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The Effects of Neonatal Immune System Activation with Lipopolysaccharide on Adolescent and Adult Anxiety Behaviours in Male and Female Rats

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

THE EFFECTS OF NEONATAL IMMUNE SYSTEM ACTIVATION WITH LIPOPOLYSACCHARIDE ON ADOLESCENT AND ADULT ANXIETY BEHAVIOURS IN MALE AND FEMALE RATS

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ABSTRACT

The present study examined the effects of neonatal (postnatal days 3 and 5) acute immune system activation with lipopolysaccharide (LPS) on adolescent and adult anxiety behaviours. The major findings suggest that neonatal LPS does not have general long-lasting effects on adolescent anxiety and locomotor behaviours. Rather, early endotoxin treatment has highly specific effects on certain anxiety behaviours that vary depending on the development period. Moreover, neonatal LPS does not seem to influence adult voluntary and non-voluntary locomotor activity or anhedonia, independent of, or in response to, an adult immune challenge. Finally, sex differences were observed in various responses in adulthood, independent of neonatal drug treatment. The findings of this study provide a better understanding of adolescent and adult behavioural outcomes in response to endotoxin, and suggest that early exposure to pathogens may not be a significant risk factor in the general development of later anxiety disorders.

Keywords: immune activation, endotoxin, lipopolysaccharide, neonatal, adolescence, adult, sex differences, anxiety, locomotor activity, acoustic startle response, anhedonia

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CHAPTER 1

General Introduction

1.1 General Introduction

Early life environment plays a critical role in shaping an organism's physiology and behaviour throughout the life span. Environmental events during the pre- or early post-natal developmental period may lead to physiological and biological changes, as well as alterations in the predisposition to pathology throughout development (McEwen, 2003). In humans, adverse and stressful experiences such as abuse, neglect, trauma and infection can increase susceptibility for psychopathology, including depression, anxiety, and drug abuse and addiction (Gilmer & McKinney, 2003; McEwen, 2003; Heim & Nemeroff, 2001). Rodent models are often used to examine the effects of early adverse life events on adult physiology and behaviour. Early stressful life events such as environmental temperature (Young, Weiss, & Boufath, 2002), maternal separation (daily, repeated removal of pups from mother for a length of time over the first weeks of life) (Plotsky & Meaney, 1993; Kalinichev, Easterling, Plotsky, & Holtzman, 2002), neonatal isolation (Knuth & Etgen, 2005; 2007) and handling (Durand, Sarriaeu, Aguere, Mormede, & Chaouloff, 1998) can induce long-lasing alterations in physiological and behavioural processes. These changes include increased stress response of the hypothalamic-pituitary-adrenal (HPA) axis (Kehoe, Shoemaker, Triano, Callahan, & Rappolt, 1998; Liu, Caldji, Sharma, Plotsky, & Meaney, 2000; Walker, Hodyl, Krivanek, & Hodgson, 2006), increasing levels of anxiety-like behaviour (Kalinichev et al., 2002; Daniels, Pieterson, Carstens, & Stein, 2004; Walker, March, & Hodgson, 2004b; Knuth & Etgen, 2007) and enhanced sensitivity to drugs of abuse (Kosten, Sanchez, Zhang, & Kehoe, 2004; Kikusui, Faccidomo, & Miczek, 2005).

The early postnatal period is a challenging developmental time period with regard to exposure to pathogens. The immunological system involved in the regulation of bacterial activity is not fully developed in early life (Walker, Hodyl, Krivanek, & Hodgson, 2006). Given the underdeveloped state of the neonatal immune system, a neonate has a potentially increased risk of developing a bacterial infection. Furthermore, there are bidirectional interactions between the immune and endocrine systems. Proinflammatory cytokines produced in response to immune system activation result in a response in the HPA axis, and hormones such as glucocorticoids produced in the endocrine system modulate immune system function (Imura & Fukata, 1994). As such, alterations in one system during development may have consequences for the other system and these effects may extend to adulthood (Tenk, 2007).

Accumulating evidence has emphasized the role of early bacterial and viral exposure on long-term physiological and behavioural processes. In humans, prenatal exposure to infection has been implicated in an increased risk of neurodevelopmental disorders such as schizophrenia (Brown, 2006; Ashdown, Dumont, Ng, Poole, Boksa, & Luheshi, 2006; Gilmore, Jarskog, Vadlamudi, & Lauder, 2004) and autism (Taylor & Rogers, 2005). Results of animal studies have also demonstrated an enhanced vulnerability to psychopathology in adulthood following an early life immune challenge. For example, exposure to pathogens prenatally results in deficits in behaviours relevant to schizophrenia (Shi, Fatemi, Sidwell, & Patterson, 2003; Fortier, Joober, Luheshi, & Boksa, 2004; Fortier, Luheshi, & Boksa, 2007). Furthermore, prenatal immune activation has been shown to result in increased susceptibility to drug abuse (Liu, Lee, Yee, Bresee, Poland, & Pechnick, 2004).

More recent research has demonstrated a need to more thoroughly consider the effects of neonatal infections on later physiology and behaviour. For example, neonatal bacterial exposure has been indicated to result in adult deficits in behaviour implicated in autism and schizophrenia, such as reductions in social interaction (Tohmi, Tsuda, Watanabe, Kakita, & Nawa, 2004). This research, as well as other studies examining the risk of psychopathology following neonatal immune activation, is of particular interest given the vulnerability of the neonatal developmental period with regard to pathogen exposure.

The endotoxin lipopolysaccharide (LPS) is the active component of the cell wall of Gram-negative bacteria, and stimulates the immune and endocrine systems. LPS administration activates the peripheral immune cells, monocytes and macrophages (Dantzer, 2001), and results in the release of proinflammatory cytokines such as interleukin (IL)-1β, tumor-necrosis-factor (TNF)-α, and interleukin (IL)-6. These cytokines target sites within the central nervous system and elicit a response mimicking infectious illness by producing a variety of physiological and behavioural symptoms. This characteristic set of adaptive responses is known as the acute phase sickness response. Such responses include fever (Gaykema, et al., 1998) and reduction in food (Hart, 1988) and water intake (Cross-Mellor, Kent, Kavaliers, & Ossenkopp, 2000). Additionally, LPS administration leads to hypoactivity (Engeland, Kavaliers, & Ossenkopp, 2003a; Franklin, Engeland, Kavaliers, & Ossenkopp, 2003) decreased sexual activity (Avitsur, Pollak, & Yirmiya, 1997) and exploratory behaviour (Nava, et al., 1997) and release of the stress-related hormone corticosterone as the end product of activation of the hypothalamic–pituitary–adrenal (HPA) axis (Nakano,

Suzuki, & Oh, 1987; Tenk, Kavaliers, & Ossenkopp, 2008). Sickness behaviour is considered to be the expression of an organized and adaptive strategy to combat invading pathogens and increase the likelihood of survival (Dantzer, 2001). Early exposure to LPS induces the same set of acute sickness responses observed in adulthood, including anorexia, hypoactivity, and activation of the HPA axis (Walker, Brogana, Roger, & Hodgson, 2004a; Dent, Smith, & Levine, 1999).

Immune system functioning has been shown to be sexually dimorphic in both adults and neonates. Generally, adult females show enhanced functioning of both humoral- and cell-mediated immunity relative to males (Gaillard & Spinedi, 1998). These effects are mediated by gonadal hormones, as estrogen exerts immune-enhancing effects (Friedman, Netti, & Schreiber, 1985; Giglio, et al., 1994), whereas testosterone has immunosuppressive effects (Klein, 2000; Roden, et al., 2004). Furthermore, females demonstrate greater basal levels of corticosterone and display greater and more rapid increases following stressors such as LPS administration (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Kant, Lenox, Bunnell, Mougey, Pennington, & Meyerhoff, 1983). Similar sex differences in the acute response to neonatal LPS exposure have also been observed. Consistent with adult findings, female neonates demonstrate greater HPA axis activation, as well as greater levels of adrenocorticotropic hormone and corticosterone in response to neonatal LPS administration (Shanks, McCormick, & Meaney, 1994).

A relatively smaller number of studies have explored the effects of early immune system activation on adult physiological and behavioural processes. During the neonatal period rodents demonstrate a very weak stress response following exposure to stressful stimuli (Vazquez, 1998). This period is considered the "stress hyporesponsive period" and coincides with postnatal days 4/5 to postnatal day 12. The HPA axis response is limited, including a reduction in the release of corticosterone and adrenocorticotropic hormone (Vazquez, 1998; Tenk, 2007). With regard to neonatal LPS administration, studies often use one of two different treatment protocols. The first protocol involves two administrations of LPS on postnatal days 3 and 5 (e.g. Shanks, McCormick, & Meaney, 1994; Breivik, Stephan, Brabant, Straub, Pabst, & von Hörsten, 2002; Walker, March, & Hodgson, 2004a) and is termed the "dual-exposure-to-endotoxin" (DEE) model. The first exposure to LPS on postnatal day 3 is prior to the onset of the stress hyporesponsive period, and therefore pups exhibit an increased HPA axis response to endotoxin (Shanks & Meaney, 1994; Witek-

Janusek, 1998). HPA activation following a single neonatal endotoxin injection persists for slightly less than 48 hr, and therefore, a second injection on postnatal day 5 induces an immune system activation that spans postnatal days 3 to 7 of life (Shanks & Meaney, 1994). The second neonatal endotoxin exposure regime involves a single administration of LPS on postnatal day 14, following the conclusion of the stress hyporesponsive period (Boisse, Mouihate, Ellis, & Pittman, 2004; Spencer, Heida, & Pittman, 2005; Spencer, Boisse, Mouihate, & Pittman, 2006a).

Examination of the long-term effects of neonatal immune system activation reveals a variety of physiological changes in adulthood. For instance, the DEE model induces heightened responsivity to different forms of stress (Shanks et al., 1995; Shanks, et al., 2000), as well as increased disease severity (Breivik et al., 2002) and impaired tumor resistance in adulthood (Hodgson, Knott, & Walker, 2001). LPS administration in later neonatal development also produces changes in adulthood, although these effects are not necessarily the same as those observed with the DEE model. For example, exposure to LPS on postnatal day 14 has been shown to result in immune system tolerance in adulthood. Rodents demonstrate a decrease in cytokine release when administered LPS on postnatal day 14 (Ellis, Mouihate, & Pittman, 2005), as well as an attenuation of the febrile response following LPS in adulthood (Boisse, Mouihate, Ellis, & Pittman, 2004; Spencer, Boisse, Mouihate, & Pittman, 2006a). Several studies have also examined the long-term behavioural consequences of neonatal LPS exposure, and have demonstrated alterations in anxiety-related behaviour. However, results from studies utilizing the DEE model, as well as those with later neonatal exposure, show that anxiety-related behaviour is test specific, with increases seen on some tests but decreases on others (Breivik et al., 2002; Walker, March, & Hodgson, 2004b; Spencer et al., 2005).

Given the conflicting results, as well as the limited research regarding several areas of early immune activation, it is of interest to examine the effects of early immune system activation at various developmental periods. Moreover, given the sexual dimorphism in immune system functioning and response to endotoxin, an investigation of possible sex differences in these effects is warranted. Thus, the present thesis conducted a detailed examination of neonatal immune system activation with LPS using the DEE model on adolescent and adult behaviour in male and female Long-Evans rats. This thesis investigated behaviours that have previously been shown to be altered following other adverse neonatal

manipulations, such as endotoxin exposure and maternal separation, as well as possible sex differences in these effects.

The first study presented in this thesis explored the effects of neonatal LPS treatment using the DEE model on adolescent anxiety-related behavioural responses. Previous research has demonstrated inconsistent results in terms of alterations in immune functioning and behaviour, and there is limited research examining the consequences of early immune system activation in adolescence. Given the purported anxiogenic effects of acute LPS administration (Lacosta, Merali, & Anisman, 1999; Nava & Carta, 2001), anxiety-related changes are of particular interest. Thus, this study used multiple measures of anxiety-related behaviours including the elevated plus maze (Moser, 1989; Rodgers & Dalvi, 1997; Walf & Frye, 2007), open-field (Ossenkopp & Kavaliers, 1996), and light dark tests (Crawley & Goodwin, 1980). These experiments were designed to further investigate the effects of early immune system activation on anxiety during a significant developmental period, as well as possible sex differences in these responses.

The second study described in Chapter 3 explored the effects of neonatal LPS adminstration on anxiety-related behavioural responses in adulthood. Previous research has shown altered adult immune functioning following DEE, but findings have been inconsistent (Breivik et al, 2002; Walker et al., 2006). Furthermore, there is limited research investigating the effects of early immune system challenge on non-voluntary sensorimotor reflexes. As such, the second experiment examined the effects of neonatal LPS administration on adult anxiety-related behaviours, unaccompanied by additional manipulations, as well as following an additional immune system challenge with LPS. This study used multiple indices of anxiety, including the elevated plus maze (Moser, 1989; Rodgers & Dalvi, 1997; Walf & Frye, 2007), light dark tests (Crawley & Goodwin, 1980) and taste neophobia (Merali, Levac, & Anisman, 2003; Dulawa & Hen, 2005). Additionally, the acoustic startle response and PPI (Lockey, Kavaliers, & Ossenkopp, 2009) were utilized as novel behavioural measures of the effects of early immune manipulation on non-voluntary motor activity. Possible sex differences in these anxiety responses were also explored.

The results of the current thesis provide a detailed examination of the effects of neonatal (DEE) LPS treatment on behaviour throughout development, including the behavioural consequences during adolescence and adulthood. Furthermore, possible sex differences in these outcomes are also investigated. The findings presented in this thesis lead

to a better understanding of potential risk factors involved in early endotoxin exposure, such as bacterial infection with Gram negative bacteria. These studies have clinical implications for the effects of early bacterial exposure on later infections at various developmental periods, as well as the development of anxiety-disorders.

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CHAPTER 2

Effects of Neonatal Immune System Activation with Lipopolysaccharide on Adolescent Anxiety Behaviours

2.1 Introduction

Events occurring during development, such as early immune system activation can result in long term neurological and behavioural changes in adulthood. Administration of the endotoxin lipopolysaccharide (LPS) results in the stimulation of the immune and endocrine systems and the release of proinflammatory cytokines to produce physiological and behavioural symptoms known as the acute phase sickness response (Dantzer, 2001). This behavioural suite enables an organism to combat invading pathogens and increase likelihood of survival. For example, hyperthermia is an adaptive response that provides a hostile, antimicrobial environment for unwanted pathogens (Romanovskya & Székely, 1998) and reductions in food and water intake reduce the availability of nutrients, thereby preventing pathogen growth (Tenk, Kavaliers, & Ossenkopp, 2008). In addition, there is a sexual dimorphism in adult immune system function, with immune-enhancing effects of estrogen resulting in enhanced functioning in females (Gaillard & Spinedi, 1998).

Recent studies have begun to explore the acute and long term effects of early immune activation in rats. Early neonatal exposure to LPS induces the same set of acute sickness responses in rat pups as seen in adulthood, including anorexia, hypoactivity, and activation of the HPA axis (Walker, Brogana, Roger, & Hodgson, 2004a; Dent, Smith, & Levine, 1999). Furthermore, consistent with adult findings, female rat pups exhibit greater HPA axis responses to an endotoxin challenge relative to males (Shanks, McCormick, & Meaney, 1994).

In order to activate the immune system during the neonatal period, the dual-exposure-to-endotoxin (DEE) model is utilized, which involves the administration of LPS on postnatal days 3 and 5 (Spencer, Martin, Mouihate, & Pittman, 2006; Tenk, 2007). Results of several studies have shown a variety of physiological and behavioural changes in adulthood following neonatal immune system activation. For instance, neonatal LPS exposure has been shown to lead to to elevated IL–6 levels and increased susceptibility to periodontal disease in adulthood (Breivik, Stephan, Brabant, Straub, Pabst, & von Hörsten, 2002), as well as lowered natural killer cell activity in adult male rats (Hodgson, Knott, & Walker, 2001). However, LPS treatment on postnatal days 3 and 5 has also been shown to reduce inflammation and the development of arthritis in adulthood (Shanks, et al., 2000), as well as attenuate febrile response following adult endotoxin challenge (Walker, Hodyl, Krivanek, & Hodgson, 2006).

Anxiety is an affective state, characterized by heightened physiological and behavioural arousal in response to perceived or true threats (Gray & McNaughton, 2003). Such responses include increased blood pressure and heart rate, increased avoidance behaviour, and increased checking behaviour, which serves as risk assessment behaviours in potentially threatening situations (Misslin & Cigrang, 1986). These reactions are analogous in humans and animals, and as such, provide the potential to develop animal models to assess anxiety-related behaviour (Rodgers, Cao, Dalvi, & Holmes, 1997).

Various rodent models of anxiety aim to induce a natural conflict situation, in which an animal is exposed to potential stressors such as a novel situation. The conflict arises between the tendency to avoid the unfamiliar (neophobia) and the tendency to explore a new environment (Bourin & Hascoet, 2003). Rodents have a proclivity toward dark, enclosed spaces, and an innate fear of open, well-lit areas (Walf & Frye, 2007). Thus, brightly illuminated areas and open spaces are appraised as potentially threatening situations and produce unconditioned aversive emotional reactions in rodents (Walsh & Cummins, 1976). Measures of anxiety-related behaviour are determined from this preference for dark areas, equated with protection and safety, with an animal's natural tendency to remain in safe areas related to the level of anxiety (Crawley & Goodwin, 1980). Tests such as the elevated plus maze and the light-dark test consist of aversive, brightly lit areas (open arms and light chamber) and dark, enclosed areas (closed arms and dark chamber). Anxiety is measured as the duration of time spent avoiding illuminated areas and remaining in the dark areas. Both the elevated plus maze and light-dark test have been shown to be valid measures of anxiety. Time spent in the open arms and light chamber is reduced when rodents are given anxiogenic drugs, but increased with the use of anxiolytics meant to alleviate anxiety (Bourin & Hascoet, 2003; Walf & Frye, 2007).

Similarly, a novel environment produces an anxiety-provoking situation and often leads to defensive reactions, which may be in conflict with the desire for exploration (Misslin & Cigrang, 1986). A measure of locomotor activity in a novel environment such as the open-field test is indicative of an animal's level of anxiety, with reduced activity and greater freezing behaviour suggestive of greater anxiety. Furthermore, because the novel environment is aversive to rodents, thigmotaxis is often observed, where activity is confined to the periphery (Prut & Belzung, 2003). This tendency to avoid the center areas is part of a natural defence mechanism to avoid predators, as it is more difficult to attack a thigmotaxic

rodent than one out in the open (Treit & Fundytus, 1988). Thus, measures of locomotor activity and thigmotaxis behaviour are used as other indicators of anxiety-related behaviours, and have been shown to be valid measures due to their sensitivity to anxiogenic and anxiolytic drugs (Ramos, 2008; Treit & Fundytus, 1988; Misslin & Cigrang, 1986).

Anxiety-related behaviour in rodents such as rats is modified by several factors, including developmental stage. Adolescence is a transitional developmental period between childhood and adulthood, during which animals undergo neurological and behavioural alterations (Spear, 2000). This developmental period encompasses a broad age range, which can begin shortly after weaning and last until early adulthood. Furthermore, these changes can vary depending on sex, with early adolescent onset occurring in female rats as early as 20 days, and lasting until as late as day 55 in male rats (Spear, 2000). Most researchers often determine the adolescent period in rats to range from postnatal days 28–42, a conservative age range during which animals of both genders tend to exhibit adolescent-typical neurobehavioural characteristics (Spear, 2000). Research has indicated that typically developing adolescent rats show predominantly more risk-taking behaviour and less anxiety-related behaviour than adults (Stansfield & Kirstein, 2006). This increase in sensation seeking associated with adolescence provides the opportunity to explore new situations and concurrent behaviours, necessary for appropriate development and maturation into adulthood (Spear, 2000). For example, adolescent animals have been shown to have greater novelty-induced locomotor activity, greater preference for novel objects, and greater approach and exploratory behaviours as compared to adult animals (Stansfield & Kirstein, 2006). Additionally, anxiety measures, such as those observed in the elevated plus maze, tend to be less evident in adolescence compared to adulthood (Doremus, Varlinskaya, & Spear, 2004). For instance, adolescent male rats have been shown to exhibit less anxiety-like behaviour than adult males in the elevated plus maze (Schramm-Sapyta et al., 2007; Andrade et al., 2003), the open-field test (Masur, Schutz, & Boerngen, 1980; Meyza, Boguszewski, Niko, & Zagrodzka, 2011), and the light-dark test (Schramm-Spyta et al., 2007). Similar results have been observed for females, with adolescent rats showing reduced anxiety in the elevated plus maze (Genn, Tucci, Thomas, Edwards, & File, 2003; Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993) and open-field (Masur, Schutz, & Boerngen, 1980) compared to adults.

Sex differences in anxiety-related behaviour have been found in both adult and adolescent rats, but are not always observed in a consistent direction. Adult female rats

(across the estrous cycle) have shown less anxiety-like behaviour than males in the elevated plus maze (Zimmerberg & Farley, 1993; Johnston & File, 1991) and the light-dark test (Hughes, Desmond, & Fisher, 2004). Furthermore, adult male show higher degree of anxiety-like behaviour in the defensive withdrawal test compared to females (Romana & Arborelius, 2009). In contrast, adult females show greater anxiety in a predator threat test (Meng & Drugant, 1993) and open-field test (Meng & Drugant, 1993; Archer, 1975). There is some evidence of sexual dimorphism in adolescence. For example, female rats show less anxiety-related behaviour than males during late adolescence in the open field (Masur, Schutz, & Boerngen, 1980) and the elevated plus maze (Genn et al., 2003; Imhof et al., 1993). However, such patterns vary across studies and developmental age, and there is a lack of evidence suggesting consistent sex differences in anxiety-related behaviour during adolescence.

