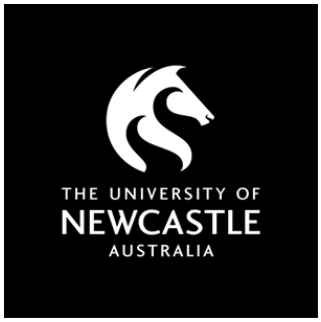
PERINATAL PROGRAMMING OF FEMALE SUBFERTILITY: THE IMPACT OF NEONATAL IMMUNE ACTIVATION ON BEHAVIOUR, OVARIAN DEVELOPMENT, AND THE BRAIN



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Declaration

I hereby certify that the work embodied in the thesis is my own original work, conducted under normal supervision. The thesis contains published scholarly work of which I am a co-author. For each such work a written statement, endorsed by the other authors, attesting to my contribution to the joint work, has been included. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Signed:

Erin Alexandra Fuller,

December 14th, 2017

This thesis consist of an introduction comprised of a written literature review and a published review paper. Experimental chapters are presented as both published works and traditional chapters, with figures and tables embedded throughout.

Published Works Incorporated in this Thesis

1. Sominsky, L.., **Fuller, E.A.**, Hodgson, D.M. (2015). Factors in early-life programming of reproductive fitness. *Neuroendocrinology*, 102 (3): 216-225. DOI: 10.1159/000431378

2. **Fuller, E.A**., Sominsky, L., Sutherland, J.M., Redgrove, K.A., Harms, L., McLaughlin, E.A., Hodgson, D.M. (2017). Neonatal immune activation depletes the ovarian follicle reserve and alters ovarian acute inflammatory mediators in neonatal rats. *Biology of Reproduction.* Accepted 7th October, 2017. DOI--: 10.1093/biolre/iox123

3. Ong, L.K., **Fuller, E.A**., Sominsky, L., Hodgson, D.M., Dunkley, P.R., Dickson, P.W. (2017). Early life peripheral lipopolysaccharide challenge reprograms catecholaminergic neurons. *Scientific Reports* (7), DOI: 10.1038/srep40475.

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Thesis Abstract.

Perinatal programming of female subfertility:
Impact of neonatal immune activation on behaviour, ovarian development, and the brain.

The early life environment prescribes long-term health and disease outcomes. Accumulating evidence suggests that female reproductive health is shaped by perinatal factors, such as immune status. The fundamentals of female reproductive success and longevity are established in early life, where the dynamics of ovarian development are co-regulated via immune pathways to establish the ovarian reserve. Additionally, the immune system is known to be especially sensitive to perinatal stressors. This suggests that the early life environment plays an important role in sustained ovarian health and female fertility. Thus, inflammatory stressors during this critical period may permanently modify female ovarian development and immune-drive reproductive functioning, altering sexual behaviour and leading to a suboptimal female phenotype.

Using a rat model, we have previously demonstrated that neonatal immune activation (NIA) with bacterial mimetic lipopolysaccharide (LPS) is associated with; altered immune milieu, hypothalamic-pituitary-adrenal axis dysfunction, adult stress vulnerability, and an anxiety-like phenotype in males. The current thesis aimed to examine both the acute and long-term alterations in reproductive parameters in female rats exposed to an intraperitoneal injection of saline (control) or LPS (0.05mg/kg) to induce NIA on postnatal days 3 and 5.

Firstly, the behavioural phenotype of females in this model was examined in order to confirm and refine previous findings pertaining to female mating behaviour deficits, and establish if these alterations were driven by altered motivational states. The results of this study indicate that NIA leads to impairments in proceptive and receptive mating behaviours and an altered reproductive developmental trajectory. Secondly, the acute effects of NIA on

female rats was analysed, where by NIA treatment was demonstrated to significantly deplete early ovarian follicle populations and increase ovarian inflammation, suggesting that immune-mediated development of the ovary is perturbed by NIA in the female rat. Thirdly, the long term ramifications of neonatal bacterial exposure was examined in the adult female rat, demonstrating that NIA led to significantly advanced puberty onset, sustained ovarian reserve depletion, exaggerated peripheral inflammatory responses to stress, and increased ovarian inflammatory pathway gene expression. Lastly, the central gene expression of mediators associated with inflammation, stress regulation, and reproductive function were examined to elucidate on potential central mechanisms that may contribute to behavioural alterations and ovarian inflammation and reserve depletion. Furthermore, prospective mechanisms are suggested and data is presented demonstrating the potential of these for investigation in a female rat model of subfertility. The findings presented in this thesis suggest that NIA has the potential to perinatally program long-term central and ovarian immune functioning to a proinflammatory bias. This may detrimentally affect female reproductive fitness, fecundity, and stress responsivity, and as such, have implications for both physiological and psychological female health.

Abbreviations

5-HT	Serotonin
AA	Arachidonic acid
ABS	Australian Bureau of Statistics
ACEC	Animal Ethics Committee
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit/hyperactivity disorder
AG	Anogenital
ANOVA	Analysis of variance
aNS	Adulthood no stress
ANS	Autonomic nervous system
APAF	Australian Proteome Analysis Facility
APC	Antigen presenting cells
ARC	Animal Resources Centre
ARTs	Assisted reproductive therapies
aST	Adulthood restraint stress
BBB	Blood-brain barrier
CASP3	Caspase 3
cDNA	Complementary deoxyribonucleic acid
CORT	Corticosterone
COX	Cyclooxygenase
CRH	Corticotropin releasing hormone
CRHR1/2	Corticotropin releasing hormone receptor 1/2
C-RP	C-reactive protein
CSF	Colony stimulating factor
CTCF	Corrected total cell florescence
DA	Dopamine
DAB	3, 3-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DEHP	Di(2-ethylhexyl)phthalate
DEPc	Diethyl pyrocarbonate

DES	Diethylstilboestrol
DEX	Dexamethasone
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origins and Health and Disease
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, version 5
DVO	Day of vaginal opening
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Epinephrine
EPM	Elevated plus maze
ER	Oestrogen receptor
Foxo3	Forkhead box O3
FSH	Follicle stimulating hormone
FWD	Forward
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GD	Gestation day
Gdf	Growth differentiation factor
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin releasing hormone receptor
GR	Glucocorticoid receptor
H&E	Hematoxylin and eosin
HC	Hippocampus
HD	Habituation day
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HTH	Hypothalamus
Iba-1	Ionized calcium-binding adapter molecule 1
IDA	Information dependent acquisition
IDO	Indolamine-2,3-Dioxygenase

IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
ip	Intraperitoneal
IPA	Ingenuity Pathway Analysis
IRAK	Interleukin-1 receptor-associated kinase 1
IUGR	Intrauterine growth restriction
IVF	In vitro fertilisation
JAK/STAT	Janus kinase/signal transducers and activators of transcription
KP	Kynurenine pathway
Kyn	Kynurenine
LBP	LPS-binding protein
LC3	Light chain 3
LC	Locus coeruleus
L-Dopa	3,4-dihydroxy-l-phenylalanine
LH	Luteinizing hormone
LPS	Lipopolysaccharide
LSV	Lateral saphenous vein
LXR/RXR	Liver X receptor/retinoic X receptor
MANOVA	Multivariate analysis of variance
MAPK	Map kinase
Mapk8/Jnk1	Mitogen activated protein kinase 8/Jun N-terminal kinase
MD	Myeloid differentiation protein
MDD	Major Depressive Disorder
MHC	Major histocompatibility complex
MIA	Maternal immune activation
mPOA	Medial pre optic area
MR	Mineralocorticoid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
mTOR	Mechanistic target of rapamycin

MyD88	Myeloid differentiation primary response 88
NCRIS	National Collaborative Research Infrastructure Strategy
NE	Norepinephrine
NF	Nuclear factor
NHMRC	National Health and Medical Research Council of Australia
NIA	Neonatal immune activation
NK	Natural killer
nLPS	Neonatal lipopolysaccharide
NMDA	N-methyl-D-aspartate
Nos1	Nitric oxide synthase-1
NSAI	Nonsteroidal anti-inflammatory pharmaceutical
nSAL	Neonatal saline
PAH	Polycyclic aromatic hydrocarbon
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCOS	Polycystic ovarian syndrome
PFC	Prefrontal cortex
PG	Prostaglandin
PMT	Paced-mating test
PND	Post-natal day
PNS	Parasympathetic nervous system
POF	Premature ovarian failure
POI	Primary ovarian insufficiency
Poly I:C	Polyinosinic:polycytidylic acid
Prkc	Protein kinase C
PRR	Pattern-recognition receptor
PTSD	Post-traumatic stress disorder
PVC	Polyvinyl chloride
PVN	Paraventricular nucleus
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

REV	Reverse
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SIT	Social interaction test
SN	Substantia nigra
SNS	Sympathetic nervous system
SPSS	Statistical Package for the Social Sciences
SPT	Sucrose preference test
STI	Sexually transmitted infection
TB	Time bin
TBS	Tris-buffered saline
T_C	T cytotoxic
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDO	Tryptophan-2,3-Dioxygenase
TGF	Transforming growth factor
T_H	T helper
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOFMS	Time of flight mass spectrometry
TRAF	Tumour necrosis factor receptor associated factor
Trp	Tryptophan
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TX	Thromboxane
VTA	Ventral tegmental area

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Chapter 1. Introduction and Literature Review

1.1 Developmental Origins of Health and Disease

It is no longer just a question of Nature versus Nurture. Ever-expanding research examining the close interaction between an individual's biology and their immediate environment indicates that the two are inextricably linked. Environmental factors have the capacity to modify an individual's biology to influence development, physiology, and function. The relative influence of environmental factors on developmental trajectories is dependent on both the timing and the nature of the environmental exposure. One developmental timeframe known to be particularly sensitive to the effects of environmental influence is the perinatal period. A key characteristic of the perinatal period is that this is a time of critical development for fundamental central and peripheral regulatory systems. As such, physiology and therefore functionality is particularly susceptible to the effects of environmental stimuli at this time. The specific area of research pertaining to the impact of environmental stimuli at-or-around the time of birth, has been termed *perinatal programming* (Hodgson & Coe, 2006).

Perinatal programming literature originates from the seminal research of Professor David Barker and Colleagues, whose focus on longitudinal epidemiological data provided the compelling foundations for the *Developmental Origins and Health and Disease* (DOHaD) hypothesis (Barker, 2004; Barker, 1995; Barker & Osmond, 1986, 1987). Originally coined *The Foetal Origins* hypothesis, Barker established clear indication that early life events were linked to later-life health outcomes and disease susceptibility. Initial studies reported that death by stroke was correlated most strongly with maternal mortality than any other cause, highlighting the importance of maternal health in offspring risk of disease (Barker & Osmond, 1987). Consequent findings demonstrate relationships between low birthweight (i.e.

intrauterine growth restriction (IUGR)) and subsequent risk of adulthood morbidity and mortality due to hypertension, diabetes, stroke, and cardiovascular disease (Barker, 1993, 1995; Barker et al., 1993; Barker & Osmond, 1986). These studies were amongst the first to associate the perinatal environmental experience with later-life disease risk and susceptibility. Since its inception, the DOHaD hypothesis has provided a framework of understanding to human development, proposing that the long term health and disease phenotype of an individual is strongly influenced by an interplay between perinatal experiences and biology (see Figure 1.1). The DOHaD framework has since evolved to encompass the effects of numerous early life physiological and psychological environmental stressors, linked not only to long term vulnerabilities to pathologies, but also psychopathologies.

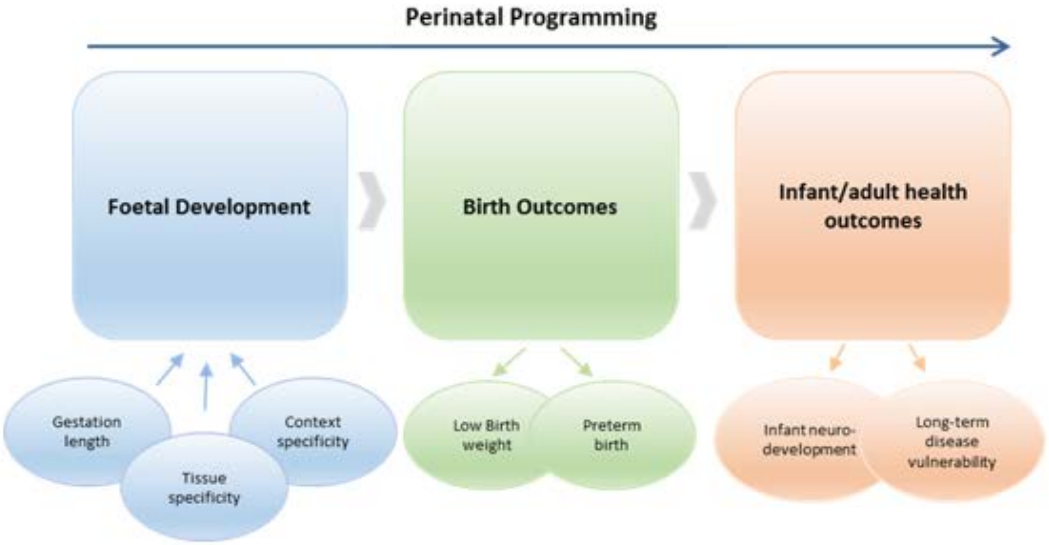

Figure 1.1. Perinatal programming of long term health and disease. Environmental influences during gestation have both context and tissue specificity, depending on the timing and nature of the stimulus. This may lead to detrimental birthing outcomes such as preterm birth or low birth weight, as well as immediate and long term developmental trajectory adjustments and adulthood disease susceptibility. Adapted from Sominsky et al, 2013.

1.2 Perinatal Programming

During critical periods of development, fundamental central and peripheral physiological systems are highly plastic, making them susceptible to environmental stimuli. This *developmental plasticity* refers to the way in which the developing brain and associated regulatory systems are able to modify their structure and function to adapt to environmental cues (Bateson et al., 2004; Crespi & Denver, 2005; McEwen & Gianaros, 2011). The resulting adaptations are enduring due to the high, but fleeting, degree of plasticity during this early developmental period, where the migration and proliferation of cells in both neural and peripheral tissue is occurring at a high rate (Fox et al., 2010). While environmental phenotypic regulation is on ongoing process through the lifespan, it is these initial periods of critical development where influences to the internal milieu can have the most effect. As these critical periods pass, the reversal of modifications become increasingly difficult, and permanent effects on morphology and function occur via these transient environmental influences (Hodgson & Coe, 2006; Korosi et al., 2012; Sominsky et al., 2013c).

The ability to adapt to one's early life environment is of evolutionary benefit. Early life phenotypical modification in response to environmental pressures ensures for optimal survival, fitness and reproductive success in the given context, the basis for which coined the *Thrifty phenotype* hypothesis (Hales & Barker, 2001). However, physiological compensations made during these critical developmental periods may prove maladaptive in the long term if the environmental insult is particularly adverse, or if the adulthood environment is incongruent with the conditions predicted in the perinatal period (Gluckman et al., 2010) (see Figure 1.2). Adverse environmental stimuli can be termed 'stressors', and may take many shapes and forms including physiological stressors such as nutritional deficits, infection or xenobiotics, as well as psychological stressors including abuse, neglect, and other traumatic

events (McEwen & Gianaros, 2011). Both physiological and psychological early life stressors have been established to perinatally program sustained alterations which affect health status, as well as behaviour (Gluckman et al., 2010). Research within the field continues to demonstrate effects of perinatal programming on an ever-increasing range of physiological systems and function.

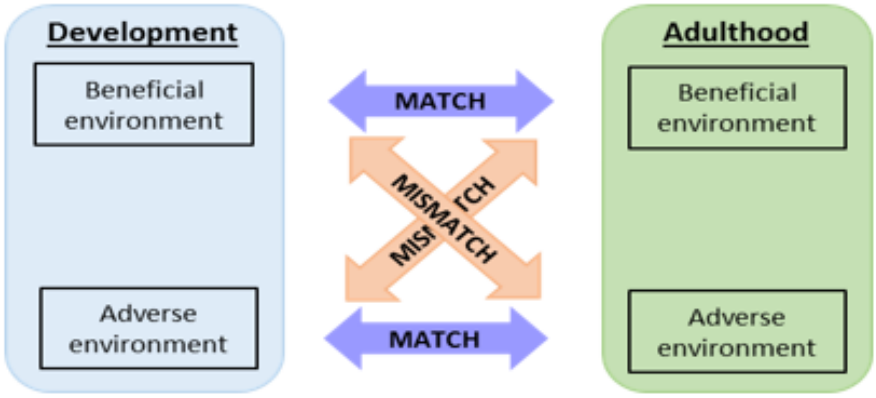

Figure 1.2. Perinatally programmed developmental alterations are adaptive, however, may become maladaptive when environmental mismatch occurs.

The concept of 'programming' is becoming vitally important when addressing the current trend of declining fertility world-wide (Aiken et al., 2015; Banerjee et al., 2014; Coall et al., 2016; Davies & Norman, 2002; Dobson & Smith, 2000; Dupont et al., 2012; Grive & Freiman, 2015; Sloboda et al., 2011; Zambrano et al., 2014). Regardless of the availability of healthcare and good nutrition, infertility levels are dramatically increasing (Inhorn & Patrizio, 2015; Kamath & Bhattacharya, 2012; Petraglia et al., 2013). 1 in 6 Australian couples experience fertility dysfunction, with 37% of sub-fertility issues being associated with female factors (AIHW, 2015; Australian Bureau of Statisitics, 2008), with these numbers approximately mirrored in American demographics (Martinez et al., 2012). Additionally, 1 in 35 births in Australia utilize assisted reproductive therapies (ARTs) (Aitken & Koppers, 2011). Increases in

the mean age for childbearing for women accounts for only a proportion of the increasing figures (Aitkin & Koppers; Martinez et al.), however recently, research has revealed the critical role of the early life environment in regards to these decreases in fecundity (Davies & Norman, 2002; Homan et al., 2007; Richardson et al., 2014; Sear et al., 2016; Sloboda et al., 2011). What's more, the incidence of reproductive disorders and dysfunction is becoming more prevalent in increasingly younger female cohorts (Hernández-Angeles & Castelo-Branco, 2016; Maheshwari et al., 2008; Norman & Moran, 2015). This suggests that the pathogenesis of unexplained sub-and infertility may have early developmental roots, particularly as the fundamentals for reproductive health are established in early life (Coall et al., 2016; Grive & Freiman, 2015; Isaksson & Tiitinen, 2004; Nepomnaschy et al., 2007; Richardson et al., 2014; Sarraj & Drummond, 2012; Sloboda et al., 2011). The building blocks for reproductive success are laid down in the highly regulated, earlier stages of development when systems that coordinate these critical developmental stages are extremely receptive to environmental influence (Banerjee et al., 2014; de Bruin et al., 1998; Dumesic et al., 2007; Grive & Freiman, 2015; Gur et al., 2015; Richardson et al., 2014; Sarraj & Drummond, 2012; Zambrano et al., 2014). The following sections outline the impacts of early-life stress on health outcomes, including physiology and psychopathology and conclude with a focus on female reproductive parameters.

1.3 The Impact of Perinatal Stress on Adult Health Outcomes

An organism's stress system coordinates the adaptive response to real or perceived stressors. McEwen (2007) outlines the concept of stress as a number of biochemical, physiological and behavioural changes that occur both centrally and peripherally, allowing the organism efficient adaptation to acute and chronic stressors. Dynamic processes are employed in response to physiological and psychological stressors that threaten the

homeostasis of an organism, provoking responses from the neuroendocrine, sympathetic and immune systems in order to return to equilibrium. Importantly, stressful events experienced early in life have the ability to program the functioning of the stress response and associated mechanisms, and facilitate the onset of later-life pathologies and psychopathologies. Since the impetus of the DOHaD hypothesis, subsequent examination of the effects of perinatal programming have been extended to include not only cardiovascular disease, stroke and diabetes, but also to other health conditions including; cancers (Johnson et al., 2009), autoimmune disorders (Dube et al., 2009), endocrine disorders (Taylor, 2010), obesity (Pico & Palou, 2013), Asthma (Miller & Ho, 2008), pain sensitivity (Zouikr et al., 2016), and- most recently, reproductive fitness (Camlin et al., 2014; Grive & Freiman, 2015; Sloboda et al., 2011). Furthermore the nature of early life environmental stressors has broadened from primarily nutritional deficiency and IUGR, to comprise other stressors such as immunological stress, including bacterial and viral infections (Estes & McAllister, 2016; Mouihate, 2013), xenobiotics (Dietert, 2009), and psychological stress and hardship (Heim & Nemeroff, 2001). Due to the diverse scope of the area, the bank of research investigating the developmental origins of health and disease is complex and varied. In order to understand how early life events shape long term health and vulnerability to disorders and disease, human studies and experimental animal research has, and continues to, examine the impact of both pre-and postnatal stressors to catalogue outcomes and elucidate potential causal mechanisms underlying disease susceptibility and onset. This evidence will be reviewed in the following sections and throughout this thesis, culminating with a particular emphasis on the role of perinatal stress in female reproductive fitness, ovarian health, and the mechanisms involved.

1.3.1 Perinatal Programming of Pathology

Epidemiological and experimental evidence has highlighted the robust link between early developmental environments and health trajectories. Considering context is particularly important when determining the effects of perinatal programming. As previously mentioned and outlined in Figure 1.2, early life adaptation to environmental stressors has adaptive value and prepares an individual for their impending future within that environmental setting. For example, being born into a context where nutritional resources are scarce, it makes ecological sense for an individual to be able to conserve the available ephemeral sustenance. Hence, a lower metabolic rate, insulin resistance and altered hormonal control of fat stores is advantageous. If the individual continued along in this environment for their lifespan, these physiological adaptations are beneficial, allowing them to survive and perhaps thrive. However, if this phenotype was then transposed into an environment with abundant nutrients, a mismatch between the early-life and later-life environments occurs, and slower metabolic rates and an altered metabolic and hormone profile now become vulnerabilities for disease. It is not just the early life adversity per se that alters development, but also the addition of contextual mismatch (Gluckman et al., 2009). This is clearly seen in research examining IUGR, low birth weight, and maternal diet. Numerous studies, particularly in famine cohorts, have consistently demonstrated links between maternal nutritional deficit and unbalance and offspring IUGR with disease, including; obesity, type 2 diabetes, hypertension, and cardiovascular disease (Barker, 2004; Barker, 1993, 1995; Fall, 2006; Ross & Beall, 2008). The foetal metabolic and hormonal developmental alterations are preparing the offspring for survival in response to this nutritionally sparse in-utero environment. Thus, if the ex-utero environment does not match previous experience, as in the case of famine resolution, this places the individual at a greater vulnerability for long term metabolic

dysfunction. These vulnerabilities are independent of, but further perpetuated by lifestyle risk factors (including smoking, alcohol and drug use, inactivity, poor diet choices) increasing risk (Bateson et al., 2004; Harris & Seckl, 2011). Importantly, this line of reasoning holds strong for not only pathologies, but also psychological aspects and disorders, where early life stress and associated central alterations facilitates behavioural changes which may be maladaptive in given contexts.

1.3.2 Perinatal Programming of Psychopathology

The perinatal period is a critical time point for the development of central and peripheral systems, and a period of immense vulnerability to environmental stimuli. The architecture of the foetal and neonatal brain is comprised of an extensive network of neuronal connections that are characterised by their developmental plasticity. Stressors that occur during this period are able to exert their influence on the design and structure of the brain. This induces changes in not only cognitive function, but also the associated behavioural output, the extent to which is depended on the nature and timing of the stress exposure, as well the genetic predisposition of the individual (Harris & Seckl, 2011).

Low birth weight, as a marker of maternal gestational stress, is associated with cognitive and affective disorders in childhood and throughout adulthood (Hack et al., 2004; Indredavik et al., 2004; Kinsella & Monk, 2009; Van den Bergh & Marcoen, 2004; Wiles et al., 2005). This includes schizophrenia, attention deficit/hyperactive disorder (ADHD), antisocial behaviour, anxiety disorders, depression, learning difficulties and post-traumatic stress disorder (PTSD) vulnerabilities (Mick et al., 2002; Nigg & Breslau, 2007; Rifkin et al., 1994; Thompson et al., 2001; Yehuda & Bierer, 2009). Research also indicates that a variety of maternal stressors influence offspring psychopathologies. Maternal psychological states including depression, anxiety and psychosis as linked to greater expression of

psychopathology and substance abuse disorders in their offspring (Beidel & Turner, 1997; Lieb et al., 2002), which has been demonstrated to be transgenerational (Weissman et al., 2005). Often, these findings are compounded by the quality of maternal care and the mother-infant relationship, which is known to effect offspring development and behaviour (Champagne & Curley, 2009; Meaney, 2001).

The biological and behaviour consequences of postnatal stressors, such as childhood abuse, neglect and deprivation, have been exemplified in longitudinal studies of orphanage settings (Chugani et al., 2001; Roy et al., 2004) as well as general populations (Bierer et al., 2003; Cohen et al., 2001; Johnson et al., 2001). Additionally, stressors during the adolescent period, which is also a critical period of maturation due to pubertal growth, shape long-term neurophysiological functioning (Chaby et al., 2017; Holder & Blaustein, 2014; Juraska & Willing, 2017). Stress is not limited to psychological or nutritional aspects, but also maternal physiological health. Maternal immune stress, via viral or bacterial exposure has been linked to an increased vulnerability for offspring development of schizophrenia, autism, and anxiety and depression (Brown & Derkits, 2010; Buehler, 2011; Meyer, 2014a; Patterson, 2002, 2011). This provides evidence of immune system, and other interconnected physiology, involvement in developmental plasticity as a mechanism for perinatal programming.

While data from human longitudinal studies and specific cohorts are able to provide robust correlational links, animal studies and associated experimental manipulations are able to give insight into underlying mechanism in order to pinpoint biological changes and hence facilitate clinical treatments and interventions (Abelaira et al., 2013; Cryan & Mombereau, 2004; Duman, 2010; Dunn et al., 2005; Maestripieri & Carroll, 1998; Minor & Smith, 2014; Steimer, 2011). Importantly, human clinical findings are echoed in experimental animal research examining developmental plasticity and perinatal programming, which solidifies the

phenomenon that numerous early life stressors increase the likelihood of psychopathology-like behaviour, and impact cognitive impairments indicative of numerous psychopathologies. Numerous animal models of prenatal and postnatal stress have been utilised in order to mimic the vast array of physiological and psychological environmental stressors, such as metabolic stressors, immune stressors, and different traumas.

In a rat model, maternal protein restriction increased anxiety-like behaviours and lead to learning impairments, and decreased motivation in their offspring (Reyes-Castro et al., 2012). Maternal calorie restriction in the week prior to conception led to an anxiogenic effect on male rat adult offspring (Levay et al., 2008). Rat dams that have been stressed by unpredictable noise and light, or unpredictable foot shocks throughout gestation bore offspring that demonstrated greater anxiety-like behaviours in adulthood (Estanislau & Morato, 2006; Poltyrev et al., 1996). Similarly, maternal restraint stress paradigms report that this psychological stressor led to sustained depressive-like and anxiety-like symptoms in the adult offspring of the stressed damn (Maccari & Morley-Fletcher, 2007), symptoms which are often attenuated with the use of antidepressants or anxiolytics (Prut & Belzung, 2003). Dams that exhibit reduced maternal care giving behaviours, such as arched back nursing, licking and grooming, had rat offspring with higher stress responsivity and showed an increase in anxiety-like behavioural symptoms in adutlhood (Uriarte et al., 2007). Interestingly, these synptoms have been shown to be reduced with handling and cross-fostering with highly maternal dams (Caldji et al., 2000), highlighting the complexities of the interaction between biology and environmental factors. Rat pups that have been seperated from there dam in early life demonstrate behavioural, cognitive, and immunoendocrine deficiets (Nishi et al., 2014). Additionally, maternal immune activation in rodent models with the viral mimetic polyinosinic:polycytidylic acid (Poly I:C), or the bacterial mimetic lipopolysaccharide (LPS), is

associated with the developmental aetiologies of symptoms correlating to the human disorders of schizophrenia, autism, depressive disorders and anxiety disorders (Meyer, 2014b; Smith et al., 2007). These studies allude to mechanisms responsible to altered parameters, and particularly indicate the involvement of the immune system, endocrine systems and autonomic involvement in pathologies characteristic of stress response dysfunction.

Cumulatively, human studies and animal models of perinatal programming provide compelling evidence that early-life stressors modify the physiology of systems that are responsible for the capacity to effectively manage later-life stress. What these studies illustrate is that a dysregulation of the stress response and associated systems is often accompanied by a gamut of associated maladaptive behaviours that occur in early-life and are continued into later-life. A number of psychopathologies and pathologies share overlapping traits, hence, similar mechanisms are thought to underlie the development of these. Maladaptive and dysfunctional biological responses are a major contributing factor to not only physical health outcomes, but also mental health outcomes (Harris & Seckl, 2011; Heim & Nemeroff, 2001; Taylor, 2010). Identifying the mechanisms implicated in perinatal programming of disease and the way in which these work in concert is therefore paramount to increasing knowledge and understanding of long term disease and disorders.

1.3.3 The Role of Stress in Perinatal Programming

An organism's stress system coordinates the adaptive response to real or perceived stressors (Skinner, 2014; Syed & Nemeroff, 2017; Taylor, 2010; Ulrich-Lai & Herman, 2009). McEwen (2008) outlines the concept of stress as a number of biochemical, physiological and behavioural changes that occur both centrally and peripherally, allowing the organism efficient adaptation to acute and chronic stressors. Dynamic processes are employed in

response to physiological and psychological stressors that threaten the homeostasis of an organism, provoking responses from the neuroendocrine, sympathetic and other interacting systems in order to return to equilibrium (McEwen, 2008). The stress response is aimed at promoting survival, regulated in part through the activity and bidirectional communication of neurotransmitters and hormones of the hypothalamic-pituitary-adrenal (HPA) axis, the autonomic nervous system (ANS) and the immune system (Harris & Seckl, 2011; Matthews & Phillips, 2012; McEwen & Gianaros, 2011). A chronically increased or dysregulated stress response disrupts internal milieu and produces detrimental effects for the organism, increasing susceptibility to a variety of illnesses such as cardiac disorders, diabetes and mental illnesses (Barker, 1995; Gluckman et al., 2009; Heim & Nemeroff, 1999, 2001; Herman et al., 2016; Mastorci et al., 2009).

Importantly, early-life stressors have the ability to program the functioning of the stress response both prenatally and postnatally, leading to alterations in later life physiology and behaviours. Perinatal programming effects of stress determining later life health complications are consistently demonstrated in both human and animal literature (Harris & Seckl, 2011; Poltyrev et al., 1996; Weinstock, 2007). Alterations have been reported in several physiological systems including; neuroendocrine, autonomic, immune, reproductive and the metabolic system (Bateson et al., 2004; Bauer et al., 2001; Bilbo & Klein, 2012; Bilbo & Schwarz, 2009; Blanchard et al., 1993; Cai et al., 2016a; Evans et al., 2016; Hodgson & Coe, 2006; Karrow, 2006; Kentner & Pittman, 2010; Meaney, 2001; Morale et al., 2001; Sloboda et al., 2011; Sominsky et al., 2013a; Sominsky et al., 2013c; Spencer et al., 2011; Spencer & Meyer, 2017; Viltart & Vanbesien-Mailliot, 2007; Won & Kim, 2016; Zakharova, 2014; Zygmunt & Stanczyk, 2010). Importantly, the timing of stress exposure, whether it be prenatal

or experienced in the postnatal period, may have differing trajectories of cause and effect, lending an additional layer of complexity (Hodgson & Coe, 2006).

1.3.4 Prenatal Stressors

It is well established in animal and human literature that the prenatal environment is a critical period of plasticity where the fetus is able to respond to the maternal environment (Gluckman et al., 2008; Hanson & Gluckman, 2008; Harris & Seckl, 2011; Hodgson & Coe, 2006; Merlot et al., 2008; Mulder et al., 2002; O'Donnell & Meaney, 2017). Stressors affecting the embryo or fetus via the maternal environment have the ability to potentiate enduring stress response alterations, which persist through to adulthood and increase susceptibility to later life dysfunction (Green et al., 2011; Gutteling et al., 2005; Maccari & Morley-Fletcher, 2007; Mastorci et al., 2009; O'Connor et al., 2005; Welberg & Seckl, 2001). Maternal stressors may be physiological, such as nutrient restriction, pain, and infection. They may also be psychological in nature, such as maternal psychological trauma, socioeconomic status, and psychological status including anxiety and depression.

Human literature explores a number of prenatal stressors that affect the future health and development of the child. Robust links have been documented between foetal health outcomes and increased maternal stress profiles induced by environmental stressors such as food availability and maternal physical and mental health (O'Donnell & Meaney, 2017). Increased maternal anxiety and depression during pregnancy lead to subsequent emotional and behavioural disturbances of their children (O'Connor et al., 2005; Welberg & Seckl, 2001). In a clinical study, Van den Bergh and Marcoen (2004) found that high levels of maternal anxiety led to an enhanced susceptibility of internalising and externalising psychological disorders in their children at age eight. Furthermore increases in circulating levels of maternal cortisol, corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) are

indicative of high maternal stress and influence the growth of the fetus, particularly glucocorticoid exposure (Entringer et al., 2009; Kinsella & Monk, 2009; Staneva et al., 2015; van Bodegom et al., 2017; von Ehr & von Versen-Hoynck, 2016). Hence, birth size has often been used as an indicative measure of maternal adversity and its consequential effects on offspring development have been assessed.

Stressors can be experimentally reproduced, creating prenatal programming animal models of psychopathology. Studies have shown that maternal calorie restriction in female rats one week before conception leads to anxiety-like behaviour in the male adult offspring compared to non-diet restricted controls (Levay et al., 2008). Pregnant dams exposed to the acute stress of electric foot shocks on during gestation produced offspring that behaved more anxiously in the elevated plus maze (EPM) than rats subjected to the postnatal stress of maternal separation (Estanislau & Morato, 2006). Furthermore, maternal restraint stress, a model of psychological stress, is consistently linked to anxiety-and depressive-like behaviour in offspring, along with biological stress response alterations (Maccari & Morley-Fletcher, 2007). Conversely, recent evidence indicates that restraint stress, administered to male and female rats pre-gestationally decreases anxiety-like behaviours in subsequent offspring (He et al., 2016). Both these conflicting findings, and the observation that these alterations are multigenerational (Grundwald & Brunton, 2015) highlight the need for a greater understanding of this concept. Interestingly, early life stress is not confined to 'typical' modes of stress, but can also be transposed to other understandings of stress including physiological sickness and infection.

Infection, sickness, and other physiological stressors such as pain, also activate a conjoint neuroimmunoendocrine response, altering hormones, immune mediators and affecting behaviour. In a maternal immune stress model, pregnant mice that were infected

with *influenza virus A* bore offspring that exhibited anxiety-like behaviour novel-object and social interaction assays, modelling for both anxiety-like and autism-like behaviours (Shi et al., 2003). Pregnant female mice that were administered with the bacterial mimetic LPS in early gestation had offspring that demonstrated increased anxiety-like and depressive-like behaviours, as well as alterations in central serotonin and catecholamine levels (Depino, 2015). Consistent and robust links have been demonstrated between maternal immune activation and schizophrenia-like behaviours in rodent models (Meehan et al., 2017; Meyer, 2014b; Spencer & Meyer, 2017), which is mirrored in epidemiological data (Brown & Derkits, 2010; Gilmore & Jarskog, 1997; Rifkin et al., 1994). Numerous studies exploring prenatal immune activation provide a robust link between maternal stress activation and immune status in the aetiology of mood disorders and psychopathology (Arsenault et al., 2014; Depino, 2015; Labouesse et al., 2015; Meyer, 2014b). In all, these studies indicate that increased stress exposure in the prenatal period is capable of producing detrimental long-term health consequences.

1.3.5 Post-Natal Stressors

Critical periods of plasticity continue throughout development. As previously mentioned, perinatal programming effects are tissue, timing, stressor, and context dependent, with differences in any of these parameters potentially culminating in alternate consequences. Sub optimal conditions in the postnatal period may lead to susceptibility to later-life health complications that are often mediated by an altered capacity to regulate responses to internal and external stressful events (Cameron & Demerath, 2002; Nesterenko & Aly, 2009). Early life events have been implicated in an increased vulnerability to later-life disease and psychopathologies in humans (Heim & Nemeroff, 2001; Taylor, 2010). Epidemiological and clinical evidence indicates that postnatal abuse, maltreatment and

neglect has been linked to a greater risk of psychiatric disorders in later-life, including PTSD (Yehuda, 2005), anxiety disorders (Heim & Nemeroff; Taylor), schizophrenia (van Os & Selten, 1998) and mood disorders (Ackerman, Newton, McPherson, Jones & Dykman, 1998).

In rodent models, the maturational state of the rat pup on different postnatal periods can be said to be compared to different periods of human perinatal development, with post-natal day (PND) 3-7 in the rat being equivalent to the third trimester of human pregnancy, and the period around PND14 being approximately equivalent to a human neonate between the age of 6 months to 2 years (Avishai-Eliner et al., 2001; Semple et al., 2013; Sengupta, 2013). In rodent models, it is well established that maternal separation is potent neonatal stressor for rats, with pups separated from the dam for 3 > hours a day displaying increases in levels of stress hormones and displaying anxiety-like behaviours later in life (Harbuz & Lightman, 1992; Hennessy et al., 2014; Hennessy et al., 2011; Lupien et al., 2009). Importantly, it is not only the amount of maternal care that is essential for development, but also the quality of maternal care is a key modulator of the infant stress response (Champagne et al., 2003). Offspring of mothers that display reduced care giving behaviours, such as arched back nursing, licking and grooming, had higher stress responsivity and also showed an increase in anxiety-like behavioural symptoms (Caldji et al., 2000; Champagne & Curley, 2009; Francis et al., 1999; Liu et al., 1997; Uriarte et al., 2007). Additionally, immune stress in the neonatal period has been demonstrated to lead to cognitive impairments, altered nociception, and psychopathology-like symptomologies in animal models (Bilbo et al., 2005b; Boisse et al., 2005; Walker et al., 2009; Zouikr et al., 2014b).

Cumulatively, both pre and post-natal, human studies and animal models, of perinatal programming provide evidence that early life stressors, both pre and post-natal, modify the physiology of systems that are responsible for the capacity to effectively manage later-life

stress. What these studies illustrate is that a dysregulation of the stress response and associated systems is often accompanied by a gamut of maladaptive behaviours that occur both early life, and are sustained into later-life.

1.4 Mechanisms of Perinatal Programming

Key factors which influence phenotypic outcomes include a person's genome and biology, their development, and the environment. These factors interact in order to maintain homeostasis and allow for swift adaptations to facilitate and maintain longevity. *Epigenetics* refers to the molecular mechanisms that occur when these key factors interact to create stable alterations in gene expression patterns without altering the deoxyribonucleic acid (DNA) sequencing (Gluckman et al., 2009). Existing genes are expressed or not expressed, via environmental influence, allowing for phenotypic variation from the same genotype (see Hochberg et al., 2011 for review). Often, epigenetic mechanisms are referred to when examining the literature surrounding the mechanisms of perinatal programming and developmental plasticity. Compelling evidence now exists that supports both DOHaD and perinatal programming literature, and the underlying epigenetic processes that contribute to physiological and functional change. Although the scope of epigenetics is extensive, it is briefly mentioned here in order to give a well-rounded picture of the perinatal programming and developmental plasticity landscape.

A number of major physiological systems remain plastic in the perinatal period to allow for maximum benefit, however, this can lead to detrimental outcomes if the early life endogenous or exogenous environment is particularly adverse or a mismatch occurs. The mechanisms of perinatal programming involve both central and peripheral regulatory systems and structures. They include the ANS, the HPA axis, and the hypothalamic-pituitary-gonadal (HPG) axis (Buynitsky & Mostofsky, 2009; Kim et al., 2015; Morale et al., 2001; Won

& Kim, 2016; Yehuda & Daskalakis, 2015). Additionally, the innate immune system is highly plastic in the perinatal period and plays a very critical role in coordinating early life development and maturation (Spencer et al., 2011; Tanriverdi et al., 2003a). Dysregulation of immune mediators, along with neuroendocrine and autonomic mechanisms, contribute to the modulation of health and disease, with numerous pathologies and psychopathologies showing consistent immune irregularities (Bilbo & Klein, 2012; Elenkov, 2008). The inextricable link between the neuroendocrine system, the immune system and the ANS is well established (Bilbo & Klein, 2012; Bilbo & Schwarz, 2009, 2012). These systems work in concert to coordinate vital physiological processes via cascades of neurotransmitters, hormones and other chemical messengers that exert tissue-dependent and timing-dependent effects and interactions, and are the central mechanisms implicated in perinatal programming (Ader et al., 1995; Karrow, 2006).

1.4.1 The Autonomic Nervous System (ANS)

The ANS is comprised of two primary branches, the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). This system plays an integral role in regulating homeostatic processes and responding to acute stressors (Elenkov, 2008). What's more, the ANS plays a critical role in the regulation, integration and orchestration of the nervous system, the endocrine system and the immune system (Calcagni & Elenkov, 2006; Kenney & Ganta, 2014). The ANS provides an immediate and ephemeral response to acute stressors via the release of catecholamine's from the sympathetic arm of the ANS, with the parasympathetic arm action working via the release of acetylcholine (Ondicova & Mravec, 2010; Ulrich-Lai & Herman, 2009). This controls both the initiation and resolution of the 'fight or flight' response. The principle neurotransmitters and hormones of the central and peripheral branches of the ANS are catecholamines, including dopamine, norepinephrine (NE;

or noradrenaline) and epinephrine (EPI; or adrenaline). Increases in catecholamines are an essential part of the stress response, synthesised from amino acid L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-Dopa) by the rate limiting enzyme tyrosine hydroxylase (TH) (Kuhar et al., 1999) (see Figure 1.3). Briefly, activation of TH is mediated via phosphorylation at several serine residues (Ser19, sSer31, and Ser40) by kinases in the N-terminal domain, but may also be regulated via transcriptional control, ribonucleic acid (RNA) stability and total protein levels (Kvetnansky et al., 2009). Alterations in TH messenger (m) RNA activity play a major role in how the catecholamine system respond to environmental stressors (Kvetnansky et al., 2009).

An activated ANS stress response leads to an immediate change in physiological states, affecting blood pressure, and increasing heart rates and breathing rates (Langhorst et al., 1981; Zygmunt & Stanczyk, 2010). Catecholamines are synthesised and released from the adrenal medulla and peripheral nerve endings, rapidly evoking physiological excitation, and influencing endocrine and immune parameters (Elenkov, 2008; Johnson et al., 2005). Interestingly, the adrenal gland is capable of producing a variety of proinflammatory cytokines, highlighting the complex nature of immune-stress interactions (Bunn et al., 2012). The counteracting parasympathetic arm of the ANS subdues the activation, promoting a 'rest and digest' state (Ulrich-Lai & Herman, 2009). Due to the ephemeral nature of the ANS stress activation, responses can be measured via means with high temporal resolution, including cardiovascular parameters, galvanic skin responses, breathing parameters, thermoregulations and blood pressure, as the state of arousal is often fleeting (Zygmunt & Stanczyk, 2010). Additionally, measurement of TH expression by gene or protein analysis provides sensitive a measure of stress-related sympathetic activation (Kumer & Vrana, 1996) Of importance for this thesis, histological examination of the ovary has described both

afferent and efferent parasympathetic and sympathetic innervation of the ovary (Burden et al., 1983; Gerendai et al., 1998, 2000), and although the involvement of these nerves in the ovary remain to be elucidated, recent research suggest that they may be involved in the rapid regulation of ovarian function to exogenous and endogenous environmental stressors (Cruz et al., 2017; Paredes et al., 1998; Uchida & Kagitani, 2015).

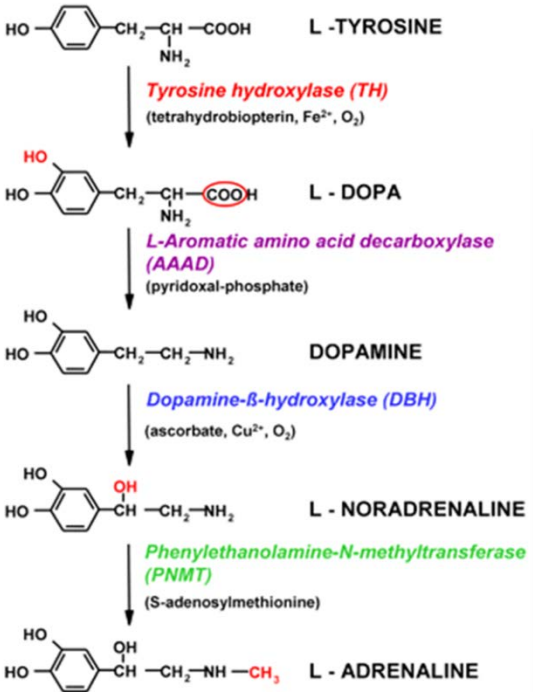

Figure 1.3. Pathway for catecholamine synthesis and enzymatic steps. Noradrenaline = norepinephrine. Adrenaline = epinephrine (Kvetnansky et al., 2009).

1.4.1.1 Programming of the ANS. Autonomic imbalance has been demonstrated as a consequence of early life stress (Wright, 2012). Peripheral catecholamines are essential for HPA axis activation (Delrueperollet et al., 1995) and it has been demonstrated that plasma concentrations of NE and EPI increase upon exposure to an immune stressor, as well as mediate stress-induced proinflammation (Johnson et al., 2005; Zhou & Jones, 1993). The SNS

branch of the ANS innervates immune organs, such as the thymus, lymph nodes and spleen, as well as the gonads, with catecholamines also having immuno-regulatory effects (Elenkov et al., 2000; Kenney & Ganta, 2014). Peripheral cytokines are able to signal the brain to trigger ANS and HPA stress responses (Elenkov et al., 2000). It has also been demonstrated that neonatal maternal separation affects the development and long term functioning of the respiratory control system, indicating common stress pathways and mediators during the neonatal period may be influencing autonomic, endocrine and immune development and increasing susceptibility anxiety-related disorders, including panic attacks (Kinkead & Gulemetova, 2010; Wright, 2012). In a rodent model, maternal separation between PND 3 and 12 was accompanied by a rapid decrease in respiration, even though temperature was controlled, showing an immediate effect neonatal stress on ANS function (Dumont et al., 2011; Kinkead & Gulemetova, 2010; Soliz et al., 2016). Conversely, Mastorci et al. (2009), demonstrated in rats that restraint in the neonatal period did not produce immediate results, however adulthood alterations were observed in circadian rhythm and heart rate when presented with an adulthood stressor, indicating ANS vulnerability to later-life stress may be perinatally programmed during the neonatal period. Findings from our laboratory suggest that the ANS response to stress can be programmed by exposure to immune activation in early life. Sominsky et al. (2013a) demonstrated that rats exposed to neonatal LPS on PND 3 and 5 displayed altered respiratory parameters when exposed to increasing intensities of mild acoustic stimuli in adulthood and upregulated TH phosphorylation in the adrenal glands of LPS treated male rats, suggesting a direct link between early life bacterial exposure and enduring autonomic alterations.

It has been demonstrated that bacteria exposure evokes activation of the immune response pathways subsequently leading to the increase of central proinflammatory

cytokines (Elenkov et al., 2000; Johnson et al., 2008) that interact with the ANS through afferent vagal nerve pathways that project to the brainstem, synapsing on catecholamine neurons (Olofsson et al., 2012). Rapid release of catecholamines such as EPI and NE activates the HPA axis through pathways that project into the paraventricular nucleus (PVN), to increase glucocorticoid release in order to overcome pathogens (Karrow, 2006; Ulrich-Lai & Herman, 2009). Minimal perinatal programming literature has examined the role of the ANS its subsequent involvement in health and disease. Studies that have, predominantly focus on male rodent models, despite the known involvement of the ANS in the aetiology of psychopathology including anxiety and depression and how these differ between sexes, as well as its involvement in ovarian functioning (Aguado, 2002; Curtis et al., 2006; Foley et al., 2014; Gerendai et al., 2000; Pinos et al., 2001; Uchida et al., 2015).

1.4.2 The Hypothalamic-Pituitary-Adrenal (HPA) Axis

The HPA axis is activated via ANS arousal and is comparatively slower and protracted in comparison to ANS arousal. The HPA axis has been a key focus in perinatal programming literature surrounding stress development, disease and disorders. Following stress exposure, the HPA axis cascade begins centrally with the hypothalamic secretion of CRH from the PVN of the hypothalamus and ACTH from the pituitary to activate the peripheral secretion of glucocorticoids (cortisol in humans and corticosterone in the rodents: CORT), which are then regulated by a negative feedback system. The HPA axis plays an important role in the regulation of the inflammatory response through the actions of glucocorticoids which limit the synthesis of proinflammatory biomarkers, such as cytokines and prostaglandins (Rook, 1999) (see Figure 1.4).

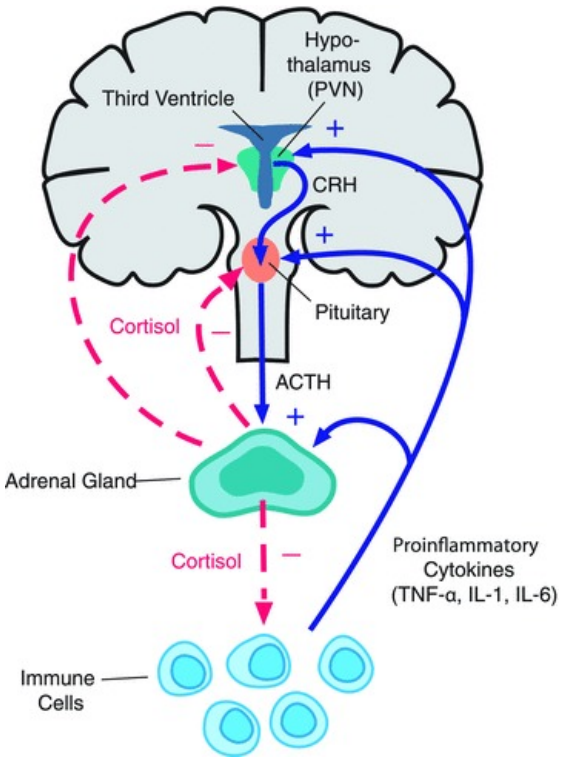

Figure 1.4. HPA axis cascade, including immune activation of cytokines and negative feedback regulation via glucocorticoids and immune mediators. Adapted from Silverman and Sternberg (2012).

***1.4.2.1 Programming of the HPA Axis*.** The HPA axis is recognised as being particularly susceptible to early-life environmental stress (Hodgson & Coe, 2006; van Bodegom et al., 2017; Yehuda & Daskalakis, 2015). Stress exposure in early life can lead to enduring alterations in the functioning of the HPA axis, including a reduction in glucocorticoid receptor (GR) and mineralocorticoid (MR) expression that may lead to variations in functioning of the negative feedback loop, thus altering CORT levels and affecting behaviours (Cottrell & Seckl, 2009; Silverman & Sternberg, 2012). In human populations, it has been reported that low birth weight babies have higher umbilical cord and urinary levels of cortisol, suggesting both maternal stress and IUGR is capable of programming the HPA axis (Clark et al., 1996). In a clinical population, Entringer et al. (2009) report that healthy young adults, whose mothers

were exposed to acute stress whilst pregnant, exhibited an increase in cortisol concentrations undertaking stressful tasks relative to controls. Children who experience a harsh early life climate exhibit elevated baseline cortisol, as well as increases in cortisol following stressors such as public speaking tasks or stressful cognitive tasks in later life (Entringer et al., 2009). Furthermore, childhood abuse is linked to the emergence of adult depression in clinical samples, as well as increased cortisol reactivity (Elzinga et al., 2010). Conversely, findings in HPA axis function have also been demonstrated in the opposite direction. Post-traumatic stress disorder has been associated with a blunted HPA axis function. Yehuda et al. (2005) reported that expectant mothers who went on to develop PTSD after the New York World Trade Tower attacks in 2001 demonstrated reduced cortisol levels, also bore children whom exhibited reduced salivary cortisol levels when examined at age one. This demonstrates a multigenerational programming effect of the PTSD phenotype, which may be mediated via the maternal line. In addition, Elzinga et al. (2008) demonstrated that healthy men with a history of childhood adversity demonstrated significantly blunted cortisol post a psychosocial task. These differing HPA axis findings highlight the complexities of early life stress in the aetiology psychopathologies in humans.

Alterations in HPA axis function have been demonstrated in a number of animal studies. Walker et al, (2009) demonstrated in a rat model, that neonatal immune stress followed by a subsequent stressor in later life lead to a blunted corticosterone response, suggesting a programmed dysregulation of glucocorticoid receptors in the hypothalamus, impairing negative feedback efficiency. Additionally, Welberg et al, (2001) found alterations in hypothalamic expression of GR and MR of rat offspring whose mothers were administered with dexamethasone (DEX), a synthetic glucocorticoid, during gestation. Maternal separation and reduced caregiving exposure in a neonatal rat model led to alterations in CRH, ACTH and

CORT (Welberg & Seckl, 2001). This implies alterations to multiple levels of HPA axis function. Furthermore, experimental exposure to bacteria in the postnatal period in the rat produced stress-mediated alterations to immune system functioning, indicating that stress induced HPA axis dysfunction also leads to neuro-immune system dysfunction (Karrow, 2006; Sominsky et al, 2012; Spenser, Galic & Pittman, 2010; Walker, Nakamura, & Hodgson, 2010). These altered HPA axis alterations in the rat are also associated with behavioural changes, including increases in anxiety-like behaviours and abnormal mating behaviours, suggesting an overall anxiety-like phenotype (Walker et al., 2012; Walker et al., 2009; Walker et al., 2004a; Walker et al., 2008; Walker et al., 2004b; Walker & Vrana, 1993). Due to the bidirectional communication between endocrine and immune function, the HPA axis interacts with the HPG axis on many levels to influence reproduction, as well as both these systems exerting effects on immune functioning (Chrousos & Kino, 2007; Chrousos et al., 1998; Dismukes et al., 2015; Morale et al., 2001; Schmidt et al., 2014).

1.4.3 The Hypothalamic-Pituitary-Gonadal (HPG) Axis

The principle regulator of reproductive function in both sexes is the HPG axis. HPG activity is closely associated with immune system and HPA axis functioning, with immune and stress status exerting influence on the production of sex hormones at numerous levels (Handa et al., 1994; Kentner et al., 2010; Spencer et al., 2006a). The HPG axis is primarily concerned with controlling the release of sex hormones, both centrally and peripherally, to influence reproductive functioning (Meethal & Atwood, 2005; Rivest, 1991; Rivest & Rivier, 1993; Viau, 2002; Zakharova, 2014). However, the HPG axis also has key roles in brain development, maturation and continued functioning throughout the lifespan. HPG hormones influence the architecture of the nervous system by having a profound effect on its development, structure and function (Gaillard & Spinedi, 1998; Meethal & Atwood, 2005). This includes processes

such as neuronal survival and synaptic pruning, neurogenesis, receptor expression, neurotransmitter synthesis and neuronal activity. During early prenatal development, sex hormones and steroids contribute to the organisation of the neuronal circuitry which remains dormant until further hormone stimulation during puberty and adulthood facilitates the appropriate adult physiology and behaviour. This view is often termed the *organisational/activation hypothesis* (Arnold & Breedlove, 1985).

The mature HPG axis cascade is centrally activated in the medial pre optic area (mPOA) of the hypothalamus, where pulses of gonadotropin releasing hormone (GnRH) are secreted from a collection of GnRH neurons called the GnRH pulse generator. This neuronal population releases GnRH in a characteristically synchronised and pulsatile, and it is this function that is the central and key element governing reproductive function (Knobil, 1990; Krsmanovic et al., 2009). GnRH in turn stimulates the releases of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary to activate the synthesis and release of steroid hormone from the gonads, hence influencing pubertal onset, female ovulation, and sexual maturation (Hiller-Sturmhofel & Bartke, 1998) (see Figure 1.5). Luteinizing hormone and FSH from the pituitary stimulate the ovarian follicle containing the mature egg to produce oestrogens. In the ovary, FSH controls follicular granulosa (germ cell supporting cells) maturation and oestradiol production, while LH facilitates oocyte (immature female germ cell, or 'egg') maturation, ovulation and follicular luteinisation (Balasch & Fabregues, 2006). A positive feedback mechanism is activated shortly before ovulation, whereby oestrogen enhances LH secretion, with the subsequent surge in LH leading to ovulation, formation of the ovarian corpus luteum, and progesterone release. Post ovulation, LH stimulates production of oestradiol and progesterone by the corpus luteum to inhibit gonadotropin releasing hormones (GnRH) and thus secretion of LH and FSH (Hiller-Sturmhofel & Bartke,

1998). Pulsatile LH and FSH are also released into the blood stream to stimulate gonadal release of sex steroids including progesterone, testosterone, oestrogen, and inhibins, whilst activin (transforming growth factor-β (TGF-β) family members) and follistatin are produced constitutively (Meethal & Atwood, 2005). Complex feedback loops regulate the activities and concentrations of these hormones and peptides at numerous levels (see Figure 1.5). The concentrations of reproductive hormones vary throughout the lifespan and depend on the reproductive state of the organism. Peripherally, gonadal steroids participate in female ovarian follicle maturation and male spermatogenesis. Centrally, they influence GnRH to facilitate sexual behaviours. Prepubertal levels of GnRH are generally low, with an increase in production occurring during puberty. In females, this also increases the production of LH and FSH and this initiates the first reproductive cycle, with concomitant immune control (Ojeda & Campbell, 1982; Ojeda et al., 2010; Sisk & Foster, 2004).

The HPG and HPA modulate each other's endocrine functioning and are highly interactive, including the physiological sharing of top-down structures (Dismukes et al., 2015). Secretions of the HPA axis block and downregulate the actions of the HPG axis at various levels (Chrousos et al., 1998). CRH and glucocorticoids inhibit the production of GnRH from the hypothalamus (Viau, 2002). Glucocorticoids limit LH secretion from the pituitary, and oestradiol and progesterone from the ovaries. Reversely, oestradiol is able to stimulate the CRH neurons of the hypothalamic paraventricular nucleus to alter stress responsivity. However, androgens have been demonstrated to have dampening and/or protective effects on the actions of both the HPA axis and the inflammatory response (Handa et al., 1994; McCormick et al., 2002), highlighting sexual dimorphisms that exist related to stress and immune responses.

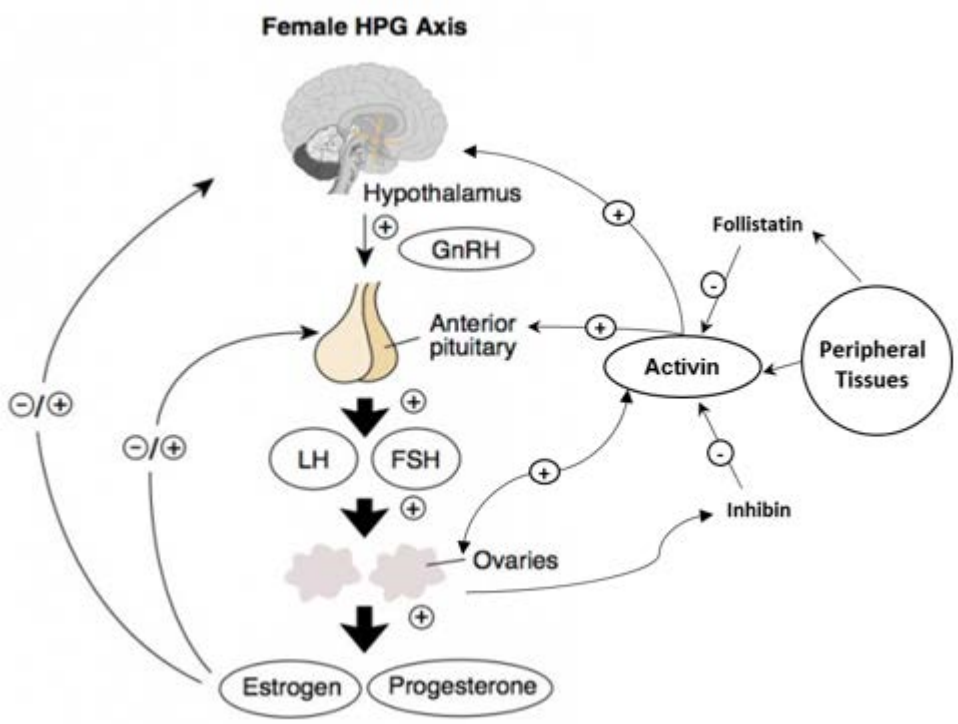

Figure 1.5. Schematic representation of the Female HPG axis and associated complex feedback mechanisms which control concentration, timing, and levels of sex steroids. Peripheral activin stimulates the hypothalamus to release GnRH, this stimulates LH and FSH synthesis from the anterior pituitary (AP). LH and FS bind to receptors on the gonads, stimulating oogenesis, sex steroid production and inhibin production. Sex steroids feedback to the hypothalamus and AP to negatively regulate gonadotrophin secretion. Inhibin has indirect control over gonadotrophin secretions and follistatin inhibits the binding of activin to its receptor (modified from Kong et al., 2014; Meethal & Atwood, 2005).

