgust behavior (i.e., forelimb flailing, chin rubbing, paw treading, and head shaking). The present findings provide further strong support for the rodent model of anticipatory nausea and its ability to model, in rodents, the same sex difference observed in the human oncology population.

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Chapter 4

4 Examining the effects of lipopolysaccharide (LPS) on the acquisition of conditioned place avoidance in the female rat and the effect of peripubertal endotoxin treatment on homotypic challenge in adulthood.

4.1 Aversive conditioning with LiCl

The toxin lithium chloride (LiCl) induces vomiting in species with an emetic reflex (Borison, 1989), and produces behavioral symptoms consistent with visceral illness in non-emetic species, such as rats and mice (Ossenkopp & Eckel, 1995; Parker, 2003). LiCl treatment leads to hypophagia (e.g., Curtis et al., 1994), including decreases in sodium consumption following sodium depletion (Chavez et al., 1995), and conditioned taste + illness associations, such as conditioned taste aversion or conditioned taste avoidance (Eckel & Ossenkopp, 1996; Ossenkopp et al., 2003; Parker, 1982; Zalaquett and Parker, 1989; Nachman and Ashe, 1973; Nachman, 1970; Parker, 2003). The nauseogenic effects of LiCl treatment have also been shown to produce conditioned disgust responses in rats re-exposed to distinct contexts that were previously paired with the toxin, such as those in the conditioned place aversion and conditioned place avoidance paradigms.

Chemotherapy treatment plays a major role in cancer survival rates; however, its cytotoxicity produces undesirable side-effects, including severe acute and delayed nausea. Such nausea can lead to the establishment of Anticipatory Nausea (AN). AN presents as a classically conditioned phenomenon (Stockhorst et al., 2006; Bovbjerg, 2006), where the pairing of a novel hospital context with nausea produced by chemotherapy leads to the establishment of a robust conditioned nausea/vomiting response upon re-entry into the hospital context on subsequent visits, prior to actual drug treatment (Morrow et al., 1997; Mollasiotis, 2005). This form of conditioned context aversion can be modelled in the rat by pairing LiCl injection with exposure to a distinct

contextual chamber. Rats will display Conditioned Disgust Responses upon re-exposure to the same context in a drug-free state. Rats lack an emetic reflex, but display a "conditioned gaping" behavior that is considered to be an index of nausea/disgust in the rat (see Limebeer et al., 2006; Parker et al., 2008).

Related to the rodent model of AN is the Conditioned Place Avoidance (CPA) paradigm. The two-chamber CPA paradigm involves the pairing of a drug treatment (e.g., LiCl) with one of two distinct chambers, and pairing saline control injections with the other, contextually distinct, chamber (see Frisch et al., 1995, Miller et al., 2000, Parker, 1992, Turenne et al., 1996 and White and Carr, 1985). Drug-paired and saline-paired exposures alternate over two consecutive conditioning cycles. On a drug-free test day (i.e., extinction phase), rats will spend significantly less time in a chamber previously paired with LiCl effects, relative to time spent in the saline-paired (i.e., "safe") chamber. Indeed, this effect has been demonstrated across a variety of LiCl doses ranging from 20 mg/kg to 128 mg/kg (Khroyan et al., 1995; Miller et al., 2000; Parker & McDonald, 2000; Tenk et al., 2005; Turenne et al., 1996; White & Carr, 1985), but not at lower doses of LiCl, such as, 10 mg/kg (Miller et al., 1999, 2000). LiCl has been shown to produce dose-dependent decreases in locomotor behavior during the conditioning phase (see Tenk et al., 2005). During the drug-free extinction phase, subjects that were treated with LiCl during conditioning spent significantly less time in the previously drug-paired chamber relative to controls. However, this drug effect was not dose-dependent (Tenk et al., 2005). Higher doses of LiCl (128 mg/kg and 96 mg/kg) produced significant dose-dependent increases in the frequency of aversion-related vertical movements, such as rearing behavior, in the formerly drug-paired chamber, relative to a lower dose of 32 mg/kg LiCl (Tenk et al., 2005). However, thus far, LiCl effects on place avoidance conditioning have only been evaluated in males.

4.2 Immune system stimulation and learning

The endotoxin lipopolysaccharide (LPS) is the active component of the cell wall of Gram-negative bacteria, and when injected into an animal, results in the production of pro-inflammatory cytokines (Dinarello, 1984) that initiate a specific set of

pathophysiological and behavioral changes collectively known as “sickness behaviors”. These “sickness behaviors” include induction of fever (Hart, 1988; O’Reilly, Vander, & Kluger, 1988; Roth et al., 1997), anorexia and adipsia (Cross-Mellor et al., 2000; Gayle et al., 1998; Langhans, 2000; Langhans et al., 1990), reductions in locomotor activity (Hart, 1988; Engeland et al., 2003a; Franklin et al., 2003; Yirmiya et al., 1994), hypersomnia (Hart, 1988), and reduction in grooming (Hart, 1988), helping the organism counter the effects of the bacterial infection (Hart, 1988).

Significant learning and memory impairments have been observed following LPS treatment in Contextual Fear Conditioning and Two-Way Active Avoidance paradigms (e.g., Pugh et al., 1998; Kranjac et al., 2012; Sparkman et al., 2005), as well as, in animal models of conditioned disgust such as Conditioned Taste Aversion (Cross-Mellor et al., 2009) and the rodent model of Anticipatory Nausea (AN; Chan et al., 2009; Chan et al., 2013; Cloutier et al., 2012 a,b; Cloutier et al., 2016 b). Male rats pre-treated with LPS 90 minutes prior to LiCl + taste pairings failed to acquire significant conditioned active and passive aversion responses (e.g., gaping, forelimb flailing, chin rubbing, head shaking, paw treading, and passive drip) when involuntarily infused with the taste in the absence of drug treatment, relative to saline controls, in the Taste Reactivity Test (TRT) for conditioned taste aversion (CTA) (see Grill & Norgren, 1978 a,b; Cross-Mellor et al., 2009). LPS, however, failed to block taste avoidance learning, actually inducing significant reductions in sucrose consumption in a 2-bottle voluntary intake test for conditioned taste avoidance (Cross-Mellor et al., 2004).

It is important to highlight that in order to produce true conditioned taste aversion, a nauseogenic unconditioned stimulus is required. Evidence supports the fact that conditioned taste aversion and conditioned taste avoidance may represent two distinct processes (see Chambers, 2015; see Parker, 2003) and this may provide a basis for the differential effects of LPS on the acquisition of the two the conditioned behaviors. Similar to what has been demonstrated with the development of CTA, LPS pre-treatment 90 minutes prior to LiCl + context pairings blocks the acquisition and expression of conditioned context aversion (i.e., disgust behavior). LPS treatment in male rats has repeatedly been shown to significantly attenuate conditioned “gaping” behavior, as well

as, chin rubbing and paw treading, relative to saline controls (Chan et al., 2009; Cloutier et al., 2012 a,b; Cloutier et al., 2016 b), even when rats were LPS-tolerant prior to LiCl-conditioning (Chan et al., 2013). The deleterious effect of LPS on context aversion learning has also been demonstrated in female rats (see Cloutier et al., 2016a), which conditioned stronger dose-dependent disgust responses, relative to males (Cloutier et al., 2016 a,b).

Thus, LPS has been shown to block the acquisition of conditioned taste and context aversions, but not conditioned taste avoidance learning (Cross-Mellor et al., 2004). However, LPS has been shown to block the simultaneous establishment of conditioned context aversion and taste avoidance following the delivery of an intravascular saccharin taste mixed with LiCl treatment, immediately prior to exposure to a distinct context (Cloutier et al., 2012 a). Although, it should be noted that intravascular administration of a tastant may conditioned weaker responses, relative to oral presentation of the Conditioning Stimulus, as it relies on transport through the blood (Fishberg et al., 1933). To date, the putative deleterious effects of LPS pre-treatment during LiCl-induced Conditioned Place Avoidance conditioning has not yet been evaluated in males or females.

4.3 Lipopolysaccharide and conditioned place avoidance learning

The current study examined LiCl-induced conditioned place avoidance (CPA) learning in adult Long-Evans female rats, using a dose of 96 mg/kg, which was previously shown to establish robust conditioned avoidant responding in male rats of the same strain, in the same 2-chamber CPA apparatus (Tenk et al., 2005). This study further examined the effects of LPS pre-treatment during conditioning on the expression of CPA behavior on a drug-free test day. It was hypothesized that 96 mg/kg LiCl-treated female rats would establish robust CPA learning; and, that pre-treatment with LPS would significantly attenuate learning in this paradigm, similar to what was observed in the rodent model of anticipatory nausea (e.g., Chan et al., 2009; Cloutier et al., 2011; Cloutier et al., 2016 a,b).

Puberty represents a period during which sexual maturity is achieved (Schulz and Sisk, 2006), and is accompanied by numerous physiological changes and significant brain reorganization (Shulz et al., 2009). Systemic LPS treatments administered during sensitive periods of development (i.e., neonatal and peripubertal periods) have been shown to produce long-term alterations in behavior. For example, LPS-treated neonatal male rat pups displayed exacerbated acute-phase responses to a second LPS challenge in adulthood, relative to saline controls, while females in the same treatment group did not (Tenk et al., 2008). Female mice injected with LPS during the peripubertal phase (6 weeks old), displayed long-term alterations in behavior, such that estrogen became anxiogenic, as opposed to producing its typically anxiolytic effects (Blaustein et al., 2011). Furthermore, it was shown that, in female mice, an LPS challenge at 6 weeks of age prevented estradiol-enhanced cognition in adulthood in social and object recognition paradigms (Blaustein et al., 2013).

In the current study, the influence of peripubertal (6 weeks old) LPS challenge in female rats on the development of CPA behavior, and on the response to a homotypic drug challenge in adulthood, were examined. It was hypothesized that an LPS challenge during puberty might alter CPA learning in adulthood, relative to females treated with saline at 6 weeks. It was hypothesized that LPS challenges in both adolescence and adulthood would alter CPA learning, as well as, the response to the endotoxin treatments.

4.4 Materials and Methods

4.4.1 Subjects

Subjects were 62 naïve female Long-Evans rats (Charles River, Canada) weighing between 150 and 200 g at the start of the experiment. Rats arrived in the laboratory two weeks prior to the commencement of the experimental manipulations. Animals were given one week to acclimatize and at least 3 separate handling sessions prior to the beginning of the experiment. The rats were housed in pairs in standard polypropylene cages (45 x 22 x 20 cm) in a temperature-controlled colony room (20 ± 1 °C) maintained

on a 12: 12 h light: dark cycle (lights on at 07:00) with ad libitum access to both food (ProLab rat chow) and tap water. All testing took place during the light phase of the light: dark cycle. All procedures were approved by the University of Western Ontario Animal Care Committee and were in accordance with the Canadian Council of Animal Care (CCAC) guidelines.

4.4.2 Drugs

Lithium Chloride (LiCl) was dissolved in distilled water to a molarity of 0.15M and given at a dose of 96 mg/kg. Isotonic saline (NaCl; 0.9%, 0.15M) was employed as the control injection. Lipopolysaccharide (LPS; derived from E. coli serotype 0111: B4, no. L-2630; Sigma) was dissolved in pyrogen-free 0.9% isotonic saline, and was administered at a dosage of 200 μg/kg (1 mL/kg). Doses were based on those employed in previous studies showing a physiological effect of the drug (e.g., Engeland, Kavaliers, & Ossenkopp, 2003). All injections were administered intraperitoneally.

4.4.3 Apparatus

The two-chamber place-conditioning apparatus consisted of eight modified Versamax Animal Activity Monitors (Accuscan Model RXYZCM-16, Columbus, OH). Each monitor consisted of a clear Plexiglas open-field (40 x 40 x 30.5 cm) covered by a Plexiglas lid with air holes. Infrared photobeams were located 2.54 cm apart and 5.7 cm above the floor along the perimeter of the box (16 beams per side). Two additional banks of 16 photobeams were located on opposite sides of the box, 2.54 cm apart and 16.4 cm above the floor. Beam breaks in lower and upper banks correspond to movements in the horizontal and vertical planes, respectively (Ossenkopp & Kavaliers, 1996). All activity monitors were connected to a Versamax data analyzer (Accuscan Model DCM-8, Columbus, OH), which then transmitted data to the computer for further analysis. Locomotor activity and its distribution within the two chambers were quantified using the Versamax Software System (Version 2.60, Accuscan, Columbus, OH).

Each Animal Activity Monitor was divided into two equal sized chambers (20 x 40 x 30.5 cm) by a clear Plexiglas partition located parallel to the elevated photobeams so

as not to interrupt theses sensors. The two chambers differed in visual wall cues and tactile floor cues. One chamber contained a removable wire mesh floor while the other chamber contained a removable clear, rough Perspex floor. The outside wall of each chamber displayed either a solid gray or black (1.8 cm) and white (2 cm) striped pattern. During conditioning trials, a solid Plexiglas partition confined the animal to one chamber and the walls of both chambers displayed the same visual pattern.

During the extinction trial, a Plexiglas partition with a doorway (10 × 15 cm) allowed passage between the chambers with each chamber displaying one of the wall patterns. During both conditioning and extinction, the gray wall pattern was paired with the rough-textured floor and the black and white striped wall pattern was paired with the wire mesh floor. Average illumination in the rough floor-gray wall chamber was 1065.5 ± 209.6 lx (measured with Digital Illuminometer, Mitchell Instruments Model YF-1065F, San Marcos, CA) and average illumination in the smooth floor-striped wall chamber was 1084.0 ± 214.2 lx.