Given the purported anxiogenic effects of acute LPS exposure, it is of particular interest to examine the behavioural consequences of neonatal endotoxin exposure on anxiety-related behaviours. Neonatal LPS administration has been shown to increase adult anxiety behaviour in the light-dark test (Lacosta, Merali, & Anisman, 1999) and elevated plus maze (Walker, March, & Hodgson, 2004b). Other findings have shown that early LPS exposure had no effect on anxiety-realted behaviours, such as activity in the open-field test (Breivik et al., 2002). Furthermore, very few studies to date have examined the influence of neonatal LPS on anxiety during adolescence. For instance, neonatal (DEE) LPS exposure was found to increase anxiety-related behaviours in the elevated plus maze in adulthood in male rats, but had no effect on anxiety in adolescence (Walker, March, & Hodgson, 2004b). Neonatal LPS treatment (DEE) has also been shown to reduce anxiety-related behaviours in the open-field and elevated plus maze, and led to increased exploratory behaviour in response to novelty (Rico, Ferraz, Ramalho-Pinto, & Morato, 2010). These studies suggest that the effects of neonatal LPS on anxiety-related behaviours may be complex and may have varying effects at different developmental stages.

Adolescence is a period of significant brain development, and early-life stress has long-term neurological and psychological consequences. Given the relatively small amount of research, as well as inconsistent results, the aims of the present study were to further investigate the effects of neonatal LPS exposure on anxiety-related behaviours in adolescent rats. The influence of neonatal LPS treatment using the DEE model on anxiety was analyzed

through observations from the elevated plus maze, open-field, and light dark tests. Given the scarcity of research examining the effects of neonatal LPS on adolescent anxiety-related behaviours, anxiety measures were collected during this critical period. Further examination of possible sex differences in anxiety responses, as well as possible interrelationships between variables were also considered.

2.2 Methods

2.2.1 Animals

Eleven primiparous female Long-Evans rats weighing approximately 250-300 g were mated with male Long-Evans rats (300-400 g, Charles River, Canada) for a total of 11 litters. Females were paired with a male overnight the night prior to behavioural estrous, identified through vaginal smear. Sperm present on a vaginal smear (hematoxylin and eosin stain) the morning after pairing indicated successful mating and this was designated as gestational day 0 (G 0). Dams were housed individually in standard polypropylene cages (45 x 22 x 20 cm) in a temperature-controlled colony room (21 ± 1 ^{o}C), and maintained on a 12:12 light – dark cycle with the lights on at 07:00 hours. Food (Prolab rat chow) and tap water were available ad libitum. Cages were checked daily until the birth of the pups and subjects were derived from a single litter from each dam. Litters (litter size ranged between 11 – 19 pups) were born on G 22 (designated as postnatal day (P) 0), toe-clipped for identification on P1, and were not culled until weaning at P 21 (M = 15.73 pups, SD = 2.24). On P21, pups were weaned and culled to a maximum of 8 animals/litter (4 males, 4 females). Following weaning, rats were pair-housed with same-sex, same-treatment littermates in standard polypropylene cages under the same conditions as dams, with behavioural testing beginning on postnatal day 38. Experimental manipulations were conducted 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 (CC guidelines).

2.2.2 Neonatal drug administration

On postnatal days 3 and 5, rat pups were injected intraperitoneally (i.p.) using a Hamilton syringe with a 30 gauge needle tip. Within a litter, two pups of each sex were randomly assigned to each treatment condition. Treatment consisted of administration of either 50 μg/kg LPS (derived from *Escherichia Coli* stereotype 0111:B4, L26030, Sigma Chemical, St. Louis, MO, USA) dissolved in 0.9% isotonic saline (NaCl; 1 μL/g), or an

equivalent volume of 0.9% saline solution on each of the two postnatal days (male-LPS *n* = 23; male-NaCl *n* = 23; female-LPS *n* = 21; female-NaCl *n* = 19). This dose and administration schedule follows a similar procedure to previous studies, and has shown it to induce long-lasting behavioural changes in adult rats (Shanks et al., 1995; Breivik et al., 20002; Walker et al., 2004b; Tenk, 2007). During the injection procedure, the entire litter was removed from the home cage and placed under a heat-lamp for the duration of the injections, approximately 15 min per litter.

2.2.3 Behavioural testing

A maximum of 8 rats from each litter was put through the battery of tests (4 males – 2 neonatal LPS, 2 neonatal NaCl; 4 females – 2 neonatal LPS, 2 neonatal NaCl per litter) beginning on postnatal day 38.

2.2.3.1 Elevated plus maze (EPM). The EPM apparatus was made of wood and painted grey with washable paint. It consisted of two open arms (54 x 12 cm) with no sides or ends orthogonal to two opposed arms of the same size, enclosed with sides and ends (54 x 12 x 48 cm). The apparatus was raised 50 cm above the floor, with the four arms extending from a center platform (12 x 12 cm). An overhead camera, connected to a television and DVD-R, recorded behaviour for later scoring.

Testing took place on the afternoon of P 38. Animals were placed on the center platform facing an open arm to begin the test. Animals were allowed to freely explore while being recorded for 5 min. After each animal, the maze was cleaned with a 20% alcohol solution.

Measures assessed included number of entries into open and closed arm and time spent (s) on open and closed arms. Percent time spent in open arms (time spent in open arms/time spent in open arms + time spent in closed arms x 100) was taken as a measure of anxiety. In addition, risk assessment behaviour was analyzed, as this provides an ethologically based assessment of anxiety; the more time an animal spends assessing the environment for potential danger, the more anxious the animal. Risk assessment was measured as: number of, and time spent (s) engaging in head stretch attends and body stretch attends (head stretching into open arms while forepaws and body remain in the closed arm or forelimbs exit the closed arm and body stretches into open arms while hindlimbs remain in the closed arm, respectively); head dips (head reaching downwards below arms towards the

floor); and double backs (exiting an arm with the forepaws and re-entering the same arm with the forepaws).

2.2.3.2 Open-field apparatus. Locomotor activity in a novel open field was monitored using eight Versamax Animal Activity Monitors (AccuScan Model DCM-8, Columbus, OH, USA). Each apparatus consists of a Plexiglas open field chamber (40 cm x 40 cm x 30.5 cm), a Plexiglas lid with air holes, and infrared beams surrounding the chamber to record horizontal and vertical locomotor movements as beam breaks (Ossenkopp & Kavaliers, 1996). There were 16 infrared beam sensors on each side (2.54 cm apart, 4.5 cm from the floor) to measure horizontal movements, and 16 upper beams located 15 cm above the chamber floor on two opposite sides to measure vertical movements. Additionally, the VersaMax software separated the open-field into discrete periphery (7.5 cm wide border) and center (30 x 30 cm square) zones to measure thigmotaxis (tendency of animals to stay close to the walls, an indication of anxiety).

Animals were placed in the open-field for 60 min (6 - 10 min time bins) on P 39, which was measured as baseline to a novel environment, and P 40 to assess locomotor activity in the now familiar open field. Horizontal activity measures analyzed were: total distance (TD) − total horizontal distance (cm); horizontal movement time (MT) − amount of time (s) an animal was engaged in horizontal movement; number of horizontal movements (NM) − number of horizontal movements separated by 1 s stop time. The vertical activity measure analyzed were: the number of vertical movements (VM) − number of vertical movements separated by 1 s stop time; vertical movement time (VT) – amount of time (s) an animal spent in a vertical position. Number of entries and duration of time spent in the periphery and center (horizontal and vertical movements) were also recorded.

2.2.3.3 Light-dark apparatus. A black Plexiglas box (40 cm x 20 cm x 30 cm) was inserted into each VersaMax Animal Activity Monitor, dividing the open-field into two equal size chambers; a “dark” chamber and a “light” chamber. The sides of the black insert contained small holes to prevent obstruction of the photo-beams. Animals were allowed unrestricted access though a 10 x 8.5 cm doorway centered along the insert wall. Animals were placed in the center of the light region and allowed to move freely between the two compartments for 30 min on P 41. Time spent in the light and dark chambers (duration; s) was analyzed, as well as number of entries into each chamber (entries), defined as a beam break once an animal moves its head through the chamber doorway. Locomotor activity was

assessed (TD, VM, VT) in both the light and dark chambers and was corrected for time spent in the corresponding chamber.

2.2.4 Procedure

As previously described, 4 male and 4 female pups from each litter were given i.p. injections of either LPS or saline on P 3 and P 5 of birth. The developmental milestone of eye opening was monitored on P 12-16 and pups were weaned on P 21. Body weight was monitored on injection days, and measured weekly throughout the testing process, as well as each day of behavioural testing. Behavioural testing occurred during adolescence (postnatal days 38-41). See Figure 2.1 for a timeline of the behavioural procedure.

On P 38 animals were placed in the elevated plus maze and recorded for 5 min. Animals were monitored in the open-field apparatus on P 39 for 60 min in order to habituate them to a novel environment, and then again on P 40 to assess any changes in locomotor activity. Finally, animals were placed in the light-dark apparatus on P 41 for 30 min in order to measure anxiety based on preference for dark places. Animals were left undisturbed following the final testing day, with the exception of body weight measurement once weekly.

2.2.5 Statistical analysis

All analyses were performed with IBM SPSS Statistics 19 (formerly PASW Statistics 18). Significance was set to $\alpha = .05$.

2.2.5.1 Eye opening. Eye opening was analyzed using a mixed design (split plot) analysis of variance (ANOVA), with the between subjects factors of Sex, Neonatal Drug (2 levels: LPS, NaCl), and Litter (11 levels), and the within subjects factor of Day (5 levels). Where appropriate, LSD post-hoc comparisons were performed.

2.2.5.2 Elevated plus maze (EPM). EPM data were analyzed using a multivariate analysis of variance (MANOVA), with the between subjects factors of Sex, Neonatal Treatment (2 levels: LPS, NaCl), and Litter (11 levels). Post-hoc comparisons of significant effects were performed where appropriate using LSD test.

2.2.5.3 Open-field, thigmotaxis, and light-dark tests. Behavioural data collected during the open-field, thigmotaxis, and light-dark tests were analyzed using a mixed design (split-plot) analysis of variance (ANOVA) with between-subjects factors of Sex (2 levels: male and female), Neonatal Treatment (2 levels: LPS or saline) and Litter (11 levels). The within subjects factors were Day and/or Time, which varied depending on the behavioural test being analyzed. For the open-field and thigmotaxis tests, there were 2 levels of Day and

6 levels of Time (6, 10-min time bins). For the thigmotaxis test, another within-subjects factor was Area (2 levels: perimeter and center). For the light-dark tests, only a within subjects factor of Time was considered (6 levels of 5-min time bins). Post-hoc comparisons of significant main effects and interactions were performed using LSD test when appropriate.

2.3 Results

2.3.1 Eye Opening

All animals showed normal eye opening patterns during development, with one or both eyes opening between postnatal days 12–16. There were no effects of neonatal LPS exposure on eye opening, as no significant delays were observed between treatment groups, $F(1, 43) = .95$, $p = .344$. Additionally, both males and females showed similar eye opening patterns, indicating no evidence of a sex difference, $F(1, 43) = 1.04$, $p = .341$, and no significant interaction was observed. Thus, it can be concluded that neonatal LPS administration did not have a significant effect on development in terms of the appropriate developmental milestone of eye opening.

2.3.2 Elevated Plus Maze

Due to the presence of outliers, eight animals were excluded from the analyses of the various behavioural measures (2 male-NaCl, 2 male-LPS, 1 female-NaCl, 3 female-LPS animals excluded). Statistical analysis did not reveal any effects of Sex for measures of anxiety in the elevated plus maze, as no significant differences between female and male groups were found in the number of entries into the open and closed arms, and the amount of time spent in the closed arms of the maze. However, a main effect of Drug was observed, $F(1, 37) = 6.24$, $p = .017$, with the vehicle animals showing a greater percent time in open arms than the LPS animals (see Figure 2.2). Additionally, no effects of sex or drug were found in the various risk assessment behaviours, inclusive of head and body stretch attends, head dips, and double backs. The MANOVA did not yield any Sex x Neonatal Treatment Interactions for any of the measures of anxiety in adolescence. For a summary of significance data see Appendix A.

A significant main effect of Litter was found for several of the EPM variables including the number of open entries ($F(10, 37) = 4.30$, $p = .001$), the number of closed arm re-entries ($F(10, 37) = 2.67$, $p = .014$), the number of head stretch attends ($F(10, 37) = 4.66$, $p < .001$), duration of head stretch attends ($F(10, 37) = 2.50$, $p = .021$), number of body stretch attends ($F(10, 37) = 9.60$, $p < .001$), and duration of body stretch attends ($F(10, 37) = 14.12$,

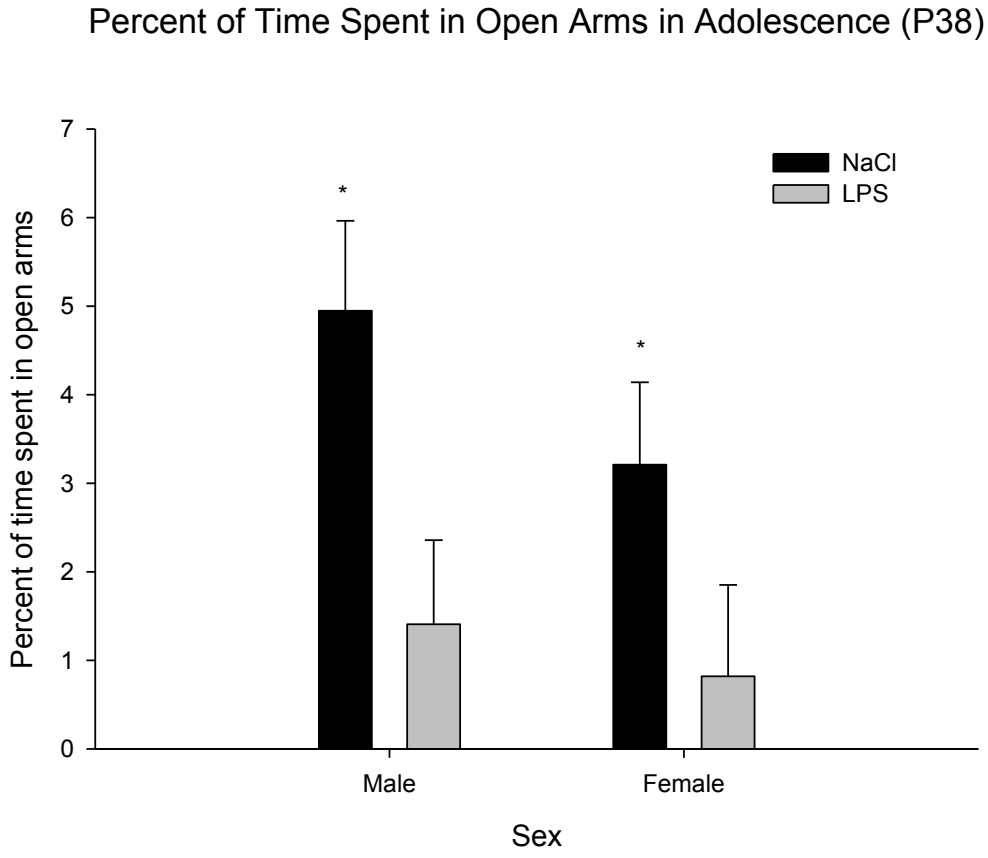

Fig. 2.2 Group mean (± S.E.M.) percent time in open arms of the elevated plus maze (time spent in open arms/time spent in open arms + time spent in closed arms x 100) during adolescence (P 38). * $p < .05$ indicates a significant overall neonatal drug effect, neonatal saline animals ($n = 39$) had greater percent time in open arms than neonatal LPS animals ($n = 39$) (male-SAL: $n = 21$, male-LPS: $n = 21$, female-SAL: $n = 18$, female-LPS: $n = 18$).

$p < .001$). These results highlight the importance of accounting for inter-litter variance (post hoc tests not reported).

2.3.3 Open Field: Overall Activity Levels

Representative measures of overall horizontal and vertical activity during adolescence are depicted in Figure 2.3A-C. Statistical analysis of these data suggests no significant effects of Sex or Neonatal Treatment in total distance, movement time, number of horizontal movements, or number of vertical movements. A significant main effect of Litter was found on Day 39 for all variables inclusive of total distance, $F(10, 43) = 8.87$, $p < .001$, total movement time, $F(10, 43) = 10.28$, $p < .001$, total number of horizontal movements, $F(10, 43) = 9.67$, $p < .001$, and total number of vertical movements $F(10, 43) = 25.16$, $p < .001$ (post hoc tests not reported). Similar Litter effects were observed on Day 40.

Habituation was demonstrated for all animals across time and day, as the overall activity level decreased over the 60 min time period, and was reduced on day 40 as compared to day 39. For a summary of significance data see Appendix B.

2.3.4 Open Field: Thigmotaxis Behaviours

Representative measures of horizontal activity (duration), vertical activity (vertical time) and number of entries in periphery and center zones on days 39 and 40 are depicted in Figure 2.4A-C. No significant differences were found between Sex and Neonatal treatment groups for any of the measures. For a summary of significance data see Appendix C. All animals travelled more, and spent greater time in the periphery than in the center areas, demonstrating consistent thigmotaxis behaviour across groups. Habituation to the open-field was demonstrated for all animals across time and day, as number of entries and overall horizontal and vertical activity levels decreased over the 60 min time period and from day 39 to day 40 (main effect of day and main effect of time, $p < .001$ for all behavioural measures). A main effect of Litter was found for all representative measures of thigmotaxic behaviour (statistics not reported), emphasizing the importance of parsing out inter-litter variance in the ANOVA.

2.3.5 Light-Dark Test

One female-NaCl animal was excluded from this analysis due to technical difficulties in obtaining data. A representative measure of horizontal activity (total distance, cm/min; duration, s) and vertical activity (total number of vertical movements, cm/min; total vertical movement time, cm/min) in both chambers is depicted in Figure 2.5A-D. The

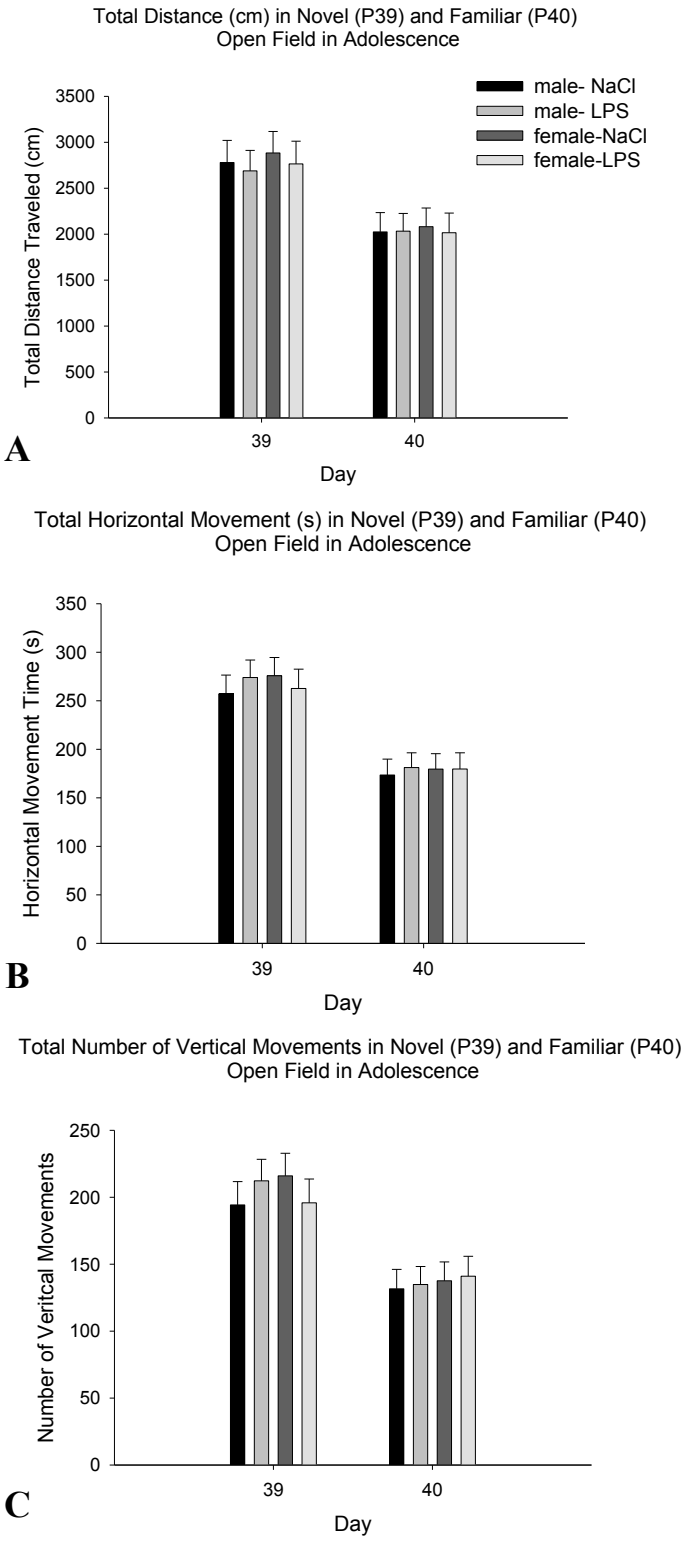

Fig. 2.3 Group mean (± S.E.M.) horizontal and vertical locomotor activity measures in the novel (P 39) and familiar open field (P 40) during adolescence. (A) total distance travelled in cm (B) movement time in s (C) number of vertical movements. * $p < .05$ (male-SAL: $n = 23$, male-LPS: $n = 23$, female-SAL: $n = 19$, female-LPS: $n = 21$).