1.4.3.1 Programming of the HPG Axis. Plasticity is normal within the HPG axis and GnRH secretion and therefore reproductive capabilities can be influenced by internal and external environmental factors (Dismukes et al., 2015; Morale et al., 2001; Schmidt et al., 2014; Walker et al., 2011). In humans, is has been consistently demonstrated that females exposed to abnormally high levels of androgenic steroids in the perinatal period show signs of masculinisation, early pubertal onset and altered oestrus cyclicity, and neuroendocrine dysfunction (Evans et al., 2016; Robinson, 2006). Teratogen exposure in early life has been

linked to HPG and HPA axis disruptions and sustained alterations in human populations. Diethylstilboestrol (DES), a synthetic oestrogen given to mothers during gestation in the 1940s-1970s to reduce pregnancy complications, is now known to produce pubertal vaginal carcinoma and breast cancer in daughters (Goldberg & Falcone, 1999; Hilakivi-Clarke, 2014). This was one of the first observations that lead to the suggestion that endocrine disruption has the ability to alter reproductive parameters. Given the importance of sex steroids throughout development, perturbations at any stage of development may have the capacity to alter reproductive functioning.

Animal studies have demonstrated that inhalation of Di(2-ethylhexyl)phthalate (DEHP), a plasticiser used in polyvinyl chloride (PVC) and consumer products, during the prepubertal period advanced vaginal opening and first oestrus in female rats, elevated HPG hormones and lead to irregular oestrus cycles (Ma et al., 2006). In rodent models, maternal exposure to environmental pollutants including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), DES, and genistein had been demonstrated to alter foetal neuronal structures associated with the reproductive axis, leading to the masculisation of the female brain, alteration in GnRH pulse generator alterations, and decreased fertility levels (Miller et al., 2004). Camlin et al. (2014) demonstrated in a murine model that teratogen exposure via maternal smoke inhalation in the perinatal period diminished ovarian follicle numbers, which would consequently negatively alter HPG axis functionality and female fertility levels. This effect has also been examined in human clinical populations, with research demonstrating that babies born to mothers who smoked during gestation display IUGR and low birth weight; which are both risk factors for long term HPG and HPA axis dysfunction and behavioural alterations (Homan et al., 2007; Nigg & Breslau, 2007).

Stress is known to have a suppressive effect on the activity of the HPG axis at many developmental and life stages (Morale et al., 2001; Walker et al., 2011). Centrally, stress inhibits GnRH pulse secretion from the hypothalamus, which alters the release of both LH and FSH from the pituitary (Whirledge & Cidlowski, 2010). Whilst at a gonadal level, the earlier altered cascade results in changes to the stimulatory effect of gonadotropins, influencing sex steroid secretion (Breen & Karsch, 2006; Rivest & Rivier, 1993; Rivier & Vale, 1990). Stressors affecting reproductive parameters include nutritional deficits, psychosocial stressors, and immune stressors. In primates, LPS exposure reliably produces an acute inflamed state and an activation of the HPA axis, as indicated by the increases in central CRH, ACTH, and cortisol release; it also results in the immediate suppression of pulsatile LH and FSH (Karsch et al., 2002; Xiao et al., 1998). A similar effect has been demonstrated using both endotoxin and psychosocial stress in the sheep (Battaglia et al., 1998; Tilbrook et al., 1999) and mice (Karsch et al., 2002; Wagenmaker & Moenter, 2017). Baker et al. (2006) demonstrated in female rodents that a number of chronic stressors, including confinement, noise exposure, food and water deprivation, temperature changes and light/dark cycle reversal; provided drastic alterations in oestrus cyclicity, with animals becoming stalled in the oestrus or diestrus phase of the cycle. Gonzales et al (1994) demonstrated that chronic stress administered in the neonatal period (handling, cold stress,) disrupted the oestrus cycle of female rats, specifically prolonging the diestrus phase, importantly as diestrus is a non-receptive phase of ovulation and the persistence of this phase of the cycle is suboptimal for healthy reproduction.

Literature presented in clinical human studies reports induced amenorrhea and menstrual cycle disruptions in response to stressors such as extreme exercise and sports training, and also nutrient restriction (Chrousos et al., 1998; Kalantaridou et al., 2004; Marinari et al., 1976). In clinical studies, early life adversity and/or stress and maternal

psychopathology is linked to precocious puberty onset (Ellis & Garber, 2000; Wierson et al., 1993). This highlights the sensitivity of the endocrine system to psychosocial environmental stimuli and the close, bi-directional relationship between HPA and HPG functionality. In clinical studies, low birth weight and higher perinatal androgen levels have been linked to advanced female pubarche and ovarian hyperandrogenism (Ibáñez et al., 1998) and has implications for *polycystic ovarian syndrome* (PCOS) development (Gur et al., 2015). PCOS is characterised by perturbed HPG axis functioning and inflammatory changes, and is associated with infertility, menstrual disorders, metabolic abnormalities, and endometrial cancer. Current evidence points towards a developmental origins theory in the aetiology of PCOS, such as genetic transmission, epigenetic alterations, IUGR and maternal androgen exposure of PCOS, indicating the involvement of the early-life environment in female reproduction (Adams et al., 2016; Dumesic et al., 2007; Gur et al., 2015).

Animal studies demonstrate HPG axis susceptibility to the programming effects of perinatal stressors including poor maternal care, maternal nutritional status, and bacterial exposure. In a rat model for instance, the Meany laboratory demonstrated that maternal care mediated HPA axis responsivity and central oestrogen alpha (ERα) receptor expression affecting maternal and sexual behaviours (Cameron, 2011). Furthermore, Cameron (2011) demonstrated that this affected female reproductive parameters including decreased sexual receptivity to males and differences in GnRH neurones and steroid production. Interestingly, cross-fostering alleviated the aforementioned affects, suggesting that multiple reproductive parameters are open to environmental influence, including reproductive strategies, behaviours and functions. This may have evolutionary benefits, and highlights the importance the thrifty phenotype/DOHaD hypothesis framework (Hales & Barker, 2001; Wierson et al., 1993). In addition, early life immune stress via LPS administration has been

demonstrated to lead to HPG axis abnormalities, ovarian and testicular morphological alterations and sexual behaviour deficits (Sominsky et al., 2012a; Walker et al., 2011), having important implications both male and female reproductive health.

Hence, both experimental data and human observational data indicate that the early life environment plays a critical role in the perinatal programming of the HPG axis and its overall function and structure. What is more, it reinforces the multidirectional relationship between neural structures, the endocrine system and the immune system, and emphasises the importance of these systems and their normal developmental trajectories for overall sustained reproductive health and well-being. The impact of early life stress on reproductive parameters is currently an emerging field, however, the importance of the developmental period is quickly becoming a focal point of research due to the increase of female reproductive disorders that have no obvious pathogenesis, including PCOS, endometriosis, and premature ovarian failure, and the currently trend in declining fertility levels worldwide. This highlights the importance of continued investigation into these systems that contribute to reproductive capabilities, including the endocrine-immune interface.

1.4.4 The Immune System

Protection from pathogens and disease is provided by both the innate and acquired components of the immune system that work together in an integrated fashion (Kuby, 1997). An inflammatory response is initiated by numerous complex interactions mediated by immune molecules and cells, which also influence the functioning of the endocrine and ANS systems at various levels (Ader et al., 1995; Kenney & Ganta, 2014). As with the endocrine system, the immune system follows an orderly developmental trajectory post conception, with immune factors contributing to the developing offspring throughout fertilisation, implantation, gestation, parturition, and continuing throughout early postnatal development

(Kuper et al., 2016; Spencer et al., 2011). The immune system also remains plastic throughout development, open to endogenous and exogenous stimuli and closely interacting with endocrine and autonomic process (Steinman, 2004; Tanriverdi et al., 2003a; Wrona, 2006; Zakharova, 2014). Inflammatory elements are critical to numerous normal homeostatic processes, particularly within reproductive parameters such as female ovulation and ovarian follicle atresia (Nash et al., 1999; Richards et al., 2008; Simon & Polan, 1994; Wu et al., 2004), and immune molecules can be secreted from cells that are not considered to be part of the immune system, such as damaged tissue (Kuby, 1997).

The architecture of the immune system is complex, forming and integrating extensive and dynamic networks both centrally and peripherally. Many molecules perform various and dynamic tasks both centrally and peripherally including; Macrophages, microglia, toll-like receptors (TLR), cytokines, chemokines and acute phase proteins. These key cells and molecular messengers of the immune system work intrinsically to identify pathogens and mount inflammatory responses when necessary, as well as maintain homeostasis (Kelso, 1998; Mire-Sluis, 1993; Nathan, 2002). The immune system is divided into two core components, consisting of innate immunity, our first line of defence, and acquired immunity, a specific defence system that is adapted to environmental stimuli and specific to certain pathogens (Kuby, 1997).

Innate immune responses provide the initial defence against invading pathogens, providing non-specific and immediate responses until acquired components are activated. The innate immune response is capable of combating the day-to-day microorganisms encountered by a healthy individual, without enlisting a specific, acquired immune response. Innate immunity is comprised of defensive barriers, which are; atomic, such as the skin and mucus membranes; physiological, including temperature, pH levels and chemical mediators;

phagocytic barriers including the cells (monocytes, neutrophils, macrophages) which break down, kill, and digests foreign material; and finally inflammatory barriers, which create a classical inflammatory response including redness, swelling, heat and pain via chemical mediations including acute-phase proteins, histamines, and kinins (Kluger, 1980; Kluger et al., 1998; Kluger & Rothenburg, 1979; Kuby, 1997). When these mechanisms fail, or immune mediation is needed, the body mounts an acquired response.

Acquired immunity reflects the exquisite versatility and adaptability of the immune system, and initially takes longer to mount an effective response (~up to a week). Once the immune system is exposed to a specific antigen, it creates antigen specificity and immunological memory in order to combat that specific antigen on repeat infection with heightened sensitivity and efficacy (Iwasaki & Medzhitov, 2004; Janeway & Medzhitov, 1998). This allows the immune system to exhibit great diversity, being able to distinguish between antigens with a difference of only a single amino acid. It is this immunological memory that enables successful vaccinations and prevents subsequent reinfection. Acquired immunity does not occur independently of innate immunity, rather the cells of the innate system are crucially and intimately involved with activation of the specific immune response. Generation of an effective immune response involves two major immune cells groups, lymphocytes and antigen presenting cells (Kuby, 1997). Lymphocytes can be further divided into B cells and T cells, depending on their maturation site. B cells mature within bone marrow, and then migrate via the circulatory and lymph system to reside in various tissues, including the spleen and lymph nodes. On encountering an antigen, naïve B cells divides, with the progeny forming both memory and effector B cells, which carry on immunological memory and antigen specificity (Supajatura, 2002). T cells are also developed in bone marrow, however immature T cells migrate to the thymus gland prior to mature release. During maturation within the

thymus, the T cell expresses a unique antigen binding receptor on its membrane which will only recognise antigens with major histocompatibility complex (MHC) molecules. When a naïve T cell encounters an antigen expressing a MCH, it proliferates into memory T cells and varied effector T cells. T cells can be further classified into T helper (T_H) and T cytotoxic (T_C) cells, which express either surface membrane glycoproteins CD4 (T_H) or CD8 (T_C) (Kuby, 1997; Tough et al., 1999). After T cells are activated, they have the ability to secrete cytokines in order to effectively activate and mediate an immune cascade in order to combat antigens and invading materials (Kelso, 1998; Schindler et al., 2007; Tracey, 2002). Antigen presenting cells ([APC], including macrophages, B lymphocytes, and dendritic cells) first phagocytise or endocytose the antigen, then re-express an antigen section along with a class II MCH molecule on their membrane, this leads to co-stimulation and further immune activation and proliferation (Tough et al., 1999; Tracey, 2002; Trinchieri, 2003).

Macrophages are constitutively expressed in a number of tissues and are activated by a variety of invading stimuli and cytokines, specifically interferon (IFN)-gamma. When activated, macrophages are able to ingest and degrade particular antigens, including bacteria (Kuby, 1997). Macrophage cell activity is aided by cytokines, the chemical messengers of the immune system, which they secrete in response to an immune assault. Cytokines are proteins or peptides that form a large group of immune molecules including IFN, IL, TNF, TGF and colony stimulating factor (CSF) (Kuby, 1997). Cytokine synthesis and distribution is elicited not only by the invasion of pathogens or tissue injury, but also in response to both physiological and psychological stress. The cytokine family includes proinflammatory molecules (T_H1) which initiate and enhance the inflammatory response, and anti-inflammatory molecules (T_H2) that dampen and regulate inflammation (Kidd, 2003) (Figure 1.5). Perturbation in the balance between cytokine levels and activity, as well as imbalances of other cells and molecules of the

immune system, can lead to immune dysregulation, and in turn, disturbances to other linked systems (Kidd, 2003). Difference in anti-and-pro inflammatory cytokine profiles and patterns lead to the T_H1/T_H2 hypothesis, which posits that an imbalance in classes of cytokines leads to disorders and disease, with a predominant T_H1 shift leading to autoimmune disorders such as arthritis, multiple sclerosis and type 1 diabetes, and a predominant T_H2 shift implicated in allergies and asthma (Calcagni & Elenkov, 2006; Dube et al., 2009; Kidd, 2003; Moser & Murphy, 2000).

Cytokines are essential to both peripheral and central processes (Besedovsky & del Rey, 2011; Calcagni & Elenkov, 2006). These immune system messages instigate and facilitate cell-to-cell communication within the immune system, as well as interact with neuroendocrine axes, the ANS and the brain (Bauer et al., 2007; Bilbo & Schwarz, 2012; Hasko & Szabo, 1998; Kenney & Ganta, 2014; Steinman, 2004). Cytokines act locally via paracrine, autocrine, and sometimes endocrine, processes to bind to specific receptors on multiple target cells, ultimately altering the gene expression in these targets (Stenken & Poschenrieder, 2015). Due to the high affinity between cytokines and their associated receptors, small concentrations (picomolar) of cytokines can mediate a large biological effect (Kuby, 1997). Additionally, cytokine receptors are present on numerous target cells, diversifying the effect cytokines may have (Rose-John & Heinrich, 1994). Cytokines mediate both the intensity and the duration of the immune response, as they have the cumulative effects of stimulating or inhibiting activation, proliferation, maturation, apoptosis, and of differentiation of other cells (Dinarello, 2000). Cytokine binding stimulates both the production of more cytokines and the expression of cytokine receptors, often influencing multiple target cells involved in the immune response and interconnected systems. As such, cytokines may be self-perpetuating, with the activities of later cytokine synthesis affecting

earlier cytokine synthesis and vice versa, perhaps with differential affect (Kelso, 1998). Via specific pathways, including the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways and kinase pathways, cytokine binding can translate an extracellular signal into a transcriptional response (Cai et al., 2013; Horvath, 2004a, 2004b; Rawlings et al., 2004; Schindler et al., 2007; Shuai & Liu, 2003), affecting many biological functions and associated behavioural outputs. Exposure to a peripheral immune challenge, such as through viral or bacterial exposure, pathogens or LPS generates an immediate acute phase immune response (Alexander & Rietschel, 2001; Chow et al., 1999). This signals and increases proinflammatory mediators, activating the cytokine cascade and proinflammatory transcription factor pathways including Map kinase (MAPK) nuclear factor (NF)-kappa beta (Corre et al., 2017; Farooq & Zhou, 2004; Mercau et al., 2014). This leads to the production of proinflammatory cytokines IL-1β, IL-6, TNFα, C-RP and IFN-γ by APCs and natural killer (NK) cells (Alexander & Rietschel, 2001; Horvath, 2004a) (Figure 1.6). The action of the inflammatory cascade activates the HPA axis in a context specific manner, causing central inflammatory and neurotransmitter alterations leading to exhibition of sickness behaviours including lethargy, social withdrawal, anhedonia, and anorexia (Bay-Richter et al., 2011).

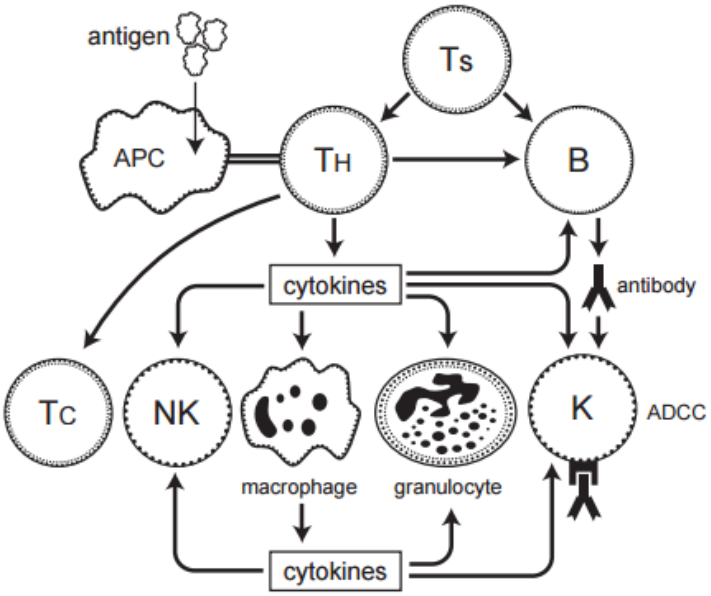

Figure 1.6. Functional flow of immunity following antigen detection adapted from (Kidd, 2003) showing how cytokines facilitate and co-ordinate the activities of multiple immune cells to mount an efficient and successful immune response. Tc = cytotoxic T lymphocyte; Ts = T suppressor cell; B = B cell; NK = natural killer cell: K = killer cell; ADCC = antibody-dependent cytotoxic cell; APC = antigen presenting cell.

Multiple pathways and processes are responsible for the transmission of information regarding peripheral immune status to central structures, including neural and non-neural relay. This peripheral-to-brain communication primarily involves the pleiotropic actions of various cytokines, and can lead to clear alterations to brain functioning and behavioural output (Besedovsky & del Rey, 2011; Corre et al., 2017; Hagberg & Mallard, 2005; Hennessy et al., 2014; Larson & Dunn, 2001; Srinivasan et al., 2004; Werner et al., 2000). Sensory neurons of the vagus nerve are stimulated by peripheral proinflammatory cytokines, namely IL-1β, stimulates catecholaminergic neurons in the ventrolateral medulla, then projects to other sites of autonomic and endocrine control including the PVN of the hypothalamus, and the hippocampus (Dantzer & Kelley, 2007; Goehler et al., 2000; Olofsson et al., 2012; Watkins

et al., 1995). This direct neural route plays a direct role in mediating the effects of peripheral inflammation, including sickness behaviours, increases in HPA axis activation, and NE alterations (Quan & Banks, 2007; Watkins et al., 1995; Wrona, 2006). Peripheral inflammatory molecules can also pass through the blood-brain-barrier (BBB) or via circumventricular organs, where the capillaries of the brain do not form the BBB (Dantzer & Kelley, 2007).

The CNS interacts with immune system via autonomic innervation and neuroendocrine means (Quan & Banks, 2007). The primary mononuclear immune cells of the brain are microglial cells, often referred to as the 'macrophages of the brain', making up around 10-15% of CNS cell populations (Galic et al., 2012; Graeber & Streit, 1990; Kim & Nagai, 2010). Microglial cells are responsible for maintaining central immune homeostasis, and are critical effectors and regulators throughout development and in health and disease (Nakamura, 2002; Paolicelli et al., 2011; Rock et al., 2004; Schlegelmilch et al., 2011). Microglia act to defend the CNS, and constantly 'scan' the brain for injury and pathogenic invasion. Upon detection of signs of insult, microglial processes rapidly move to the detection site, transforming microglia from their resting to activated state and stimulate the release of cytokines and other immune mediators (Ayoub & Salm, 2003). Microglia play an important role in the synaptic pruning and developmental remodelling during critical periods of development (Paolicelli et al., 2011; Prinz & Priller, 2014). Due to their important role in development and maturation, microglia and associated immune function are integral to models of perinatal programming involving early life immune stressors. Microglial cells express TLRs and other receptors that play roles in pathogen recognition, and are known to activate in response to LPS stimulation to stimulate the secretion of numerous central cytokines and their receptors via paracrine and autocrine processes (Rock et al., 2004). Hence, microglia expression and activity are implicated in disease and disorders that relate to

irregular immune functioning, including many psychiatric disorders such as depression, schizophrenia, anxiety, Alzheimer's, dementia, and Parkinson's (Mander & Brown, 2005; McCoy & Tansey, 2008; Nakamura, 2002; Schlegelmilch et al., 2011).

Essentially, immune activation is an innate and adaptive response to fight off invading pathogens and maintain homeostasis. Inflammation is beneficial, it helps maintain health, aid healing, and supress illness. However, when immune activation becomes chronic and imbalanced, immune related illness and dysfunction of the immune system and related systems occur (Boots & Jungheim, 2015; Harrison, 2013; Jessop, 2008; Kumar & Wakefield, 2010; Lukewich et al., 2014; Weiss et al., 2009a). For instance, increased inflammation is a hallmark of many disease states and disorders, particularly female reproductive disorders and mental health disorders (Erlebacher et al., 2004; Halis & Arici, 2004; Jabbour et al., 2009; Weiss et al., 2009a). Chronic or dysregulated immune activation can modulate endocrine function and neurotransmitter function, leading to both physiological and psychological disorders. Immune dysfunction can occur to due numerous factors, including being perinatally programmed via early life environmental conditions (Alexander & Rietschel, 2001; Galic et al., 2009; Mouihate, 2013; Spencer et al., 2011).

***1.4.4.1 Immune Mediation of Female Reproductive Parameters*.** It is known that immuno-endocrine factors are key regulators of continued female reproductive success and longevity, with immune components being particularly crucial to the rudiments of ovarian follicular development, as well as throughout the female reproductive lifespan (Bukovsky & Caudle, 2012). Female reproductive health and success is reliant on normal and uninterrupted establishment of a finite pool of ovarian primordial follicles (McGee & Hsueh, 2000). In the human, ovarian follicle development occurs after 11 or 12 weeks of gestation. Females are born with a finite number of germ cells, or *oocytes* contained within primordial ovarian

follicles (Baker, 1963). The non-renewing pool of ovarian follicles dictates the female reproductive lifespan, serving as the foundation for all developing oocytes (Banerjee et al., 2014; Findlay et al., 2015; Kim, 2012; Oktem & Urman, 2010; Richardson et al., 2014). These oocytes are surrounded by supporting somatic cells, termed *granulosa* cells that change morphologically and proliferate as the oocyte/egg matures (Hage et al., 1978). In rodents, the early neonatal period during the first week of birth is the critical time point for the final stages of ovarian follicular pool development, called folliculogenesis (Hirshfield & DeSanti, 1995; Rajah et al., 1992). During the early gonadotropin-independent ovarian developmental stages, complex interactions of chemokines, cytokines, neurotrophins and growth factors mediate the maturation of the primordial follicular pool, as well as govern the maintenance of resting or dormant follicles (Cai et al., 2013; Hirshfield, 1991; Hirshfield & DeSanti, 1995; Kerr et al., 2013; McGee & Hsueh, 2000; McLaughlin & McIver, 2009; Skinner, 2005a; Terranova & Rice, 1997; Tingen et al., 2009). Therefore, early life stressors that disrupt or perturb the delicate immune processes occurring during critical periods of immune-driven reproductive development, such as the formation of the gonads and folliculogenesis, may play a key role in shaping female fertility outcomes and longevity (Evans et al., 2016; Nepomnaschy et al., 2007; Sominsky et al., 2012a; Sominsky et al., 2013b; Walker et al., 2011).

Immune mechanisms play a critical role in protecting the female reproductive tract from microbial infections that may cause abortions, preterm labour, disease and infertility (Jabbour et al., 2009; Sheldon et al., 2016; Song & Shi, 2014). Furthermore, inflammatory mechanisms facilitate and often govern physiological events of the normal ovarian cycle, such as implantation, fertilisation and parturition (Nash et al., 1999; Simon & Polan, 1994; Turner et al., 2012; Wu et al., 2004). The female reproductive tract has developed in such a way that both innate and adaptive immunity functions in concert to protect against invading pathogens

whilst maximising the chances for reproductive success (Wira & Fahey, 2004). Immune cells are present and functional throughout the female reproductive tract; these include epithelial cells, macrophages, dendritic cells, neutrophils and NK cells (Rivier & Vale, 1990; Robertson et al., 2015; Sheldon et al., 2016; Wira & Fahey, 2004). Epithelial cells of the female reproductive tract are involved in both the innate and adaptive immune response and express TLRs to detect pathogens (Bromfield & Sheldon, 2013; Spanel-Borowski, 2011). Epithelial cells are also involved with the physiological processes of the menstrual cycle due to their ability to secrete cytokines and chemokines (Wira & Fahey, 2004). Through NF-κβ pathways activated by TLRs, stimulation of endocervical epithelial cells induces the secretion of cytokines, such as IL-6, IL-8 and TNFα (Auersperg & Woo, 2004) . Additionally, catecholamines are present in ovarian nerve fibers and are essential for steroidogenesis, ovulation and the follicular response to gonadotropins, whilst ovarian denervation leads to decreases in oestrus cyclicity, conception and offspring numbers (Risvanli et al, 2004). Given the importance of these inflammatory mediators in normal reproductive development and their close functional capabilities with related systems, alteration in their physiological levels during critical periods of gonadal development may predispose to an increased risk of subfertility in later life.

As with immune-HPA axis interactions, there is a strong association between immune function and the functioning of the HPG axis, where immune processes modify sex hormone actions and their receptors (Morale et al., 2001). GnRH receptors (R) are expressed locally by some immune cells, such as lymphocytes, as well as GnRH and sex hormones being strongly implicated in the early modulation and development of the immune system (Tanriverdi et al., 2003b). GnRH is involved in immune system development; androgen and oestrogen receptors are expressed on immune cells, as well as the ovary and reproductive tract being an immune privileged site (Herath et al., 2007; Sheldon et al., 2014; Sheldon et al., 2016; Turner et al.,

2012). Oestrogen receptors have also been demonstrated to be present in a number of immune cells, including those in the primary lymphoid organs, thymocytes, and mature peripheral T and B cells (Tanriverdi et al., 2003b). Moreover, GnRH has been demonstrated to enhance the production of proinflammatory cytokines, IL-2 and IFN–γ *in vivo* (Batticane et al., 1991). Hence, the development and functionality of these systems can be affected by the immune system.

Bacterial and viral infection is a common environmental immune stressor that affects pregnant women and newborns, particularly with maternal-foetal immune modifications in place (Levy, 2005, 2007; Song & Shi, 2014). In the newborn rat, the immune system is functionally immature (Kuper et al., 2016; Vosters et al., 2010) and highly susceptible to the effects of perinatal programming by environmental stimuli. Interruptions to the delicate immune processes during critical stages of follicular formation may alter physiology including normal ovarian follicular establishment and growth, as well as alter general neuroimmunoendocrine functionality (Bilbo & Schwarz, 2012; Spencer et al., 2011).

***1.4.4.2 Perinatal Programming of the Immune System*.** The immune system is highly sensitive to exogenous and endogenous influences during critical periods of maturation, including endocrine and sympathetic signalling (Fagundes et al., 2013). As such, stressors, whether they be immune or otherwise, affect immune functioning during critical periods of plasticity. The perinatal programming of the immune system has been linked to various disease phenotypes and profiles, including asthma, allergies, autoimmune disorders and metabolic disorders including cardiovascular disease (Bilbo & Schwarz, 2009; Morale et al., 2001; Mouihate, 2013; Spencer et al., 2011). Research continues to elucidate immune involvement in numerous disorders, including female reproductive diseases such as PCOS and

endometriosis (Halis & Arici, 2004; Weiss et al., 2009b) and psychological disorders (Buehler, 2011; Harrison, 2013; Maes, 1995; Raison et al., 2006; Yong-Ku & Sang Won, 2017).

The following aims to give a brief summary on the background of immune programming. Previous research has often highlighted how immune-related diseases and disorders are associated with an atypical T_H1/T_H2 imbalance or inadequate immune responses. Allergic responses have been linked with a skew towards T_H2 mediated inflammatory profiles in individuals, which is associated with excess anti-inflammatory molecules and signalling of cytokines (IL-4, IL5, IL-11, IL-12) and immunoglobulin (IgE), leading to atopy (Kidd, 2003). This T_H1/T_H2 hypothesis is driven by diseases including asthma, allergies, dermatitis, and lupus, however it has limitations that include the pleiotropic nature of cytokines and current literature is acknowledging the restrictions of this hypothesis. Other views regarding the immune systems role in disease states include the *hygiene hypothesis* (Strachan, 1989) which posits that a lack of childhood exposure to infections, microorganisms, bacteria, parasites and helminths lead to increased susceptibility to atopic diseases and an inability to mount sufficient immune responses. Evidence does suggest that microbial exposure has a protective effects (Von Ehrenstein et al., 2000), however others find little support for this hypothesis (Backman et al., 1984). While perinatal exposure to immune activation may have a protective effect in some instances and be beneficial for developing immune tolerance and resistance, inappropriate exposure to immune activation, as well as other stressors, at critical periods of development has been linked to later life pathologies. Differing theories and evidence highlight the complexity of development and the interplay of physiological systems, suggesting that differential health trajectories may be depended on timing and extent of exposure, as well as a genetic susceptibility.

The neonatal immune system is relatively naïve and functionally immature, generating a less robust immune response in comparison to adults (Vosters et al., 2010). However during this time, immune functionality, including patterns of cytokines, chemokines and growth factors is critically important for the growth and maturation of vital systems, including female ovarian development, and central development including microglia maturation. The expression of cytokines in the developing brain is significantly increase in order to facilitate maturation and neuronal development, even in the absence of immune activation (Bilbo & Schwarz, 2012). The distinct cytokine profiles and microglial morphologies during early life reflect the increased sensitivity of the developing brain to outside immunological interference. Perturbations of immune processes that are essential to neurodevelopmental processes and cell maturation may result in permanent alterations to developmental processes and lead to long term programming of functions and behaviours both in the short and the long term.

1.4.4.3 Perinatal Programming of the Immune System via Neural-Endocrine-Immune Interactions. Reciprocal communication between physiological systems govern development and maturation. The neuro-immune-endocrine pathways which govern development, maturation, and ongoing function are established during the pre and perinatal period. Hence, perturbations to the development of any of these systems may result in disturbances to not only the individual systems, but also have an effect on the bidirectional communication between them, leading to pathologies. Epidemiological evidence suggests that numerous early life stressors, including immune stress and psychological stress, perinatally program long term functional alterations in the immune system, as well as associated neuro-endocrine dysfunction. Childhood stress and trauma have been associate with increased immune

responses to stressors later in life, as well as general increased immune pathway upregulation (Jessop, 2008).

Evidence for perinatally programmed alterations in immunity has been observed in human experimental and epidemiological research. Male adults with depression who experience early life stress had a more pronounced proinflammatory IL-6 response to experimental stressors than controls (Pace et al., 2009), as did healthy adult males who reported childhood trauma (Carpenter et al., 2010). Women currently presenting with depression or PTSD who reported childhood abuse had higher levels of NF-κB, a signalling molecule that controls proinflammatory cytokine gene expression and inflammatory pathways (Pace et al, 2012). Taylor et al (2006) links low socio-economic-status and a harsh childhood family climate to elevated basal adult levels of C-RP, which is a major acute phase inflammatory biomarker associated with increased risk to cardiovascular disease, metabolic diseases, immune disorders and depression (Bassuk et al., 2004). Children who experience psychological abuse or neglect demonstrates higher levels of proinflammatory cytokines in childhood and this cytokine profile continues into adulthood (Fagundes et al., 2013). In addition, a proinflammatory phenotype and concomitant stress-axis dysfunction has been associated with low childhood socioeconomic status, indicated by altered GR mRNA and TLR4 mRNA in adolescence (Miller & Chen, 2007; Miller & Chen, 2010). These relationships between early life stress and immune dysfunction has been further been explored in animals studies, allowing for the manipulation of stressors and the exploration of their effects on various systems.

In primate studies, Reys and Coe (1997) demonstrated that ACTH endocrine stimulation in mid to late gestation led to offspring that displayed blunted plasma and cerebral spinal fluid IL-6 levels and a diminished febrile responses to IL-1β injections at 2 years

of age. Additionally, Coe et al., (2002) demonstrated that gravid monkeys exposed to six weeks of brief acoustic startle manipulations at either early or late gestational periods resulted in their offspring displaying a blunted blood leukocyte TNFα and IL-6 response to LPS stimulation at 2 years of age, demonstrating the long term effect of psychological stress on immune parameters. In a rodent study, Vanbesien-Mailliot et al. (2007) exposed pregnant dams to a restraint procedure for the last 11 days of gestation and analysed inflammatory markers in their offspring at six weeks and again at six months of age. It was demonstrated that prenatally stressed offspring exhibited increases in T_C ($CD8^+$) and NK cells, indicating an increased proinflammatory profile of progenies at later developmental stages, signifying the long term effects of maternal stressors on their offspring (Vanbesien-Mailliot et al). Nakamura et al. (2011) demonstrated that maternal separation in early life impaired NK cytotoxicity in adult rats, and that this was independent of corticosterone patterns, suggesting that immune parameters may be affected without an additional life stressor. Additionally, the same study demonstrated that maternal separation paired with chronic stress in later life compromised tumour immunity (Nakamura et al., 2011). Chandra (1975) demonstrated that female rats that were malnourished prior to pregnancy gave birth to pups that displayed a significant reduction in antibody response, even though the offspring had access to food ad libitum. Interestingly, Chandra also demonstrated that this impaired immunocompetence continued intro subsequent generations. Furthermore, Barreau et al. (2004) demonstrated rat pups that underwent maternal separation during PND 2-14 displayed exaggerated adult immune responses evidenced by increases of mRNA expression of the cytokines IFN-γ, IL-1β, IL-2, IL-4 and IL-10 in colonic mucosa, the liver and spleen, which is further indication of an altered inflammatory phenotype and has implications for not only physiological pathologies, but

psychopathologies such as autism and other neurodevelopmental disorders associated with gut microbiota.

The dichotomous relationship of action between immune and endocrine elements is interesting and inextricably linked. Immune mediators are supressed by neuroendocrine activity at multiple levels, however, in some instances, stress hormones may bolster the immune response via the actions of particular cytokines, perpetuating inflammation into chronic levels with an allosteric load. The mechanisms and effects of perinatal programming of the immune system and associated systems by various early life stressors remains to be fully elucidated. Animal studies have utilised many models of early life stress and perinatal immune activation, including the central and peripheral administration of immune products such as cytokines and HPA axis products, bacterial and viral mimetics including LPS, Poly I:C, live pathogens such as Escherichia coli (*E. coli*) and Chlamydia, and other reagents such as endotoxins and exotoxins. The following section will focus on the impact of perinatal immune challenge by LPS administration as work using this model will be presented in the current thesis.

1.5. **Animal Models of Early Life Stress**

Experimental manipulations in animal models allow for the preclinical examination of specific mechanisms and basic scientific gain. Human studies are limited in their capacity, due to both ethical constraints and variable interference. Although large epidemiological studies aid in the development of specific hypothesis and understanding of phenomena and relationships, they lack the nuances and specificity that animal model are able to provide. Rodent models are typically utilised in the examination of early life stress on developmental trajectories, and allow for the examination of biological mechanisms with relative ease, elegance and relatability. Numerous rodent models of early life stress have been utilised and

described in previous sections of this thesis, including maternal and neonatal restraint, maternal separation, maternal and neonatal caloric and nutrient manipulation, exposure to neuroendocrine products and immune activation. The following sections will focus on the impact of early life immune stress utilising bacterial mimetic LPS.

1.5.1 Lipopolysaccharide (LPS): An Immunological Stressor

Lipopolysaccharide is a gram-negative bacterial mimetic, comprised of the cell wall of Gram-negative bacteria (derived usually *Salmonella entreitidid*s or *E.coli*) that provokes an innate immune response, activating the proinflammatory cascade via binding to the LPS-specific TLR4 which is expressed constitutively. This includes TLR4 expression in the female reproductive tracts, where it is expressed on ovarian supporting (granulosa) cells (Herath et al., 2007) as well as monocytes, macrophages, and adipocytes (Iwasaki & Medzhitov, 2004). Immune stimulation by LPS and the associated inflammatory-driven behavioural symptomology is largely identical to that of a live bacterial infection (Karrow, 2006) and thus is considered a systemic immunological stressor (Beishuizen & Thijs, 2003). Unlike a live bacterial challenge, LPS does not replicate, allowing for the control of dose and therefore the confounding nature of an actual infection. As such, LPS is commonly used to explore the complexities of neuroimmune-neuroendocrine relationships and has been demonstrated as a reliable immune and stress activator (Alexander & Rietschel, 2001; Beishuizen & Thijs, 2003; Bilbo & Schwarz, 2009; Depino, 2015; Galic et al., 2009; Spencer et al., 2007a; Walker et al., 2009; Wu et al., 2011b).

Via TLR4 binding, key proinflammatory transcription factor pathways are activated including MAPK and NF- κβ, which translocates to the nucleus to produce proinflammatory cytokines IL-1β, IL-6, TNFα, CRP, IFN-γ and cyclooxygenase (COX)-2 prostaglandin (PG) pathway (PGE_2) activation (see Figure 1.7) (Alexander & Rietschel, 2001; Peri & Piazza, 2012;

Zarember & Godowski, 2002; Zhang et al., 2008a). Central prostaglandin and cytokine actions contribute to the initiation of the febrile response and associated sickness behaviours (Bay-Richter et al., 2011; Eliopoulos et al., 2002; Mercau et al., 2014). Anti-inflammatory mediators and the HPA axis respond to immune stimulation to control the magnitude and longevity of the immune activation (Dinarello, 2000; Han & Ulevitch, 1999; Lawrence et al., 2002). The immune system, HPA axis and ANS work in concert to resolve the immune activation. The HPA axis is activated following the administration of LPS in order to subdue the inflammatory response. It is well established that glucocorticoid release quell inflammatory mediators (Elenkov, 2008). ANS activation, via vagus nerve stimulation, aids the alleviation of inflammation and the stress response in order to return the organism to homeostasis. Therefore, these mechanisms are all activated with LPS exposure; during sensitive periods of development this activation may be capable of reprogramming the system to lead to dysfunction.

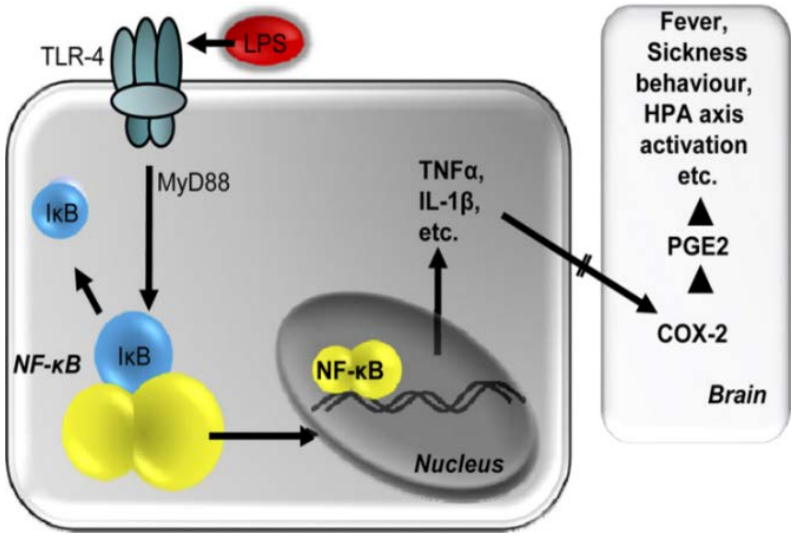

Figure 1.7. LPS immune activation mechanisms via toll-like receptor 4 (TLR4) binding and subsequent release of cytokines and prostaglandin E_2 (PGE_2), stimulating the febrile response and HPA activation. Image via Spencer et al. (2011).

1.5.2 Lipopolysaccharide: Animal Models of Neonatal Immune Activation (NIA)

The LPS model of immune activation has been utilised for a number of decades and as such, is a validated and reliable immunological stressor. Witek-Janusek (1988) conducted the primary research that demonstrated the sensitivity of neonatal rats to bacterial endotoxin and the accompanying marked corticosterone and ACTH elevations between PND 1-5, despite this being a hypo-responsive stress period for the neonatal rat. Following on from this work, Shanks et al (Shanks et al., 1995; Shanks & Meaney, 1994) demonstrated the LPS exposure on PND 3 and 5 at a relatively low dose of 0.05mg/kg produced a rapid, sustained immune and stress response in the neonatal period and that these animals treated with LPS as neonates exhibited a significantly greater HPA axis response to restraint stress in adulthood (PND 85-90) when compared to controls. This research implicated LPS immune activation in the long term programming of stress sensitivity and predisposition to stress related pathologies. The findings from this study have been replicated in numerous subsequent experiments, including those from our laboratory (Sominsky et al., 2012b; Walker et al., 2011; Walker et al., 2009; Walker et al., 2010; Walker et al., 2004a; Walker et al., 2003). Response variations such as differing amplitudes of ACTH and CORT that have been observed (Takemura et al., 1997) have been attributed to dosage and LPS preparation differences (Beishuizen & Thijs, 2003).

A long spanning and well-rounded body of evidence has characterised the rodent model of PND 3 and 5 LPS dose and timing efficacy for inducing sustained neuro-immuno-endocrine alterations. Evidence suggests that a singular dose is insufficient in producing long term changes in stress responsivity. Takemura et al. (1997) demonstrated that a single LPS dose significant upregulated the staining c-Fos mRNA in catecholaminergic nuclei, associated with upregulated hippocampal GRs which would inhibit glucocorticoid production and switch off the HPA axis via negative feedback loops prior to changes taking place. This does not occur

with the dual LPS dose method. A robust body of evidence has been accumulated using this PND 3 and 5 LPS administration model, and confirms the efficacy and reliability of the model for immediate immune activation and long terms stress axis alterations. This PND 3 and 5 model has been well characterised in our laboratory and findings include metabolic alterations, immune alterations, neuroendocrine alterations, changes in pain sensitivity, behavioural alterations, and most recently; changes in both male and female reproductive parameters (Sominsky et al., 2012a; Sominsky et al., 2012b; Walker et al., 2011; Walker et al., 2009; Walker et al., 2010; Walker et al., 2004a; Walker et al., 2003; Zouikr et al., 2015; Zouikr et al., 2014a; Zouikr et al., 2014b).

1.5.2.1 Impact of Neonatal LPS on Metabolic Function. Postnatal day 3 and 5 LPS exposure has been demonstrated to alter long term metabolic activity (Walker et al., 2004a). Iwasa et al. (2010) reported that neonatal LPS administration in rodents significantly elevated body weight post-weaning, increased food intake and elevated serum leptin levels. Spencer et al. (2007b) however demonstrated that neonatal exposure to LPS has no long term effects on growth, birthweight, fat distribution or leptin level. Alternatively, Walker et al. (2009) demonstrated that neonatally treated LPS animals gained significantly less weight between adolescence to adulthood. These changes have been suggested to be related to changes in glucose tolerance, insulin sensitivity and pancreatic functioning (Jaworek et al., 2007; Nilsson et al., 2002). Resent research examining other forms of early life stress on leptin levels and metabolic functioning indicate there are differences occurring through metabolic pathways of early life stress, and that timing and mode of stressor may impact on the presentation of symptomology (Hanson & Gluckman, 2008; Iwasa et al., 2010; Kim et al., 2007; Mitchell et al., 2005; Sominsky et al., 2016).

1.5.2.2 Impact of Neonatal LPS on Endocrine Function. The bank of literature examining the effects of early life LPS on endocrine function is varied and robust. These studies focus on hormone and neurotransmitters from the HPA and their receptors. Research demonstrates both central and peripheral endocrine alterations with the administration of neonatal LPS (Beishuizen & Thijs, 2003; Iwasa et al., 2009; Karrow, 2006; Spencer et al., 2006b; Takemura et al., 1997; Wu et al., 2011a). Both short and long-term alterations to the HPA axis have been demonstrated at multiple levels of HPA functioning following PND 3 and 5 LPS administration and other neonatal time points. These include CORT and ACTH alterations (Hodgson & Coe, 2006; Kentner et al., 2010; Shanks et al., 1995; Shanks & Meaney, 1994; Walker et al., 2009; Walker et al., 2004a; Zouikr et al., 2016; Zouikr et al., 2014b) and alterations to MR, GR, and CRH receptor (R) 1 receptor densities (Sapolsky & Meaney, 1986; Shanks & Meaney, 1994; Webster & Sternberg, 2004). Changes in receptor densities and functionality have been implicated in the dysregulation of the HPA negative feedback system, which have been reported as both blunted (Walker et al., 2009) and hyperactive (Iwasa et al., 2009). This implies that glucocorticoid pathways and HPA axis signalling is sensitive to early life LPS stimulations leading to the programming of development, as well as having implications for immune-endocrine interactions and overall behavioural output.

1.5.3 Impact of LPS Administration on Behaviour

***1.5.3.1 Anxiety-like behaviours*.** LPS administration stimulates behavioural responses both short and long-term. Research suggests that neonatal LPS administration perinatally programs an anxiety-like phenotype, particularly following a subsequent stressor in adulthood. Several studies from our laboratory and others indicate that adult animals treated with neonatal LPS exhibit reduced exploratory behaviour and increased hypervigilance in numerous tests including the hole board, open field and elevated plus maze (Sominsky et al.,

2012b; Spencer et al., 2005; Walker et al., 2012; Walker et al., 2009; Walker et al., 2010; Walker et al., 2004b) as well as in response to acoustic startle (Walker et al., 2008). Conversely, some studies report that neonatal immune stress leads to greater exploratory behaviour in adolescence (Rico et al., 2010), but also greater emotionality (Dinel et al., 2014). Early LPS treatment has also demonstrated sexually dysmorphic results regarding anxiety-like behaviours (Bernardi et al., 2014; Donner & Lowry, 2013; Tenk et al., 2008), indicating that males and females may respond differently and manifest differing symptomology to neonatal immune stress, particularly as it is known that females and males differ in immune responses (Cai et al., 2016b; Klein & Flanagan, 2016; Yee & Prendergast, 2010). However, some studies indicate that LPS administration has been shown to affect febrile responses in females and males similarly, however the neonatal dose was at a later time point (Spencer et al., 2006a).

1.5.3.2 Sickness behaviours and depressive-like behaviours. Generally, LPS administration is known to produce a distinct set of adaptive and physiological response behaviours termed 'sickness behaviours' (Aubert, 1999; Dantzer, 2001, 2004; Dantzer, 2009). These include hypo-activity, anorexia, hyperthermia, hypodipsia, anhedonia, fatigue and lethargy, malaise, hyperalgesia, disinterest in social behaviour or environmental interactions, and decreased sexual activity, finalised with HPA axis activation (Maes et al., 2012). These sickness behaviours are exhibited in order to combat pathogen invasion and are known to be driven by the release of proinflammatory cytokines including; interleukin (IL) 1β, tumour necrosis factor (TNF)α, and IL-6 from activated peripheral immune cells, macrophages and monocytes targeting central areas (van Dam et al., 1992; Yirmiya, 1996).

As chronic or abnormal stress activation can result in psychopathology such as anxiety; as can abnormal, exaggerated and chronic sickness behaviours. Due to their strikingly parallel nature, sickness behaviours have been associated to symptoms of clinical depression. This has

led to the postulation that clinical depression and depressive-like behaviour is an immune-inflammatory disorder (Dantzer, 2009; Dantzer & Kelley, 2007; Dantzer et al., 2008; Treadway et al., 2012). Human evidence supports this proposal. Clinically, research indicates that major depression and mood disorders are associated with significant elevations in circulating levels of proinflammatory cytokines and other inflammatory mediators including cyclooxygenase (COX)-2 and c-reactive protein (CRP) (Dowlati et al., 2010; Liu et al., 2012; Maes, 1995; Maes et al., 1992; Pariante, 2017; Song et al., 1994; Zunszain et al., 2011). Chronic activation of the immune system can anticipate the development of depressive disorders in human populations (Capuron et al., 2004; Dantzer, 2001; Dantzer, 2009; Dantzer & Kelley, 2007; Dantzer et al., 2008). Additionally, a variety of human medical conditions and pathologies that are associated with inflammatory phenotypes are also associated with sexual dysfunction, including neurological, endocrine, vascular, and infectious diseases (Avitsur & Yirmiya, 1999b).

Yirmiya (1996) demonstrated that rodents exposed to LPS demonstrated a depressive-like episode, which was attenuated or ameliorated by antidepressant medication, suggesting immune involvement in the aetiology of depression with others demonstrating this same effect (see Dunn et al., 2005; Stepanichev et al., 2014). What's more, neonatal stress such as maternal separation, has been shown to perpetuate sickness behaviours in adulthood when immunologically challenged (Avitsur & Sheridan, 2009). Sickness behaviours and depressive-like episodes have been likened to motivational states and behaviours, triggered by the peripheral and central innate immune response (Aubert, 1999; Barch et al., 2016; Dantzer, 2009; Dantzer & Kelley, 2007; Lang et al., 1998; Treadway et al., 2012). Animal studies have demonstrated that damns exposed to LPS display sickness behaviours such as diminished nursing and nesting, however are only motivated to actively nurse their pups and nest when

the external environment is manipulated in such a way that biological material care motivation takes over (Aubert, 1999). Lipopolysaccharide administration has been demonstrated to inhibit sexual behaviour in female but not male rats, which has been suggested as a motivation state (Avitsur et al., 1997). Additionally, treatment with cytokines and immune products result in general suppression of female rat sexual behaviour and also social withdrawal (Avitsur et al., 1999; Avitsur & Yirmiya, 1999a; Bluthé et al., 1994; Hennessy et al., 2014). This has been suggested as an evolutionary adaptation to reduce conception during illness or infection, as it may result in maternal or foetal dangers and mortality (Avitsur & Yirmiya, 1999b) but also as a state of de-motivation (Dantzer, 2009). Inhibition of sexual behaviour, as well as social withdrawal, is included in the repertoire of depressive-like symptomology in animal models, comprised of both motivational and anhedonic elements.

Neonatal immune activation has been demonstrated to adversely affect female sexual behaviours in the long term. Walker et al. (2011) demonstrated that neonatal LPS affected the mating behaviours of both males and female rats in an open field test, however the most robust effects were demonstrated in female animals. Neonatally treated females participated in significantly less receptive mating behaviours with a male stud and displayed inappropriate behavioural cues needed for successful mating practices. Additionally, Walker et al. (2011) found these behaviours to be unrelated to anxiety-like behaviours, suggesting that this was not a cofounding effect of an anxiety-like state affecting reproductive behaviours in these LPS treated female rats.

As both clinical and experimental animal evidence suggests, male and females could be responding differently to this neonatal immune stressor. Clinical evidence suggests that women currently experience major depressive disorder at a higher rate than men, perhaps LPS administration during the neonatal period is differentially affecting males and female and

what presents as an anxiety-like phenotype in males, could perhaps be a depressive-like phenotype in female animals. Depression and anxiety are often co-morbid clinical conditions and there are parallels in symptomology of both disorders. Due to the clinical similarities, sexually dysmorphic behavioural results, and the known differences in male and female immune responses, it is important to further explore the role of NIA in female development, and determine if there are immediate and long term alterations in both behaviour and physiology.

1.5.4 Impact of Neonatal LPS on Immune Function

The early life microbial environment plays a critical role in foetal and neonatal maturation, priming the system for later life. Exposure to bacterial and viral infections in early life is a common occurrence in humans, having both physiological and behavioural effects (Bay-Richter et al., 2011). Few human studies explore the effects of early life immune activation with LPS and subsequent later life alterations in immune functioning, obviously due to ethical constraints. It was reported that children who were exposed to higher levels of endotoxin from household dust samples had a significantly lower risk of developing hay fever, asthma and wheezing, compared to those that had limited exposure (Braun-Fahrlander et al., 2002). Moreover, there is consistent literature linking maternal viral exposure (i.e. maternal immune activation (MIA)) to the development of schizophrenia and other neurodevelopmental disorders in the offspring (Aftab et al., 2016; Babenko et al., 2015; Brown & Derkits, 2010; Cheslack-Postava et al., 2015; Estes & McAllister, 2016; Karanikas, 2011; O'Donnell & Meaney, 2017). Clinically, people that have been diagnosed with schizophrenia, have been reported to have altered cytokine profiles, indicative of long term alterations in immune functioning and suggestive of an inflammatory basis for the disorder

and that this may be perinatally programmed (Gilmore & Jarskog, 1997; Karanikas, 2011; Maes et al., 1995a; Maes et al., 1997b; Potvin et al., 2008; Shi et al., 2003).

In rodents, a single immune challenge during the neonatal period is able to alter both neuroendocrine and behavioural responses in adulthood (Walker et al, 2004, 2009, 2011). This pattern of activation is known to proceed from the release of proinflammatory cytokines from the stimulated immune cells, and as a consequence, activate the ANS and the HPA axis stress response (Karrow, 2006). The administration of proinflammatory cytokines (IL-1, IL-2 and TNFα) in early life produces similar effects to that of LPS injection (Dunn et al., 2005; Larson & Dunn, 2001; Swiergiel & Dunn, 2007). Schwarz and Bilbo (2011) demonstrated that LPS elicited a broad and more robust inflammatory action than a live bacteria, hence confirming the efficacy of LPS as a reliable immune activator, particularly during the neonatal period.

Animal models have explored the effects of LPS administration on the immune system, with experimental manipulations in timing, dose and exposure length. Hodyl et al. (2008) report that prenatal LPS administration in rats stimulated maternal immune responses throughout gestation day ((GD): 16, 18 and 20) and a subsequent neonatal LPS dose on PND 5 led to neonatal rats displaying a lower white blood cell profile with decreased numbers of neutrophils, monocytes and eosinophils, as well as an attenuated proinflammatory cytokine response (IL-1β, and TNFα). Boisse et al. (2004) injected PND 14 rat pups with LPS and observed that this neonatal exposure led to an attenuated febrile response in adulthood when challenged with a second LPS dose, but not when challenged with IL-1β or prostaglandin E_2 (PGE_2) in adulthood, suggesting that the alterations are stimulus specific. Additionally, Boisse et al demonstrated that neonatally LPS treated rats displayed elevated levels of basal hypothalamic COX-2 expression in adulthood, indicating that prostaglandins play a role in a

proinflammatory phenotype as a result of NIA. Ellis et al. (2006) demonstrated that a single neonatal exposure of LPS on PND 14 lead to altered innate immune responses in adulthood evidenced by a reduced febrile response and reduced plasma IL-6 and TNFα after adulthood LPS stimulation. Bilbo et al reported that a neonatal LPS challenge altered central levels of IL-1β, as well as exhibiting more rapid and prolonged cortical IL-1β responses to LPS injection in adulthood, but reported no peripheral differences (Bilbo et al., 2005a; Bilbo et al., 2005b; Schwarz & Bilbo, 2011). Hodgson et al. (2001) demonstrated that neonatal LPS exposure on PND 1, 3, 5, and 7 lead to a decrease in tumour resistance in adulthood, as well as attenuated natural killer (NK) cell activity and hyper-responsiveness of the HPA axis.

The PND 3 and 5 LPS model utilised in our laboratory coincides with critical stages of endocrine, immune, vagus nerve development and microglial maturation, as well as reproductive development (Sominsky et al., 2013c) and provides an ecological valid mode for the examination of early life immune perturbation. In addition to alterations in immune functioning and stress responsivity in adulthood, early life immunological stressors are also capable of programming the HPG axis to influence reproductive functioning (Iwasa et al., 2012; Knox et al., 2009; Watanobe & Hayakawa, 2003; Wu et al., 2011b; Yoo da & Lee, 2016). Excess inflammation has deleterious effects on reproductive function; hence, it is logical to propose that an inflammatory phenotype programmed by early life stress is capable of having an adverse effect on the development of the reproductive system.

1.5.5 Impact of LPS on Reproductive Parameters.

Early life stress has been shown to alter reproductive parameters in both males and females. Infection and experimental animal studies indicate that neonatal immune stress alters reproductive parameters including puberty onset, downregulates HPG hormones including LH and FSH, and impairs mating (Iwasa et al., 2012; Sominsky et al., 2012a; Walker

et al., 2011). Knox et al. (2009) determined that LPS administered at PND 3 and 5 was the critical window for the programming of female reproductive development as exposure at later neonatal time points (PND 7, 14 & 16) did not yield cyclicity or puberty onset differences. The impact of early life LPS on reproductive parameters is briefly outlined in the following section within this chapter, however is covered in greater depth in the co-authored review article which supplements this chapter (Sominsky et al., 2015). This paper is presented at the conclusion of this chapter.

1.5.5.1 Endocrine alterations. Alterations to endocrine regulation of reproductive function has been demonstrated in research from our laboratory using the PND 3 and 5 LPS model. Walker et al. (2011) demonstrated that neonatal LPS exposure in the rat supressed LH levels in the female during puberty and testosterone levels in the male during adolescence. In the same study, both testosterone and LH pulses were diminished during mating in LPS treated animals of both sexes, however no differences in LH levels were seen between the ages of 9-12 months in either sex. Additionally, a blunted circulating CORT response was seen in male adult rats only subjected to neonatal LPS and adult stress, when blood was taken directly following a sexual behaviour assay (Walker et al., 2011). Alternatively, Sominsky et al. (2012a) reported that female rats had significantly higher levels of corticosterone on PND 14, day of vaginal opening (DVO; signalling puberty onset), and during adolescence and adulthood, without being paired with a subsequent stressor. No differences in circulating LH levels during puberty onset and during adolescence were observed, however, LPS treated females displayed significantly reduced FSH levels during adolescence, but not at DVO (Sominsky et al., 2012a). These findings suggests that the early postnatal period is critical to reproductive functioning that is dependent on endocrine function, such as LH, FSH and stress

hormone pulsatility and that the addition of subsequent stressors in adulthood may further interfere with endocrine mediated reproductive parameters.

1.5.5.2 Morphological alterations. Gonadal morphological alterations have been reported in both males and females exposed to LPS in early life. In neonatally LPS treated males, Walker et al (2011) demonstrated reduced neonatal gonocyte populations and greater epithelial disorganisation and delayed spermatogenesis in adult males. A depleted primordial follicle reserve was previously observed in prepubescent female at PND 14 (Sominsky et al, 2012a), and has also been reported adult females (Wu et al, 2011) following PND 3 and 5 LPS exposure. Bromfield and Sheldon (2013) demonstrated that acute systemic exposure to LPS in adulthood led to a reduced primordial follicle pool, accompanied by upregulated follicle atresia in 8 week old mice. This indicates that LPS does indeed have negative effects on ovarian follicles, and also gives credence to the suggestion that neonatal LPS exposure may be detrimental to ovarian follicles at this critical time point of female reproductive development. Interestingly, these findings seemed to be mediated via the specific receptor, TLR4, as $Tlr4^{-/-}$ C57BL/6 mice used in Bromfield's study showed no primordial follicle deficits. Sominsky et al (2013) demonstrated that neonatal LPS exposure upregulated immune–related and LPS stimulated pathways via microarray analysis in the PND 7 female rat following PND 3 and 5 LPS stimulation, including upregulated TLR4 expression. Ovarian development and follicle maturation is governed by many factors in the early-life period, including inflammatory mediators, growths factors and transcription factors. However, the exact processes and interactions of mediators remains to be fully elucidated. Further exploration of how LPS NIA can modify the ovarian follicle pool, both in the short term and the long terms will aid the understanding of these rudiments of female reproductive health.

1.5.5.3 Ovarian alterations and reproductive aging. In the female rat, the finite pool of ovarian follicles continues to develop until during the first week of birth, coordinated in concert by the oocyte and its surrounding somatic cells, growth factors, transcription factors, cytokines and neutrophils (Skinner, 2005b). This includes the final formation of the primordial follicle pool and the beginnings of follicular maturation to the primary stage (see Figure 1.8).

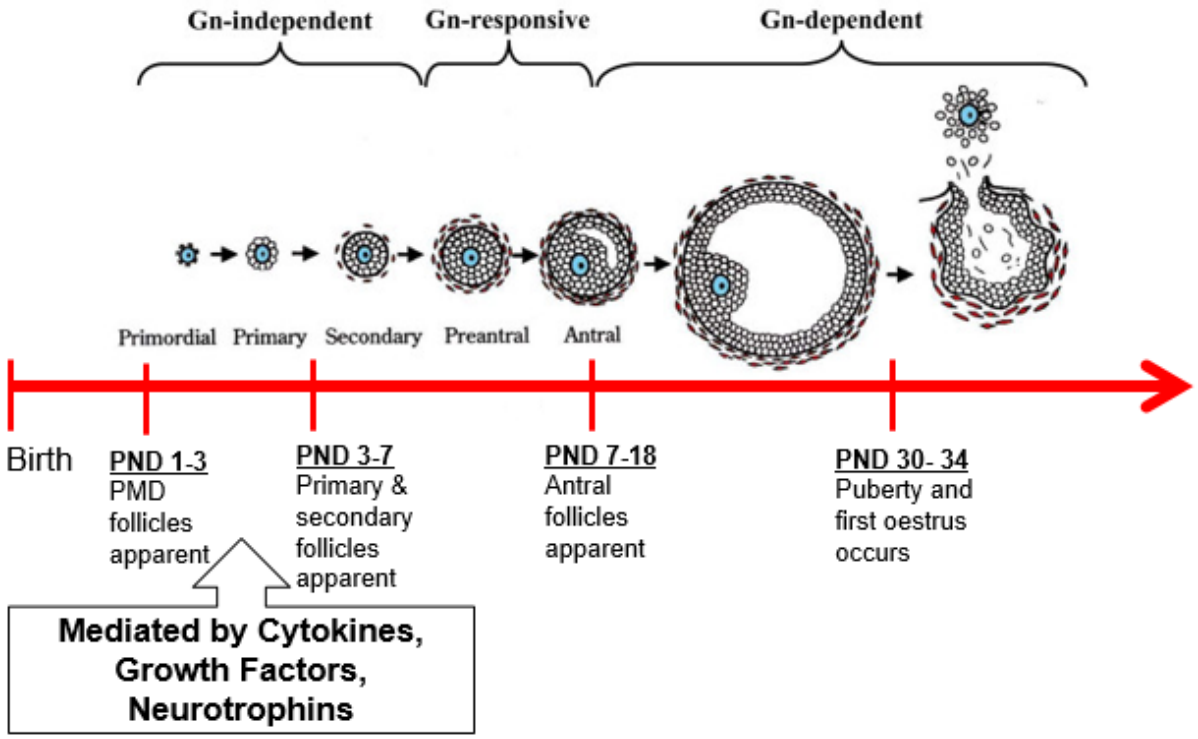

Figure 1.8. Identification and corresponding postnatal development of the ovarian follicle pool. From birth to PND 3, primordial follicles are evident. These follicles are identified as the oocyte is surrounded by one layer of flatly shaped granulosa cells. As these follicles mature, the surrounding granulosa cells become activated, changing from a flattened to a cuboidal shape. Primary and secondary follicles types are evident in the ovary from PND 3, and are identified by one layer of cuboidal granulosa cells. Pre antral and antral follicles become apparent from PND 7 onwards. These processes are driven by non-hormonal factors. Following puberty, mature follicles are selected for ovulation via sex hormone depended mechanisms (adapted from Orisaka et al., 2009).