The place preference and activity variables were quantified directly by the Versamax analyzer for each of the two chambers. The place preference measure was Time (s) spent in each chamber during extinction trials. Horizontal activity measures included Total Horizontal Distance (cm), Horizontal Movement Time (s), and Number of Horizontal Movements. Vertical activity measures included Vertical Time (s) and Number of Vertical Movements.

4.4.4 Procedure

4.4.4.1 Peripubertal Immune Challenge

At 6 weeks of age, all subjects were injected (intraperitoneal) with either 200 µg/kg LPS or 0.9% isotonic NaCl (1 mL/kg), and returned to the home cage. Body weight was measured prior to injection and 24 h following treatment. Vaginal lavage samples were collected the day of and 24 h following treatment.

4.4.4.2 Conditioning phase

Subjects received two conditioning cycles, each consisting of four, 30 min conditioning trials. Conditioning trials were 30 min in length because LiCl exerts maximal effects on behaviour 15–30 min following administration (e.g., Parker et al., 1984). The conditioning trials were separated by 24 h and alternated between drug and saline trials. Thus, each conditioning cycle was comprised of two drug and two saline trials. The two conditioning cycles were separated by 72 h. Prior to conditioning trials, animals were weighed and pre-treated with LPS or NaCl. 90 minutes later, following administration of LiCl or saline, animals were quickly placed in the apparatus. Each apparatus and all removable floors were cleansed with a mild detergent solution and rinsed with a baking soda solution after each conditioning and extinction trial.

On odd-numbered conditioning trials (Trials 1, 3, 5, 7; Days 1, 3, 7, and 9), different groups of rats received intraperitoneal injections of either 200 µg/kg LPS or 0.9% NaCl, followed 90 minutes later by an injection of 95 mg/kg LiCl or 0.9% NaCl, and were then confined to the right chamber for 30 min. Drug-paired chambers were counter-balanced so that half consisted of the striped wall-wire mesh floor pairing, and the other half consisted of the gray wall-rough floor pairing. On even-numbered conditioning trials (Trials 2, 4, 6, 8; Days 2, 4, 8, and 10), rats received control pre-treatment injections of 0.9% 0.15 M NaCl in the same volume as the LPS injection, followed 90 minutes later by a second injection of 0.9% NaCl equal to that of LiCl, and were confined to the left chamber for 30 min, with respective counter-balancing of chamber patterns and flooring. A procedural schedule is given in Figure 1. Locomotor activity was measured during each of the conditioning trials. Body weight was measured on each conditioning day and 24 h following each conditioning day. During the entire conditioning period, vaginal lavage samples were collected each day.

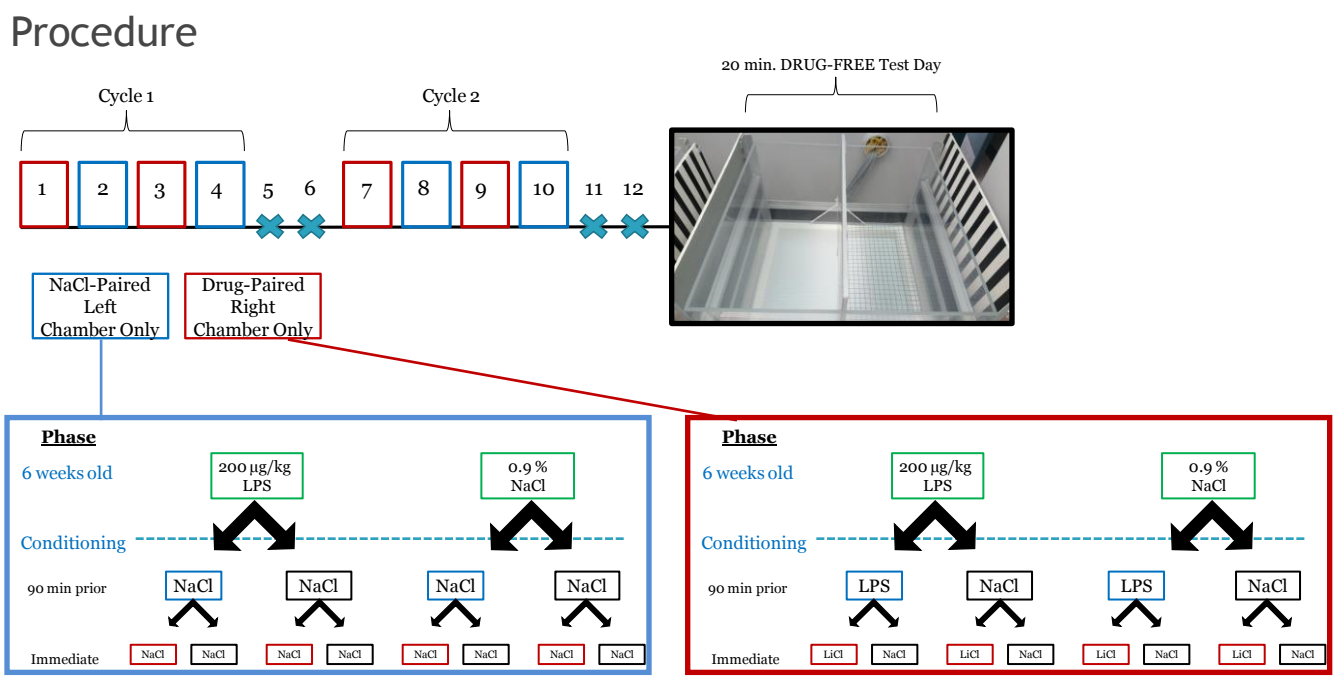

Figure 1. Injection schedule and procedure.

4.4.4.3 Drug-free test day

Seventy-two hours following the last conditioning trial, rats received one, 20 min test trial. The test trial was 20 min in length to minimize the effects of habituation on locomotor activity which tends to occur after 20 min (Engeland et al., 2003). Rats received no injections prior to the extinction trials. The animals were initially placed in the centre of the chamber previously paired with LiCl and allowed unrestricted access to both chambers. Locomotor activity and the spatial distribution of behaviour were assessed in each chamber.

4.4.5 Statistical analyses

4.4.5.1 Six-week-old (peripubertal) treatment

Following LPS treatment, significant reductions in body weight are typically observed 24 h post-treatment, relative to controls (e.g., Engeland et al., 2003). Percent of body weight change was calculated by subtracting the Conditioning Day body weight from the 24 h post-treatment weight, then dividing by the Conditioning Day body weight and multiplying by 100.

A one-way between-subjects Analysis of Variance (ANOVA) was used to analyze percent changes in body weight 24 h after the peripubertal (6 weeks), with one factor of Drug 1 (at two levels: LPS or NaCl). All statistical analyses used a significance criterion of $\alpha = 0.05$.

4.4.5.2 Conditioning Phase

Dependent variables examined during the Conditioning Phase included: percent changes in body weight (as calculated above), Total Distance Travelled (cm), Horizontal Movement Time (s), Vertical Movement Time (s), and Number of Vertical Movements. This set of variables was examined on both Drug-paired (days 1, 3, 7, 9) and NaCl-paired (days 2, 4, 8, 10) conditioning days. Body weight was analyzed for Drug-paired days and NaCl-paired days separately, in two Mixed Design Repeated Measures ANOVAs. The

within-subjects factor was Day (at 4 levels: Drug-paired Days 1, 3, 7, 9; or, NaCl-paired Days 2, 4, 8, 10), and 3 between-subjects factors consisting of Drug 1 (at 2 levels: peripubertal LPS or NaCl), Drug 2 (at 2 levels: adult LPS or NaCl), and, Drug 3 (at 2 levels: NaCl or LiCl). Each remaining dependent variable was analayzed using a 2 x 2 x 2 Between-subjects ANOVA, with 3 between-subjects factors consisting of Drug 1 (at 2 levels: peripubertal LPS or NaCl), Drug 2 (at 2 levels: adult LPS or NaCl), and, Drug 3 (at 2 levels: NaCl or LiCl).

Since this study was exploratory, all post hoc pairwise comparisons were performed using Fisher's Least Significant Difference (LSD), except for changes in body weight, which are well-documented, and thus examined with the more conservative Tukey's Honestly Significant Difference (HSD) post hoc test. All statistical tests used a significance criterion of $\alpha = 0.05$. All analyses were run in IBM SPSS 20.0 for Windows.

4.4.5.3 Drug-free Test Day

On a drug-free Test Day, each animal was reintroduced into the drug-paired chamber, but this time allowed to escape into the previously NaCl-paired chamber (i.e., the "safe" chamber) through an opening in the wall dividing the two chambers of the apparatus. Since subjects spend different amounts of time in each chamber, dependent variables (excluding Duration) were corrected to reflect the frequency of behavior per minute spent in a given chamber. Dependent variables measured on the Extinction Day included: Duration (min), Total Distance Travelled (cm/min), Horizontal Movement Time (s/min), Vertical Movement Time (s/min), Number of Vertical Movements (movements/min). Each variable was analyzed using a 2 x 2 x 2 x 2 Mixed Design Repeated Measures ANOVA, with a within-subjects variable of Chamber (at 2 levels: previously Drug-paired or NaCl-paired); and, three between-subjects factors of Drug 1 (at 2 levels: peripubertal LPS or NaCl), Drug 2 (at 2 levels: adult LPS or NaCl), and, Drug 3 (at 2 levels: NaCl or LiCl). All post hoc pairwise comparisons were performed using Fisher's Least Significant Difference (LSD).

All statistical tests used a significance criterion of $\alpha = 0.05$. All analyses were run in IBM SPSS 20.0 for Windows.

4.5 Results

Results demonstrated robust conditioned place avoidance learning in female rats. Furthermore, LPS pre-treatment block the formation of CPA. However, in those subjects treated with LPS during the peripubertal period and adulthood, tolerance-like effects to the acute-phase response, as well as, the previously observed deficit in learning and memory, were observed.

4.5.1 Peripubertal percent change in body weight

The one-way ANOVA revealed a significant main effect of Drug 1 (LPS or NaCl), $F(1, 60) = 100.88$, $p < 0.0001$), where LPS-treated females lost significantly more body weight, relative to NaCl-treated females, 24 h following the peripubertal immune challenge. Means and their standard errors (S.E.M.) are depicted in Figure 2.

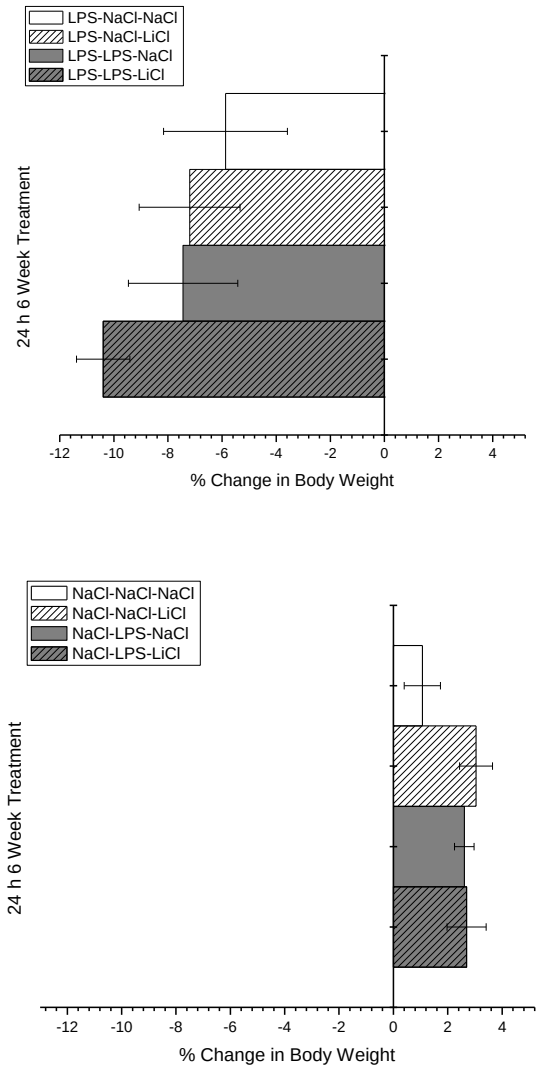

Figure 2. Peripubertal Percent Change in Body Weight. Means and their standard errors (S.E.M.) for percent of body weight change. The one-way ANOVA revealed a significant main effect of Drug 1 (LPS or NaCl), $F(1, 60) = 100.88$, $p < 0.0001$), where LPS-treated females lost significantly more body weight, relative to NaCl-treated females, 24 h following the peripubertal immune challenge.

4.5.2 Adult conditioning phase body weight

4.5.2.1 Drug-paired days

24 h percent changes in body weight following each Drug-paired Day or NaCl-paired Day were analyzed with a mixed factor repeated measures analysis of variance (ANOVA). The ANOVA revealed significant main effects of Day (days 1, 3, 7, and 9), $F(1, 125) = 20.258$, $p < 0.0001$; Drug 1 (pubertal LPS or NaCl), $F(1, 54) = 9.102$, $p < 0.01$; Drug 2 (adult LPS or NaCl), $F(1, 54) = 41.279$, $p < 0.0001$; and, Drug 3 (LiCl or NaCl), $F(1, 54) = 6.738$, $p < 0.02$. Significant interactions were observed between Day x Drug 1 (pubertal LPS or NaCl), $F(1, 125) = 7.897$, $p < 0.0001$; Day x Drug 2 (adult LPS or NaCl), $F(1, 125) = 38.402$, $p < 0.0001$; Day x Drug 1 x Drug 2, $F(1, 125) = 11.622$, $p < 0.0001$; and, Drug 1 x Drug 2, $F(1, 54) = 7.641$, $p < 0.01$.