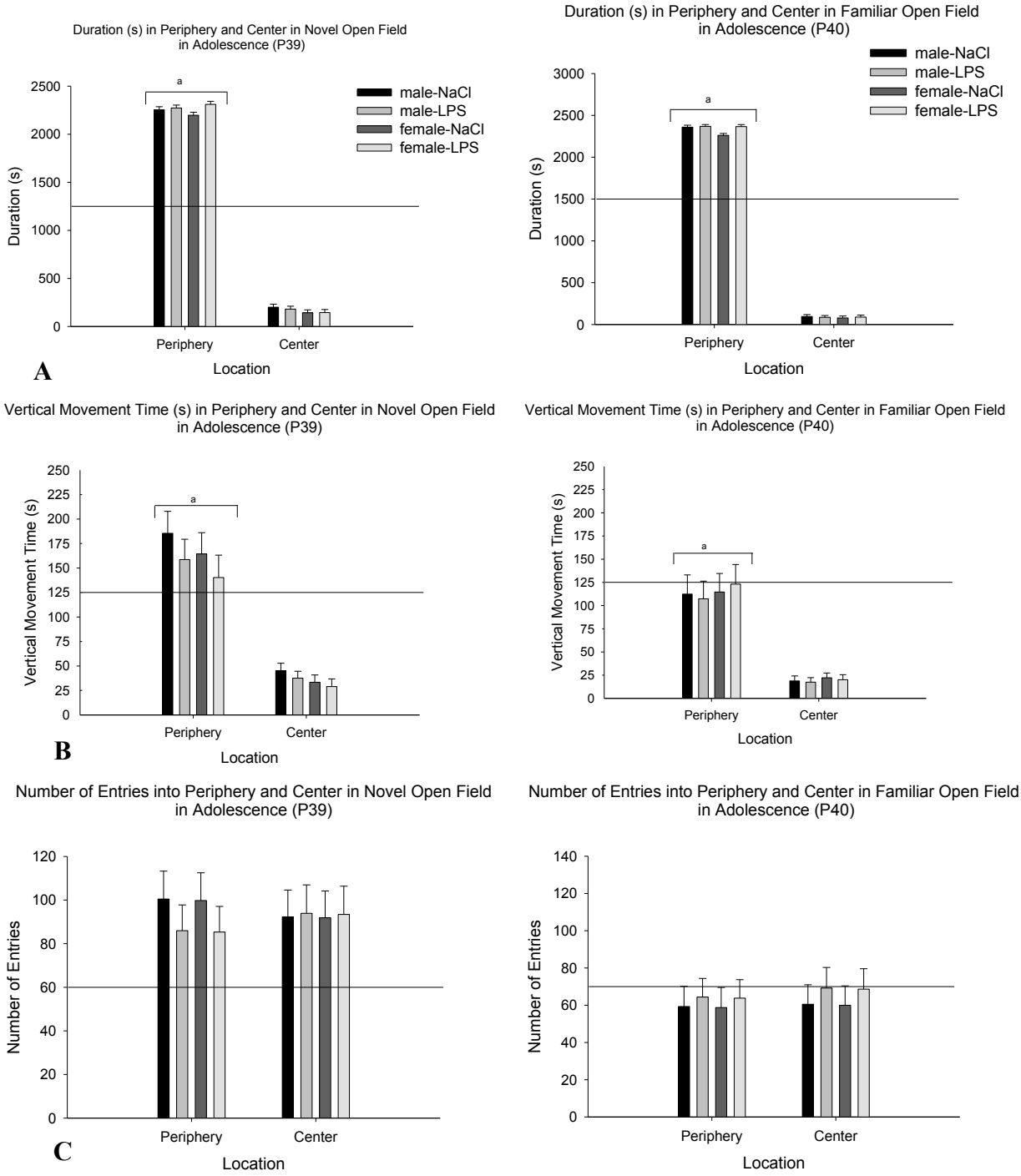

Fig. 2.4 Group mean (± S.E.M.) thigmotaxis behavioural measures in the novel (P 39) and familiar open field (P 40) during adolescence. (A) duration in periphery and center areas in s (B) vertical time in periphery and center areas in s (C) number of entries in periphery and center areas. Horizontal lines represent the point at which equal time would be expected to be spent in each area. * $p < .05$ (male-SAL: $n = 23$, male-LPS: $n = 23$, female-SAL: $n = 19$, female-LPS: $n = 21$).

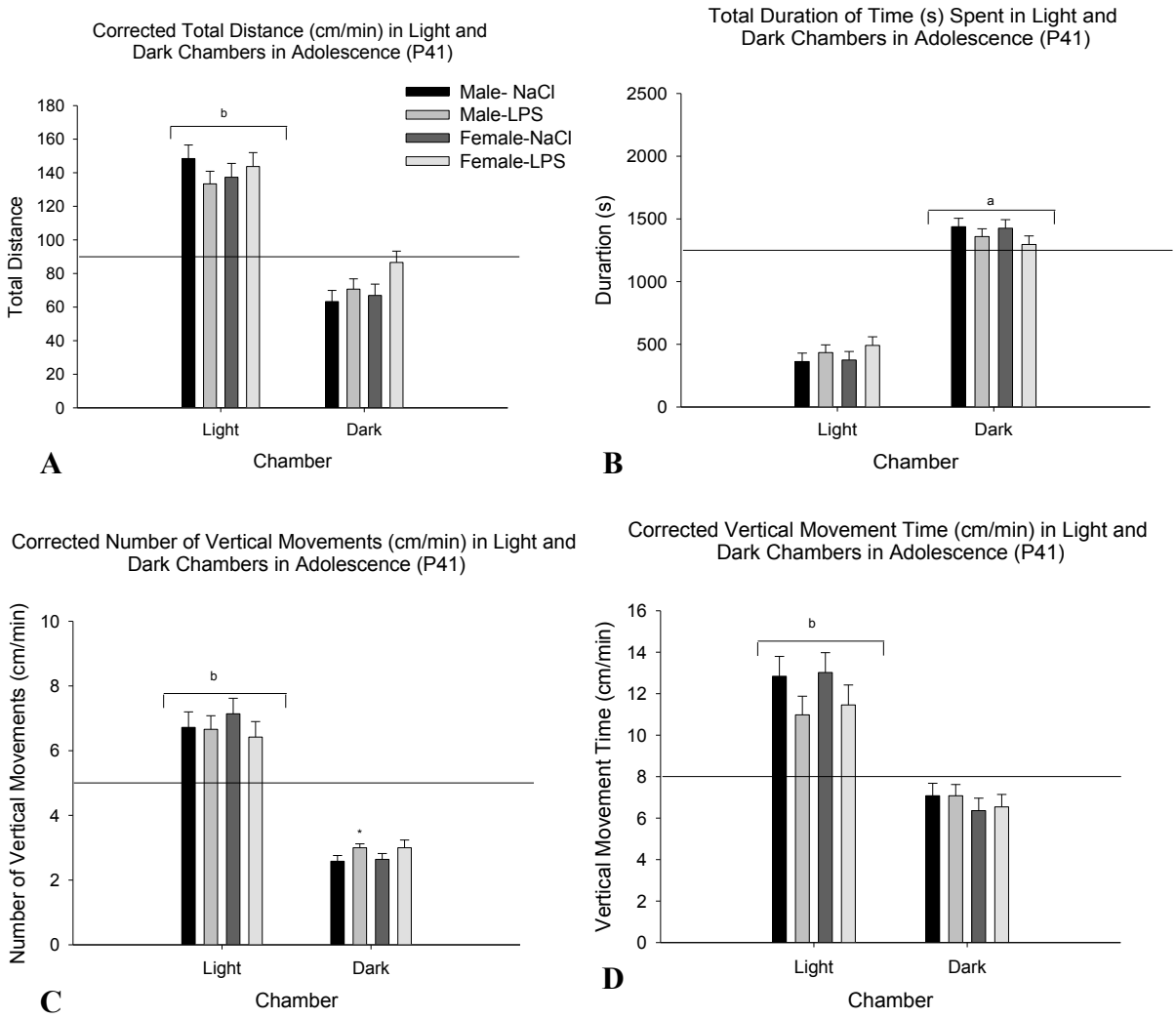

Fig. 2.5. Group mean (± S.E.M.) activity behaviours in the light-dark test during adolescence (P41). (A) corrected total distance (cm travelled per min) in light and dark chambers (B) total duration (s) spent in light and dark chambers (C) corrected number of vertical movements (cm per min) in light and dark chambers (D) corrected vertical time (cm per min) in light and dark chambers. Horizontal lines represent the point at which equal time would be expected to be spent in each chamber. *$p < .05$ indicates significant Neonatal Drug x Sex interaction. Male-LPS animals had a greater number of vertical movements than male-NaCl animals in the light chamber. 'a' indicates a significant main effect of chamber ($p < .05$) with all treatment groups spending more time in the dark chamber. 'b' indicates a significant main effect of chamber ($p < .05$) with all treatment groups travelling more, having greater number of vertical movements and greater vertical time in the light chamber (male-SAL: $n = 23$, male-LPS: $n = 23$, female-SAL: $n = 18$, female-LPS: $n = 21$).

ANOVA conducted on these data yielded no significant interactions or main effects for measures of horizontal activity in either the light or dark chambers. A significant Sex x Neonatal Drug interaction was observed for the total number of vertical movements in the dark chamber, $F(1, 42) = 5.95$, $p = .019$, with male LPS animals demonstrating a greater number of vertical movements than male NaCl animals ($p = .017$). No significant effects were observed for total duration of vertical movements. There were no differences between the sexes or neonatal treatment groups in the number of entries into the light or dark chambers. For a summary of significance data see Appendix D.

A main effect of Chamber was observed for all animals across the various activity measures. All animals showed greater total distance in the light chamber, $F(1, 42) = 122.72$, $p < .001$. A main effect of Chamber was observed for Duration, $F(1, 42) = 197.83$, $p < .001$, as all animals spent more time in the dark chamber as compared to the light chamber. As well, animals showed a greater amount of vertical activity in the light chamber, as compared to the dark chamber. All animals demonstrated a greater number of vertical movements, $F(1, 42) = 238.65$, $p < .001$, and greater duration of vertical movements, $F(1, 42) = 108.54$, $p < .001$, in the light chamber.

Finally, all animals entered the light chamber more often than the dark chamber, $F(1, 42) = 35.66$, $p < .001$. Analysis revealed a main effect of Time for number of entries in the light chamber ($F(4, 155) = 160.43$, $p < .001$) and dark chamber ($F(3, 135) = 76.04$, $p < .001$), as well as duration into the light ($F(3, 139) = 48.34$, $p < .001$) and dark ($F(3, 132) = 37.16$, $p < .001$) chambers. Thus, all animals demonstrated habituation to the environment, as the number of entries into the chambers decreased over the 30 min period. Additionally, a main effect of Litter was observed for several variables in the dark chamber, inclusive of number of entries ($F(10, 42) = 3.26$, $p = .003$), total distance ($F(10, 42) = 2.53$, $p = .017$), total number of vertical movements ($F(10, 42) = 8.75$, $p < .001$), and total duration of vertical movements ($F(10, 42) = 3.26$, $p = .003$). Finally, a main effect of Litter was observed for the number of entries into the light chamber, $F(10, 42) = 19.70$, $p < .001$ (post hoc tests not reported).

2.4 Individual Differences Analysis

Given the within-litter differences observed in the statistically analyses, individual differences were examined. In order to assess the validity of the various behavioural tests of anxiety, Pearson correlations were conducted between variables within a test for each

treatment group. Furthermore, Pearson correlations were also conducted to examine differences in behavioural measures between the various tests of anxiety.

2.4.1 Correlations Within Tests

2.4.1.1 Elevated plus maze correlation results. Pearson correlation analysis revealed several significant correlations between the classic measures of anxiety. Specifically, the number of open entries was significantly correlated with the percent time in open arms for all treatment groups (ps < .01), as well as negatively correlated with the duration in the closed arms for all groups (ps < .01). In addition, the duration in the closed arms was negatively correlated with percent time in open arms across sex and neonatal treatment groups (ps < . 01). These patterns of results indicate that duration in the closed arms decreased as animals entered and spent more time in the open arms.

Significant correlations were also observed for various risk assessment behaviours, particularly the number of body stretch attends, which correlated highly with many of the classic variables. More specifically, the number of body stretch attends was positively correlated with the number of open arm entries (for treatment groups: female-LPS, female-NaCl, and male-LPS), the number of closed arm entries (for treatment group: female-LPS, female-NaCl, and male-NaCl) and percent time in open arms (for treatment groups: female-LPS, female-NaCl, and male-LPS). This general pattern is suggestive of greater risk assessment behaviour as an animal moves to various parts of the maze, but also highlights that such behaviours vary between individuals. Additionally, the number of body stretch attends correlated highly with the duration of body stretch attends (ps < .01), and a similar pattern of significant correlations was observed between number and duration of head stretch attends (ps < .01).

The significant relationships between classic EMP variables demonstrate validity and suggest that a similar construct of anxiety is measured within the test. Furthermore, certain risk assessment variables, such as body stretch attends correlate highly with the classic EMP variables, while others, such as the number of closed arm re-entries (double backs) did not correlate with any of the other measures. This finding suggests that while a variable may seem to indicate a particular behaviour, this may not always be the case, and emphasizes the importance of examining these relationships. Finally, the correlation analyses show that all treatment groups generally demonstrate similar patterns of relationships. However, there are

some important individual differences across various measures of the same construct. For a summary of the correlations see Appendix J.

2.4.1.2 Open field and thigmotaxis correlation results. Convergent validity was demonstrated for variables in the open field. Pearson correlation analysis yielded significant positive correlations between all five locomotor activity variables, inclusive of total distance, duration of horizontal movements, number of horizontal movements, number of vertical movements, and duration of vertical movements (ps < .01). These correlations were observed in both the novel and familiar open field and across sex and neonatal drug treatment groups, suggesting high validity of these measures. For a summary of the correlations see Appendix K1 and K2.

Furthermore, significant correlations were observed for the various thigmotaxis behaviours in the open field. For both the novel and familiar open field, the number of entries into the center was positively correlated with duration of vertical movements in periphery (ps < .01) for all treatment groups. However, different patterns were observed for the duration of time in the periphery on each of the test days. For the novel open field, duration in periphery only correlated with the number of center entries for the female-saline and male-LPS groups (ps < .05), and was only significantly correlated with the duration of vertical movements in the periphery for the female-saline group (ps < .05). In the familiar open field, periphery duration correlated significantly with the number of entries into the center area for both male treatment groups (ps < .05), and with duration of vertical movements in the periphery for all treatment groups except the female-saline animals. For a summary of the correlations see Appendix L1 and L2. It should be noted that in the center areas in both the novel and familiar open field, all variables correlated significantly with all other variables across both sex and neonatal drug treatment (ps < .01). These results emphasize the importance of considering individual differences within treatment groups when measuring constructs such as anxiety-related behaviours.

The results of these analyses suggest that while there are similar patterns of significant relationships between the number of center area entries and duration of vertical movements in the periphery for all treatment groups, individual differences appear when examining the duration of time spent in the periphery in the novel and familiar open field. Overall, these findings suggest that despite measuring behaviour in a similar area of the environment, variables may be measuring different behaviours. For instance, duration in the

periphery may be measuring general anxiety, whereas vertical time may be indicative of escape behaviours, and these may be related in some, but not all, treatment groups.

2.4.1.3 Light-dark test correlation results. For a summary of the correlations between light-dark test variables, see Appendix M. Pearson correlation analysis revealed significant correlations between behavioural measures in the light-dark test. Within the light chamber, analysis yielded strong negative correlations between duration and total distance, number of vertical movements, and duration of vertical movements (ps < .05). These significant correlations were observed for all treatment groups, with the exception of the female-saline animals for total distance and number of vertical movements. Such a pattern is expected, as animals move significantly less the more time they spend in a chamber. Additionally, total distance was significantly correlated with the number of vertical movements for all treatment groups, highlighting the relationship between two variables measuring similar constructs of movement. Furthermore, the number and duration of vertical movements in the light chamber were positively correlated for all treatment groups, emphasizing the validity of these behaviours in measuring the same construct.

In the dark chamber, the number of entries was negatively correlated with the duration of time spent in the dark chamber (ps < .05) for all treatment groups, and positively correlated with total distance travelled in the dark chamber for both female treatment groups and male-LPS animals (ps < .05). Furthermore, total distance was negatively correlated with duration for all treatment groups, and positively correlated with the number of vertical movements for all groups except the female-LPS animals. Finally, the number and duration of vertical movements in the dark chamber were positively correlated for all treatment groups, similar to the correlations observed in the light chamber.

Several significant correlations were also observed between the light and dark chambers. Duration in the light chamber was negatively correlated with duration in the dark chamber for all treatment groups (ps < .01). Furthermore, duration in the light chamber was positively correlated with number of entries and total distance in the dark chamber for all groups (ps < .01), suggesting that animals made a greater number of entries and travelled more in the dark chamber as the time spent in the light chamber increased. Finally, significant negative correlations were observed between the number of vertical movements in the light chamber and the number of entries (ps < .05) and total distance (ps < .05) in the dark chamber, indicated that as animals showed more vertical movements in the light chamber,

they had fewer entries and travelled less in the dark chamber. Such behaviour may be indicated of greater escape behaviour in the light chamber, which could not be accomplished in the dark chamber. However, a positive correlation was observed between the number of vertical movements in the light chamber and duration in the dark chamber for all groups with the exception of the female-saline animals. Thus, as escape behaviour increased through a greater number of vertical movements, duration in the dark chamber also increased, suggesting that when escape is not possible, hiding in the dark chamber increases.

Overall, there are many significant relationships between variables within and across the light and dark chambers. This emphasizes the validity of the light-dark test and the behavioural measures used as indicators of anxiety. However, differences between treatment groups in these relationships are evident.

2.4.2 Correlations Across Tests

2.4.2.1 Novel open field compared to familiar open field. Pearson correlation analysis yielded many significant correlations between locomotor activity variables in the novel and familiar open field. For all treatment groups, total distance, number and duration of horizontal movements, and number of vertical movements in the novel open field correlated positively with the same variables in the familiar open field (ps < .01). For instance, a significant correlation was found between total distance in the novel open field and in the familiar open field for all sex and neonatal treatment groups. These findings suggest strong temporal consistency. Furthermore, significant positive correlations were observed between these variables on the two test days. For example, a significant correlation was found between total distance in the novel open field and the duration of horizontal movements in the familiar open field, suggestive of similar behaviour patterns on both test days. For the duration of vertical movements, similar patterns were observed, although not for all treatment groups. The results suggest similar patterns of behaviour in both the novel and familiar open field, although some variance in these relationships was evident between treatment groups.

2.4.2.2 Thigmotaxis in novel open field compared to thigmotaxis in familiar open field. Significant correlations were observed for the various thigmotaxis behaviours between the novel and familiar open field. Duration in periphery, vertical time in periphery and number of entries into the center area in the novel open field all correlated significantly with their familiar open field variable equivalent (ps < .01). Furthermore, the number of entries into the center was positively correlated with duration of vertical movements in periphery (ps

< .01) across both the novel and familiar open fields. These results were observed for all treatment groups. However, different patterns were observed for the duration of time in the periphery. Male treatment groups showed significant correlations between duration in the periphery and number of center area entries between both test days (*p*s < .05). A similar pattern was observed for male-LPS animals between duration and vertical time in the periphery across the novel and familiar open fields. These correlations were not found to the same extent with the female animals.

Overall the results suggest temporal consistency, as behaviours observed in the novel open field were significantly correlated with those in the familiar open field for all treatment groups. Similar to the results observed within tests, similar patterns of relationships were found between the number of entries into the center area and the duration of vertical movements in the periphery. However, duration in the periphery may better allow for individual differences to be detected, as different patterns of relationships were observed across sex and neonatal treatment groups.

2.4.2.3 Novel open field compared to thigmotaxis in novel open field. Significant positive correlations were observed for the female groups and the male-NaCl group between the vertical time in the periphery and the locomotor activity variables (*p*s < .05). Moreover, the male animals and the female-LPS animals showed significant correlations between the number of center area entries and the locomotor variables. However, there were almost no significant correlations between duration in the periphery and any of the locomotor variables in the novel open field. These differences in results highlight the importance of examining individual differences in the variables across a battery of tests and suggest that indices of activity may be measuring different behavioural constructs. For instance, thigmotaxis may be an index of anxiety as measured by duration in the periphery, whereas locomotor activity may suggest exploration and/or escape behaviour.

2.4.2.4 Novel open field compared to thigmotaxis in familiar open field. Correlation analysis of locomotor activity variables in the novel open field with thigmotaxis variables in the familiar open field showed that vertical time in the periphery did not significantly relate to any of the locomotor variables. Similar results were found for the duration in periphery, although a single positive correlation was found between total distance in novel open field and duration in periphery of familiar open field found for female-NaCl animals. However, number of entries into the center area was significantly correlated with all

locomotor variables from the female-LPS and male-NaCl animals. This finding provides evidence for relationships between various exploratory behaviours in novel and familiar environments.

2.4.2.5 Familiar open field compared to thigmotaxis in familiar open field. Similar to the analysis between novel open field and thigmotaxis variables, no significant correlations were found between duration in the periphery of the familiar open field and any of the locomotor variables. Vertical time in the periphery was significantly correlated with all locomotor variables for female-LPS animals, and a similar pattern was observed between number of center area entries and locomotor activity (ps < .05). Male animals showed significant correlations between several open field and thigmotaxis variables, particularly the male-NaCl animals. Overall, the results suggest that various indices of activity in the open field may be measuring different behavioural constructs, and these relationships may change depending on sex and neonatal treatment.

2.4.2.6 Familiar open field compared to thigmotaxis in novel open field. Contrary to findings in comparisons between locomotor variables in the novel open field and thigmotaxis behaviours in the familiar open field, different patterns of correlations were found for the familiar open field and thigmotaxis variables in the novel open field. Duration in the periphery in the novel open field did not yield significant correlations with any of the locomotor variables in the familiar open field. However, female-LPS and male-NaCl animals showed significant positive correlations between locomotor variables and vertical time in the periphery, as well as number of center area entries. These findings suggest relationships between exploratory behaviours that may vary depending on the novelty of the environment and behaviours assessed.