Neonatal exposure to LPS lead to prolonged ovarian dysfunction in the female rat. Utilising a similar model to that employed in our laboratory, it has been demonstrated that neonatal exposure to LPS in the first week of life significantly delayed vaginal opening and day of first vaginal oestrus and disrupted normal cyclicity immediately post puberty and in adulthood (Knox et al., 2009; Wu et al., 2011a; Wu et al., 2011b). Conversely, in our laboratory, Sominsky et al (2012a) demonstrated that PND 3 & 5 LPS treatment lead to the early onset of puberty, as well as an earlier onset of senescence by 12 months of age, when compared to saline animals. Additionally, Sominsky et al (2012a) demonstrated a reduction in primordial follicles at PND 14. Yoo da and Lee (2016) demonstrated that the ovaries of rats exposed to LPS on PND 20-25 were smaller and weighed less than controls, and DVO was delayed. Wu and colleagues (2011) found the theca interna layer that surround antral sized follicles was significantly thicker in adult animals neonatally treated with LPS compared to control animals at differing time points throughout the female rat's oestrus cycle. This thickening was accompanied by an increase in immune-reactivity of nerve growth factor and its receptor (p75NGFR) in the ovaries of LPS treated animals, indicating an increased amount of ovarian sympathetic activity (Wu et al., 2011a; Wu et al., 2011b). Increases in sympathetic activity have been shown to lead to aberrations in follicular development, disrupt oestrus cyclicity and reduce ovulation in stress animal models (Greiner et al., 2005; Ricu et al., 2008). Interestingly, previous findings from our laboratory have shown sympathetic activation in the adrenals following LPS treatment in both male and female neonates (Sominsky, 2013) and further investigation in male and females animals would aid in the determinations of long term changes and contribution to female reproductive alterations.

Amongst the other effects of NIA, it is possible that an immune challenge may perturb ovarian growth and primordial follicle assembly during a critical period of reproductive

development during that first postnatal week. Sominsky et al. (2013b) demonstrated ovarian upregulation of LPS stimulated inflammatory pathways on PND 7 following PND 3 and 5 LPS administration. This suggests that this model of NIA coincides with acute activation of inflammation that is sustained throughout the entire first week of birth. Hence, NIA is implicated in alterations to pubertal and senescence onset, aberrant ovarian development and perhaps the premature decline of the ovarian follicular pool.

1.5.5.4 Central alterations. Central regions important to reproductive functions include the hypothalamus, hippocampus, prefrontal cortex and amygdala (Angoa-Perez & Kuhn, 2015; Rivest & Rivier, 1993; Rivest & Rivier, 1995; Rudolph et al., 2016; Wilson & Davies, 2007). Sex differences are known to exist in these regions (de Vries & Södersten, 2009; Lajud & Torner, 2015; Mahmoud et al., 2016). Additionally, differing neuronal populations influence reproductive parameters (Angoa-Perez & Kuhn, 2015; Lara et al., 1990; Lonstein & Blaustein, 2004; Melón & Maguire, 2016; Ojeda et al., 2010; Uphouse, 2014). Exposure to peripheral LPS stimulation in the perinatal period also has repercussions for central structures that are implicated in reproductive behaviours, as well as psychopathologies (Depino, 2015; Jenkins et al., 2009; Knox et al., 2009; Pang et al., 2016; Schwarz & Bilbo, 2011; Sominsky et al., 2012b; Zavitsanou et al., 2013). Connections are found between CRH neurons and GnRH neurons in the medial preoptic area (mPOA) of the hypothalamus (Clarke et al., 2015; Han et al., 2005; Krsmanovic et al., 2009; Melón & Maguire, 2016; Tanriverdi et al., 2003b; Whirledge & Cidlowski, 2010), with certain GnRH receptor–rich regions of the mPOA receiving extensive projections from the ventral lateral septum, the medial nucleus of the amygdala and the posteromedial aspect of the bed nucleus of the stria terminalis, hence implicating the mPOA as a potential central structure associated with changes in reproductive function (Pompolo et al., 2005). In ewes, Fergani et al. (2013) demonstrated that LPS administrated increased

immediate early gene cFos expression in the hypothalamus within the mPOA, along with CRHR type 2 immunoreactivity compared to controls. Furthermore, the percentage of kisspeptin cells co-expressing c-Fos was lower in mPOA. Early life bacterial exposure may perturb the development of both peripheral and central structures that are critical to reproductive functions, and hence negatively impact reproductive parameters. Specifically, these alterations may be mediated by mechanisms involved in the inflammatory response to an immune stressor during a critical period of development.

1.6. Mechanisms of the LPS Inflammatory Response: Involvement in female Reproduction

Inflammation involves alterations to vascular and immune cell functionality in order to restore homeostasis. It is known that a number of female reproductive processes display characteristic inflammatory characteristics such as ovulation, menstruation, implantation and parturition (Jabbour et al., 2009; Richards et al., 2002; Sheldon et al., 2014; Sheldon et al., 2016; Turner et al., 2012). Inflammatory mediators contribute to physiological events with the ovary, and immune cells are present throughout the reproductive tract in order to protect the gonads from infection, hence, dysregulation to these mediators at any level may negatively impact female reproductive parameters. An integrated approach is needed to understand how stress and the environment modulates interactions between the immune system, inflammation and female reproductive parameters. Animal models of immune activation, particularly during sensitive periods of development, will help aid this understanding.

Lipopolysaccharide produces acute inflammation in low, controlled doses, and its specific receptor is present throughout the reproductive tract and ovary. As it is a gram negative bacterial mimetic, it produces an almost parallel response as many infections that are commonplace not only during the perinatal periods, but also throughout the lifespan. In

particular, it mimics infections of the uterus, pelvis and reproductive tract, all of which can have negative ramifications on ovarian health and follicle reserve status. Additionally, LPS exposure and neonatal immune activation is associated with subsequent low grade chronic inflammation. The mediators of the proinflammatory response are prime candidates for consideration when examining the mechanisms responsible for perinatal programming, particularly considering the relevance of these mediators to lifelong reproductive success, and mental and physical health (Banks, 2005; Bilbo & Schwarz, 2012; Harrison, 2013; Jabbour et al., 2009; Richardson et al., 2014). The following sections detail the involvement of some key inflammatory mediators in reproductive functioning and the endotoxin response, particularly focusing on the roles of these mediators in ovarian processes and development throughout the life span.

1.6.1 Cytokines

Lipopolysaccharide stimulates cytokine synthesis (Karrow, 2006). As previously outlined, cytokines exert their effect both peripherally and centrally, where they interact and influence the expression of hormones from the endocrine system. Cytokines are small proteins and glycoproteins with a mass of less than 30kDa, and perform as intercellular messengers between target cells, rarely acting alone (Kuby, 1997). Rather, a mixture of cytokines and other factors work in concert and, when combined, have differential synergistic or antagonist affects depending on the combination (Dinarello, 2000; Kelso, 1998). Defects in the complex, inflammatory regulatory networks that govern cytokine production and the expression of their receptors, lead to both cytokine unbalance. This over expression and/or under expression, has been implicated in a number of diseases and disorders.

It is well established that ovarian tissue contains cells capable of producing and being receptive to cytokines (Nash et al., 1999). Cytokines play a critical role within the normally

functioning ovary, contributing to processes during initial development and well as throughout the lifespan (Bornstein et al., 2004; Brannstrom, 2004; Eddie et al., 2012; Hill, 2000; McLaughlin & McIver, 2009; Norman & Brännström, 1996; Skinner, 2005b). What remains to be determined is the pathway through which peripheral, in vivo inflammation caused by early bacterial exposure during critical periods of development is able to change the overall tone of the female reproductive system and, as such, lead to ovarian dysfunction, subfertility and related disorders later in life. Taking into account their involvement in immune activation, as well as evidence suggesting that proinflammatory cytokines are involved female reproductive disorders and psychopathology, their study within this framework is important.

1.6.1.1 Interleukin 1 (IL-1). Cytokines are vitally important for the development and continued function of the ovary (Herath et al., 2007; Jabbour et al., 2009; Nash et al., 1999). Interluekin-1 α and β are potent inflammatory cytokines secreted by monocytes, macrophages, B cells, dendritic cells, endothelial cells, and other cells types where it has a wide range of biological functions including aiding and enhancing immune stimulation. Interleukin 1 also actively participates in the maturation and proliferation of cells, as well as clonal expansion. In the ovary, IL-1 has been implicated in the facilitation of gonadotropin actions and luteinisation (Evron et al., 2015). The intra-ovarian presence and origin of IL-1 remains to be fully understood, however, current evidence indicates that it may be produced via ovarian macrophages, which are present in ovarian thecal, stromal and luteal regions (Wu et al, 2014). Additionally, IL-1 and its receptor expression has been immunohistochemically located in a murine model in the theca-interstitial layers of growing follicles, as well as receptor staining present in the cytoplasm and the plasma membrane. During ovulation, IL-1 receptor staining was present in cumulus cells and granulosa cells prior to follicle rupture and

subsequent ovulation (Simon et al., 1994). Uri-Belapolsky et al. (2014) localised IL-1 α and β to the oocytes and granulosa cells of developing follicles. These findings indicate an autocrine-paracrine role of IL-1 within the ovary, especially during maturation, ovulation and luteinisation (Terranova & Rice, 1997). In an IL-1 knockout mouse model, IL-$1^{-/-}$ female mice demonstrated high pregnancy rates and bigger litter sizes compared to wild type females, as well as a greater ovarian follicle reserve in adulthood compared to wild type and lower apoptosis signalling (Uri-Belapolsky et al., 2014). This evidence indicates that IL-1 may be involved in the age-related depletion of the ovarian reserve in a murine model. Indeed, inflammatory mediators and excess and chronic inflammation have been suggested to be involved in the untimely elimination of the follicle reserve via apoptotic processes (Herath et al., 2007; Jabbour et al., 2009), particularly as IL-1 is known to have deleterious effects in the male testes (Ganaiem et al., 2009).

1.6.1.2 Interleukin 2 (IL-2). Interleukin-2 is synthesised by activated T-cells, with usual peak activity approximately 4 hours following immune stimulation (such as infection) (Flaherty, 2012). IL-2 produces the cytokines TNFα and IFN-γ, and has been described as a neurotoxic cytokine which can be induced by LPS stimulation (Girard et al., 2008). IL-2 has been identified as being produced by granulosa cells in the ovary, and increased expression has been linked to advanced stage ovarian cancer (Barton et al., 1994). Additionally, lower levels of IL-2 in ovarian follicular fluid have been associated with reproductive failure and ineffective in vitro fertilization (Ostanin et al., 2007). Furthermore, altered IL-2 expression is implicated in major depressive disorder, schizophrenia, and anxiety disorders (Liu et al., 2012; Maes et al., 1995a; Maes et al., 1991; Maes et al., 1995b; Song et al., 2007).

1.6.1.3 Interleukin-6 (IL-6). Interleukin-6 is a 21kDa pleiotropic cytokine that is produced by various lymphoid and non-lymphoid cells (Naka et al., 2002) including but not

limited to monocytes, macrophages, T and B cells and endothelial cells. Interleukin-6 is an important factor involved in the acute inflammatory response, especially to endotoxin (Kopf et al., 1994), however it has a broad effect on many cells both within and external to the immune system (Hunter & Jones, 2015). This includes the differentiation and proliferation of cells (Cai et al., 2013), antibody secretion, prostaglandin synthesis, as well as anti-inflammatory properties (Hunter & Jones). Additionally, IL-6 has been involved in chronic inflammatory states of many disorders and diseases (Ishihara & Hirano, 2002; Kanda & Takahashi, 2004; Schafer & Brugge, 2007)

The ovary exhibits cells, including resident immune cells, which secrete IL-6. Within the ovary, IL-6 and its receptor is secreted and expressed in the epithelium and by follicular granulosa cells, where it participates in follicle development by reducing FSH binding capacity of granulosa cells and reducing LH mediated ovulation rates (Bornstein et al., 2004; Kumari et al., 2016). It has also been implicated in follicular development, atresia, ovulation, steroidogenesis, and co regulation of ovarian sex steroid production (Bornstein et al., 2004; Terranova & Rice, 1997), as well as a having a similar role in the testes (Huleihel & Lunenfeld, 2004). Altered circulating levels, follicular levels, and gene polymorphisms of IL-6 been detected in women with PCOS (Fulghesu et al., 2011; Peng et al., 2016; Vgontzas et al., 2006), as well as endometriosis (Imaizumi et al., 1993; Li et al., 2014; Martinez et al., 2007) and ovarian cancers (Isobe et al., 2015).

1.6.1.4 Tumour Necrosis Factor alpha (TNFα). Tumour necrosis factor-α is a key, yet complex, regulatory cytokine that is implicated in a diverse range of proinflammatory states and diseases (Bradley, 2008; Camara et al., 2015; Camara et al., 2013; Himmerich et al., 2013). Produced by macrophages, monocytes, mast cells and various other immune cells, TNFα in its 26 kDa protein is expressed on the plasma membrane, where it can be cleaved to form a

17KDa soluble form (Kuby, 1997). Both membrane-associated and soluble forms of TNFα are bioactive and are not usually detected in healthy states Elevated serum and tissue levels are demonstrated in inflammatory disease states and infectious conditions (Bradley, 2008). TNFα signalling is activated by LPS stimulation, triggering a signalling cascade and activating map-kinase pathways that converge on NF-κB transcription factor activation (Andreakos et al., 2004; Bouwmeester et al., 2004).

Cells that secrete TNFα and cells expressing TNF receptors are constitutively expressed in the normally functioning ovary and throughout the female reproductive tract (Marcinkiewicz et al., 2002b; Wu et al., 2004). In murine ovaries, TNFα mRNA and protein has been identified in oocytes of all developmental stages, particularly in the ooplasm (oocyte cytoplasm) in adult animals and neonatal animals, but not foetal animals (Marcinkiewicz et al., 1994). It has also been demonstrated to be present in the human ovary during follicular growth and in the oocyte of primordial follicles, where it participates in follicle maturation, regression, atresia and apoptosis both in the absence of inflammation, but also during the inflammatory processes of ovulation and in cancerous ovarian cells (Kondo et al., 1995; Terranova, 1997).

In the ovary, TNFα is known to be involved in the development and regulation of the ovarian follicle reserve, in which the final stages occur in the first post-natal week in the rat (Marcinkiewicz et al., 2002a; Morrison & Marcinkiewicz, 2002; Skinner, 2005b). Importantly, this timing coincides with the administration of our model of neonatal immune activation on PND 3 and 5. TNFα affects steroid and progesterone production by inhibiting gonadotropin-induced steroidogenesis, and as such has a critical functional role in the stimulation of ovulation and follicular growth (Terranova, 1997; Williams et al., 2008). In vivo TNFα and LPS administration also decreased ovulation rates in bovines (Williams et al., 2008).

It also promotes granulosa cell proliferation and induces granulosa and oocyte cell death (Greenfeld et al., 2007; Kaipia et al., 1996; Son et al., 2004). In human studies, women with PCOS expressed higher intrafollicular expression of cytokines and chemokines, particularly in obese patients (Adams et al., 2016). Additionally, women with PCOS who were of a normal weight display elevated serum levels of TNFα (Gonzalez et al., 1999). TNFα has also been associated with endometriosis (Azuma et al., 2017; Halis & Arici, 2004; Harada et al., 1999; Khan et al., 2013), the progression of ovarian cancers (Kulbe et al., 2005; Muthukumaran et al., 2006; Szlosarek et al., 2006), premature ovarian failure (POF, sometimes referred to as primary ovarian insufficiency, POI) (Erlebacher et al., 2004; Kim et al., 2012; Naz et al., 1995) and poor assisted conception outcomes (Field et al., 2014). Further research concerning TNFα and its receptors is needed in order to fully elucidate the involvement of this cytokine in ovarian process, inflammatory disorders and behaviours.

Cytokines and their bionetworks are key regulators of physiology, particularly within the female reproductive system. These acute phase mediators are concomitantly activated in the protective inflammatory response, but are also implicated in chronic proinflammatory states, and are of consequence for the state of health and disease, including psychopathologies (Dowlati et al., 2010; Maes, 1995; Maes et al., 1997a; Maes et al., 1999; Voorhees et al., 2013). What is more, these proinflammatory cytokines, known to be activated by LPS, have important roles not only immune function, but also in the modulation of stress as well as ovarian function throughout the lifespan (Field et al., 2014). As such, further examination of the role of cytokines is paramount to facilitate understanding the long term effects on neonatal immune activation of female reproductive parameters and stress responsivity.

1.6.2 Toll-Like Receptors (TLRs)

Toll-like receptors (TLRs) are specialised transmembrane-signalling pattern-recognition receptor (PRR) proteins that facilitate the activation of APCs in the innate immune system. They highly evolutionarily conserved and play a crucial role in pathogen defence, belonging to a large receptor superfamily that includes IL-1 receptors (Akira & Takeda, 2004). Hence, TLRs are fundamental to the induction of innate immune response, the initiation of inflammation and the establishment of adaptive immunity (Barton & Kagan, 2009). Toll-like receptors are expressed on the surface of antigen presenting cells, such as dendrites and macrophages, as well as constitutively expressed on most tissue types (Barton & Kagan, 2009; Zarember & Godowski, 2002). What is more, they are present throughout the female reproductive tract and in ovarian cells (Bromfield & Sheldon, 2011; Kumar et al., 2009) where they are crucial to reproductive functioning (Chow et al., 1999). TLRs are specific to certain pathogen-associated molecular patterns (PAMP), with some playing dual roles (see table 1.1). Lipopolysaccharide produces a proinflammatory immune response through dependent or independent myeloid differentiation primary response protein 88 (MyD88) pathways (Akira & Takeda, 2004) binding to TLR4, aided by the coordination of specific surface protein cofactors such as LPS-binding protein (LBP), cluster differentiation antigen 14 (CD14) and myeloid differentiation (MD) protein 2. This leads to the activation of MAPK and NF-κB pathways and cytokine synthesis (Arbour et al., 2000; Peri & Piazza, 2012). TLRs play a role in cell death signalling and have been implicated in mediating the innate and adaptive immune response to non-immunological stressors, including psychological stress (Xiang et al., 2015; Zhang et al., 2008a; Zhang et al., 2008b). Research aimed at facilitating our understanding of specific TLR activation, expression and regulation is of paramount importance in elucidating the repercussions of early life immune stress.

Table 1.1. TLR expression and specificity. Adapted from Liu et al. (2010).

TLR	Cell expression	Specificity
TLR 1/2	Plasma membrane	Triacyl lipopeptides (bacteria and mycobacteria)
TLR2	Plasma membrane	Peptidoglycan (Gram-positive bacteria)
TLR3	Endosome	ssRNA virus , dsRNAvirus (reovirus), including Poly I:C
TLR4	Plasma membrane	LPS (Gram-negative bacteria)
TLR5	Plasma membrane	Flagellin (flagellated bacteria)
TLR6		Diacyl lipopeptides (mycoplasma), LTA (Streptococcus), zymosan saccharomyces)
TLR 7/8	Endosome	ssRNA viruses (VSV, influenza virus)
TLR9	Endosome	dsDNA viruses (HSV, MCMV), CpG motifs from bacteria and viruses, haemozoin (plasmodium)

***1.6.2.1 Toll-like Receptor 4 and Female Reproductive Function*.** It is well established that the footmarks of innate immunity are essential to female reproductive processes. Ovulation, menstruation, implantation, and also the onset of parturition display proinflammatory characteristics that are coordinated by immune pathways and endocrine pathways. In addition, bacterial infections are able to perturb mammalian follicular growth and alter oestradiol production, leading to aberrations in reproductive function including ovulation, conception and pregnancy (Bromfield & Sheldon, 2011; Herath et al., 2007). Although the role of TLRs in infection control has been widely research, the emerging role that TLRs, specifically TLR4, plays in female reproduction is emerging (Girling & Hedger, 2007; Liu et al., 2008; Zhou et al., 2009).

Granulosa cells, which are expressed within the basement membrane of the ovarian follicle, have been demonstrated to express TLR4, along with CD14 and MD-2, all of which are

essential cofactors for LPS responsiveness (Bromfield & Sheldon, 2011; Liu et al., 2008; Sheldon et al., 2014). During folliculogenesis where ovarian follicles mature, TLR4 is also expressed on the theca layer during the secondary phase, with differing mammalian species displaying differential TLR expression patterns (Kannaki et al., 2011). Toll-like receptor 4 is expressed in cells of the human ovary, including the epithelium, granulosa cells and also cumulus cells (Bromfield & Sheldon, 2011). These pathogen receptors have been implicated in protecting the female reproductive system, but also the non-defence roles of tissue remodelling and the modulation of ovarian follicle functioning in the absence of pathogenic stimulation (Kannaki et al., 2011).

Zhou et al. (2009) observed increases in TLR4 gene expression and function in the cumulus cells of patients with ovarian cancer. Additionally, Zhou et al. (2009) and Woods et al. (2011) demonstrated that TLR4 is expressed on cancerous human granulosa cells, implicating TLR4 expression and its signalling pathways in the pathogenesis of ovarian cancers. Moreover, recent experimental studies have highlighted the critical role of maternal-foetal TLR4 signalling pathways in infection-induced premature parturition. Li et al. (2010) demonstrated that a TLR4-neutralising monoclonal antibody significantly decreased LPS-induced preterm delivery and foetal death in a murine model. Using the gravid rhesus monkey, Adams Waldorf et al. (2008) demonstrated that an intra-amniotic infusion of LPS upregulated levels of TNFα, IL-8, PGE_2, $PGF_{2\alpha}$ and leukocytes, signifying TLR4 binding and activation of inflammatory pathways. In contrast, gravid females that were pre-treated with a TLR4 antagonist displayed inhibited cytokine and prostaglandin expression in response to the endotoxin. Research from our laboratory has demonstrated that female rats exposed to LPS on PND 3 and 5 exhibit TLR4 alterations (Sominsky et al., 2013a). Specifically, upregulated TLR4 mRNA expression on PND 7 was seen in the ovaries of LPS treated animals as well as the

upregulation of several inflammatory pathways concerned with the LPS inflammatory response, including NF-kB and LPS-stimulated MAPK pathway.

It is apparent that the LPS/TLR4 pathway may provide insight into the ways inflammation via bacterial exposure is controlled, particularly in relation to female reproduction. Aberrations in the regulation of the LPS/TLR4 pathways in both male and females have the potential to lead to an inflammatory phenotype, increasing risk for chronic inflammatory disorders and predisposing to disease. Importantly, the role of early life bacterial exposure may play a role in programming the expression of TLR4 in peripheral tissue, specifically in the reproductive organs. Together, this evidence highlights the importance of the TLR4 as a mechanism linking early life bacteria exposure to subfertility, specifically through direct LPS/TLR4 communication by immune factors in the ovary, which may lead to imbalances in later life ovarian function and oocyte quality (Bromfield and Sheldon, 2011).

1.6.3 Prostaglandins and Cyclooxygenase (COX) Enzyme Pathways

1.6.3.1 Prostaglandins. Prostaglandins play a key role in the generation and regulation of the inflammatory response, as well as modulating important physiological systems such as the CNS, endocrine and immune systems via autocrine and juxtacrine signalling. As such, PGs have been implicated in diseases such as cancer, cardiovascular disease, hypertension and chronic inflammation (Hata & Breyer, 2004). Prostaglandins are formed when arachidonic acid (AA) is released from the plasma membrane by phospholipases and metabolised by a sequence of actions via COX enzyme pathways. The principal bioactive PGs generated in vivo include; PGE_2, prostacyclin (PGI_2), PGD_2, $PGF_{2\alpha}$ and thromboxanes (TX) (Tilley et al., 2001). Each of these PGs and their receptors are ubiquitously produced in tissues and aid in the maintenance of homeostasis and the resolution of acute inflammation (Kalinski, 2012; Ricciotti & FitzGerald, 2011). The overall impact of PGs in the individual inflammatory

response depends on several factors, including the level of immune cell activation, the presence of other mediators, and the physiological state of the organism. Additionally, PGE_2 had the paradoxical status of a proinflammatory factor; however it also has immunosuppressant effects and the ability to induce anti-inflammatory IL-10 (Kalinski, 2012; Ricciotti & FitzGerald, 2011; Tilley et al., 2001).

Prostaglandins are implicated in both ovarian and uterine function (Sales & Jabbour, 2003a; Sales & Jabbour, 2003b; Sugimoto et al., 2015) In the ovary, prostaglandin synthesis, induced by the COX-2 pathway and mediated by the LH surge, is a critical step of the inflammatory events of ovulation. The process of ovulation consists of a series of biochemical and biophysical events, leading to the rupture of the pre-ovulatory follicle and the release of the female germs cell. Espey (1980) was the first to recognise that the biochemical processes of ovulation are not unlike a controlled inflammatory event. Hence, the exploration of the relationship between prostaglandins, their pathways, and their role in both inflammation and reproductive processes proves logical.

1.6.3.2 Cyclooxygenase (COX) Enzyme Pathways. The COX pathways play an important role in the regulation of inflammation by producing PGs and therefore are a target for a number of widely used nonsteroidal anti-inflammatory pharmaceuticals (NSAIs). Furthermore, COX pathways have been implicated in a number of human pathologies including cancers, chronic inflammation, and Alzheimer's and Parkinson disease (Garavito & Mulichak, 2003). Whilst COX-1 is constitutively expressed in most tissues and is responsible for mainly homeostatic regulation, the COX-2 enzyme is expressed in induced-stimulated cells and upregulated by proinflammatory stimuli such as cytokines, growth factors and PGE_2 in cells such as endothelial cells (Morita, 2002)

COX-2 is responsive to LPS and proinflammatory cytokines such as IL-1α, IL-1β, IL-2, and TNFα (Eliopoulos et al., 2002; Mercau et al., 2014; Morita, 2002). In rodents neonatally treated with LPS, the COX-2 enzyme is expressed more readily in the liver and plasma of adult rats, compared to saline-treated controls where the enzyme must be induced, mediated by TLR4 (Mouihate et al., 2010). COX-2 was also found to be more constitutively expressed in the CNS of postnatal LPS-treated animals (Boisse et al., 2004; Boisse et al., 2005). This increased ability of COX-2 enzymes and speed of PGE_2 production leads to a HPA axis response that is also amplified following stress or infection (Ma et al., 2013; Spencer et al., 2011; Spencer & Meyer, 2017). Both anti-inflammatory cytokines (IL-4, IL-10, IL-13) and glucocorticoids have been demonstrated to down regulate the induction of COX-2 (Barnes, 1998) and the use of selective COX-2 inhibitors has been demonstrated to have a positive, normalising effect on glucocorticoid receptor functioning by inducing GR nuclear localisation and enhancing GR-mediated gene transcription (Hu et al., 2005). COX enzymes are also distributed in the CNS, with COX-1 distributed throughout the brain and are most prevalent in the forebrain where PGs are needed for the facilitation and modulation of autonomic and sensory processes (Niiro et al., 1997). Central COX-2 expression is limited to the hippocampus, the cortex, hypothalamus and the spinal cord, with human brain tissue containing equal amounts of mRNA for COX-1 and COX-2 (Simmons et al., 2004). The importance of the COX-2 pathway in reproduction is evidenced by studies showing that mice null for COX-2 have impaired fertility due to a failure to ovulate (Lim et al., 1997). Minimal research has included investigation into the impact of early life bacterial exposure on the programming of COX pathways and the role these enzymes play in the expression of an inflammatory phenotype, female reproduction and a later life vulnerability to subfertility (Adelizzi, 1999).

1.7 Conclusion: Rationale Summary and Aim of Thesis

The evidenced presented here exemplifies the critical role the early life environment plays in the development of health and disease. In particular, early life immune stress alters the developmental trajectories of critical systems involved in the pathogenesis of psychological disorders including anxiety and depression, as well as female reproductive disorders. The high comorbidity between these disorders in human populations, as well as the shared peripheral and cortical structures, suggests that similar mechanisms may be involved their pathogenesis. Additionally, female reproductive disorders are becoming increasingly prevalent in a younger females, with no obvious known cause. Mounting evidence suggests that the early life environment may play a role in the aetiology of reproductive disorders and general female subfertility (Borghese et al., 2015; Dumesic et al., 2007; Hernández-Angeles & Castelo-Branco, 2016; Maheshwari et al., 2008; Norman & Moran, 2015).

Numerous clinical and experimental studies have demonstrated female reproductive disorders are associated with a proinflammatory phenotype and endocrine dysfunction, both systems which are known to be highly susceptible to the effects of perinatal programming. Considering the importance of normal immune functioning in the development, maturation and continuation of normal female reproductive health and wellbeing, little research has focused on the acute and long term impact of early life immune stress on female reproductive health. This solidifies the importance of aiming to identify the underlying causes and mediators involved, in order to facilitate translational research. This thesis aims to further examine the female subfertility phenotype that is emerging using a model of NIA, in the hope of further understanding the role of the early life environment in female reproductive development and behaviour.

Using an animal model of PND 3 and 5 LPS exposure, we aimed firstly to characterise the female subfertility behavioural phenotype previously touched upon in our laboratory using this model. By utilising a more refined mating paradigm and additional assays, female mating behaviours, anxiety-like behaviours and motivational aspects were examined in differing contexts in order to confirm and extend on previous results. This included a particular focus on motivational aspects in order to delineate between a depressive-like or anxiety-like phenotype in the female rat exposed to neonatal LPS. Secondly, we aimed to examine the acute effects of NIA on the final stages of ovarian development in order to ascertain the immediate effects of neonatal immune activation. This included a particular focus on peripheral inflammatory mediators as they are known to play a critical role in ovarian development during the timing of our LPS challenge. Both circulating inflammation and local ovarian inflammation was examined by proteomic and genetic assessment, as well as the morphological assessment of neonatal ovaries. Thirdly, we investigated the long term effect of NIA on inflammatory and endocrine mediators in the periphery, with a focus on the ovary, in order to determine if the acute changes demonstrated were sustained. This also included examination of the impact of an additional 'second hit' of stress in adulthood in order to determine if an immune-vulnerability existed within this female phenotype; particularly considering the known negative effects psychological stressors exert on female reproductive parameters and the propensity of NIA to create immune vulnerabilities to stressors in male rodents. Lastly, we examined the long term consequences of NIA in fundamental central regions associated with stress responsivity and female reproductive function, focusing on the genetic expression of known mediators of stress, inflammation and reproduction, and their relation to the establishment of the female subfertility phenotype.

By examining the impact of early life immune stress on female reproductive parameters, we are able to facilitate the understanding of the mechanisms and pathways involved in the naturalistic aetiology of female reproductive disorders, subfertility and associated psychopathologies. PND 3 and 5 LPS exposure in the male rat has previously provided robust links to the development of an anxiety-like phenotype, however the examination of the female phenotype presenting within this model remains to be well-characterised. The studies presented in this thesis elucidate on the reciprocal regulation of immune and endocrine functioning during early life, and the consequences of immune activation by bacterial exposure on female reproductive health and longevity. The early neonatal period in the female rat coincides with the final stages of immune mediated ovarian growth and maturation, and central and peripheral immune and endocrine maturation. This parallels third trimester development in humans, where a similar final maturation state is occurring for these systems. As such, this model of early life immune activation allows for the examination of central and peripheral alterations to female reproductive parameters that could be occurring in humans and determining overall reproductive health and longevity. Therefore, it is hypothesised that LPS treatment in the early postnatal period in the female rat alters immune and endocrine mediated development both acutely and in the long term. Specifically, it is hypothesised that LPS exposure alters female mating behaviours due to immune driven changes in ovarian development, peripheral and central immune system development and endocrine development as a consequence of perinatal LPS. Additionally, it is hypothesised an immunological insult in early life programs the long term functioning of the immune system and leads to altered central and peripheral immune mediators both chronically and in the presence of an additional stressor

1.8 Overview of papers

The current thesis contains three published papers, presented both as chapters and as chapter sections.

Paper 1. Forming section 1.5.5 of the introduction

Sominsky, L.., **Fuller, E.A.,** Hodgson, D.M. (2015). Factors in early-life programming of reproductive fitness. *Neuroendocrinology*, 102 (3): 216-225. DOI: 10.1159/000431378

This first publication provides a review of the evidence that perinatal exposure to an immunological challenge by LPS influences the HPG-axis and results in long term alterations in reproductive function. As such, conclusions are drawn suggesting that a disposition to infertility and subfertility may have developmental origins.

Paper 2. Presented as Chapter 4

Fuller, E.A., Sominsky, L., Sutherland, J.M., Redgrove, K.A., Harms, L., McLaughlin, E.A., Hodgson, D.M. (2017). Neonatal immune activation depletes the ovarian follicle reserve and alters ovarian acute inflammatory mediators in neonatal rats. *Biology of Reproduction.* Accepted 7th October, 2017. DOI--: 10.1093/biolre/iox123

This second publication forms a chapter examining the direct, acute impact of early life immune stress on the final stages of ovarian development of the neonatal rat. This paper demonstrates that PND 3 and 5 LPS leads to an immediate activation and depletion of the ovarian follicle pool, which has implication for female reproductive longevity. Furthermore, it is demonstrated the NIA upregulates both circulating inflammatory mediators, as well as increases inflammation within the ovary itself, suggesting perturbation to the final stages of immune mediated ovarian development and suggesting that this may lead to long term inflammatory changes and an overall proinflammatory subfertile phenotype.

Paper 3. Presented as Chapter 7

Ong, L.K., **Fuller, E.A.**, Sominsky, L., Hodgson, D.M., Dunkley, P.R., Dickson, P.W. (2017). Early life peripheral lipopolysaccharide challenge reprograms catecholaminergic neurons. *Scientific Reports* (7), DOI: 10.1038/srep40475.

This publication is presented at the end of chapter 7. This paper examines the long term catecholaminergic alterations in the male rodent brain in regions associated with sympathoadrenomedullary activation by NIA on PND 3 and 5. This publication provides novel evidence for sustained alterations to central regions implicated in catecholaminergic synthesis and sympathetic stress mediation, as well as the modulation of both endocrine and immune responses. Additionally, the evidence presented here provided novel indication that long-term central activation of astrocyte and microglia populations that are also able to modulate stress responses, and behavioural output. Importantly, this publication provides an auspicious platform for the examination of these mediators in in the female rodent. This is of specific pertinence due to the known contribution catecholaminergic-immune modulation of female reproduction during normal development. Perturbation to these systems during early development may be an underlying mechanism contributing to the current female subfertile phenotype that has emerged during the examination of impact of early life immune stress in the female rat.

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Paper 1

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Factors in Early-Life Programming of Reproductive Fitness

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Abstract
Fertility rates have been declining worldwide, with a growing number of young women suffering from infertility. Infectious and inflammatory diseases are important causes of infertility, and recent evidence points to the critical role of the early-life microbial environment in developmental programming of adult reproductive fitness. Our laboratory and others have demonstrated that acute exposure to an immunological challenge early in life has a profound and prolonged impact on male and female reproductive development. This review presents evidence that perinatal exposure to immunological challenge by a bacterial endotoxin, lipopolysaccharide, acts at all levels of the hypothalamic-pituitary-gonadal axis, resulting in long-lasting changes in reproductive function, suggesting that disposition to infertility may begin early in life.

Introduction

Over the past few decades, there has been a dramatic decline in fertility rates worldwide [1, 2]. While a trend for delayed childbearing has a substantial impact on fecundity, the increasing number of young couples who suffer from impaired fertility [3] suggests other factors are involved. The development of the reproductive system begins early in life, and similar to other physiological systems, adverse experiences during the sensitive periods of development may produce long-lasting alterations to its functioning, and thus impede reproductive success [4]. This is particularly relevant to the female reproductive system, whereby the initial quantity and quality of the ovarian follicular pool are crucial to continued normal fertility [5]. A wide variety of environmental factors experienced during early development have been demonstrated to induce a longstanding impact on fertility; these include perinatal exposure to smoking [6, 7], endocrine-disrupting chemicals [8, 9], including excess prenatal testosterone exposure [10], and poor nutrition [11]. Recent advances in our understanding of the impact of early-life events on later-life predisposition to pathology have begun to elucidate a critical role for immunological factors. Perinatal immune activation has been shown to affect reproductive physiology long term [12]. Of note is the finding that aberrant activation of the immune response during early development, triggered by a bacterial endotoxin, has been shown to alter the trajectory of sexual maturation and affect reproductive outcomes in adulthood [13–18].

This review will discuss evidence obtained from animal models for the developmental programming of reproductive maturation and function with particular attention to the significance of the early-life microbial environment.

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Early-Life Programming of Health and Disease

Changes in the early-life environment can have long-term physiological and behavioural impacts through the process of *perinatal programming*, preparing the foetus for specific extra-uterine demands [19]. The increased sensitivity of the developing organism to environmental inputs during the perinatal period is due to enhanced plasticity during this time, allowing phenotypic modification in response to environmental influences. The ability of an environment to influence long-term health outcomes is at its highest during the perinatal period, with each physiological system demonstrating a critical sensitive period, after which the level of plasticity is reduced/altered.

Enhanced plasticity is thought to promote greater functional adaptation, thus facilitating survival under challenging environmental conditions. Early-life adversity has however been associated with both positive and negative long-term health outcomes. These outcomes often depend, to a large extent, on the degree of match between the early- and later-life environmental conditions, rather than entirely on the nature of the insult itself [20]. For instance, in response to inadequate nutrient supply, a foetus is able to restrict its growth in utero, allowing a unique metabolic adaptation to the adverse prenatal environment and enhancing its chances of survival in a scarce postnatal environment, where the access to food is limited [21]. However, the same adjustments may become maladaptive when the foetus is born into conditions of abundant food supply. For example, intrauterine growth restriction in developed countries has been linked to a range of long-term health consequences, including increased susceptibility to develop cardiovascular disease, systolic hypertension, obesity, insulin resistance and diabetes type 2 [22, 23]. The early-life environment is thus an important determinant of later-life health status. It is important to view this concept of *perinatal programming* as an adaptive mechanism, with the ultimate goal to promote organismal survival in an anticipated environment [24]. However, a lack of correspondence between early- and later-life environmental conditions may lead to maladaptive programming and unwanted consequences, by interfering with the formation of essential physiological function and leading to poor health outcomes [25].

Many early-life adversities elicited by changes in the nutritional environment, or due to exposure to stressful and traumatic events, have recently received increasing attention. One particular focus has been the impact of the early-life environment on the immune milieu during defined developmental periods. Immune responses can be divided into innate and adaptive immunity. *Innate immune* responses are the first line of defence, characterised by rapid non-specific responses to invading pathogens. Innate immune responses are initiated by macrophages, dendritic cells, monocytes, neutrophils and other phagocytes. Pathogen-associated molecular patterns trigger specific pattern recognition receptors on the innate immune cells, alerting the host to the presence of infectious agents by inducing the expression of inflammatory cytokines and chemokines [26, 27]. *Adaptive immune responses* on the other hand are driven by antigen-specific defence mechanisms, which may take days to develop, and are characterised by immunological memory, such that a subsequent exposure to the same antigen results in an accelerated response. Adaptive responses are mediated by lymphocytes, T cells (thymus-derived) or B cells (bone marrow-derived). The innate immune recognition plays a major role in the activation and direction of adaptive immune responses, through presentation of antigens by antigen-presenting cells, such as dendritic cells [27]. Exogenous antigens are typically displayed by the major histocompatibility complex class II molecules on the surface of antigen-presenting cells, promoting antigen-specific T-cell proliferation [28]. The immune system of the neonate is functionally immature, resulting in an increased susceptibility to infections during this period of life [29, 30]. This increased sensitivity to infections is caused primarily by very limited exposure to antigens in utero and hence the lack of immunological memory, as well as quantitative differences in the number of immune cells as compared to an adult [31]. For instance, neonatal dendritic cells demonstrate limited antigen presentation capacity due to deficient expression of major histocompatibility complex class II molecules [32]. Dendritic cell-derived cytokines play a major role in determining the type of immune response. Through the production of IL-12, dendritic cells drive the development of T helper (Th) 1 pro-inflammatory responses, and these responses are diminished in neonates [33]. Given that Th1 responses also contribute to autoimmune pathology, such diminished responses may be beneficial during the establishment of tolerance to common antigens in the environment [31], however rendering the neonate susceptible to infections. Suboptimal Th1 responses and diminished antigen-presenting capacity in the neonate result in a global hypo-inflammatory state. These maturational differences affect the ability of the neonatal immune system to mount an appropriate immune response and to main-

tain immune system homeostasis [34]. Exposure to immunogenic stimuli in early life may disturb the trajectory of immune maturation and has been associated with a number of inflammatory diseases, including increased susceptibility to allergic and autoimmune diseases later in life, with increased prevalence of these diseases in developed countries [35–38]. This susceptibility may in part be associated with limited exposure to certain bacteria and microorganisms, once ubiquitous in our environment [39]. The 'hygiene' or 'old friends' hypothesis suggests that diminished exposure to innocuous environmental organisms in developed countries results in impaired immunoregulation due to decreased activity of regulatory T cells (Treg). Treg play a major role in maintaining tolerance against harmless antigens, by supressing Th1 and Th2 cell responses. The 'hygiene hypothesis' proposes that reduced presence of agents that drive expansion and activity of Treg cells may lead to overactive immune responses to allergens and self-antigens [40]. Since the perinatal phase of life represents a critical window of vulnerability for the developing immune system [41], absence of essential microorganisms that play an important role in the maturation of the immune response in the perinatal environment, may contribute to the increased prevalence of chronic inflammatory conditions throughout life [40].

Not only may altered development of individual systems affect later-life functioning, but disturbances to the nature of the interaction between several physiological systems during critical periods may also be relevant to a variety of pathologies. For instance, development of the interplay between the immune system and the hypothalamic-pituitary-gonadal (HPG) axis has a significant impact on later-life functioning of both systems. It is well established that systemic infections and chronic inflammatory diseases (e.g. endometriosis, polycystic ovarian syndrome) are associated with diminished reproductive function [42–44]. In adult animals, acute immune challenge with lipopolysaccharide (LPS), as a common model of infection, has been shown to inhibit ovarian steroidogenesis [45, 46], impair testicular steroidogenesis [47] and reduce male testosterone levels [48]. It is now increasingly apparent that immune activation in early life can perturb the establishment of relationships between the immune and reproductive systems, inducing long-term alteration in reproductive function [12].

Perinatal Immune Challenge: A Model of Infection

Neonatal responses to an immune challenge are distinct from those of adults due to the immaturity of the immune system, rendering the neonates more susceptible to infections [29, 49]. Gram-negative bacterial infections are a common cause of morbidity and mortality in newborns [29]. LPS, a principal pathogenic component of Gram-negative bacterial cell walls, is widely used to mimic the inflammatory response associated with bacterial infections.

LPS is recognized by the Toll-like receptor 4 (TLR4), an innate immune pattern recognition receptor. TLR4 is expressed by a variety of cell types, including monocytes, macrophages, dendritic cells, microglia, adipocytes, granulosa ovarian cells and testicular Sertoli cells [50, 51]. Activation of TLR4 by administration of LPS initiates a downstream intracellular signalling cascade, resulting in immune activation and a subsequent inflammation-induced behavioural symptomology, which is largely identical to that induced by live bacterial infection [52, 53]. As opposed to live bacteria, LPS does not replicate, and as such, as a model it has the advantage of allowing tight control over dosage and limiting the confounding nature of replicating infection.

Inflammatory responses associated with perinatal LPS exposure have been implicated in long-term programming of a variety of physiological and behavioural outcomes, such as adult immune responses [54–57], metabolic function [58, 59] and neurobehavioural outcomes [60–62]. Only a few studies, however, have investigated the impact of perinatal immune challenge on reproduction. These studies, reviewed below, have demonstrated that perinatal LPS exposure can affect all levels of the HPG axis, leading to life-long changes in reproductive function.

Impact of Perinatal LPS Exposure on Reproductive Function

Impact of Perinatal LPS Exposure on HPG Hormones

Accumulating evidence has shown that reproductive hormones are sensitive to the impact of perinatal bacterial exposure. Prenatal exposure to LPS has been demonstrated to alter foetal GnRH neuronal migration into the forebrain, leading to suppression of GnRH synthesis before and after puberty [63, 64]. This effect has been suggested to be mediated via the maternal and foetal inflammatory response to an immune challenge, given the possible regulatory role of pro-inflammatory cytokines in GnRH migration [65]. However, while the overall content in the adult hypothalamus was suppressed by 25% as compared to control animals [64], this reduction is not

sufficiently severe to cause infertility. A loss of more than 85% of GnRH neurons is required to result in female infertility [66].

Postnatal LPS administration on days 3 and 5 in the rat has also been reported to induce long-term alterations in GnRH signalling, leading to pre- and post-pubertal downregulation of the hypothalamic mRNA levels of kisspeptin (Kiss1), a potent regulator of GnRH neurons [16]. Altered Kiss1 expression was accompanied by a delayed onset of puberty, supporting an important role of Kiss1 signalling in pubertal onset and its sensitivity to neonatal immune challenge [16]. Importantly, no such effects occurred when LPS was administered after 7 days of age, pointing towards the criticality of the timing of a perinatal insult [12].

Perinatal LPS exposure has also been shown to result in long-term suppression of HPG hormones. Immediate HPG responses to LPS administration on postnatal days 3 and 5 were observed in the neonatal period, whereby circulating testosterone and LH in males and LH in females were decreased [14]. In the same model, LH and FSH suppression was observed at puberty in females [13, 14], and suppression of testosterone and LH surges was observed in both sexes during mating [14]. Similarly, LPS exposure during gestation induced a decline in circulating testosterone levels in pubertal male offspring [67]. Moreover, in late adulthood, neonatally LPS-treated males have been reported to exhibit decreased testosterone levels [14], while adult females had lower circulating progesterone levels and a tendency to increased testosterone levels [68]. Suppressed LH pulses were also observed in neonatally LPS-treated females in response to a subsequent LPS challenge in adulthood, along with an increased expression of corticotropin-releasing hormone (CRH) receptor 1 in the medial preoptic area [69]. In rodents, the hypothalamic-pituitary-adrenal (HPA) axis completes its development postnatally during the 1st week of life [70], and neonatal exposure to LPS has been repeatedly shown to program long-term changes in the HPA axis activity, with increases in basal and stress-induced corticosterone levels, increases in hypothalamic expression of CRH and reduced negative feedback sensitivity in adulthood [13, 14, 60, 71]. The HPA and HPG axes are known to co-regulate one another, and stress has been shown to suppress reproduction via CRH-mediated inhibition of GnRH release and glucocorticoid-mediated inhibition of pituitary hormones and adrenal sex steroids [72, 73]. Therefore, LPS-induced stress response may represent one of the mechanisms by which perinatal immune challenge induces long-term perturbation of the HPG axis. These endocrine perturbations have been associated with a disruption to puberty onset and impairment in sexual behaviours in both male and female animals [13–16], suggesting that postnatal exposure to LPS during the 1st week of life can induce long-term programming of neuroendocrine regulation of reproductive function.

Impact of Perinatal LPS Exposure on Pubertal Development, Mating and Maternal Behaviours

Neonatal immune challenge by administration of LPS has been reported to both delay [15, 16] and advance [13, 14] puberty onset. These discrepancies may stem from strain differences in which the studies were conducted; however, it is clear that there is an apparent disruption of the mechanisms governing pubertal maturation. Metabolic cues influence the timing of puberty. As such, accelerated growth and increased body fat typically predict advanced puberty [74]. Both an increase [13] and no change [15, 16] in the body weight gain have been found during the pubertal period in the neonatally LPS-treated animals. One study, however, demonstrated that neonatal LPS exposure disrupts the typical linear relationship between body weight and the timing of puberty [14], suggesting that the HPG axis fails to respond to metabolic cues that play a regulatory role in puberty onset [74]. The ability of neonatal LPS exposure to affect pubertal onset appears to be critically dependent on the timing of exposure. While LPS treatment on postnatal days 3 and 5 has been shown to alter the timing of pubertal onset [13–16], LPS administered on postnatal days 7 and 9, or 14 and 16 produced no such effect [16]. LPS administration initiates the transcription of inflammatory cytokines. The release of these cytokines stimulates the synthesis of cyclooxygenase-2, the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandin E_2 (PGE2) [75]. PGE2 contributes to the initiation of the febrile response and has also been shown to mediate the effects of oestradiol on brain masculinisation during the critical period of sexual differentiation of the brain [76]. In the rat, this period occurs during the 1st postnatal week of life [77]. Therefore, while further investigation is warranted, it is plausible that the time-dependent effects of neonatal LPS exposure on puberty are due to its acute inflammatory actions in the developing brain. Interestingly, while neonatal LPS administered on postnatal days 7 and 9 had no effect on the onset of puberty [16], a single LPS injection on postnatal day 10 and a subsequent LPS challenge in adulthood significantly prolonged the oestrous cycle. This effect has been attributed to increased hypothalamic

expression of CRH-related peptides induced by neonatal LPS treatment, due to their involvement in stress-induced suppression of GnRH release [18]. This further suggests reciprocal interactions between the neuroendocrine and immune systems can be programmed early in life, altering the sensitivity of reproductive function to immunological stress in later life.

Mating behaviours in male and female rodents can also be affected by perinatal exposure to an immune challenge. Dual LPS exposure on days 3 and 5 impaired sexual behaviour in both sexes; however, the strongest effects were observed in female animals. Female rodents treated with LPS as neonates exhibited decreased receptivity during mating, leading to diminished performance by untreated male studs, as demonstrated by increased interaction times, significantly more mating attempts, but fewer successful mounts of the female, as well as fewer ejaculations [14]. These findings further support the hypothesis for the involvement of PGE2-related mechanisms in the effects of neonatal LPS on reproductive maturation, given that administration of PGE2 to newborn female rats has also been shown to impair adult sexual behaviour through masculinisation [76].

With regard to male sexual behaviour, male rats prenatally exposed to LPS exhibited impaired sexual performance, as demonstrated by increased latency to the first ejaculation and reduced number of ejaculations. While certain motor aspects of sexual behaviour, such as the number of mounts, appeared to be affected by prenatal LPS, no changes in behaviours reflective of sexual motivation, such as the mount and intromission latency, were observed. LPS-induced sickness behaviour in pregnant dams has been proposed to underlie these behavioural changes through the potential suppression of GnRH release and thus a possible reduction in GnRH content in the dams' milk, subsequently interfering with the process of brain masculinisation in the offspring [78].

It is important to note that animals perinatally treated with LPS are not infertile. Therefore, the impairment in mating strategies characterises a subfertile, rather than infertile, phenotype. Neonatal LPS exposure has been repeatedly shown to predispose to increased stress responsivity and anxiety-like behaviours in adulthood [60, 79]. An anxiety-like phenotype is often associated with anhedonia-like symptoms. Specifically, deficits in initiation of sexual behaviour have been previously reported to coincide with increased anxiety-like behaviours, in sexually naïve animals [80, 81]. Thus, the impaired mating strategies of neonatally LPS-treated rats may also be reflective of an anxious state.

Interestingly, corresponding with the increased anxiety-like phenotype as described above, female animals neonatally exposed to LPS were shown to exhibit significantly reduced maternal care towards their offspring [82], suggesting that an anxiety-like phenotype incorporates a wide range of behavioural alterations that are typically induced under potentially stressful conditions. Given that maternal behaviours in rats can be learned [83], the reduced quality of maternal care provided by females neonatally treated with LPS has the capacity to become a recurring phenomenon in subsequent generations. Importantly, variations in maternal care have previously been shown to influence the reproductive fitness of the female offspring [84, 85].

Despite the alterations in the HPG axis activity and reproductive behaviours in animals perinatally exposed to LPS, as described above, the influence of gonadotropins and consequently sex steroids on reproductive functioning is largely exerted after pubertal maturation. Importantly, the initial ovarian follicular growth and development are mediated by complex interactions between the oocyte and peripheral immune factors, such as cytokines, chemokines and growth factors [86–88]. Similarly in males, growth factors and cytokines are crucial in the regulation of testicular spermatogenesis and steroidogenesis [89, 90]. Therefore, the impact of perinatal immune challenge on the early development of reproductive organs may be mediated via peripheral factors to a greater extent than via central mechanisms. The emerging evidence for the programming effect, at the gonadal level, of an early-life immunological challenge is discussed below.

Impact of Perinatal LPS Exposure on Gonadal Development

Both models of pre- and postnatal LPS exposure have been recently implicated in impaired testicular development and spermatogenesis in male rodents, indicated by reduced testicular weight, decreased sperm number, delayed development of seminiferous tubules, increased disorganisation of seminiferous epithelium, as well as reduced gonocyte presence per tubule. These changes have been associated with decreased circulating testosterone [14, 67], as well as abnormal aggregation of Leydig (testosterone producing) cells in foetal testes [67]. It is quite plausible that perinatal LPS challenge disrupts testosterone synthesis, leading to long-term changes in gonadal morphology and function.

Robust alterations in gonadal morphology in response to postnatal LPS challenge during the first week of life have also been documented in female rodents, in which

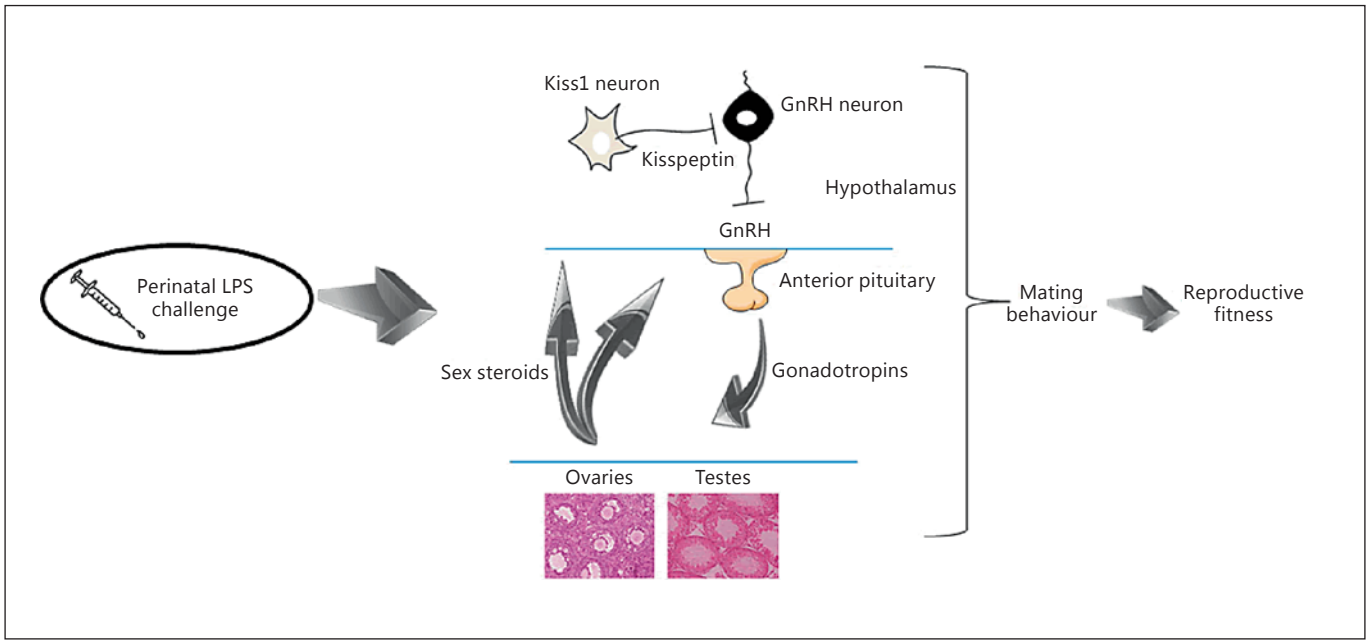

Fig. 1. Perinatal LPS challenge acts at all levels of the HPG axis by suppressing the GnRH release, inhibiting the release of gonadotropins, affecting folliculogenesis and spermatogenesis, reducing the production of sex steroids and impairing mating behaviours. These alterations lead to diminished reproductive fitness long term.

this timing of exposure coincides with the final processes in the formation of the primordial follicle pool [88]. Diminished follicular reserve has been detected in the ovaries of neonatally LPS-treated female rats [15], with reduced population of primordial follicles being evident as early as 2 weeks of age [13]. Moreover, this timing of LPS exposure resulted in an increased expression of ovarian nerve growth factor receptor (p75NGFR) along with increased thickness of the theca interna layer of the ovarian follicle [15]. p75NGFR is a marker of ovarian sympathetic innervation that regulates several important aspects of ovarian function, including follicular development, ovulation and steroidogenesis [91]. Its increased expression in the ovaries of adult females neonatally exposed to LPS suggests an increase in ovarian sympathetic activity may underlie the depletion of ovarian follicular reserve, as seen in these animals [15]. These findings further point towards the possible involvement of peripheral neuroimmune interactions in gonadal development and functioning [15].

Neonatal LPS challenge is known to induce an acute rise in pro-inflammatory cytokines (i.e. TNF-α, IL-6, IL-1β), and these are known to play a role in the initial gonadotropin-independent stages of follicular development [92, 93]. This may suggest that neonatal LPS treatment acutely disrupts the assembly of the follicular pool through an induction of an acute pro-inflammatory response. While this might be a transient disruption, it may potentially lead to the previously reported persistent reduction in the follicular pool [13, 15].

Additionally, a more direct pathway through which LPS may produce its effects on the ovary is possible. The receptor for LPS, TLR4, is present in the ovary and expressed by several cell types, such as ovarian epithelial cells, granulosa/cumulus cells and ovarian macrophages [50, 94–96]. In the ovary, these immune receptors regulate fertility, by contributing to ovulatory processes and mediating sperm capacitation [95, 97]. In response to an in vitro challenge with LPS, ovarian granulosa cells respond acutely, with rapid phosphorylation of TLR4 signalling components, such as p38 and ERK1/2 and other NF-κB components, resulting in increased expression of IL-6, IL-1β, IL-8, IL-10 and TNF-α mRNA [98, 99]. Exposure to LPS in vivo in adult animals or in vitro has been shown to result in impaired follicular function, inducing follicular atresia in cattle [94, 100] and in rodents [100, 101]. A recent study has shown that 2 days after in vivo neonatal LPS challenge, there is a substantial upregulation of inflammatory genes in the neonatal ovary. Specifically, the expression of TLR4 transcript, a major com-

ponent of the LPS-stimulated mitogen-activated protein kinase signalling pathway, was found to be significantly increased [102]. These data indicate that peripheral administration of LPS during the perinatal period in the rat results in activation of ovarian TLR4 signalling, which may directly intervene with the formation and establishment of the finite primordial follicle pool via activation of inflammatory pathways.

Prenatal and Postnatal Models of LPS Exposure: Mechanisms of Action

It is important to note that while the evidence presented here suggests both models of prenatal and postnatal LPS administration influence the reproductive system long term, they appear to be mediated by different mechanisms. Prenatally, LPS does not cross the placenta, and its effects on the foetus are mediated by the maternal immune response, including an increase in pro-inflammatory cytokines, fever and the glucocorticoid feedback on the immune system [103, 104]. Maternal cytokines and glucocorticoids can then cross the placental barrier to some extent, with placental permeability to these factors changing across the course of pregnancy [105]. In addition to inflammatory changes in maternal circulation, exposure of pregnant rodents to LPS has been shown to induce placental inflammation, facilitating the transfer of inflammatory markers to the foetus [106]. Foetal ability to produce cytokines in response to maternal infection appears to be dependent on a myriad of factors including the gestational age, the nature of the inflammatory stimulus, its dosage as well as tissue-specific and sex-dependent differences [107]. While the exact pathways responsible for the influences of prenatal LPS exposure on the reproductive function of the offspring remain to be established, it is unlikely that LPS has a direct TLR4-mediated effect on the developing gonads prenatally, as opposed to this being possible in a postnatal model.

Finally, the research presented in this review emphasises the imperative need to focus future studies on the mechanisms through which programming of adult reproductive function occurs, in order to identify potential therapeutic targets and develop successful interventions. Witek-Janusek [108] was the first to demonstrate the sensitivity of developing rat neonates to endotoxic shock and the sensitivity of the neonatal endocrine system to LPS. Since then, for more than 20 years, models of perinatal LPS exposure have been used to investigate the development and function of neuroendocrine, metabolic, immune and, recently, reproductive systems. However, the mechanisms underpinning the long-term influence of this challenge and its relevance to human health have not been fully explored. Future research focusing on the time-dependent role of PGE2 in reproductive maturity following perinatal LPS exposure as well as on the role of gonadal TLR4 and other novel central and gonadal inflammatory pathways may expand our understanding of the development of neuro-immune-endocrine interactions and thus represents promising lines of investigation, with important implications for understanding the mechanisms underlying reproductive dysfunction.