Post hoc pairwise comparisons (Tukey's HSD) showed that subjects treated with LPS during conditioning in adulthood-only (Groups NaCl-LPS-NaCl and NaCl-LPS-NaCl) lost significantly more body weight following the initial treatment relative to all other groups ($p's < 0.01$). Females treated with LPS at both time points (Groups LPS-LPS-NaCl and LPS-LPS-LiCl) showed changes in body weight that were not significantly different from non-LPS-treated controls, except Group LPS-LPS-NaCl, which showed significantly greater body weight losses after Day 1, relative to Groups LPS-NaCl-LiCl and NaCl-NaCl-LiCl ($p's < 0.05$), where LiCl-treated animals showed minor but greater gains in weight following treatment. Means and S.E.M are depicted in Figure 3 A-D.

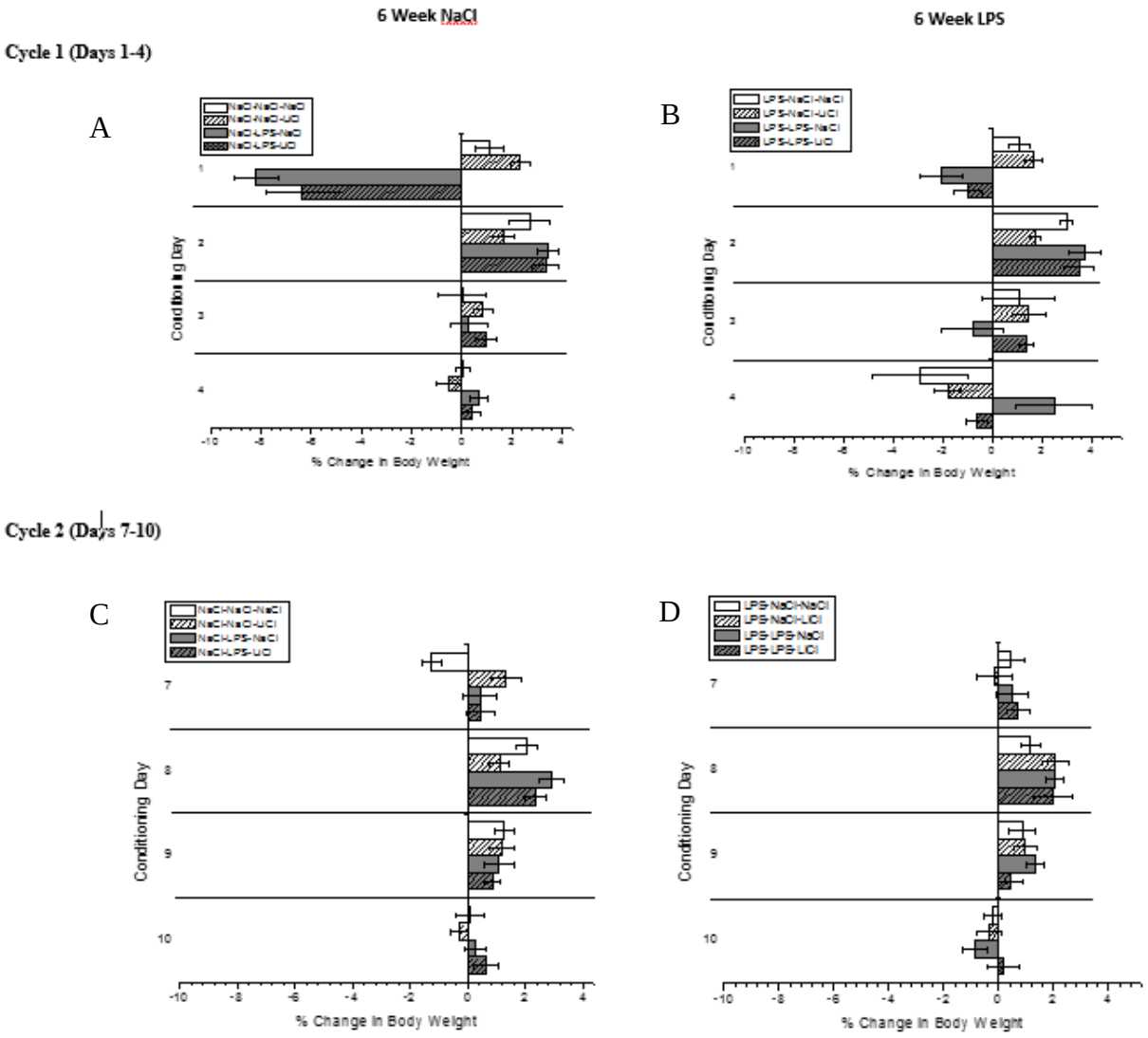

Figure 3 A-D. Adulthood percent change in body weight. A mixed factor repeated measures ANOVA revealed a significant **Day** x **Drug 1** (6 wk LPS or NaCl) x **Drug 2** (adult LPS or NaCl) interaction, $F(1, 54) = 7.641$, $p < 0.01$. Following Day 1, adult LPS-treated rats showed significant body weight loss (anorexia) relative to NaCl-treated rats, but there were no significant differences when rats were treated with LPS in both adolescence and adulthood (tolerance).

4.5.2.2 Saline-paired days

The ANOVA revealed significant main effects of Day (days 2, 4, 8, and 10), $F(3, 123) = 53.087$, $p < 0.001$; and, Drug 2 (adult LPS or NaCl), $F(1, 54) = 18.759$, $p < 0.001$. Significant interactions were observed between Day x Drug 2, $F(3, 123) = 3.213$, $p < 0.05$; and, Day x Drug 1 (pubertal LPS or NaCl) x Drug 2, $F(1, 54) = 3.029$, $p < 0.05$. No significant pairwise comparisons were obtained following any of the saline-paired treatment days (Tukey's HSD).

4.5.3 Adult conditioning phase locomotor behavior

During conditioning, the following variables were recorded and analyzed separately for drug- and saline-paired days: Total Distance Travelled (cm), Horizontal Movement Time (s), Number of Vertical Movements, and Vertical Movement Time (s). Each variable was analyzed using a mixed design repeated-measures ANOVA.

4.5.3.1 Total Distance Travelled (cm)

4.5.3.1.1 Drug-paired days

The ANOVA revealed significant main effects of Day (days 1, 3, 7, and 9), $F(3, 162) = 11.616$, $p < 0.0001$; and, Drug 2 (adult LPS or NaCl), $F(1, 54) = 27.421$, $p < 0.0001$. Significant interactions were found between Day x Drug 2, $F(3, 162) = 3.00$, $p < 0.05$; and, Day x Drug 3 (LiCl or NaCl), $F(3, 162) = 12.74$, $p < 0.0001$. Means and S.E.M are depicted in Figure 4 A-D.

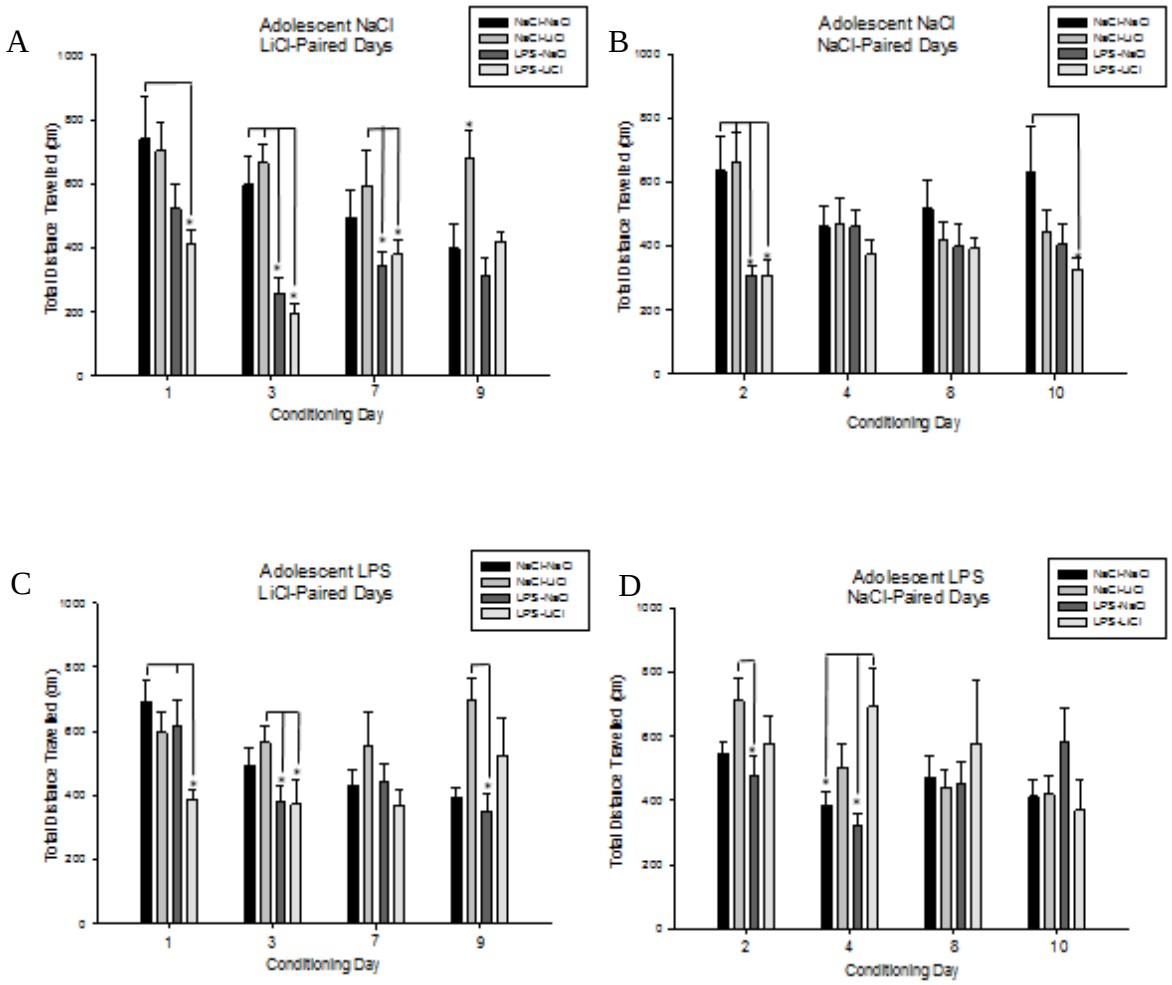

Figure 4 A-D. Total Distance Travelled (cm/s). Rats treated with LPS in adulthood-only displayed significant decreases in locomotor activity at the beginning of conditioning (relative to controls) that were not as robust as those observed in rats treated with LPS both in adolescence and adulthood. Towards the end of conditioning, LiCl-treated rats show significantly further distances travelled (aversive behaviour) relative to NaCl controls and LPS-pretreated rats (adulthood only). Rats pre-treated with LPS in both adolescence and adulthood did not differ significantly from other LiCl-treated groups that were not treated with LPS ($p's < 0.05$), evidence of tolerance to adult homotypic LPS challenge.

4.5.3.1.2 Saline-paired days

The ANOVA revealed a significant main effect of Day (days 2, 4, 8, and 10), $F(3, 162) = 3.738$, $p < 0.05$, as well as, significant interactions between Day x Drug 2 (adult LPS or NaCl), $F(3, 162) = 7.788$, $p < 0.001$; Day x Drug 3 (LiCl or NaCl), $F(3, 162) = 6.661$, $p < 0.001$; Day x Drug 1 (pubertal LPS or NaCl) x Drug 2 x Drug 3, $F(3, 162) = 3.337$, $p < 0.05$; and Drug 1 x Drug 2, $F(1, 54) = 4.255$, $p < 0.05$.

4.5.3.2 Horizontal Movement Time (s)

4.5.3.2.1 Drug-paired days

The ANOVA revealed significant main effects of Day (days 1, 3, 7, and 9), $F(3, 162) = 14.379$, $p < 0.0001$; and, Drug 2 (adult LPS or NaCl), $F(1, 54) = 21.800$, $p < 0.0001$. Significant interactions were found between Day x Drug 2, $F(3, 162) = 3.32$, $p < 0.05$; and, Day x Drug 3 (LiCl or NaCl), $F(3, 162) = 10.512$, $p < 0.0001$.

4.5.3.2.2 Saline-paired days

The ANOVA revealed a significant main effect of Day (days 2, 4, 8, and 10), $F(3, 162) = 9.412$, $p < 0.0001$. Significant interactions were found between Day x Drug 2 (adult LPS or NaCl), $F(3, 162) = 7.498$, $p < 0.0001$; and, Day x Drug 3 (LiCl or NaCl) $F(3, 162) = 4.302$, $p < 0.01$.

4.5.3.3 Post hoc pairwise comparisons (Fisher's LSD): horizontal activity

Rats treated with LPS in adulthood-only displayed significant decreases in locomotor activity at the beginning of conditioning (relative to controls) that were not as robust as those observed in rats treated with LPS both in adolescence and adulthood. Towards the end of conditioning, LiCl-treated rats show significantly further distances travelled (aversive behaviour) relative to NaCl controls and LPS-pretreated rats (adulthood only). Rats pre-treated with LPS in both adolescence and adulthood did not

differ significantly from other LiCl-treated groups that were not treated with LPS, evidence of tolerance to adult homotypic LPS challenge. Means and S.E.M are depicted in Figure 5 A-D.