2.4.2.7 Elevated plus maze compared to novel open field. When the variables from the elevated plus maze were compared with those from the novel open field, only a few significant correlations emerged and these varied depending on the treatment group. Significant positive correlations were observed for the female-LPS animals between the number of entries into the open arm and total distance in the open field (r = .44, p < .01) as well as the number of vertical movements (r =. 47, p < .01). This suggests that some exploratory behaviour in the elevated plus maze correlates with exploratory behaviour in the novel open field for the females treated with neonatal LPS. Conversely, open arm entries may indicate checking behaviour, which correlates positively with the number of vertical

movements indicative of escape behaviour. Males treated neonatally with LPS show significant negative correlations between the number of head stretch attends in the EMP and the number of vertical movements in the novel open field (r = -.56, p < .01), as well as the duration of vertical movements (r = -.43, p < .05). Similarly, a significant negative correlation was observed between the number of head stretch attends in the EMP and the number of vertical movements in the novel open field for the female-saline animals (r = -.57, p < .01). These results suggest that as risk assessment behaviour in the elevated plus maze increase, the number and duration of vertical movements in the open field decrease. Taken together, the few correlations between the behaviours in the elevated plus maze and those in the novel open field suggest that these two tests may measure different constructs of anxiety.

2.4.2.8 Elevated plus maze compared to thigmotaxis in novel open field. Pearson correlation analysis yielded significant correlations between variables in the elevated plus maze and thigmotaxis behaviours in the novel open field. Specifically, negative correlations were found for all treatment groups between the number and duration of body stretch attends and the duration in the periphery of the novel open field (ps < .05). This suggests that as typical thigmotaxis behaviour increases, checking/risk assessment behaviour decreases. A similar pattern emerged for males, particularly the male-LPS animals, with the number and duration of head stretch attends negatively correlating with the number of center area entries. This result indicates that risk assessment behaviour increases with decreases in entries into the center area.

2.4.2.9 Elevated plus maze compared to familiar open field. A pattern of correlations between variables in the elevated plus maze and the familiar open field emerged for some of the treatment groups. Specifically, as anxiety behaviour in the elevated plus maze increased, locomotor activity in the familiar open field decreased, particularly in the male-LPS and female-NaCl animals. These results were observed between the duration of time in the closed arms, the number of closed arm re-entries and head stretch attend behaviours and the various locomotor variables. For example, a significant negative correlation was observed between the number of closed arm re-entries and the duration of horizontal movements (r = -.53, p < .01) for male-LPS animals. The negative correlation suggests behavioural consistency between tests, where freezing behaviour, or the decline in locomotor activity, is related to risk assessment and anxiety-related behaviours in the elevated plus maze. This pattern was not observed for the female-LPS animals, but rather significant positive

correlations were observed between the number of closed arm entries and total distance (r = .46, p < .05), as well as the number of horizontal movements (r = .49, p < .05). This suggests that anxiety behaviour in the elevated plus maze is correlated with increases in locomotor activity for female-LPS animals. Overall, the results highlight the importance of examining individual differences between treatment groups, as the sex and neonatal treatment groups demonstrated different patterns of relationships between EPM and locomotor variables.

2.4.2.10 Elevated plus maze compared to thigmotaxis in familiar open field. A pattern of significant negative correlations was observed between duration in the periphery of the familiar open field ad the number and duration of body stretch attends. These results were observed for all treatment groups, and suggest that as thigmotaxis behaviour increases, risk assessment behaviour decreases. Similar patterns were observed for head stretch attend behaviours for both male treatment groups, with significant negative correlations with duration and vertical time in periphery. Furthermore, males show significant negative correlations between number and duration of head stretch attends and number of entries into the center. Finally, females show a significant positive correlation between the number of open arm entries and the number of entries into the center area. These results are suggestive of similar anxiety-like behaviour, as well as common exploratory behaviour across tests.

2.4.2.11 Elevated plus maze compared to light-dark tests. Very few correlations were observed between variables in the elevated plus maze and light-dark tests. Furthermore, the analysis yielded different correlations for each treatment group. For instance, a significant negative correlation was observed between the number of closed arm entries in the EMP and the number of vertical movements in the dark chamber for females treated with LPS (r = -.55, p < .05). Generally, body stretch attends were correlated with duration and total distance in the light and dark chambers for female groups, whereas for male groups, head stretch attends were correlated with these variables in the dark chamber only. Finally, the number of head stretch attends was negatively correlated with the number of entries into the light chamber for LPS-males only (r = -.58, p < .01). This finding suggests that an increase in risk assessment is associated with a decrease in anti-anxiety behaviour. The small number of correlations between the EMP and light-dark test may suggest a lack of behavioural consistency across tests, or possibly that the two tests measure different constructs of anxiety.

2.4.2.12 Light-dark test compared to novel open field. Pearson correlation analysis yielded several patterns of significant correlations between variables in the light-dark test and

the novel open field. For males treated with LPS or saline control, entries into the light chamber correlated significantly with all locomotor variables in the novel open field, inclusive of total distance, number and duration of horizontal movements, and number and duration of vertical movements (ps < .05). The same pattern of correlations was observed for entries into the dark chamber for both groups of males (ps < .05). Furthermore, similar correlations were observed for females treated with saline, with entries into the light chamber correlating with all locomotor variables, and entries into the dark chamber correlating significantly with some, but not all, of these variables (total distance, number and duration of horizontal movements). LPS-females showed significant correlations only between entries into the light chamber and the number of vertical movements (r = .51, p < .05), as well as vertical time in the light chamber and vertical time in the novel open field (r = .46, p < .05). Taken together, these findings suggest behavioural consistency across these tests for some groups, particularly the males regardless of neonatal treatment.

2.4.2.13 Light-dark test compared to thigmotaxis in novel open field. Very few significant correlations were observed between variables in the light-dark test and thigmotaxis behaviours in the novel open field. However, several significant correlations emerged for the different treatment groups. For instance, duration in the periphery was positively correlated with entries into the dark chamber and total distance in the dark chamber, and negatively correlated with total distance in the light chamber. These results suggest that anxiety-related behaviours between tests are related. Male, but not female, animals show significant positive correlations between the number of entries into the light chamber and the number of entries into the center, suggestive of checking and exploratory behaviour. Furthermore, a significant correlation was also found between the number of entries into the light chamber and the duration of vertical movements in the periphery for male-LPS animals, indicative of similar escape behaviours. Taken together, these results suggest similar patterns of anxiety and exploratory behaviours between the measures of the light-dark and novel thigmotaxis tests.

2.4.2.14 Light-dark test compared to familiar open field. Pearson correlation analysis yielded several significant correlations between variables in the light-dark test and the familiar open field. Entries into the light chamber correlated with all locomotor variables for male-NaCl animals (total distance, number and duration of horizontal movements, and number and duration of vertical movements), as well all locomotor variables for female-NaCl

animals with the exception of duration of vertical movements (*p*s < .05). However, for the female-LPS animals, a significant correlation was only observed between number of entries into the light chamber and number of vertical movements ($r = .47$, $p < .05$). Furthermore, a similar result was observed for male-LPS animals, with number of entries into the light chamber correlating with the number ($r = .63$, $p < .01$) and duration ($r = .46$, $p < .05$) of vertical movements in the familiar open field. These results suggest that the LPS treated animals demonstrated behavioural consistency between measure of escape (vertical movements) and checking behaviour (entries into the light chamber). Finally, for the male-saline animals only, significant negative correlations were observed between duration in the dark chamber and locomotor activity variables in the familiar open field, suggesting that as duration in the dark chamber increase, locomotor activity in the open field decreased. These findings highlight the necessity of examining individual differences, as the male-saline animals showed far greater number of correlations than the other treatment groups. Furthermore, they emphasize the importance of understanding how different tests can assess various aspects of the same construct.

2.4.2.15 Light-dark test compared to thigmotaxis in familiar open field. Similar to thigmotaxis behaviours in the novel open field, the Pearson analysis yielded very few correlations between measures in the light-dark tests and thigmotaxis behaviours in the familiar open field. Significant results were observed for the female-LPS animals. For instance, positive correlations were found between duration in the periphery and entries in the dark chamber, as well as total distance in the dark chamber (*p*s < .05). Furthermore, female-LPS animals showed negative correlation between duration in the periphery and total distance in the light chamber. These results suggest that anxiety-like behaviours are similar across tests. Female-LPS animals also demonstrated a significant positive correlation between duration in periphery of the familiar open field and duration in the light chamber. This result may suggest that the familiar open field measures different aspects of anxiety than the novel light-dark test. Finally, male-NaCl animals showed similar patterns of exploratory and escape behaviour between the tests, with the number of entries into the light chamber correlating with number of entries into the center area and vertical time in the periphery. Taken together, these results suggest that the tests may measure similar constructs of anxiety, but also highlight the unique aspects produced by each test.

2.4.3 Conclusions

There are clear relationships between variables both within and across tests of anxiety-related behaviours. As such, it is important to examine these relationships, as they promote a better understanding of the different constructs of anxiety that are found across tests. Overall, the correlation analyses within the various tests suggest that the elevated plus maze, open-field, and light-dark tests are reliable measures, in that the variables within a test appear to show many significant relationships. However, different patterns of relationships emerged between the sex and neonatal drug treatment groups, highlighting the role of individual differences in altering behavioural observations of well-established variables. For instance, males treated neonatally with saline and female treated neonatally with LPS showed the greatest number of significant correlations between tests. However, the patterns of relationships vary depending on the tests being examined. This may suggest that neonatal treatment may have sex-specific effects on activity and anxiety-related behaviours, although these specific effects remain unclear.

Furthermore, analyses between tests show that the various tests may be assessing different aspects of anxiety. While these correlations are generally informative, to gain a better understanding of these relationships, factor analysis should be conducted. In this way, the large number of variables both within and across tests may be reduced to a small number of underlying dimensions of anxiety. Using factor analysis will allow for a better understanding of the constructs of anxiety being measured from each individual test.

2.5 Discussion

The current study examined the effects of neonatal LPS exposure on anxiety-related behaviour in male and female adolescents, as well as potential sex differences in these effects. The findings of this study suggest that LPS treatment on postnatal days 3 and 5 may have highly specific effects on certain anxiety behaviours in adolescence. Neonatal LPS administration resulted in increased anxiety in the elevated plus maze, as measured by reduced percent time in the open arms relative to neonatal saline treatment. Furthermore, males treated neonatally with LPS showed more vertical movements in the dark chamber than those treated with saline. However, no significant differences were observed between treatment groups in locomotor activity in the novel or familiar open field. Finally, no sex differences were observed in any of the various anxiety-related tests.

Males and females treated neonatally with LPS showed less percent time in the open arms of the elevated plus maze than animals treated with saline, although no other significant

differences between treatment groups were found in other anxiety-related behaviours. Nevertheless, the percent time in the open arms is a well-established index of anxiety, as it relies upon animals' proclivity toward dark, enclosed spaces and an unconditioned fear of open spaces. Behaviour in this test reflects a natural conflict between preference for the safety of the closed arms and an innate tendency to explore a novel environment. Furthermore, anxiogenic drugs result in reduced time spent in the open arms, whereas anxiolytic drugs increase the time spent in the open arms, thereby demonstrating the validity of this measure in assessing anxiety (Walf & Frye, 2007). The findings of the current study suggest that neonatal endotoxin exposure results in highly specific changes in adolescent anxiety behaviour, as demonstrated by the significant behavioural differences between neonatal treatment groups in percent time spent in the open arms.

Neonatal LPS treatment did not seem to significantly affect adolescent locomotor activity behaviour in the novel or familiar open field, as animals treated with LPS as neonates demonstrated similar activity relative to animals treated with saline. Moreover, all adolescent animals showed similar thigmotaxic behaviours, as both neonatal LPS and neonatal saline groups spent more time in the periphery than the center area on both test days. Finally, all of the animals demonstrated habituation to the open field, with reduced activity in the familiar environment relative to the novel environment. Similarly, adolescent animals demonstrated similar patterns of behaviour in the light-dark test. All animals spent more time in the dark chamber than the light chamber, but exhibited greater activity levels in the light chamber.

No sex differences were observed for any of the tests of anxiety. This finding is unique as previous research suggests that female adolescents tend to exhibit less anxiety-related behaviour than males in the elevated plus maze (Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993), the open-field (Masur, Schutz, & Boerngen, 1980), and the light-dark tests (Tenk, 2007). This sexual dimorphism has been shown to continue into adulthood for some tests, such as the elevated plus maze (Johnston & File, 1991; Zimmerberg & Farley, 1993) and the light-dark test (Hughes, Desmond, & Fisher, 2004). The developmental period of adolescence is not commonly studied, and there is limited data on sex differences during this developmental period, indicating that further research is warranted. It is possible that the increased exploratory behaviour, characteristic of adolescence, results in similar behavioural responses in both sexes, despite some anxiety produced by the testing environments common to both sexes.

Anxiety-related behaviour may be modulated by developmental stage. The adolescent period is a transitional developmental stage between youth and adulthood, and the age range spans from shortly after weaning until as late as 60 days (Spear, 2000). The majority of research indicates that male and female adolescent rats exhibit less anxiety-related behaviour than adults. Such results have been found in the light-dark test (Schramm-Sapyta, Cha, Chaudhry, Wilson, Swartzwelder, & Kuhn, 2007), the elevated plus maze (Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993; Meyza, Boguszewski, Niko, & Zagrodzka, 2011), the open field test (Masur, Schutz, & Boerngen, 1980), and hole board exploration (Meyza, Boguszewski, Niko, & Zagrodzka, 2011). Adolescents also tend to show more exploratory and risk-taking behaviour than adults, and this is observed across many mammalian species. Adolescent rodents are hyperactive and demonstrate greater exploration in a novel environment, exhibit higher levels of novelty seeking, and elevations in social interactions relative to their adult counterparts (Spear, 2000; Doremus, Varlinskaya, & Spear, 2004; Stansfield & Kirstein, 2006). Adolescent rats also show hyper-reactivity to stimuli, with greater startle response amplitude than adults. Such age-related changes may help adolescents to successfully negotiate the transition from dependence in childhood to independence in adulthood and provide the opportunity for acquisition of necessary survival skills (Spear, 2000; Meyza, Boguszewski, Niko, & Zagrodzka, 2011).

These differences in behaviour during adolescence may explain why animals treated neonatally with LPS showed similar behaviours in some tests of anxiety to animals treated with saline. Sickness behaviours are considered to be the expression of an organized strategy critical to survival (Dantzer, 2001). Infection results in altered behaviour such as lethargy and reductions in food and water intake in order to overcome the disease. As such, sickness is considered a motivational state that enables individuals to reorganize their behaviour depending on the circumstances. For instance, fear-motivated behaviour takes precedence over sickness behaviours in a dangerous situation order to satisfy more urgent needs. This phenomenon has been demonstrated in studies that show that the depressing effects of IL-1β are more prominent when animals are in the safe surroundings of home cages compared to a novel environment (Dantzer, 2001). It is therefore possible that neonatal LPS treatment is not sufficient to induce increased anxiety in the open field and light-dark tests because the motivation to experience new and intense stimuli through increased exploratory and risk-taking behaviour in adolescence take precedence (Meyza, Boguszewski, Niko, & Zagrodzka,

2011). As such, in the novel environments (unique from the home cages), adolescent animals show some anxiety-related behaviours, such as thigmotaxis and greater duration in the dark chamber, but also show increased exploration common to this developmental period regardless of neonatal treatment.

Most studies to date use one of two treatment protocols to explore the effects of early immune system activation on later responses. The dual-exposure-to-endotoxin (DEE) model involves two administrations of LPS on postnatal days 3 and 5 (e.g. Shanks et al., 1994; Breivik et al., 2002; Walker et al., 2004a). Results of studies that utilize this protocol have shown that DEE model alters anxiety-related behaviour in adulthood such that anxiety is increased on some tests but decreased on others (Breivik et al., 2002; Walker et al., 2004b). The second neonatal LPS regime involves a single injection of LPS on postnatal day 14 (Boisse et al., 2004; Spencer et al., 2005; Spencer et al., 2006a). This protocol seems to result in adult immune system tolerance, such as decreased cytokine release (Ellis et al., 2005) and attenuation of the febrile response (Boisse et al., 2004; Ellis et al., 2005; Spencer et al., 2006). However, the results of other studies have shown more variable results, with LPS administration on postnatal day 14 resulting in reduced exploration of novel objects, but having no effect on anxiety-related behaviours in the elevated plus maze, forced swim test, or open-field (Spencer et al., 2005).

These two protocols have investigated adult anxiety behaviours following neonatal endotoxin exposure. Thus, the current experiment advances this area of research and further develops these protocols by providing a novel investigation of the effects of neonatal LPS treatment (DEE model) on adolescent anxiety behaviours. Given that adolescence is a transitional period associated with many changes and new experiences, this development stage may be considered extremely stressful, and many psychopathological disorders emerge during this period. However, results from previous studies have demonstrated that stress (e.g. novel environment and predator odor) during early adolescence results in decreased anxiety-like behaviour and increased risk taking and novelty seeking behaviour during late adolescence in the open field, elevated plus maze, and novel object tests (Toledo-Rodriguez & Sandi, 2011). This suggests that seeking out and learning from novel stressors is necessary for growth towards independence in adulthood. Furthermore, adolescents tend to show greater extremes in mood, which may be adaptive, as heightened emotionality may increase animals' vigilance to potential threats in novel environments (Meyza, Boguszewski, Niko, &

Zagrodzka, 2011). Thus, the results of the present study are consistent with previous research and add novel findings, which suggest that neonatal experiences may alter certain adolescent anxiety-related behavioural responses to stressors, but not others.

Variability among individual animals and differences in behaviour can be interpreted as reflecting differences in personality. The combination of genetic factors, along with the contribution of early physical and social environment, help to shape individual differences in personality and behaviour (Trillmich & Hudson, 2011). Moreover, psychopathological anxiety is considered to a product of this interaction between early environmental factors and genetic susceptibility that produce structural and functional changes in the brain that underlie this disorder (Gross & Hen, 2004). Such precursors of personality may begin prenatally, including nutritional and endocrine conditions in utero. Postnatally, such influences may include litter size and body mass, maternal care, and other environmental conditions or potential stressors. It is assumed that differences in behavioural temperament develop in order to enhance an individual's ability to survive challenges presented by the early environment. Such differences develop through interactions of endocrine, neuronal and immunological process, and are maintained throughout development, leading to differences in adult behaviour and reactivity to stressors (Trillmich & Hudson, 2011). For instance, results of previous research have shown that heavier rat pups (i.e. larger body mass) are bolder and more explorative in later stages of development (Rödel & Meyer, 2011). Furthermore, large and small litter sizes resulted in animals with greater anxiety, relative to medium sized litters, due to the adoption of behavioural styles depending on the size of the litter. This suggests that early environmental factors such as body mass and litter size influence changes in behavioural responses and the development of personalities.

Thus, understanding individual differences in behavioural temperament or personality may help to explain variability that remains in experimental research despite standardization efforts (Lewejohann, Zipser, & Sachser, 2011). Behavioural consistency across time and context can be observed through correlations between behavioural variables and may reveal distinct animal personality traits that are maintained throughout the life cycle (Groothuis & Trillmich, 2011). The results of the correlation analyses in the current study provide evidence for individual differences in the animals tested, and suggest that the development of behavioural temperament from early environmental influences may play a role in the behavioural responses to stressors in adolescence.

Results suggest that the elevated plus maze, open-field and light-dark tests are reliable measures of anxiety due to the significant relationships between behavioural measures within a test. The elevated plus maze shows strong relationships between classic anxiety variables such as duration in the closed arms, as well as number of entries and percent time in open arms. Furthermore, certain risk assessment behaviours (body stretch attends) were also correlated with the traditional measures. Similarly, the locomotor variables in the novel and familiar open field were significantly correlated with one another, as well as variables assessing thigmotaxis behaviours. There were also significant correlations between variables within the light and dark chamber respectively, as well as significant relationships between variables across the two chambers. Such results suggest that these tests are reliable indices of anxiety. However, there was variability in the relationships, as not every treatment group demonstrated significant correlations for every test. Such variance emphasizes that individual differences in behavioural temperament play a role in how anxiety-related behaviours are expressed and observed.

Many significant correlations were observed between tests, suggesting that such measures are assessing anxiety. However, not all tests demonstrated considerable correlation. These findings suggest that the various tests might measure different constructs of anxiety, which may be influenced by individual differences in the animals. This highlights the importance of using multiple tests to assess behaviour, because while measures of multiple ethological tests may be inter-related, they may also assess independent constructs. Results from previous research have found that variables from the elevated plus maze and open field do not produce common anxiety-related factor in rats (Ramos, 2008). Results of the current study are consistent with this, as only a few significant correlations were found between measures of the elevated plus maze and the open field. Similarly, very few significant relationships between the elevated plus maze and the light-dark test emerged. The inter-test variations within the study indicate construct differences between tests and more detailed analyses, such as factor analysis, should be conducted to better understand the constructs being tested. Moreover, the variations in correlations between treatment groups suggest that individual differences in emotionality also play a role in the observed constructs of anxiety. This may explain why certain treatment groups show a greater number of significant correlations in measures of anxiety between tests than others. For instance, males treated neonatally with saline and females treated neonatally with LPS show a greater number of

significant correlations between measures in the light-dark test and the novel and familiar open field, as well as various thigmotaxis behaviours. However, all treatment groups show a relatively equal number of correlations when comparing the elevated plus maze with other tests. Such analyses provide a better understanding of how early environmental factors interact with genetic susceptibilities to influence the expression of various anxiety behaviours in later life. The results also emphasize that a battery of tests may provide more valuable information on various overlapping constructs of the same trait.