Conclusions

This review highlights recent studies that demonstrated a robust impact of perinatal immune challenge on different components of the HPG axis and the subsequent functional outcomes, emphasising the criticality of a perinatal immune activation in a variety of physiological systems and the reciprocal communication between them (fig. 1).

Infections and inflammations of the genital tract are considered the most frequent causes of reduced fertility in both males and females [42, 109]. However, the role of systemic infection in fertility throughout the lifespan is only recently attracting attention. Specifically, the unique sensitivity of the developing organism to environmental impacts supports the possibility that inflammatory challenge during this time is able to disrupt ongoing formation of reproductive circuitry, leading to prolonged health consequences.

It is important to view these outcomes in a context-specific manner. The diminished fertility of LPS-treated animals or their offspring may serve to prevent proliferation of this potentially 'maladaptive' phenotype in species with a short lifespan. However, the consequences for the human population, where bacterial infections are commonly experienced throughout life, may be different. It is possible that severe infections in infancy and childhood may produce detrimental effects on fertility, such as the rare complications of orchitis and oophoritis [110, 111]. This review, however, focused on the impact of a subtle immune activation that can be readily elicited by other deleterious environmental factors such as smoking, endocrine disruptors, high fat diet and other unhealthy habits. Therefore, future work is needed to understand how common inflammatory conditions may contribute to individual vulnerability to disorders associated with reproductive dysfunction, with a focus on preventative health strategies.

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Chapter 2. General Methods

2.1 Animal Ethics Approval

The research conducted within this thesis was approved by the University of Newcastle Animal Ethics Committee (ACEC), protocol number A-2012-218, under the guidelines of the National Health and Medical Research Council of Australia (NHMRC). All parameters concerning the welfare of the animals were considered, ensuring that there was minimal distress, harm and impact to the animals that were housed in the facility and throughout the undertaking of these thesis studies. Where possible, multiple parameters were assessed in order to minimise the number of animals used, without the loss of statistical power. All experimental testing was conducted in the biological psychology laboratory and the reproductive life science laboratory at the University of Newcastle, Callaghan, Australia, unless stated otherwise.

2.2 Animals and Housing

The research carried out for this thesis was conducted using the Wistar outbred strain of rat. For breeding purposes, experimentally naïve Wistar rats were supplied from an out-bred colony at the Animal Resources Centre (ARC), Canning Vale, Western Australia, or the University of Newcastle Animal House, Callaghan, NSW. The Wistar outbred rat allows for the genetically diverse, yet standardised examination of both behaviour and physiology. The Wistar rat provides for an excellent animal model within a health and disease framework to examine the long-term consequences of early life stress due to their homologous physiological systems and functioning; as well as their relatively quick maturation rate that is comparable and translational to human developmental trajectories (Semple et al., 2013; Sengupta, 2013). Both psychological and medical translational research has effectively

employed the used the Wistar rat for research purposes to greatly progress our understanding of many diseases and disorders.

2.2.1 Housing

All experimental and breeding animals were housed at the University of Newcastle Psychology Vivarium. Breeding animal were held for a minimum of two weeks upon arrival in order to acclimatise to the facility before any breeding procedures. All experimental and breeding animals were housed at constant temperature of 21 ± 2°C and 34 ± 2 % humidity on a 12-h light/dark cycle (0600-1800) with standard rat chow (autoclaved Rat and Mouse pellets, Glen Forest, Western Australia) and water available *ad libitum*. All rats were same-sex paired with littermates and housed in wire topped cages Mascot cages (41.5 cm x 28.0 cm x 22.0 cm; Mascot Wire Works, Sydney, Australia) that were lined with compressed paper bedding.

2.2.2 Breeding

In order to obtain experimental litters for studies within this thesis, an experimentally naïve female aged 10 - 12 weeks old would be mated with an experimentally naïve male aged 12 - 20 weeks. This would occur either in the home cage during one-on-one mating, or in a large harem cage (80 cm x 65 cm x 55.5 cm, as above) where one male stud would service a maximum of three females. In order to mate the animals, female animals were placed in a new cage with the male animals where they would remain together for a 12 day period to ensure the passing of at least 2 female oestrus cycles. Following these 12 days, the gravid female was removed from the stud's cage and paced in her own home cage containing bedding, shredded paper, sunflower seeds and woodchips for nesting. The female would remain in this cage throughout gestation, pregnancy, and with her litter until weaning. Pregnant dams were monitored twice daily in the period of expected birth and the day of

birth was recorded as post-natal day (PND) 1. Dams were culled following weaning, or prior to weaning if whole litters were exhausted for neonatal studies.

Following breeding, the breeder-only males were returned to same-sex pair housing in cages as outlined above, however their cages contained a red Perspex tube for enrichment. Male studs were used a maximum of 5 times for breeding purposes for ethical reasons. It is important to note here that these males and females were used for breeding purposes only, therefore the addition of enrichment to their cages is for ethical reasons only and did not interfere with the stress protocol of their litters. All animal cages, including experimental animals, were cleaned once a week, apart from the dam's cage, which were left undisturbed for 10 days post-birth of litter bar-visual monitoring.

2.2.3 Housing of Experimental Animals.

Rats born to naïve females become experimental litters. Day of birth was labelled as PND 1 and they were randomly allocated to either experimental (Lipopolysaccharide (LPS) treatment) or control (saline treatment) groups. These litters remained undisturbed in their home cage with the dam until weaning, apart from neonatal immune stress or saline control administration on PND 3 and again on PND 5. On PND 22, litters were weaned from their dam and group housed four per cage with same-sex litter mates. On PND 29, animals were further divided into same-sex litter-mate housing with two animals per cage, as outlined in our ethical protocol. All experimental handling was minimised to once per week when animals were monitored and weighed as per ethical protocol. No enrichment was provided.

2.3 Animal Weights and Monitoring

All animals were monitored weekly during housing as per ethical protocol. During this monitoring process, the animals were also weighed on a set of electric scales and their weights recorded in grams (g). This ensured constant awareness of any extreme weight loss

of the animals, such as when teeth were misaligned. Any animals that lost or gained excessive weight due to variables outside of our control were excluded from the experimental groups. In order to record weight on PND 3 and 5, pups were removed from the home cage and placed on the scale that had been covered with a thick cotton padding in order to maintain temperature and provide comfort. From PND 22 onwards, animals were weighed in a plastic bowl and the average weight of a three second period was taken, to ensure a correct weight record as the animal moved around freely.

2.3.2 Monitoring During Experimental Procedures

Specialised monitoring was set in place during specific experimental procedure in order to monitor the animal's continued welfare. Following neonatal treatment for both control and LPS treated groups, animals were visually monitored 2h and 4 h after the injection, and then twice a day (AM/PM) for 10 days following their last injection. This monitoring included looking for visual and auditory signs of extreme sickness including proximity of pups to litter and dam, skin tone, and vocalisation. It any pups were deemed extremely unwell, they were culled in order to minimise distress and their data were excluded from the analysis. Other altered and increased monitoring schedules included following the finalisation of behavioural assays, non-terminal blood collections and subsequent stress protocols.

2.4 Early life Stress Paradigm: Neonatal Lipopolysaccharide Administration

The mode of early life stress utilised in this thesis is neonatal immune activation (NIA) using LPS immune activation. As outlined in chapter 1 (section 3.1, & 4), LPS is an elegant tool to investigate the repercussion of immune activation, mimicking that of bacterial infection. The model of early life immune activation carried out in our laboratory consists of a dual dose of LPS (Salmonella enterica, serotype Enteritidis: Sigma-Aldrich Chemical Co., USA in sterile

pyrogen-free Saline) at a dose of 0.05 mg/kg in 0.02mL, administered on PND 3 and again PND 5.

Whole rat pup litters were removed from their home cage and placed in an incubator to maintain body temperature at 34°C, where they were then weighed and administered an intraperitoneal (ip) microinjection of LPS or equivolume of saline (Livingstone International, Australia) according to weight using a 3/10cc:0.3mL ultra-fine II insulin syringe (BD Medical, Australia). All injections occurred between the hours of 09:00 h and 10:00 h and litters were immediately returned to the home cage following drug or saline injections. This model has been previously successfully employed in our laboratory (Sominsky et al., 2012a; Sominsky et al., 2012b; Walker et al., 2012; Walker et al., 2011; Walker et al., 2009; Walker et al., 2010; Walker et al., 2004a; Walker et al., 2008; Walker et al., 2004b; Zouikr et al., 2015; Zouikr et al., 2016; Zouikr et al., 2014a; Zouikr et al., 2014b) and others (Nilsson et al., 2002; Shanks et al., 1995; Shanks & Meaney, 1994; Wu et al., 2011).

The dosage and timing used here has been demonstrated to elicit a rapid, sustained immune and endocrine response, with no mortality in litters (Shanks et al., 1995; Shanks & Meaney, 1994). The timing of this LPS challenge coincides with a stress hypo-responsive period (SHRP) in the neonatal rat pup, ranging from PND 1 to ~PND 14 (Sapolsky & Meaney, 1986; Vazquez, 1998; Walker & Vrana, 1993; Witek-Janusek, 1988). This SHRP is an early developmental time point characterised by a markedly reduced stress response, offering a certain degree of protection from environmental stressors for biological conservation (Walker & Vrana, 1993). The perinatal period in mammals represents a period of bacterial exposure (nasopharynx, intestinal, vaginal), as well as a time period where the newborn and mother are quite susceptible to both bacterial and viral infections (Lamagni et al., 2017; Shanks et al., 1995). Importantly, our model of neonatal LPS exposure falls into a period of plasticity for

critical endocrine, immune and central development, including development of the gonads and microglial development (Karrow, 2006; Rivest, 1991; Sapolsky & Meaney, 1986; Sominsky et al., 2013a; Spencer et al., 2006) (see Figure 2.1).

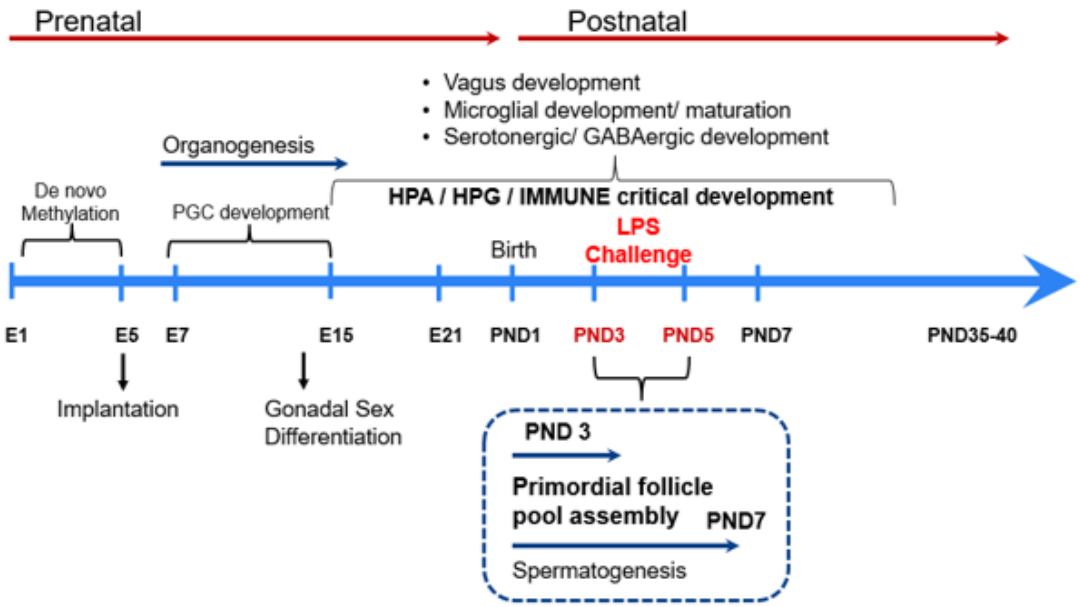

Figure 2.1. The model of early life immune activation utilised in our laboratory coincides with the neonatal rodents stress hypo responsive period (SHRP), but also critical periods of developmental plasticity for the immune system, the HPA and HPG axis, central development of key neuronal cells and synapses, as well as the final stages of gonadal development in the male and female. Figure adapted from (Sominsky et al., 2013b).

2.5 Neonatal Blood and Tissue Collection

A subset of neonatal animals were culled within each study on PND 5 in order to examine the acute effects of LPS administration.

2.5.1 Blood Sampling

2.5h post LPS or saline administration, neonatal animals were euthanized via rapid decapitation and trunk blood was collected into EDTA coated tubes. This timing was chosen as it has been demonstrated as an average optimal time point for both peripheral and central

genetic and protein expression of cytokines and immune mediators (Kakizaki et al., 1999; Saban et al., 2001). All blood samples were centrifuged at 1000g for 20 minutes at 4°C and stored at -20°C until assessment.

2.5.2 Tissue Collection

Tissue was collected from neonatal animals at the same time as blood collection. Peripheral tissue including ovaries and spleen, were dissected out via an abdominal incision using fine point forceps and dissection scissors. All neonatal tissue was excised and placed in a 8.7cm^2 vented petri dish (Greiner Bio-One, Germany) filled with 4°C molecular grade phosphate buffered saline (PBS) which had been filtered through a sterile minisart-plus syringe filter (Sigma-Aldrich/Merck, Australia). In PBS, tissue was further dissected off all surround connective tissue under a dissection microscope at 20X magnification, to ensure only specific target tissue was used for analysis. Following microdissection, tissue was snap frozen in 1.5mL Eppendorf tubes on dry ice and stored at -80°C until further analysis. Neonatal brains were also collected at this stage by removing skull tissue and bone with micro-rongeurs. Brains were cleaned as detailed above. The cerebellum was removed from the cerebrum and both were snap frozen on dry ice, then stored as mentioned above.

2.6 Adult Blood and Tissue collection

2.6.1 Non-terminal and Terminal Blood Sampling

Blood was collected from adult animals at multiple time points throughout experiments. This consisted of both terminal and not terminal collections. All non-terminal blood collections were performed using a lateral saphenous vein (LSV) puncture on non-sedated animals. In order to access the LSV, the area was firstly sanitised with antiseptic (chlorhexidine in 70% alcohol in water), hair was then covered with a small amount of paraffin oil in order to identify the vein site. Once the vein was located, it was swiftly punctured with

a 22 gauge needle and blood was collected into an EDTA coated tube, centrifuged at 1000g for 20 minutes and stored at -20°C until analysis. Haemostasis was effected through direct pressure applied to the puncture site with a piece of sterile surgical gauze applied continuously for at least 60 seconds. In a single bleed, no more than 10% of the animal's blood volume was extracted. For multiple samplings, the maximum removed volume was no more than 1% of the animal's blood volume at a time. This method is humane and practical, and is approved for use by the University of Newcastle ACEC. Following saphenous blood sampling, animals were returned to the home cage after haemostasis and were monitored 2 hours and 6 hours after for signs of blood loss and distress. Terminal blood sampling was achieved via cardiac puncture on adult animals that were deeply anaesthetised for euthanasia using two methods. Firstly, the heavily sedated animal was laid on its right hand side and the left ventricle was punctured with an 18 gauge needle attached to a 10cc:10mL Syringe. 5mL of blood was drawn into the syringe and deposited into an EDTA coated tube via puncture of the vacuum membrane atop the tube, where it was promptly centrifuged and stored as mentioned above. Once blood was exsanguination, the animal was prepared for tissue extraction. Secondly, once the animal was deeply sedated for euthanasia, a surgical incision was made along the abdomen through to the top of the chest area. Prior to perfusion, the right ventricle was pierced with an 18 gauge needle and 2mL of blood was exsanguination into a 5cc:10mL needle. Following this, the needle was left in place and a syringe containing chilled PBS was connected for subsequent saline transcardial perfusion of the animal.

2.6.2 Tissue Collection

Animals were euthanised via deep anesthetisation, using 1ml of Lethabarb (Virbac Pty Ltd, Australia) administered via ip injection. Animals were deemed unresponsive and fully sedated when they become unresponsive to foot reflex stimulation. Depending on

experimental needs, cardiac punctures were performed via one of the two methods outlined above, and tissue was collected. In order to assess specific tissue, animals were transcardially perfused with approximately 600 - 800mL of chilled PBS. In order to perform a saline perfusion, an 18 gauge perfusion needle was inserted into the lower left ventricle of the animal's heart, ideally whilst it is still beating, and secured with a fine tip haemostat. A small incision was then made with a fine scalpel blade in the right atrium, allowing for blood to exit the heart. Saline was then perfused into the body at a very slow and steady rate of approximately 5-10 cc volume until fluids ran clear and tissue was visually cleared of blood. Formaldehyde perfusions were not carried out within the scope of this thesis. Following saline perfusion, tissue was collected from the animal using fine, sterilized, RNaseZap (Sigma Aldrich) cleaned dissection tools and deposited into chilled, molecular grade, filtered PBS. Here, tissue was then excised of all surrounding fat and connecting tissue, and snap frozen in sterile micro tubes/sample tubes on dry ice, and stored at -80°C until analysis. For brain tissue collection, the animal's skull was removed using multiple sized rongeurs, taking extreme care in order to not pierce the soft, unfixed brain tissue. Once the brain has been sufficiently exposed, it was extracted using a sterile, RNaseZap treated micro spatula into chilled, molecular grade PBS to be rinsed. The cerebellum was then dissected from the cerebrum and both were placed in a sample tube, snap frozen on dry ice and stored as previously mentioned until assayed.

2.7 Tissue Preparation and Analysis

2.7.1 Ovarian tissue

One ovary (counterbalanced across animals) was collected, cleaned of superfluous tissue under a dissection microscope, snap frozen on dry ice, and stored at -80°C. The remaining ovary was placed in a fixative solution (Bouins, Polysciences, Pennsylvania, USA)

for twenty-four hours (adult ovaries) or four hours (neonatal ovaries). After this fixing time, ovaries were washed in 3 x 15 min 70% ethanol baths to clear fixing fluid from the tissue. Fixed ovaries were then stored in 70% ethanol at 4°C prior to being prepared for immunohistochemical analysis.

2.7.1.1 Histological Evaluation of Ovaries. For immunohistochemical analysis, ovaries were dehydrated, embedded in paraffin and sectioned at 4µm. Every 4th slide was stained with hematoxylin and eosin (H&E) for quantification of ovarian follicles, resulting in approximately 8-10 H&E slides per rat neonatal ovary (Sobinoff et al., 2012; Sominsky et al., 2013a). The samples were examined by an experimenter blind to experimental groups, and only follicles with a visible oocyte were counted. Primordial, activated primordial, and primary follicles only were classified on H&E sections as follows (see Sobinoff et al., 2012); (1) *Primordial follicle*: an oocyte surrounded by one layer of flattened cuboidal granulosa cells; (2) *Activated primordial follicle*: a maturing oocyte surrounded by both flattened granulosa and one or more cuboidal granulosa cells in a single layer; (3) *Primary follicle*: an oocyte surrounded by 4 or more cuboidal granulosa cells in a single layer; (4) *Preantral/Secondary follicle*: follicles without an antral cavity and with two or more layers of cuboidal granulosa cells; (5) *Antral follicle*: follicles with an antral cavity and with two or more layers of cuboidal granulosa cells; (6) *Preovulatory follicle*; largest type of follicle possessing a cumulus granulosa layer (Figure 2.2). Total counts were carried out on the first and third section of every H&E stained slide, resulting in the quantification of all visible follicles (Myers et al., 2004) (Figure 2.3).

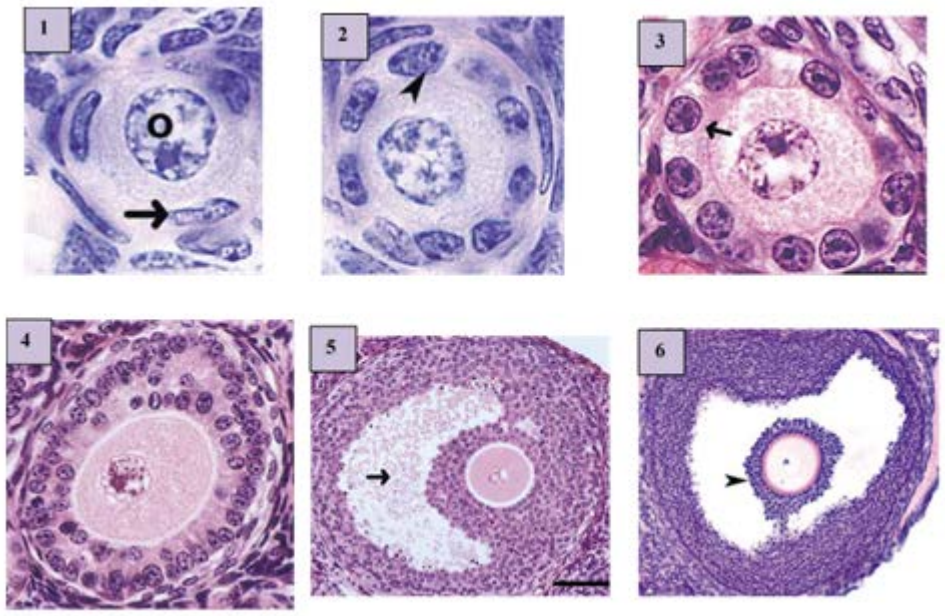

Figure 2.2. Pictorial representation of rat ovarian follicles for histological quantification. Numbers align to those mentioned in text. Images adapted teaching slide provided by (Myers et al., 2004).

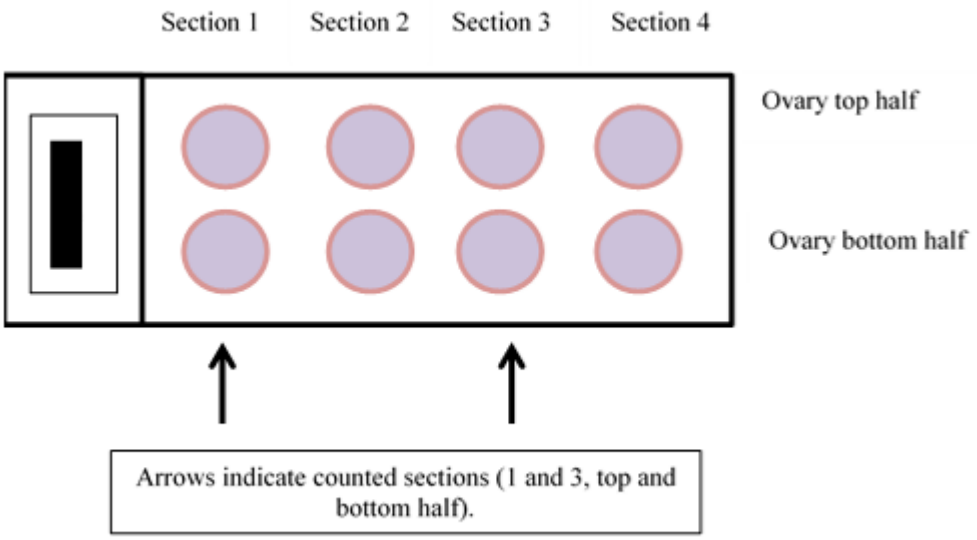

Figure 2.3. Schematic representation of the H & E stained ovarian sections mounted on a microscope slide. In this experiment, the first and third sections of each stained slide were counted by an experimenter blind to treatment allocation.

2.7.2 Frozen Tissue

2.7.2.1 RNA extraction. Frozen ovaries were prepared for ribonucleic acid (RNA) extraction by thawing and placing them individually or in pooled same treatment/condition groups (depended on age of animal) in a 1.5mL Eppendorf tube with 500mL of lysis reagent (QIAzol, Qiagen). Ovaries were hand-lysed then homogenised using a small plastic pestle, cleaned with RNaesap and diethyl pyrocarbonate (DEPc) water. RNA was extracted from ovarian tissue using an RNeasy mini kit (Qiagen) in accordance with the manufacturer's instruction. Nucleic acid purity and concentration was then assessed in a 1µl volume of each sample with a NanoDrop™ Spectrophotometer 2000c (Thermo Fisher Scientific, DE USA) unless otherwise stated.

2.7.2.2 Reverse Transcription. RNA was converted to complementary (c) DNA using a SuperScript ® VILO cDNA synthesis kit (Life Technologies, Thermo Fisher Scientific) by combining the kit components according to the manufacturer's instructions, with the extracted RNA sample, creating a total volume of 20µl of cDNA per reaction. Converted cDNA was stored at -20°C in preparation for quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2.7.2.3 Quantitative Real Time PCR. Expression of mRNA levels in ovarian tissue was determined by qRT-PCR (Sigma Aldrich, Australia, see Appendix F for sequence and efficiency details). qRT-PCR was performed using SYBR Green reagents (Life Technologies, Thermo Fisher Scientific) and was conducted on a 7500 RT-PCR Fast instrument (Applied Biosystems, CA, USA). The 20µl PCR mixture for each well consisted of 10 µl of SYBR Green, 0.4 µl of each primer (forward and reverse) and 4.6 µl of water, which was added to the 5 µl (at 5µg/µl) cDNA template. All reactions were performed in triplicate and were accompanied by a RT-blank sample. The reference genes β-actin, glyceraldehyde 3-phosphate dehydrogenase

(GAPDH), tubulin or Cyclophilin (Life Technologies, Australia) were used to normalise the data. A relative quantitative measure of the target gene expression was then calculated by comparing the expression level of the target gene mRNA to that of the housekeeping gene. Final gene expression changes were presented as a normalised fold change relative to the control group.

2.7.2.4 ELISA and Corticosterone RIA Assays. All enzyme-linked immunosorbent assays (ELISA) and corticosterone (CORT) assays were conducted according to the manufacturer's instructions for both tissue and blood analysis. Individual detection rates and ELISA/RIA information is supplied in specific chapters/papers. All assays contained biological samples from at least three different litters per treatment group. All samples were assayed in duplicate.

2.8 Determination of Puberty Onset

Female animals were assessed daily for puberty onset from PND 29 – 40 between the hours of 0900am – 1100am. Onset of puberty was determined by day of vaginal opening (DVO) (Evans, 1986). Vaginal opening is an apoptosis mediated event (Rodriguez et al., 1997) that can be used as a non-invasive, external index of puberty onset, with minimal harm and handling. Timing of puberty can be divided into two categories, an early period at PND 31-35, and a late period at PND 36-40 (Rivest, 1991). In order to assess DVO, the animals was briefly inverted by gently but firmly holding the loose skin at the nape of the neck between ones thumb and forefinger ensuring the full weight of the rat is based in the palm and the rat is fully supported. The vagina of female animals was visually and manually inspected for opening of the vaginal cavity. The DVO was recorded if the vaginal cavity was opened.

2.9 Female Reproductive Anatomy, Oestrus Cycle and Oestrus Monitoring

In the female rat, the reproductive system consists of two ovaries and the genital tract; consisting of the oviducts, uterus, cervix and vagina. The vaginal is an external structure, internally, the path divides into the two uterine horns that extend rostrally towards the ovaries, positioned laterally near each kidney. Each ovary is connected to the uterine horn via the oviduct, part of which forms a complete capsule surrounding each ovary (Hamid & Zakaria, 2013). Oestrus cycling begins simultaneously with vaginal opening in the female rat, signalling ovarian and sexual maturity. The oestrus cycle is relatively short, lasting between 4 – 5 days, occurring consistently with no seasonal effects. The female rat will continue to cycle through the initial and continued recruitment until the end of the reproductive life span, where oestrus cycling will become noticeably elongated in non-receptive phases and senescence will occur (Lu et al., 1979). This signals the exhaustion of the ovarian reserve (see Figure 2.4).

The rat oestrus cycle consists of four phases; proestrus, oestrus, metestrus (diestrus I) and diestrus (diestrus II) (See Table 2.1). Sex hormone signalling excreted from the anterior pituitary controls oestrus cyclicity, regulating the oestrus cycle with varying levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH). This results in ovarian and follicular morphological alterations, as well as alterations in vaginal cytology and visual appearance. Within the ovary, FSH stimulates dominant follicle growth, LH stimulates ovulation and corpus luteum formation. Progesterone is secreted from the corpus luteum during metestrus, with levels declining in diestrus (Ojeda et al., 1980) (see Figure 2.5).

Oestrus phases were monitored daily following DVO and through to end of experiment using the vaginal lavage technique examining vaginal cytology (Cora et al., 2015), considered the gold standard of rat cyclicity measurement. Between the hours of 1300 – 1500pm, the

non-sedated female rat was placed in an opaque coloured, close fitting 'hood' that covered the rat's entire body. The rat was then secured and inverted against the experimenter's body using the non-dominant arm. A small glass pipette was filled with ~200ul of sterile saline then carefully inserted into the vagina (2-4mm depth), where the saline was flushed in and out of the pipette and into the vagina approx. 3 times. The sample was collected and deposited onto a clean glass slide. The animal was then returned to its home cage and the samples were immediately viewed under a light microscope at 10x magnification to determine oestrus phase according to vaginal cell morphology outlined in Table 2.1 and Figure 2.6. When vaginal samples were not conclusive, cyclicity was also assessed using the impedance method, where a small probe was gently inserted into the vagina (2-4mm depth) and vaginal wall electrical impedance was measured in Ω as follows; oestrus = 3.5 ± 0.4 Ω, proestrus = 2.0 ± 0.3 Ω, and diestrus = 1.4 ± 0.3 Ω (Bartoš, 1977; Jaramillo et al., 2012; Ramos et al., 2001; Taradach, 1982). Irregular cycling was classified as a period of diestrus lasting longer than three days or having samples that were indeterminable for 3 or more days.

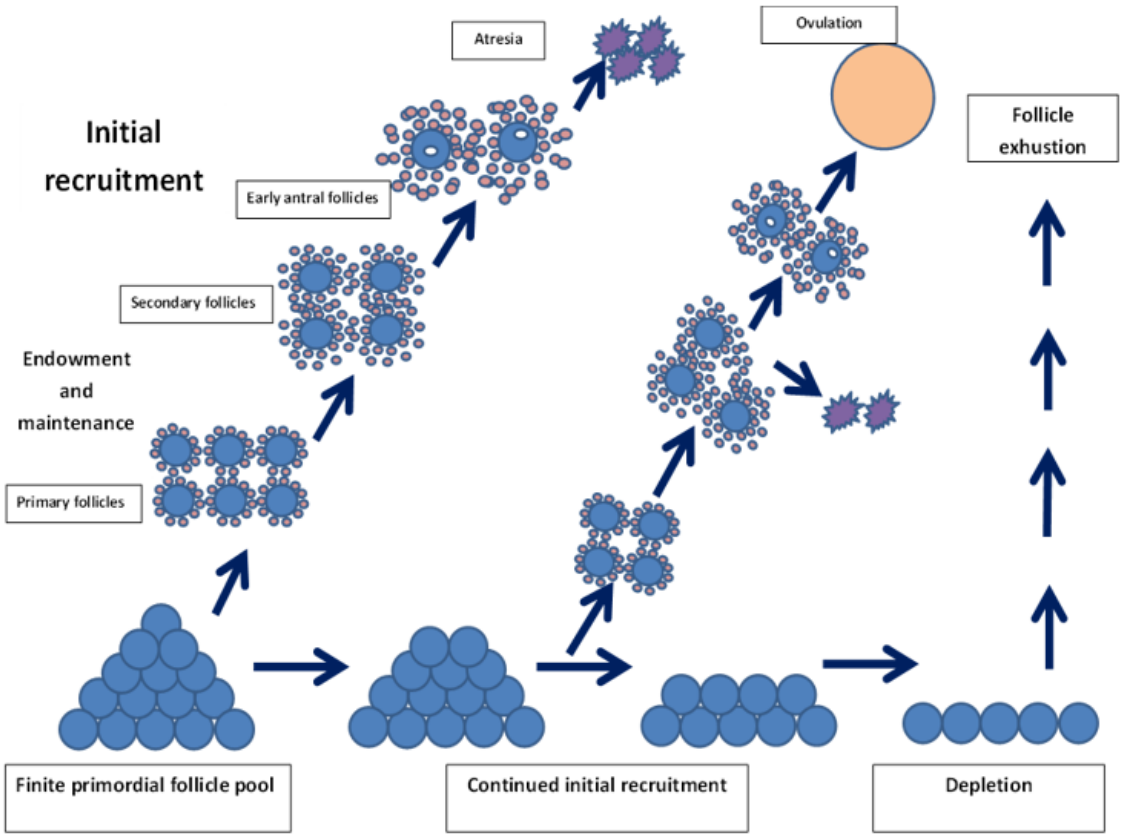

Figure 2.4 Schematic representation of ovarian follicle recruitment in the female rat. Primordial follicles form during early life (PND 3 in rodents/mid gestation in humans), the majority remain arrested in dictyate meiotic I phase. Progression through primordial, primary, secondary and antral stages occurs throughout a female's reproductive life starting with initial recruitment. The majority undergo atresia at differing stages. Following puberty, gonadotropin stimulation enables a few follicles to be rescued for cyclic recruitment to continue to develop to the pre-ovulatory stage and participate in ovulation. Depletion heralds a female's reproductive senescence. Adapted from (McGee & Hsueh, 2000).

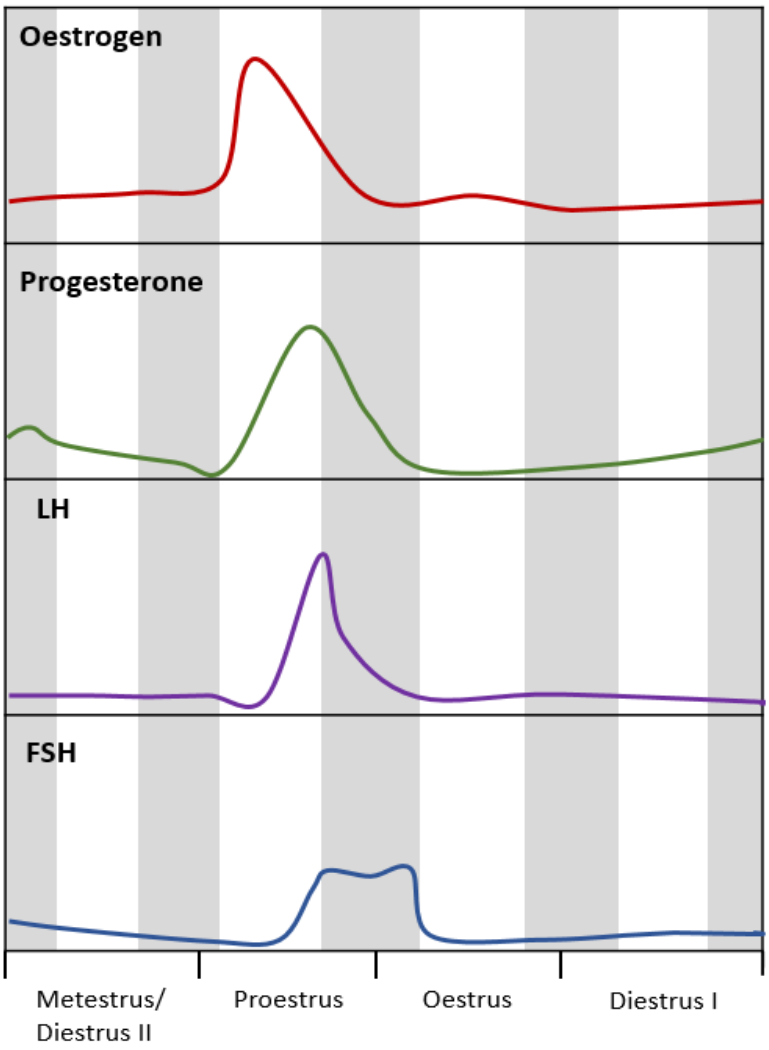

Figure 2.5 Graphical representation of the 4-5 day oestrus cycle of the female rat depicting the fluctuations in Oestrogen (top, red line), Progesterone (2nd from top, green line), LH (2nd from bottom, purple line), and FSH (bottom, blue line). The grey bars indicate night hours between 1800 – 0600. Adapted from Staley and Scharfman (2005).

Table 2.1. Summary of oestrus phase and duration, behaviour and vaginal cell morphology

Cycle Phase	Duration (h)	Behaviour	Vaginal Cell Morphology
Proestrus	12	Lordosis; Receptive to male	Nucleated epithelial cells
Oestrus	12	Lordosis; Receptive to male	~75% nucleated cells; ~25% cornified cells
Metestrus	21	Unreceptive to male	Leukocytes with nucleated and some cornified cells
Diestrus	57	Unreceptive to male	Leukocytes

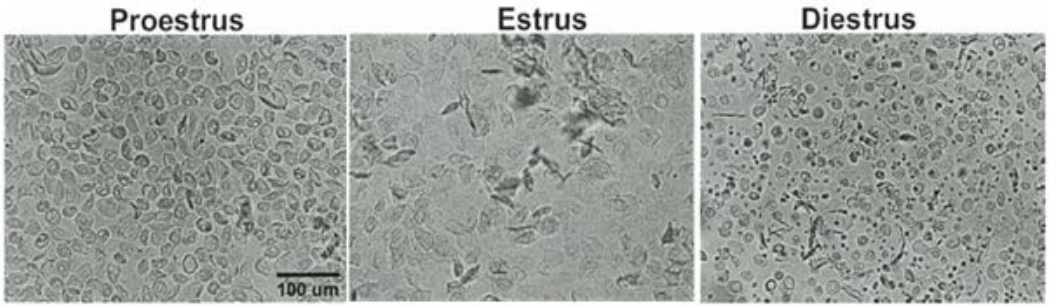

Figure 2.6 Photomicrograph at 10x magnification showing stages of the rodent oestrus cycle which typically lasts between 4 -5 days. Proestrus is characterised by the presence of round nucleated epithelial cells. Oestrus (oestrus) is characterised by the predominate presence of non-nucleated, cornified epithelial cells. Ovulation in the rat occurs approximately 10 hours after the beginning of oestrus. Diestrus contains mostly lymphocytes and represents the non-receptive phase of the rodent reproductive cycle. Representative images adapted from Goldman et al. (2007).

2.10 Adult Behavioural Tests

In adulthood (PND 85 >) animals underwent behavioural testing procedures. All animal testing was carried out between the hours of 0800am-1300pm where possible during the rats' active phase. All testing was counterbalanced where possible, and animals were tested within the same oestrus cycle where applicable to control for possible oestrus cyclicity effects. A latency period of 5 days between all behavioural tests was included to negate carry-over effects.

2.10.1 Sucrose Preference Test

We have previously demonstrated that our early life immune challenge model contributes to a later life anxiety-like phenotype in the male rat, with concomitant Hypothalamic-pituitary –adrenal stress response alterations (Walker et al., 2009; Walker et al., 2008). Anxiety and depression are very often co-morbid psychopathologies present in a human population, sharing similar neurobiological pathways (Kircanski et al., 2017; Krishnan & Nestler, 2011; Ramirez et al., 2017; Wohleb et al., 2016). However, despite the symptomology and neurobiological parallels, we have not previously investigated the depressive-like phenotype within our early life inflammation model. Current research indicates a well-established link between elevated immune activation, stress activation and symptoms of depression (depressed mood, anhedonia, psychomotor retardation, sleep abnormalities, decreased libido fatigue, and sickness behaviours, outlined in chapter 1). Previous studies from our laboratory have demonstrated both acute and prolonged stress and immune activation within the current model (Sominsky et al., 2012a; Sominsky et al., 2012b; Walker et al., 2009; Walker et al., 2004b), yet depressive-like parameters have yet to be assessed. Considering the differing immune response pattern and disorder aetiology in females, this test aimed to determine if this NIA manifests in adult depressive-like tendencies

in the female rat. The sucrose preference test (SPT) is a valid and reliable behavioural assay that models the reduced sensitivity to reward, or anhedonia, which is a key symptom in both human and animal depressive states (Strekalova et al., 2011). This test is widely used in conjunction with chronic, low grade stressors (Eagle et al., 2016; Forbes et al., 1996). The rat's responsivity and motivation to reward is measured by preference for a palatable sucrose solution over tap water (regular drinking water). A typical response is a preference towards sucrose, with failure to do so interpreted as anhedonia-like behaviour.

2.10.1.1 Sucrose Preference Test Protocol. Sucrose preference testing was carried out in a modified home cage using the 2- bottle choice protocol (Eagle et al., 2016). This ensured the abolishment of any variables that may confound the test, such as those arising from a novel environment. Two regular drinking bottles with sipper caps were placed side by side in the food cache area of the cage lid, and food placed in the usual drinking cache. These bottles contained plain room temperature tap water (rats normal drinking water), and a room temperature 2% sucrose solution made by dissolving 2g sucrose crystals into 100g water. Drinking bottles were filled with 200mL of both liquids. Bottles were inverted in order for air bubble release and were marked with (A; H_2O) or (B; sucrose) prior to testing to distinguish between sucrose solution and H_2O. Bottles were carefully placed through the wire spaces with minimal shaking to avoid drips and to ensure the rubber stopper was flush with the wire portion of the cage (see Figure 2.7). The positioning of the drinking bottles was alternated each day for each animal to eliminate learning and place preference effects. The contents of both drinking bottles were both weighed (in grams) and measured (in ml) in a volumetric cylinder before and after testing to obtain a standard volume amount of liquid to accurately measure ingestion. This cylinder was dried between weighs to ensure accuracy.

All animals were habituated to the testing apparatus for 1 hour over 3 consecutive days prior to testing. This habituation period was additionally used as a baseline measurement for liquid consumption. Following the third habituation day, animals were placed on a restricted water schedule for 12 hours to motivate liquid consumption (no water overnight during non-active phase) (Larson & Dunn, 2001). The following morning (0800 h), animals were transferred into their individual SPT cage, with one animals per cage, and given access to both drinking bottles for a total of 4 hours, with food available ad libitum. Both cage-mates were used for SPT testing. Sucrose preference was calculated as a percentage of the amount of sucrose consumed from the total volume of liquid consumed, using a standard equation: Sucrose Intake / Sucrose + Water Intake (Papp et al., 1991).

Figure 2.7. Top view of individual SPT cage setup. Animal was unrestricted within their home testing cage with ad libitum access to bottles containing water and a 2% sucrose solution and chow.

2.10.2 Social Interaction Test

An unwillingness to engage in social behaviour is a characteristic of both depression and social anxiety, and has been extensively studied (Hennessy et al., 2014; Lightowler et al., 1994; Yee & Prendergast, 2010; Yirmiya, 1996; Zhan et al., 2014). Rats are social creatures by nature; it has been demonstrated in rat models that social isolation leads to the display of depressive-like behaviours and increased inflammation and illness (Eisenberger et al., 2010; Liu & Wang, 2005; Stepanichev et al., 2014; Van den Berg et al., 1999). Hence interactions with others can be interpreted as a rewarding experience (Trezza et al., 2011) and specific parameters within a social interaction test (SIT) can be used to measure both motivational, depressive-like, and anxiety-like behaviours. Previous research indicates that rodents who were exposed to an LPS injection demonstrated a significant decrease in social exploratory behaviours in the SIT and significant anhedonic responses in other tests (sucrose preference and mating) (Yirmiya, 1996). This suggests the involvement of immune activation and inflammation in depressive-like behaviours.

The SIT has been extensively used in animal models of anxiety, depression and schizophrenia, particularly to examine the effects of developmental or chronic stressors (Beery & Kaufer, 2015; Sandi & Haller, 2015; Wilson & Koenig, 2014) in both rats and mice. This assay provides an ethologically valid and reliable measure of an animal's motivation and willingness to engage in a social context, as well as assessing neophobic anxiety and depressive-like behaviours (Trezza et al., 2011). Interestingly, sex differences have been demonstrated while using tests of depression and anxiety, including the SIT (Carrier & Kabbaj, 2012; Kokras & Dalla, 2014; Palanza & Parmigiani, 2017; Stack et al., 2010), which may involve differing neurobiological profiles. As anxiety and depression research typically focus on male cohorts, it is important to further explore our model of early life immune stress in female

cohorts, focusing on assays that allow for multidimensional measurement particularly considering psychopathological overlap.

***2.10.2.1 Social Interaction Test Protocol*.** The social interaction test area consisted of four raised walls made from white opaque Perspex (100cm x 40cm x 50cm) enclosing a grid of 16 squares on the floor (Duxon et al., 1997; File et al., 2001; Lightowler et al., 1994). Prior to testing, rats were weight and oestrus cycle matched where applicable to the naïve animal. To begin the assay, the experimental rat was placed in one corner of the arena, while a naïve conspecific of the same sex was placed in the opposing corner (see Figure 2.8 & 2.9). Placement was counter balanced between all tests and the apparatus was sanitised between subsequent assays. Undisturbed social interaction was recorded by a camera mounted above the arena for 15 minutes and later scored. Social behaviours of aggressive and non-aggressive behaviours were assessed, including frequency of approaching, following, leaving and sniffing behaviours towards the naïve conspecific rat. Following previous literature (Wesson, 2013), differential sniffing behaviours were assessed. Face and anogenital (AG) sniffs were scored as well as non-specific sniffing (i.e. sniffing on alternative areas to the face and AG regions, usually flank). Stress and anxiety-like behaviours of the experimental rat were also assessed including self-grooming and rearing (Dunn & File, 1987; File et al., 2001). Behaviour was scored using the software JWatcher ™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia available at http://www.jwatcher.ucla.edu/), by a scorer blind to treatment conditions.

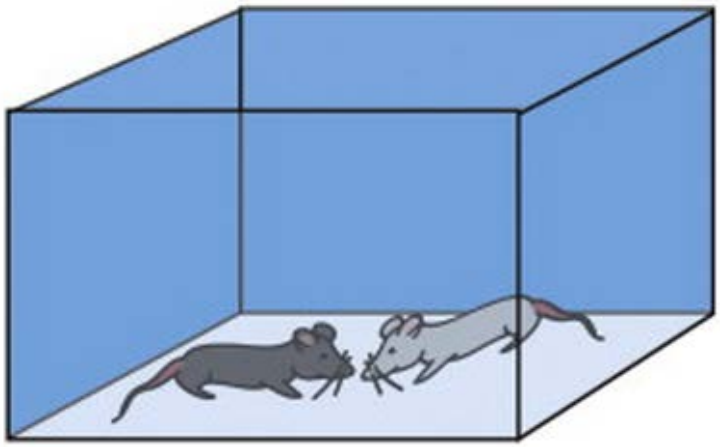

Figure 2.8. In the social interaction test, an experimental rat is placed in the arena with a conspecific. Investigation represents a willingness to engage socially. Adapted from Franklin et al., 2012.

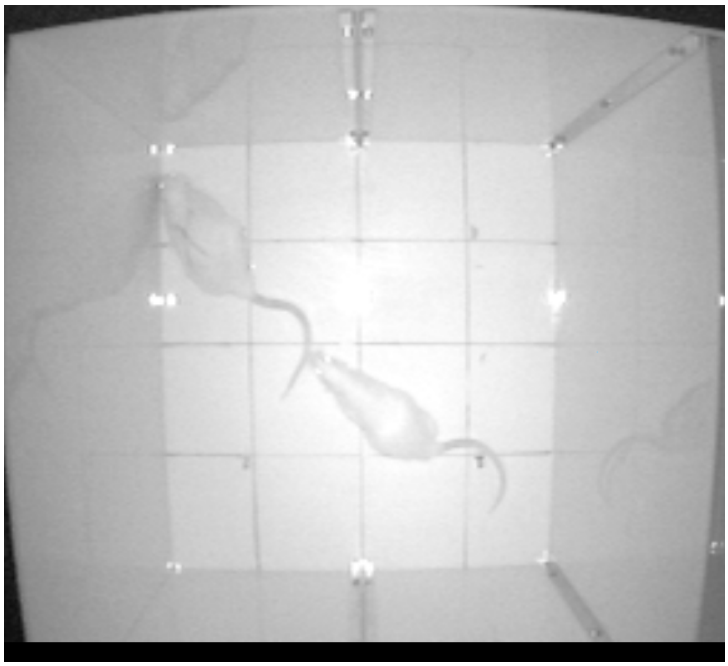

Figure 2.9. Photographic representation of the social interaction arena used for the social interaction test. The boxed arena is comprised of four raised walls enclosing a grid of 16 squares. Animals were left undisturbed for 15 minutes to interact.

2.10.3 Female Sexual Behaviour Testing

Rodents are an internally fertilising species that require sexual behaviour for reproductive success (Wallen & Zehr, 2004), where optimal reproductive fitness is critical for biological success and longevity of the species. Female rat mating behaviours are appetitive and consummatory, and can be further divided into two categories, these include proceptive and receptive behaviours (Ball & Balthazart, 2008). Receptive behaviour describes the female lordosis reflex in response to a male mount, characterised by immobility of the female; along with arching of the back and hind leg extension in order to elevate and expose the vaginal area. Proceptive behaviours include precopulatory or 'courting' behaviours that display the female's willingness to mate. These include approaching the male, orientating towards the male in order to sniff or groom the anogenital region, ear wiggling, hopping and darting (Erskine, 1989). Through these behaviours, the female strongly influences the pattern of copulation and these behavioural have also been used as an index of female sexual motivation in the rat (Erskine, 1989; Paredes & Vazquez, 1999). Studies has demonstrated that particular mating assays where the male has constant access to the female can have an aversive, non-reinforcing effect on the female rat and inhibit their behaviour and motivation (Brandling-Bennett et al., 1999; Fitzroy Hardy & Debold, 1971; Martínez & Paredes, 2001; Paredes & Vazquez, 1999; Zipse et al., 2000). Our laboratory has previously examined both male and female mating behaviours (Walker et al., 2011), however, as mentioned above, the copulatory timing was not paced by the female and therefore lack a particular focus on female motivation to mate. As such, this thesis examines female sexual behaviour in a *paced mating* apparatus, where the female controls or 'paces' the timing of copulations, where these interactions become reinforcing and rewarding for the female, hence allowing for motivational assessment. A lack of motivation to mate may be used as indicator of sub-

optimal reproductive behaviours, as well as an indicator of depressive-like behaviours (i.e. loss of libido).

2.10.3.1. Paced Mating Protocol. The paced mating test (PMT) arena was constructed from opaque white Perspex box (60 x 70 x 54 cm) and elevated 40 cm above ground level. All testing took place 2 h > the onset of the rats dark cycle. The main arena was then divided into 2 compartments by a clear, removable Perspex partition that has 4 passages cut into the base (4 x 4 cm), allowing for the female to pass through easily, however they are too small for the male to pass through (see Figure 2.10). Clean regular house bedding was placed on both sides of the partition, along with samples of used bedding from the males home cage. A proven male stud from a pool selected for testing was placed on one side of the chamber. This positioning of male side was counter balanced between all experimental animals to control for place preference. If more than one female was tested per day, male animals were changed in order to combat any carry-on exposure effects. A sexually naïve experimental female in the proestrus phase of her cycle was placed on the other side of the chamber. Both male and female rats were previously acclimatised to the chamber for 2 x 15 minutes time periods in the week prior to testing. The test was completed after a 30 minute period (as per Zipse et al, 2000). All testing took place in a dark, undisturbed room in the animal's natural active time, under infrared lighting and was recorded using an infrared camera. All female mating behaviours were visually quantified by an experienced experimenter blind to treatment groups using the software JWatcher ™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia available at http://www.jwatcher.ucla.edu/). These included lordosis, hops, kicks, darts, male mounts, attempted mounts, entries and exits into the male chamber, sniffing, and rearing. Immediately following the assay, a blood sample was taken from the female via saphenous bleed and animals were returned to the home cage.

All bedding was discarded between runs and the PMT chamber was sanitised between subsequent assays.

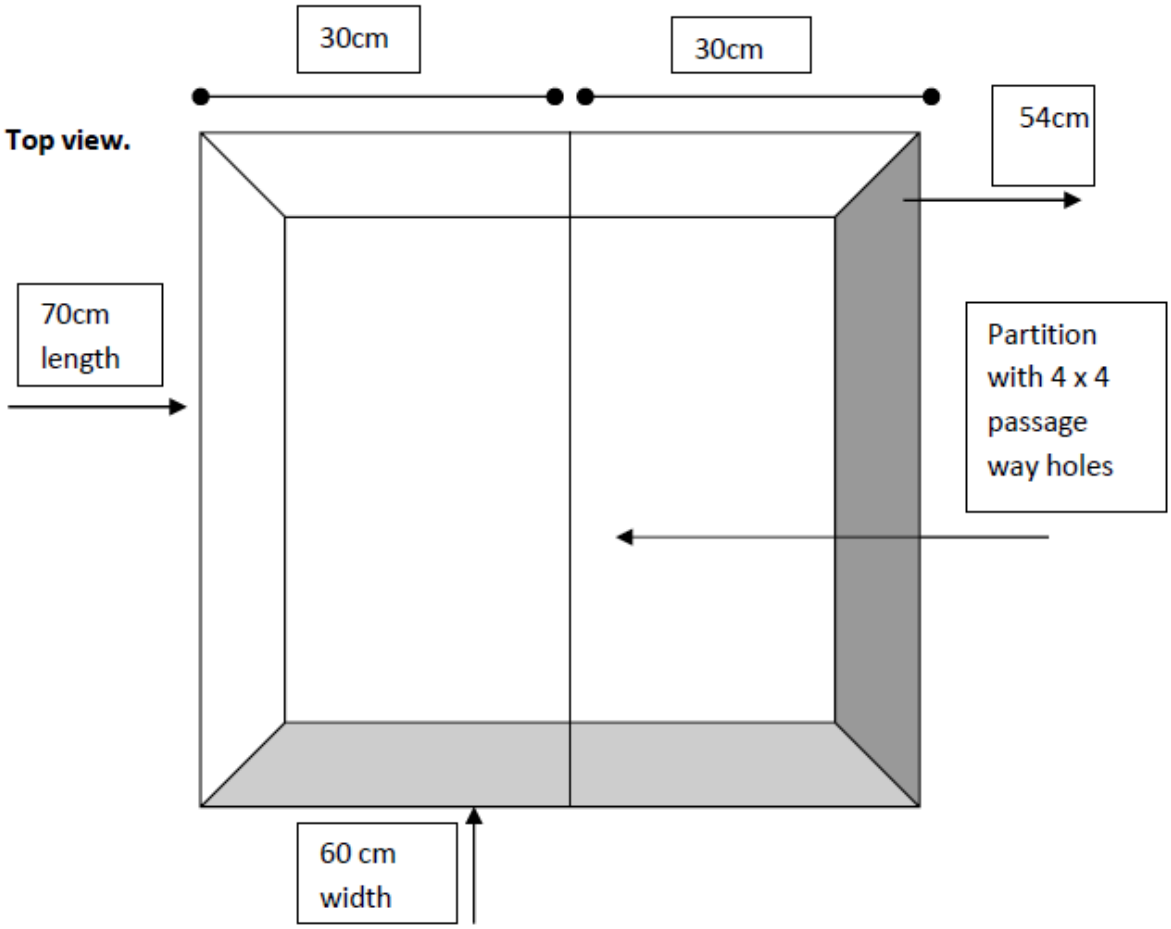

Figure 2.10. Diagram representing dimensions and layout of the paced mating apparatus. The female mouse was able to move freely through 4 passageways (4 x 4cm) along the base of the partition, however the male animal was unable to leave the allocated side he was placed in.

2.10.4 Restraint Stress

Restraint stress is a typical experimental stressor used to mimic the effects of an acute psychological stressor in rodent models (Bauer et al., 2001; Buynitsky & Mostofsky, 2009; Chu et al., 2016; Gameiro et al., 2006; Nukina et al., 2001; Sántha et al., 2016; Walker et al., 2009; Zhang et al., 2008a; Zhang et al., 2008b). By altering the timing, intensity and the duration of

the protocol, this experimental technique is an effective, efficient and valid model of both chronic and acute stress. This restraint stress protocol has previously been employed in our laboratory (Barouei et al., 2012; Walker et al., 2009) when examining stress vulnerability, or a '2nd hit' in later life. Walker et al (2009) demonstrated that male animals who were exposed to both neonatal immune stress and a 2nd hit of adult stress demonstrated blunted corticosterone responses, as well as anxiety-like behaviour. Additionally, alterations in immune functioning have been demonstrate in rodents who have been exposed to restraint stress (Voorhees et al., 2013), which parallels the immune profile of patients with major depression. As such, restraint stress was used in this thesis to examine immune alterations both in central and peripheral parameters, in adulthood following NIA or control. We hypothesise that early life immune activation creates a chronic, altered immune profile in later-life, particularly when paired with a subsequent stressor in adulthood. Importantly for the current model of NIA, altered immune imbalances between anti and proinflammatory markers have been linked to depression, anxiety, female fertility impairments and reproductive disorders, and depression.

2.10.4.1 Restraint Stress Protocol. In adulthood (PND 85 >) animals were randomly allocated into either a three-day stress, or no stress condition, which, when accounting for neonatal treatment, resulted in four treatment groups i.e. Neonatal (n) saline (SAL)/adult (a) no stress (NS), nSAL/a stress (ST), nLPS/aNS and nLPS/aST. Animals allocated to the adulthood stress condition underwent three consecutive days of acute stress exposure, consisting of 30 minutes of restraint stress on the first and second day, and 30 minutes of restraint stress followed by 30 minutes of isolation housing on the third (and last) day. All stress protocols were conducted in a separate, darkened room to minimise distress. The restraint apparatus was constructed using soft yet pliable wire mesh (25.0 cm × 20.0 cm) with no sharp edges

folded around the animal and secured using large fold back clips to restrict movement and secure the animal. Isolation housing consisted of a 41.5cm x 28cm x 22cm home cage (Mascot Wire Works, Australia) identical to those used to house the animals, where they were given food and water ad libitum. Animals in the 'No Stress' conditions were briefly handled before being placed back in their home cages where they remained undisturbed for the duration of the restraint session.

On the first day of the three-day restraint protocol, saphenous bleeds were taken from all 4 treatment groups. A baseline sample was taken immediately before the animal entered the restraint apparatus and a post-test sample was taken 2.5 hours after the animal was removed from the apparatus as previously described. Those not undergoing restraint had blood taken at the same time after brief handling.

2.11 Data Analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Version 22 (SPSS Inc.) Data were analysed using factorial analysis of variance (ANOVA), and repeated measures ANOVAs with significance level set at $p \leq 0.05$. Student's independent t-test were used where appropriate, with pairwise comparisons between treatments carried out using the Bonferroni correction where applicable. Litter size, litter ratio, weight and oestrus phase when applicable were included as covariates and reported where significant. Where covariates did not significantly impact dependent variables, they were removed from the analysis to maximise statistical power. Outliers present in the data that were more than ± two standard deviations away from the group mean for that particular measure were removed from the analysis. All ANOVA assumptions were tested and violations reported. Log transformations (Ln or Log10) were reported when used to transform the data in the case of extreme violations to Shapiro-Wilk or Levine's

assumptions, however it is known that ANOVA is robust to minor violations. Pairwise comparisons and independent samples t-test were used where significant outcomes were present.

Chapter 3. Neonatal Immune Activation Alters the Female Behavioural Phenotype: Motivational, Social, and Reproductive Behaviours.

3.1 Introduction

One in four people will experience some form of mental health condition in their lifetime. The Australian Bureau of Statistics (ABS) (2008) lists anxiety as the most common mental health condition in Australia. Furthermore, 3 million Australians are living with depression and comorbid anxiety, affecting their wellbeing, relationships, health and productivity (Australian Bureau of Statisitics). These statistics are echoed in the United States, with approximately 40 million adults (~18% of the population) suffering from anxiety, with approximately half of those numbers suffering from comorbid depression (Anxiety & Depression Association of America, 2016). From epidemiological statistics and extensive experimental literature available, it is obvious that anxiety and depression, although clinically differing diagnoses, share a high rate of comorbidity, and as such, hypothesised neurobiological pathways (Anderson & Hope, 2008; den Hollander-Gijsman et al., 2010; Fava et al., 2000; Kircanski et al., 2017). Hence, it becomes important in animal models examining the impact of early life stress to examine and delineate between specific psychopathology-like behaviours in order to understand their neurogenesis, facilitate specific interventional strategies, and aid clinical translation (Andreatini & Bacellar, 1999; Cryan & Holmes, 2005; Kalueff et al., 2007; Steimer, 2011).

Early life is a critical developmental time point during the human lifespan where plasticity is heightened to environmental input and stress (Lupien et al., 2009). Stress during this time is known to exert both immediate and sustained effects on central and peripheral systems, influencing physiology, behaviours and holistic health (Langley-Evans et al., 2012). Clinical studies indicate that early life stress impacts neuroendocrine and immune

development, and is associated with later life physiological and psychological diseases and disorders (Fagundes et al., 2013; Kajantie, 2006; Kinsella & Monk, 2009; Kloet et al., 2005; McEwen & Gianaros, 2011; Mueller et al., 2010). Human literature indicates that there is a robust link between early life adversity and stress, and an increased vulnerability to the development of mood disorders and anxiety (Heim & Nemeroff, 2001; Zannas & West, 2014).