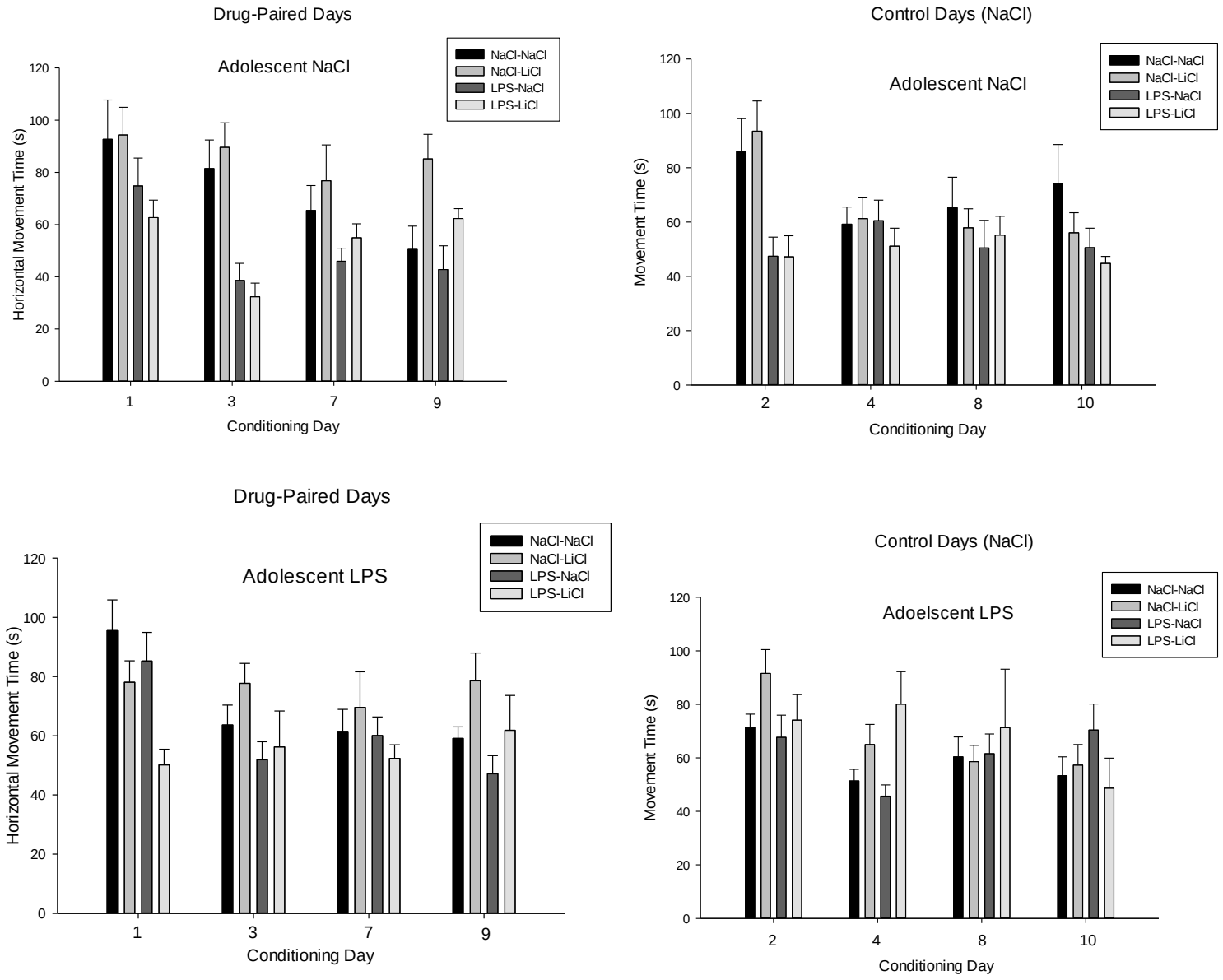

Figure 5 A-D. Horizontal Time (sec/min). Rats treated with LPS in adulthood-only displayed significant decreases in locomotor activity at the beginning of conditioning (relative to controls) that were not as robust as those observed in rats treated with LPS both in adolescence and adulthood. Towards the end of conditioning, LiCl-treated rats spend significantly more time moving horizontally (indicative of agitation) relative to NaCl controls and LPS-pretreated rats (adulthood only). Rats pre-treated with LPS in both adolescence and adulthood did not differ significantly from other LiCl-treated groups that were not treated with LPS ($p's < 0.05$), evidence of tolerance to adult homotypic LPS challenge.

4.5.3.4 Number of Vertical Movements

4.5.3.4.1 Drug-paired days

The ANOVA revealed significant main effects of Day (days 1, 3, 7, and 9), $F(3, 162) = 8.952$, $p < 0.0001$; Drug 2 (adult LPS or NaCl), $F(1, 54) = 32.573$, $p < 0.0001$; and, Drug 3 (LiCl or NaCl), $F(1, 54) = 7.75$, $p < 0.01$. Significant interactions were found between Day x Drug 3, $F(3, 162) = 25.204$, $p < 0.0001$, Day x Drug 2 x Drug 3, $F(3, 162) = 3.729$, $p < 0.05$; and, Drug 2 x Drug 3, $F(1, 54) = 8.768$, $p < 0.01$. Means and S.E.M are depicted in Figure 6 A-D.

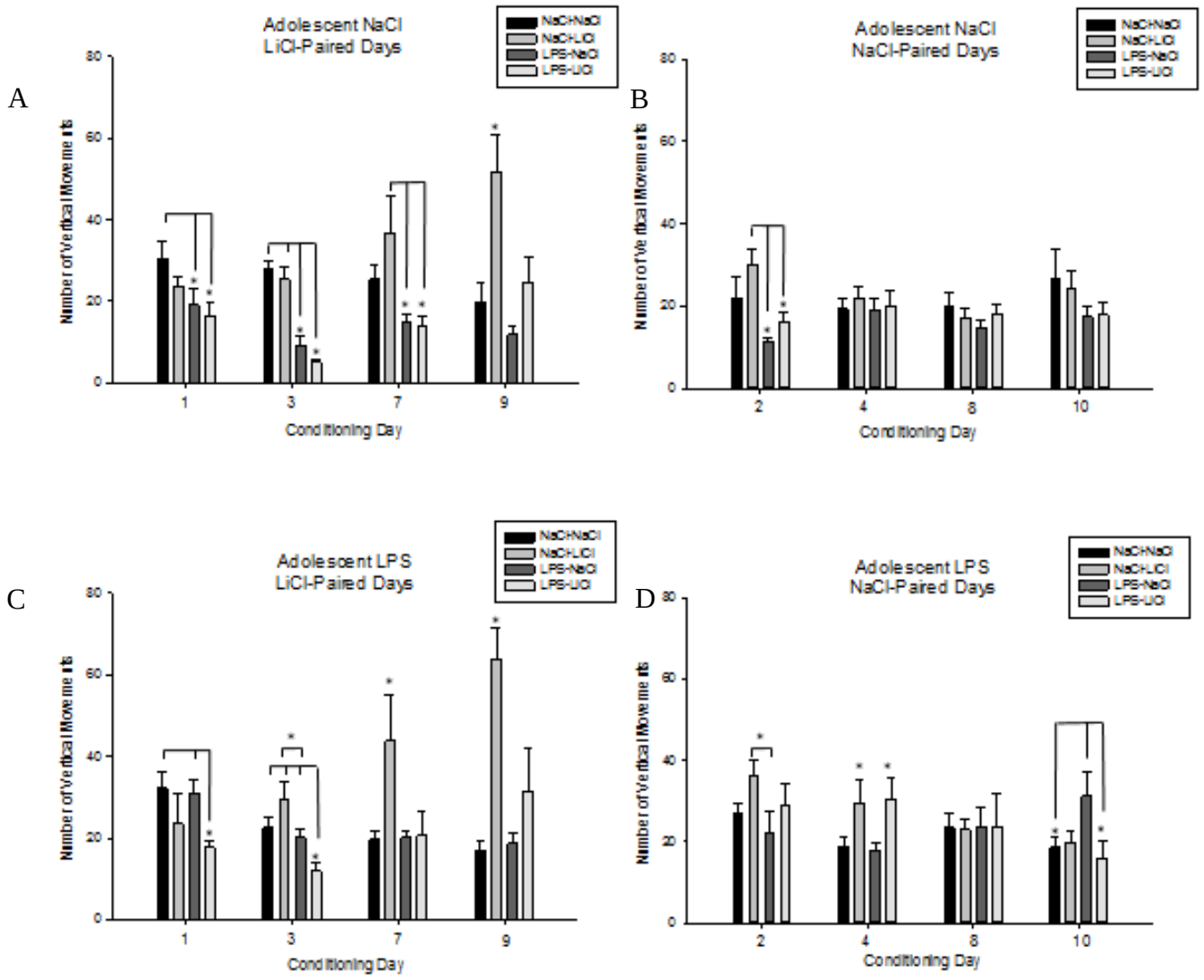

Figure 6 A-D. Number of Vertical Movements. Means and S.E.M for number of vertical movements displayed during the conditioning phase. Rats treated with LPS in adulthood-only displayed significantly less rearing behaviour (aversive behaviour) at the beginning of conditioning (relative to controls) that was not as robust as that observed in rats treated with LPS in both adolescence and adulthood. Towards the end of conditioning, LiCl-treated rats exhibited significantly more rearing behaviour relative to NaCl controls and LPS-pretreated rats. Rats treated with LPS in adolescence and adulthood, although they did not display significant tolerance-like effects to the deleterious effects of LPS on conditioning, they displayed trending increases in rearing behaviour over the course of conditioning that were significant on the drug-free test day.

4.5.3.4.2 Saline-paired days

The ANOVA revealed a significant main effect of Drug 1 (pubertal LPS or NaCl), $F(1, 54) = 6.054$, $p < 0.05$. Significant interactions were found between Day (days 2, 4, 8, and 10) x Drug 2 (adult LPS or NaCl), $F(3, 162) = 3.056$, $p < 0.05$; and, Day x Drug 3 (LiCl or NaCl), $F(3, 162) = 5.353$, $p < 0.01$.

4.5.3.5 Vertical Movement Time (s)

4.5.3.5.1 Drug-paired days

The ANOVA revealed significant main effect of Day (days 1, 3, 7, and 9), $F(3, 162) = 4.237$, $p < 0.05$. No further significant main effects or interactions were observed.

4.5.3.5.2 Saline-paired days

The ANOVA revealed a significant main effect of Day (days 2, 4, 8, and 10), $F(3, 162) = 6.405$, $p < 0.05$. No further significant main effects or interactions were observed.

4.5.3.5.3 Post Hoc Pairwise Comparisons (Fisher's LSD): vertical activity

Rats treated with LPS in adulthood-only displayed significantly less rearing behaviour (aversive behaviour) at the beginning of conditioning (relative to controls) that was not as robust as that observed in rats treated with LPS in both adolescence and adulthood. Towards the end of conditioning, LiCl-treated rats exhibited significantly more rearing behaviour relative to NaCl controls and LPS-pretreated rats. Rats treated with LPS in adolescence and adulthood, although they did not display significant tolerance-like effects to the deleterious effects of LPS on conditioning, they displayed trending increases in rearing behaviour over the course of conditioning that were significant on the drug-free test day (p's < 0.05).

4.5.4 Drug-free Test Day

On a drug-free Test Day, each subject was re-exposed to the context in the absence of any drug treatment, but this time allowed to move between the two previously drug- or saline-paired chambers through an opening in the dividing wall. Each variable (excluding Duration) was corrected for frequency of behavior per amount of time spent in a given chamber, analyzed using a 2 x 2 x 2 x 2 mixed design repeated measures ANOVA.

4.5.4.1 Duration (min)

The ANOVA revealed a significant main effect of Chamber (drug-paired or saline-paired), $F(1, 54) = 26.156$, $p < 0.0001$ on the duration spent in the drug-paired chamber. Significant interactions were also observed between Chamber x Drug 3 (LiCl or NaCl), $F(1, 54) = 54.619$, $p < 0.0001$; Chamber x Drug 1 (pubertal LPS or NaCl) x Drug 2 (adult LPS or NaCl), $F(1, 54) = 13.043$, $p < 0.001$; and, Chamber x Drug 2 x Drug 3, $F(1, 54) = 4.545$, $p < 0.05$.

Post hoc pairwise comparisons (Fisher's LSD) showed that subjects in Groups NaCl-NaCl-LiCl, LPS-NaCl-LiCl, and LPS-LPS-LiCl spent significantly less time in the previously drug-paired chamber relative to rats treated with NaCl during conditioning, as well as, rats pre-treated with LPS prior to LiCl treatment (Group NaCl-LPS-LiCl), $p's < 0.05$. Group NaCl-LPS-LiCl spent significantly less time in the previously drug-paired chamber, relative to Groups LPS-NaCl-NaCl and NaCl-LPS-NaCl ($p's < 0.01$), but did not differ significantly from other controls groups (Groups NaCl-NaCl-NaCl and LPS-LPS-NaCl). Means and S.E.M are depicted in Figure 7.