The results of the current experiment show that neonatal LPS treatment has effects on certain anxiety behaviours, which may be observed in certain tests, but not in others. Furthermore, typical adolescent behaviours common to both males and females, such as exploration and risk-taking may take precedence over anxiety and alterations due to neonatal endotoxin exposure. Finally, individual differences in personality and behavioural temperament may affect the way in which anxiety-related behaviours are expressed. Future research is warranted in this area to better understand these influences. Taken together, these results have potential clinical significance given that neonatal exposure to infection is common, and identifies potential risk factors in infants exposed to early bacterial infection. Furthermore, this study has empirical significance, as adolescence is a time of numerous developmental changes that has not been studied in great detail to date.

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CHAPTER 3

Effects of Neonatal Immune System Activation with Lipopolysaccharide on Voluntary and Non-Voluntary Adult Anxiety Behaviours

3.1 Introduction

Immune system activation in early life can affect disease susceptibility and have long lasting neurological and psychological effects during later developmental periods. The endotoxin lipopolysaccharide (LPS) is the active component of the cell wall of Gram-negative bacteria and elicits a response mimicking responses to bacterial infection. Administration of LPS stimulates the immune and endocrine systems and induces the expression of proinflammatory cytokines responsible for the inflammatory response (Dantzer, 2001). The variety of adaptive physiological and behavioural symptoms produced is known as the acute phase sickness response.

Sickness behaviour is considered to be the expression of an organized and adaptive strategy to combat invading pathogens and increase the likelihood of survival (Dantzer, 2001). For instance, illness-induced inactivity allows the animal to avoid predation in a weakened condition, as well as conserve energy and body heat, thereby facilitating the production and maintenance of fever. Fever increases body temperature thus impairing bacterial and viral proliferation and survival, and enhances existing immune defence mechanisms (Galic, Spencer, Mouihate, & Pittman, 2009). When animals are prevented from developing fever, they have higher pathogen loads and are more likely to die from the infection (Hart, 1988).

Results from previous research have provided evidence for a sex difference in immunity, with females showing enhanced functioning relative to males across the life span (Shanks, McCormick, & Meaney, 1994; Gaillard & Spinedi, 1998). Research has also demonstrated that immune system activation in the first week of life results in similar sex differentiated acute sickness response (Walker, Brogana, Roger, & Hodgson, 2004a; Dent, Smith, & Levine, 1999). Early acute LPS exposure results in long-term changes in behavioural and physiological processes, such as increased disease severity in adulthood following an immune challenge, elevated IL–6 levels, and increased susceptibility to periodontal disease in adulthood (Breivik, Stephan, Brabant, Straub, Pabst, & von Hörsten, 2002). Furthermore, LPS administration on postnatal days 1, 3, 5, and 7 has been shown to result in reduced natural killer cell activity and decreased resistance to tumor colonization in adult male rats (Hodgson, Knott, & Walker, 2001). However, the effects of neonatal LPS treatment may be more complex. For instance, the results of some studies have shown that LPS administration on postnatal days 3 and 5 results in the reduction of inflammation and

development of arthritis in adulthood (Shanks, et al., 2000). Additionally, as observed in previous studies, early immune system activation also attenuates the febrile response following adult endotoxin challenge (Walker, Hodyl, Krivanek, & Hodgson, 2006).

In contrast, LPS administration during later neonatal development appears to result in immune system tolerance. LPS exposure on postnatal day 14 has been shown to lead to decreased cytokine release (Ellis, Mouihate, & Pittman, 2005), as well as reduced fever response in adulthood following an LPS challenge (Boisse, Mouihate, Ellis, & Pittman, 2004; Ellis, Mouihate, & Pittman, 2005). Both male and female rats show attenuated hyperthermia in response to an LPS challenge in adulthood, although this response in females occurs without the accompanying upregulation of fever-mediating enzymes observed in males (Spencer, Boisse, Mouihate, & Pittman, 2006a). Furthermore, a sex difference is observed in how such neonatal LPS treatment affects adult ability to combat a more severe immune challenge in adulthood. Adult males treated neonatally with LPS show reduced hypothermia and enhanced hyperthermia following both a low and high dose of LPS in adulthood. In contrast, neontally LPS-treated females show a similar response with a low, but not high dose of LPS (Spencer, Field, & Pittman, 2010).

Beyond altered immune functioning, neonatal LPS administration also exerts many long-term changes on anxiety-related behaviours in adulthood, such that anxiety behaviour is increased on some tests but decreased on others (Tenk, 2007). LPS exposure in early development has been shown to lead to enhanced responsivity to restraint stress (Shanks, Larocque, & Meaney, 1995), increased sensitivity to noise (Shanks, et al., 2000) and pain (Boisse, Spencer, Mouihate, Vergnolle, & Pittman, 2005) in adulthood. Additionally, acute neonatal LPS has been shown to increase adult anxiety behaviour in the light-dark test (Lacosta, Merali, & Anisman, 1999) and the elevated plus maze (Breivik et al., 2002; Walker, March, & Hodgson, 2004b). Other studies report no effect of neonatal LPS treatment on performance in the elevated plus maze, but reductions in exploration of novel objects in an open-field (Spencer, Heida, & Pittman, 2005). Reserach has also shown that adult males treated neonatally with LPS show hypoactivity in a non-novel open field test in adulthood in response to an LPS challenge (Tenk, Kavaliers, & Ossenkopp, 2008), while other findings have shown that early LPS exposure does not result in increased anxiety in an open-field test (Breivik et al., 2002). Furthermore, studies have also shown that female rats neonatally exposed to LPS show hyperactivity in a novel open field (Nilsson, Jennische, Ho, Eriksson,

Bjorntorp, & Holmang, 2002). It is clear that while some studies demonstrate effects of neonatal LPS exposure in adulthood without re-activation of the immune system, other studies purport that re-activation with a "second hit" of LPS is necessary to produce such behavioural changes (Williamson, Sholar, Mistry, Smith, & Bilbo, 2011). Thus, these studies not only highlight the inconsistent effects of early immune system challenge, but also call into question the necessity of activating the immune system again in adulthood in order to observe these changes.

A number of animal models used to assess anxiety-related behaviour in rodents have been discussed earlier (Chapter 2). These include the elevated plus maze and/or light-dark test, in which an animal is exposed to stressors such as a novel situation, open space, and/or bright light (Walsh & Cummins, 1976; Crawley & Goodwin, 1980). However, additional anxiety-related behavioural measures were considered for this study. For instance, animals experience taste neophobia, particularly those that lack the ability to vomit, which causes them to consume only a small amount of a novel food or fluid until the food has been proven safe to ingest (Corey, 1978). Taste neophobia is an ethologically valid measured used to assess anxiety-related behaviours in rodents (Dulawa & Hen, 2005; Merali, Levac, & Anisman, 2003), demonstrated by its sensitivity to both anxiogenic (Shephard & Broadhurst, 1982) and anxiolytic (Cooper, 1980; Shephard & Estall, 1984) compounds.

LPS administration has been found to lead to reductions in voluntary locomotor activity. In addition, LPS has been shown to induce non-voluntary motor responses. The acoustic startle response is a defensive sensorimotor reflex in response to a sudden burst of auditory stimulation, which is hypothesized to have evolved as an adaptive response to predator attacks (Hebb, Zacharko, Gauthier, & Drolet, 2003; Lockey, Kavaliers, & Ossenkopp, 2009). The resulting reflex is a non-voluntary contraction of the skeletal muscles such that the subject will appear to jump (Hoffman & Ison, 1980). The prepulse inhibition (PPI) measure is a well-studied startle paradigm, which is used to operationally measure "sensorimotor gating". PPI refers to a level of neural processing at which irrelevant sensory stimuli are disallowed access to sensory processing areas. Normal PPI performance requires adequate sensory detection and processing in order for the animal to allocate attentional resources to more salient stimuli (Swerdlow, Geyer, & Braff, 2001). In research studies, PPI refers to the relative decrease in the magnitude of the startle response when the startling stimulus is preceded by a non-startle inducing sensory input (Lockey, Kavaliers, &

Ossenkopp, 2009). Results have shown that LPS treatment in adult rats resulted in significant reductions in startle response magnitude, suggesting an ability to modulate sensorimotor reflexes, but had no effect on sensory processing (PPI) (Lockey, Kavaliers, & Ossenkopp, 2009).

There are inconsistent findings not only in the behavioural responses to neonatal immune system manipulations in adulthood, but also in the observed sex differences in such responses. Furthermore, there is a lack of consideration of the possible effects of early immune system challenge on non-voluntary sensorimotor responses in adulthood. As such, the current experiment examined the effects of neonatal LPS administration on male and female adult anxiety-related behaviours. This study examined anxiety behaviours in adulthood unaccompanied by additional manipulations, as well as following an additional immune system challenge with LPS. Observations in the elevated plus maze, light-dark test and taste neophobia test were used as behavioural indices of adult anxiety. Additionally, acoustic startle response and PPI were measured, in order to gain better understanding of the effects of neonatal LPS administration on non-voluntary motor activity.

3.2 Methods

3.2.1 Animals

Following adolescent behavioural testing in experiment 1, animals were left undisturbed until adulthood. Animals remained pair-housed (same-sex, same-treatment littermates) in standard polypropylene cages (45 x 22 x 20 cm) in a temperature-controlled colony room (21 ± 1 ^{o}C), and maintained on a 12:12 light – dark cycle with the lights on at 07:00 hours. Food (Prolab rat chow) and water were available ad libitum, unless otherwise specified. On postnatal day 74, animals were single-housed for the final testing procedures. Experimental manipulations were conducted during the light phase of the light – dark cycle and body weight was monitored during testing. All procedures were approved by the University of Western Ontario Animal Care Committee and were in accordance with the Canadian Council of Animal Care (CC guidelines).

3.2.2 Drug Administration

All animals received two challenge doses of LPS (200 μg/kg) in adulthood on P 71 and P 72, derived from *Escherichia Coli* stereotype 0111:B4, L26030, Sigma Chemical, St. Louis, MO, USA) dissolved in 0.9% isotonic saline. Injections were given 1 ½ hours prior to testing.

3.2.3 Behavioural testing

3.2.3.1 Startle apparatus. All acoustic startle response and prepulse inhibition (PPI) testing was conducted in 3 separate startle chambers (SRLAB, San Diego Instruments, San Diego, CA). Each chamber consisted of a cylindrical, clear acrylic rat enclosure (10.2 cm outside diameter) mounted on an acrylic platform. The platform sat on a piezoelectric accelerometer, which transduced the force of animal movement. This was placed inside a ventilated, sound attenuating box containing a mounted fluorescent light and a speaker which emitted the background noise, prepulse and acoustic startle stimuli. Data were recorded for 100 ms immediately following the onset of the acoustic startle stimulus. Testing occurred in adulthood on P 70 (no LPS challenge) and P 72 (LPS challenge).

In a testing session, lasting approximately 22 min, a 5 min acclimation period with background noise (70 dB) was followed by a 17 min (67 trials) testing session in which the 70 dB background noise was maintained. Eleven trial types were used in the testing session; startle-alone trials (consisting of a 115 dB burst of white noise stimulation lasting 40 ms in duration), six different prepulse inhibition trial types (prepulse-pulse, categorized by the intensity of the prepulse and the inter-stimulus interval (ISI) between the prepulse and startle pulse; prepulses 3, 6 or 12 dB louder than the 70 dB background noise (73, 76 and 82 dB, respectively), each consisting of a 20 ms burst of white noise presented with an onset either 120 ms prior to the startle pulse (100 ms ISI) or 80 ms prior to the startle pulse (60 ms ISI), and four control trial types (no pulse, 73, 76, or 82 dB prepulse only – to ensure animals responded only to startle pulse). The first 10 trials were startle-alone trials, which served to reduce the amount of variability measured for the startle response; these trials were not used in later analysis. The middle 52 trials (presented in pseudo-random order) consisted of 10 startle-only, 30 PPI trials (5 each of 6 different PPI trial types), and 12 control trials (3 each of no pulse, 73, 76, or 82 dB only). The session ended with 5 startle-alone trials. All of the trials were separated by an inter-trial interval (ITI) of 8–23 s in length (average ITI = 15 s). Percent PPI to a particular prepulse-ISI trial type was calculated as response to startle alone trials (in the middle 52 trials of a session) minus the response to prepulse-pulse trials (i.e. 73 db prepulse-pulse trial with ISI 100 ms) all divided by the response to startle-alone trials times 100.

3.2.3.2 Elevated plus maze and light-dark tests. Behavioural variables were collected in the elevated plus maze and light dark tests described in experiment 1 (Chapter 2).

3.2.3.3 Drinking apparatus. Drinking behaviour of adult animals was recorded using eight automated drinking boxes (8 Channel Lickometer, DiLog Instruments, Tallahassee, FL). Each Lickometer consisted of a clear Plexiglas chamber (31 x 31 x 41 cm) with a removable Plexiglas lid containing airholes. A graduated drinking tube was mounted in the center of each chamber, with the spout 8 cm above the floor. Contact with the spout by the animal's tongue completed a very low voltage electric circuit. These electrical responses were analyzed using the Lickometer system software (Qlick) to provide a microstructural analysis of licking patterns. Testing in the Lickometer took place on P 76 – 81 where animals were allowed to drink freely for 30 min. Variables generated included total volume ingested during each session in mL (volume) measured by noting the initial and final volumes in the graduated tubes recorded to the nearest half millimeter, and total number of licks per session (number of licks). Additionally, microstructural analysis of drinking behaviour illustrated the frequency of licks at each drinking spout. Licking burst was defined as the number of licks that occurred before an inter-lick interval of 250 ms (burst number) and the number of licks in each burst was also calculated (burst size). Bursts separated by pauses of approximately twice the duration of an inter-lick interval (500 ms) are grouped into clusters. The number of clusters, each measured as a group of bursts that have been interrupted by transient pauses in drinking, were calculated (cluster number), as well as the number of bursts within a cluster (cluster size).

3.2.4 Procedure

As previously described, 4 male and 4 female pups from each litter were left alone following tests in adolescence until adulthood. Body weight was measured weekly throughout the testing process, as well as on each day of behavioural testing. Behavioural testing occurred during adulthood on postnatal days 70- 72 and 75-82.

Rats were tested in the startle apparatus on P 70 for approximately 22 min. On P 71 all animals were given a challenge i.p. injection of LPS and tested in the elevated plus maze and the light-dark apparatus. Additionally, a second LPS challenge was administered on P 72 and all animals were again tested in the startle apparatus. All behavioural testing occurred 1 ½ hr following LPS injections.

Taste neophobia and intake of a novel sucrose solution was assessed for animals from litters 5- 11 only (male-LPS n = 14; male-NaCl n = 14; female-LPS n = 14; female-NaCl n = 13). Following the three test days, animals were single-housed in preparation for water

habituation necessary in the final testing period. On P 75 rats were adjusted to a water restriction schedule. Animals received 30 min daily access to water in their home cages at the end of testing each day. Four days of habituation to the drinking boxes began on P 76. Animals were given access to distilled water in the Lickometer for 30 min each morning. The final day of habituation (P 79) was designated as the baseline day. Following habituation, animals were given access to 0.3M sucrose solution for 30 min daily on two consecutive days (P 80 and P 81). The first exposure to the novel sucrose provided measures of taste neophobia, while the second produced an index of intake (Tenk, 2007). See Figure 3.1 for a timeline of the behavioural procedure.

3.2.5 Statistical analysis

All analyses were performed with IBM SPSS Statistics 19 (formerly PASW Statistics 18). Significance was set to $\alpha = .05$.

3.2.5.1 Changes in body weight. A univariate analysis of variance (ANOVA) was used to analyze changes in body weight, with the between subjects factors of Sex, Neonatal Treatment (2 levels: LPS or saline) and Litter (11 levels). When appropriate LSD post hoc test were used.

3.2.5.2 Startle and prepulse inhibition. A univariate ANOVA was used to analyze average startle response, with the same between subjects variables mentioned above and Chamber used as a co-variate. Chamber refers to the 3 startle chambers used for testing; there were differences in the sensitivity of the chambers. Additionally, a repeated measures ANOVA was conducted to analyze differences in average startle response across the two test days, with the same between subjects factors mentioned previously, and a within subjects factor of Day (2 levels). Prepulse inhibition (PPI) data was analyzed using a repeated measures ANOVA for each ISI, with the same between subjects factors as average startle and the addition of prepulse level (73, 76, or 82 dB) as a within subjects factor. LSD test served as a post-hoc comparison when appropriate.

3.2.5.3 Elevated plus maze (EPM). EPM data were analyzed using a multivariate analysis of variance (MANOVA), with the between subjects factors of Sex and Neonatal Treatment (2 levels: LPS, NaCl), and Litter (11 levels). Where appropriate, LSD test was used as a post-hoc comparison.

3.2.5.4 Light-dark and lickometer tests. Behavioural data collected during the light-dark and lickometer tests were analyzed using a mixed design (split-plot) analysis of variance

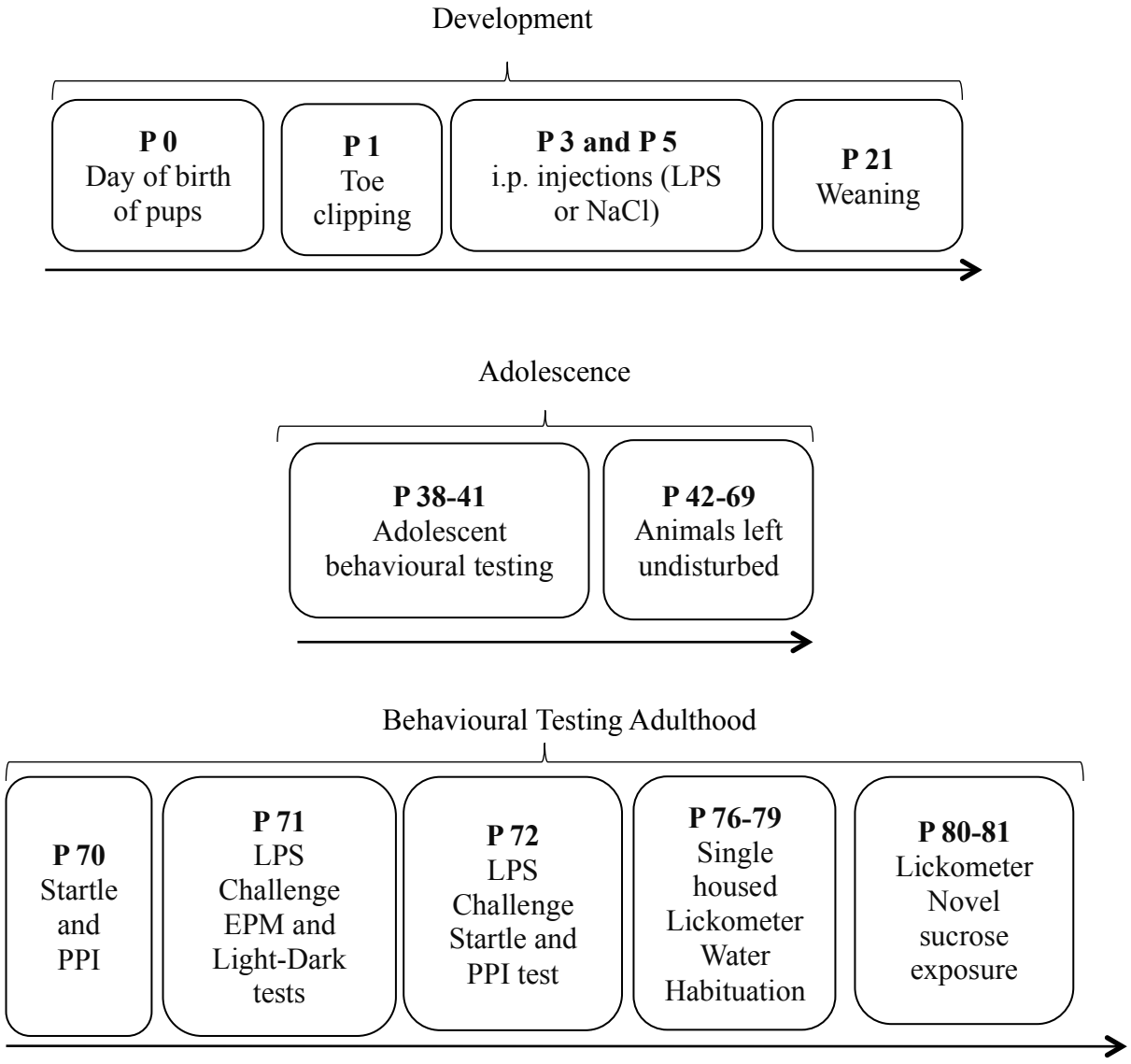

Fig. 3.1. Timeline for experiment 2. Neonatal drug treatment occurred on postnatal days 3 and 5, followed by the testing period during adolescence (experiment 1), and behavioural testing during adulthood (postnatal days 70 – 81). Testing without adult immune system challenge occurred on postnatal day 70 in the startle apparatus and on postnatal days 76 – 81 in the lickometer test. Testing adult immune system challenge occurred on postnatal days 71 and 72.

(ANOVA) with between-subjects factors of Sex (2 levels: male and female) and Neonatal Treatment (2 levels: LPS or saline). Litter was also considered a between subjects factor with 11 levels for the light-dark test and 6 levels for the lickometer test. The within subjects factors were Day and/or Time, which varied depending on the behavioural test being analyzed. For the light-dark tests, only a within subjects factor of Time was considered (6 levels of 5- min time bins). For the lickometer tests, the within subjects factor of Day (3 levels) was measured. Post-hoc comparisons of significant main effects and interactions were performed using LSD test when appropriate.