The Diagnostic and Statistical Manual of Mental Disorders (DSM-V) outlines anxiety and depression as clearly separate disorders (American Psychiatric Association, 2013), yet controversy also exists as to the degree of similarity and dynamics between the two (Beard et al., 2016; Blanco et al., 2014; Hettema, 2008). Complexity is added, as each general disorder has multiple facets which are distinct, yet often comorbidly expressed. In general, anxiety disorders are categorised by a shared feature of excessive fear and related behavioural disturbances, such as hypervigilance and overactive stress responses (American Psychiatric Association, 2013). Although varied, the common features of many depressive disorders include depressed mood and sadness, anhedonia, fatigue, agitation, social and cognitive impairment, and feelings of worthlessness (American Psychiatric Association, 2013). Dysfunction within the same physiological mechanisms are involved in the aetiology of both anxiety and depression, and are linked with experiencing adverse early life conditions (Syed & Nemeroff, 2017). Interestingly, both anxiety and depression are recorded as being more prevalent in females than males (Albert, 2015; McLean et al., 2011). However, human studies examining the impact of early life stress on psychopathology development often fail to examine gender differences (Weinstock, 2007) and until recently, the majority of experimental human and animal research typically examines male animals (Weinstock, 2007). Ipso facto, the bias towards male subjects in both human and animal psychological and neurodevelopmental early life stress research seems in part contrary to the ultimate goal of

clinical translation. This is critical as it is known that gender dimorphism exists in the stress and immune mechanisms responsible for behavioural responses and outcomes, both peripherally and centrally (Bouman et al., 2005; Klein & Flanagan, 2016; Morale et al., 2001; Verma et al., 2011; Wang et al., 2007).

Animal models allow for the distillation of specific behaviours and mechanisms relating to psychological disorder symptomology. However, when there is such physiological and behavioural interrelatedness not only between disorders, but also within disorders, it becomes increasingly tricky to separate out distinct behavioural patterns and identify isolated biological determinants. This is particularly true of comorbid depressive-like behaviours in animal models of stress and anxiety. The use of pharmacologic interventions within animal studies allows for a degree of validation, however, anxiety and depression are often both treated with similar medications targeting pathways associated with both disorders, including GABAergic, serotonin and opioid pathways (Vaswani et al., 2003), as well similar neuroendocrine and immune mechanisms being implicated in both disorders. Therefore, multiple assays have proven necessary in order to examine and label specific behavioural hypotheses (Cryan & Holmes, 2005), with a particular focus on the assessable and specific aspects of both depressive-like behaviours, such as anhedonia and motivation (Willner et al., 1992), and anxiety-like behaviours including hypervigilance.

Experimental animal studies indicate that males rodents typically display anxiety-like phenotypes following a stress protocol, however anxiety-like alterations in females are less apparent an may manifest as an alternate phenotype. Pohl et al. (2007) exposed rats of both sexes to either chronic mild or severe stress in early life, and while male animals demonstrated exaggerated anxiety-like behaviours in adulthood, female animals demonstrated behaviours analogous to a depressive-like symptomology. This sexual

dimorphism is echoed in a number of other studies examining stress-induced behavioural and physiological alterations (Gaillard & Spinedi, 1998; Kokras et al., 2012; Lajud & Torner, 2015; Tenk et al., 2008). This highlights the importance of considering gender in how stress treatment typically manifests. Regardless of consistent sexually dimorphic findings in animal models and clinical epidemiological research, considerably less is known about the consequences of early life stress in female animal models, including the aetiology and manifestation of a depressive-like and/or anxiety-like phenotype due to early life immune stress or infection.

Infection is a common stressor encountered in early life, whether it be gestational or during the perinatal period (Bilbo & Schwarz, 2009; Labouesse et al., 2015; Levy, 2005). Immune stress activates both the innate immune response via proinflammatory actions, as well as the hypothalamic-pituitary-adrenal (HPA) axis in order to subdue and/or potentiate inflammation, depending on homeostatic need (Steinman, 2004; Webster & Sternberg, 2004). Numerous studies have examined the long-term impact of bacterial or viral immune activation in early life (Boisse et al., 2004; Galic et al., 2009; Mouihate et al., 2010; Spencer et al., 2011; Spencer et al., 2005; Spencer et al., 2006b; Spencer & Meyer, 2017). Immune and stress activation by bacterial exposure in the early neonatal period has been linked to anxiety and depression in both human studies (Du Preez et al., 2016; Goodwin, 2011) and animal models (Bilbo & Schwarz, 2009; Depino, 2015; Spencer et al., 2005). Our laboratory and others have established in a rat model that a neonatal immune challenge with lipopolysaccharide (LPS) in the perinatal period leads to anxiety-like behaviours in male animals, as well as male HPA axis and immune hyperactivity that is further dysregulated following subsequent adulthood psychological stress. This is indicative of a specific male anxiety-like phenotype. The findings in female rat models are less clear.

Immune activation during critical periods of development in both humans and animals has been linked to perinatally programmed alterations in neuroimmunoendocrine functioning (Heim & Nemeroff, 1999; van Bodegom et al., 2017). Furthermore, it has been demonstrated that sex differences exist in perinatally programmed HPA axis reactivity, with hypothalamic-pituitary-gonadal (HPG) axis hormones modifying the HPA axis stress response. This has been suggested as a basis for the sexually dimorphic results demonstrated in stress and psychopathology research (Handa et al., 1994; Kudielka & Kirschbaum, 2005; Verma et al., 2011).

Along with robust HPA axis alterations, immune system dysfunction has consistently been established in depressive and anxiety disorders (Leonard & Song, 1996), with stress demonstrated to effect immune functioning in both human (Heim & Nemeroff, 2001; Segerstrom & Miller, 2004) and animal studies (Bilbo & Schwarz, 2009; Dhabhar, 2002). Immune dysfunction, in particular inflammation, has been associated with major depressive disorder (MDD) and anxiety (Kiecolt-Glaser et al., 2015; Leonard & Song, 1996; Lotrich, 2015; Maes, 1995; Wohleb et al., 2016). Heightened inflammatory activity has been demonstrated in patients with depression, including upregulated proinflammatory cytokine levels both peripherally (Maes et al., 1997; Maes et al., 1990; Maes et al., 1995; Pariante, 2017) and centrally (Miller & Raison, 2016). What is more, the link between immune dysfunction and psychological disorders has been strengthened with evidence from human studies and animal models demonstrating hyperactive proinflammatory responses following both early life and adulthood stressors (Capuron & Dantzer, 2003; Dantzer, 2004; Fagundes et al., 2013; Kiecolt-Glaser et al., 2015; Slavich & Irwin, 2014; Sominsky et al., 2012b; Walker et al., 2010). Therefore, early life immune activation and other perinatal stressors may contribute to chronic inflammation.

Upregulated inflammatory signalling and illness creates a suite of *sickness behaviours*, which are dramatic modifications to behavioural output and responses, in order to facilitate healing (Aubert, 1999; Dantzer, 2004; Hennessy et al., 2014; Johnson, 2002). This response to infection includes behaviours such as feeling nauseated and feverish, lack of appetite, increased and fragmented sleep, increased pain sensitivity, and a loss of interest in physical and social environments and pleasurable activities (Dantzer et al., 2008). These behavioural modifications parallel many symptoms of depression and certain aspects of anxiety disorders, and have been linked to increases in circulating and central proinflammatory mediators (Connor & Leonard, 1998; Leonard & Song, 1996). Lipopolysaccharide administration, has been consistently demonstrated to induce sickness behaviours and a depressive-like episode in animal rodent models on exposure (Leonard & Song, 2002; Yirmiya, 1996). Additionally in human studies, experimental cytokine increase, the main immunological effectors of a LPS response, immediately leads to a negative mood and the display of sickness behaviours (Brydon et al., 2009; Vollmer-Conna et al., 2004). These behaviours have been characterised as motivational states (Aubert, 1999; Dantzer, 2001, 2004) and play a key role in the governance of behaviours driven by physiological alterations, particularly in the context of psychopathologies.

An important aspect in both depression and anxiety is motivation. In an evolutionary context, emotional expression in man developed as a way of facilitating survival and can be characterised as motivational states of readiness that orientates both perceptions and actions (Aubert, 1999; Lang et al., 1998a). As such, a motivational framework provides a useful scaffold to understand behaviours in both anxiety and depression, and aids the delineation between the two. Under a biphasic theory of emotion and motivation, the brain needs to adaptively respond to either aversive or appetitive stimulation in order to preserve (i.e.

ingestion, mating, nurturing) or protect (i.e. withdrawal or rejection of harmful physiological, psychological or social consequences) (Lang & Davis, 2006; Olatunji & Fan, 2015). When motivational, emotional, and behavioural responses are disproportionate to the threat or if no risk is actually associated, the response is maladaptive and may be associated with psychiatric illness. It is known that motivation plays a modulatory role in anxious and depressed states via complex neurobiology including immune and endocrine involvement (Lang et al., 1998b). Motivational behaviours including sickness behaviours and hypervigilance behaviours are associated with depression and anxiety, and can be induced in animal models with LPS and cytokine administration. Interestingly, LPS administration inhibits some motivational behaviours in the female and not male rat, for example decreasing appetitive sexual behaviour (Avitsur et al., 1997). Additionally, direct cytokine exposure results in general suppression and withdrawal of female sexual and social behaviours, not observed in the male (Avitsur et al., 1999; Avitsur & Yirmiya, 1999; Bluthé et al., 1994; Hennessy et al., 2014). This indicates that LPS administration in the female may differentially impact the female, and potentially cause a state of state de-motivation and change in readiness, akin to depressive-like symptomology.

Our laboratory has previously demonstrated in a rat model that neonatal exposure to LPS on post-natal day (PND) 3 and 5 leads to long-term alterations in central proinflammatory cytokine expression and microglia activation in male animal cohorts (Sominsky et al., 2012b; Walker et al., 2010), indicating sustained immune alterations such as those implicated in depression and anxiety. These findings are in line with results from other laboratories, indicating long term central and peripheral immune and neuroendocrine alterations following an LPS challenge in the early neonatal period (PND 1 through to 14) (Harré et al., 2008; Spencer et al., 2006c). Additionally, our laboratory has continuously demonstrated a robust

anxiety-like phenotype in male animals treated with LPS on PND 3 and 5 (Walker et al., 2009; Walker et al., 2004a; Walker et al., 2008; Walker et al., 2004b). However this phenotype has been less obvious in our laboratory in female cohorts, with Walker et al. (2012) showing no difference in anxiety-like behaviours of LPS treated females in classical measures of rodent anxiety-like behaviour, including the elevated plus maze (EPM) and hole-board apparatus, as well as an attenuated difference in acoustic startle response when compared to male animals of the same treatment. This was regardless of greater circuiting corticosterone (CORT) and Adrenocorticotropic hormone (ACTH) concentration levels in female adult rats compared to males. Also, female rats were demonstrated to spend less time in the hide-box and less time partaking in hypervigilance behaviours in the hide-box/open field test, when compared to males (Walker et al., 2011; Walker et al., 2009). This indicates that although HPA axis functionality appears to be dysregulated in females treated with an early life immunological stressor, it is not specifically manifesting as an anxiety-like phenotype in traditional behavioural tests as it does in males. Interestingly, what has been previously demonstrated in our laboratory in neonatally treated LPS females, is less resistance to restraint, suggesting learned helplessness, significant decreases in maternal care given to F2 generation born of mothers neonatally LPS treated, and reproductive behavioural impairments in an open field test (Walker et al., 2012; Walker et al., 2011).

This previous research from our laboratory suggests that neonatal immune activation (NIA) with LPS, may have differential long-term consequences for female and male animals. The immunological stressor utalised in our laboratory is administered at a critical time point for endocrine, immune and neurobiological development, and it is hypothesised that this may lead to a female behavioural phenotype that differs from the male in regards to the motivational behaviour of preference and protection. Specifically, that this may be skewed

towards a depressive-like state. Hence, the current study aimed to utilise a rodent model of early life bacterial exposure to examine how neonatal immune stress manifests behaviourally in the adult female rat, by means of assays not previously examined in our laboratory. Specifically, we examined the impact of PND 3 and 5 LPS exposure on motivational aspects associated with female rodent sexual behaviour, and anxiety-and-depressive-like behaviours. This consist of assessment of anhedonic and motivational behaviour of the adult female rat in a suite of behavioural tests including the sucrose preference test (SPT), the social interaction test (SIT), and the paced-mating test (PMT), allowing for both the refinement and expansion of the examination of the specific female phenotype that has emerged in previous research from our laboratory. Additionally, HPA and HPG axis assessment was carried out, as well as the assessment of female developmental parameters including puberty onset.

3.2 Methods

3.2.1 Animals

All animal experimental procedures were carry out under the approval of the University of Newcastle Animal Care and Ethics Committee (ACEC, A-2012-2813). 8 experimentally naïve female Wistar rats were obtained from the University of Newcastle animal house and mated with proven male studs in the Laboratory of Neuroimmunology Vivarium. This resulted in 8 litters and a total of 108 pups. For this study, 38 female pups (19 saline derived from 3 litters, 19 LPS derived from 4 litters) were randomly allocated this study. Animals were same-sex and treatment pair housed under normal housing conditions at 21-22°C on a 12 hour light/dark cycle (0600-1800) with food available *ad libitum*. As previously described (Sominsky et al., 2012a; Sominsky et al., 2013; Sominsky et al., 2012b; Walker et al., 2011; Walker et al., 2010; Walker et al., 2004a), at birth (PND 1) whole litters were randomly allocated to either LPS or saline treatment conditions. Whole litters were exposed

to LPS (Salmonella enterica, serotype Enteritidis: Sigma-Aldrich Chemical Co., USA in sterile pyrogen-free Saline, 0.05mg/kg) or Saline (equivolume; Livingstone International, Australia) on PND 3 and again on 5. Briefly, pups were removed from the home cage and transferred to an incubator to maintain body temperature at 36°C, weighed, administered with an intraperitoneal injection of LPS or vehicle, and then returned to the home cage and dam. Animals remained undisturbed until weaning (PND 21) where they were then pair housed with same sex litter mates. Animals were monitored weekly and their weights taken. Females were monitored for day of vaginal opening, and daily oestrus checks via vaginal smears began following puberty onset, taken daily between 1300 - 1500 pm. Female weight was also taken on day of vaginal opening (DVO). The phase of oestrus cycle was determined according to the predominant cell type visible in the vaginal smear at 10x under a light microscope, as per chapter 2 and previous studies from our laboratory (Sominsky et al., 2012a).

3.2.2 Behavioural Testing

Behavioural testing began in early adulthood (PND 70 >). Sucrose preference testing (3 days habituation and 1 day test phase) and SIT was carried out prior to PMT in females. Sucrose preference testing and SIT was counterbalanced in both sexes. Paced mating was carried out as the last assay in order to control for female pregnancy as a confounding variable (Viau, 2002; Walker et al., 2011). Following the paced mating assay, all females were checked for sperm plug presence. A one week rest period was implemented between each test in order to mitigate carry over effects/stress effects of testing (Beery & Kaufer, 2015). Oestrus cycle was controlled for in all behavioural testing where possible. All female animals were tested in the social interaction apparatus during diestrus, and in the paced mating apparatus during proestrus, which is the receptive phase of the oestrus cycle in order to test true biological mating motivation (Mora et al., 1996; Zipse et al., 2000). Sucrose preference testing

habituation was initiated during proestrus, in order for the test day to fall during the diestrus phase of the natural cycle. Differences in oestrus cycling were factored into the model as covariates when cycles deviated during the beginning of habituation to the last test phase of the SPT, due to natural variations in individual cycling. However studies have demonstrated no effect of oestrus cyclicity on sucrose consumption in the SPT (Kentner et al., 2010).

3.2.2.1 Sucrose preference. As described in Chapter 2, animals underwent the SPT using a modified home cage using the 2- bottle choice protocol (Eagle et al., 2016). Briefly, two regular drinking bottles with sipper caps were placed side by side in the food cache area of the cage lid, and food placed in the usual drinking cache. These bottles contained plain room temperature tap water (rats normal drinking water), and a room temperature 2% sucrose solution and were filled with 200mL of both liquids. Bottles were carefully placed through the wire spaces to avoid drips and to ensure the rubber stopper was flush with the wire portion of the cage. Positioning of the drinking bottles was alternated each day to eliminate place preference effects. The contents of both drinking bottles were both weighed (in grams) and measured (in ml) in a volumetric cylinder before and after testing to obtain a standard volume amount of liquid to accurately measure ingestion. All animals were habituated to the testing apparatus for 1 hour over 3 consecutive days prior to testing and this was used as a baseline measurement for liquid consumption. Following the last habituation day, animals were placed on a restricted water schedule for 12 hours to motivate liquid consumption (no water during lights on cycle) (Larson & Dunn, 2001). The following morning (0800 h), animals were transferred into their individual SPT cage, with one animal per cage, and given access to both drinking bottles for a total of 4 hours, with food available ad libitum. Sucrose preference was calculated as a percentage of the amount of sucrose consumed from the total volume of liquid consumed, using a standard equation: (Sucrose

Intake / Sucrose + Water Intake) (Papp et al., 1991) and accounting for differences in individual body weight(Sucrose Intake / Sucrose + Water Intake) /(body weight) x 100) (Forbes et al., 1996; Kentner et al., 2010; Strekalova et al., 2004).

3.2.2.2 Social interaction. The SIT area consisted of an arena with four raised walls made from white opaque Perspex (100cm x 40cm x 50cm) (Duxon et al., 1997; File et al., 2001; Lightowler et al., 1994). All rats, including the naïve conspecific, we habituated to the social interaction chamber for 2 x 15 minute sessions in the two days leading up to the assay. Prior to testing, rats were weight, sex and oestrus cycle (diestrus) matched with a naïve conspecific rat. To begin the assay, the experimental rat was placed in one corner of the arena with the naïve conspecific in the opposing diagonal corner. Placement was counter balanced between all animals and the apparatus was sanitised between subsequent assays. Undisturbed social interaction was recorded by a camera mounted above the arena for 15 minutes and scored on video following testing. Social behaviours were assessed, including frequency of approaching, following, leaving and sniffing behaviours towards the naïve conspecific rat (see Table 3.1) (File & Seth, 2003). Following previous literature (Wesson, 2013), differential sniffing behaviours and timing of behaviours throughout the test were assessed. Face, anogenital (AG), and non-specific (i.e. sniffing on alternative areas to the face and AG regions) sniffs were scored and frequencies compiled. Stress and anxiety-like behaviours of the experimental rat were also assessed including self-grooming and rearing frequency (Dunn & File, 1987; File et al., 2001). Behaviour was scored using the software JWatcher ™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia available at http://www.jwatcher.ucla.edu/), by a scorer blind to treatment conditions.

Table 3.1 Definition of social interaction behavioural variables measured.

Behaviour	Definition
Latency to 1st contact	Latency to first interaction with naïve conspecific
Approach	number of times the experimental rat approached the naïve rat
Follow	total number of times the experimental rat followed the naïve rat
Leave	number of times the experimental rat left the naïve rat
Rear	Number of times the experimental rat reared
Kicks	Number of times the experimental rat kicked the naïve rat
Facial sniff	Number times the experimental rat sniffs the naïve rats face
Non-specific sniff	Number of times the experimental rat sniffs the naïve rat in regions other than the face and A.G, including flank and tail.
Anogenital sniff	Number of times the experimental rat sniffs the naïve rats anogenital area

3.2.2.3 Paced mating. The paced mating arena was constructed from opaque white Perspex box (60 x 70 x 54 cm) and elevated 40 cm above ground level. All testing took place 2 h > the onset of the rats dark cycle. The main arena was then divided into 2 compartments by a clear, removable Perspex partition that has 4 passages cut into the base (4 x 4 cm), allowing for the female to pass through easily, however they are too small for the male to pass through (see Figure 2.11). A proven male stud was placed on one side of the chamber. This positioning of male side was counter balanced to control for place preference. A sexually naïve experimental female in the proestrus phase of her cycle was placed on the other side of the chamber. Both male and female rats were previously acclimatised to the chamber for 2 x 15 minutes time periods in the week prior to testing to minimise novel environment

behaviours. The test was completed after a 30 minute period (as per Zipse et al, 2000). All bedding was discarded and then refreshed between individual runs and the PMT chamber was sanitised between subsequent assays. All testing took place in a dark, undisturbed room under infrared lighting and was recorded using an infrared camera. All female mating behaviours (see Table 3.2) were visually quantified by an experienced experimenter blind to treatment groups using the software JWatcher ™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia available at http://www.jwatcher.ucla.edu/).

Table 3.2 Definition of paced mating behavioural variables measured.

Behaviour	**Definition**
Darts	Total number darts in either male and female chamber
Hops	Total number hops in either male and female chambers
Lordosis	Total times female displays lordosis
Female grooming	Total number of grooming behaviours exhibited in either chamber
Rears	Total number of times the experimental female rat reared in either chamber
Kicks	Total number of times the experimental rat kicked the male stud
Entries and exits	Number times the female entered and exited the male chamber
Male mounts	Number of times male attempts to mount the female
Female sniff male	Number of times the female rat sniffs male stud
Male sniff female	Number of times the male stud sniffs the female
Exploratory sniffing	Number of times female sniffs arena area of either chamber
Freezing	Time spent immobile/frozen by female animal

3.2.3 Blood and Tissue Sampling

A subset of animals were culled on PND 5, 2 hours following injections for analysis of circulating tumour necrosis factor alpha (TNF-α) in order to assess inflammation from LPS injection (Kakizaki et al., 1999; Saban et al., 2001). Neonatal animals were culled by rapid decapitation and trunk blood was collected into EDTA coated tubes, centrifuged at 1000g at 4°C for 20 minutes. Plasma supernatant was then collected and stored at -20 until assessment of proinflammatory cytokine TNF-α by ELISA (R&D Systems Rat TNF alpha Quantikine ELISA Kit, RTA00, minimum detection rate <5 pg/mL, intra- and inter-assay variability 2.1 - 5.1% and 8.8 - 9.7% respectively, n = 7 per group derived from 3-4 litters per group).

In adulthood, blood was sampled via saphenous bleed both prior to social interaction test (baseline) and 30 min following social interaction testing (post-test) for assessment of HPA axis activation via CORT radioimmunoassay (Herman et al., 2016). Blood samples were centrifuged at 1000g at 4°C for 20 minutes, and plasma supernatant was collected and stored at -20 until assessment. Circulating levels of plasma CORT were assessed using a rat CORT 125I radioimmunoassay kit (MP Biomedicals, CA, USA). The recovery of free CORT was 100%, with a mean inter- and intra-assay variability of 4.4 and 6.5%, respectively. saphenous blood was taken from female animals prior to and post paced mating testing for assessment of luteinising hormone (LH) (Abnova, Sapphire Bioscience, KA2332; mean inter- and intra-assay variability of 5.16 and 5.4%, respectively) and follicle stimulating hormone (FSH) (Abnova, Sapphire bioscience, KA2330; mean inter- and intra-assay variability of 5.88 and 6.35%, respectively). All samples were assayed once in duplicate. Blood was not taken from animals during the SPT due to the ethical considerations of the water restriction protocol associated with the test phase of the SPT. Following the end of all assays, rats were deeply anesthetised and tissue and cardiac blood was collected.

3.2.4 Statistical Analysis

Data were analysed using IBM SPSS statistics (Version 24, IBM Australia) with a repeated measures analysis of variance (ANOVA), analysis of covariance (ANCOVA), and student independent sample t-test where appropriate, with pairwise comparisons between treatments groups carried out using the Bonferroni correction. Covariates including litter size, body weight, and gender ratio were included in the analysis and where non-significant, they were removed from the model analysis and analysis adjusted accordingly. All assumptions were met unless otherwise mentioned in results, with appropriate correction applied and reported. Data are presented here as the mean $\pm$ standard error of the mean (SEM). Significance was assumed at $p \leq .05$.

3.3 Results

3.3.1 Neonatal Weight Gain

There was a main effect of treatment ($F(1,25) = 4.657$, $p = .041$), with pairwise comparisons indicating that saline allocated animals weighed significantly less than LPS treated animals prior to treatment on PND 3, however this difference was < 1 gram, ($t(25) = 2.85$, $p = .009$) (Figure 3.1, A). No significant difference in weight gain was observed between treatment days PND 3 and PND 5 between saline and LPS treated animals, furthermore no significant time x treatment effect existed ($F(1,25) = 1.14$, $p = .296$). An expected significant main effect of age existed, with PND 5 animals weighing more than PND 3 animals overall ($F(1,25) = 640.81$, $p < .005$).

3.3.2 Neonatal Circulating Tumour Necrosis Factor Alpha (TNFα).

As expected, LPS treated animals had significantly higher circulating TNFα levels on PND 5, 2 hours following injections, compared to saline treated controls, ($t(12) = 3.35$, $p = .014$) (Figure 3.1, B).

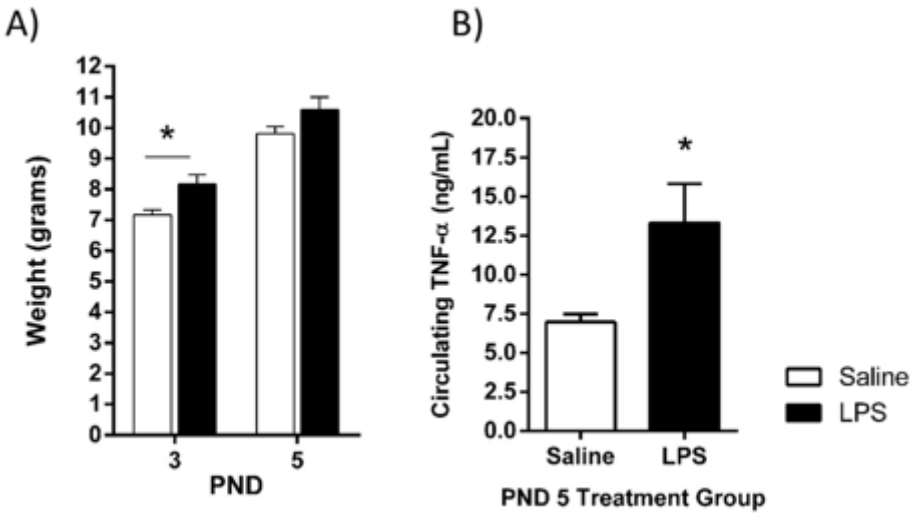

Figure 3.1. A) Average neonatal female weights (g) taken prior to treatment on PND 3 and PND 5. Saline animals weighed significantly less on PND 3. B) LPS animals had significantly higher circulating levels of TNFα two hours post treatment on PND 5. Hollow bars depict saline treated animals, filled bars depict LPS treated animals, means ± SEM graphed. * denotes significance at $p < 0.05$.

3.3.3 Developmental Weight Gain

A repeated measures ANOVA of weight gain differences indicated a significant main effect of time ($F(4.14, 103.85) = 18.39$, $p = 0.001$) indicating all animals increasingly gained weight on subsequent weeks. A significant time x treatment interaction effect existed ($F(4.14, 103.85) = 2.55$, $p = .042$), using the greenhouse-Geisser correction for violation of sphericity. Pairwise contrasts indicate that LPS treated animals gained significantly more weight than saline treated animals between weaning (PND 22) and pre-puberty (PND 29), ($t(25) = 2.39$, $p = .024$). A trend existed between PND 29 – 36, with LPS treated animals gaining more weight during this week than saline animals, however this was non-significant, ($t(25) = 1.90$, $p = .069$). In early adulthood (between PNF 57 and PND 64), LPS treated females gained significantly less weight than saline treated controls, ($t(25) = 2.84$, $p = .014$) (Figure 3.2).

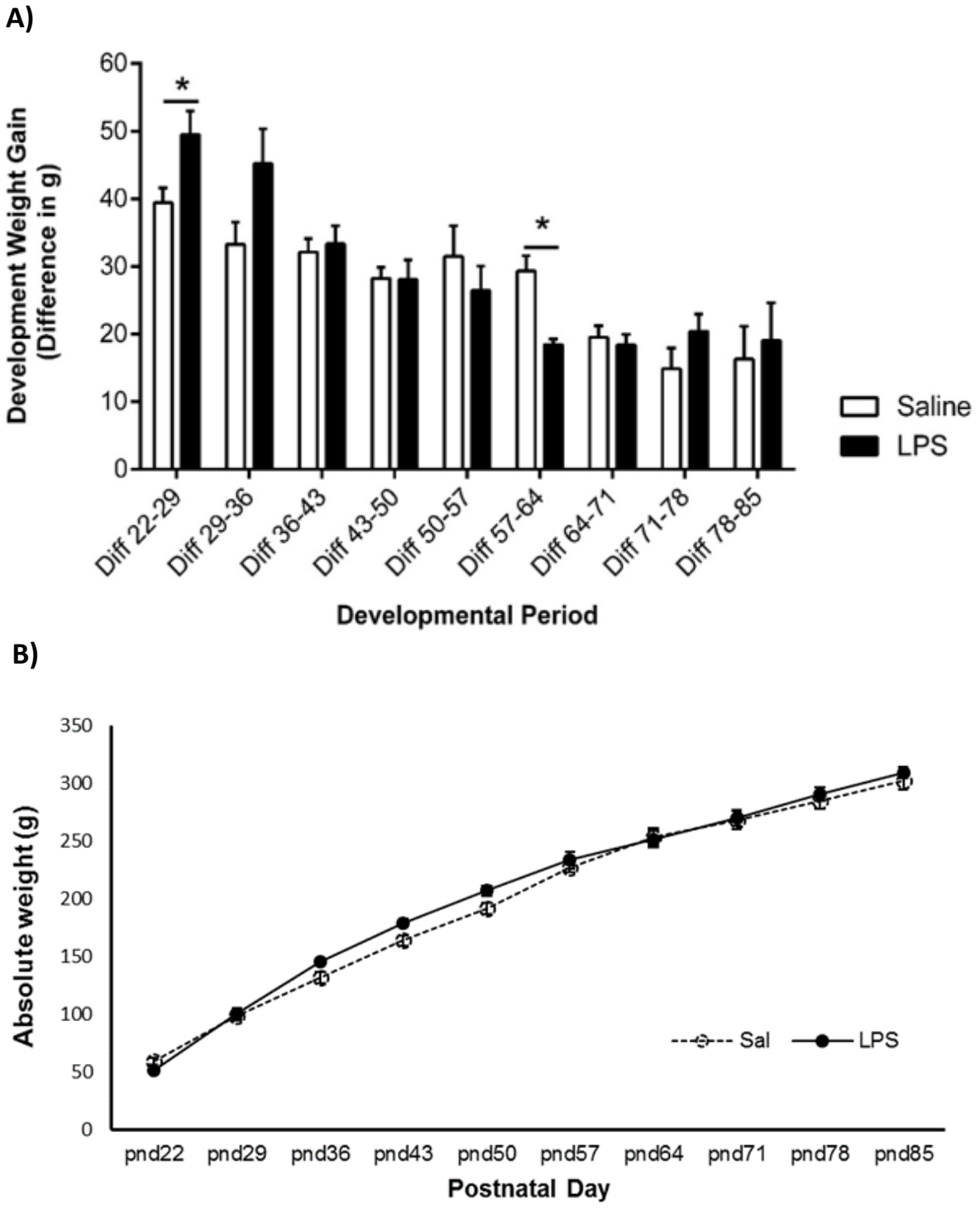

Figure 3.2. A) Difference in weight gain (g) between saline and LPS animals during weekly developmental weight monitoring. LPS animals gained significantly more weight between PND 22 and PND 29 than saline treated controls (LPS, M = 49.48, SEM = ± 3.48; saline M = 39.40, SEM = ± 2.21). LPS animals (M = 18.45, SEM = ± 0.8) gained significantly less weight than saline animals (M = 29.34, SEM = ± 2.24) between PND 57 and PND 64. Hollow bars depict saline controls, filled bars depict LPS treated animals. Mean ± SEM graphed. * denotes significance at $p < 0.05$. B) Average absolute weight gain (g) and SEM of saline and LPS treated animals. Hollow markers/dashed line represents saline treated controls, filled markers and solid line represents LPS treated animals.

3.3.4 Day of Vaginal Opening, Weight at Puberty and Oestrus Cyclicity

LPS treated animals came into puberty significantly earlier than saline treated animals, (F(1, 25) = 4.46, p = .045) with DVO in LPS animals an average of 2 days earlier than saline treated controls (Figure 3.3, A). There was no significant difference in weights between groups observed on DVO (Figure 3.3, B). Shapiro-Wilk test of normality was violated for DVO, however ANOVA is reported to be particularly robust to this violation (Maxwell & Delaney, 2004). The day of first proestrus followed by a normal phase of oestrus cyclicity was significantly earlier in LPS treated animals (F(1, 25) = 14.8, p = .001) Figure 3.3, C). Cycle regularity did not differ between LPS-treated or saline-treated females at any time point (data not shown).

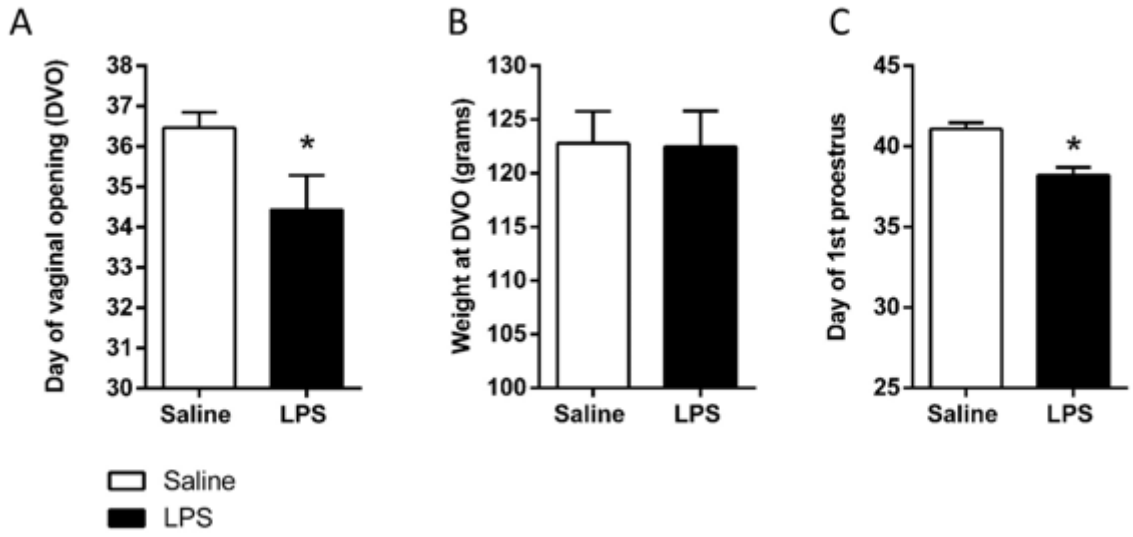

Figure 3.3. A) LPS treated animals demonstrated significantly earlier onset of puberty, as marked by DVO. B) There was no significant difference in weights between treatment groups on DVO (LPS, M = 117.83, SEM = ± 0.85; saline, M = 122.79, SEM = ± 0.38). C) LPS treated animals demonstrated a significantly earlier day of 1st proestrus followed by a normal oestrus phase. Hollow bars depict saline controls, filled black bars depict LPS treated animals means ± SEM graphed. * denotes significance at p < .05.

3.3.5 Sucrose Preference Assay

Repeated measures ANOVA using LPS and saline as between subject variables, and habituation and test days as within subject variables demonstrated a significant day x treatment effect for sucrose preference (in mL) ($F(3, 75) = 5.685$, $p = .001$, and significant effect of day ($F(3, 75) = 12.766$, $p < .001$). Independent samples t-tests indicated that saline treated animals drank significantly more sucrose on habituation day (HD) 1 compared to LPS animals ($t(25) = 2.849$, $p = .009$), however LPS animals consumed *more* sucrose on HD 2 ($t(25) = 2.105$, $p = .046$) (Figure 3.4, A) with body weight on all corresponding test days being a significant cofactor. As such, percentage of sucrose preference in mLs per individual body weight was analysed in order to control for differences in consumption due to individual body weight and give a truer representation of preference. Repeated measures ANOVA indicated a significant day x treatment effect ($F(3, 75) = 4.048$, $p = .010$), and main effect of day ($F(3, 75) = 11.133$, $p < .001$) and treatment ($F(1, 25) = 13.937$, $p = .001$), with animals consuming less sucrose on HD 1, compared to HD 3 and test day. Pairwise comparisons indicated that LPS treated animals consumed a greater percentage of sucrose (in mLs) per body weight, when compared to saline treated controls on HD 2 ($t(25) = 4.438$, $p < .001$, HD 3 ($t(25) = 2.463$), $p = .014$, and during the SPT test phase ($t(25) = 3.865$, $p = .001$ (Figure 3.4, B, continue on next page).

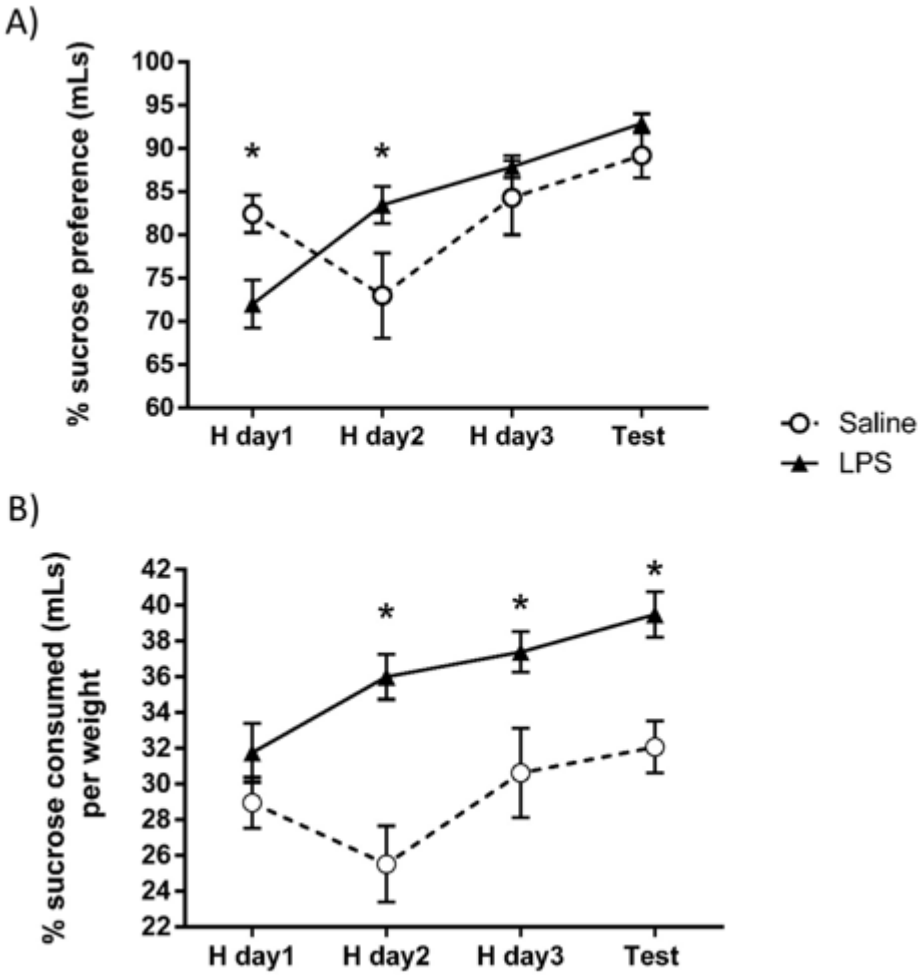

Figure 3.4. Graphs plot sucrose preference over a 3 day habituation period of 1 hour per day, and 4 hour test phase on day 4. A) Indicates sucrose preference as a % of total liquid consumed per day by saline (open circles, dotted line) and LPS (Filled triangles, solid line) treated animals. LPS treated animals consumed significantly less saline on HD 1 (H day 1) and significantly greater sucrose on HD 2 when compared to saline controls. B) Depicts % sucrose consumed by body weight, indicating LPS treated animals consumed a significantly greater % of sucrose per body weight than saline treated controls on HD 2, HD 3 and during the test phase. Mean $\pm$ SEM graphed, * denotes $p < .05$ between treatment groups on that particular habituation or test phase.

3.3.6 Social Interaction Behaviours

3.3.6.1 Analysis of complete duration behavioural totals. Independent samples t-tests on total counts for behaviours during the complete duration of the social interaction assay were carried out. These behaviours included; approaching, following and leaving the naïve rat, non-specific sniffing (flank, tail), facial sniffing and AG area sniffing of the naïve rat, rearing of test rat, and kicks towards naïve rat (see Table 3.1). LPS treated animals reared significantly more during the entire test than saline treated animals ($t(22) = 3.931$, $p = .001$) (Figure 3.5, A). Additionally, LPS animals delivered a significantly greater amount of kicks towards the naïve conspecific when compared to saline treated controls ($t(22) = 2.145$, $p = .047$) (Figure 3.5, B). There were no significant difference in total counts for other behaviours (Figure 3.5, C). No differences existed in latency of first contact between groups. Furthermore, no significant difference existed between LPS and saline treatments for any sniffing behaviours or grooming behaviours. See Table 3.3 for all variable mean, SEM and SD.

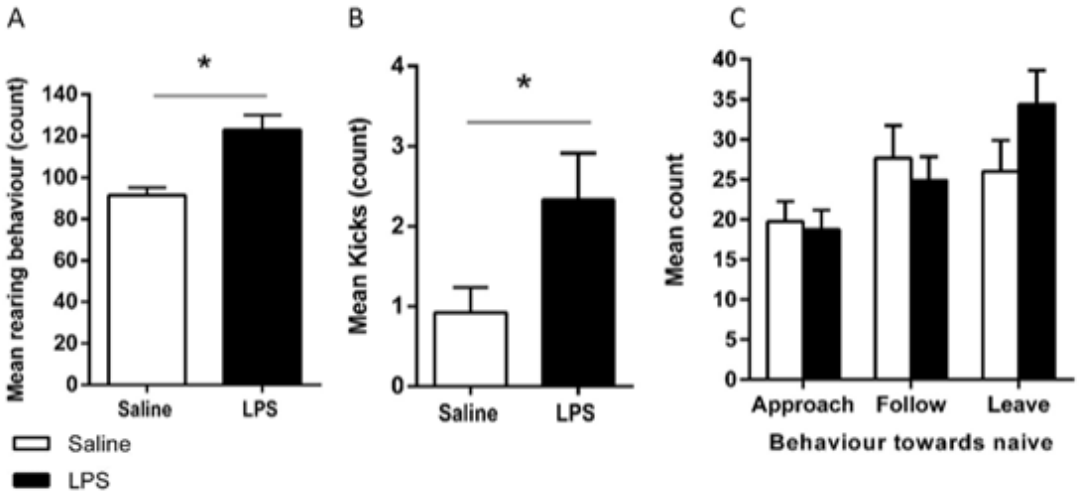

Figure 3.5. Mean counts of rearing behaviour (A) and mean kicks (B) towards naïve conspecific during the complete duration (15 min) of the social interaction test. C) No significant difference existed for other behaviours. Mean +SEM graphed, * denotes $p < .05$.

3.3.6.2 Time bin analysis of social interaction behaviours. Time bin (TB) durations were analysed in order to discern differences in behaviours throughout testing to reflect the dynamic nature of rodent social interaction. A main effect of time existed for approach behaviours ($F(4, 88) = 4.48$, $p = .002$) with pairwise comparisons indicating that this difference was driven by both treatments approaching naïve animals significantly more in the first 3 minutes of the test (TB 1) compared to the last 3 minutes (TB 5) ($p = .013$) (Figure 3.6, A). A main effect of time existed for leaving behaviours of the test rat towards the naïve rat ($F(4, 88) = 7.48$, $p = .000$), with both treatments leaving the naïve rats significantly less in the latter part of the test (TB 4, $p = .005$; TB 5, $p = .007$) (Figure 3.6, B). A main effect of time was evident for following behaviours ($F(4, 88) = 5.33$, $p = .001$), with both treatment groups following the naïve conspecific more in TB 1, compared to TB 4 ($p = .03$) and TB 5 ($p = .002$) (Figure 3.7, A). A main effect of time ($F(4, 88) = 5.09$, $p = .001$) and treatment ($F(1, 22) = 15.45$, $p = .001$) existed for rearing behaviours, with less rears in the latter part of the test (TB 5) compared to the beginning (TB 1) ($p = .047$) for all animals (Figure 3.7, B). Furthermore, LPS animals rearing significantly more in TB 1 ($t(22) = 3.82$, $p = .001$), compared to saline controls, as well as significantly greater rearing behaviour of LPS animals in TB 2 ($t(22) = 3.28$, $p = .003$), TB 3 ($t(22) = 2.74$, $p = .012$), and TB 5 ($t(22) = 2.25$, $p = .035$) (Figure 3.7, B). No significant interactions or main effects of kicking behaviour were demonstrated between groups in any TB (data not shown). There was a significant main effect of time ($F(4, 88) = 4.265$, $p = .003$) on grooming behaviours only, with all animals displaying increased grooming in TB 2 compared to TB 4 ($p = .012$), and TB 5 ($p = .047$) (Figure 3.8).

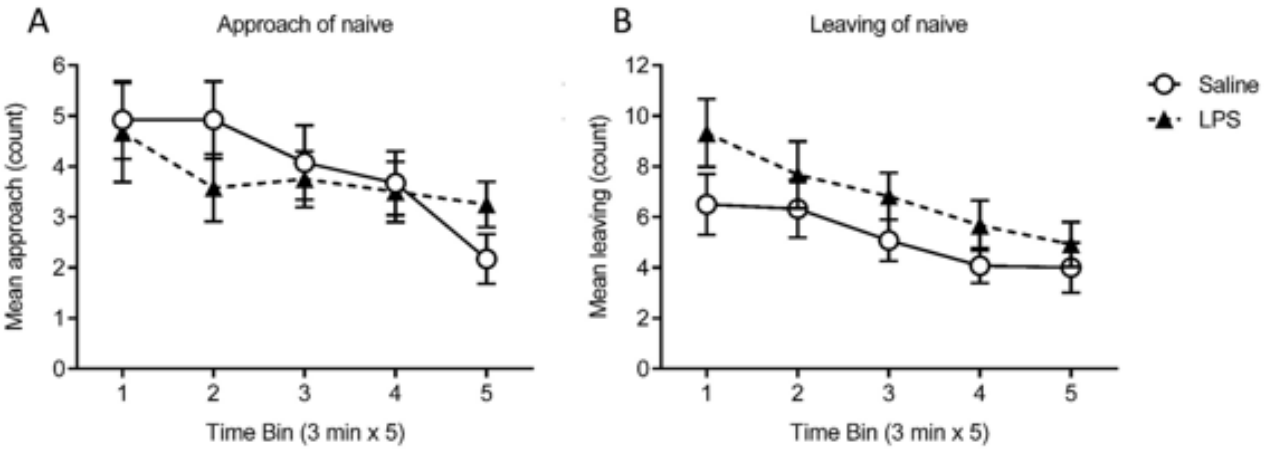

Figure 3.6. Mean frequency of behaviours of test rat toward naïve conspecific, as defined by 3 min time bin (TB) (total time 15 min). A) Approaching behaviours of test rat. B) Leaving behaviour of the test rat. Opened circles denote saline treated animals. Filled black triangles denote LPS treated animals. Mean ± SEM per time bin graphed.

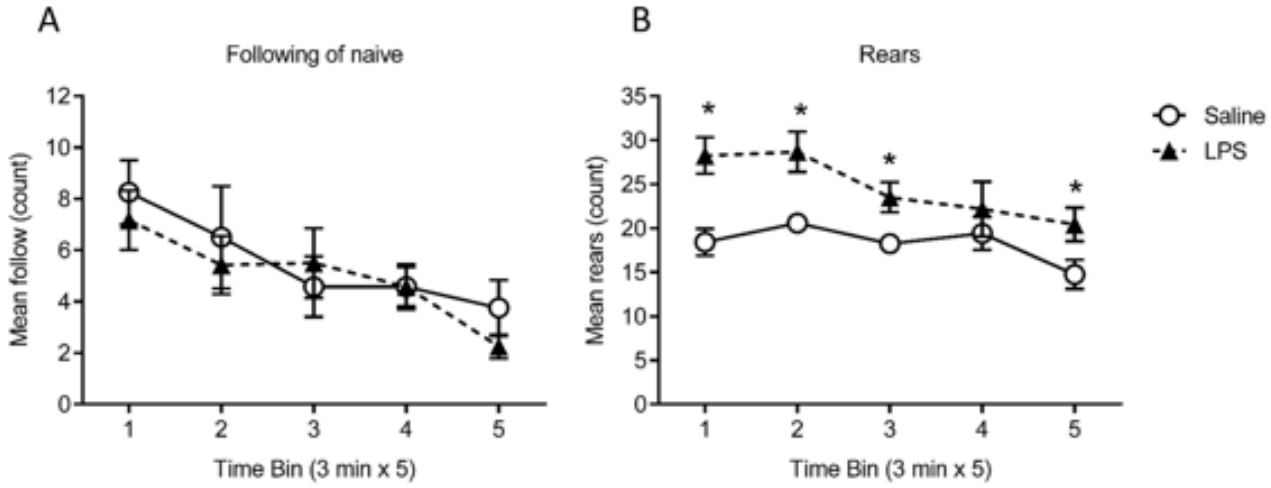

Figure 3.7. Mean frequency of behaviours of test rat toward naïve conspecific, as defined by 3 min time bins (TB) (total time 15 min). A) Following behaviour of test rat. B) Rearing behaviour of the test rat, where LPS animals reared a significantly greater amount in TB1, TB2, TB3, and TB5. Opened circles denote saline treated animals. Filled black triangles denote LPS treated animals. Mean ± SEM per time bin graphed, * denotes significant difference between treatment groups of $p < .05$.

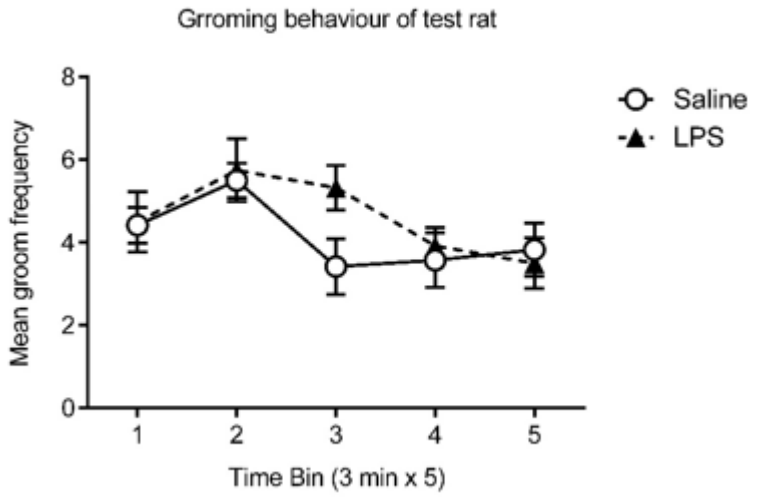

Figure 3.8. Graph depicts frequency of grooming behaviour of test rat. A significant effect of time existed, where rats in both treatments groomed less in time bin (TB) 4 and TB 5 compared to TB 2. Mean ± SEM per time bin graphed.

3.3.6.3 Time bin analysis of social interaction sniffing behaviours. There was significant main effect of time on sniffing behaviour ($F(4, 88) = 9.66$, $p = .0001$), with animals demonstrating a greater number of non-specific and flank sniffs in TB 1 compared to TB 4 ($p = .0001$) and TB 5 ($p = .0001$) (Figure 3.9, A). A significant main effect of time was demonstrated in AG behaviours ($F(4, 88) = 8.26$, $p = .0001$) with less AG sniffing occurring in both treatments in TB 5 ($p < .05$) compared to all others (Figure 3.9, B). A significant time x treatment interaction existed for facial sniffing ($F(4, 88) = 3.5$, $p = .011$), driven by difference between saline and LPS groups in TB 3 where controls demonstrated greater face sniffing behaviours ($t(16.5) = 2.15$, $p = .045$), and the opposite in TB 5 ($t(22) = 2.3$, $p = .031$) with greater facial sniffing exhibited by LPS females (Figure 3.9, C).

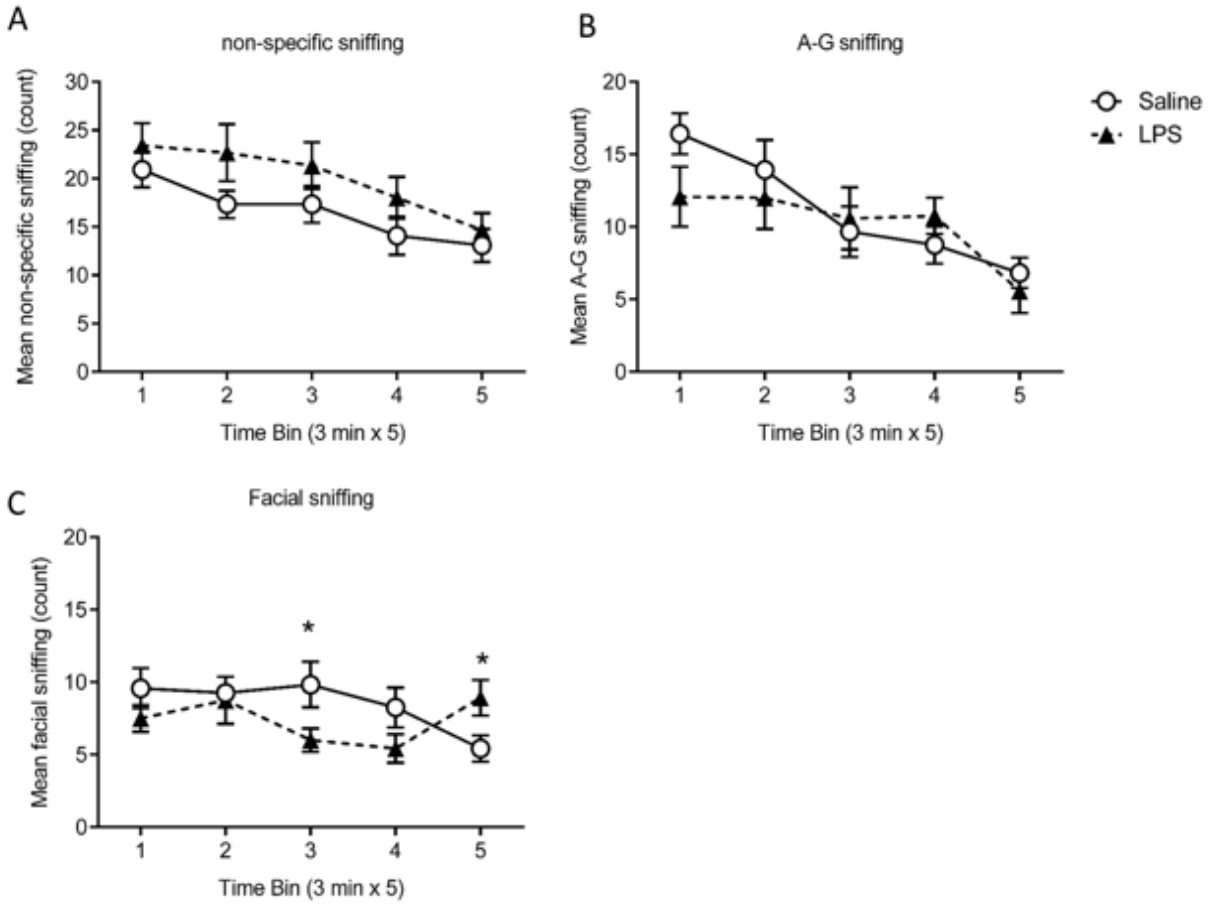

Figure 3.9 A) & B) Both treatments performed more sniffs in TB 1, compared to the later time bins. C) Saline females demonstrated a significantly greater number of facial sniffs in TB 3, however LPS animals demonstrated greater facial sniffs in TB 5. Mean ± SEM per TB graphed, * denotes significant difference between treatment groups of $p < .05$.

3.3.6.4 Social interaction circulating corticosterone levels. Analysis of CORT levels in samples taken both prior to and post SIT indicated a significant main effect of time (pre or post-test) ($F(1, 17) = 50.715$, $p < .0001$) and treatment ($F(1, 17) = 8.23$, $p = .011$). Pairwise comparisons indicate that LPS treated females had significantly greater circulating levels of CORT both before and after the SIT ($p < .05$) (Figure 3.10, A), and post-test CORT levels were significantly greater than pre testing CORT levels across both groups (Figure 3.10, B. See Table 3.3 for mean, SEM, & SD). No significant interaction effect existed ($F(1,17) = .336$, $p = .570$).

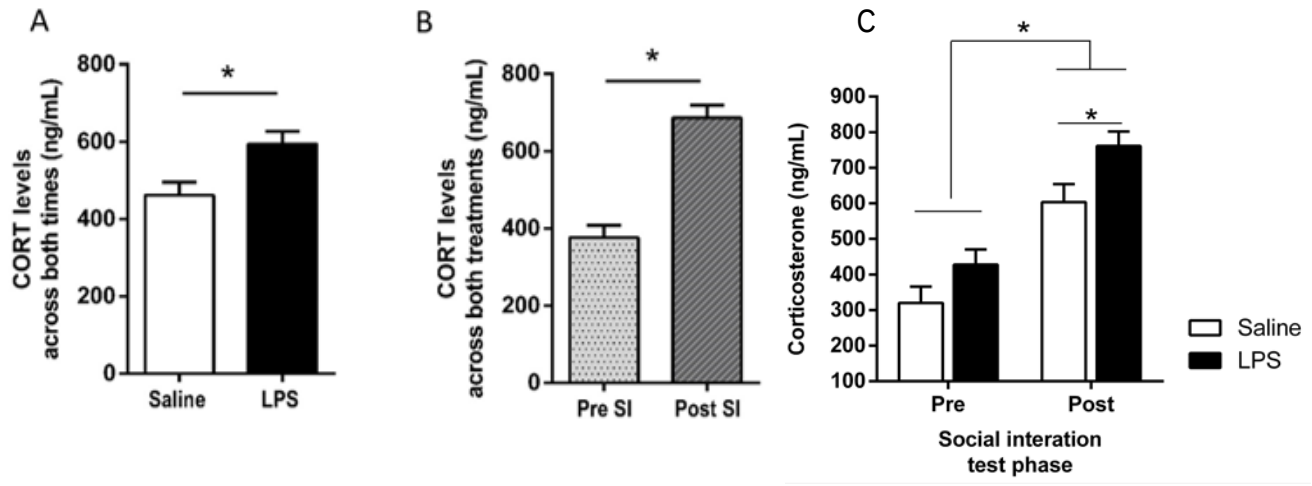

Figure 3.10. A) Corticosterone levels in saline and LPS animals collapsed across time, where LPS females had significantly higher circulating CORT levels both pre and post-test. B) Significant effect of time on CORT levels (Pre-test = light grey dot, post-test = dark grey diagonal line). Both treatments demonstrated significantly higher CORT levels following social interaction testing, compared to pre-testing levels. Mean ± SEM graphed, * denotes significant difference of $p < .05$. C) pre and psot CORT levels for both treatment groups.

Table 3.3 Means, SEM and SD of frequency of social interaction total variables.

SIT Variable (totals)	**Saline**			**LPS**		
	Mean	***SEM***	***S.D***	***Mean***	***SEM***	***S.D***
Latency 1st contact (s)	0.59	0.06	0.21	0.53	0.10	0.17
Approach	19.75	2.538	8.792	18.75	2.419	8.379
Follow	27.67	4.053	14.041	24.92	2.419	10.131
Leave	26	3.894	13.491	34.42	4.206	14.569
Rear	91.42*	3.789	13.125	123*	7.084	24.539
Kicks	.92	0.313	1.084	2.33	0.582	2.015
Facial sniff	42.33	3.283	11.372	36.50	3.397	11.767
Non-specific sniff	82.75	6.734	23.328	100.08	9.018	31.239
Anogenital sniff	55.58	3.496	12.109	50.92	6.362	22.039
Grooming	20.75	1.871	6.482	23	1.784	6.179
CORT PRE ng/mL	320.04	45.54	136.59	427.53	42.766	135.235
CORT POST ng/ml	603.61	50.79	152.35	761.39	40.455	127.933

***** denotes significant difference between treatments, $p < .05$.

3.3.7 Paced Mating Behaviours

3.3.7.1 Motivational, proceptive and receptive behaviours. Females neonatally treated with LPS demonstrated a significantly greater number of entries ($t(22) = 16.21$, $p = .012$ and exits ($t(22) = 17.2$, $p = .009$) of the male chamber, compared to saline treated females (Figure 3.11, A). LPS treated females performed significantly less hops ($t(22) = 2.98$, $p = .007$) than saline treated animals (Figure 3.11, B) and kicked the male stud significantly greater than saline treated controls ($t(16.26) = 5.98$, $p < .000$) (Figure 3.11, C). Additionally, there was a strong trend for LPS animals to perform less darts than saline treated female ($t(21.93) = 2.03$, $p = .054$) (Figure 3.11, D). Receptive lordosis behaviour was performed significantly less in LPS treated females, compared to saline controls ($t(15.23) = 3.73$, $p = .002$) (Figure 3.11, E). However, male studs attempted to mount LPS treated female significantly more than saline treated females ($t(11.92) = 3.746$, $p = .003$) (Figure 3.11, F). There was a trend for male studs to sniff LPS treated animals a greater number of times, however this was non-significant ($t(16.26) = 2.03$, $p = .06$). Equal variance not assumed values reported for Levene's Test variations (See Table 3.4 for mean, SEM and SD of variables).