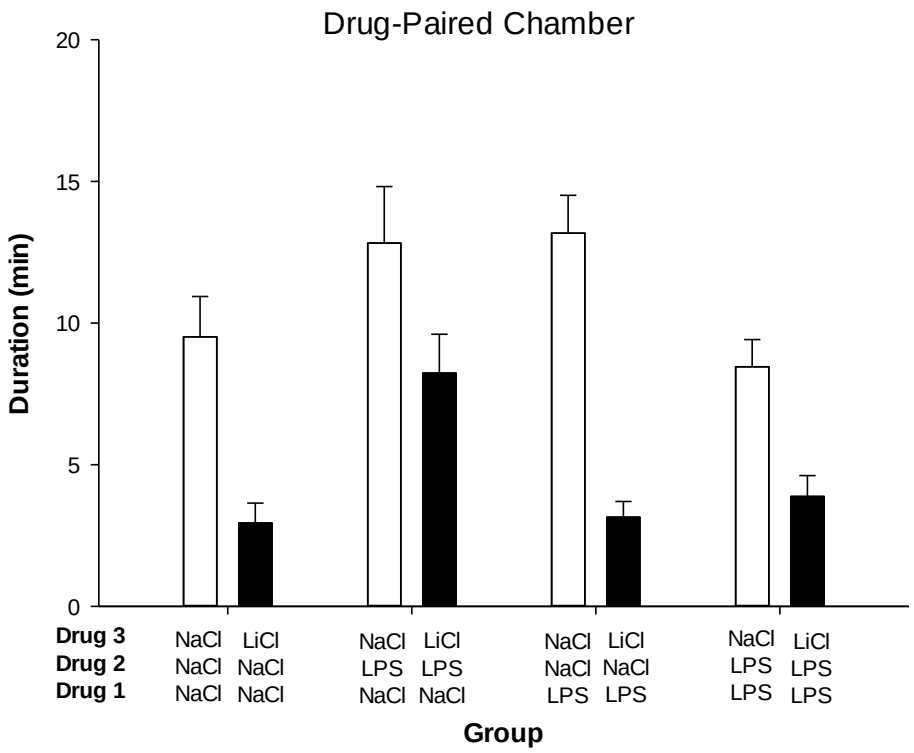

Figure 7. Duration in drug-paired chamber. Means and S.E.M for duration spent in LiCl-paired chamber. LiCl-treated rats spent significantly less time in the Drug-paired chamber relative to NaCl controls. Rats pre-treated with LPS (adulthood only) followed by LiCl did not differ significantly from controls. Rats treated with LPS in both adolescence and adulthood did not show this effect (p's< 0.05)

4.5.4.2 Total Distance Travelled (cm/min)

The ANOVA revealed a significant main effects of Chamber (drug-paired or saline-paired), $F(1, 54) = 28.837$, $p < 0.0001$; and, Drug 3 (LiCl or NaCl), $F(1, 54) = 10.318$, $p < 0.01$. Significant interactions were found between Chamber x Drug 3, $F(1, 54) = 58.136$, $p < 0.0001$; Chamber x Drug 1 (pubertal LPS or NaCl) x Drug 2 (adult LPS or NaCl), $F(1, 54) = 12.285$, $p < 0.001$; Chamber x Drug 2 x Drug 3, $F(1, 54) = 4.625$, $p < 0.05$; Drug 2 x Drug 3, $F(1, 54) = 5.110$, $p < 0.05$; and, Drug 1 x Drug 2 x Drug 3, $F(1, 54) = 4.091$, $p < 0.05$.

Post hoc pairwise comparisons (LSD) showed that subjects in Groups NaCl-NaCl-LiCl, LPS-NaCl-LiCl, and LPS-LPS-LiCl travelled significantly further in the previously drug-paired chamber, relative to all other groups (p's< 0.01). Rats pre-treated with LPS during LiCl conditioning (Group NaCl-LPS-LiCl) travelled a significantly shorter distance in the previously drug-paired chamber, relative to all other LiCl-treated groups (p's< 0.01), and did not differ significantly from NaCl controls.

In the previously saline-paired chamber, females in Groups NaCl-LPS-NaCl and LPS-NaCl-NaCl travelled significantly further, relative to all other groups (p's< 0.05), except that Group LPS-NaCl-NaCl also travelled significantly further relative to Group NaCl-NaCl-NaCl ($p < 0.05$). Means and S.E.M are depicted in Figure 8.

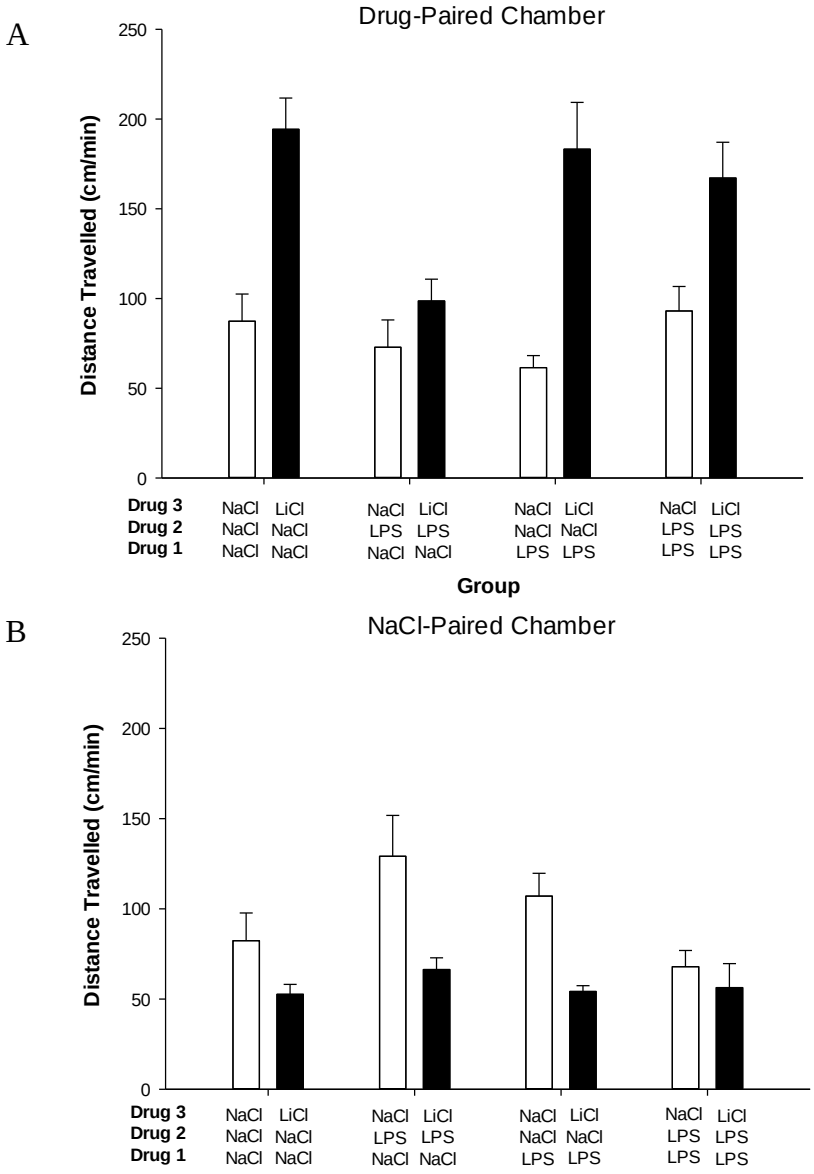

Figure 8. Means and S.E.M. Total Distance Traveled on test day (cm/min). LiCl-treated rats travelled significantly further in the Drug (LiCl)-Paired chamber relative to NaCl controls, unless rats were pre-treated with LPS (adulthood only) 90 minutes prior to conditioning (p's < .05). Rats treated with LPS in adolescence and adulthood did not show this effect (p's< 0.05)

4.5.4.3 Horizontal Movement Time (s/min)

The ANOVA revealed significant main effects of Chamber (drug-paired or saline-paired), $F(1, 54) = 31.310$, $p < 0.0001$; Drug 2 (adult LPS or NaCl), $F(1, 54) = 4.563$, $p < 0.05$; and, Drug 3 (LiCl or NaCl), $F(1, 54) = 13.571$, $p < 0.001$. Significant interactions were found between Chamber x Drug 3, $F(1, 54) = 60.860$, $p < 0.0001$; Chamber x Drug 1 (pubertal LPS or NaCl) x Drug 2, $F(1, 54) = 15.433$, $p < 0.0001$; Chamber x Drug 2 x Drug 3, $F(1, 54) = 5.917$, $p < 0.05$; Drug 2 x Drug 3 = 4.548, $p < 0.05$; and, Drug 1 x Drug 2 x Drug 3, $F(1, 54) = 7.474$, $p < 0.01$.

Post hoc pairwise comparisons (LSD) showed that subjects in Groups NaCl-NaCl-LiCl, LPS-NaCl-LiCl, and LPS-LPS-LiCl spent significant more time moving horizontally in the previously drug-paired chamber, relative to all other groups (p's< 0.001). Rats pre-treated with LPS during LiCl conditioning (Group NaCl-LPS-LiCl) spent significantly less time moving horizontally relative to all other LiCl-treated groups (p's< 0.001), and did not significantly differ from NaCl controls.

In the previously saline-paired chamber, rats in Group NaCl-LPS-NaCl spent significantly more time moving horizontally relative to all other groups (p's< 0.01), except Groups LPS-NaCl-NaCl and NaCl-NaCl-NaCl. Means and S.E.M are depicted in Figure 9.

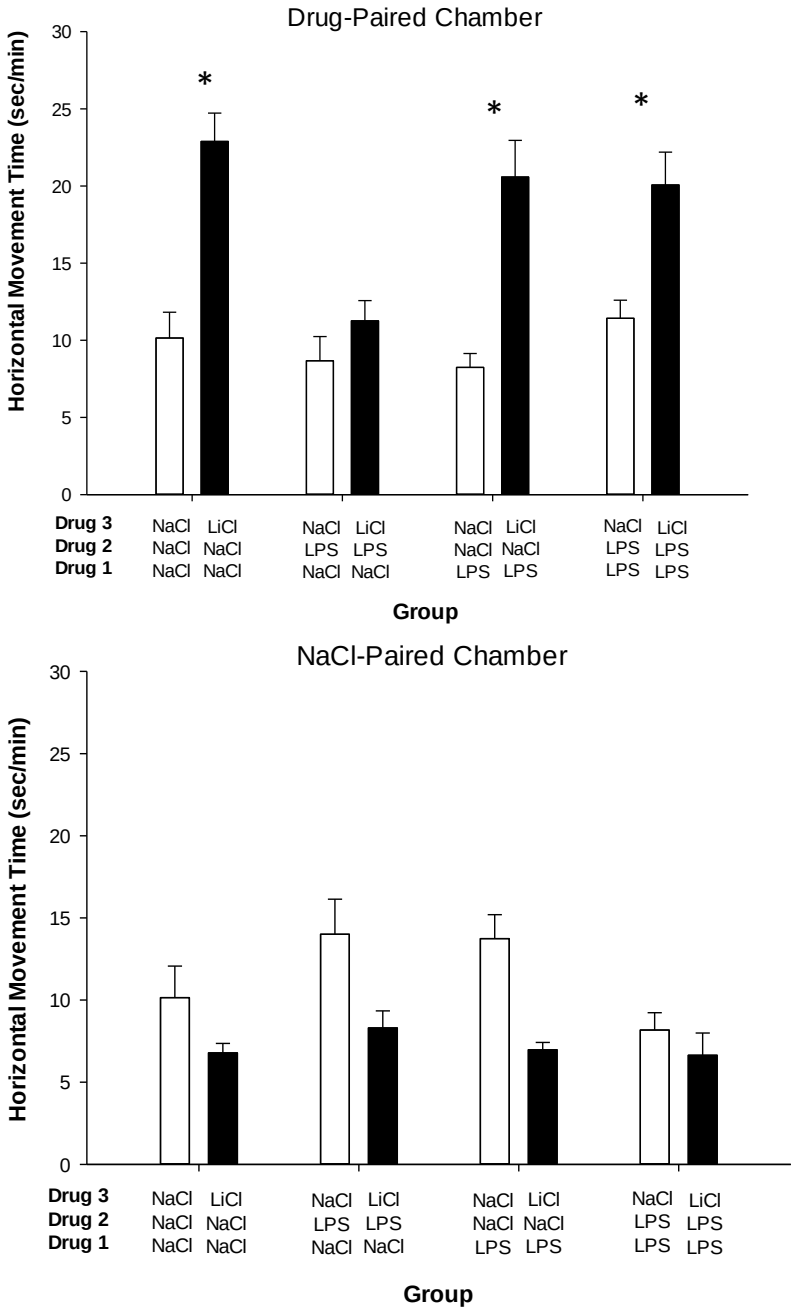

Figure 9. Means and S.E.M for time spent moving horizontally in each chamber on the test day. LiCl-treated rats spent significantly more time moving in the Drug (LiCl)-Paired chamber relative to NaCl controls, unless rats were pre-treated with LPS (adulthood only) 90 minutes prior to conditioning (p's < .05). Rats treated with LPS in adolescence and adulthood did not show this effect (p's< 0.05)

4.5.4.4 Number of Vertical Movements (movements/min)

The ANOVA revealed significant main effects of Chamber (drug-paired or saline-paired), $F(1, 54) = 27.148$, $p < 0.0001$; and, Drug 3 (LiCl or NaCl), $F(1, 54) = 16.778$, $p < 0.0001$. Significant interactions were found between Chamber x Drug 3, $F(1, 54) = 62.046$, $p < 0.0001$; and, Chamber x Drug 1 (pubertal LPS or NaCl) x Drug 2 (adult LPS or NaCl), $F(1, 54) = 11.044$, $p < 0.01$.

Post hoc pairwise comparisons (LSD) showed that subjects in Groups NaCl-NaCl-LiCl, LPS-NaCl-LiCl, and LPS-LPS-LiCl displayed a significantly greater number of vertical movements, relative to all other groups (p's< 0.05), in the previously drug-paired chamber. Rats pre-treated with LPS during LiCl conditioning (Group NaCl-LPS-LiCl) showed a significantly smaller number of vertical movements relative to all other LiCl-treated rats (p's< 0.05), but a significantly higher number of vertical movements relative to rats in Groups LPS-NaCl-NaCl and NaCl-LPS-NaCl (p's< 0.05), and did not differ significantly from Groups NaCl-NaCl-NaCl and LPS-LPS-NaCl.

In the previously saline-paired chamber, rats in Group LPS-NaCl-NaCl displayed significantly more vertical movements, relative to all other groups (p's< 0.05), except Groups NaCl-LPS-NaCl and NaCl-LPS-LiCl; and, Group NaCl-LPS-NaCl displayed significantly more vertical movements relative to all other groups (p's< 0.05), except for Group LPS-NaCl-NaCl. Means and S.E.M are depicted in Figure 10.