3.3 Results

3.3.1 Changes in Body Weight

All animals demonstrated a reduction in body weight 24 hours following the initial injection of LPS on postnatal day 71 (see Figure 3.2). However, no significant differences were observed between treatment groups, as statistical analysis did not yield main effects of Sex ($F(1, 43) = .171$, $p = .682$) or Neonatal Treatment ($F(1, 43) = .193$, $p = .662$), or a significant interaction ($F(1, 43) = .995$, $p = .334$). Furthermore, as body weight was not collected after the second dose of LPS on postnatal 72, evidence of LPS tolerance could not be assessed.

3.3.2 Elevated Plus Maze

Due to the presence of outliers, five animals were excluded from the analyses of the various behavioural measures (2 male-NaCl, 1 male-LPS, 1 female-NaCl, 1 female-LPS animals excluded). Analysis revealed no differences between Neonatal Treatment groups in traditional anxiety behaviours in adulthood, despite receiving a challenge dose of LPS. However, analysis of the data yielded a significant main effect of Sex for the number of entries into the open arms, $F(1, 38) = 5.51$, $p = .024$, and the percentage of time spent in the open arms, $F(1, 38) = 8.74$, $p = .005$. Males showed reduced entries and percent time in the open arms as compared to females (Figure 3.3 A and B). No effects of Sex or Drug were found in the various risk assessment behaviours, inclusive of head and body stretch attends, head dips, and double backs. The MANOVA did not yield any Sex x Neonatal Treatment Interactions for any of the measures of anxiety in adulthood. For a summary of significance data see Appendix E.

A significant main effect of Litter was found for several of the EPM variables

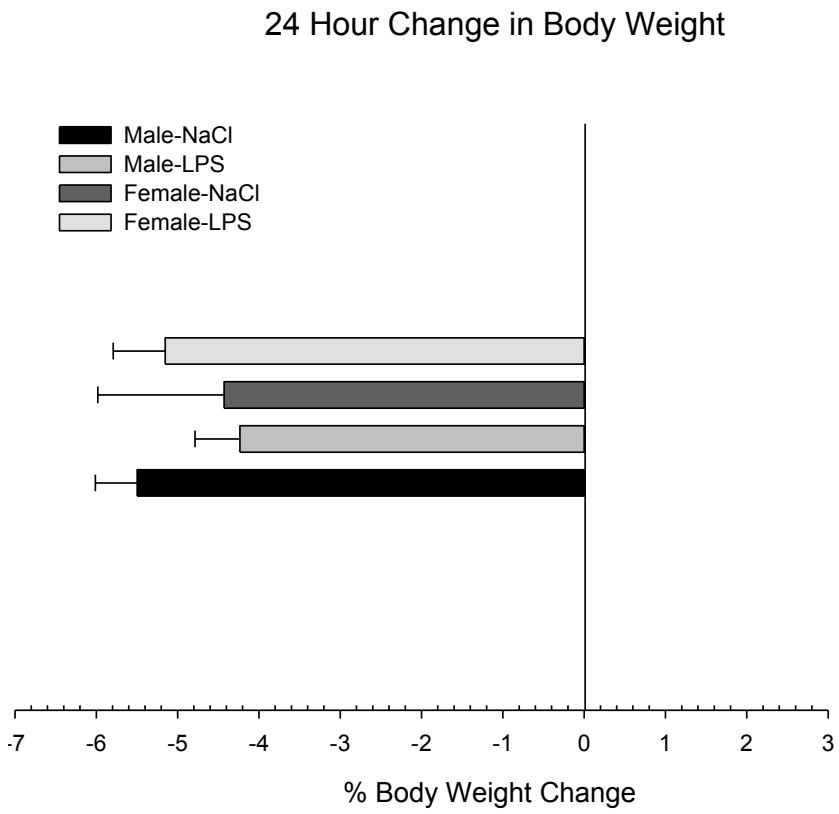

Fig. 3.2 Twenty-four hour group mean (± S.E.M) change in body weight following i.p. challenge injection of lipopolysaccharide (LPS; 200 μg/kg) in adulthood. All treatment groups show similar reductions in body weight (male-SAL: $n = 23$, male-LPS: $n = 23$, female-SAL: $n = 19$, female-LPS: $n = 21$).

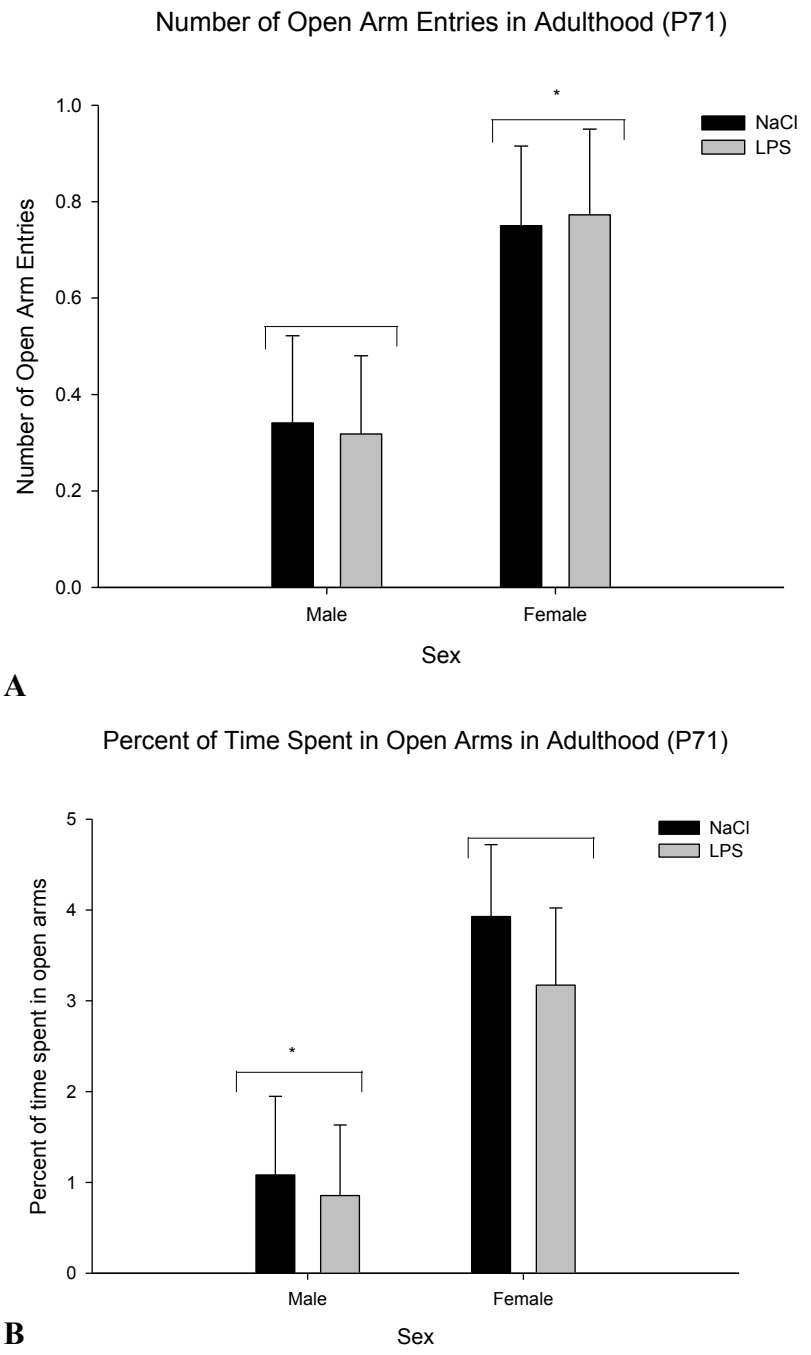

Fig. 3.3 Group mean (± S.E.M.) anxiety-related behaviours in the elevated plus maze in adulthood (P71). (A) number of entries into the open arms (B) percent time in open arms of the elevated plus maze (time spent in open arms/time spent in open arms + time spent in closed arms x 100). * $p < .05$ indicates a significant overall sex effect, females showed greater number of entries and percent time in the open arms as compared to males (male-SAL: $n = 21$, male-LPS: $n = 22$, female-SAL: $n = 18$, female-LPS: $n = 20$).

including the number of open entries ($F(10, 38) = 2.15$, $p = .001$, the number of closed arm entries ($F(10, 38) = 3.80$, $p = .007$), the duration of time in the closed arms ($F(10, 38) = 2.98$, $p = .007$), the number of head dips ($F(10, 38) = 2.80$, $p = .011$), and duration of body stretch attends ($F(10, 38) = 4.23$, $p = .001$).

3.3.3 Light-Dark Test

Due to technical difficulties in obtaining data, two animals were excluded from this analysis (1 male-NaCl animal and 1 female-LPS animal excluded). A representative measure of horizontal activity (total distance, cm/min; duration, s) and vertical activity (total number of vertical movements, cm/min; total vertical movement time, cm/min) in both chambers is depicted in Figure 3.4A-D and a summary of significance data is represented in Appendix F.

Statistical analysis indicated that all of the groups of animals spent significantly more time in the dark chamber during adulthood, $F(1, 41) = 147.5{,}6$ $p < .001$, although no differences between sexes and neonatal treatment groups were observed. All animals travelled more in the light chamber, $F(1, 42) = 87.90$, $p < .001$, and analysis of these data revealed a significant Sex x Neonatal Treatment interaction in the light chamber, $F(1, 41) = 4.41$, $p = .042$. Males treated with NaCl travelled significantly more in the light chamber than males treated with LPS ($p = .022$). Additionally**,** females treated with LPS travelled significantly more in the light chamber than males treated with LPS ($p = .010$).

Animals showed a greater amount of vertical activity in the light chamber, as compared to the dark chamber. All animals demonstrated a greater number of vertical movements, $F(1, 42) = 207.01$, $p < .001$, and greater duration of vertical movements, $F(1, 42) = 98.21$, $p < .001$, in the light chamber. A main effect of Litter was observed for the total distance travelled ($F(10, 41) = 2.68$, $p = .013$) and duration of vertical movements ($F(10, 41) = 2.12$, $p = .045$) in the light chamber, as well as duration of vertical movements in the dark chamber, $F(10, 41) = 4.34$, $p < .001$.

Finally, all animals entered the light chamber more often than the dark chamber, $F(1, 41) = 9.61$, $p = .004$. Analysis revealed a main effect of time for number of entries in the light chamber ($F(3, 131) = 101.48$, $p < .001$) and dark chamber ($F(3, 102) = 68.34$, $p < .001$), as well as for duration in the light ($F(3, 127) = 12.96$, $p < .001$) and dark chamber ($F(3, 127) = 13.00$, $p < .001$). Thus, all animals demonstrated habituation to the environment, as the number of entries into the chambers decreased over the 30 min period. In addition, a main

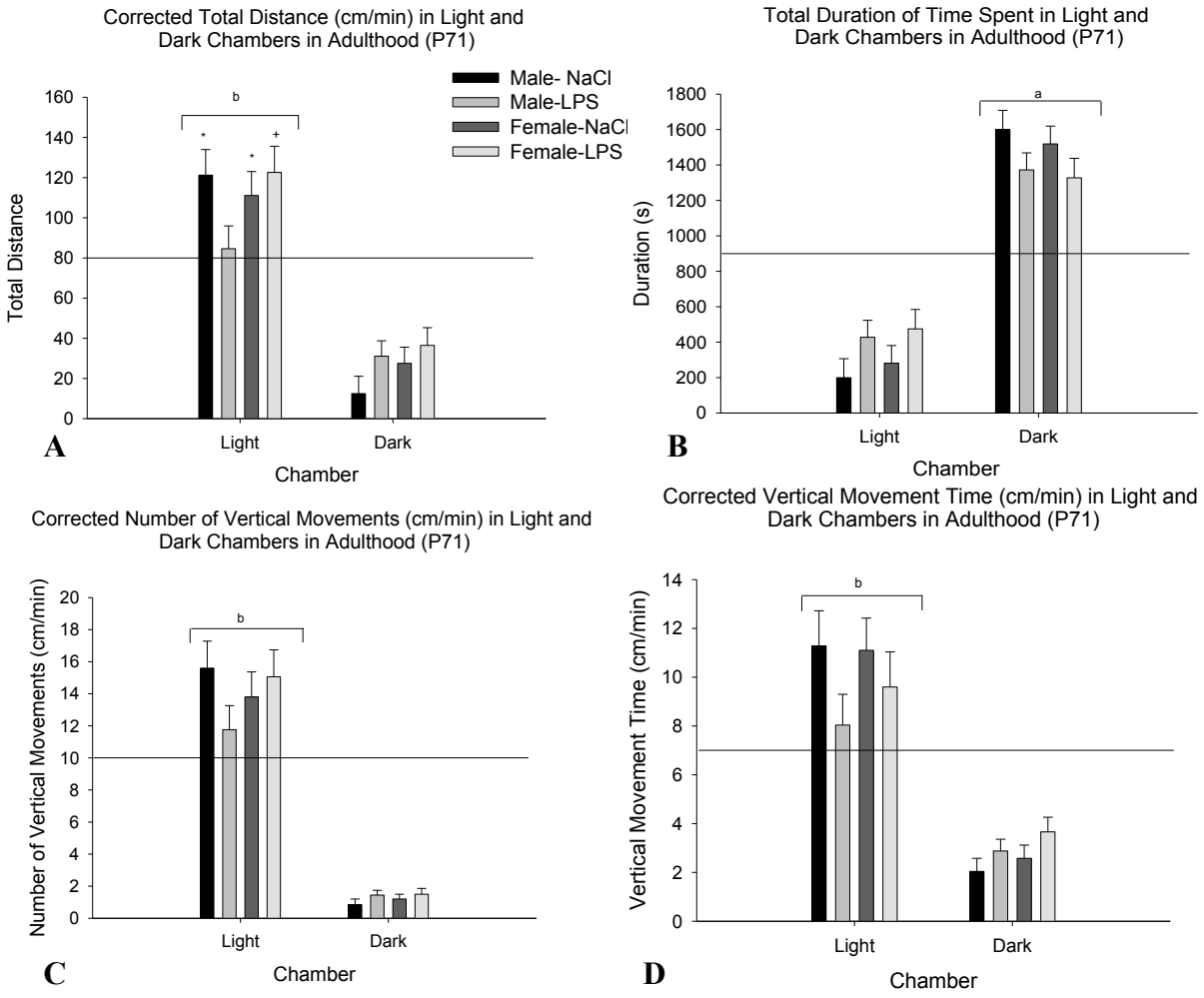

Fig. 3.4. Group mean (± S.E.M.) activity behaviours in the light-dark test during adulthood (P71). (A) corrected total distance (cm travelled per min) in light and dark chambers (B) total duration (s) spent in light and dark chambers (C) corrected number of vertical movements (cm per min) in light and dark chambers (D) corrected vertical time (cm per min) in light and dark chambers. Horizontal lines represent the point at which equal time would be expected to be spent in each chamber. *$p < .05$ indicates significant Neonatal Drug x Sex interaction, where male-NaCl animals travelled more than male-LPS animals in the light chamber. + $p < .05$ female-LPS travelled more in the light chamber than male-LPS. 'a' indicates a significant main effect of chamber ($p < .05$) with all treatment groups spending more time in the dark chamber. 'b' indicates a significant main effect of chamber ($p < .05$) with all treatment groups travelling more, having greater number of vertical movements and greater vertical time in the light chamber (male-SAL: $n = 22$, male-LPS: $n = 23$, female-SAL: $n = 19$, female-LPS: $n = 20$).

effect of Litter was observed for number of entries into the light chamber, $F(10, 41) = 4.18$, $p = .001$ and dark chamber $F(10, 41) = 6.17$, $p < .001$.

3.3.4 Startle and Prepulse Inhibition

Analysis of average startle responses to startle-only trials yielded a significant main effect of Sex on both Day 70, $F(1, 42) = 11.39$, $p = .002$ and Day 72, $F(1, 42) = 11.74$, $p = .001$, where animals received a challenge injection of LPS 90 min before testing (Figure 3.5 A and B). A main effect of Litter was also observed on Day 70, $F(10, 42) = 3.23$, $p = .004$ and Day 72, $F(10, 42) = 2.77$, $p = .010$ (post hoc tests not reported). On both test days, males showed a larger startle response than females. Neonatal LPS treatment had no effect on average startle response. Furthermore, no main effect of Day was found for average startle between the two test days, $F(1, 41) = .108$, $p = .744$. There were no significant effects of Sex or Neonatal Treatment on percent prepulse inhibition, for each of the ISIs of 60 ms and 100 ms on either test day (graphs not shown). A significant main effect of Litter was observed for the ISI of 60 ms on Day 70 ($F(10, 42) = 2.21$, $p = .036$) and Day 72 ($F(10, 42) = 5.75$, $p < .001$) (post hoc tests not reported). For a summary of significance data see Appendix G.

3.3.5 Novel Sucrose Taste Neophobia

Only seven litters were tested in the Lickometer, and due to technical difficulties in obtaining data, four animals were excluded from these analyses (2 male-NaCl, 1 female-NaCl and 1 female-LPS animals excluded). Data analysis of taste neophobia examined changes in intake and licking patterns between baseline day and the first exposure to the sucrose solution. Each variable was computed as a difference between the values of the measure collected on the two days. Measures of overall consumption for each group are depicted in Figure 3.6 A and B. Neonatal LPS treatment had no significant effect on the volume consumed or the Number of Licks as evidenced by the lack of significant interactions or main effects on these measures. Statistical analysis of the microstructural variables (Size of Burst, Number of Bursts, Size of Cluster, and Number of Clusters) revealed no significant effects of Sex or Neonatal Treatment on drinking behaviour. A summary of significance data is depicted in Appendix H.

3.3.6 Familiar Sucrose Intake

Variable totals collected during the second exposure to sucrose were used as the measures of familiar sucrose intake. Consumption measures on this day for each group are depicted in Figure 3.6 A and B. A main effect of Sex was observed for volume consumption,

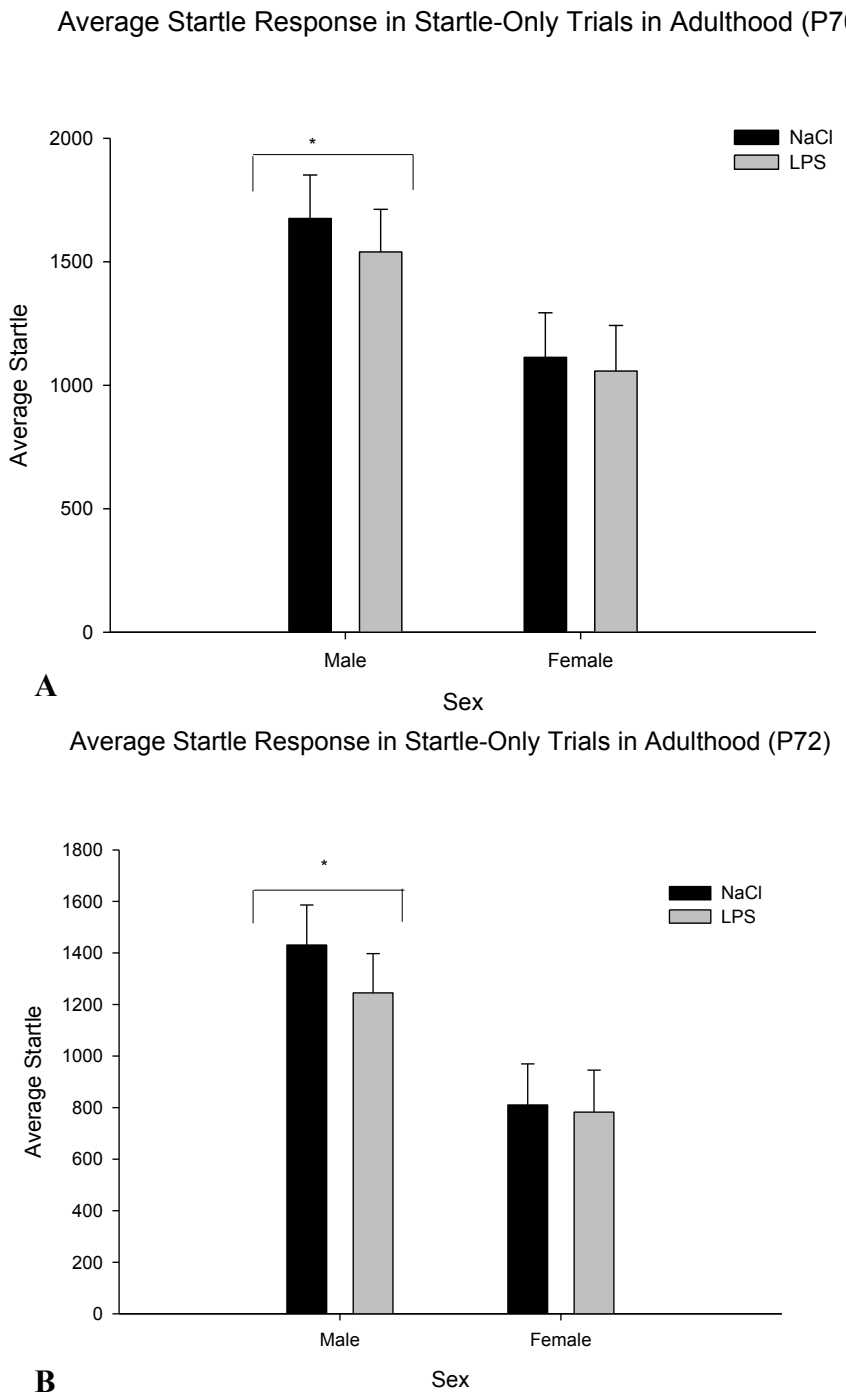

Fig. 3.5. Group mean (± S.E.M) amplitude of the acoustic startle response on Startle-Only trials (A) without adult LPS challenge (P70) and (B) 90 min following adult immune LPS challenge (P72). *$p < .05$ indicates significant main effect of sex, with males showing greater startle response than females (male-SAL: $n = 23$, male-LPS: $n = 23$, female-SAL: $n = 19$, female-LPS: $n = 21$).