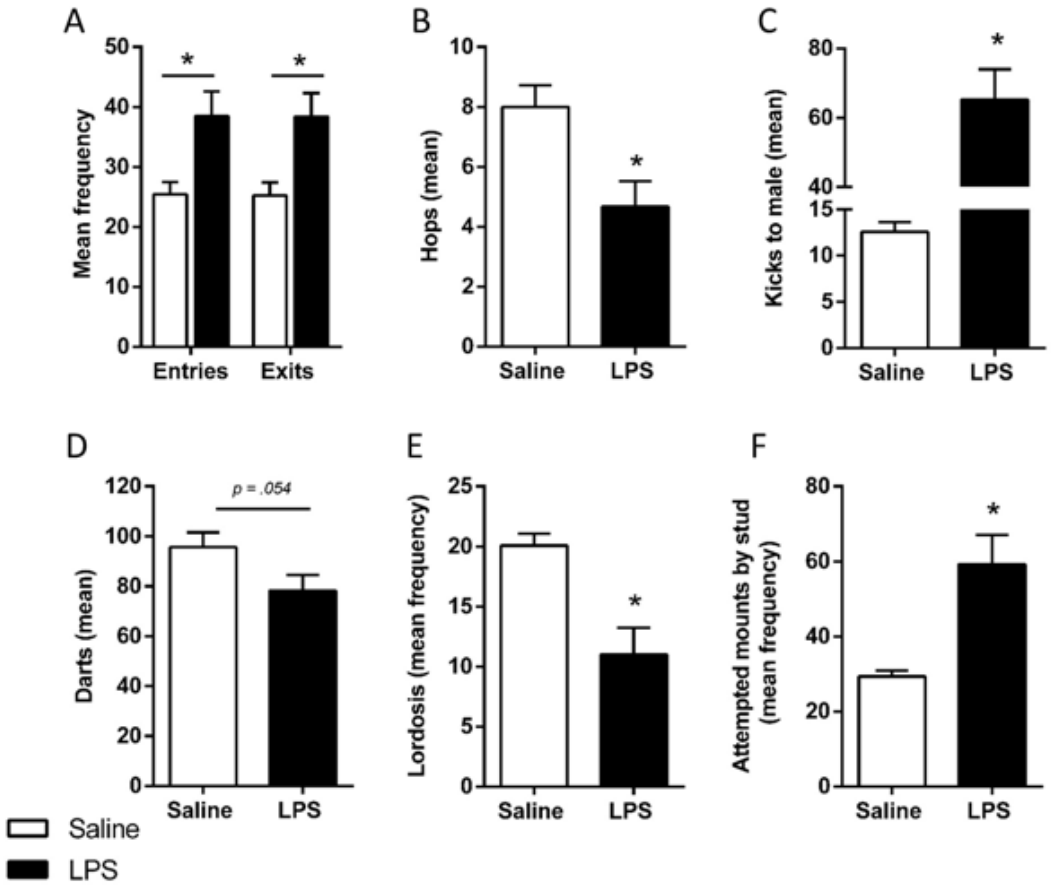

Figure 3.11. A) LPS treated females visited the male chamber significantly more than saline treated animals, however demonstrated significantly less hops (B) and significantly more kicks (C). D) A strong trend existed for LPS animals to perform less darts than saline counterparts. E) Female animals performed significantly fewer lordosis behaviours than saline treated animals, regardless of the significantly greater attempted mounts by the male stud towards LPS treated females (F). Mean ± SEM graphed, * denotes significant difference between treatment groups of $p < .05$.

3.3.7.2. Anxiety-like and hypervigilance behaviours. Females treated with LPS displayed a significant increase in total rearing behaviours ($t(21.99) = 3.89$, $p = .001$) (Figure 3.12, A). Further discrimination indicated that females reared significantly more in their private chamber ($t(22) = 3.24$, $p = .004$, than when with the male in his chamber ($t(18.59) = 1.85$, $p = .08$) (see Figure 3.12, B). There were no significant difference in overall grooming time between treatment groups, however LPS female did groom themselves significantly

longer in the male chamber compared to saline control animals ($t(22) = .46$, $p = .044$) (Figure 3.12, C). There were no significant difference in freezing behaviours or exploratory sniffing behaviours between the two treatment groups (see Table 3.4 for mean, SEM and SD of variables). Equal variance not assumed values reported for Levene's test variations.

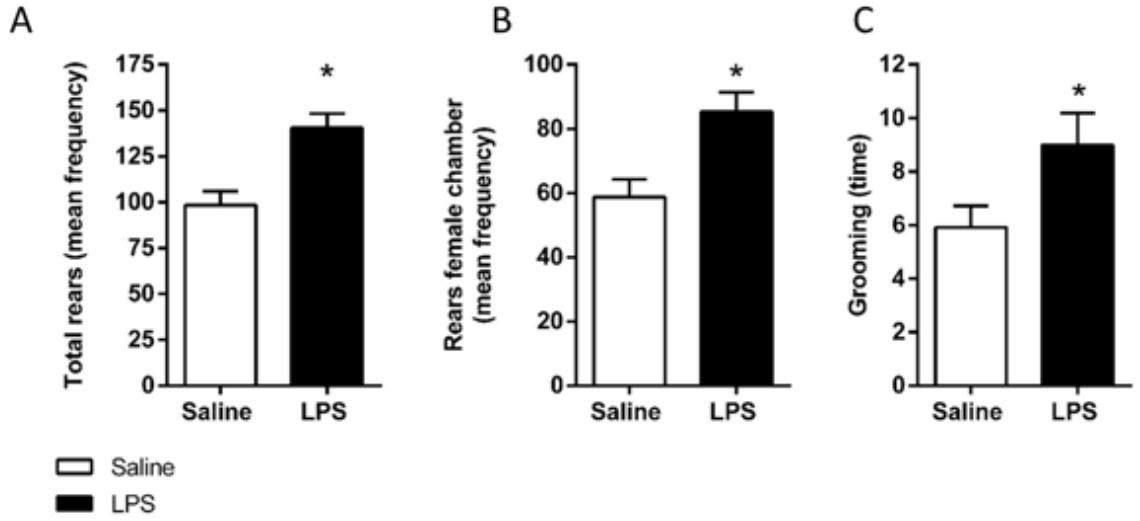

Figure 3.12. A) Total female rears for entire test duration. B) LPS treated female reared significantly more in the female chamber, compared to saline controls. C) LPS animals groomed more frequently than saline treated females. Mean ± SEM graphed, * denotes significant difference between treatment groups of $p < .05$.

***3.3.7.3 Sperm plug detection*.** Sperm plugs were detected by visual inspection and confirmed by vaginal smear. Saline treated animals displayed sperm plugs and presence 100% of the time following paced mating, indicating a successful mount by the male following female lordosis behaviour. Sperm plugs were detected in LPS treated females 92% of the time, with one animal not performing lordosis during the test phase and therefore zero successful mounts were considered accomplished by the male on this female (data not shown).

***3.3.7.4 Female HPG axis assessment during paced mating: luteinising hormone and follicle stimulating hormone*.** Repeated measures analysis of circulating LH level in samples

taken prior to and following paced mating indicate no significant integration effect ($F(1, 10) = .204$, $p = .664$) or main effect of time ($F(1,10) = 2.053$, $p = .19$), however it was observed that LH levels were higher in both treatments following paced mating (see Figure 3.13 A). Data from 4 animals was excluded from the LH analysis due to high deviation in replicates and results greater than 2 standard deviations away the mean. For circulating FSH levels, no significant interaction ($F(1,14) = 1.096$, $p = .313$) or main effect of time ($F(1,14) = .602$, $p = .358$) existed (see figure 3.13 B). M, SEM and SD are reported in table 3.4.

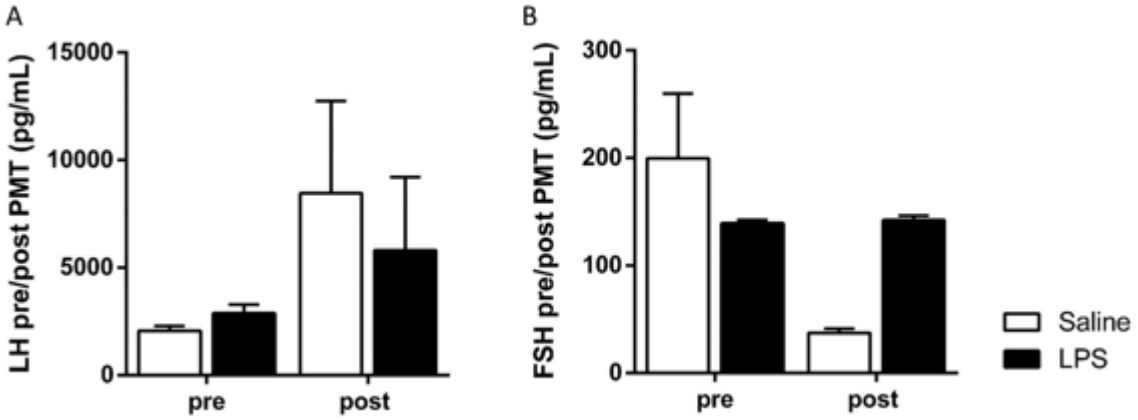

Figure 3.13. A) Mean circulating LH pre and post PMT (pg/mL) ± SEM. B) Mean circulating FSH pre and post PMT (pg/mL) ±SEM. Hollow bars represent saline treated females. Filled bars represent LPS treated females.

Table 3.4. Mean, SEM and SD of paced mating variables.

PMT Variable	**Saline**			**LPS**		
	Mean	***SEM***	***S.D***	***Mean***	***SEM***	***S.D***
Darts	95.67	5.866	20.322	78.33	6.19	21.44
Hops	8*	0.718	2.486	4.67*	0.856	2.964
Lordosis	20.08*	0.996	3.45	11*	2.226	7.71
Female grooming	23	1.813	6.281	24.5	3.302	11.438
Rears	98.42*	7.624	36.411	140.58*	7.684	26.617
Kicks to male	12.58*	1.062	3.679	65.25*	8.783	30.269
Entries	25.42*	2.072	7.179	38.5*	4.128	14.299
Exits	25.25*	2.168	7.509	38.42*	3.9	13.514
Male attempted mounts	29.33*	1.602	5.549	59.25*	7.824	27.103
Female sniff male	94.75	5.497	19.041	106.83	9.054	19.583
Male sniff female	66.92	11.49	39.803	118.58	22.771	78.88
Exploratory sniffing	241.83	23.403	81.07	297.25	36.421	126.164
Freezing	3.58	0.811	2.811	3.5	0.723	2.505
LH PRE (pg/mL)	2070.32	223	499	2887	397	1124
LH POST (pg/mL)	8450	4367	11555	5794.81	3433	7676.9
FSH PRE (pg/mL)	199.56	60	172.31	139.09	3.9	11.06
FSH POST (pg/mL)	137.21	4	13.38	142.12	4.01	13.18

***** denotes significant difference of means between treatment groups, $p < .05$.

3.4 Discussion

The early life environment plays a key role in shaping both developmental and long-term outcomes (Bilbo & Klein, 2012; Bilbo & Schwarz, 2009, 2012; Ellis et al., 2006; Galic et al., 2008; Patterson, 2002; Spencer et al., 2006a; Spencer et al., 2005). The current study aimed to extend on previous work by examining the behavioural phenotype in adult females rat associated with LPS exposure in the early neonatal period. This included the assessment of the motivational aspects of both anxiety-like and depressive-like behaviours in a female cohort, reproductive development, and endocrine measurements associated with HPA and HPG axis function. Here, we demonstrate that female adult animals treated with LPS as neonates do not express anhedonic behaviour in a sucrose preference test as expected, however consumed greater sucrose. Additionally, assessment of motivational and anxiety-like behaviours in the SIT and PMT indicate that female rats treated with LPS do not display decreases in the motivational aspects of these tests, however female rats do demonstrate alterations in some anxiety-like behaviours and behaviours associated with social communication and social cues in both the SIT and PMT, that are particularity associated with reproductive parameters. Furthermore, female rats exposed to NIA demonstrated general elevated HPA axis activity in response to the SIT, and HPG hormone alterations following the PMT assay (+hormones). These findings suggest that exposure to immune perturbation in the early life period may perinatally program a suboptimal reproductive behavioural phenotype in the female adult rat.

In the neonatal period, weights were monitored and immune activation assessed for efficacy of treatment. The neonates randomly allocated to the LPS treatment group were significantly smaller on the first day of treatment (PND 3), however the mean difference was less than 1 gram and falls within a normal distribution of offspring weight. Furthermore, litter

size was not a significant covariate in the model, suggesting that this difference may not be attributed to slight fluctuations in litter size. In order to maintain a naturalistic, ecologically valid and non-confounding model of early life stress, our laboratory does not disturb litters prior to injections on PND 3 and PND 5. This ensure minimisation of confounding stress variables that may alter the validity of the immune stressor. This is in line with previous research from our laboratory, where no culling of litters has taken place. Neonatal rats treated with LPS and saline controls did not differ in regards to amount of weight gained between treatment days, and the initial differences in weight were maintained through to PND 5. This suggests that maternal care was constant thought neonatal immune treatment, ensuring that changes in maternal behaviours cannot be attributed to any behavioural or mechanistic findings reported here. Previous findings from our laboratory have demonstrated that LPS animals tend to gain less weight between treatment days PND 3 and PND 5, as an indication of ephemeral sickness behaviours demonstrated by the pup after LPS injections. In a confirmation of efficacy of immune treatment, LPS treated neonates demonstrated a significantly increased TNF-α proinflammatory response, compared to saline treated animals, indicating activation of the innate immune system. TNF-α is a key proinflammatory cytokine involved with the rapid activation and potentiation of the immune activation, as well as activation of the HPA axis (Beishuizen & Thijs, 2003).

Weight gain was assessed from weaning throughout development to adulthood. In the current study, LPS treated female gained significantly more weight than saline treated controls between PND 22-29 in the pre-pubertal period, a trend that was continued through to PND 36. This is in line with previous research from our laboratory that demonstrated females treated with NIA gained significantly more weight from PND 22 to PND 50, or adolescence to early adulthood (Sominsky et al., 2012a). The weight gain demonstrated in the

current study is in line with literature detailing a period of significant 'catch up growth', following an early life infection (Samuels & Baracos, 1992). In humans, a period of catch up growth has been linked with elevated metabolic rates in adulthood (Criscuolo et al., 2008), having implications not only for metabolic syndrome, cardiovascular disease, obesity and diabetes, but also hormone alterations (Criscuolo et al., 2008; Dunger et al., 2006; Huxley et al., 2000; Stout et al., 2015). Importantly, increases in weight during the prepubertal period are known to contribute to pubertal onset and sexual maturation in both humans and in animals models (Baker, 1985; Newnham et al., 2002; Sloboda et al., 2007; Wang et al., 2012), and may have an effect on reproductive lifespan. This is particularly pertinent for females, considering the transgenerational implications of perinatal stressors (Manikkam et al., 2012; Skinner, 2014; Skinner et al., 2013). LPS animals gained significantly less weight in early adulthood (PND 57-64), however this amount was in line with weight gain from the previous weeks, and normalised in subsequent weeks to that of controls. Other studies using similar NIA models have found conflicting results, with both no difference in weight and adiposity (Spencer et al., 2007), and differences in weight regulation (Iwasa et al., 2010) being reported. Further research may investigate metabolic and endocrine alterations at this stage, including leptin and ghrelin pathways (Meier & Gressner, 2004).

In regards to female reproductive development, neonatal treatment with LPS resulted in advanced onset of puberty, as indicated by earlier DVO and emergence of 1st proestrus, with weight taken on DVO showing no differences. These findings have been previously demonstrated in our laboratory (Sominsky et al., 2012a), however were previously accompanied with an increased weight in LPS treated animals. These studies differs however, as weights were taken on actual DVO to compare groups in the current study, whereas Sominsky et al (2012a) used weekly monitoring weights to asses weight at this stage. The

current results differ from others who have demonstrated similar models of neonatal LPS exposure leading to delays in puberty onset in female rats. Knox et al. (2009) used a model of LPS exposure on PND 3 and 5, however there were subsequent injections at later neonatal time points with increasing LPS doses. Additionally, Knox et al. (2009) manipulated litter sizes and genders, treatments were allocated to half litters only, and a differing rat breed was used. Wu et al. (2011) and also demonstrated results contrary to the current findings, with LPS treated animals demonstrating a delayed pubertal onset and first proestrus, however Sprague-Dawley rats were utilised for this study, and litter manipulation was carried out at birth. Similar to our findings though, Wu et al. (2011) observed no weight differences at this time. The differences seen here in the current study maybe due to differing litter treatment, dosage timing, LPS strain and rat strain methods. Wistar rats generally demonstrate a bimodal distribution of age at vaginal opening, with differing peaks occurring at PND 34 and PND 39, which may account for variability seen (Rivest, 1991). Regardless of advancement of 1st proestrus, cycle regularity did not significantly differ between treatment groups, similar to previous findings from our laboratory (Sominsky et al. 2012a). These findings differ from others (Iwasa et al., 2009; Knox et al., 2009; Wu et al., 2011) who demonstrate delayed first proestrus and altered normal cyclicity following LPS exposure, however Nilsson et al. (2002), using the same dose, LPS strain, and rat breed as the current study, echoed the current cyclicity findings. Precocious pubertal onset in female children has been linked to developmental origins and the adult onset of disease (Anderson, 2003; Ibáñez et al., 1998; Wierson et al., 1993).

A central question of this study was to determine whether outcomes were due to changes in motivational behaviour. We examined anhedonic behaviours using the SPT, a classical test for depressive-like behaviour in a rodent model. Regarded as a deficit in gaining

pleasure from pleasurable experiences, anhedonic behaviours are also a reflection of a motivation, decision making, and reward evaluation (Barch et al., 2016; Treadway et al., 2012; Treadway & Zald, 2011). Converse to predications, neonatal LPS treatment did not yield a long-term anhedonic effect in adult female animals, in line with those of Kentner et al. (2010), who demonstrated no changes in female sucrose preference following PND 14 LPS exposure, at any stage of oestrus cyclicity. Previous findings from our laboratory has demonstrated an anxiety-like phenotype in male animals which seems to not be present in female cohorts. To this effect, we hypothesised that perhaps neonatal LPS was differentially affecting female animals, and predisposing to a phenotype that has more depressive-like qualities relating to motivational behavioural aspects, in line with Pohl et al. (2007), particularly considering the frequent comorbidity and gender distribution observed between the two psychopathologies in human literature (Cameron, 2006; Cryan & Holmes, 2005; Depino, 2015; Fava et al., 2000; Kornstein, 1997; Syed & Nemeroff, 2017). The SPT is often paired with a chronic mild stress paradigm, which has been demonstrated to result in anhedonia and depressive-like behaviours in rodent models (Forbes et al., 1996; Papp et al., 1991; Willner, 1997; Willner et al., 1992). Using a mouse model of LPS exposure on PND 3 and 5, Doosti et al. (2013) demonstrated that NIA was sufficient to produce depressive-like behaviours in the forced swim and tail suspension test, however sucrose preference was not assessed. Although early life stressors have been shown to result in similar health outcomes as later-life chronic stress exposure (Hammen, 2005), perhaps NIA alone is not sufficient in generating an anhedonic effect or depressive-like behaviour without a subsequent later-life hit of stress.

In contrast to the current predictions, LPS treated female rats actually consumed more sucrose solution compared to saline treated controls. Furthermore, LPS animals consumed a progressively greater amount of sucrose solution on subsequent habituation days and during

the test phase, compared to saline treated controls. Food is a potent natural reward, actually increasing pleasure and reward signalling pathways in the brain, particularly in hypothalamic, striatal, and other limbic/basal ganglia circuitry regions associated with homeostatic regulation (de Macedo et al., 2016a; Haber, 2011). Literature demonstrating a preference for sweet over that of addictive drugs in rats (Lenoir et al., 2007; Madsen & Ahmed, 2015). Sugar intake has been demonstrated to activate similar central pathways to that of drug-dependence, including alterations in binding and expression of dopamine (DA) 1 and 2 receptors, opioid receptor binding, and increased extracellular DA and serotonin (5-HT) levels (Avena et al., 2008; Hone-Blanchet & Fecteau, 2014). Our lab and others have demonstrated dopaminergic alteration in rat models following similar models of neonatal LPS exposure, including dopaminergic system injury and persistent inflammation (Fan et al., 2011; Zavitsanou et al., 2013). Moreover, Tien et al. (2011) demonstrated that neonatal LPS exposure on PND 5 enhanced adulthood sensitization to methamphetamine, highlighting implications for later life addiction-like behaviours following NIA. As it is known that numerous early life stressors are implicated in the pathogenesis of reward-seeking behaviours (Brake et al., 2004; Yehuda & Daskalakis, 2015), perhaps neonatal LPS exposure is altering reward pathways during this critical periods and increasing sensitivity, especially as both dopaminergic and serotonergic development is known to continue throughout the post-natal period (Money & Stanwood, 2013). Interestingly, drug addiction studies in a rat model have demonstrated that toll-Like receptor (TLR) 4 in part mediates drug-induced neurochemical and behavioural alterations (Hutchinson et al., 2012; Kashima & Grueter, 2017; Northcutt et al., 2015). As TLR4 is the main receptor for LPS, this has implications for the perinatal programming of motivational and addictive behaviours, particularly via LPS

immune pathways (Mouihate et al., 2010), and this warrants further examination in the NIA model.

Asociality is a core motivational behaviour associated with psychopathologies and neurodevelopmental disorders including depression, anxiety and schizophrenia (Kaidanovich-Beilin et al., 2011). In rodents, social interactions are also a fundamental element in determining hierarchy. In the current study, behaviours in the SIT were assessed as both totals, as well as being compartmentalised to evaluate nuances in dynamic social behaviours (Beery & Kaufer, 2015). We observed no overt alteration in motivation to socially interact. Analysis of behaviours during the complete duration of the SIT indicated that LPS treated female displayed a significantly greater number of rears in the arena, compared to controls. Additionally, LPS treated animals kicked their naïve conspecific a greater number of times, however the total mean counts were quite low. No overall differences in interaction behaviours existed between treatments when assessing complete duration totals. Breakdown of behavioural assessment into time bins indicated that rats in both treatment groups demonstrated significantly higher performance of interaction behaviours earlier, compared to the later. This is not surprising, considering the novelty of the naïve animal. Both treatments groomed more in the first 6 minutes, compared to the last 6 minutes of the test, as well as performed more non-specific and AG sniffing in the first three minutes compared to the last 6 minutes. Significant differences between treatments were seen at differing times for facial sniffing and rearing behaviours only. In addition to rearing and sniffing alterations in this test, LPS animals demonstrated higher overall circulating corticosterone levels before and after the test compared to saline controls, indicative of hyperactive HPA axis dysregulation. Previous studies exploring postnatal stress have demonstrated long-term deficiencies in social interaction. Maciag et al. (2002) previously demonstrated that rats who experienced

neonatal maternal separation displayed significantly fewer social interaction behaviours and higher ACTH levels in adulthood compared to controls, an effect that was ameliorated by the administration of a corticotropic releasing hormone (CRH) antagonist.

Although social motivation does not seem to be impacted, the current study indicated a significant increase in overall rearing frequency of LPS treated females in the social interaction chamber. In human epidemiological samples, it has been found that women are more likely to suffer from social anxiety disorders than men (Kessler et al., 1994), and although we have not previously seen an anxiety-like phenotype in female animals, increased rearing behaviours in LPS animals may be a marker of anxiety-like hypervigilance within a social context. Rearing is an ethological rat behaviour typically suggested as in indicator of environmental novelty, associated with investigation, information gathering and environmental assessment (Lever et al., 2006). In the current study, rearing behaviours, although continuously elevated in LPS animals, diminished in the latter parts of the test in both treatment, suggesting that environmental information gathering and assessment decreased as the test progressed. Conversely, others have found an increase in frequency of rears in other traditionally tests of anxiety-like behaviours, with Brown and Nemes (2008) demonstrating that female rodents reared more as the hole board test progressed, suggesting that the exploratory behaviours increased as fear decreased with familiarity of environment. In the current study, all animals were habituated to the social interaction chamber prior to testing, hence increased rearing in LPS treated females may be a hypervigilance behaviour in this particular social setting independent of environmental novelty. This postulation may be corroborated by the overall elevated levels of CORT in LPS treated females. Although, as Weiss et al. (1998) suggests, rearing may be also been seen as a measure of general activity, with less anxious rats suggested as being more active. Landgraf and Wigger (2002)

demonstrated that a high-anxiety rat breed locomoted significantly less than a low-anxiety rat breed, but both studies were performed with male animals. In this sense, the female rats here could be displaying a protective effect of early life LPS exposure to mild social stress, as previously suggested by Bilbo et al. (2008) who demonstrated exposure to live *E.coli* on PND 4 protected against the negative impact of a 2nd exposure to stress.

Lastly in the SIT, females across both treatments performed significantly more sniffing behaviours in the earlier parts of the test compared to the latter. Interestingly, LPS females in the current study showed significantly less facial sniffing in differing time bins than saline animals. In rats, sniffing behaviours are not only essential for odour acquisition and information processing (Uchida et al., 2006; Welker, 1964), but are essential for motivation and social communication (Clarke & Trowill, 1971; Deschênes et al., 2012; Kepecs et al., 2007). Wesson (2013) demonstrated a similar effect in male and ovariectomised female rats, with first social interactions characterised by high and rapid sniffing behaviour, exploration and rearing that decreased over time, which is in line with the changes we see with rearing and sniffing in the current study. No differences in general/flank or AG were seen between females of either treatment group in this study. These findings parallel those of a recent study by Farrell et al. (2016), who demonstrated an increased latency of only nose-to-nose social contact in adolescent female rats, not males, following neonatal maternal separation. Facial interaction between rodents serves multiple communicative purposes, including transmission of food preference, and social signals and intent (Wolfe et al., 2011). Both the current findings in female adults and those of Farrell et al. (2016) may indicate impaired or sub-optimal social interaction rather than a blatant decrease in social interaction, having implications within a neurodevelopmental framework. Interestingly, Wesson (2013) notes that face sniffing and investigation may covey greater communicative information regarding hierarchy and conflict

resolution, and reflects the appeasement strategies seen in other animals (Aureli et al., 2002). Wesson (2013) demonstrated that socially dominate rats display increased facial sniffing and investigation, whereas subordinate rats will decrease their sniffing in the presence of a dominate rat. Perhaps in this sense, LPS treated females may be displaying a more subordinate role within the SIT, compared saline treated animals. Early life immune activation is known to alter endocrine functionality (Morale et al., 2001), and neuroendocrine functioning is also a major contributing factor involved in determining social status within hierarchies (Hamilton et al., 2015). Kozorovitskiy and Gould (2004) demonstrate that socially subordinate rats displayed decreased adult neurogenesis, a phenomena also seen as a result of early life immune stress (Korosi et al., 2012; Lajud & Torner, 2015; Musaelyan et al., 2014). Considering the overlap in endpoints of both early life immune stress and social subordination, further social hierarchy testing use the LPS exposure model may be warranted (Blanchard et al., 1993).

Motivational behaviours associated with female mating success were evaluated in this study, as loss of libido is often a symptom of depression and a repercussion of increased inflammation and stress (Winkler et al., 2004). In the PMT, LPS females entered the male chamber a significantly greater number of times than controls, suggesting that motivation to spend time with the male stud was not decreased by neonatal LPS treatment. Others have demonstrated a clear conditioned place preference for the male chamber in the PMT where the female is able to control coitus and interaction with the stud, highlighting the rewarding aspects of this test (Martínez & Paredes, 2001; Paredes & Vazquez, 1999). The findings here are in accordance with the current results from the SIT, demonstrating no changes in motivational state to interact with a novel animal of the same sex, as well as SPT findings, where the LPS treated animals actually seemed inclined to hedonic states. Regardless of their

apparent drive to mate with the proven male stud, LPS treated females displayed deficits in both proceptive and receptive mating behaviours in this study. The female rat in proestrus has a suite of precopulatory and courtship behaviours that signal their willingness to mate. These solicitation behaviours ensure male engagement and subsequent copulation with the female. The LPS treated rats in the current study demonstrated decreases in these precopulatory behaviours, performing significantly less hops and a strong trend for less darts. The findings from the current study are in line with previous results from our laboratory. In an opened field setting, Walker et al. (2011) observed that LPS treated females demonstrated a stronger effect of NIA on sexual behavioural deficits than that of male animals, with LPS females also displaying reduced proceptive behaviours yet no decrease in time spent with the male. This suggest deficits in overall social behavioural signalling or communicative cues to the male during reproductive behaviours, and taken together with alterations in the SIT, provide evidence for suboptimal communicative behaviours that may negatively affect fecundity.

Converse to predictions, no significant differences were demonstrated in LH and FSH levels between groups during proestrus and in response to paced mating. Previous findings from our laboratory have demonstrated an attenuation of the LH surge in LPS treated females both during mating and in response to restraint stress (Walker et al., 2011). However, our laboratory has also demonstrated no changes to LH on DVO or in adolescence, with only FSH levels dampened in NIA treated females at adolescence (Sominsky et al., 2012). In the current study, a surge of LH in both treatment groups is observable following the PMT. Furthermore, LPS animals maintain a level of FSH before and after testing. Due to the variations in data, additional analysis of LH and FSH levels is needed. Central gene analysis of receptor levels for these hormones would elucidate on changes and give grounds for a more solid interpretation.

Additionally, future research may examine the immunohistochemical protein localisation of these receptors in the ovary.

Adding to previous findings from Walker et al (2011), the current study also examined lordosis behaviours, previously unexamined in the current model. We demonstrate here that NIA resulted in a significant decrease in lordosis behaviours, however male attempted mounts increased, presumably due to the altered behavioural cues by the female. Regardless, high sperm detection rates indicates no differences in successful male mounts between treatments. Others have demonstrated decreases in lordosis and a general attenuation in female rat mating behaviours following LPS and IL-1β treatment in adulthood, which is suggested to be modulated via central cytokine pathways, including IL-1β and TNFα a (Avitsur et al., 1999; Avitsur & Yirmiya, 1999; Yirmiya et al., 1995). Hence, determination of these cytokines is needed in the current model, in order to gain an understanding of their contribution to female mating deficits following a neonatal immune challenge with LPS.

A significant body of literature suggests that the lordosis reflex is regulated not only by hormonal and immune influence, but also by 5-HT production and the associated neuronal circuitry in the hypothalamus and the medial pre-frontal cortex, two areas known to regulate female reproductive and maternal behaviours (Angoa-Perez & Kuhn, 2015; Snoeren et al., 2014). Increases in 5-HT by pharmacological agents (SSRIs, MAOIs and 5-HT precursors) and 5-HT receptor agonists led to decreases lordosis behaviours, with depletion of 5-HT associated with increased lordosis (Mendelson, 1992; Uphouse, 2014). This negative regulation of lordosis by 5-HT has implications for a number of systems that may be perturbed by neontal LPS exposure including central 5-HT pathway signalling and perhap kyneurine signalling (Williams et al., 2017; Zavitsanou et al., 2013).

Of note, increased 5-HT is also associated with reward pathway stimulation, not only in drug addiction but also with other rewards such as palatable food, sociality, and sex (de Macedo et al., 2016b; Fortuna, 2010; Jacobs & Azmitia, 1992; Li et al., 2016). In this study, females exposed to neonatal LPS demonstrated significantly increased consumption of sucrose, and also seemed to display an increased motivation to visit the male chamber during paced mating. These behaviours may be construed as a higher drive or motivation for reward, considering the known rewarding nature of both sucrose and paced mating for the female rat as previously discussed. Speculatively speaking, perhaps reward pathway activation via paced mating stimulation is triggering 5-HT release, however this increase is in turn diminishing lordosis in LPS treated animals, and leading to subfertility outcomes. Further studies may investigate this link considering the current findings, as well examine mechanisms involving 5-HT synthesis and related behaviours, such as the tryptophan/kynurenine pathway and associated mediators including Indolamine-2,3-Dioxygenase (IDO) and Tryptophan-2,3-Dioxygenase (TDO).

In conclusion, the current study indicates that female rats exposed to neonatal LPS demonstrate a long-term behavioural phenotype specific to suboptimal behavioural cues that is specific to mating and subtly expressed during social interaction. Additionally, NIA treatment impaired female sexual performance. Furthermore, the current findings indicate that these suboptimal mating behaviours are not due to decreased motivation in LPS treated females, suggesting that other factors such as inflammation, endocrine, or gonadal alterations, may be mediating these behaviours. The current research follows on from the initial female behavioural findings demonstrated in our laboratory (Walker et al., 2011), and refines and confirms the existence of a suboptimal reproductive behavioural phenotype in

female animals following neonatal immune stress with LPS. This includes a precocious pubertal onset, precopulatory behavioural cue deficits, and a diminished lordosis response.

Neonatal LPS exposure alters immune and neuroendocrine parameters, which can affect and determine reproductive development and viability. This is particularly important considering the involvement of immune and endocrine mediators in female reproductive development, health, success, and longevity (Bouman et al., 2005; Jabbour et al., 2009; Klein & Nelson, 1999; Weiss et al., 2009) and the association between advanced puberty and increased susceptibility to adulthood disease (Anderson, 2003; Ibáñez et al., 1998; Juraska & Willing, 2017; Thomas-Teinturier et al., 2013; Wierson et al., 1993). Further investigation into what may be driving these sexual behavioural changes is needed. This includes a focus on ovarian mediated alterations that may be established during the initial immune challenge, and the long-term examination of these mediators. Further investigation into the bi-directional communication between immune and endocrine mechanisms with give greater insight into the female phenotype that is emerging due to early life immune stress. Critically, these results highlight the importance of understanding the contribution of the early life environment to female reproductive development and function if infection remains unchecked or prolonged, especially due to current literature indicating an increased prevalence of postnatal bacterial infection (Darville, 2005; Koumans et al., 2012; Lamagni et al.; Simonsen et al., 2014).

Chapter 4. Neonatal Immune Activation Depletes the Ovarian Follicle Reserve and Alters Ovarian Acute Inflammatory Mediators In Neonatal Rats.

4.1 Publication Introduction.

The previous chapter of this thesis demonstrated that neonatal female rats exposed to an LPS challenge exhibit an adult behavioural phenotype that differs from the male (Walker et al., 2011; Walker et al., 2008; Walker et al., 2004). Following neonatal LPS exposure, female rats predominantly displayed deficits in mating behaviours, including decreased lordosis and reduced proceptive behaviours, as well as an earlier onset of puberty. Given these alterations, it becomes important to examine the immediate effects of LPS on physiology which may be driving these behavioural and pubertal alterations and determine the mechanisms that may be contributing to these changes.

Successful reproduction relies on the precise coordination between the brain and the ovaries via endocrine and immune communication (Marchetti et al., 1990). Alterations in the physiology of these pathways may result in altered behaviours. In female rodents, a series of ovarian-mediated events occur, relaying information to hypothalamic regions of the brain in order to stimulate initial ovulation (signalling puberty), as well as sexual behaviours to coincide with ovulation (Christensen et al., 2012). The fundamentals of ovarian functioning are determined in early life, setting the tone for reproductive success and longevity (Smith et al., 2014). This includes the quantity and quality of the ovarian reserve, which dictates the reproductive lifespan and participates in ovary-brain communication (Grive & Freiman, 2015; Richardson et al., 2014). This initial establishment of the ovarian follicle reserve is largely governed by immune mediators (Hirshfield, 1991; McLaughlin & McIver, 2009; Tingen et al.,

2009), hence, immune stress that coincides with this maturation process may have a detrimental impact on ovarian functioning and influence sexual development and behaviours.

As such, this paper aimed to explore the initial alterations in the ovary as a consequence of a neonatal LPS immune challenge. This allows for further insight into the mechanisms contributing to a suboptimal reproductive phenotype, which includes earlier onset of puberty and suboptimal mating behaviours. The findings of the paper presented in this chapter indicate that LPS exposure had an acute detrimental effect on the neonatal ovary, significantly depleting the early ovarian reserve. Circulating inflammatory mediators and ovarian inflammatory mediators were significantly upregulated, coinciding with increased apoptosis of follicular cells within the ovary. These findings indicate that the ovarian immune milieu is disturbed by early life LPS exposure, which may form the basis for long-term detrimental modifications in not only ovarian immune functioning, but overall inflammatory status, contributing to puberty onset and behavioural changes. Further investigation is needed to establish if these alterations are sustained into adulthood, which will form the premise of subsequent chapters within this thesis.

Paper 2

Neonatal immune activation depletes the ovarian reserve and alters ovarian acute inflammatory mediators in the neonatal rat.

Erin A. Fuller, Luba Sominsky, Jessie M. Sutherland, Kate A. Redgrove, Lauren Harms, Eileen A. McLaughlin, Deborah M. Hodgson.

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Statement of author contributions to the publication.

Author	**Description of contribution to manuscript**	**Signature**
Erin A Fuller	Designed and performed the experiments Analysed and interpreted the data Wrote the manuscript	
Luba Sominsky	Assisted in experimental design, data analysis and interpretation Provided intellectual contribution and critical input Revised the manuscript	
Jessie M Sutherland	Assisted in technical and experimental procedures Provided intellectual contribution and critical input Revised the manuscript	
Kate A Redgrove	Assisted in technical and experimental procedures Provided intellectual contribution and critical input Revised the manuscript	
Lauren Harms	Revised the manuscript Provided intellectual contribution and statistical input	
Eileen A McLaughlin	Assisted in experimental design and data interpretation Contributed reagents/materials/analysis tools Provided intellectual contribution and critical input Revised the manuscript	
Deborah M Hodgson	Assisted in experimental design and data interpretation Contributed reagents/materials/analysis tools Provided intellectual contribution and critical input Revised the manuscript	

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Research Article

Neonatal immune activation depletes the ovarian follicle reserve and alters ovarian acute inflammatory mediators in neonatal rats†

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Abstract

Normal ovarian development is crucial for female reproductive success and longevity. Interruptions to the delicate process of initial folliculogenesis may lead to ovarian dysfunction. We have previously demonstrated that an early life immune challenge in the rat, induced by administration of lipopolysaccharide (LPS) on postnatal day (PND) 3 and 5, depletes ovarian follicle reserve long term. Here, we hypothesized that this neonatal immune challenge leads to an increase in peripheral and ovarian inflammatory signaling, contributing to an acute depletion of ovarian follicles. Morphological analysis of neonatal ovaries indicated that LPS administration significantly depleted PND 5 primordial follicle populations and accelerated follicle maturation. LPS exposure upregulated circulating interleukin 6, tumor necrosis factor alpha (TNFa), and C-reactive protein on PND 5, and upregulated ovarian mRNA expression of *Tnfa,* mitogen-activated protein kinase 8 (*Mapk8/Jnk1*), and growth differentiation factor 9 (*Gdf9)* ($P < 0.05$). Mass spectrometry and cell signaling pathway analysis indicated upregulation of cellular pathways associated with acute phase signaling, and cellular survival and assembly. Apoptosis assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling indicated significantly increased positive staining in the ovaries of LPS-treated neonates. These findings suggest that increased proinflammatory signaling within the neonatal ovary may be responsible for the LPS-induced depletion of the primordial follicle pool. These findings also have implications for female reproductive health, as the ovarian reserve is a major determinate of female reproductive longevity.

Summary Sentence

Neonatal immune activation acutely impacts immune-mediated early ovarian development, depleting the primordial follicle pool and upregulating inflammatory mediators.

Key words: early life immune stress, lipopolysaccharide, inflammation, cytokines, follicular development, oocyte development, developmental origins of health and disease.

Introduction

Despite significant medical advances, idiopathic infertility and the prevalence of reproductive disorders in younger female cohorts is increasing [1–4]. As the fundamentals of reproductive health and longevity are established in early life, the pathogenesis of female reproductive dysfunction may have developmental roots. Abnormalities occurring during this critical period of development may lead to sustained ovarian pathophysiology, including premature ovarian failure (POF) and other fertility issues [5–7]. Recent evidence indicates that the female reproductive system is sensitive to early life stressors, such as xenobiotics, infections, and malnutrition, which can perturb reproductive development and negatively impact long-term fertility levels [8–12].

Mammalian female reproductive health and success is reliant on the normal establishment of the nonrenewing ovarian primordial follicle reserve, which is the foundation of all future follicles and determines the reproductive lifespan [as reviewed in 13, 14]. This initial folliculogenesis occurs prenatally in humans, however finalizes during the first postnatal week in rodents. Early follicles containing oocytes ultimately develop from the primordial stage through to the ovulatory stage, until the reserve is diminished and menopause, or senescence in rats, occurs [15]. Initial folliculogenesis is governed by numerous mechanisms that remain to be fully elucidated; however, current evidence indicates that complex interactions of chemokines, cytokines, neurotrophins, growth factors, and transcription factors mediate the bidirectional communication between the oocyte and its supporting granulosa cells [16–19]. This oocyte-granulosa crosstalk controls the quiescence, activation, and maturation of the primordial follicular pool to regulate both the quantity and quality of the ovarian reserve [20, 21]. Variation or perturbation to these delicate developmental processes, via immune activation for example, can potentially lead to sustained changes in ovarian development, and overall reproductive health [6, 22].

Immune activation is a common perinatal environmental stressor that is modeled experimentally using gram-negative bacterial mimetic, lipopolysaccharide (LPS). LPS provokes an innate immune response by binding to toll-like receptor-4 (TLR4). This instigates a proinflammatory cascade via activation of nuclear factor kappa beta (NFkB) and mitogen-activated protein kinase (MAPK) pathways, and the subsequent secretion of proinflammatory cytokines interleukin (IL) 6, IL1beta (B), tumor necrosis factor alpha (TNFa), C-reactive protein (CRP), and interferon gamma (IFNy) from activated macrophages and immune cells [23, 24]. Animal models of neonatal immune stress via LPS exposure have demonstrated a broad range of long-term physiological and behavioral alterations, including neuroendocrine dysfunction, brain morphological alterations, and innate immune system dysfunction [25–28].

The ovary expresses innate immune cells including monocytes, macrophages, and adipocytes [29]. Additionally, cytokines and their receptors, and TLRs are locally expressed in ovarian cells and participate in immune functioning essential to ovarian processes [30, 31]. Importantly, LPS exposure has been demonstrated to detrimentally affect female reproductive outcomes [32, 33] including premature puberty and senescence onset, downregulation of hypothalamic-pituitary-gonadal hormone expression, and impairments in mating and maternal behaviors [8, 11, 26, 34, 35]. Moreover, neonatal and adulthood LPS exposure has been demonstrated to lead to in vitro follicular atresia, reduced prepubertal ovarian follicle reserve, and upregulated ovarian TLR4 expression [36–38]. Taken together, these findings suggest that early life LPS exposure produces sustained detrimental effects on ovarian functioning.

To date, little research has focused on the acute in vivo impact of early life immune activation on ovarian morphology and inflammation. Previous studies from our laboratory indicate ovarian inflammatory pathway activation on postnatal day (PND) 7 from LPS administration at PNDs 3 and 5 [37], but none to our knowledge have examined the immediate effect of this LPS exposure. Considering the importance of immune involvement for ovarian development and continuing reproductive health [29], the current study aims to examine the acute inflammatory mediators activated by neonatal administration of LPS and associated growth and transcription factors that may underpin the sustained ovarian morphological and behavioral reproductive alterations seen previously in our laboratory with this model, including the early onset of reproductive senescence in female rats, indicated by premature cessation of oestrus cycling [11]. Given that our PND 3 and 5 model of neonatal immune activation falls within the critical period of ovarian development and sensitivity to immune stress for the rodent ovary [35], we propose that LPS exposure may directly perturb the critical, gonadotropin-independent final stages of neonatal primordial folliculogenesis via excessive immune stimulation occurring both at a systematic and local level.

Methods

Animals and neonatal immune challenge

All animal experimental procedures were undertaken with the approval of the University of Newcastle Animal Care and Ethics Committee (ACEC, A-2012-2813). Twenty-one experimentally naïve female Wistar rats were obtained from the University of Newcastle animal house and mated with proven male studs in the Laboratory of Neuroimmunology Vivarium. This resulted in 18 litters and a total of 62 female pups used for this study. Animals were maintained under normal housing conditions at 21°C–22°C on a 12-h light/dark cycle (0600-1800) with food available ad libitum. As previously described [11, 26–28, 37, 39], at birth (PND 1) whole litters were randomly allocated to treatment conditions, either LPS (derived from 10 litters) or saline (derived from eight litters). Whole litters were exposed to LPS (*Salmonella enterica*, serotype Enteritidis: Sigma-Aldrich Chemical Co., USA in sterile pyrogen-free saline, 0.05 mg/kg) or saline (equivolume; Livingstone International, Australia) on PND 3 and again on 5. This low dose and timing of LPS administration has been demonstrated in our laboratory and others to elicit a rapid, sustained, and controlled immune and endocrine response during a critical developmental period, without inducing mortality seen at higher doses [26, 28, 37, 39–43]. Briefly, pups

were temporarily removed from the home cage and transferred to an incubator to maintain body temperature, weighed, administered with an intraperitoneal injection of LPS or vehicle, and then returned to the home cage/dam.

Blood and tissue collection

Female pups were euthanized by rapid decapitation. A subset of female animals were culled on PND 3 for analysis of ovarian morphology ($n = 6$ per group derived from three litters per group). The remaining females were euthanized on PND 5, at 2 h following the last neonatal injection, as this has been demonstrated to be the optimal time point for peripheral, central, and genetic cytokine expression following LPS [44, 45]. PND 5 trunk blood was collected into ethylenediaminetetraacetic acid-coated tubes and centrifuged for 20 min at 1000*g*. Plasma was collected and stored at –20°C for assessment of plasma cytokine levels. Ovaries were dissected with fine tip forceps in 4°C sterile phosphate-buffered saline (PBS, Sigma) under a dissection microscope, where all surrounding tissue was removed. One ovary from a subset of animals was randomly chosen and placed in Bouins fixative for histological examination (PND 5; $n = 6$ LPS, 6 saline, derived from three to four litters per group). Remaining ovaries were snap-frozen on dry ice, and stored at –80°C. All remaining tissue was randomly pooled within treatment conditions to create sufficient tissue for biological samples and allocated to either real-time quantitative (qRT)-PCR or proteomic analysis. Male and female animals remaining in litters were allocated to other experiments.

Blood analysis for peripheral inflammatory cytokines

PND 5 plasma was analyzed by ELISA according to the manufacturer's instructions for proinflammatory markers IL6 (Abcam, ab119548 rat ELISA kit, minimum detection rate 12 pg/mL, intra- and interassay variability <5% and <10% respectively), TNFa (R&D Systems Rat TNF alpha Quantikine ELISA Kit, RTA00, minimum detection rate <5 pg/mL, intra- and interassay variability 2.1%–5.1% and 8.8%–9.7% respectively), and CRP (Abcam, ab108827, minimum detection rate 0.7 ng/mL, intra- and interassay variability 3.8% and 9.6%, respectively). Each ELISA contained biological samples from at least three different litters per treatment group, with $n = 6$–12 per group. All samples were assayed in duplicate.

Histological evaluation of ovarian follicles

Ovaries were fixed in Bouins fixative (Sigma Aldrich, castle Hill, Australia) solution for 4 h, then washed four times in 70% ethanol, dehydrated, embedded in paraffin and sectioned at 4 μm. Every fourth slide was stained with hematoxylin and eosin (H&E) for quantification of ovarian follicles, resulting in approximately 8–10 H&E slides per rat neonatal ovary [12, 37]. An experimenter blind to experimental groups examined the samples, and only follicles with a visible oocyte were counted. Primordial, activated primordial, and primary follicles only were classified on H&E sections as follows [see 12]: (1) *primordial follicle*: an oocyte surrounded by one layer of flattened cuboidal granulosa cells; (2) *activated primordial follicle*: a maturing oocyte surrounded by both flattened granulosa and one or more cuboidal granulosa cells in a single layer; (3) *primary follicle*: an oocyte surrounded by four or more cuboidal granulosa cells in a single layer. Total counts were carried out on the first and third section of every H&E stained slide, resulting in the quantification of all visible follicles [46].

Proteomic identification in ovarian tissue

Postnatal day 5 ovarian protein (10–20 μg) was submitted to the Australian Proteome Analysis Facility (APAF) for proteomic analysis. Prior to submission, protein was extracted from each sample containing 15–20 pooled neonatal ovaries using a modified sodium dodecyl sulfate (SDS) extraction method. Briefly, neonatal ovaries were manually homogenized in extraction buffer (0.375 M Tris, pH 6.8, 2 mL 10% SDS, 3 mL MQ H_2O, 1 g sucrose), heated at 100°C for 5 min and centrifuged at 13 000 rpm. Supernatant was removed, then stored and shipped at –80°C. For proteomic analysis, excised gel bands were resized, destained, dried, and then digested with trypsin in ammonium bicarbonate (pH 8) overnight. Supernatant from gel was made up to 40 μL in electrospray ionization loading buffer then was injected onto a peptide trap (Michrome peptide Captrap) for preconcentration and desalted with 0.1% formic acid, 2% ACN, at 8 μL/min. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using a linear solvent gradient, with steps, from H_2O: CH3CN (100:0, +0.1% formic acid) to H_2O: CH3CN (10:90, +0.1% formic acid) at 500 nL/min over an 80 min period. The liquid chromatography eluent was subject to positive ion nanoflow electrospray mass spectrometry (MS) analysis on QSTAR that was operated in an information dependent acquisition mode (IDA). In IDA mode, a TOFMS (time of flight mass spectrometry) survey scan was acquired (m/z 400–1600, 0.5 s), with the three largest multiply charged ions (counts >25) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (m/z 100–1600). The data were processed using the database search program, Mascot (Matrix Science Ltd, London, UK) with peaklists searched against Rattus in the SwissProt database [47]. High scores in the database search indicate a likely match that was confirmed or qualified by operator inspection. Search results were generated with a significance threshold of $P < 0.05$ with an ion score cut-off of 25 for all samples. This work was undertaken at APAF, the infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS). LPS and saline groups were compared and Ingenuity Pathway Analysis (IPA: Ingenuity Systems, Redwood City, CA) software was used to identify top canonical signaling protein pathways and upstream regulators affected by neonatal treatment.

RNA extraction, reverse transcription and real-time quantitative-PCR

In order to isolate sufficient quantity and quality mRNA from whole PND five ovaries, six to eight ovaries from the same treatment group were randomly pooled within treatment groups to create biological replicates. Total RNA was isolated from ovaries using a modified acid guanidinium thiocyanate-phenol-chloroform protocol, followed by an isopropanol precipitation as previously described [48, 49] and DNase treated prior to reverse transcription for the removal of genomic DNA. Reverse transcription was performed as outlined in Sobinoff et al. [48] with 2 μg of total isolated RNA, 500 ng oligo(dT), 15 μg of primer (FWD and REV), 40 μg of RNasin, 0.5 mM dNTPs, and 20 μg of M-MLV-Reverse Transcriptase (Promega; Madison, WI, USA). Reverse transcription reactions were verified by Actin beta *(Actb)* or Cyclophilin qPCR using cDNA amplified with GoTaq Flexi (Promega). Quantitative RT-PCR was performed in 20 μl reactions using SYBR Green GoTaq qPCR master mix (Promega) according to manufacturer's instructions on a LightCycler 96 SW 1.0 (Roche, Castle Hill, NSW, Australia) for transcription factor Forkhead box O3a (*Foxo3a*) and growth differentiation factor 9 (*Gdf9*), as well as

Table 1. qRT-PCR primer information.

Target Gene	Forward	Reverse	Efficiency
Mapk8/Jnk1	*CGGAACACCTTGTCCTGAAT*	*GAGTCAGCTGGGAAAAGCAC*	1.94
Prkcb	*ATCAGCCCTACGGGAAGTCT*	*CGTTGTGCTCCATGATTGAC*	1.91
Tlr4	*ACTGGGTGAGAAACGAGCTG*	*CGGCTACTCAGAAACTGCCA*	1.97
Actin B	*TCTGTGTGGATTGGTGGCTCTA*	*CTGCTTGCTGATCCACATCTG*	1.94
Cyclophilin A	*CGTCTCCTTCGAGCTGTTT*	*ACCCTGGCACATGAATCCT*	1.9
Gdf9	*CAACCAGATGACAGGACCC*	*AGAGTGTATAGCAAGACCGAT*	1.83
Foxo3a	*CACAGAACGTTGTTGGTTTG*	*CAGTTTGAGGGTCTGCTTTG*	1.84

Forward and reverse sequence and efficiency for target genes analyzed involved in early ovarian follicular control and development, and inflammation.

inflammatory markers; *Tnfa*, mitogen-activated protein kinase 8/Jun N-terminal kinase (*Mapk8/Jnk1*), protein kinase C beta (*Prkcb*) and *Tlr4*. These markers are associated with both LPS-activated inflammatory pathways and are essential to steroidogenesis during this critical time point in gonadotropin-independent ovarian development, as well as continued ovarian-immune functioning throughout the lifespan [18, 50–60]. Gene and protein markers assessed were chosen to reflect the nature and timing of our early life immune stress exposure model, based on previous research from our laboratory [37] and in line with broad proteomic identification of factors in the current study associated with ovarian cell proliferation, migration, apoptosis, and LPS-induced inflammation (see Supplementary Table S5). Each sample was accompanied by a RT-negative replicate as a negative control. Quantitative RT-PCR data were normalized to the housekeeping control gene Cyclophilin as per Sutherland et al. [49] and analyzed using the comparative C_T method equation $2^{-\Delta\Delta C(t)}$ (where C(t) is the threshold cycle at which fluorescence is first detected as statistically significant above background) and presented as a fold increase relative to the saline control group [61]. Experiments were replicated a minimum of three times prior to statistical analysis, with all PCR performed on at least three separate tissue isolations/biological replicates [as per 62]. Primer sequences are supplied (Table 1) and were optimized by qPCR both here and previously [37].

Immunohistochemistry

Immunohistochemistry was used to localize TNFa protein and DNA damage via Phosphorylated histone gamma H2AX (yH2AX) expression. Caspase 3 (CASP3) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining were used to quantify apoptosis. TLR4 localization was assessed to confirm LPS activation via TLR4 binding in the neonatal ovary. TNFa was localized in PND 5 ovarian tissue using a Vector 3, 3-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA) following manufacturer's instructions. Slides were deparaffinized in xylene and rehydrated in ethanol washes. Antigen retrieval was carried out in preheated Na citrate buffer (10 mM, pH 6), microwaved for 12 min. Endogenous peroxidase quenching was performed (0.3%) for 20 min; slides were rinsed in PBS-TX and blocked in 3% bovine serum albumin (BSA) in PBS for 1 h. Slides were incubated with primary antibody overnight at 4°C (anti-TNFa, Abcam ab6671, 1:200 dilution), then rinsed with PBS-TX and incubated with biotinylated secondary antibody (rabbit IgG; Abcam ab191866, 1:500) for 30 min. Sides were rinsed, incubated with Vectorstain ABC prepared to manufacturer's instructions for 30 min at room temperature, then incubated with DAB for 2 min, counterstained with Carezzis blue for 2 min, rinsed in bluing solution, dehydrated in ethanol and xylene, then mounted and viewed using an Axio imager A1 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Images were taken using an Olympus DP70 microscope camera (Olympus America, Centre Valley, PA, USA). For TLR4, yH2AX, immunohistochemistry was carried out following the dewaxing, rehydrating, and antigen retrieval previously mentioned. Cooled slides were blocked in 3% BSA/Tris-buffered saline for 1 h at room temperature. Sections were incubated overnight at 4°C with anti-TLR4 (1:100; Santa Cruz, sc-16240), anti-yH2AX (1:200; Abcam ab26350) and anti-CASP3 (cleaved form, ~17kDa, 1:100; Abcam, ab13847). Slides were washed in TBX (0.1% Triton X-100) and incubated with appropriate fluorescent conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit/goat anti-mouse IgG, 1:200, Abcam; ab150080, ab150120 respectively). TUNEL was performed using an ApopTag Fluorescein in situ Apoptosis Detection Kit (Millipore, S7110) according to the manufacturer's instructions. Sections for immunofluorescence were counterstained with either YOYO-1 nuclear stain (green) or DAPI (blue), mounted with Mowiol, and viewed as described above. Positive controls included treated mouse and rat reproductive tissues and spleen for antibody specificity and DNase-treated tissue where appropriate, and no primary antibody/TdT enzyme negative controls on target tissue (see Supplementary Table S1 for antibody information and Supplementary Table S6 for immunohistochemical control imaging). TUNEL positive cells were quantified by an experimenter blind to treatment conditions. CASP3 corrected total cell florescence (CTCF) was measured in Image J (National Institutes of Health, MD, USA) and calculated using the formula CTCF = integrated density – (area of selected section × mean fluorescence of background readings) [63].

Statistical analysis

Data were analyzed using IBM SPSS statistics (Version 24, IBM Australia) with a two-way analysis of covariance design, repeated measures analysis of variance, and student independent *t*-test where appropriate, with pairwise comparisons between treatment groups carried out using the Bonferroni correction. Where covariates including litter size, body weight, and male-to-female ratio did not significantly impact dependent variables, they were removed from the analysis to maximize statistical power. Statistical assumption violations were corrected by a log10 transformation (IL6 only). Data are presented here as the mean + standard error of the mean (SEM). Significance was assumed at $P \leq 0.05$.

Results

Neonatal weight gain

No significant weight difference was observed between treatment groups on PND 3 or PND 5; however, LPS females gained significantly less weight between PND 3 and 5 compared to controls (neonatal treatment × age: $F_{(1, 57)} = 42.02$, $P \leq 0.0001$; pairwise

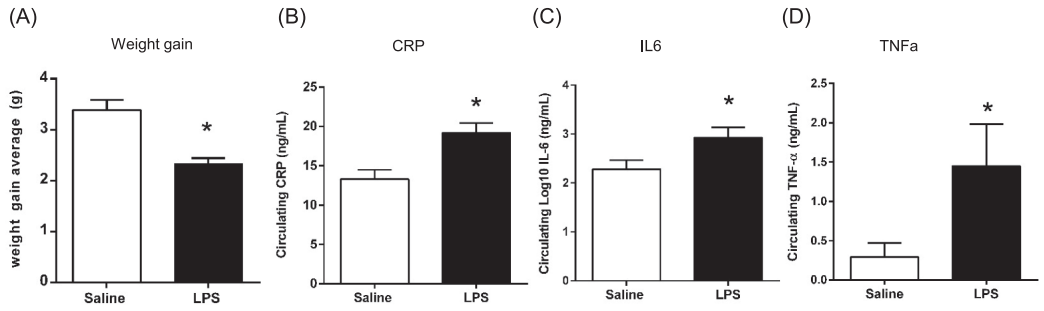

Figure 1. Weight gain between PND 3 and PND 5 and circulating proinflammatory cytokines. (A) Neonatal females treated with LPS ($n = 29$) gained significantly less weight between treatment days compared to saline-treated females ($n = 31$), mean difference in weight gain between groups represented in grams. LPS-treated animals displayed significantly increased plasma levels of (B) CRP, (C) IL6, and (D) TNFa. White bars represent saline controls, filled bars represent LPS-treated animals. '*' indicates $P < 0.05$.

contrast: $t(43) = 4.5$, $P < 0.0001$; Figure 1A). An expected significant main effect of age ($F_{(1, 57)} = 105.49$, $P < 0.0001$) was also observed, with PND 5 animals weighing more than PND 3 animals.

Impact of lipopolysaccharide on peripheral inflammatory markers on postnatal day 5

As anticipated, circulating CRP, IL6, and TNFa was significantly upregulated in LPS-treated animals compared to controls ($F_{(1, 17)} = 11.782$, $P = 0.003$ [Figure 1B], $F_{(1, 15)} = 10.47$, $P = 0.006$ [Figure 1C], $F_{(1, 24)} = 4.71$, $P = 0.04$ [Figure 1D], respectively).

Impact of lipopolysaccharide on early ovarian follicle pool

On PND 5, LPS-treated animals had significantly reduced primordial follicles compared to saline controls (t (10) = 4.02 $P = 0.002$, Figure 2A; neonatal treatment effect: $F_{(1, 20)} = 18.78$, $P < 0.001$; neonatal treatment × age effect: $F_{(1, 20)} = 10.90$, $P = 0.004$). Neonatal LPS exposure altered the quantity of activated primordial follicles, with *reduced* numbers of follicles in LPS-treated rats compared to saline controls on PND 3 (t (10) = 2.39, $P = 0.03$, Figure 2B), but significantly *increased* activated primordial follicles on PND 5 (t (10) = 2.31, $P = 0.044$); age × treatment effect: $F_{(1, 20)} = 10.97$, $P = 0.003$; Figure 2B). There were no significant differences in primary follicle numbers on either treatment day (Figure 2C). As expected, a significant effect of age was seen on all follicle types (primordial: $F_{(1, 20)} = 104.4$; activated primordial $F_{(1, 20)} = 13.48$; primary; $F_{(1, 20)} = 26.47$, $P < 0.005$ for all), with PND 5 ovaries containing more follicles overall compared to PND 3.

Impact of lipopolysaccharide on ovarian proteome

There was significant expression of 598 proteins in the ovaries of PND 5 LPS females ($P \leq 0.05$). Protein expression was compared to saline control levels, resulting in 29 proteins differentially expressed in LPS-treated animals. Functional analysis of these identified several molecular networks, canonical pathways and cellular functions implicated in acute phase response signaling and innate immune responses, amino acid and lipid metabolism, molecular transport, and cellular movement, signaling, assembly and survival (Figure 3A and B; Supplementary Tables S2–S5).