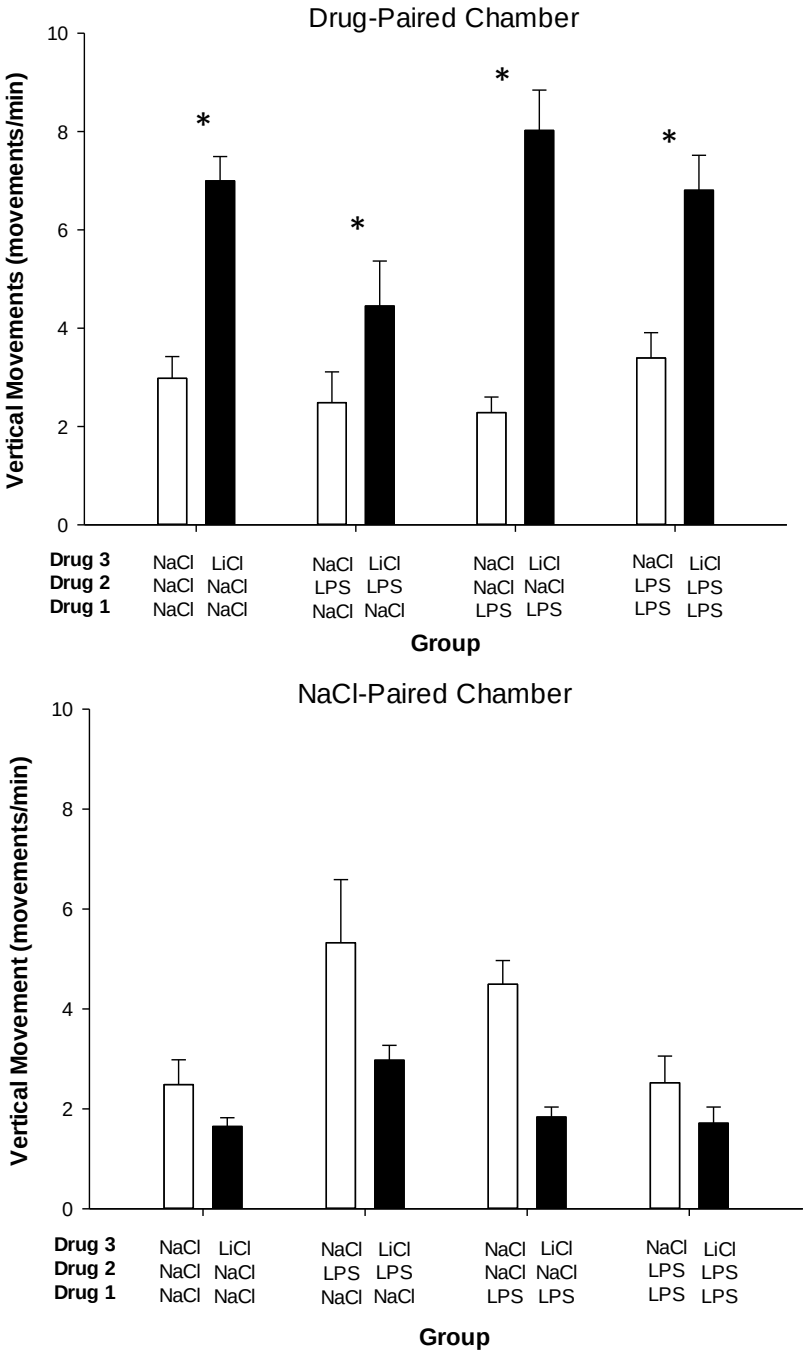

Figure 10. Means and S.E.M for the number of vertical movements on the test day. LiCl-treated rats reared significantly more in the Drug (LiCl)-Paired chamber relative to NaCl controls, regardless of LPS treatment (*p*'s< 0.05)

4.5.4.5 Vertical Movement Time (s/min)

The ANOVA revealed significant main effects of Chamber (drug-paired or saline-paired), $F(1, 54) = 26.698$, $p < 0.0001$; and, Drug 3 (LiCl or NaCl), $F(1, 54) = 9.467$, $p < 0.01$. Significant interactions were found between Chamber x Drug 3, $F(1, 54) = 61.445$, $p < 0.0001$; and, Chamber x Drug 1 (pubertal LPS or NaCl) x Drug 2 (adult LPS or NaCl), $F(1, 54) = 7.617$, $p < 0.01$.

Post hoc pairwise comparisons (LSD) showed that subjects in Groups NaCl-NaCl-LiCl, LPS-NaCl-LiCl, and LPS-LPS-LiCl spent significantly more time moving vertically, relative to all other groups (p's< 0.05), in the previously drug-paired chamber. Rats pre-treated with LPS during LiCl conditioning (Group NaCl-LPS-LiCl) spent significantly less time moving vertically, relative to all other LiCl-treated groups (p's< 0.05), but spent more time moving vertically relative to Groups LPS-NaCl-NaCl and NaCl-LPS-NaCl (p's< 0.05), and did not differ significantly from Groups NaCl-NaCl-NaCl and LPS-LPS-NaCl.

In the previously saline-paired chamber, Groups LPS-NaCl-NaCl and NaCl-LPS-NaCl spent significantly more time moving vertically, relative to all other groups (p's< 0.05), except for Group NaCl-LPS-LiCl. Due to the similarity of this variable with Number of Vertical Movements, no figure is provided.

4.6 Discussion

The aims of the present study were 4-fold: to examine Conditioned Place Avoidance (CPA) behavior in adult female Long-Evans rats, and the effects of Conditioning Phase pre-treatment with LPS on the establishment of these conditioned behaviors; and, to examine the effects of a peripubertal (6 weeks old) LPS challenge on the acquisition of LiCl-induced context conditioning, as well as, to a homotypic LPS challenge in adulthood. Our hypotheses were mainly supported. As expected, female rats successfully acquired conditioned place avoidance, evidenced by shorter durations of

time spent in the previously drug-paired chamber, as well as, increased frequencies of rearing and horizontal movements, relative to controls. As predicted, LPS pre-treatment was shown to block the formation of CPA, evidenced by similar durations spent in each chamber on the drug-free test day, durations that did not differ significantly from a saline control. The hypothesis that peripubertal LPS challenge would alter CPA learning in adulthood was unsupported. Female rats that were administered a pubertal LPS challenge showed no sign of learning decrements, relative to females treated with saline at 6 weeks old.

Interestingly, it was found that females treated with LPS during the peripubertal period displayed tolerance-like behavior following a homotypic LPS challenge in adulthood. Females treated with LPS at both time points failed to show significant reductions in body weight, nor did LPS in adulthood successfully block CPA conditioning. The current study provides the following novel findings: a) LiCl conditions robust Conditioned Place Avoidance in female rats; b) LPS pre-treatment blocks the acquisition of Conditioned Place Avoidance; c) LPS treatment during the sensitive peripubertal phase of development leads to tolerance to further immune challenge with LPS in adulthood, including the acute-phase response and learning impairments that have been shown to follow LPS treatment. Each finding is further discussed below.

4.6.1 LiCl-induced conditioned place avoidance

It was found that adult female Long Evans rats condition place (i.e., context) avoidant and aversion-related behaviors following LiCl conditioning, which is consistent with previous studies demonstrating CPA in male subjects (Tenk et al., 2005). LiCl-treated female rats spent significantly less time in the previously drug-paired chamber, relative to saline controls; and, during their time spent in the previously drug-paired chamber, LiCl-treated subjects also showed significantly higher frequencies of aversion-related behaviors, such as, further distances travelled, more time moving around the chamber horizontally, and more time spent rearing, relative to saline controls and, again, Group NaCl-LPS-LiCl. In fact, the current results present robust conditioning effects of

LiCl at a dose of 96 mg/kg, which were not as clear in male subjects (Tenk et al., 2005), highlighting the need to examine place avoidance responses in female subjects.

The results of the current study are consistent, in part, with previous studies that have examined conditioned disgust behavior in female rats. Females have been shown to display dose-related increases in conditioned 'gaping' behavior on the drug-free Test Day of the rodent model of Anticipatory Nausea. Anticipatory Nausea (AN) is a classically conditioned response in humans where the previous pairing of a salient context (e.g., hospital) with a nausea-inducing stimulus (e.g., chemotherapy treatment) leads to the establishment of anticipatory nausea and/or vomiting upon re-exposure to that environment, prior to actual drug treatment (Molassiotis, 2005). This phenomenon has been modelled in the rat using a context-based conditioning paradigm, wherein rats learn to associate a distinct contextual chamber with "feelings" of nausea produced by LiCl treatment, and subsequently retrieve these associations by displaying aversion-related behaviors, such as, "conditioned gaping", in response to re-exposure to the distinct context in the absence of drug treatment (see Chan et al., 2009; Cloutier et al., 2012 a,b; Limebeer et al., 2006; Parker et al., 2008)

4.6.2 Lipopolysaccharide effects

4.6.2.1 Acute LPS treatment during LiCl conditioning

LPS treatment significantly reduced body weight in both groups pre-treated with LPS during adulthood. Subjects in Groups NaCl-LPS-NaCl and NaCl-LPS-LiCl lost significantly more body weight following the first conditioning day, relative to controls. This is consistent with prior reports of tolerance development in female rats. Females treated with LPS have been shown to develop tolerance to the acute-phase sickness behaviors after the first treatment, unless each of the two treatments occurs during the proestrous phase of the cycle (Engeland et al., 2006).

LPS pre-treatment in adulthood, prior to LiCl conditioning, prevented the establishment of CPA. Subjects in Group NaCl-LPS-LiCl spent significantly more time in the previously drug-paired chamber relative to other LiCl-treated groups and was

similar to that of saline controls. LPS treatment has been shown to interfere with the establishment of other forms of conditioned behavior, such as, conditioned taste aversion and anticipatory nausea (Chan et al., 2009; Chan et al., 2013; Cloutier et al., 2012 a,b; Cloutier et al., 2016 a). However, until now the effects of LPS have not been investigated in the CPA paradigm, in males or females. Thus, this study demonstrates that LPS disrupts CPA acquisition which adds to previous work showing that LPS disrupts place/context aversion (i.e., anticipatory nausea).

4.6.2.2 Peripubertal LPS treatment and learning in adulthood

Body weight loss consistent with previous studies in adult subjects was observed 24 h following LPS treatment at 6 weeks old. Peripubertal females treated with LPS at this time point lost significantly more weight relative to saline controls. Furthermore, no disruptions in regular estrous cycling were observed following peripubertal LPS challenge. LPS-treated females exhibited 4-day cycles in adulthood comparable with those of non-LPS treated controls.

Treatment with LPS at 6 weeks old had no effect on the acquisition of CPA behavior in adulthood. Group LPS-NaCl-LiCl (i.e., subjects treated with LPS in adolescence and conditioned with LiCl in adulthood) spent similar amounts of time in the previously drug-paired chamber relative to other LiCl-treated groups, which was also significantly less time spent in the chamber relative to controls. Thus, a single LPS challenge at 6 weeks old does not influence learning in the CPA paradigm in the adult female Long Evans rat.

4.6.2.3 LPS challenge at 6 weeks old and adult homotypic challenge during CPA conditioning

Interestingly, subjects treated with LPS at both time points- in adolescence and adulthood- behaved differently than predicted. It was hypothesized that subjects treated

with LPS at both 6 weeks old and in adulthood might show exacerbated acute-phase responses to LPS in adulthood, relative to subjects treated with LPS in adulthood only.

Female rats treated with LPS at 6 weeks old displayed tolerance-like behavior to homotypic challenge in adulthood. Females in Groups LPS-LPS-NaCl and LPS-LPS-LiCl did not exhibit significant losses in body weight relative to saline controls, and retained body weight relative to subjects treated with LPS in adulthood only (Groups NaCl-LPS-NaCl and NaCl-LPS-LiCl). Likewise, female rats treated with LPS at both time points failed to show locomotor decrements that are typically observed during the acute-phase response as the animal conserves energy. It was hypothesized that a homotypic challenge with LPS in adulthood might lead to exacerbated immune responses; however, subjects given a homotypic drug challenge actually failed to demonstrate the acute-phase response sickness behaviors that typically follow LPS treatment. This finding was inconsistent with previous work carried out in male and female rat neonates, where it was shown that LPS treatment in early life led to exacerbated responses to homotypic challenge in male rats, but normal responses in female rats (Tenk et al., 2005).

In addition, LPS treatment at 6 weeks of age followed by adult homotypic challenge during the conditioning phase of the CPA paradigm lead to tolerance-like effects to the learning and memory impairments (i.e., central effects) that have been shown to follow treatment. Females in Group LPS-LPS-LiCl, those treated at both time points with LPS and conditioned with lithium, spent significantly less time in the previously drug-paired chamber relative to saline controls and subjects in Group NaCl-LPS-LiCl. Furthermore, time spent in the previously drug-paired chamber did not differ significantly from other LiCl-conditioned subjects that were pretreated with saline. Although LPS pretreatment during conditioning was shown to block CPA learning in those subjects treated with saline at 6 weeks old, this learning impairment was not observed when subjects were treated with LPS during the peripubertal phase. Thus, these results demonstrate that a single immune challenge with LPS at a sensitive developmental time point, followed by LPS pretreatment during LiCl-induced CPA conditioning,

prevents LPS from attenuating conditioned avoidant and aversive behavior in this paradigm, evidence for tolerance-like effects to LPS treatment.