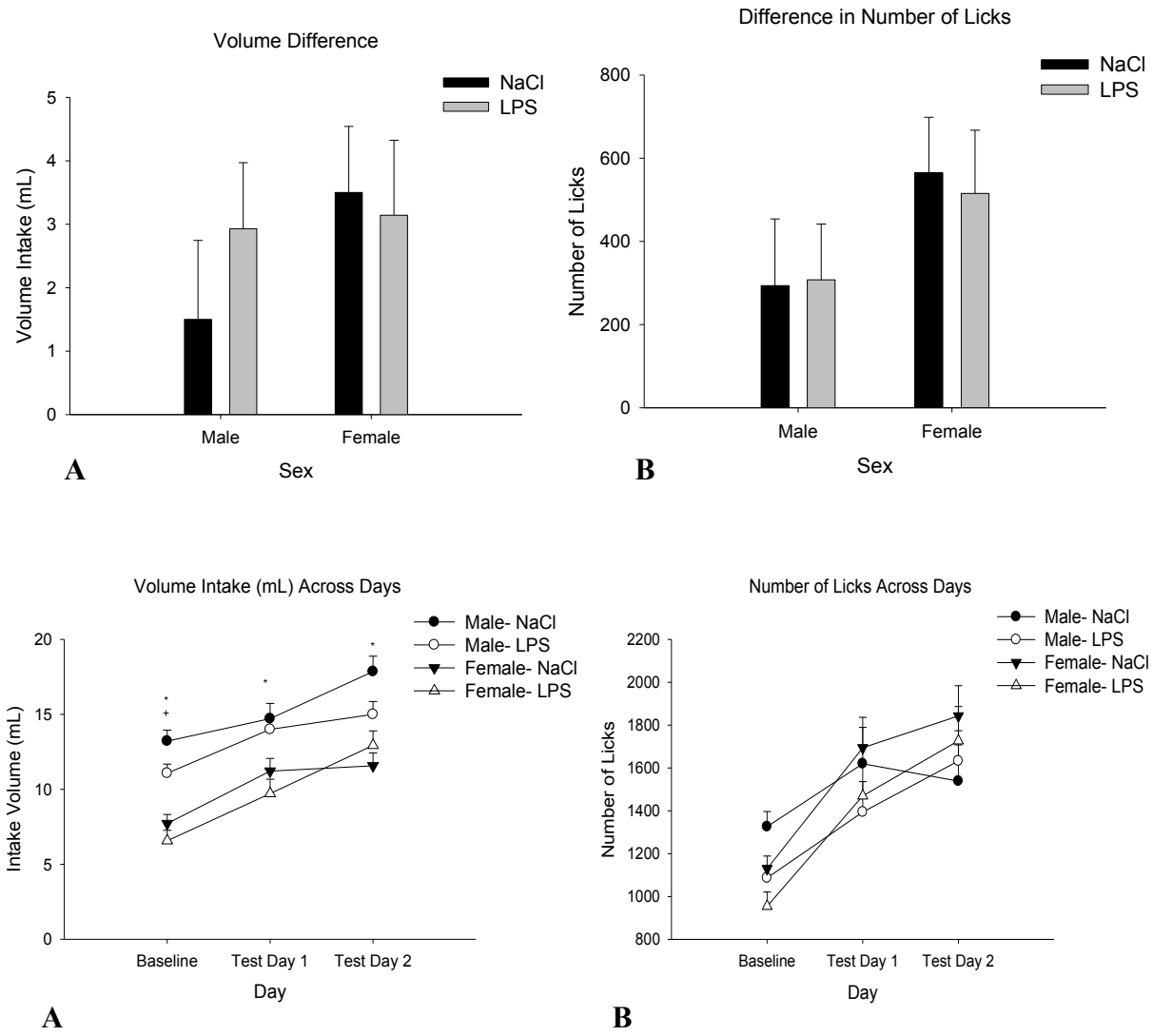

Fig. 3.6. Measures of overall consumption displayed both as difference between baseline and initial exposure to sucrose (top), as well as totals consumed across days (bottom). (A) Group mean (± S.E.M**)** volume consumed (B) group mean (± S.E.M) number of licks**.** **p* < .05 indicates main effect of sex, as males consumed more fluid across days compared to females. +*p* < .05 indicates main effect of neonatal drug treatment, with saline-animals showing greater volume intake on baseline day than LPS-animals (male-SAL: *n* = 11, male-LPS: *n* = 14, female-SAL: *n* = 14, female-LPS: *n* = 12).

$F(1, 23) = 65.12$, $p < .001$, as males drank significantly more fluid across all days compared to females. Finally, a main effect of Day was found, $F(2, 46) = 35.10$, $p < .001$), where consumption in all treatment groups increased from baseline to the final test day. The analysis did not yield any significant interactions. Statistical analysis revealed no significant effects of Sex of Neonatal Treatment for the Number of Licks or any of the microstructural variables. For a summary of significance data see in Appendix I. A significant main effect of Litter was found for volume, $F(6, 23) = 16.36$, $p < .001$ and the Number of Licks, $F(6, 23) = 6.10$, $p < .001$ (post hoc tests not reported).

3.4 Discussion

The current study examined the effects of neonatal LPS exposure on voluntary and non-voluntary behavioural responses in adults with and without an accompanying immune challenge. Furthermore, potential sex differences in these effects were explored. The findings of this study suggest that LPS treatment on postnatal days 3 and 5 may have specific long-lasting effects on general anxiety behaviours in adulthood, as adult immune challenge did not significantly affect behaviours in the elevated plus maze for any of the treatment groups, but did result in differences in light dark test. Furthermore, adult anxiety-related behaviours in the elevated plus maze and light-dark tests were influenced by sex. Neonatal LPS exposure did not significantly affect non-voluntary startle reflex regardless of whether or not animals received an immune challenge in adulthood. Finally, neonatal LPS treatment did not have an effect on taste neophobia, but sex-specific differences in drinking behaviour were observed.

Adult animals with and without neonatal LPS exposure showed similar patterns of heightened anxiety and risk assessment behaviours in the elevated plus maze. Acute LPS has been shown to increase anxiety-related behaviour in the elevated plus maze in adult rats (Nava, et al., 1997; Nava & Carta, 2001) and the light-dark test in adult mice (Lacosta, Merali, & Anisman, 1999). Thus, the effects of the adult immune challenge on anxiety behaviours in adulthood are consistent with results from previous research. However, neonatal LPS exposure did not significantly affect adult anxiety behaviours in the elevated plus maze in response to the adult immune challenge, as the animals treated neonatally with LPS showed similar behaviour patterns as the animals treated with saline.

LPS challenge in adulthood similarly increased anxiety-related behaviours in the light-dark test in animals treated with and without neonatal LPS, with a greater duration of time spent in the dark chamber, and more escape-related behaviours in the light chamber.

However, neonatal LPS had a significant effect on distance travelled, as males treated neonatally with saline travelled more in the light chamber than males treated with LPS in response to an adult immune challenge. Results from prior research have been variable. Some studies report no influence of neonatal LPS exposure on anxiety-related behavioural (Walker, Knott, & Hodgson, 2008). Furthermore, results also show no effect of maternal endotoxin exposure (viral mimetic, Poly IC) on various adult behaviours, including those in the elevated zero maze, open-field, and light-dark test (Vorhees, et al., 2012).

However, other studies present opposing findings that show neonatal LPS to result in increased adult anxiety in the elevated plus maze (Walker, March, & Hodgson, 2004b). Furthermore, repeated adult endotoxin treatment has been shown to cause an increase in sickness sympoms, corticosterone release, and anxiety behaviours (Schmidt, Janszen, Wouterlood, & Tilders, 1995; Hayley, Brebner, Lacosta, Merali, & Ainsman, 1999; Hayley, Lacosta, Merali, van Rooijen, & Ainsman, 2001). Although these findings suggest that neonatal LPS may intensify disease severity and increase the risk for anxiety following an immune challenge in adulthood, this was not observed in the current study in terms of anxiety-related behaviours in the elevated plus maze.

All adult animals showed reductions in body weight 24 hours after the adult LPS injection, highlighting the physiological effects of acute LPS. However, no differences were found between those that received neonatal LPS or saline treatment. As all animals demonstrated similar physiological changes in terms of body weight loss, it may suggest that rather than increasing disease severity in adulthood, neonatal LPS may result in immune system tolerance. This has been found in previous studies demonstrating that treatment with neonatal LPS on postnatal days 3 and 5 results in attenuated fever following adult LPS adminstration (Walker, Hodyl, Krivanek, & Hodgson, 2006) and reduces the development of arthritis following an adult inflammatory challenge (Shanks, et al., 2000).

Neonatal LPS may not exert general long-lasting effects on anxiety in adulthood, as the treatment groups did not show differences in many of the anxiety-related behaviours. However, the finding that males treated neonatally with saline travelled significantly more in the light chamber than males treated with LPS following acute adult immune stimulation suggests that neonatal endotoxin exposure has highly specific effects on certain adult behavioural responses. The inconsistent findings from the various warrant more research on the various effects of neonatal endotoxin exposure on adult anxiety behaviours.

Sex differences in anxiety in response to adult immune challenge were found in the current study. Female rats from both the neonatal saline and LPS treatment groups similarly demonstrated more open arm entries and greater percent time in the open arms of the elevated plus maze compared to males. This finding is consistent with reports from previous studies suggesting that female rats have greater levels of basal activity in novel environments, indicative of less anxiety (Engeland, Kavaliers, & Ossenkopp, 2003a; Franklin, Engeland, Kavaliers, & Ossenkopp, 2003), and display less anxiety-related behaviour in the elevated plus maze (Zimmerberg & Farley, 1993). Furthermore, females treated neonatally with LPS travelled more in the light chamber than males treated with LPS. Results from previous research have demonstrated that females display less anxiety-related behaviour in the light dark test (Hughes, Desmond, & Fisher, 2004). Furthermore, while males treated neonatally with LPS were found to display significantly lower amounts of locomotor activity in the open field in response to adult LPS adminstration, neonatal LPS treatment did not induce similar hypoactivity in females (Tenk, 2007). This result is consistent with reports that males may be more susceptible to the effects of endotoxin than females in certain behavioural responses associated with a fear-inducing situation (Engeland, Kavaliers, & Ossenkopp, 2003; Franklin, Engeland, Kavaliers, & Ossenkopp, 2003). Previous reports have also suggested that sex differences in activity disappear once an environment is no longer novel (Engeland et al., 2003a; Franklin et al., 2003). However, this was not the case in the current study, suggesting that novelty is dependent on the duration of time between exposures. It is therefore possible that the length of time between the initial exposure to the elevated plus maze and light-dark tests (in adolescence) was enough to render the testing environment novel by the time animals reached adulthood.

The mechanism underlying the observed sex difference in hypoactivity following an adult challenge remains unclear. It has been hypothesized that the immunosuppressive effects of testosterone may influence this response (Gaillard & Spinedi, 1998). As females have been shown to have enhanced immune functioning, it is possible that the adult LPS challenge resulted in greater immune system tolerance in females treated neonatally with LPS than in males. However, given that this sex difference was only found in one measure of locomotor activity, it is possible that it is another finding demonstrating greater levels of activity in females relative to males.

The present study found no significant effects of neonatal LPS administration on acoustic startle response and prepulse inhibition, either with or without endotoxin challenge in adulthood. All animals demonstrated similar patterns of average startle response when tested in the startle apparatus without adult challenge. This result is consistent with those from previous studies, which suggest that neonatal endotoxin exposure alone is not sufficient to produce abnormal startle responses (Walker, Knott, & Hodgson, 2008). However, results from previous studies have also shown deficits in adult startle response and PPI associated with neonatal endotoxin exposure (Tohmi, Tsuda, Watanabe, Kakita, & Nawa, 2004), as well as reductions in female, but not male, startle response associated with prenatal endotoxin exposure (Vorhees, et al., 2012). Therefore, effects of neonatal LPS treatment on startle response seem to be inconclusive.

Results of the current study also show that neonatal LPS exposure did not significantly affect startle response following adult immune activation. Acute LPS treatment has been shown to significantly reduce startle response magnitude (Lockey, Kavaliers, & Ossenkopp, 2009). Furthermore, previous studies have suggested that neonatal LPS treatment results in exaggerated startle following exposure to stress (Walker, Knott, & Hodgson, 2008). Therefore, it was expected that the neonatal saline-treated animals would show reduced startle responses relative to the neonatal LPS-treated animals following an adult immune challenge. It should be noted that animals received an injection of LPS less than 24 hours prior to the second injection of LPS. Animals develop rapid physiological and behavioural tolerance to LPS immediately following the first administration (Zeisberger & Roth, 1998), and this may suggest that all animals showed similar startle patterns due to tolerance development. Furthermore, given that no differences were observed between startle responses on the first and second test days for any treatment groups, it is unlikely that behavioural tolerance to the startle apparatus was developed.

Sexual dimorphism in average startle response was observed on both test days, with males demonstrating greater average startle than females. This result is consistent with results from some previous research, which found males to have greater acoustic startle response than females (Lehmann, Pryce, & Feldon, 1999). This is also consistent with findings indicating that males showed enhanced anxiety relative to females (Zimmerberg & Farley, 1993). However, no sex differences were observed in prepulse inhibition, which is inconsistent with previous studies that found males to have enhanced PPI relative to females

(Lehmann, Pryce, & Feldon, 1999). This effect may vary depending on the strain of animal tested. For instance, previous research has shown that Sprague-Dawley rats are more sensitive than Long Evans rats to the PPI disruptive effects of dopamine agonists (Qu, Saint Marie, Breier, Ko, Stouffer, & Parsons, 2009). Furthermore, results of prior studies have shown that restraint stress decreases PPI in female Long Evans rats, but does not affect males or other rat strains (Faraday, 2002). Finally, research has also shown Long-Evans rats to have high mean startle amplitude relative to many other strains of rat (Glowa & Hansen, 1994). As such, results seem to be inconsistent across studies, and the observed effects of acoustic startle response and PPI may be influenced by rat strain.

Neonatal LPS administration did not appear to alter drinking behaviour during the taste neophobia task, as all animals showed similar patterns of drinking in response to the novel sucrose solution. This suggests that early endotoxin exposure may not influence novel taste-related anxiety in adulthood. Furthermore, neonatal LPS treatment did not affect drinking behaviours in adulthood in response to familiar sucrose solution. These findings are similar to results from some previous research, which found no effect of neonatal LPS on initial or familiar consumption behaviours (Tenk, 2007). However, other studies have demonstrated that prenatal psychological stress (e.g. novel environment) leads to increased taste neophobia in adulthood (Pfister, Golus, & McGee, 1981). These inconsistent findings suggest that the developmental period during which animals are exposed to pathogens may have differing effects on anxiety behaviours in adulthood. Results from previous studies have also demonstrated differences in licking patterns, and suggest that neonatal LPS may influence situational anxiety, such that animals treated with LPS neonatally show fewer risk assessment behaviours in licking patterns than those treated with saline (Tenk, 2007). The current study did not replicate these results, and further research is required to determine whether neonatal LPS has significant effects on anxiety-related drinking behaviours.

All animals showed consistent drinking patterns, whereby volume consumed increased across test days. Thus, all animals drank more of the sucrose solution on the first day compared to water consumption on baseline day, and drank significantly more sucrose on the second test day compared to the initial exposure. This suggests that while animals liked the palatable sucrose during the initial exposure, they all showed expected taste neophobia, which disappeared by the second sucrose exposure (Corey, 1978).

Depressive-like behaviours are associated with increased secretion of cytokines, indicative of a relationship between immune system activation and depression (Connor & Leonard, 1998). While non-treated rats show a preference for the palatable fluids such as sucrose, acute LPS administration results in anhedonia (inability to experience pleasure), demonstrated by reductions in preference for and consumption of saccharine (Yirmiya, 1996). Furthermore, such effects are reversed with chronic administration of antidepressants (Yirmiya, 1996). However, early life manipulations such as handling, separation, and deprivation have been shown to have varying effects on depressive behaviours. While there are some reports of decreased sucrose preference following early life stressors, other findings report no effects of such stressors on sucrose preference (Schmidt, Wang, & Meijer, 2011). Furthermore, postnatal LPS treatment has been shown to have no effect on anhedonia and sucrose preference in adulthood, as well as other behavioural indices of depression (Kentner, McLeod, Field, & Pittman, 2010; Lucchina, Carola, Pitossi, & Depino, 2010). This is consistent with the results from the current study, and suggests that neonatal LPS administration does not have long-lasting effects on anhedonia in adulthood.

The current study revealed some evidence of sexual dimorphism in drinking behaviour across days, whereby males consumed more fluid than females (both water and sucrose solution). Results from previous research have shown that females have a heightened preference for, and consume greater amounts of sucrose than males (Sclafani, Hertwig, Vigorito, & Feigin, 1987; Curtis, Davis, Johnson, Therrien, & Contreras, 2004), although this finding was not replicated in the current study. Results from previous research have highlighted a small, but significant relationship between larger body weight and increased fluid consumption (Cizek & Nocenti, 1965). As all animals showed similar preference for the sucrose solution, the current sex difference may suggest that males consumed more fluid than females due larger body weight, regardless of solution preference.

The sex differences that were observed in the various tests are consistent with results from previous studies. Females showed greater activity levels in the elevated plus maze and light dark test, while males demonstrated heightened acoustic startle response and increased fluid consumption. It is of interest to note that such sex differences only emerged in adulthood, as the results of experiment 1 (Chapter 2) did not indicate sexual dimorphism in any of the anxiety-related behaviours in adolescence. This suggests that sex differences may be dependent on developmental time period. The adolescent period commonly involves

heightened exploratory and risk-taking behaviour, and as such, sex differences may not appear until adulthood, when such behaviour is reduced.

In summary, the present study demonstrated neonatal endotoxin exposure may not have long-lasting effects on voluntary and non-voluntary behavioural responses in adulthood, including general anxiety- and depression-related behaviour. However, early endotoxin exposure may have highly specific effects on certain anxiety-related behaviours assessed in independent ethological tests. Furthermore, acute LPS administration in adulthood does increase various anxiety-related behaviours. Moreover, sex differences were observed in the various tests of anxiety-related behaviours. The current study has important implications for determining adult immune outcomes for neonates exposure to bacterial infection.

3.5 References

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CHAPTER 4

General Discussion

4.1 General Discussion

The present thesis examined the long-term effects of neonatal endotoxin exposure on various behaviours in adolescent and adult Long-Evans rats. Such studies may help to gain a better understanding of these effects and further identify any potential risk factors of acute pathogen exposure during infancy and later development, such as alterations in immune system functioning, anxiety and depression behaviours, and non-voluntary and voluntary activity.

The dual-exposure-to-endotoxin (DEE) model of neonatal lipopolysaccharide (LPS) administration was utilized, which included administration of LPS on postnatal days 3 and 5, prior to the neonatal stress hyporesponsive period. Results of previous research have shown that neonatal endotoxin exposure alters various behavioural responses in adulthood following other adverse neonatal manipulations such as neonatal handling and maternal separation. However, the effects of early immune system activation during adolescence have not been explored in detail. The results of the first experiment suggested that early immune system activation resulted in increased anxiety in the elevated plus maze for both male and female adolescents, but had no effect on behaviours in the novel and familiar open field and light dark tests. Furthermore, no sex differences were observed for any of the anxiety-related behaviours in adolescence.

The second experiment evaluated the effects of neonatal LPS treatment on behaviours in adulthood, inclusive of non-voluntary startle response and sensorimotor gating with and without an immune challenge in adulthood; anxiety-related behaviours following acute LPS administration; and taste neophobia and anhedonia independent of additional immune system activation. Acute LPS administration in adulthood was found to increase anxiety behaviours in the elevated plus maze and light-dark test, with this effect being stronger in males than females. Results suggested that neonatal LPS did not significantly affect adult anxiety-related behaviours in the elevated plus maze following adult immune system challenge. However, neonatal LPS resulted in reduced activity in the light chamber in adulthood related to neonatal saline treatment. Neonatal endotoxin treatment did not significantly affect non-voluntary startle behaviours with or without an immune challenge in adulthood. Furthermore, no effect of neonatal treatment was found for taste neophobia or sucrose preference in the lickometer drinking test in adulthood. To better understand whether neonatal LPS administration has effects in a sex-specific manner, sex differences in adult behaviours were

investigated. Females demonstrated greater levels of activity than males in the elevated plus maze and light-dark test. In the startle test, male animals showed greater average startle responses than females, although no sex differences in prepulse inhibition were observed. Finally, males drank more water and sucrose solution on all test days relative to females, suggesting that males have a greater fluid intake regardless of solution preference.

The varying results observed during the adolescent period are consistent with previous research. In a brief test of unconditioned exploration of an aversive environment (i.e. the elevated plus maze), neonatal LPS resulted in increased anxiety. However, in tests interpreting activity and locomotion as indices of anxiety, no effects were observed. The findings highlight the importance of using a battery of ethological tests to assess anxiety-related behaviours. They suggest that a set of different tests may measure various aspects of anxiety, which may overlap with one another to produce a general index of anxiety (Ramos, 2008). These ethological tests are all based on the natural conflict between the drive to explore a new environment and the tendency to avoid potentially dangerous situations. However, the correlation analyses between tests also indicate that the various tests may measure different aspects of this conflict and the resulting anxiety-related behaviour (Bourin & Hascoet, 2003; Walf & Frye, 2007). In adolescence, there were relatively few correlations between measures on the elevated plus maze and the open field and light dark tests, which reflect anxiety possibly unique from the confound of motor activity. In contrast, more correlations were found between variables in the open field (novel and familiar) and variables in the light-dark test, which reflect exploration and locomotor activity. This understanding may provide better insight into the nature and degree of overlap between tests and the constructs of anxiety they are measuring.