Ovarian real-time quantitative-PCR

PND 5 ovaries were probed for mRNA expression of proinflammatory markers and growth and transcription factors. Fold-change mRNA expression of *Gdf9* was significantly upregulated in the ovaries of PND 5 LPS-treated females (t (4) = 8.05), compared

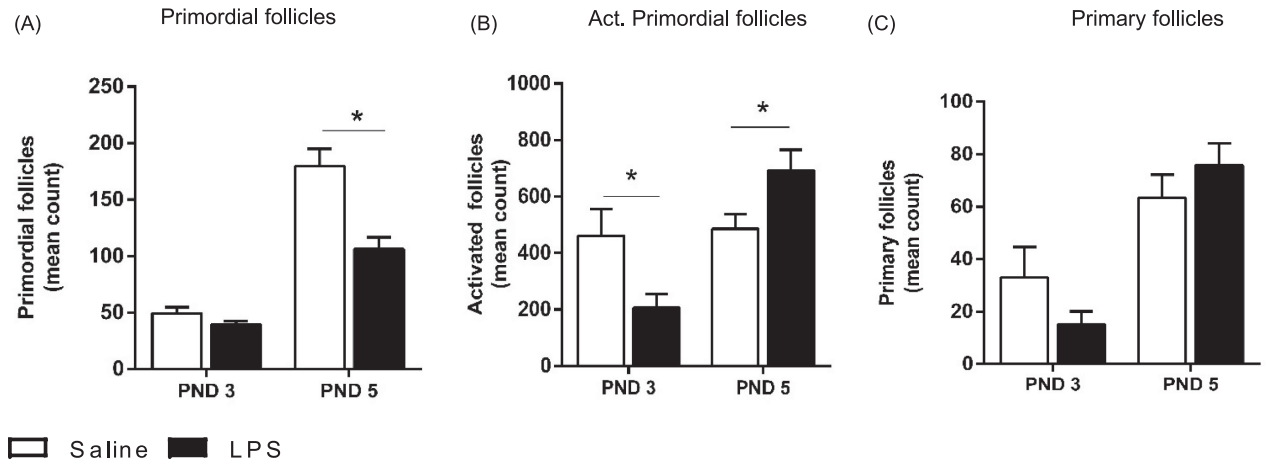

Figure 2. PND 3 and 5 mean ovarian follicle counts. Mean counts of early follicle populations (A) primordial follicles on PND 3 and PND 5; (B) activated primordial follicles on PND 3 and PND 5; (C) primary follicles on PND 3 and PND 5. Mean ovarian follicle count + SEM is graphed. White bars represent saline-treated controls, filled bars represent LPS-treated animals. '*' indicates $P < 0.05$.

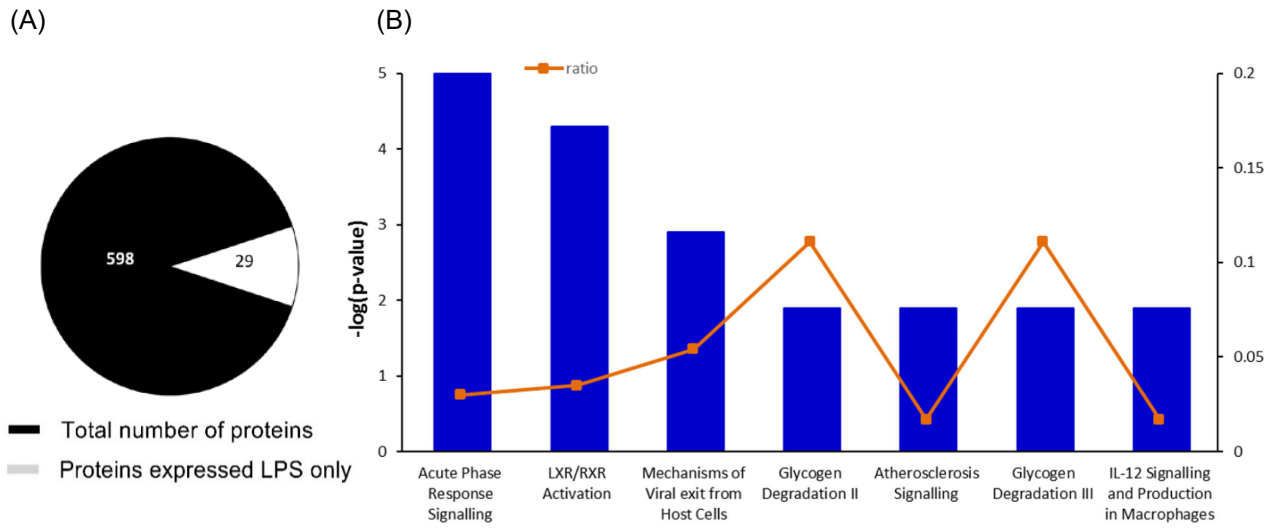

Figure 3. Mass Spectrometry and Top Canonical Pathways significantly upregulated by neonatal LPS treatment on PND 5, as identified by Ingenuity Pathway Analysis (IPA). (A) Representation of mass spectrometry information obtained from PND 5 LPS-treated ovaries. Twenty-nine proteins were significantly differentially expressed in the ovaries of LPS-treated females out of the total number of proteins significantly expressed, compared to controls. (B) Blue bars (column graph, x-axis) represent the significance of associated upregulated expressed proteins and the canonical pathway assessed using a right-tailed Fisher's exact test to calculate p-values determining the probability that the association is explained by chance alone. Ratio score (line graph, z-axis) indicates the proportion of coverage from a pathway related to total number of molecules, subject to pathway size bias.

to saline controls, $P \leq 0.001$ (Figure 4A). There were no significant changes in expression of *Foxo3a* (Figure 4B). We observed a trend towards downregulated *Tlr4* mRNA expression in LPS-treated animals ($P = 0.061$) (Figure 4C). Expression of *Tnfa* and *Mapk8/Jnk1* were significantly upregulated in LPS-treated animals on PND 5 (t (4) = 3.54 and t (4) = 0.14, respectively; both $P \leq 0.05$) (Figure 4D and E). LPS females displayed a nonsignificant increase in *Prkcb* mRNA expression ($P = 0.170$, Figure 4F).

Ovarian protein localization and quantification

Immunohistochemical processing was carried out on the PND 5 ovaries of LPS and saline-treated females to detect the localized expression of TLR4 and TNFa protein, yH2AX as a marker of double-stranded DNA damage, and cleaved CASP3 and TUNEL for assessment of apoptosis. Gamma H2AX was detected in oocytes of both LPS and saline animals (Figure 5A and B). TLR4 immunolabeling was detected surrounding the oocyte (Figure 5C and D). Tumor necrosis factor alpha expression was detected both in the granulosa cells and oocytes in both treated and control samples (Figure 5E and F). CASP3 quantification in the ovaries of LPS-treated animals demonstrated a 2.3× fold change increase in CTCF compared to saline-treated controls. However, this increase was not statistically significant (t (4) = 2.291, $P = 0.084$ [Figure 6A–C]). LPS-treated animals demonstrated a significantly greater number of TUNEL positive ovarian cells (t (4) = 5.191, $P = 0.007$, particularly in oocytes [Figure 6D–F]).

Discussion

Early life is a critical period for fundamental ovarian development and associated neuroendocrine and immune system maturation. Disruption of developmental trajectories via immune activation during this sensitive period may have persisting detrimental effects. Here, we demonstrate in female neonatal rodents that a low dose of LPS in the first week of life has an acute effect on the neonatal ovary during the final stages of follicular pool formation in vivo. LPS administration on PND 3 and 5 upregulated circulating inflammatory mediators, altered early follicle populations, and had an immediate effect on ovarian immune status on PND 5. This study is one of the first to show the immediate effect of perinatal immune stress on early ovarian follicle populations and associated ovarian transcriptome.

Neonatal LPS exposure resulted in reduced weight gain between PND 3 and PND 5, consistent with our previous findings [11, 27, 28]. As expected, administration of LPS on PND 3 and 5 caused significant increases in circulating acute phase protein CRP, IL6, and TNFa. These results confirm the efficacy of LPS treatment in what is considered a hypo-responsive period for immune responses and stress in the neonatal rodent [64, 65]. Peripheral increases in proinflammatory cytokines may stimulate and exacerbate normal inflammatory mediator levels within the ovary at this critical time, regardless of ovarian immune privilege.

Neonatal LPS exposure altered early follicle populations in the PND 5 ovary, leading to a significant decrease in primordial follicle numbers and a significantly greater number of activated primordial follicles. This suggests that early life immune activation may prematurely activate quiescent follicles, leading to follicle depletion. Previous findings from our laboratory demonstrated a depleted PND 14 primordial follicle pool [37], as well as advanced senescence at 1 year of age [11], indicating that the morphological changes seen here are sustained through to prepubescence and may contribute to an early fertility decline. Premature follicle diminishment reduces the number of viable follicles for later ovulation, in turn affecting the quality of the dominate follicle. The untimely depletion of primordial follicle pool seen here may be occurring via intraovarian autocrine and paracrine immune signaling [66] or via gap junctions operating within the oocyte-granulosa cell complex [67], stimulated by excessive immune perturbation. Rapid follicle formation, proliferation, maturation, and atresia is typical in the PND 2 to 5 female rodent, where oogonia migration is occurring [15]. LPS administration may

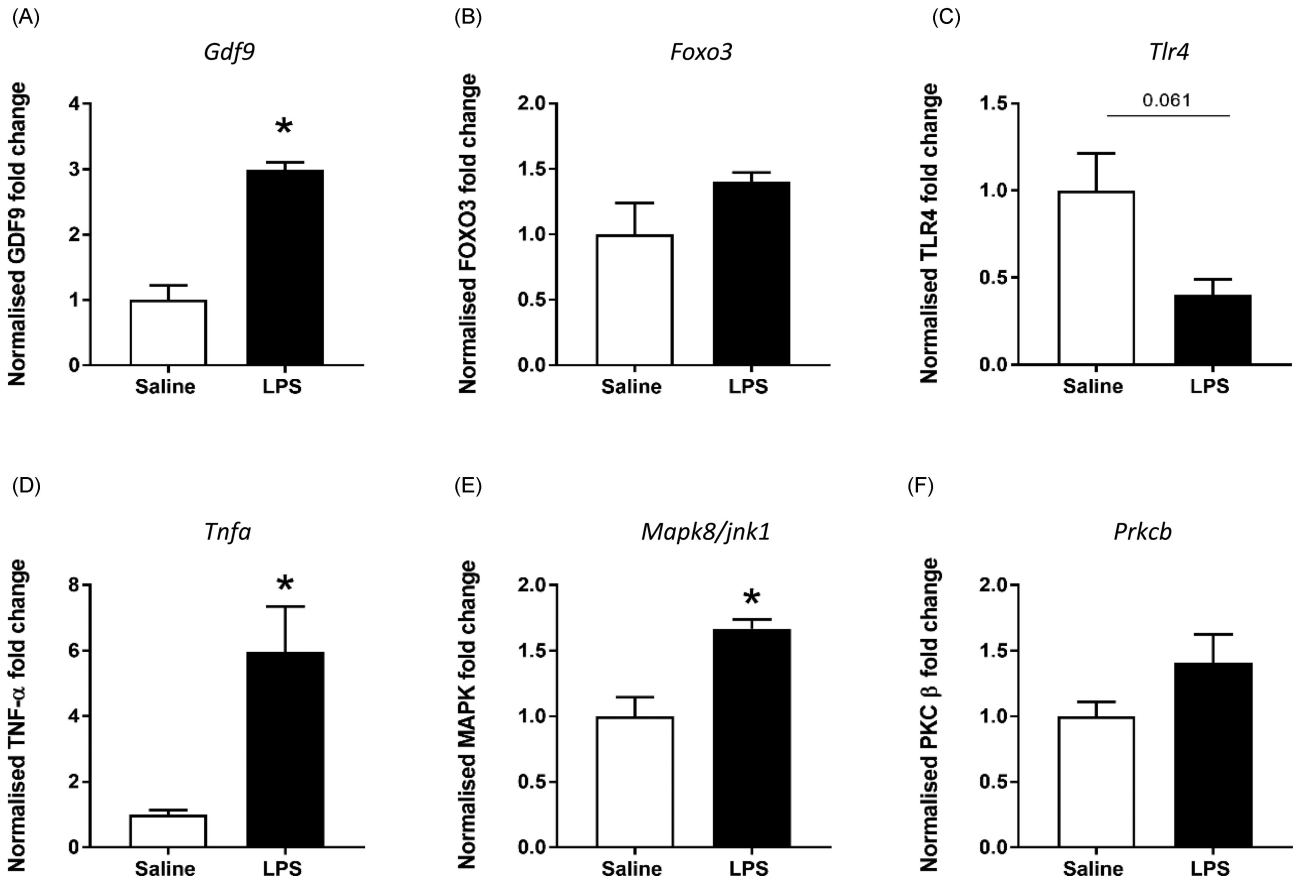

Figure 4. qRT-PCR analysis of growth and transcription factors, and inflammatory pathways associated with initial folliculogenesis. Fold change mRNA expression of ovarian growth factor *Gdf9* (A) and transcription *Foxo3*a (B). Fold change mRNA expression of inflammatory mediators associated with ovarian follicular development and function; *Tlr4* (C), *Tnfa* (D), *Mapk8/Jnk1* (E), and *Prkcb* (F). White bars represent saline controls, filled bars represent LPS-treated animals. '*' indicates $P < 0.05$.

exacerbate these typical ovarian processes, particularly as LPS stimulation has been demonstrated to cause oocyte and granulosa cell apoptosis in fully developed ovaries [36, 68]. This is substantiated in the current study by the localization of yH2AX in the oocyte complex, indicative of rapid DNA damage [69], and the significant increase in TUNEL positive oocytes in LPS-treated animals, demonstrating a combined effect of PND 3 and PND 5 LPS injections. Primary follicle populations did not differ, suggesting that primordial follicles may have already undergone apoptosis postactivation, and that the primordial follicle population may have a heightened vulnerability to the effects of LPS. Likewise, Bromfield and Sheldon [36] demonstrated that the primordial follicle pool is particularly sensitive to LPS-driven apoptosis, whereas larger follicles display some resilience.

Using MS, we identified 29 proteins differentially expressed in the PND 5 ovaries of LPS-treated animals. Pathway analyses indicated that these proteins were associated with acute-phase response signaling, liver X receptor/retinoic X receptor (LXR/RXR) activation, mechanism of viral exit, and glycogen degradation signaling (see Figure 3; Supplementary Tables S2–S5). This indicates transcription of acute phase mediators within the PND 5 ovary, confirmed here with qRT-PCR. These results are congruent with our previous microarray findings demonstrating LPS stimulated upregulation of immune pathways in PND 7 ovaries [37]. Activation of the LXR/RXR pathway is of novel interest, as LXR/RXRs are involved in the macrophage response to TLR4 activation and are expressed in the ovary [70, 71], with LXR null mice displaying subfertility and oocyte meiotic incompetence [72]. Interestingly, LXR/RXR activation inhibits inflammatory signaling [73]; hence, activation of LXR/RXR seen here may serve to protect the developing ovary from excessive inflammation. Further investigation is needed as this may be a novel pathway to examine in the early life immune stress model, particularly considering the association between early life stress, fertility dysfunction, and metabolic and inflammatory diseases.

In the current study, LPS administration significantly upregulated the mRNA expression of inflammatory mediators MAPK8/JNK1 and TNFa in the ovaries of LPS-treated animals on PND 5. MAPK8/JNK1 signaling is a major component in acute phase responses, cell survival and apoptosis [60] and both LPS and proinflammatory cytokines, particularly TNFa, activate the MAPK8/JNK1 pathway [74, 75]. The MAPK8/JNK1 pathway is also implicated in the activation and maturation of early follicles [60, 76]. Recent evidence indicates MAPK8/JNK signaling contributes to follicle activation via the mechanistic target of rapamycin signaling, which may merit further investigation within the current model [77]. The significantly increased expression seen here may indicate that a downstream acute phase response is activated within the ovary, with immune mechanisms contributing to early follicle depletion. Concomitant to MAPK8/JNK1 stimulation, is the activation of other inflammatory pathways and mediators including NFKB, PI3K/AKT, PKCB,

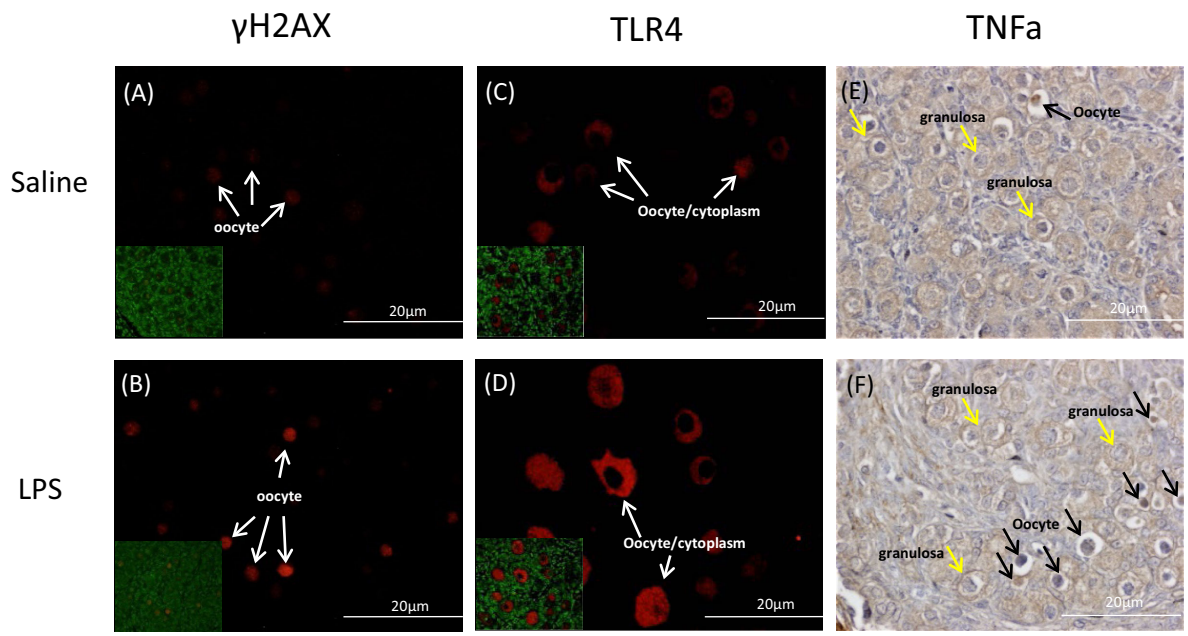

Figure 5. Observational fluorescent and Immunohistochemical protein localization of γH2AX, TLR4 TNFa. (A and B) Representative γH2AX immunolabeling detected in oocytes in both treatment groups; (C and D) TLR4 immunolabeling was expressed in the oocytes and oocyte cytoplasm in both treatment groups; (E and F) TNFa expression was localized to the oocyte and surrounding granulosa cells in both LPS and saline. Fluorescent green (YOYO) represents nuclear staining, fluorescent red staining represents specific staining for protein of interest (A–D). White arrow = area of interest. DAB TNFa staining is indicated in brown, counterstained with Carezzis blue (E and F). Yellow arrow = granulosa, black arrow = oocyte.

IL1B, IL6, nitric oxide synthase-1 (NOS2), and cyclooxygenase-2 [78], which are all associated with both normal and pathological ovarian function [66, 79–82], and early life activation of MAPK pathways may lead to long-term functional differences with these mediators.

It is known that TNFa activates and exacerbates the LPS-driven immune and stress responses, and plays a fundamental role in the immature ovary, facilitating normal oocyte atresia and follicular assembly to define the size of the primordial follicle pool [58, 83]. As TNFa alone can impair ovarian functions and override factors that inhibit follicle activation [59, 84], these higher gene expression levels of *Tnfa* seen here may be a driving factor increasing primordial follicle activation in LPS-treated animals, leading to superfluous and premature depletion. Tumor necrosis factor alpha protein was localized to granulosa cells, oocytes, and the surrounding complex in both groups, indicating that expression may be facilitating bidirectional crosstalk between the oocyte-granulosa matrixes. The strong trend for *Tlr4* downregulation seen here is most probably due to a habituation effect of the temporal proximity of dual LPS administration and the time point at which tissue was taken. These findings were contrary to those hypothesized, as we previous demonstrated a significant upregulation of *Tlr4* gene expression in the PND 7 ovary; however, TLR4 staining here was localized to the oocyte cytoplasm as previously demonstrated [37]. The current and previous findings suggesting that the ovary may adapt to the level of LPS signaling and display altered TLR4 expression as a way to manage a predicted high-immune-stress or bacterial-rich environment. Permanent alterations in TLR4 expression may be detrimental, particularly considering that TLR4 overexpression is involved in the growth and survival of ovarian cancer cells [85] and poor ovarian response [86], and TLR4 under expression is implicated in polycystic ovarian syndrome (PCOS) and endometriosis [87, 88]. Protein kinase C beta aids the regulation of early follicular proliferation, survival, and activation [89], and although upregulation here is nonsignificant, it may be contributing to the perpetuation of cytokine secretion [90].

Growth factor GDF9 and transcription factor FOXO3A are implicated in early follicle primordial growth, maturation, and apoptosis. The significantly increased *Gdf9* mRNA expression demonstrated here may indicate that immune activation is prematurely instigating the maturation signaling between the oocyte and its granulosa cells, particularly as GDF9 stimulates small follicle proliferation of granulosa cells in rats in vivo [91, 92] and promotes growth of human ovarian follicles in vitro [93]. Abnormal expression of GDF9 and *Gdf9* mutations of are apparent in women with PCOS and POF [53, 94]. Additionally, the *Gdf9* increases seen here may also be an attempt to downregulate inflammation, as *Gdf9* activation stimulates transforming growth factor (TGF)-beta-like SMAD2/3 intracellular pathways [95] that have been shown to inhibit immune cells, including macrophage activation, with LPS exposure [96]. As macrophages that express TLR4 are present in the ovary, the increase in *Gdf9* expression in LPS challenged animals may indicate a protective mechanism undertaken by the ovary in order to self-modulate inflammatory signaling, with activation and atresia of some early follicles being compensation for overall ovarian reserve defense. Neonatal immune insults may not only impact the quantity, but also the long-term quality of the remaining follicular pool. As GDF9 and associated TGF-b superfamily members are modulators of sex hormone sensitivity, aberrant GDF9 signaling during ovarian development may have long-term effects on follicle stimulating hormone and luteinizing hormone receptor densities and sensitivities, affecting postpubertal ovarian processes [97, 98]. Forkhead box-O3a has been suggested as a key mediator of naked oocyte and primordial follicle apoptosis within the neonatal rat ovary [56]. As

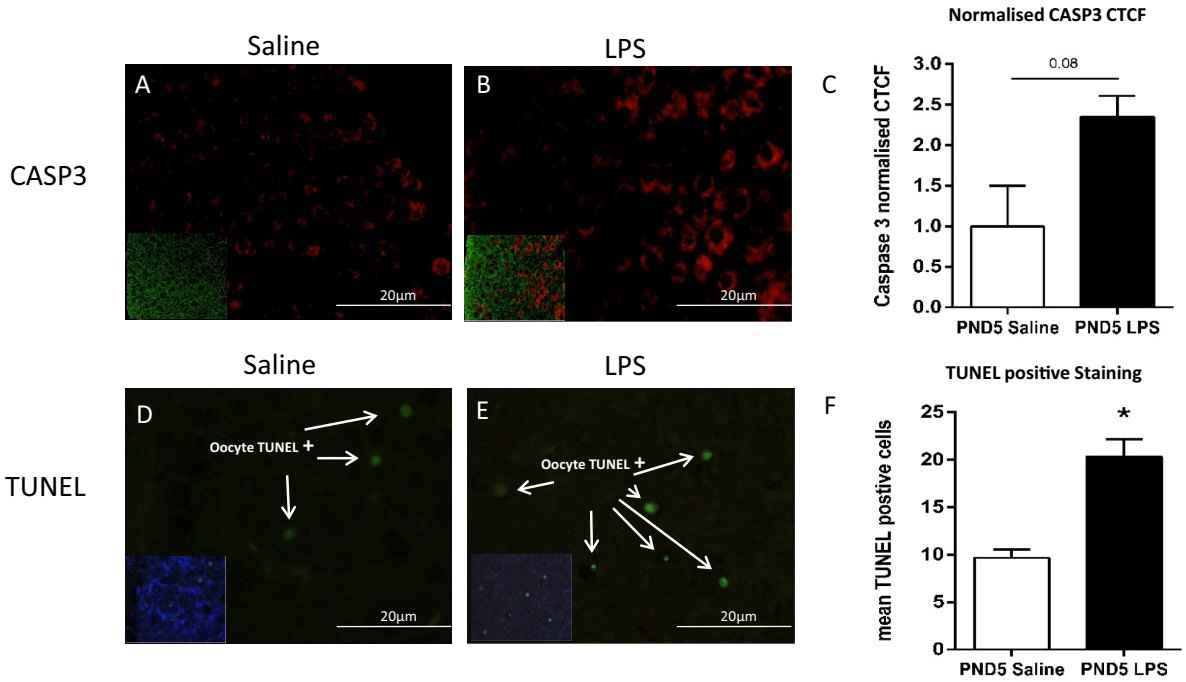

Figure 6. Quantification of cleaved CASP3 and TUNEL positive cells in PND 5 ovaries. Localization representative images of cleaved CASP3 in (A) saline-treated females and (B) LPS-treated females. Fluorescent green (YOYO) represents nuclear staining, fluorescent red staining represents CASP3 staining. (C) Quantification of CASP3 represented as normalized fold change CTCF compared to saline controls + SEM. Localization representative images of TUNEL positive ovarian cells in (D) saline-treated females and (E) LPS-treated females. (F) Quantification of TUNEL positive cells in saline and LPS-treated groups represented as mean count + SEM. Fluorescent blue (DAPI) represents nuclear staining. White bars represent saline controls, filled bars represent LPS-treated animals. '*' indicates $P < 0.05$.

FOXO is negatively regulated by PKB/AKT/PI3K and MAPK8/JNK1 activation [55, 99], this may be contributing to our unexpected non-significant findings as previous findings with this LPS model indicate significant upregulation of *Prkb/Akt/Pi3k* pathways in PND 7 ovaries [37]. The nonsignificant difference in *Foxo3a* at this current time point indicates that analysis at an alternate time point is needed to capture *Foxo3a* translocation, transcription, and apoptosis induction. Forkhead box O3-null mice demonstrate normal follicle assembly, followed by global activation and early depletion of the primordial follicle pool, leading to POF and infertility [100]. This implicates FOXO3A in the maintenance of quiescent follicles, as transgenic models of constitutively active *Foxo3a* expression demonstrate suppression of follicular maturation and infertility [101], meriting future investigation in this model. Future studies are necessary to examine FOXO3A and additional growth/transcription factor gene and protein expression in the neonatal LPS model, particularly given the links between FOXO3A and LPS-induced innate inflammatory pathway upregulation, ovarian follicular development, and studies demonstrating LPS inactivating FOXO3A in other tissues [102–104].

Our current findings indicate that LPS exposure activates and depletes the early primordial follicle pool. This may be occurring via immune pathways to affect transcription and growth factor-mediated early follicular control, particularly considering the shared, complex immune-regulatory properties and capabilities of TNFa, MAPK8/JNK1, and GDF9. These mediators influence early follicle formation, development, and maintenance during a critical period, where follicle dynamics are both intricate and elusive in nature. The development of the finite ovarian follicular pool is dependent on homeostatic processes, which if disrupted, may lead to sustained alterations to ovarian physiology. Premature loss of the ovarian reserve not only has a detrimental effect on the female reproductive lifespan, but is associated with a myriad of heath complications due to deficiency of ovarian-produced oestrogen, including osteoporosis, cardiovascular disease, autoimmunity, and psychological disorders. Early life stress is already a well-established risk factor for morbidity and mortality from a range metabolic, immune, and neuroendocrine disorders [105], and is known to lead to a physiological vulnerability to stressors in later life [43, 106]. The pathogenesis of idiopathic reproductive disorders may have developmental origins, as well as be intensified by the additional stress these disorders exert. The current study provides further insight into the link between early life immune disturbances and ovarian development. A number of female reproductive disorders associated with skewed inflammatory profiles such as; PCOS, endometriosis, and POF are increasingly presenting in a younger female demographic, often with no apparent origin. Examining stressful events in the early life environment can therefore provide valuable insight into the pathogenesis and progression of reproductive disorders, as well as aid in the understanding of mechanisms regulating the formation and longevity of the ovarian reserve.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1. Antibody information.

Supplementary Table S2. Significantly altered protein expression in the ovaries of LPS-treated animals classified according to molecular and cellular functions/biological processes.

Supplementary Table S3. Significant differentially expressed proteins in LPS and control groups via Mass Spec., $P < 0.05$.

Supplementary Table S4. Ingenuity pathway analysis (IPA) information including top networks, top biological functions, top canonical pathways and top upstream regulators for both saline and LPS treatment groups, and molecular pathway visualization links for top LPS pathways.

Supplementary Table S5. Function/role of the 29 differentially expressed proteins in LPS-treated animals according to immune (IM) and/or ovarian developmental functions (OV) (retrieved from uniprot).

Supplementary data S6. Immunohistochemistry positive and negative control images. (A) Gamma H2AX controls; (B) CASP3 controls; (C) TLR4 controls; (D) TUNEL controls; (E) TNFa DAB controls.

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Chapter 4. Supplementary Information

S1. Antibody information

S2. LPS mass spec data

S3.Saline mass spec data

S4. Top canonical pathways upregulated

S5. LPS protein expression functions table

S6. Immunohistochemistry control data/images.

Supplementary data can be found at *BIOLRE online: https://academic.oup.com/biolreprod/article/97/5/719/4430632#supplementary-data*

Chapter 5. Neonatal Immune Activation Leads to Sustained Ovarian Reserve Depletion and Altered Peripheral Inflammatory Mediators.

5.1 Introduction

The previous chapters of this thesis indicate that immune activation in early life alters sexual development and performance, and disturbs initial ovarian development via inflammatory pathways. This has implications for long-term reproductive fitness and ovarian function. Female reproductive health is fundamental to women's overall health and wellbeing. Despite advances in female reproductive biology and treatments, the origins of female subfertility and infertility are still not completely clear (Evers, 2002; Kamath & Bhattacharya, 2012). Reproductive disorders and dysfunction is becoming increasingly prevalent in younger female cohorts and developed societies, regardless of the availability of healthcare and good nutrition (Beck-Peccoz & Persani, 2006; Hernández-Angeles & Castelo-Branco, 2016; Maheshwari et al., 2008; Norman & Moran, 2015; Weiss & Clapauch, 2014). One in 6 Australian couples experience fertility dysfunction, with 37% of subfertility issues being associated with female factors (O'Rouke, 2008). Increases in the mean age of childbearing in women accounts for only a proportion of these increasing figures (Aitken & Koppers, 2011). Current evidence suggests that the physical, psychological and social environment influences not only overall health, but are critical determinants of female reproductive outcomes (Dobson & Smith, 2000; Klein & Nelson, 1999; Vrekoussis et al., 2010). Interestingly, mounting evidence also suggests the early life environment as a major factor influencing lifelong female reproductive health and success (Richardson et al., 2014; Sloboda et al., 2011; Sominsky et al., 2015). It is known that the early life environment contributes towards variations in developmental trajectories and later life health and behaviour, including a vulnerability to later life stressors (Barker, 2004; Bateson et al., 2004; Crespi & Denver, 2005;

Gluckman et al., 2010). Developing cells, organs and systems, most notably the immune and endocrine system, are particularly sensitive to exogenous and endogenous environmental stimuli during maturation (Hochberg et al., 2011; McEwen & Gianaros, 2011; Zakharova, 2014). Importantly, these immune-endocrine systems are key regulators of initial and continued female reproductive success and longevity, with immune components being particularly crucial to the rudiments of early ovarian follicular development (Bukovsky & Caudle, 2012; Richardson et al., 2014). Therefore, early life stressors that disrupt or perturb the delicate immune processes during critical periods of immune-driven reproductive development, such as the formation of the gonads and long-term immune function, play a key role in shaping ovarian functionality, female reproductive longevity, and overall health (Dobson & Smith, 2000; Inhorn & Patrizio, 2015; Lutz et al., 2001; Sominsky et al., 2015).

The fundamentals of female reproductive health are laid down in the early life period. Female gonadal development is reliant on the normal and non-perturbed establishment of a finite, pool of ovarian primordial follicles (McGee & Hsueh, 2000). In the human ovary, this initial ovarian follicle development, or initial folliculogenesis, occurs after 11 or 12 weeks of gestation (Gougeon, 2004; Oktem & Urman, 2010; Sarraj & Drummond, 2012). Females are born with a finite number of germ cells, called ‘oocytes’ contained within primordial ovarian follicles (Baker, 1963). In female mammals, the non-renewing pool of ovarian follicles dictates the female reproductive lifespan and potential, serving as the foundation for all developing oocytes (Banerjee et al., 2014). In rodents, the early neonatal period is a critical time point for the final stages of initial ovarian folliculogenesis, making it an ecologically valid animal model to explore the impact of early life stress on immune-mediated ovarian development (Gougeon, 2004; Zeleznik, 2004). In rodents, the 1st postnatal week is where oocyte ‘nests’, tightly gathered clusters of oocytes, break down to form the primordial follicle reserve (Grive

& Freiman, 2015; Skinner, 2005). This process is typically characterized by a period of mass proliferation and programmed follicle atresia (Dissen et al., 2004). After formation of the ovarian reserve, a large proportion of newly formed follicles will remain in a mainly quiescent state until puberty (day of vaginal opening [DVO] in the female rat), where selected follicular cohorts will be recruited through gonadotropin-dependent processes and cross communication between the oocyte and surrounding granulosa cells for maturation throughout the antral and pre-ovulatory stages for ovulation selection or atresia, completing the 4-5 day rat oestrus cycle (Hirshfield, 1991; Hirshfield, 1994; McLaughlin & McIver, 2009). Less than 1% of all recruited follicles will proceed through to ovulation, as follicular atresia at these stages is a normal process of dominant follicle selection (Matsuda et al., 2012; McGee & Hsueh, 2000). However, research has demonstrated that premature or abnormal atresia and apoptosis may be triggered via environmental stimuli such as xenobiotics (Camlin et al., 2014; Sobinoff et al., 2013; Sobinoff et al., 2010; Sobinoff et al., 2012), ovarian dysfunction (Borghese et al., 2015; De Vos et al.; Dumesic et al., 2007), and immune stressors (Besnard et al., 2001; Bromfield & Sheldon, 2011; Bromfield & Sheldon, 2013; Sominsky et al., 2013; Wu et al., 2011b). Environmental stressors experienced by the neonatal rodent may influence the fundamentals of follicular maturation and selection, and have long-term effects on the systems that govern early and later life ovarian processes, and hence, reproductive health. What is more, early life stress may perinatally program a susceptibility to later life stress, which may also compound the detrimental effects on female reproductive health (Belsky & Pluess, 2009; Spencer et al., 2006c).

It is known that stress has a negative impact upon female reproductive parameters and fecundity (Chrousos et al., 1998; Dobson & Smith, 2000; Kalantaridou et al., 2004; Shalev & Belsky, 2016; Vrekoussis et al., 2010; Wingfield & Sapolsky, 2003). Female reproductive

capacity is governed by endocrine and immune factors that are involved in a carefully orchestrated temporal sequence (Moberg, 1985). The hypothalamic-pituitary-gonadal (HPG) axis, hypothalamic-pituitary-adrenal (HPA) axis, and the immune system work in concert to maintain female reproductive homeostasis. These systems are also the main biological mechanism implicated in the perinatal programming of pathologies, psychopathologies, and later life stress vulnerability (Belsky & Pluess, 2009; Bilbo & Schwarz, 2009; Colodro-Conde et al., 2017; Daskalakis et al., 2013; Green et al., 2011; McCreary et al., 2016; Shanks et al., 1995; Walker et al., 2009). Products of the HPA axis have an inhibitory effect on immune and HPG axis regulation of female reproduction, altering necessary reproductive process such as ovulation (Vrekoussis et al., 2010; Weiss & Clapauch, 2014). Research indicates that females who report excessive stress from infertility issues, stress while trying to conceive, or who experience stress and psychopathologies whilst undergoing in vitro fertilization (IVF) techniques have poorer pregnancy, IVF, and mental health outcomes than those who do not (Beydoun & Saftlas, 2008; Boivin & Takefman, 1995; Matthiesen et al., 2011; Mulder et al., 2002; Reis et al., 2013; Smeenk et al., 2001; Staneva et al., 2015; Terzioglu et al., 2016). Additionally, inflammatory exacerbation and chronic immune dysregulation is associated with both reproductive disorders that effect the functioning of both gonadal and stress hormones (Jabbour et al., 2009; Straub, 2007; Weiss et al., 2009), as well as psychopathologies such as anxiety and depression (Dantzer et al., 2008; Kiecolt-Glaser et al., 2015; Leonard & Song, 1996; Miller & Raison, 2016).

It is significant to note that inflammatory functions are central to normal female reproductive processes, including ovulation, menstruation, implantation, gestation and labour (Hutchinson et al., 2011). Furthermore, inflammatory dysregulation is a major characteristic of reproductive disorders such as polycystic ovarian syndrome (PCOS),

endometriosis and premature ovarian insufficiency/failure (POI/POF) (Benson et al., 2009; Bukovsky & Caudle, 2012; De Vos et al., 2011; Figueroa et al., 2015; Gonzalez et al., 1999; Greene et al., 2014; Halis & Arici, 2004; Harada et al., 1999; Khan et al., 2013; Martinez et al., 2007; Nash et al., 1999; Norman & Brännström, 1996; Peng et al., 2016; Wu et al., 2004; Zhou et al., 2009). As such, perinatally programmed changes to one system may skew the function of others and establish a predisposition to inflammatory-driven reproductive dysfunction that is exacerbated when paired with a second hit of stress in later life, particularly as it is known that the immune system is extremely sensitive to early life environmental influences (Bilbo & Klein, 2012; Bilbo & Schwarz, 2009, 2012)

Bacterial or viral infection is a common environmental immune stressor that affects pregnant women and newborns, particularly with maternal-foetal immune modifications in place (Dupont et al., 2012; Hodyl et al., 2008; Levy, 2005). In the newborn rat, the immune system is functionally immature and highly susceptible to the effects of perinatal programming by environmental stimuli (Kuper et al., 2016). Animal models of infection and immune stress using bacterial mimetic lipopolysaccharide (LPS) exposure in the perinatal period have shown a range of sustained physiological and behavioural alterations, including immune dysfunction (Boisse et al., 2004; Ellis et al., 2006; Spencer et al., 2006b; Walker et al., 2010) metabolic alterations (Iwasa et al., 2010; Walker et al., 2004a), pain sensitivity (Zouikr et al., 2016; Zouikr et al., 2014b), brain morphological changes (Bilbo et al., 2005b; Cardoso et al., 2015; Walker et al., 2003; Zavitsanou et al., 2013), and stress hyper-responsivity and anxiety-like behavioural outcomes (Rico et al., 2010; Shanks et al., 1995; Sominsky et al., 2012b; Spencer et al., 2006a; Tenk et al., 2013; Walker et al., 2012; Walker et al., 2009; Walker et al., 2004a; Walker et al., 2008; Walker et al., 2004c; Walker & Vrana, 1993). Lipopolysaccharide activates an inflammatory cascade by binding to toll-like-receptor 4

(TLR4), triggering multiple inflammatory pathways and mediators. LPS exposure has been demonstrated to be detrimental to ovarian functioning and female reproductive health outcomes (Sheldon et al., 2014; Sheldon et al., 2016; Turner et al., 2012; Williams et al., 2008). The ovary and reproductive tract are immune privileged sites that contain immune cells, receptors, and mediators for reproductive processes, but are also capable of mounting immune responses to pathogen invasion (Herath et al., 2007; Iwasaki & Medzhitov, 2004; Liu et al., 2008; Marcinkiewicz et al., 1994; Norman & Brännström, 1996; Williams et al., 2008; Wu et al., 2004; Zhou et al., 2009). Infections of the reproductive tract, including sexually transmitted infections (STIs), pelvic inflammation and other immune-related disorders are known to result in poorer female reproductive outcomes if remaining unchecked or untreated (Khan et al., 2017; Lanari et al., 2007; Sheldon et al., 2014; Sheldon et al., 2016; Williams et al., 2008).

Infection and experimental animal studies indicate that neonatal LPS alters female reproductive parameters including puberty onset, downregulates HPG hormones including luteinizing hormone (LH) and follicle stimulating hormone (FSH), and impairs mating and maternal behaviours (Iwasa et al., 2012; Iwasa et al., 2009; Sominsky et al., 2012a; Walker et al., 2011). Wu et al. (2011a) demonstrated that LPS on day 3 and 5 altered oestrus cyclicity, and delayed female puberty onset, contrary to the findings of Sominsky et al. (2012a) and chapter 3 of this thesis, where female puberty onset and senescence was precocious in LPS-treated animals using the same model. Knox et al. (2009) demonstrated that LPS administered on PND 3 and 5 was the critical window for the programming of female reproductive development as exposure at later neonatal time points (PND 7, 14 & 16) did not yield cyclicity or puberty onset differences. A diminished primordial follicular reserve has been observed in prepubescent female at PND 14 (Sominsky et al, 2012), as well as in adult females (Wu et al,

2011) following PND 3 and 5 LPS exposure. Bromfield and Sheldon (2013) demonstrated that acute systemic exposure to LPS in adulthood led to a reduced primordial follicle pool, accompanied by upregulated follicle atresia in 8 week old mice mediated via TLR4, as $Tlr4^{-/-}$ C57BL/6 mice used in Bromfield's study showed no primordial follicle deficits. In our laboratory, Sominsky et al. (2013) demonstrated that neonatal LPS exposure upregulated mRNA expression of immune-related and LPS-stimulated pathways via microarray analysis in the PND 7 female rat, following PND 3 and 5 LPS stimulation. Additionally, we demonstrate here in this thesis (Chapter 4, Fuller et al., 2017) that acute alterations to the ovarian immune milieu and diminishment of the primordial follicle pool occur in the neonatal period immediately following PND 3 and 5 LPS stimulation. These studies highlight the sensitive nature of immune-driven female reproductive development and the long-term alterations in reproductive functionality that they can be associated with.

Interruptions to the delicate immune processes during critical stages of follicular formation may alter immune processes responsible for normal ovarian follicular establishment and growth, leading to abnormal follicular assembly, premature follicular activation, excessive atresia, and apoptosis of the limited follicular pool (Hirshfield, 1991; Hussein, 2005; Morrison & Marcinkiewicz, 2002; Skinner, 2005). Evidence from our laboratory indicates that neonatal immune activation (NIA) with LPS has an acute effect on ovarian immune status and negative implications for primordial follicle numbers in the early and late neonatal period (Fuller et al,. 2017, Ch4; Sominsky et al., 2013). Additionally, our laboratory has demonstrated deficits in mating behaviours in the female rat (Walker et al., 2011), which appears to not be driven by motivational factors or excessive HPG axis alterations (Fuller at al., 2017, Chapter 3; Walker et al., 2011). What remains to be examined within this model is the long term effects of NIA on immune mediators in the periphery, with a particular focus

on the ovarian immune environment which may be a factor contributing to the altered behaviours and precocious puberty onset previously demonstrated. Additionally, as NIA is known to create a programmed immune vulnerability, the addition of a later life '2nd hit' of psychological stress will allow for the examination of an immune vulnerability to later life psychological stress that may compound the effects of NIA. We hypothesize that NIA will have long-term effects on ovarian morphology and immune functionality, particularly in the presence of a 2nd hit of psychological stress in adulthood. Furthermore, it is hypothesized that LPS treated females will display altered patterns of ovarian inflammatory gene expression and variations in ovarian follicle populations due to programmed immune and stress vulnerabilities, providing mechanistic insights into the perinatal programming of a female subfertility phenotype.

5.2 Method

5.2.1 Animals

Twelve experimentally naïve female outbred Wistar rats were obtained from the University of Newcastle animal house and mated with proven male studs in the University of Newcastle Behavioural Sciences vivarium, resulting in 12 litters and a total of 137 pups. Sixty-five female pups were allocated to this study (32 saline derived from 4 litters, 33 LPS derived from 6 litters) the remaining pups were male and not utalised in this study. As previously described (Sominsky et al., 2012a; Sominsky et al., 2012b; Walker et al., 2012; Walker et al., 2011; Walker et al., 2009; Walker et al., 2010; Walker et al., 2004a; Walker et al., 2008; Walker et al., 2004c; Zouikr et al., 2015; Zouikr et al., 2014a; Zouikr et al., 2014b), animals were housed under normal conditions (0600-1800) with controlled temperature (21±1 °C) and humidity (34±2%). Animals were housed in plastic wire top cages (41.5cm x 28cm x 22cm; Mascot Wire Works, Australia) containing recycled compressed paper bedding with food

(standard rat chow) and water available *ad libitum*. As previously described (Sominsky et al; Walker et al; Zouikr et al.) litters were randomly allocated at birth (post-natal day [PND] 1), to either a LPS or a saline treatment. On PND 3 and PND 5, whole litters were briefly removed from their home cage and administered either LPS (Salmonella enterica, serotype Enteritidis: Sigma-Aldrich Chemical Co., USA in sterile pyrogen-free saline, 0.05mg/kg) or an equivolume of sterile saline (Livingstone International, Australia) via intraperitoneal injection (ip). Briefly, entire litters were removed from the dam, incubated to maintain body temperature, weighed, and administered with either a LPS or saline injection. Litters were then placed back with the dam in the home cage and left undisturbed until PND 21, when they were weaned into same-sex, same-litter pair housing. Weekly weighing and monitoring commenced at weaning and continued until the conclusion of the experiment. Post weaning, animals were monitored daily for day of vaginal opening (DVO) signalling puberty, at which time daily oestrus checks (as previously described) began until experiment conclusion. All procedures were carried out under the approval of the University of Newcastle Animal Care and Ethics Committee (ACEC, A-2012-2813) and are in accordance with the 2004 NH&MRC Australian Code of Practice for the Care and Use of Animals for Scientific Practice under the protocol.

5.2.2 Oestrus cycle monitoring.

As previously described, animals were removed from their home cage and a sterile glass pipette containing sterile saline (~200µl) was gently inserted and flushed into the vaginal opening, then collected and transferred to a glass slide for assessment. Phase of oestrus cycle was identified under light microscope at 10x magnification according to the predominate population of vaginal cells displayed (Goldman et al., 2007; Sominsky et al., 2012a). Oestrus cycle was measured from day of vaginal opening to end of experiment.

5.2.3 Acute adult stress protocol.

In adulthood (PND 85) animals from both groups were randomly allocated into a '2nd hit' stress or no stress condition, resulting in the formation of four treatment groups (i.e. neonatal (n) Saline/adult (a) Stress, nSaline/aNoStress, nLPS/aStress and nLPS/aNoStress) (See Figure 5.1; NS = no stress, ST = restraint stress). Animals allocated to the adulthood stress condition underwent a consecutive three-day acute stress protocol, previously utilised in our laboratory (Barreau et al., 2004; Walker et al., 2009) consisting of 30 minutes restraint stress per day for three days, followed by 30 minutes isolation housing on day three. All stress protocols were conducted between 0900h-1100h in a separate room removed from all other animals. The restraint apparatus consisted of soft wire mesh (25.0 cm × 20.0 cm) folded around the animal and clipped to restrict movement. Isolation housing was identical to the animals' home cage, and female rats were given food and water ad libitum. Animals in the 'No Stress' conditions were briefly handled and weighed before being returned to their home cage.

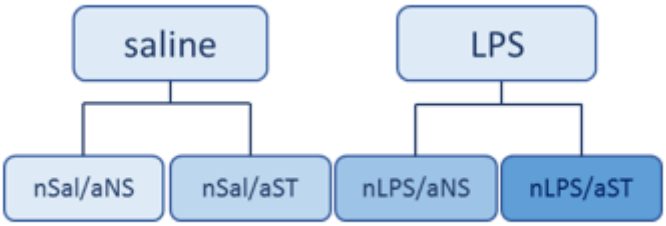

Figure 5.1. Representation of treatment group allocation. n = neonatal, a = adult, Sal = saline, LPS = lipopolysaccharide, NS = no stress, ST = restraint stress.

5.2.4 Blood and Tissue Collection

***5.2.4.1 Blood collection and assessment*.** A subset of animals were culled on PND 5 (saline *n* = 11, LPS n = 11, min 1 per litter) via rapid decapitation 2.5 hours following treatment to confirm efficacy of NIA. Trunk blood was collected into EDTA coated tubes, centrifuged at 1000g at 4°C for 20 minutes, and plasma supernatant was collected and stored at -20°C until assessment. In adulthood on day one of the three-day restraint protocol, non-terminal

saphenous bleeds were taken from all animals to assess immune response at baseline (pre restraint) and 2.5 hours post restraint. Samples were taken from animals in the NS conditions at the same time points. All blood samples were centrifuged at 1000g at 4°C for 20 minutes, plasma supernatant was collected and stored at -20°C until further assessment. Plasma was analysed for neonatal immune activation using markers IL-6 (Abcam, ab119548 rat ELISA kit, minimum detection rate 12pg/mL, intra- and inter-assay variability <5% and <10% respectively) and TNFα (R&D Systems Rat TNF alpha Quantikine ELISA Kit, RTA00, minimum detection rate <5 pg/mL, intra- and inter-assay variability 2.1 - 5.1% and 8.8 - 9.7% respectively) and IL-2 (Abcam, ab100769 rat ELISA kit, minimum detection rate 0.1ng/mL, intra- and inter-assay variability <10% and <12% respectively). IL-6 and IL-2 only were measured adulthood. These T helper (T_H) 1 immune markers have been established as being involved in the stress-mediated, neuroendocrine modulated cytokine changes and the pathogenesis of psychiatric disorders and female reproductive disorders (An et al., 2015; Glaser et al., 1990; Himmerich et al., 2013; Rybka et al., 2016; Tanebe et al., 2000; Tian et al., 2014).

***5.2.4.2 Tissue collection*.** Animals were humanely euthanized via ip injection of Lethabarb (2ml/kg of body weight) 24 hours post-stress protocol. Upon complete euthanasia, a sample of cardiac blood was taken, and the animal then perfused with ~600ml of 4°C sterile PBS to clear tissue of blood. One ovary (counterbalanced across animals) was excised, dissected of superfluous tissue, snap frozen on dry ice, and stored at -80°C. The remaining ovary was dissected as previously mentioned and fixed in Bouins fixative (Polysciences, Pennsylvania, USA) for 24 hours then rinsed in 4 x 70% ethanol rinses, stored in 70% ethanol prior to immunohistochemical analysis (See Figure 5.2 for schematic of methodology).

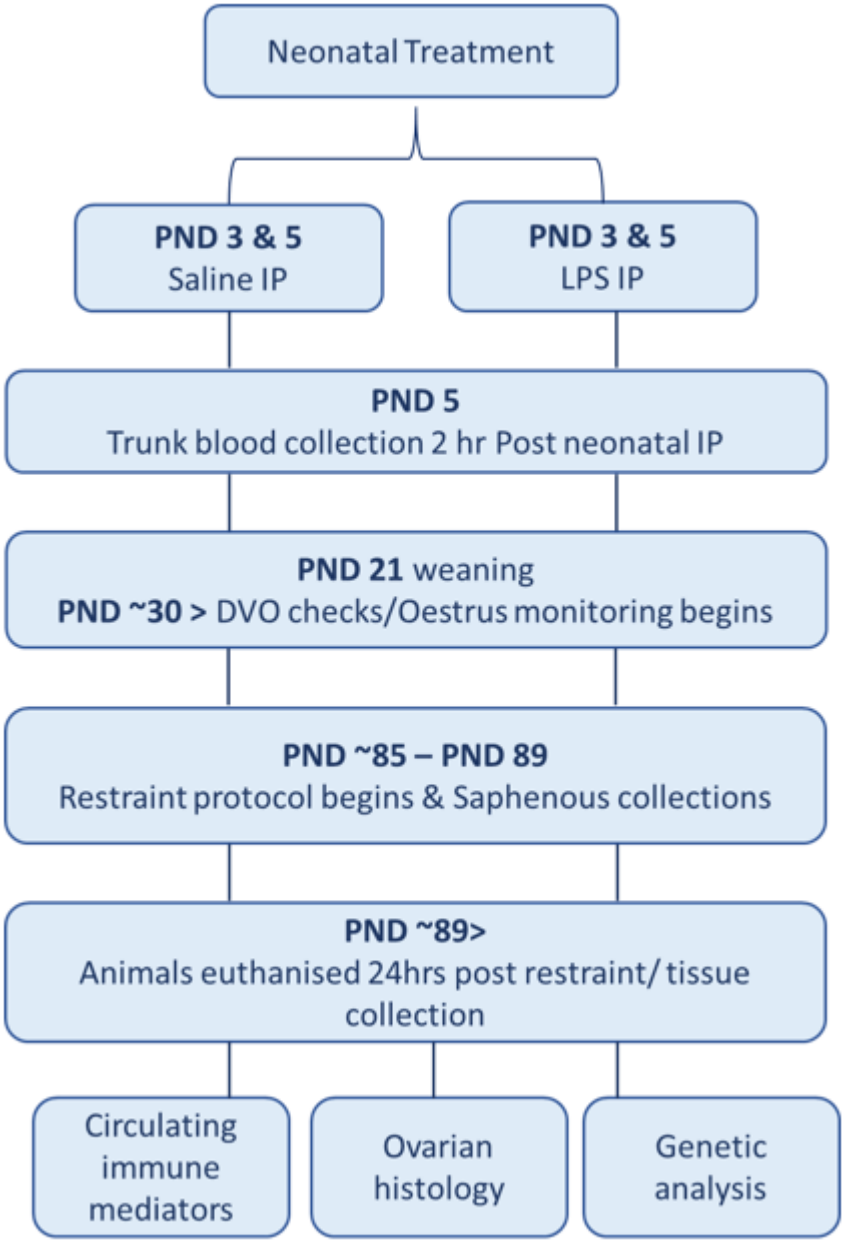

Figure 5.2. Timeline of the experimental protocol. Following neonatal injections (PND 3 and PND 5) a subset of animals were culled (PND 5) to evaluate the efficacy of LPS treatment. Weaning occurred at PND 21, followed by DVO monitoring. Oestrus checks commenced following DVO and continued until the conclusion of the experiment. Exposure to the acute stressor occurred in adulthood (PND ~85>) and continued for 3 consecutive days. Baseline and post-test bleeds were taken from all animals on the first day of restraint. All animals were euthanized and tissue collected on PND ~89.

5.2.5 Tissue Preparation and Analysis

5.2.5.1 Fixed ovarian tissue. Fixed ovarian tissue was dehydrated, embedded in paraffin, sliced at 4µm, and mounted four ovarian sections per slide (Sominsky et al., 2012a). Slides were stained with hematoxylin and eosin (H&E) for morphometric quantification of the 1st and 3rd section of every 4th slide (Myers et al., 2004). Follicle types were classified by an experimenter blind to the experimental conditions according to the number and shape of granulosa cells surrounding the oocyte as previously described (Chapter 2; Sobinoff et al., 2013; Sobinoff et al., 2010; Sobinoff et al., 2012; Sominsky et al., 2013) with only follicles demonstrating a visible oocyte being quantified.

5.2.5.2. RNA extraction, Reverse Transcription and qRT-PCR. Total ribonucleic acid (RNA) extraction was performed on thawed ovarian tissue. Ovaries were hand-lysed and homogenized with 500mL of lysis reagent (QIAzol, Qiagen). RNA was extracted from whole ovarian tissue using an RNeasy mini kit (Qiagen) in accordance with the manufacturer's instructions with added DNase treatment (Invitrogen, CA, USA) protocol in accordance with manufactures instructions. Nucleic acid purity and concentration was assessed in a 1µl volume by NanoDrop™ Spectrophotometer 2000c (Thermo Fisher Scientific, DE USA). RNA was converted to cDNA using a SuperScript ® VILO cDNA synthesis kit (Life Technologies, Thermo Fisher Scientific) by combining the kit components according to the manufacturer's instructions with the extracted RNA sample, creating a total volume of 20µl of cDNA per reaction. Quantitative RT-PCR was performed in 20µl reactions (10 µl of SYBR Green, 0.4 µl of each primer (forward and reverse), 4.6 µl nuclease free H_20, and 5 µl cDNA template (5ng/µl)) using SYBR Green reagents (Life Technologies, Thermo Fisher Scientific) and conducted on a 7500 RT-PCR Fast Instrument (Applied Biosystems, California, USA). All reactions were performed in triplicate accompanied by a RT-negative replicate as a negative control.

Qualitative RT-PCR data were normalized to reference gene β-actin or tubulin (Life Technologies, Australia) and analysed using the comparative C_T method equation $2^{-\Delta\Delta C(t)}$ (where C(t) is the threshold cycle at which fluorescence is first detected as statistically significant above background), and presented as a fold increase relative to the saline control group (Schmittgen & Livak, 2008). All PCR was performed on at least 6 separate tissue isolations/biological replicates, with final gene expression presented as a normalised fold change relative to the nSAL/aNS control group. Primer sequences are supplied (see Table 5.1) and were optimized by qPCR both here and previously (Fuller et al., 2017, Chapter 4; Sominsky et al., 2013).

Table 5.1 Primer forward and reverse sequence and efficiency for primer pairs used.

Target Gene	Forward Primer Sequence	Reverse Primer Sequence	Primer Efficiency
β-actin	TCTGTGTGGATTGGTGGCTCTA	CTGCTTGCTGATCCACATCTG	94%
Tubulin	GAGGCCGAGAGCAACATGAA	CTTCCGACTCCTCGTCGTCA	95%
Mapk8/Jnk1	CGGAACACCTTGTCCTGAAT	GAGTCAGCTGGGAAAAGCAC	94%
Tlr4	ACTGGGTGAGAAACGAGCTG	CGGCTACTCAGAAACTGCCA	97%
Il-6	TGCCTTCCCTACTTCACAAG	CCATTGCACAACTCTTTTCTCA	103%
Il-6 r	CGGAAGAACCCCCTTGTAAA	GGTGGTGTTGATTTTCTTTGC	106%
Tnfα	CGAGATGTGGAACTGGCAGA	CGATCACCCCGAAGTTCAGT	108%
Tnfα r	AACCTCAAATGGAAACGTGA	CAGGATGCTACAAATGCGG	106%
Il-1β	AACATAAGCCAACAAGTGGT	TTCATCACACAGGACAGGTA	100%
Cox-2	CAAGACAGATCAGAAGCGAG	TCCACCGATGACCTGATATT	107%
Fshr	TGGCTGTGTCATTGCTCTAA	TGAGCACAAACCTCAGTTCA	93%

5.2.6 Data Analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Version 22 (SPSS Inc.) Data were analysed using appropriate factorial or repeated measures ANOVAs, with 'neonatal treatment' and 'adult stress' as dependent

variables, with significance level set at $p \leq 0.05$. Litter size, litter ratio, weight and oestrus phase were included as covariates in analyses and reported where significant. Outliers present in the data that were more than ± two standard deviations away from the group's mean for that particular measure were removed from all analyses. All ANOVA assumptions were tested and violations reported. Pairwise comparisons and independent samples t-test were used where significant outcomes were present. Log transformations (Ln or Log10) were reported when used to transform the data in the case of extreme violations to Shapiro-Wilk or Levene's assumptions, however ANOVA is known to be robust to some of these violations (Howell, 2010).

5.3 Results

5.3.1 Neonatal Weight Gain

A significant Age x treatment effect existed ($F(1, 60) = 76.52$, $p < .0001$), with LPS animals weighting significantly less on PND 5 compared to saline controls ($t(60) = 5.87$, $p < .0001$) (See Figure 5.3). No differences existed between groups prior to treatment on PND 3, however LPS female pups gained significantly less weight between treatment days (PND 5 minus PND 3), compared to saline animals ($t(60) = 8.75$, $p < .001$).

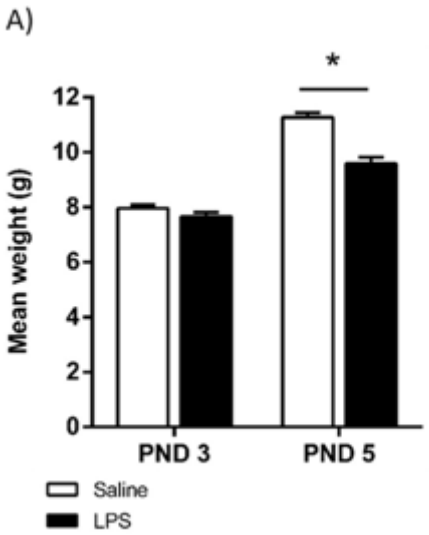

Figure 5.3. Mean weights of pups on PND 3 & PND 5 taken prior to injections. Hollow bars = saline, filled bars = LPS, mean ± SEM graphed, *= $p < .05$.

5.3.2 Neonatal Circulating Inflammation

On PND 5, two hours after injections, LPS treated animals had significantly elevated levels of circulating TNFα ($t(20) = 8.56$, $p < .0001$ (Figure 5.4, A) and IL-6 ($t(19) = 4.704$, $p < .0001$) (Figure 5.4, B), however IL-2 expression was significantly downregulated ($t(20) = 3.44$, $p = .003$) (Figure 5.4, C).

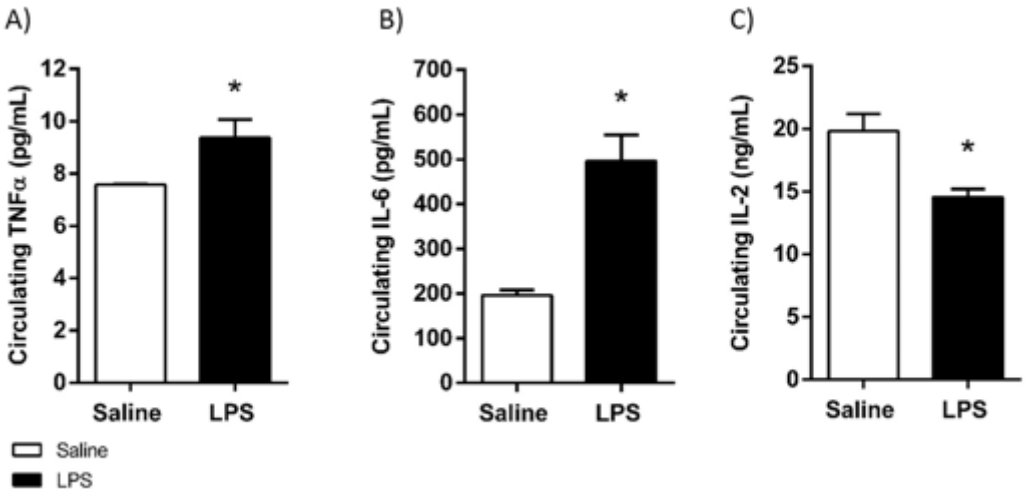

Figure 5.4. A) LPS animals demonstrated significantly increased circulating TNFα & B) IL-6 levels on PND 5, compared to controls. C) IL-2 was significantly decreased in LPS females. Hollow bars = saline, filled bars = LPS, mean + SEM graphed, *= $p < .05$.