This finding was unexpected, and in part, inconsistent with previous work. It has been shown that tolerance to the acute-phase behavioral response following LPS treatment does not influence the learning and memory effects of the drug. Firstly, during the conditioning phase of either the CTA or anticipatory nausea paradigms, subjects develop tolerance to LPS, such that significant losses in body weight and decrements in locomotor behavior are only observed after the first one or two treatments. In these experiments, the drug-free test day for conditioned aversive responding always occurs during a state of tolerance to the acute-phase behavioral response (i.e., anorexia) produced by LPS, yet the learning and memory impairments persist (Chan et al., 2009; Chan et al., 2013; Cloutier et al., 2012a, b; Cross-Mellor et al., 2009).

One study examined the effects of LPS pretreatment on the acquisition of AN in rats that were tolerant to LPS prior to the conditioning phase treatments (Chan et al., 2013). Adult male rats were treated with LPS on four separate days (72 h apart) and returned to the home cage. Following the tolerance phase, LPS-tolerant and non-tolerant male rats were then pretreated with LPS or saline during LiCl-induced anticipatory nausea conditioning. It was shown that although subjects were tolerant to LPS just prior to conditioning, and remained tolerant to the acute-phase behavior (i.e., body weight loss), they continued to display marked learning and memory impairments, evidenced by significantly attenuated frequencies of conditioned gaping and other aversion-related behaviors, relative to controls. Thus, results of the current study demonstrate a unique effect of an adolescent immune challenge with LPS on the effects subsequent LPS treatment, and how homotypic challenge influences learning and memory in the CPA paradigm.

The rodent model of AN measures aversion-related responses to involuntary exposure to a context, and the CPA test allows the subject to move between the previously drug- or saline-paired chambers freely, and thus measures voluntary avoidance behavior. However, in both paradigms, the negative association formed

between a salient context and the effects of LiCl treatment is blocked by pre-treatment with LPS during the Conditioning Phase. In regards to taste + illness associations, LPS has been shown to significantly attenuate the formation of active aversion-related disgust behavior in the Taste Reactivity Test (TRT) for Conditioned Taste Aversion (CTA; Cross-Mellor et al., 2009), but has also been shown to serve as an unconditioned stimulus in the condition taste avoidance paradigm (Cross-Mellor et al., 2004). Conversely, LPS treatment blocked the formation of conditioned taste avoidance in a simultaneous conditioning paradigm for taste avoidance and context aversion (i.e., AN) (see Cloutier et al., 2012). As aforementioned, it should be noted that the administration of a taste cue by an intravascular route might provide a weakened stimulus, relative to stimuli administered orally, and thus, might produce weaker conditioned responses to the Conditioned Stimulus (Cloutier et al., 2012).

Immune challenge with LPS during the peripubertal phase of development, and how it affects future behavioral outcomes, has not been extensively examined in males or females. Many inflammation-derived developmental models of disorders involve perinatal LPS challenges, making the current findings hard to compare. To the best of our knowledge, this is the first study to show that peripubertal immune challenge with LPS at 6 weeks old in the rat leads to tolerance-like behavior to homotypic challenge in adulthood. In neonatal male rats, LPS challenge on postnatal days 3 and 5 led to an exacerbated acute-phase response following homotypic challenge in adulthood; however, normal acute-phase responses are observed following the same treatment in females (Tenk et al., 2008). Adult male rats have been shown to exhibit greater losses in body weight, relative to female rats, following LPS treatment in adulthood, with female rats showing faster tolerance rates (Engeland et al., 2003; Engeland et al., 2006). Furthermore, long-term alterations in the anxiolytic effects of sex hormones, such as, estrogen, have been observed following peripubertal immune challenges in female mice (Blaustein et al., 2011), in addition to the loss of estrogen-induced cognitive enhancement in some tasks (Blaustein et al., 2013).

4.6.3 Putative Mechanisms

Similar to AN, the acquisition of CPA relies on the association of feelings of nausea with a specific context, thus, it is likely that this task is largely hippocampus-dependent (Kranjac et al., 2012; Limebeer et al., 2006). Here, we discuss the putative mechanisms of LPS-induced memory disruptions in the form of inhibited consolidation processes in the hippocampus. Indeed, there is evidence suggesting that LPS treatment inhibits aversion conditioning by disrupting memory consolidation processes. Examination of LPS-induced chronic neuroinflammation on the induction of NMDA-dependent, and NMDA-independent, long-term potentiation (LTP) shows that intracerebroventricular administration of LPS produces significant spatial memory impairment in the Morris water maze (Min et al., 2009). LPS treatment also impaired the ability to form representations of distinct contexts in a contextual fear conditioning paradigm, as demonstrated by a reduction in freezing responses upon re-exposure to a context previously paired with an aversive foot shock in LPS-treated rats (Pugh et al., 1998). In both studies, it was suggested that LPS could affect the functioning of the hippocampus. Recordings of postsynaptic potentials showed that the induction of NMDA-dependent and NMDA-independent LTP were impaired in the Schaffer collateral-CA1 synapse of the hippocampus (Min et al., 2009).

Contextual fear conditioning has been shown to be, at least in part, a hippocampus-dependent learning paradigm, as demonstrated by the elimination of contextual fear conditioned responses after hippocampal lesions one day following conditioning (Kim & Fanselow, 1992). Tanaka et al. (2006) reported that LPS administration to the CA1 region of the hippocampus activated microglial cells and resulted in an increased production of IL-1β and TNF-α in this region. After 5 days of injections, it was found that long-term activation of microglia, induced by LPS, resulted in a decrease of glutamatergic transmission and learning and memory impairments without neuronal cell death (Tanaka et al., 2006).

Kranjac et al. (2012) showed that a single LPS injection, following contextual fear conditioning training, not only impaired memory consolidation, but could also disrupt memory reconsolidation processes in mice. This was evidenced by a decrease in freezing responses in LPS-treated mice, along with heightened peripheral and central cytokine and

chemokine levels, and significantly decreased brain-derived neurotrophic factor (BDNF) mRNA expression in the hippocampus and cortex (Kranjac et al., 2012).

It has been shown that peripheral inflammation by LPS causes a reduction of trophic supply in the brain (Schnydrig et al., 2007). Neurotrophins, such as BDNF and nerve growth factor (NGF), are known to play an important role in synaptic plasticity and long-term potentiation (Schnydrig et al., 2007). An experiment by Hennigan, Trotter, & Kelly (2007) demonstrated that synaptic plasticity in the dentate gyrus of the hippocampal complex is related to neurotrophin signaling changes, and that the disruption of these changes in plasticity by LPS may be partially due to a strong effect on these signaling cascades. Guan and Fang (2006) found that LPS treatment decreased BDNF expression in not only the hippocampus, but also the frontal, parietal, temporal, and occipital cortices. LPS also exerts a depressive effect on the expression of other neurotrophins, such as, NGF and neurotrophic factor 3 (NT-3), where expression was significantly reduced in cortical regions, as well as, the hippocampus (Guan & Fang, 2006).

4.6.4 Conclusions

The current study examined conditioned place avoidance (CPA) learning in adult female Long Evans rats, the effect of acute LPS treatment on CPA learning, and, the effects of peripubertal immune challenge on CPA learning, as well as, responses to homotypic drug challenge. It was found that females condition robust LiCl-induced place avoidances, and that this form of conditioned behavior is blocked following immune system stimulation with LPS. Furthermore, LPS challenge at 6 weeks old failed to alter CPA learning in adulthood, and led to peripheral and central tolerance-like behavior in those subjects administered a homotypic drug challenge in adulthood. To best of our knowledge, this was the first study to demonstrate these findings in female rats. Future studies should evaluate the effects of peripubertal immune system challenges in males, and whether locomotor variables observed in the CPA paradigm might correlate to active aversion-related behavior (i.e., gaping frequencies) in the rodent model of anticipatory nausea.

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Chapter 5

5 General Discussion

The current thesis examined sex differences in context-based conditioned disgust behaviours in the rat, using the rodent models of anticipatory nausea and conditioned place avoidance. Among cancer patients receiving chemotherapy treatment, anticipatory nausea and taste avoidance produce aversive conditioning consequences to the treatment (Molassiotis, 2005; Morrow et al., 1998; Morrow, Roscoe, Korshner, Hynes, & Rosenbluth, 1998). Importantly, evidence suggests that there is a sex difference in the onset and severity of conditioned nausea responses, where females are shown to be more susceptible relative to males (Fetting et al., 1983; Hilarius et al., 2012; LeBaron et al., 1988; Williams et al., 1980).

The second aim of this thesis was to evaluate the effects of Gram-negative endotoxin, lipopolysaccharide (LPS), treatment on the establishment of such forms of conditioned aversion-related disgust behavior. Previous studies have shown that LPS blocks the acquisition of LiCl-induced aversion-related behaviours, such as conditioned gaping behavior in the rodent models for anticipatory nausea and conditioned taste aversion, in male rats (Chan et al., 2009; Cloutier et al., 2012 b; Cross-Mellor et al., 2009). However, until now, these effects had not yet been evaluated in female subjects, even though sex differences in the prevalence and severity of inflammation-related disease exist in the human population.

For example, Women comprise 60% and 80% of the populations suffering from asthma (Tam et al., 2011) and autoimmune disease (Voskuhl, 2011), respectively; and,

are 2-6 x more likely to die from H5N1 Avian influenza, relative to men (Klein, Pekosz, Passaretti, Anker, & Olukoya, 2010). Furthermore, a greater incidence of anxiety/depression-related disorders (for review, see Altemus, Sarvaiya & Epperson, 2014), as well as, neurodegenerative disease (For review, see Rocca, Mielke, Vemuri & Miller, 2014), are observed in women- all of which have been associated with pro-inflammatory pathology (Abbott et al., 2015; Raz, Knoefel & Bhaskar, 2016; Vivekanantham et al., 2015).

In Chapter 2, the rodent model of anticipatory nausea (AN) was employed to examine the ability of female rats to associate "feelings" of disgust with a distinct context and to test the effects of immune system stimulation on the development these conditioned behaviours (e.g. conditioned gaping, paw treading, chin rubbing). As previous studies have shown, LPS pretreatment during the LiCl conditioning phase led to significantly attenuated frequencies of aversion-related disgust behavior, relative to males pretreated with saline prior to LiCl. LPS also significantly attenuated conditioned disgust in LiCl-treated adult female rats. However, females pretreated with saline followed by 128 mg/kg LiCl displayed significantly higher conditioned gaping frequencies when re-exposed to the distinct context, relative to males treated with the same dose of LiCl. To the best of our knowledge, this was the first study to demonstrate a significant sex difference in conditioned disgust using the rodent model of anticipatory nausea.

In order to investigate this sex difference further, in Chapter 3 male and female rats were conditioned with either 0 (saline), 64, 96, or 128 mg/kg and exposed to a distinct context. The results showed a dose-related sex difference, evidenced by significantly higher gaping frequencies in females relative to males in those subjects

treated with either 96 or 128 mg/kg LiCl, but not the 64 mg/kg dose. In this study, not only was the previous sex difference in gaping behaviour replicated, but it was shown to be dose- and sex- dependent.

To date, very few studies have examined sex differences in conditioned disgust, even though human examples strongly suggest that nausea has more of an impact on female health and well-being, relative to males. In Chapter 4, we examined conditioned place avoidance (CPA) behavior in adult female rats. It was found that LiCl, at a dose of 96 mg/kg, conditioned significant avoidant behavior, relative to saline-treated subjects. LiCl-treated females spent significantly less time in the previously drug-paired chamber relative to controls, and relative to the amount of time spent in the previously saline-paired chamber. Also, LiCl-treated rats displayed significantly higher frequencies of locomotor behavior, relative to controls, indicative of agitation or aversion.

Taken together with the findings in Chapter 2, a robust sex difference in the establishment of context-based, LiCl-induced conditioned disgust and conditioned avoidance behaviours has been demonstrated. Females show significantly higher, dose-related increases in conditioning gaping behavior, relative to males, which provides a valuable preclinical tool for evaluating not only the efficacy of anti-nausea medications, but also the noxious side-effects of therapeutic drug treatment, such as, lithium used to treat depression-related mental health disorders.

The effects of LPS on the establishment of CPA were also examined. As predicted, LPS blocked CPA behavior, evidenced by similar lengths of time spent in both chambers that did not differ from saline controls. The third aim of this experiment was to

examine the effects of a single immune challenge with LPS during an important stage of development (peripubertal phase) on the development of CPA behavior in adulthood, as well as, on the response to a subsequent LPS challenge in adulthood. Results showed that a single LPS challenge at 6 weeks of age had no effect on the establishment of CPA in adulthood.

Interestingly, female rats that were treated with LPS at 6 weeks of age and again in adulthood displayed a tolerance-like response the adult challenge. Indeed, female rats treated with LPS at both time points did not lose significant amounts of body weight, relative to rats treated with LPS in adulthood only; and, these subject also did not show significant reductions in locomotor behavior. Furthermore, in female rats that were treated with LPS at 6 weeks of age, LPS treatment in adulthood failed to block the establishment of CPA.

To the best of our knowledge, this was the first study to evaluate CPA learning in female subjects, and the first study to examine the effects of LPS on CPA learning and memory. Results from this experiment support prior evidence (see Blaustein et al., 2011; Blaustein et al., 2013), that suggests that immune challenge during this developmental time period leads to long-lasting changes in behavior, by showing that the acute-phase response and typical memory impairments previously observed following LPS treatment are absent. The measures in this study were behavioral in nature, and hormone concentrations were not measured at any time point, but estrous cycles were tracked and found to not be disrupted by any drug treatment. However, it has been shown that in female Wistar rats that received an adolescent stressor, LPS challenge in adulthood led to increased levels of circulating estrogen (Pyter et al., 2013). In healthy controls, estrogen

was positively correlated to microglial gene expression in the hippocampus, but this relationship was not observed in females who received an adolescent stressor (Pyter et al, 2013). It was suggested that estradiol may have a protective effect against stress-induced neuroinflammation (Pyter et al., 2013), and if so, it is plausible that a second immune challenge with LPS in adulthood may increase estradiol levels without the associated increases in microglial gene expression in the hippocampus — a structure known to play a critical role in the formation of context-based memories.