Variable results were also observed in adulthood, as neonatal LPS did not significantly affect behaviours in the elevated plus maze or startle apparatus, but did result in specific differences in the light-dark test. Males treated neonatally with LPS travelled less in the light chamber than males treated with saline. This finding supports the argument that different tests may measure various aspects of anxiety. Furthermore, it suggests that neonatal LPS may have varying effects on the different constructs of anxiety at certain developmental stages, as anxiety-related behaviour was altered in the elevated plus maze in adolescence, but was altered in the light-dark test in adulthood.

Experiment 1 examined behaviours during the transitional developmental period of adolescence. No significant differences were observed between animals treated neonatally with LPS and those treated with saline on measures of locomotor activity in the open field and light-dark tests. Furthermore, no sex differences in the various behaviours were observed in either experiment. These findings illustrate the behavioural responses unique to the adolescent developmental stage, whereby all animals show less anxiety than adults, as well as heightened exploratory behaviour, increased risk-taking behaviour, and reduced neophagia (Spear, 2000; Doremus, Varlinskaya, & Spear, 2004; Stansfield & Kirstein, 2006). As such, the findings of the current study may represent typical adolescent behaviour across all animals, regardless of early immune system manipulation or sex, which is necessary for appropriate development and transition into adulthood (Spear, 2000; Meyza, Boguszewski, Niko, & Zagrodzka, 2011). Therefore, neonatal endotoxin exposure in experiment 1 did not result in significant changes in activity because of motivational reorganization, whereby adaptive behaviours associated with adolescence may have taken precedence over other responses such as severely enhanced anxiety.

While the anxiety associated from the novel experiences during adolescence was suppressed by the increased exploratory and risk-taking behaviour common to this developmental period, different findings were observed in adulthood. Consistent with results from previous findings (e.g. Nava, et al., 1997; Lacosta et al., 1999; Nava & Carta, 2001) acute LPS in adulthood resulted in increased anxiety in specific behavioural measures of the elevated plus maze and the light-dark tests. Sickness behaviours associated with acute immune activation are a set of physical and behavioural symptoms to overcome the infection and increase survival by conserving energy and avoiding predation in a weakened state (Dantzer, 2001). Such a strategy is an adaptive motivational state that can be reorganized depending on the consequences. A fear-inducing situation may invoke escape behaviours that overcome sickness in order to avoid potential threat (Dantzer, 2001). Given the motivational state of sickness behaviour, it is possible that adult animals in experiment 2 all demonstrated similar anxiety-related behaviours in the elevated plus maze and light-dark tests because of the potentially threatening experience of a novel, anxiety-provoking environment. Furthermore, sex differences consistent with previous research emerged in adulthood, with females showing greater activity levels and less anxiety. This suggests that motivational reorganization can be demonstrated in a sex-specific manner.

Many of the results presented in this thesis revealed some inter-litter differences in the dependent variables. The impact of this variability is moderately reduced because of the within-litter nature of the experimental design, whereby each litter contained both LPS- and saline-treated animals, either neonatally or during adolescence. These inter-litter differences are likely the result of a combination of genetic, intrauterine environment and/or maternal behaviour influences, which have all been shown to have effects on behaviours later in life (e.g. Geerse, van Gurp, Wiegant, & Stam, 2006a; 2006b; Hernandez-Tristan, Leret, & Almeida, 2006; Liu, Diorio, Day, Francis, & Meaney, 2000).

However, such interactions between genes and the prenatal and postnatal environment also influence the developmental of individual behavioural temperament (Trillmich & Hudson, 2011). The correlation analyses from experiment 1 highlight the importance of examining such individual differences and interpreting variations behaviour in terms of differences in animal behavioural style. Individuals adopt particular behavioural styles that increase the risk of survival depending on various environmental factors such as litter size, body mass, and maternal care, as well as potential environmental stressors (Rödel & Meyer, 2011). Such temperaments are often maintained throughout development and result in the emergence of different behavioural responses to various ethological tests (Groothuis & Trillmich, 2011). Results of the correlation analyses showed similar patterns of relationships between males and females treated neonatally with LPS or saline in the elevated plus maze in adolescence. However, differences between treatment were found in comparisons of the novel and familiar open field, associated thigmotaxis behaviours, and the light-dark test, with female-LPS and male-NaCl animals showing more significant correlations than the other treatment groups. These emphasize the importance of examining individual differences and highlight the effects of early environmental factors on the development of behavioural temperament and the resulting differences in responses to stressors in later life.

The results of the correlation analysis also provide evidence for the importance of using multiple ethological tests when investigating behavioural responses. Very few significant correlations were observed between the elevated plus maze and the open field and light dark tests, suggesting that these may be measuring different constructs of anxiety. Consistent with this observation, results of previous studies have suggested that the variables

from the elevated plus maze and the open field do not produce a common anxiety-related factor in rats (Ramos, 2008).

A more detailed analysis is required in this area to determine if behavioural temperaments remain stable across time. An examination of relationships between variables in adulthood is necessary, as well as comparisons of such correlations with those found in adolescence. Furthermore, future research should focus on conducting factor analysis to determine which tests measure similar or different constructs of anxiety and gain a better understand of whether these vary across developmental stage.

These results of this thesis have potential clinical significance given that neonatal exposure to infection is common, particularly in the Gram negative bacterial form (Washburn, Medearis, & Childs, 1965). The results identify potential risk factors in infants exposed to early bacterial infection, including increased susceptibility to specific aspects of anxiety disorders at different developmental stages. Furthermore, the results also highlight the considerable influence of the motivation to behave in a typical adolescent manner**,** which may overcome other influences, such as immunological manipulation and heightened fear and anxiety. While no sex differences were observed in adolescence, sex differences were found in adulthood. This suggests that that sexual dimorphism may only emerge at certain developmental stages. The results of the current study provide an in-depth analysis of a developmental period that has not been explored in great detail to date, as well as resulting behavioural alterations in later life.

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Appendix A:

Significance values for EPM anxiety behaviours in adolescence: Interaction and main effects of Sex and Neonatal Drug Treatment

Anxiety Behaviours	Sex x Neonatal Drug Interaction	Main Effect of Sex	Main Effect of Neonatal Drug
Number of entries in open arms	.172	.487	.054
Number of entries in closed arms	.455	.896	.894
Duration of time in closed arms	.386	.196	.443
Percent time in open arms	.242	.527	.017 *
Number of head stretch attends	.811	.665	.189
Duration of head stretch attends	.711	.300	.247
Number of body stretch attends	.434	.557	.949
Duration of body stretch attends	.768	.062	.941
Head dips	.212	.098	.759
Number of closed arm re-entries	.123	.959	.816

Note. Significance is set at $\alpha = .05$ and * indicates significance.

Appendix B:

Significance values for locomotor activity: Interaction and main effects of Sex and Neonatal Drug Treatment, main effects of time and day

Locomotor Activity	Sex x Neonatal Drug Interaction		Main Effect of Sex		Main Effect of Neonatal Drug		Main Effect of Time	Main Effect of Day
	Day 39	Day 40	Day 39	Day 40	Day 39	Day 40		
Total Distance (cm)	.744	.951	.981	.981	.919	.986	.001*	.001*
Movement Time (s)	.698	.904	.879	.982	.647	.702	.001*	.001*
Horizontal Movements	.701	.949	.255	.389	.563	.269	.001*	.001*
Vertical Movements	.588	.991	.961	.679	.886	.794	.001*	.001*

Note. Significance is set at $\alpha = .05$ and * indicates significance.

Appendix C:

Significance values for thigmotaxis: Interaction and main effects of Sex and Neonatal Drug Treatment

Thigmotaxis behaviours	Sex x Neonatal Drug Interaction		Main Effect of Sex		Main Effect of Neonatal Drug	
	Day 39	Day 40	Day 39	Day 40	Day 39	Day 40
Total Duration in Periphery (s)	.648	.551	.120	.705	.906	.861
Total Duration in Center (s)	.648	.551	.120	.705	.906	.861
Total Vertical Time in Periphery (s)	.735	.763	.268	.631	.355	.904
Total Vertical Time in Center (s)	.720	.980	.148	.611	.527	.838
Total Number of Entries in Periphery	.392	.778	.857	.818	.747	.444
Total Number of Entries in Center	.393	. 780	.869	.815	.744	.445

Note. Significance is set at $\alpha = .05$ and * indicates significance.

Appendix D:

Significance values for light-dark activity measures in adolescence: Interaction and main effects of Sex and Neonatal Drug Treatment

	Light			Dark		
Activity Measures	Sex x Drug	Main Effect of Sex	Main Effect of Drug	Sex x Drug	Main Effect of Sex	Main Effect of Drug
Number of entries	.847	.714	.949	.400	.496	.303
Duration of time in chamber (s)	.855	.492	.458	.892	.467	.368
Total distance in chamber (cm/min)	.178	.952	.808	.899	.064	.335
Vertical movements in chamber (cm/min)	.744	.886	.662	.019*	.399	.361
Duration of vertical movements in chamber (cm/min)	.644	.828	.158	.746	.419	.695

Note. Significance is set at $\alpha = .05$ and * indicates significance.

Appendix E:

Significance values for EPM anxiety behaviours in adulthood: Interaction and main effects of Sex and Neonatal Drug Treatment

Anxiety Behaviours	Sex x Neonatal Drug Interaction	Main Effect of Sex	Main Effect of Neonatal Drug
Number of entries in open arms	.782	.024*	.850
Number of entries in closed arms	.918	.187	.710
Amount of time spent in closed arms	.793	.987	.889
Percent time in open arms	.875	.005*	.470
Number of head stretch attends	.434	.513	.675
Duration of head stretch attends	.483	.549	.961
Number of body stretch attends	.803	.134	.576
Duration of body stretch attends	.675	.817	.961
Head dips	.434	.538	.989
Double backs	.296	.205	.286

Note. Significance is set at $\alpha = .05$ and * indicates significance.

Appendix F:

Significance values for light-dark activity measures in adulthood: Interaction and main effects of Sex and Neonatal Drug Treatment

	Light			Dark		
Activity Measures	Sex x Drug	Main Effect of Sex	Main Effect of Drug	Sex x Drug	Main Effect of Sex	Main Effect of Drug
Number of entries	.280	.870	.531	.678	.106	.072
Duration of time in chamber (s)	.590	.451	.123	.582	.459	.129
Total distance in chamber (cm/min)	.042*	.269	.492	.930	.419	.162
Vertical movements in chamber (cm/min)	.102	.618	.602	.781	.573	.199
Duration of vertical movements in chamber (cm/min)	.455	.629	.152	.683	.314	.068

Note. Significance is set at $\alpha = .05$ and * indicates significance.

Appendix G:

Significance values for average startle response and percent PPI in adulthood: Interaction and main effects of Sex and Neonatal Drug Treatment

	Day 70			Day 72		
Activity Measures	Sex x Drug	Main Effect of Sex	Main Effect of Drug	Sex x Drug	Main Effect of Sex	Main Effect of Drug
Average startle	.278	.002*	.341	.349	.001*	.248
Percent PPI – ISI of 60 ms	.975	.445	.682	.297	.935	.311
Percent PPI – ISI of 100 ms	.978	.377	.941	.329	.227	.181

Note. Significance is set at $\alpha = .05$ and * indicates significance.

Appendix H:

Significance values for novel sucrose drinking behaviours in adulthood: Interaction and main effects of Sex and Neonatal Drug Treatment

Drinking Behaviours	Sex x Neonatal Drug Interaction	Main Effect of Sex	Main Effect of Neonatal Drug
Volume intake	.438	.338	.640
Number of Licks	.830	.114	.905
Size of Bursts	.985	.709	.551
Number of Bursts	.716	.483	.416
Size of Clusters	.928	.755	.803
Number of Clusters	.589	.663	.519

Note. Significance is set at $\alpha = .05$ and * indicates significance

Appendix I:

Significance values for familiar sucrose drinking behaviours across days (baseline, sucrose day 1 and sucrose day 2) in adulthood: Interaction and main effects of Sex and Neonatal Drug Treatment

Drinking Behaviours	Sex x Neonatal Drug Interaction	Main Effect of Sex	Main Effect of Neonatal Drug
Volume intake	.185	.001*	.041*
Number of Licks	.777	.761	.095
Size of Bursts	.339	.253	.911
Number of Bursts	.803	.592	.493
Size of Clusters	.368	.825	.980
Number of Clusters	.253	.537	.498

Note. Significance is set at $\alpha = .05$ and * indicates significance

Appendix J: Correlation table within elevated plus maze variables in adolescence

		A		B		C		D		E		F		G		H		I		J	
A	Number of open arm entries	**1**	**1**	**.548***	**.067**	**-.734****	**-.604****	**.961****	**.878****	**-.180**	**-.405**	**.643****	**.329**	**-.194**	**-.217**	**-.399**	**-.265**	**.549***	**.649****	**.521***	**.193**
		1	1	.674**	.248	-.760**	-.735**	.935**	.924**	-.292	-.210	-.023	.582**	-.094	.312	-.305	-.134	.603**	.248	.467*	.121
B	Number of closed arm entries			**1**	**1**	**-.707****	**.179**	**.367**	**-.071**	**.360**	**.146**	**.479***	**.199**	**.489***	**-.015**	**.250**	**-.240**	**.737****	**.345**	**.513***	**.066**
				1	1	-.532*	-.302	.613**	.160	.188	.221	-.098	.038	-.138	.406	-.223	.045	.823**	.540*	.431	.307
C	Duration in closed arms					**1**	**1**	**-.609****	**-.624****	**.066**	**.094**	**-.814****	**-.395**	**-.090**	**.038**	**.229**	**.084**	**-.717****	**-.375**	**-.608****	**-.262**
						1	1	-.804**	-.730**	.270	.136	-.356	-.357	.300	-.405	.367	-.107	-.702**	-.456*	-.855**	-.328
D	Percent time in open arms							**1**	**1**	**-.252**	**-.049**	**.435**	**.357**	**-.063**	**.156**	**-.263**	**.107**	**.616****	**.481***	**.762****	**.064**
								1	1	-.257	-.232	.069	.591**	.076	.238	-.241	-.202	.522*	.188	.512*	.132
E	Number of closed arm re-entries									**1**	**1**	**-.165**	**-.331**	**.317**	**.286**	**.679****	**.206**	**.241**	**-.267**	**.064**	**-.245**
										1	1	-.327	-.265	.072	.379	.032	.149	.045	.332	-.135	.208
F	Number of head dips											**1**	**1**	**.101**	**-.130**	**-.184**	**-.265**	**.475***	**.113**	**.482***	**.160**
												1	1	.060	.161	-.292	.019	-.016	.123	.185	-.054
G	Number of head stretch attends													**1**	**1**	**.674****	**.678****	**.454***	**.065**	**.352**	**.307**
														1	1	.676**	.592**	-.300	.578**	-.361	.388
H	Duration of head stretch attends															**1**	**1**	**.154**	**-.014**	**.103**	**.207**
																1	1	-.380	.356	-.224	.204
I	Number of body stretch attends																	**1**	**1**	**.887****	**.699****
																		1	1	.685**	.854**
J	Duration of body stretch attends																			**1**	**1**
																				1	1

N*ote.* Significance is set at α = .05; **p* <.05, ***p* <.001

Left – females; Right – males; Bolded – neonatal LPS treatment; Unbolded – neonatal NaCl

Appendix K1: Correlation table within novel open field variables in adolescence

		A		B		C		D		E	
A	Total distance in novel open field	**1**	**1**	**.920****	**.941****	**.979****	**.990****	**.830****	**.884****	**.805****	**.655****
		1	1	.959**	.922**	.983**	.970**	.897**	.890**	.813**	.883**
B	Horizontal movements in novel open field			**1**	**1**	**.948****	**.956****	**.783****	**.820****	**.896****	**.726****
				1	1	.958**	.965**	.879**	.882**	.855**	.913**
C	Movement time in novel open field					**1**	**1**	**.833****	**.877****	**.821****	**.631****
						1	1	.924**	.904**	.776**	.870**
D	Vertical movements in novel open field							**1**	**1**	**.694****	**.683****
								1	1	.701**	.793**
E	Vertical time in novel open field									**1**	**1**
										1	1

Appendix K2: Correlation table within familiar open field variables in adolescence

		A		B		C		D		E	
A	Total distance in familiar open field	**1**	**1**	**.961****	**.900****	**.983****	**.959****	**.909****	**.754****	**.687****	**.689****
		1	1	.946**	.891**	.986**	.973**	.866**	.836**	.862**	.700**
B	Horizontal movements in familiar open field			**1**	**1**	**.958****	**.942****	**.883****	**.705****	**.753****	**.736****
				1	1	.956**	.901**	.832**	.798**	.881**	.846**
C	Movement time in familiar open field					**1**	**1**	**.889****	**.762****	**.686****	**.619****
						1	1	.892**	.846**	.843**	.702**
D	Vertical movements in familiar open field							**1**	**1**	**.609****	**.664****
								1	1	.654**	.554**
E	Vertical time in familiar open field									**1**	**1**
										1	1

Note. Significance is set at $\alpha = .05$; $*p < .05$, $**p < .001$

Left – females; Right – males; Bolded – neonatal LPS treatment; Unbolded – neonatal NaCl

Appendix L1: Correlation table within novel thigmotaxis variables in adolescence

		A		B		C	
A	Duration in periphery in novel open field	**1**	**1**	**.303**	**.401**	**.202**	**.479***
		1	1	.506*	.199	.618**	.310
B	Vertical time in periphery in novel open field			**1**	**1**	**.877****	**.724****
				1	1	.892**	.731**
C	Number of entries into center area in novel open field					**1**	**1**
						1	1

Appendix L2: Correlation table within familiar thigmotaxis variables in adolescence

		A		B		C	
A	Duration in periphery in familiar open field	**1**	**1**	**.508***	**.511***	**.331**	**.523***
		1	1	.348	.595**	.369	.586**
B	Vertical time in periphery in familiar open field			**1**	**1**	**.820****	**.697****
				1	1	.917**	.712**
C	Number of entries into center area in familiar open field					**1**	**1**
						1	1

Note. Significance is set at α =.05; *p <.05, **p <.001

Left – females; Right – males; Bolded – neonatal LPS treatment; Unbolded – neonatal NaCl

Appendix M: Correlation table within light-dark test variables in adolescence

		A		B		C		D		E		F		G		H		I		J	
A	Number of entries into light chamber	**1**	**1**	**.173**	**-.130**	**-.345**	**.206**	**-.094**	**.172**	**.253**	**.268**	**.445***	**.323**	**-.140**	**.152**	**.174**	**.026**	**.030**	**-.082**	**.097**	**.053**
		1	1	.173	.349	.351	-.401	-.450*	-.384	-.370	-.121	.706**	.627**	-.173	-.349	.343	.042	.131	.113	.154	.186
B	Duration in light chamber			**1**	**1**	**-.820****	**-.831****	**-.795****	**-.775****	**-.536***	**-.596****	**.852****	**.718****	**-.996****	**-.997****	**.967****	**.760****	**.374**	**.853****	**.173**	**.700****
				1	1	-.337	-.743**	-.431	-.821**	-.515*	-.620**	.493*	.689**	-1.000**	-1.000**	.816**	.663**	.527*	.319	.118	-.041
C	Total distance in light chamber					**1**	**1**	**.592****	**.692****	**.162**	**.501***	**-.734****	**-.489***	**.815****	**.827****	**-.698****	**-.488***	**-.377**	**-.700****	**-.286**	**-.552****
						1	1	-.551*	.517*	-.469*	.332	.217	-.420	.337	.743**	-.015	-.219	.035	-.107	.201	.014
D	Vertical movements in light chamber							**1**	**1**	**.711****	**.837****	**-.699****	**-.586****	**.791****	**.791****	**-.760****	**-.628****	**-.240**	**-.643****	**-.131**	**-.504***
								1	1	.952**	.657**	-.599**	-.674**	.431	.821**	-.505*	-.540*	-.366	-.178	-.318	.101
E	Duration of vertical movements in light chamber									**1**	**1**	**-.426**	**-.445***	**.546***	**.616****	**-.550***	**-.494***	**-.081**	**-.431***	**.077**	**-.204**
										1	1	-.600**	-.546*	.515*	.620**	-.564**	-.326	-.435	-.067	-.338	.434*
F	Number of entires into dark chamber											**1**	**1**	**-.834****	**-.711****	**.830****	**.485***	**.097**	**.514***	**.012**	**.484***
												1	1	-.493*	-.689**	.571**	.401	.305	.218	.333	-.072
G	Duration in dark chamber													**1**	**1**	**-.962****	**-.771****	**-.367**	**-.852****	**-.166**	**-.692****
														1	1	-.816**	-.663**	-.527*	-.319	-.118	.041
H	Total distance in dark chamber															**1**	**1**	**.339**	**.876****	**.127**	**.740****
																1	1	.748**	.755**	.437	.409
I	Vertical movements in dark chamber																	**1**	**1**	**.922****	**.822****
																		1	1	.732**	.709**
J	Duration of vertical movements in dark chamber																			**1**	**1**
																				1	1

Note. Significance is set at α =.05; *p <.05, **p <.001

Left – females; Right – males; Bolded – neonatal LPS treatment; Unbolded – neonatal NaCl

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Zaltzman, A., Foley, K. A., Kavaliers, M., Ossenkopp, K.-P. (2011). The Effect of Neonatal Immune System Activation with Lipopolysaccharide on Adolescent and Adult Anxiety Behaviours in Female and Male Rats. Washington, DC: Society for Neuroscience, Online.

Foley, K. A, **Zaltzman, A.**, Cross-Mellor, S. K., Kavaliers, M., Ossenkopp, K.-P. (2010). Pre- exposure to saccharin inhibits subsequent quinpirole-induced locomotor sensitization in rats. San Diego, CA: Society for Neuroscience, Online.

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