5.1 Conclusions

In the present thesis, sex differences in the establishment of conditioned gaping in the rodent model of anticipatory nausea and the response to LPS treatment were examined. Furthermore, the effect of LPS pretreatment on CPA learning was evaluated for the first time in female subjects, including the influence of an adolescent (i.e., peripubertal) immune challenge with LPS on subsequent LPS administrations in adulthood.

It was shown that female rats condition significantly stronger conditioned gaping responses to a context previously paired with LiCl, and that this difference is dose-related. LPS disrupted the establishment of conditioned gaping behaviour in both male and female groups, although a ceiling effect cannot be ruled out. Future studies might examine lower doses of LPS in order to determine if sex differences in the response to endotoxin might be present.

Future studies should also evaluate putative changes in circulating estradiol levels following adolescent immune challenge and measure corresponding microglial expression in the brain. Given that anxiety and depression-related disorders are more common in females (see Fitelson & McGibbon, 2016 for review), and that the onset of these behaviours often correlates to adolescence (see Guyer et al., 2016 for review), it follows that this time period might provide a valuable time point at which to test the effects of significant stressors on mental health outcomes in those disorders associated with neuroinflammation, such as, depression and anxiety (Chesnokova et al., 2016), for which sex differences in the population are observed. Furthermore, the effects of a peripubertal immune challenge on the response to LPS treatment in adulthood should be evaluated in males, as male rats have shown exacerbated responses to homotypic challenge if they were treated neonatally with LPS (Tenk et al., 2008).

There is still much to be learned about nausea- and inflammation-induced changes in behavior; however, the current thesis was the first to demonstrate a robust sex difference in the development of conditioned nausea-related behaviour in the rodent model of anticipatory nausea; and, it was the first to examine CPA learning in female Long Evans rats, as well as, the first study to examine the influence of peripubertal and adult immune system stimulation on CPA acquisition.

In the human population, evidence suggests that the observed sex differences in the onset and severity of nausea begin to emerge in adolescence (Lebaron et al., 1988), which indicates the influence of sex steroids. Since it has also been shown that anxiety increases the likelihood of developing Anticipatory Nausea (Andrykowski, 1990) it would follow that significant immune system stimulation during a sensitive period of

development might lead to changes in circulating hormone concentrations, and, in the corresponding immune responses to further immune challenges in adulthood. It is likely that the nausea-associated malaise following LiCl treatment is responsible for the development of conditioned place avoidance in female rats; however, this should be further evaluated using a method that allows for the observation of both chamber dwell times, as well as, specific conditioned disgust behavior, such as gaping.

The current results demonstrate a method with which sex differences in the efficacy of anti-nausea drug treatments can be tested, or the evaluation of noxious drug side-effects. In addition, this thesis has demonstrated that a peripubertal immune challenge with LPS at 6 weeks of age leads to a tolerance-like response to both the acute-phase response and memory impairments typically associated with LPS treatment, and thus provides support for the notion that adolescence presents a critical time point in development when the experience of a stressor may alter the course of future development, leading to lasting changes in behaviour.

5.2 References

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Caylen J. Cloutier

ACADEMIC BACKGROUND

PhD. Candidate Psychology- Behavioural and Cognitive Neuroscience
University of Western Ontario
2011-Present

- Supervisors: Dr. Klaus-Peter Ossenkopp & Dr. Martin Kavaliers

Master of Science (M.Sc.) Psychology- Behavioural and Cognitive Neuroscience
University of Western Ontario
2009-2011

- Thesis Title: Simultaneous Conditioning of Anticipatory Nausea and Taste Avoidance and the Influence of Immune System Stimulation
- Supervisors: Dr. Klaus-Peter Ossenkopp & Dr. Martin Kavaliers

B.A. (Hons.) Specialization in Psychology
University of Western Ontario
2004-2009

- Thesis Title: Administration of Muramyl Dipeptide (MDP) Inhibits the Acquisition of Lithium-induced Anticipatory Nausea in the Rat
- Supervisors: Dr. Shelley Cross-Mellor, Dr. Klaus-Peter Ossenkopp, & Dr. Martin Kavaliers

PROFESSIONAL/ACADEMIC HONOURS/AWARDS

International Behavioral Neuroscience Society (IBNS) Travel Award 2014

- Value: $1000

Natural Sciences and Engineering Research Council of Canada (NSERC)
Alexander Graham Bell Canada Graduate Scholarship 2012-2015

- Value: $105,000 ($35,000/year)

Western Graduate Research Scholarship 2011-2012

- Value: $11,000

ACADEMIC/RESEARCH INTERESTS

Western Graduate Research Scholarship 2010-2011

- Value: $8,000

Western Graduate Research Scholarship 2009-2010

- Value: $8,000

Dean's Honour List 2007-2009

Nomination for Honours Thesis McLelland Award 2009

ACADEMIC/TEACHING EXPERIENCE

- The effects of immune system stimulation on learning and memory
- Neuro-immune and neuro-endocrine interactions
- LiCl-induced conditioned "gaping" behaviour in the rodent model of anticipatory nausea
- LiCl-induced conditioned taste avoidance/aversion
- Intravascular gustatory conditioning
- Sex differences in learning and memory
- Early life infection and the development of psychological disorders
- Evolution of sex differences

Nomination- Teaching Assistant of the Year 2012

Invited Guest Lecture- Intro Psychology March 2011
University of Western Ontario, *Brescia University College*, Instructor: Dr. Cross-Mellor

- Two hour lecture on "The Treatment of Psychological Disorders"

Invited Guest Lecture- Research in Behavioural Neuroscience 2011
University of Western Ontario, *Main Campus*, Instructor: Dr. Cross-Mellor

- One hour lecture on therapeutic uses of drugs related to course

RELATED EXPERIENCE

Undergraduate Honours Student thesis supervision 2011-2014

- Aided in the development of research project
- Gave instruction and hands on aid in the experiment
- Provided feedback on thesis writing

Teaching Assistant- Behavioural Pharmacology 2015
University of Western Ontario, Main Campus

Teaching Assistant – Human Sex Differences 2014
University of Western Ontario, Main Campus

Teaching Assistant- Brain, Behaviour, and Immune System 2014
University of Western Ontario, *Main Campus*

Teaching Asssistant- Honours Thesis Course 2013
University of Western Ontario, Main Campus

- Ran tutorial presentation and graded thesis sections
- 2nd reader for 3 thesis students, graded theses and questioned them at oral presentation

Teaching Assistant- Evolution of Human Behaviour 2012
University of Western Ontario, *Main Campus*

Teaching Assistant- Research in Behavioural Neuroscience 2011
University of Western Ontario, *Main Campus*

- Aided in sheep brain dissections, provided guidance in carrying out rodent experiments and writing scientific reports

Teaching Assistant- Introductory Psychology 2011
University of Western Ontario, *Main Campus*

- Forty-five minute presentation of two review chapters on "Motivation/Emotion" and "Development Through the Lifespan"

Teaching Assistant- Human Sex Differences 2010
University of Western Ontario, *Main Campus*

TECHNICAL/SPECIALIZED SKILLS

Teaching Assistant- Sensation and Perception 2009-2010
University of Western Ontario, *Main Campus*

Graduate level course in Functional Connectivity 2011

- fMRI techniques, introduction to Graph Theory, Diffusion Tensor Imaging

Graduate level course in Statistics 2009

- Analysis of Variance, Hierarchical Linear Modelling, Multiple Regression/Correlation, Factor Analysis

PROFESSIONAL LICENCES/CERTIFICATES

Research Assistant Position Summer 2009

- Ossenkopp/Kavaliers lab
- Conducted multiple experiments:
  - Effects of various ginger solutions on the acquisition of anticipatory nausea; using rotation and the taste reactivity paradigm
  - Effects of acute stress (corticosterone) on the acquisition of anticipatory nausea
  - Performed dissections and drew blood samples for assays
  - Social conditioning of disgust reactions in the rat

VOLUNTEER EXPERIENCE

Undergraduate introductory course in fMRI and ERP design and analysis

- *Instructor*: J. Bruce Morton, University of Western Ontario, 2008
- Focus on pre- and post-processing of fMRI and ERP data from a cognitive developmental perspective

AFFILIATIONS

Work Study Position in ERP Lab

- Participant recruitment and EEG testing of adolescent population in ERP study
- *Ph.D. Candidate Researcher*: Matt Waxer, University of Western Ontario, 2008

PUBLICATIONS

I) REFEREED JOURNALS

- Experienced with statistical analyses program SPSS

- Sigma Plot graphing program
- OriginLab graphing program
- Noldus Observer 5.0 program
- Noldus Ethovision
- Microsoft Office
- Versamax

University of Western Ontario Animal Care and Veterinary Services Certification

- WebCT Animal Care & Use Course
- Basic Rat training
- Experience with intraperitoneal and subcutaneous injection techniques
- Supported by the Canadian Council on Animal Care, 2008

Psychology Graduate Student Association Executive Committee 2010-2011

- Organization of incoming graduate student orientation
- Planning psychology graduate student events and fundraisers

Brain Bee Competition 2010-2011

- Helped with organizational meetings and preparations for the event

International Behavioral Neuroscience Society Member 2014 - present

Society for Neuroscience Member 2009 - present

Cloutier, C.J., Kavaliers, M., Ossenkopp, K.-P. (2015). The effects of immune system stimulation with the bacterial endoxtoxin lipopolysaccharide (LPS) on learning and memory in rodent models of disgust: a review. *In preparation*.

Cloutier, C.J., Kavaliers, M., Ossenkopp, K.-P. (2015). Adolescent immune challenge with lipopolysaccharide (LPS) to tolerance to

II) PUBLISHED ABSTRACTS

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Cloutier, C.J., Kavaliers, M., Ossenkopp, K.-P. (2015). Sex- and Dose-dependent differences in the establishment of LiCl-induced anticipatory nausea. *In preparation.*

Kavaliers, M., Banasikowski, T.J., **Cloutier., C.J.**, Ossenkopp, K.-P. (2014). Repeated exposure of male mice to low doses of lipopolysaccharide: dose and time dependent development of behavioral sensitization and tolerance in an automated light-dark anxiety test. *Behavioural Brain Research, 286*, 241-8.

Kavaliers, M., Colwell, D.D., **Cloutier, C.J.**, Ossenkopp, K-P., Choleris, E. (2014). Pathogen threat and unfamiliar males rapidly bias the social responses of female mice. *Animal Behaviour, 97*, 105-111.

Cloutier, C.J., Kavaliers, M., and Ossenkopp, K.-P. (2012). Lipopolysaccharide inhibits the simultaneous establishment of LiCl-induced anticipatory nausea and intravascularly conditioned taste avoidance in the rat. *Behavioural Brain Research, 232,*278-286.

POSTER PRESENTATIONS

Cloutier, C.J., Rodowa, M.-S., Chan, M.Y.T., Cross-Mellor, S.K., Kavaliers, M., and Ossenkopp, K.-P. (2012). Inhibition of LiCl-induced conditioning of anticipatory nausea in rats following immune system stimulation: comparing the immunogens lipopolysaccharide, muramyl dipeptide, and polyinosinic: polycytidylic acid. *Physiology & Behavior, 106*, 243-51.

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Ossenkopp, K.-P., Biagi, E., **Cloutier, C.J.**, Chan, M.Y.T., Kavaliers, M., Cross-Mellor, S.K. (2011). Acute corticosterone increases conditioned spontaneous orofacial behaviors but fails to influence dose-related conditioned “gaping” responses in the rodent model of

PROFESSIONAL PRESENTATIONS

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Cloutier, C.J., Kavaliers, M., & Ossenkopp, K.-P. Conditioned disgust and social behaviour in rats treated with the toxin LiCl. Program No. 401.05. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

Cloutier, C.J., Cross-Mellor, S.K., Kavaliers, M., & Ossenkopp, K.-P. The effects of the bacterial immune stimulant lipopolysaccharide (LPS) on the simultaneous acquisition of anticipatory nausea and conditioned taste avoidance. Program No. 407.22. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Cloutier, C.J., Cross-Mellor, S.K., Kavaliers, M., & Ossenkopp, K.-P. Simultaneous conditioning of anticipatory nausea and taste avoidance in the rat. Program No. 806.7. 2010 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2010. Online.

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Cloutier, C.J., Kavaliers, M., & Ossenkopp, K.-P. Sex differences in the acquisition of conditioned disgust behaviour in the rat. Presented at the International Behavioral Neuroscience Society Meeting (Victoria, BC).

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Cloutier, C.J., Ossenkopp, K.-P., & Kavaliers, M. Social modulation of LiCl-induced “disgust” responses in rats. Presented at the International Behavioral Neuroscience Meeting (Malahide, Ireland).

Cloutier, C.J., Cross-Mellor, S.K., Kavaliers, M., & Ossenkopp, K.-P. (2010). Simultaneous conditioning of anticipatory nausea and taste avoidance in the rat. Presented at the International Behavioral Neuroscience Meeting (Sardinia, Italy).

Frewen, P.A., Evans, B., Lanius, R., Moran, G., Goodman, J., Boylan., J, & **Cloutier, C.J.** (2009). Development of a retrospective computerized attachment & relational trauma scale. Presented at the CIHR NET Research in Family Violence meeting (Toronto, ON).

Travel Award Winner Mini Data Blitz: The effects of immune system stimulation on toxin (LiCl)-induced conditioned place avoidance in the female rat

- Presented at the 2014 International Behavioral Neuroscience Society meeting in Las Vegas, USA