5.3.3 Developmental Weight Gain

Repeated measures ANOVA demonstrated a significant main effect of age ($F(4.76, 162.05) = 63.335$, $p < .0001$), a significant main effect of neonatal treatment ($F(1, 34) = .406$, $p = .012$), and a significant age x neonatal treatment interaction ($F(4.76, 162.05) = 3.97$, $p = .002$) using the greenhouse-Geisser correction for sphericity violation. No significant main effect or interaction effects existed for adult treatment. Pairwise comparisons indicated that LPS animals gained significantly more weight than saline animals between PND 29 - 36 ($t(31.21) = 5.05$, $p = .013$; corrected for Levene's violation), and PND 36 - 43 ($t(36) = 4.95$, $p < .001$) (see Figure 5.5).

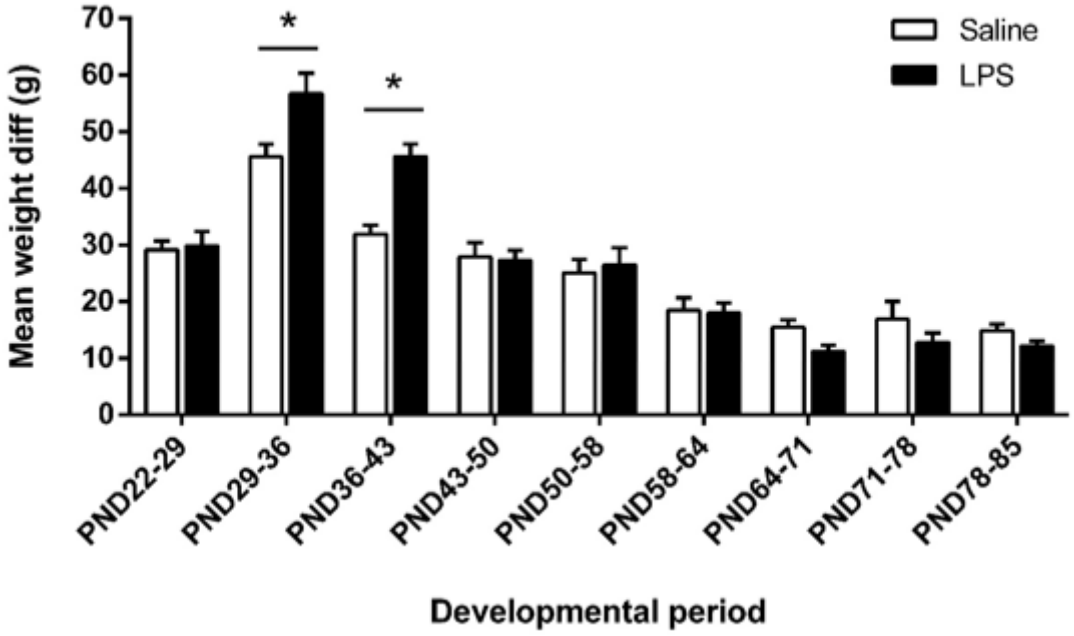

Figure 5.5. Difference in weight gain (g) between saline and LPS animals during weekly developmental weight monitoring. Hollow bars depict saline controls, filled bars depict LPS treated animals means + SEM graphed. * denotes significance at $p < 0.05$.

5.3.4 Day of Vaginal Opening, Weight at Puberty and Oestrus Cyclicity

A significant effect of neonatal treatment was demonstrated for DVO ($F(1, 42) = 12.91$, $p = .001$) but not weight at DVO ($F(1, 42) = .948$, $p = .336$) (Figure 5.6; A, B). Pairwise comparisons indicated that LPS treated females had an earlier onset of DVO compared to saline controls (LPS, $M = 34$; saline, $M = 36.435$). Neonatal treatment had a significant effect on first proestrus followed by a regular cycle, with 1st proestrus occurring earlier in LPS treated animals ($F(1, 42) = 15.15$, $p < .0001$) (Figure 5.6, C). No differences were determined between treatment groups for oestrus cyclicity.

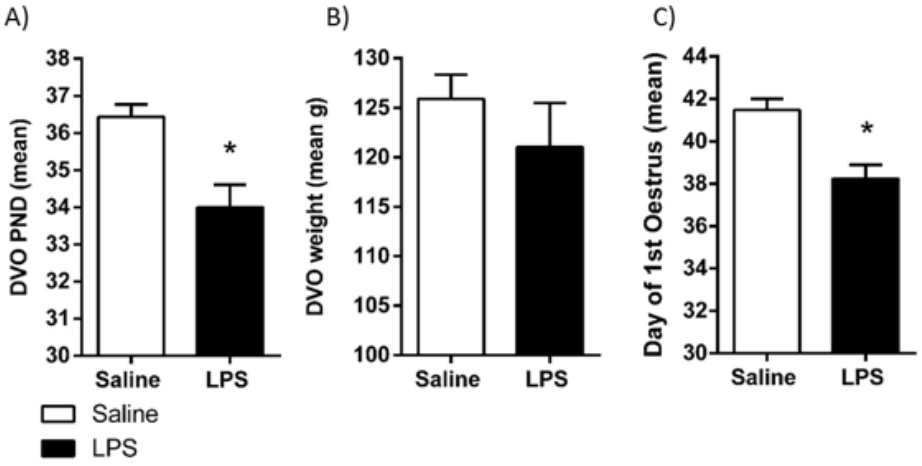

Figure 5.6. A) LPS treated animals demonstrated significantly earlier onset of puberty, as marked by DVO. B) There was no significant difference in weights between treatment groups on DVO (LPS, M = 121.02, SEM = ± 4.47; saline, M = 125.88, SEM = ± 2.48). C) LPS treated animals demonstrated a significantly earlier day of 1st proestrus, approximately 2 days before saline controls, followed by a normal oestrus phase. Hollow bars depict saline controls, filled black bars depict LPS treated animals means + SEM graphed. * denotes significance at $p < 0.05$.

5.3.5 Adult Circulating Inflammation

5.3.5.1 Circulating Interleukin-6 (IL-6) prior to and following restraint stress. As expected, a significant main effect of time was demonstrated ($F(1, 24) = 45.12$, $p < .0001$), with overall elevated IL-6 levels post stress (post $M = 145.49$) compared to pre stress levels (pre $M = 123.99$). A significant time x adult treatment interaction ($F(1, 24) = 4.55$, $p = .043$) and time x neonatal x adult treatment interaction ($F(1, 24) = 5.741$, $p = .025$) existed. Pairwise comparisons indicated nLPS/aST treated animals demonstrated significantly elevated IL-6 levels following restraint stress than those treatment groups not exposed to adulthood stress (p .05), and a strong trend for elevated post stress IL-6 levels compared to nSAL/ST treated females ($p = .066$) (Figure 5.7).

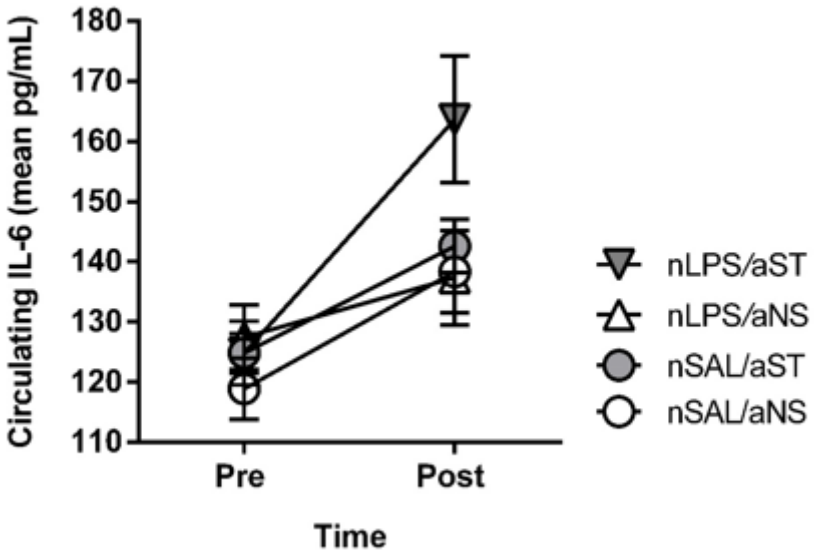

Figure 5.7. nLPS/aST treated females demonstrated elevated IL-6 levels following a 2nd hit of psychological restraint stress, compared to all groups. Legend defines groups.

5.3.5.1 Circulating Interleukin-2 (IL-2) 24 hours post restraint. Circulating IL-2 levels were analysed 24 hours following restraint stress. A significant neonatal x adult treatment interaction existed (F (1, 24) = 4.35, p = .005). Pairwise comparisons indicated a significant effect of neonatal treatment (F(1,24) = 16.12 p = .001), with neonatal LPS treated females demonstrated significantly higher levels of IL-2 compared to neonatal saline groups (saline M = 6.695, LPS M = 17.83), and females in the nLPS/aNS displaying significantly elevated levels of IL-2 compared to the nSAL/NS (p =.001) and nSAL/aST (p = .042) (Figure 5.8).

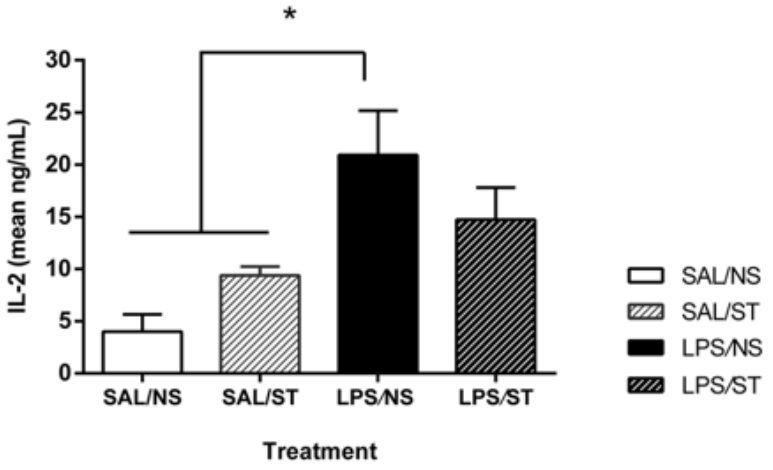

Figure 5.8. Neonatal LPS treatment significantly upregulated circulating IL-2 levels, compared to saline groups. No significant effect of adult treatment was demonstrated. * = p <.05. Legend on graph describes groups.

5.3.6 Adulthood Ovarian Follicle Quantification 24 Hours Post-Restraint

5.3.6.1 Early ovarian follicle populations.

Primordial follicles. A significant effect of neonatal treatment on primordial follicle numbers existed (F(1,28) = 8.312, p = .007). There was no significant effect of adult treatment, or neonatal x treatment interaction. Overall, the ovaries of nLPS animals contained a depleted population of primordial follicles (M = 107.37, SEM = 10.75) compared to ovaries from nSAL animals (M = 166.81, SEM = 17.33).

Activated primordial follicles. A significant effect of neonatal treatment (F(1,28) = 16.09, p = .0001) and a neonatal x adulthood treatment interaction existed (F(1,28) = 9.48, p = .005) existed. Pairwise comparisons indicated that nSAL/aNS animals (M = 91.57, SEM = 11.2) had significantly less activated primordial follicles, compared to all other treatment groups (nSAL/aST, M = 147.5, SEM = 12.1, p = .01; nLPS/aNS, M = 171.73, SEM = 7.3, p = .001; nLPS/aST, M = 158, SEM = 13.38, p = .002).

Primary follicles. Neonatal treatment was found to significantly influence primary follicle populations ($F(1,28) = 8.26$, $p = .001$). Overall, the ovaries of nLPS treated females contained an average depleted primary follicle population ($M = 65.93$, $SEM = 5.02$) when compared to the ovaries of nSal animals ($M = 88.81$, $SEM = 6.32$) (see Figure 5.9). Pairwise comparisons revealed that this main effect was driven predominately by the significant simple effect of adult stress exposure on nLPS and nSal groups ($F(1,28) = 3.69$, $p = .023$) with nLPS/aST displaying further reductions in primary follicle populations following adulthood stress (nLPS/aST $M = 56.45$, $SEM = 3.37$; nSAL/aST $M = 88.75$, $SEM = 7.35$) (Figure 5.9).

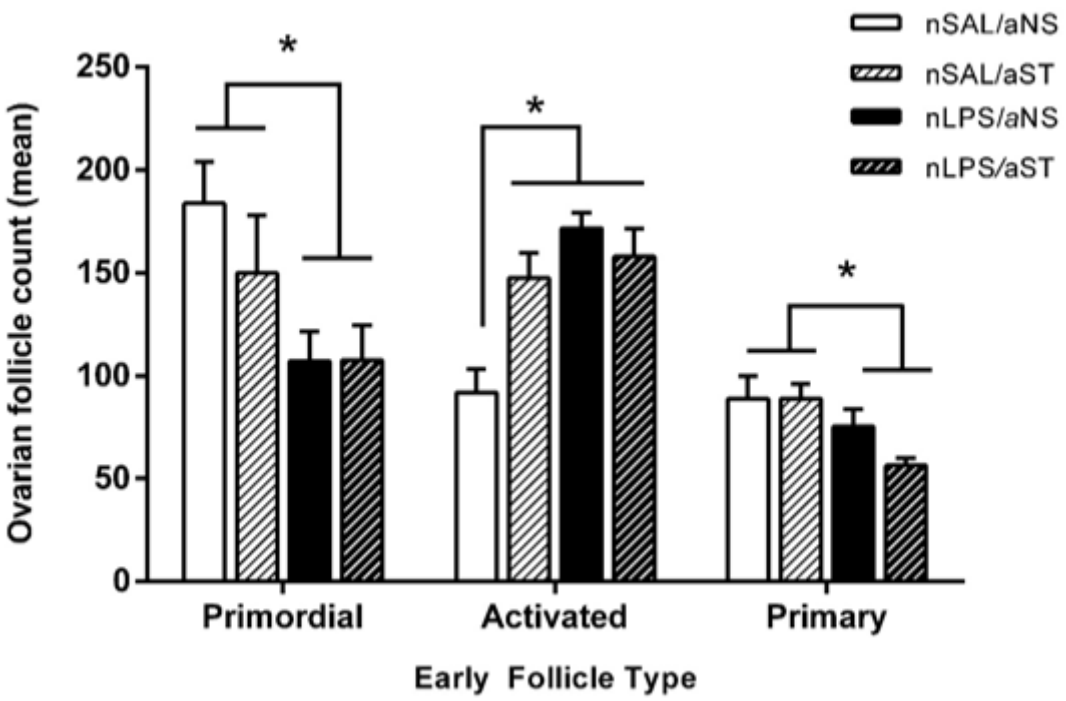

Figure 5.9. The effect of neonatal treatment and adulthood stress group allocation on primordial, activated and primary follicle populations with the ovary. Neonatal LPS animals exhibited a significant reduction in the quantity of primordial and primary follicles. Adulthood stress exposure resulted in a significant increase in activated follicles in nSAL/aST animals, relative to nSAL/aNS animals. Data is expressed as the mean + SEM, * $p < .05$, legend displayed on graph.

5.3.6.2 Late ovarian follicle populations. No significant effect of neonatal treatment, adulthood treatment, or interaction effects existed (see Table 5.2). An adult treatment effect on pre-ovulatory numbers neared significance, partially driven by nSAL/aNS antral follicle numbers (Figure 5.10).

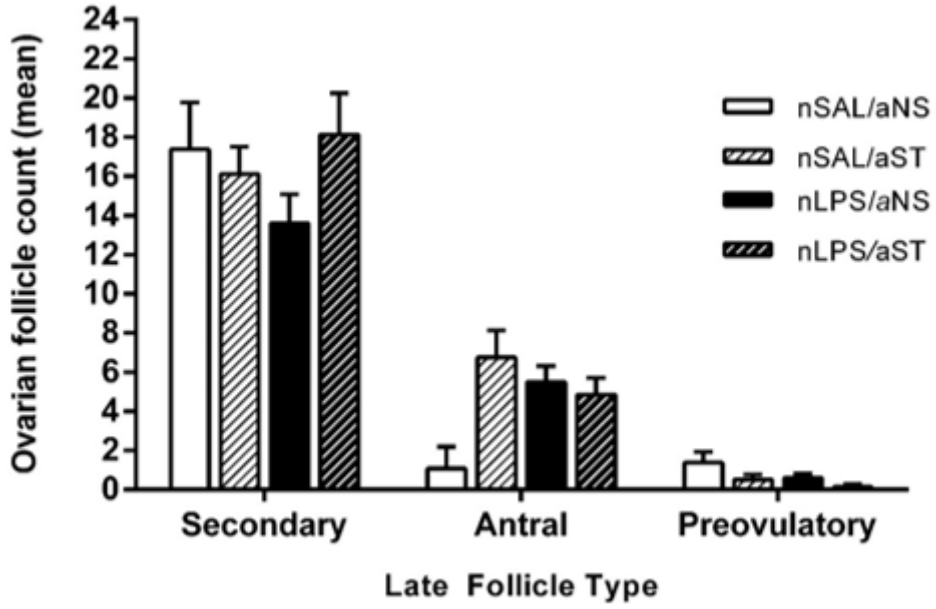

Figure 5.10. Graphical representation of mean ovarian late follicle populations. Data is expressed as the mean + SEM, data legend on graph.

Table 5.2 Analysis of variance statistical information for neonatal, adult and interaction treatment effects on late follicle types and total late follicle numbers.

	Follicle Type			
Treatment	*Secondary*	*Antral*	*Pre-ovulatory*	*Total Number*
Neonatal Treatment	$F(1,29) = 0.22$ $p = 0.64$	$F(1,29) = 1.20$ $p = 0.28$	$F(1,29) = 2.78$ $p = 0.11$	$F(1,16) = 0.30$ $p = 0.59$
Adult Treatment	$F(1,29) = 0.78$ $p = 0.38$	$F(1,29) = 0.004$ $p = 0.95$	$F(1,29) = 3.85$ $p = 0.06^{\dagger}$	$F(1,16) = 0.01$ $p = 0.92$
Neonatal X Adult	$F(1,29) = 2.42$ $p = 0.13$	$F(1,29) = 0.224$ $p = 0.64$	$F(1,29) = 0.379$ $p = 0.54$	$F(1,16) = 0.39$ $p = 0.54$

† Denotes trend.

5.3.7 Ovarian mRNA expression 24 hours post restraint

Ovarian mRNA was assessed for markers of inflammation and associated receptor expression following adult treatment. A significant main effect of neonatal treatment existed for *Il-6 receptor (r)* and mitogen activated protein kinase 8/Jun N-terminal kinase (*Mapk8/Jnk1*). A significant main effects of adult treatment was demonstrated on ovarian *Tnfα*, *Tnfα R, Il-1ϐ, and Mapk8/Jnk1.* There was a statistically significant neonatal x adult treatment interaction effect on ovarian mRNA expression of *Il-6* only (see Table 5.3 for all ovarian mRNA ANOVA statistics, and Figure 5.11, A → I).

Table 5.3. Analysis of Variance (ANOVA) statistics for normalised fold change ovarian mRNA expression.

	ANOVA Statistics		
Ovarian mRNA	Neonatal Treatment	Adult Treatment	Neonatal x Adult
*Tnfα**	$F(1, 24) = 1.077$ $p = .312$	$F(1, 24) = 5.205$ $p = .034$*	$F(1, 24) = 1.077$ $p = .312$
*Tnfα receptor**	$F(1, 24) = .429$ $p = .429$	$F(1, 24) = 36.098$ $p = .001$*	$F(1, 24) = 2.385$ $p = .138$
*Il-6**	$F(1, 24) = 2.348$ $p = .141$	$F(1, 24) = 2.371$ $p = .139$	$F(1, 24) = 5.616$ $p = .028$*
*Il-6 receptor**	$F(1, 24) = 11.710$ $p = .003$*	$F(1, 24) = 3.266$ $p = .086$†	$F(1, 24) = 2.908$ $p = .104$
*Il-1ϐ**	$F(1, 24) = 1.945$ $p = .178$	$F(1, 24) = 5.11$ $p = .035$*	$F(1, 24) = .059$ $p = .810$
Cox2	$F(1, 24) = 3.038$ $p = .097$	$F(1, 24) = 2.885$ $p = .105$	$F(1, 24) = 2.915$ $p = .103$
*MapK**	$F(1, 24) = 9.841$ $p = .005$*	$F(1, 24) = 20.921$ $p = .001$*	$F(1, 24) = 3.072$ $p = .095$
Tlr4	$F(1, 24) = .261$ $p = .615$	$F(1, 24) = .366$ $p = .552$	$F(1, 24) = .348$ $p = .562$
Fsh receptor	$F(1, 24) = 1.103$ $p = .306$	$F(1, 24) = 1.814$ $p = .193$	$F(1, 24) = 3.072$ $p = .095$

* Denotes significance of $p < .05$ † Denotes trend.

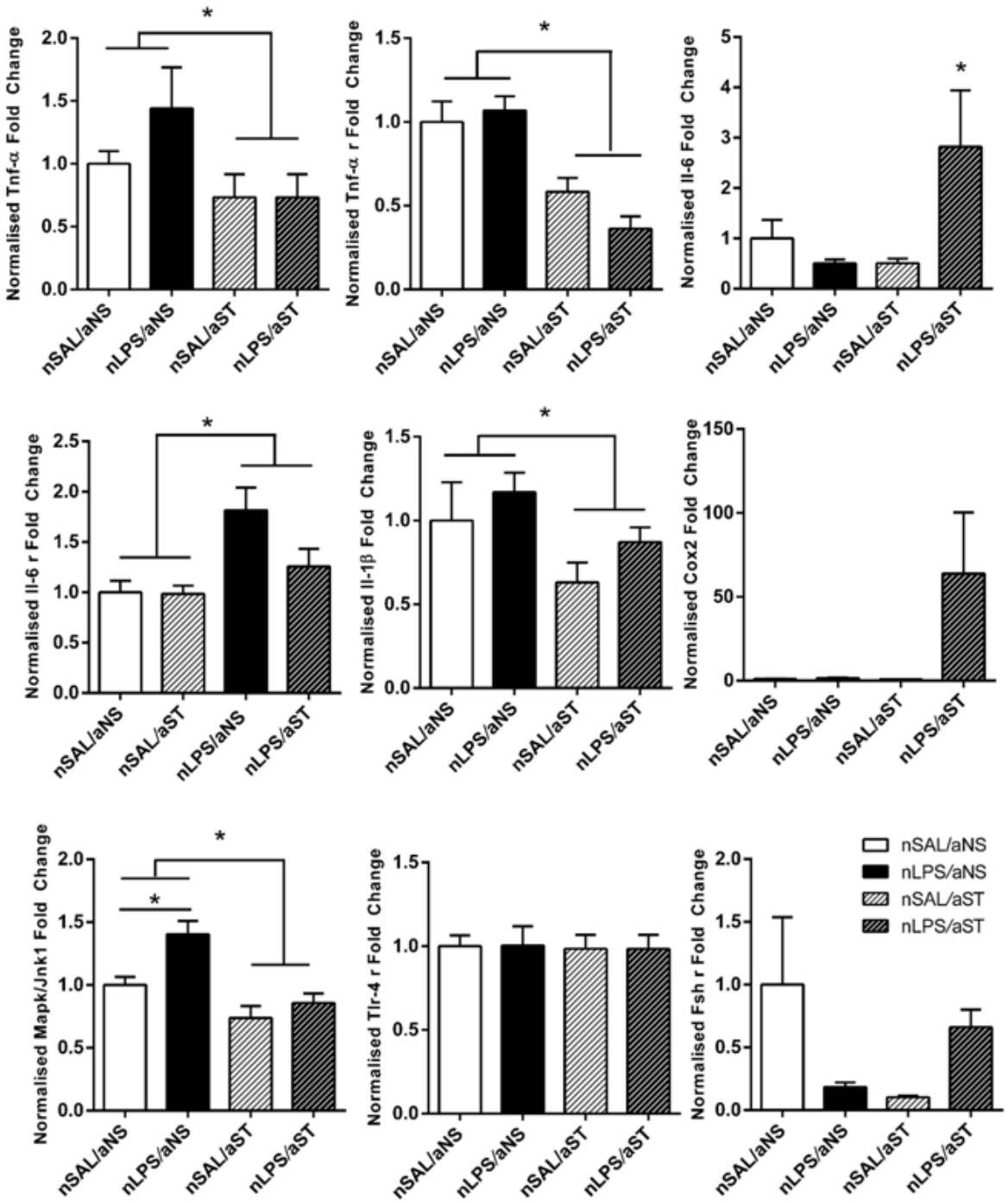

Figure 5.11. Normalised fold change mRNA expression of ovarian inflammatory mediators and receptors essential to ovarian processes. Note deviated IL-6R formatting to appropriately display groups differences.* Denotes significance of $p < .05$

5.4 Discussion.

The early life immune environment is critically important for female reproductive development, health and longevity. Immune stressors encountered during early life have the potential to inflict both immediate and sustained physiological alterations to biological systems involved in reproductive processes and stress vulnerability, such as the immune and endocrine systems, modifying their operations. What is more, susceptibilities produced by early life immune stress may be triggered or potentiated by later life stress, exacerbating affects. The current study demonstrates that early life immune activation with a low-level dose of LPS altered female reproductive parameters including long-term depletion of the early ovarian follicle pool and augmentation of inflammatory mediators within the adult ovary, particularly in the presence of a later life psychological stressor. Additionally, neonatal immune stress followed by later life stress upregulated circulating acute phase inflammatory mediator IL-6 following exposure, and increased circulating IL-2 levels. We also observed acute inflammatory upregulation immediately in response to NIA, as well as precocious pubertal onset. This study is among the first to demonstrate the long-term effects of NIA on immune-regulated development of female reproduction and persistent ovarian follicular and peripheral immune alterations in the rat.

Regardless of similar PND 3 weights between groups, LPS treated neonates gained less weight than their saline counterparts between treatment days, leading to significantly smaller NIA treated pups on PND 5. This follows previous findings from our laboratory, demonstrating a lesser weight gain of LPS treated pups between PND 3 and PND 5 (Sominsky et al., 2012a; Walker et al., 2009; Walker et al., 2004a; Walker et al., 2004b). However, we have also previously demonstrated no differences in weight between treatment days (Fuller et al, 2017; Walker et al, 2011). Lipopolysaccharide is used extensively in our lab and others to reliably

induce a controlled immune response and associated endocrine and autonomic nervous system activation, with no mortality (Bilbo et al., 2005a; Bilbo et al., 2008; Galic et al., 2008; Karrow, 2006; Mouihate et al., 2010; Shanks et al., 1995; Shanks & Meaney, 1994; Spencer et al., 2006c; Spencer et al., 2007). The inflammatory properties of LPS lead to a suite of physiological and behavioural responses, including increased cytokine production, fever and sickness behaviours (Karrow, 2006; Karrow et al., 2010; Spencer et al., 2011). Lipopolysaccharide given at this low dose in the current study is known to induce a mild and ephemeral, acute-phase fever response, with associated sickness behaviours including anorexia and inactivity which may affect pup feeding behaviours (Walker et al., 2004c), and hence weight gain. Additionally, similar models of neonatal immune activation have previously shown no differences in maternal behaviour and pup vocalizations following NIA (Galic et al., 2009; Spencer et al., 2006c; Spencer et al., 2007).

We demonstrate here that administration of LPS on PND 3 and 5 caused significant increases in peripheral proinflammatory cytokines IL-6 and TNFα, two hours following PND 5 treatment. These results confirm the efficacy of the neonatal treatment and indicates that a relatively small dose of 0.05mg/kg dose of LPS stimulation leads to a significant proinflammatory immune response in what is considered the immune and stress hypo-responsive period in the neonatal rodent (Sapolsky & Meaney, 1986). The early neonatal period is characterised by a predominant bias to T_H2-type immune responses and a diminished proinflammatory response to LPS in both animal models and human cord blood samples (Chelvarajan et al., 2004; Hodyl et al., 2008). This is observed regardless of comparable protein and mRNA expression levels of CD14 LPS receptor binding complex and TLR4 as adults (Levy, 2005). TNFα and IL-6 share activation pathways and biological activities with IL-1β, and, although not measured here, IL-1β is typically expressed in the

proinflammatory cascade alongside with TNFα and IL-6. Interleukin-1β has been shown to be normally increased in the neonatal brain in the first week post birth (Giulian et al., 1988), providing evidence for cytokine necessity in typical, neuronal maturation trajectories. As circulating proinflammatory cytokines are able to cross the blood brain barrier (Banks, 2005; Banks & Erickson, 2010; Banks et al., 2004; Quan & Banks, 2007; Yarlagadda et al., 2009), peripheral LPS stimulation may potentially exacerbate or impede the developmental effects of normally occurring cytokine levels at this time, not only in peripheral tissues such as ovary and spleen, but also contribute to central immune-regulated perinatal programming that may led to long-term behavioural and functional alterations (Kiecolt-Glaser et al., 2015).

Importantly, IL-6, TNFα, and other immune mediators are critically involved in ovarian developmental signalling during the final stages of initial folliculogenesis (PND 1-5 in the neonatal rat), where oogonia (germ cells) that are clustered in nests/cysts, break down and transition into primordial follicle populations (Bornstein et al., 2004; Feeney et al., 2014; Greenfeld et al., 2007; Marcinkiewicz et al., 1994; Terranova, 1997). Peripheral increases in proinflammatory cytokines by immune challenge may further stimulate those normal levels within the ovary at this critical period of development, regardless of ovarian immune privilege, as demonstrated previously (Fuller et al., 2017 [Chapter 4]). It has been suggested that females are more susceptible to the detrimental effects of inflammation (Derry et al., 2015; Gaillard & Spinedi, 1998; Kiecolt-Glaser et al., 2015), and although inflammatory mechanisms are necessary for key long-term reproductive processes such as ovulation and steroidogenesis, chronic pro-inflammation programmed by early life immune activation may contribute to the onset of ovarian disorders and diseases that are associated with a chronic proinflammatory phenotype, such as PCOS and endometriosis (Dumesic et al., 2007; Vannuccini et al., 2016; Weiss et al., 2009) ovarian cancers (Lane et al., 2016; Maccio &

Madeddu, 2012) , and psychopathologies (Fagundes et al., 2013; Kiecolt-Glaser et al., 2015).

Unexpectedly, IL-2 was significantly downregulated in LPS treated neonates two hours following exposure, this may be due to the pleiotropic attributes of IL-2 itself, as well as that of other cytokines such as IL-6 (Liao et al., 2011b; Scheller et al., 2011). A lower level of IL-2 is consistent with a reduced T_H1 response associated with mediating tolerance (Liao et al., 2013), which may be a result of the previous PND 3 injection. It has also been demonstrated that IL-2 inhibits IL-6 receptor expression, hence downregulation of IL-2 may be needed during the immediate acute phase response in order to facilitate responsiveness of other proinflammatory cytokines and modulate helper T cell differentiation (Liao et al., 2011a) to effectively clear pathogens. Additionally, other studies have demonstrated LPS evoked IL-2 production at 4 and 6 hours only, and not 2 hours, hence, the time point in the current study may not be insufficient to detect IL-2 upregulation in the neonatal period (Costalonga & Zell, 2007; De Groote et al., 1992). Interestingly, genetic expression of IL-2 spans a chromosomal area that is implicated in maintenance of immune homeostasis and ovarian dysgenesis (Yamanouchi et al., 2007), therefore further examination of this cytokine in NIA models may be warranted.

Developmentally, female animals in this study demonstrated a precocious pubertal onset, as indexed by an earlier DVO. This is in line with other work in this thesis (Chapter 3), as well as earlier studies from our laboratory (Sominsky et al., 2012a). As previously discussed (Chapter 3 of this thesis), our results are contrary to others who have demonstrated a delayed puberty onset following neonatal LPS immune activation, however timing and severity of LPS dose differs, as does methodology and rat breed (Iwasa et al., 2009; Knox et al., 2009; Wu et al., 2011b). Additionally, the aforementioned studies delivered LPS at a later time point, closer to the general timing of female puberty onset. Ecologically, it makes sense that animals who

are immunologically unfit delay, or display plasticity of, major developmental trajectories to ensure optimal individual survival and reproductive success, particularly as infection is known to lead to poor reproductive outcomes in both human and animal models (Sheldon et al., 2014; Sheldon et al., 2016; Turner et al., 2012).

As expected due to previous results, LPS treated females also demonstrated advanced 1st proestrus, however oestrus cycle regularity did not significantly differ between treatment groups, similar to previous findings from our laboratory (Chapter 3; Sominsky et al. 2012a). These findings differ from others (Iwasa et al., 2009; Knox et al., 2009; Wu et al., 2011b) who demonstrate delayed first proestrus and altered oestrus cyclicity, however Nilsson et al. (2002), using the same animals and manipulations as the current study, demonstrated findings in line with our own. These discrepancies highlight the delicate nature of early life stress during critical windows of development on different developmental outcomes. As critical periods of plasticity occur at differing time points for specific cells, alterations may be time, tissue, and also challenge specific, culminating in varied phenotypic modification (Bauer et al., 2007; Crespi & Denver, 2005; Harris & Seckl, 2011; Meyer et al., 2006).

Regardless of puberty and 1st proestrus onset differences, females in this study did not demonstrate significant weight differences on the actual day of DVO, suggesting that mediators other than endocrine and metabolic may also contribute to the control of the female reproductive lifespan. However, a period of what could be considered as 'catch-up growth' was observed here in female animals, where LPS treated females displayed significantly greater weight gains than saline treated animals over a two week period, ranging from PND 29 to 43. This timeframe is typically considered the adolescent period of the rat developmental trajectory (Spear, 2000) and although weight on DVO did not differ, adolescent weight increases are known to contribute to pubertal onset and sexual maturation

in both humans and in animals models (Baker, 1985; Wang et al., 2012). Catch up growth is a known risk factor for later life metabolic, hormonal and immune system dysfunction, all of which are common to female reproductive complications, including hyper-insulinism, ovarian hyper-androgenism, and chronic low grade inflammation (Ibanez et al., 1998). Interestingly, it is currently known that the initial establishment, maintenance and regulation of the ovarian primordial follicle pool is also a key factor dictating the female reproductive lifespan (McLaughlin & McIver, 2009; Tingen et al., 2009a; Tingen et al., 2009b). Perhaps the quantity and quality of the ovarian reserve is partially dictating the precocious puberty onset we are seeing, bringing forward maturation for optimal reproductive success.

This is one of the first studies to indicate that immune perturbation in the early postnatal period has a significant long term, detrimental effect on specific follicle populations within the ovarian reserve that may be potentiated by later life psychological stress. Examination of early ovarian follicle populations in adulthood revealed that neonatal LPS treatment significant downregulated the long-term primordial follicle pool, regardless of adulthood stress. Additionally, adulthood stress significantly increased the number of activated follicles in both neonatal saline and LPS treatment groups. Interestingly, this result was also present in females treated with neonatal LPS alone. Lastly, primary follicle numbers were significantly downregulated by neonatal LPS treatment, with further examination highlighting that this effect was driven by a more prominent downregulation of primary follicles in female rats exposed to a double hit of stress. As mentioned, we have previously seen a diminished primordial follicle pool LPS treated neonates immediately (PND 5) following LPS and in the later neonatal/prepubescent period (PND 14) Both of these periods within female development, in rats and humans, are characterised by high levels of follicular loss due to normal atretic processes (Bristol-Gould et al., 2006; Tingen et al., 2009b). However,

greater loss induced by environmental insult, such as those seen in our laboratory by neonatal LPS, may change long term ovarian functioning. As the reproductive lifespan of a mammalian female is largely defined by non-renewing primordial follicle numbers within the ovarian reserve, loss of this population may be detrimental to female reproductive health and be associated with disorders such as POF/POI and health complications that accompany reproductive decline. Indeed, results from our laboratory demonstrate that neonatal LPS treatment leads to earlier onset of oestrus cyclicity changes associated with senescence (Sominsky et al., 2012b). In human cohorts, excessive prepubertal ovarian follicle loss is associated with earlier onset menopause, reducing the fertility window (Chemaitilly et al., 2017; Thomas-Teinturier et al., 2013; van Dorp et al., 2016). Excessive immune stimulation during initial folliculogenesis may be inducing changes to ovarian dynamics and prompting early reproductive aging, perhaps via premature activation and hence atresia of otherwise quiescent follicles.

Lipopolysaccharide exposure has previously been demonstrated to reduce primordial follicle number in the ovaries of both adult cattle and mice, in vitro and in vivo (Bromfield & Sheldon, 2011; Bromfield & Sheldon, 2013). Interestingly, Bromfield and Sheldon (2011) also demonstrated that LPS exposure led to accelerated primordial maturation in vitro. In the current study, adulthood stress appeared to trigger the activation of primordial follicles, a trend that was seen to be increased in animals also exposed to neonatal LPS treatment. These results suggest that inappropriate activation of primordial follicles would be likely to diminish the ovarian reserve, thus compromising fertility and reproductive health. What's more, acute psychological stress may have a more profound effect on the ovarian reserve in the presence of a predisposed stress or immune vulnerability, programmed in early life. Interestingly, a double hit of immune stress paired with psychological stress, in the current study, also led to

a depletion of the primary follicle population. The overall lower number of primary follicle numbers may indicate that after initial activation, primordial follicles are entering atresia or apoptosis. This implication is supported by our finding of no observable significant differences in larger follicle populations (secondary to preovulatory) between any treatment groups. Primary follicles are formed when oocytes leave the primordial resting pool and become surrounded by a single layer of cuboidal granulosa cells. The direct mechanisms regulating the activation of otherwise quiescent primordial follicles into preantral stages remain unclear (Fortune, 2003; Morohaku et al., 2017). However, it is known to be largely gonadotropin-independent event, and involves growth factors, immune mediators and biological cross-talk between the oocyte and its surrounding granulosa cells (Orisaka et al., 2009). Considering the nature of the neonatal stressor utalised here and the mechanisms involved in primordial to primary follicle activation, perinatally programmed alterations to immune mediators of follicle development may be implicated (Fuller at al., 2017; Sominsky et al., 2013). The significantly elevated circulating IL-6 concentrations following the adult psychological stressor in this study supports this theory, where nLPS/aST females demonstrated a heightened immune sensitivity to a 2^{nd} hit of stress, above those of all other treatment groups.

In the current study, circulating IL-2 levels were also elevated in nLPS/NS stress animals compared to the neonatal saline groups, in terminal blood samples 24 hours following restraint stress. Interleukin-2 is a T_H1 mediated proinflammatory cytokine that indices proliferation, enhances immune activity, and is secreted alongside interferon-Gamma (IFN-γ) and other proinflammatory cytokines (Kuby, 1997). Previous studies have linked elevated IL-2 levels with sickness behaviours, depression, and ovarian cancers (Barton et al., 1994; Capuron et al., 2004; Dantzer & Kelley, 2007; Dowlati et al., 2010; Liu et al., 2012; Maes et al., 1990; Maes et al., 1991; Maes et al., 1995; Xia et al., 1996; Yee & Prendergast, 2010).

Furthermore, psychological stress has been demonstrated to modulate both circulating levels and gene expression of IL-2 and IL-2 receptors (Glaser et al., 1990; Heinz et al., 2003) and lower levels of IL-2 are correlated with severe psychological symptoms in patients with post-traumatic stress disorder (PTSD) (Song et al., 2007). Presently, whilst circulating IL-2 was significantly upregulated in the adult neonatal LPS group, a differing pattern of activation was present between those treated with neonatal saline and those treated with neonatal LPS following a 2nd hit. Stress led to a slight upregulation of IL-2 in the saline animals, however a downregulation was evident in nLPS animals with additional stress. These results differ from those of Himmerich et al. (2013), who demonstrated increases in rat IL-2 following the forced swim test, however they did not observe alterations following restraint stress. However, Liu and Wang (2005) demonstrate elevated levels in mice stressed with social isolation, which similarly was a component of the adulthood stress procedure in the present study. This differing pattern observed here may be due to perinatally programmed immune mechanisms due to NIA, such as $CD4^{+}/CD25^{+}$ Treg cell dysregulation, which is known to be implicated in the TLR4 mediated LPS response and IL-2 homeostatic immune control (Dietert, 2014; Liao et al., 2013; Liao et al., 2011b). These results, along with the increased IL-6 levels demonstrated here, strongly support the notion of perinatally programmed immune dysregulation, susceptible to a 2nd hit of stress (Galic et al., 2009; Spencer et al., 2011; Spencer & Meyer, 2017). Importantly, this dysregulation may be altering immune activities essential to ovarian reproductive processes.

The importance of immune mediators and pathways to both normal and dysregulated ovarian and reproductive function is well known (Bornstein et al., 2004; Bukovsky & Caudle, 2012; Nash et al., 1999; Richards et al., 2008; Sheldon et al., 2016; Wu et al., 2004; Ye et al., 2016; Zhou et al., 2009). Immune dysregulation has consistently been demonstrated to be

linked with female reproductive disorders, including PCOS, endometriosis, and POF/POI (Adams et al., 2016; Halis & Arici, 2004; Harada et al., 1999; Weiss et al., 2009). What is more, a sustained proinflammatory phenotype is associated with early life exposure to LPS (Bilbo & Schwarz, 2009; Mouihate, 2013; Spencer et al., 2011; Walker et al., 2010). We suggest that early life immune activation via LPS exposure may be altering immune pathways and mediators essential to ovarian function, and in turn, contributing to the detrimental alterations to female reproductive parameters we have previously demonstrated.

Here, we demonstrate for the first time that NIA during the postnatal period in the rat alters the long-term genetic expression of local immune mediators and pathways in the ovary, modulated by the addition of later life psychological stress. Firstly, neonatal LPS exposure significantly altered ovarian *Il-6* receptor gene expression, and upregulated *Il-6* expression in NIA females with the addition of restraint stress. Interleukin-6 and its receptor are a major immunoregulatory pathway associated with ovarian cancers, with demonstrated roles in tumour formation, progression, angiogenesis and prognosis (Dijkgraaf et al., 2012; Kumar & Ward, 2014; Nilsson et al., 2005; Robinson-Smith et al., 2007; Setrerrahmane & Xu, 2017). Furthermore, IL-6 is a downstream mediator of the Janus kinase/signal transducer and activation of transcription (JAK/STAT) pathway, which is essential for normal immune function, development, and homeostasis (Rawlings et al., 2004). Holt et al. (2006) demonstrated JAK/STAT signalling is implicated in intracellular ovarian signalling channels between the oocyte and its supporting granulosa cells, regulating primordial follicle activation. This finding was further substantiated by Sutherland et al. (2012), who demonstrated that this pathway and its associated molecules play an active role in regulating the inhibitory and stimulatory factors coordinating early follicle maturation and maintenance of the follicle pool. Further investigation is needed in order to evaluate JAK/STAT pathway

activation, involvement and potential dysregulation in the NIA model, however it may be a key peripheral mechanism initiating the premature activation and depletion of the primordial follicle pool demonstrated both here and previously (Fuller et al, 2017; Sominsky et al., 2012a). JAK/STAT signalling may potentially be altered due to neonatal immune stress exposure, considering this pathway's role in development and maintenance of the ovarian follicle reserve, and the crosstalk between this pathway and others (including MAPK/JNK1 and NF-κβ) that are activated with LPS exposure (Horvath, 2004b; Schindler et al., 2007; Shuai & Liu, 2003). Of note, JAK/STAT activation is also associated with IFNγ and IL-2 signalling (Horvath, 2004a), having implications for the circulating IL-2 findings demonstrated here and solidifying this pathway as worthy of further examination.

In the current study, neonatal LPS exposure upregulated the long-term gene expression of the *Mapk8/Jnk1* inflammatory pathway in the ovary, however *Mapk8/Jnk1* was downregulated following adult stress exposure of LPS treated animals, with saline treated animals maintaining relative levels. Map-kinases play a key role in the induction, regulation and potentiation of the pro-inflammatory cascade (including; IL-1β, TNFα, IL-6, COX2) and is activated upon LPS stimulation via TLR4 (Bachstetter & Van Eldik, 2010; Davis, 2000; Dong et al., 2002; Farooq & Zhou, 2004; Geppert et al., 1994). Within the ovary, MAPK/JNK has been identified in murine models to be associated with primordial follicle activation and progression both during neonatal initial and subsequent folliculogenesis (Du et al., 2012; Li-Ping et al., 2010; Yang et al., 2013). Previously, our laboratory has demonstrated ovarian *Mapk8/Jnk1* pathway upregulation on PND 7 (Sominsky et al., 2013) and gene upregulation on PND 5 (Fuller et al., 2017). Neonatal LPS exposure may be heightening *Mapk8/JNK1* signalling channels within the ovary and altering these processes long term, or perhaps even decreasing environmental stress sensitivity as a protective effect. MAPK8/JNK1 plays a role in

adaptive responses to intra and extra-cellular environmental stressors, and is a regulator of cellular migration, proliferation and survival (Corre et al., 2017; Leppa & Bohmann, 1999). The downregulation seen here may be an intracellular attempt to conserve the ovarian follicle pool from detrimental effects of excess immune stimulation. Central upregulation of *Mapk8/Jnk1* has been demonstrated in rats following both restraint stress and forced-swim stress (Liu et al., 2004), converse to the findings here; where stress led to a downregulation within the ovary, however differing central vs peripheral mechanisms may be in place and the immune privilege of both these sites must be taken into consideration. Early life stress-induced modulation to the MAPK/JNK1 pathway within the ovary may have implications for ovarian health both generally and in response to psychological and physiological stressors, especially considering the association between MAPK/JNK1, stress exposure and dysregulated steroidogenic tissue response (Choi et al., 2003; Manna & Stocco, 2011).

To our knowledge, this is one of the first studies to demonstrate immune mRNA alterations local to the ovary as a consequence of an adult psychological stressor. Previous studies from our laboratory have demonstrated a significant increase in acute ovarian expression of *Tnfα*, following neonatal immune activation (Fuller et al., 2017 Chapter 4). This study aimed to examine if long term dysregulation to this proinflammatory mediator and related markers was present. Acute or time-limited stressors have been associated with cytokine balance shifts and upregulating immune parameters for adaptive short terms defence, while chronic stress is associated with increased HPA axis activation and immune suppression (Segerstrom & Miller, 2004). Interestingly, in this study we demonstrate that ovarian mRNA expression of *Il-1β*, *Tnfα*, and *Tnfα receptor* were significantly downregulated following restraint stress, regardless of neonatal treatment as originally hypothesised, with no differences seen in *Tlr4* expression within any groups. Although, a trend for slightly

increased proinflammatory expression is observable for the nLPS/aNS group overall. Hence, we may be seeing an adaptive protective mechanism regulating both the quality and quantity of the ovarian reserve as these cytokines are implicated in oocyte-to-granulosa maturation cross-talk (Albertini & Barrett, 2003; Kidder & Mhawi, 2002; Kidder & Vanderhyden, 2010). Interestingly, in line with this suggestion, IL-1 knockout mice demonstrate increased fecundity, greater ovarian reserve numbers, higher levels of serum anti-mullerian hormone (marker of follicle reserve), and a prolongation of ovarian lifespan, compared to wild type females (Uri-Belapolsky et al., 2014). Additionally, Uri-Belapolsky et al. (2014) observed an attenuated *Il-6* and *Tnfα* ovarian gene expression in these IL-1 knockout females, similar to those demonstrated here with the addition of adulthood stress. Studies have indicated that these markers are involved in follicular development, atresia, ovulation, and steroidogenesis (Bornstein et al., 2004; Greenfeld et al., 2007; Simon et al., 1994; Terranova, 1997; Terranova & Rice, 1997) and are constantly demonstrated to be altered in patients with PCOS, POF/POI and endometriosis (Adams et al., 2016; Erlebacher et al., 2004; Nash et al., 1999; Norman & Brännström, 1996). This study importantly provides a platform for further analyses; most notably additional studies are needed to determine the specific mechanisms involved with long-term inflammatory and apoptotic signalling pathways within the ovary.

Alterations to the immune milieu in early life may be detrimental to long-term ovarian functionality and normal fertility. The novel findings presented here suggest that an immune challenge during the final stages of initial ovarian folliculogenesis may locally perinatally program long-term ovarian immune homeostasis, thus having an impact on the quantity and perhaps the quality of the ovarian reserve. This in turn has repercussions for the female reproductive lifespan. Immune processes are critical to not only the establishment of the ovarian reserve, but also the sustained health and longevity of the ovarian follicle pool and

the response to environmental stress. These findings provide insight into the governance of follicle dynamics throughout the lifespan and elucidate on peripheral immune vulnerabilities stemming from the early life environment. Furthermore, we demonstrate that the ovary has a local immunological response to an acute, *in vivo* psychological stressor and may in fact deploy immunological-suppression based mechanisms in response, modifiable by early-life immune stress. The examination of central mediators will assist in determining the specific nature of the immune phenotype demonstrated in female animals following NIA. This will aid in understanding the link between early life stress, inflammation, and sexual behaviour deficits in the female rat.

Chapter 6. Neonatal Immune Activation and a 'Second Hit' of Adult Psychological Stress Alters Central Inflammatory Mediators: Implications for Female Reproduction

6.1 Introduction

Interactions between the brain and the immune system are essential to many aspects of physiological and psychological development, including female reproductive fitness. Alterations to this complex bidirectional network has implications for disease and disease susceptibility, including reproductive disorders and fertility issues. The inextricably linked brain-immune communication pathways are influenced at multiple operational levels by interactions with endocrine mediators and endogenous and exogenous environmental stimuli (Fagundes et al., 2013; Marques-Deak et al., 2005; Quan & Banks, 2007). Understanding the links between the central nervous system (CNS), the immune system, and the endocrine system is critical to elucidating mechanisms responsible for female physiological and psychological reproductive health. An evolving focus of reproductive biological research is aimed at gaining an understanding of how these systems interact with the early life environment in a way that modulates their functions and therefore impacts upon later life fertility and reproductive health (Camlin et al., 2014; Grive & Freiman, 2015; Richardson et al., 2014; Sloboda et al., 2011; Sominsky et al., 2013c). The previous chapters of this thesis indicate that early life immune activation results in short and long-term alterations in peripheral inflammation, depletes the ovarian follicular pool, and impairs female sexual behaviour in the female rat. Both central and peripheral systems are responsible for the early life determinants of female reproductive fitness via tightly controlled processes, therefore examination of central parameters is needed in order to increase understanding (Findlay et al., 2015; Nelson & Lenz, 2017; Richardson et al., 2014).

The early life microbial environment plays a key role in priming the immune and endocrine systems for adaptive functioning and sets the tone for subsequent physiological regulation. This *plasticity* is most critical during sensitive periods where environmental perturbation can interrupt immune-mediated development, such as the final stages of immune-driven brain and ovarian maturation (Goenka & Kollmann, 2015; Grive & Freiman, 2015; Nelson & Lenz, 2017; Reemst et al., 2016; Schlegelmilch et al., 2011; Sominsky et al., 2015). Experimental animal studies demonstrate alterations following neonatal immune activation (NIA) to both the central and peripheral functioning of the hypothalamic-pituitary-adrenal (HPA) axis (Beishuizen & Thijs, 2003; Walker et al., 2009; Webster & Sternberg, 2004), the hypothalamic-pituitary-gonadal (HPG) axis (Morale et al., 2001; Schmidt et al., 2014) and the immune system (Bilbo & Schwarz, 2009, 2012; Galic et al., 2009; Spencer et al., 2006b). Persistent central alterations as a result of NIA include impaired cognitive capacity, learning and memory (Bilbo et al., 2005; Dinel et al., 2014; Harré et al., 2008; Williamson et al., 2011), and exaggerated microglial activation and cytokine upregulation (Cardoso et al., 2015; Holder & Blaustein, 2017; Sominsky et al., 2012b; Walker et al., 2010), which may culminate in behavioural alterations (Kohman et al., 2008; Rico et al., 2010; Sominsky et al., 2013a; Spencer et al., 2005; Tenk et al., 2008; Walker et al., 2009; Walker et al., 2004). Of particular significance, NIA is linked to alterations in female reproductive parameters and decreased fertility, including ovarian follicle deletion, pubertal onset alterations, and deficits in female rat mating behaviours, (Chapter 3; Chapter 5; Fuller et al., 2017; Knox et al., 2009; Sominsky et al., 2012a; Sominsky et al., 2013b; Walker et al., 2011; Wu et al., 2011).

Central and peripheral immune systems are particularly sensitive to environmental stimulation, impacting overall immune profiles and influencing the function of interconnected neuroendocrine processes (Spencer et al., 2011). The CNS contains resident immune cells

including microglia and astrocyte populations, and a diverse range of macrophages, endothelial cells, oligodendrocytes and neurons (Korin et al., 2017; Sousa et al., 2017). These central immune mediators are critical for normal brain maturation, participating in dynamic developmental processes including neurogenesis, synaptogenesis, synaptic pruning, and homeostasis (Hanamsagar & Bilbo, 2017; Reemst et al., 2016). Central immune cells produce local cytokines and chemokines which are vital to homeostasis and normal functioning, the central response to infection and disease, and the direction of behaviours. Cytokines and their pathways have been lauded as the major mediators of the immune-brain interface, with cytokine dysregulation participating in the onset of disease and psychopathology (Besedovsky & del Rey, 2011; Galic et al., 2012; Mire-Sluis, 1993). Thus, immune disturbances during critical periods of plasticity may compromise important central processes, leading to dysfunction, affecting behaviour, and predisposing to physiological and psychological disease. These same immune pathways are implicated as mediators of female reproductive dysfunction and are known to be vulnerable to NIA (Bornstein et al., 2004; Jabbour et al., 2009; Wu et al., 2004).

Peripheral cytokine production impacts central inflammatory levels to modulate behaviour, including sickness-behaviours and reproductive behaviours (Avitsur & Yirmiya, 1999b; Bay-Richter et al., 2011; Dantzer, 2009; Turrin et al., 2001a; Vollmer-Conna et al., 2004). Neonatal immune activation has been demonstrated to lead to sustained upregulation of peripheral and central inflammation and altered cytokine expression in clinical studies and experimental animal models (Boisse et al., 2004; Bossu et al., 2012; Ellis et al., 2006; Galic et al., 2009; Mouihate et al., 2010; Sominsky et al., 2012b; Walker et al., 2010). Additionally, immune dysregulation and altered cytokine profiles are major hallmarks of many female reproductive disorders and poor reproductive outcomes (Adams et al., 2016; Bukovsky &

Caudle, 2012; Gonzalez et al., 1999; Halis & Arici, 2004; Harada et al., 1999; Jabbour et al., 2009; Robertson et al., 2015; Vgontzas et al., 2006; Weiss et al., 2009). Despite this, central immune alterations stemming from NIA and their subsequent effects on, and contribution to, female reproductive parameters and behaviours remains largely unexamined.

Central regions implicated in the control of female reproduction and reproductive behaviours include the hypothalamus, hippocampus, and the medial preoptic area (mPOA) (Clarke et al., 2015; Giraldi et al., 2004; Maffucci & Gore, 2009; Mahmoud et al., 2016; McKenna, 2002). Within these regions, female reproductive functioning is organised by tightly regulated endocrine, immune and neurochemical processes (Aguado, 2002; Lenz & McCarthy, 2015; McKenna, 1999). Alterations to the functioning of any of these regulatory systems may have detrimental consequences to reproductive health. Due to the integrative, dynamic, and reciprocal nature of these systems, the aberrant functioning of one may potentially skew others, leading to multiple levels of dysfunction (Ader et al., 1995; Glaser & Kiecolt-Glaser, 2005).

There has been relatively little research into the interaction of NIA and long-term central alterations of immune and endocrine factors relating to female reproduction. Knox et al. (2009) demonstrated that hypothalamic expression of *Kiss1* mRNA, a potent regulator and activator of the reproductive and GnRH system, was downregulated in female rats at postnatal day (PND) 32, following PND 3 and 5 lipopolysaccharide (LPS) exposure. Li et al. (2007) demonstrated that female rats exposed to PND 3 and 5 LPS exhibited no basal mPOA alterations in corticotropin-releasing hormone (CRH) and its receptors (CRHR1/2), however expression of CRHR1 significantly increased in LPS females with the addition of a later life-stressor. Importantly, the CRH system is a core component of stress-mediated suppression of the female reproductive system (Joseph & Whirledge, 2017; Rivest & Rivier, 1995). Research

examining the effects of neonatal LPS exposure on long-term central immune system alterations produces constantly sexual dimorphic results (Spencer et al., 2006a). Additionally, these differences are often only observed following a subsequent challenge, or *second hit*, in later life (Bland et al., 2010; Spencer et al., 2006a; Walker et al., 2010). Kentner et al. (2010) demonstrated that female rats did not show increases in basal levels of COX2 in the hypothalamus in adulthood following PND 14 LPS exposure, however Boisse et al. (2004) demonstrated an elevated basal level in male rats using the same model. Spencer et al. (2006a) demonstrated that female rats treated with LPS on PND 14 exhibited no basal alteration in hypothalamic COX2 protein expression or IL-1β, however COX2 was attenuated in neonatal LPS treated females following a subsequent LPS challenge. Osborne et al. (2017) demonstrated that both males and female rats exposed to LPS on PND 4 exhibited increased basal levels of hippocampal IL-1β gene expression on PND 24, with females, and not males, demonstrating an exaggerated IL-1β response following a subsequent juvenile LPS stressor.

As early life stress is known to perinatally program an exaggerated central proinflammatory response and a vulnerability to stressors, it becomes important then to investigate the potential central inflammatory processes that may in part drive the sustained female reproductive changes associated with NIA. Regardless of the plethora of literature focused on immune and endocrine health, the impact of early life immune stress on female reproduction and associated central mediators is fairly limited. The current study aims to investigate alterations in genetic expression of key proinflammatory and stress mediators that are known to be vulnerable to NIA, and established as products of LPS activation, and additionally, are known regulators of female reproduction. We propose that early life stress upregulates central inflammation, and this in turn is critically linked to deficits in sexual behaviours, alterations in pubertal timing and contributes to ovarian inflammation and

depletion of the ovarian reserve. Whilst we cannot demonstrate causal links, the gene associations are aimed at providing a preliminary examination of potential pathways to facilitate future studies. An additional later-life psychological stressor was including in the experimental design based on previous studies from this laboratory and others demonstrating that a *second hit* is often critical in revealing subsequent later life pathology induced by early life immune stress in the female rat (Li et al., 2007; Shalev & Belsky, 2016; Walker et al., 2010).

6.2 Methods

All animal and experimental procedures carried out for this current chapter are described in chapter 5 of this thesis. This chapter is concerned with the examination of the centrally mediated immune and stress related mediators induced by neonatal immune activation with lipopolysaccharide (LPS) and the determination of any additional vulnerabilities and/or alterations provoked by exposure to a 2nd hit of adulthood psychological stress . Hence, brains were collected and analysed from the previous cohort of female animals utilised in chapter 5 of this thesis (N = 6 - 8 per treatment group from at least three different litters per treatment group). As previously described (Chapter 5), neonatal and adult treatments resulted in four treatment groups that consisted of neonatal (n) saline (SAL) or lipopolysaccharide (LPS), and adult (a) no stress (NS) or restraint stress (ST) resulting in 4 treatment groups; nSAL/aNS, nSAL/aST, nLPS/aNS, nLPS/aST. All experimental protocols and treatments are as previously described in chapter 5, with additional procedures outlined below.

6.2.1 Brain Dissection

All animals were euthanised 24 hours following the last day of the 3-day stress protocol as previously described (Chapter 5). Transcardial perfusion was performed using

~600mL of 4°C sterile PBS to clear all central tissue of blood. Whole brains were then rapidly extracted using ribonucleic acid (RNA) free fine-tipped rongeurs (*RNaseZap*, Sigma Aldrich, Australia). Brains were rinsed in 4°C sterile, filters, and RNA-free PBS, then snap frozen in dry ice and stored at -80°C until sectioning and subsequent analysis.

6.2.2 Brain Sectioning

Frozen brains were prepared for sectioning using a cryostat (Leica, Wetzlar, Germany). Whole brains (cerebellum removed) were mounted and a series of coronal cryosections were obtained following the rostral-caudal extent of the prefrontal cortex (PFC), medial pre optic area (mPOA), the hippocampal region (HC), and the hypothalamus (HTH) (see Table 6.1 for Bregma measurements and Figure 6.1 & 6.2) (Paxinos & Watson, 2007). Sections were then placed onto chilled, sterile microscope slides and regions of interest were bilaterally excised using 1mm-5mm tissue punches depending on region. Punched issue sections were transferred into 2mL PCR-grade micro-tubes (Eppendorf South Pacific, Australia) containing 1 mL of QIAzol® Lysis Reagent (Qiagen, Netherlands) and a 5 mm diameter stainless steel bead (Qiagen, Netherlands). Samples were then homogenized using a TissueLyzer® (Qiagen, Netherlands; 4 min at 20 Hz) and prepared for RNA extraction and PCR.

Table 6.1. Brain sectioning Bregma levels.

Brain region	Bregma region start	Bregma region end
mPOA	-0.2	-1.35
HC	-1.70	-3.12
HTH	-1.6	-3.6

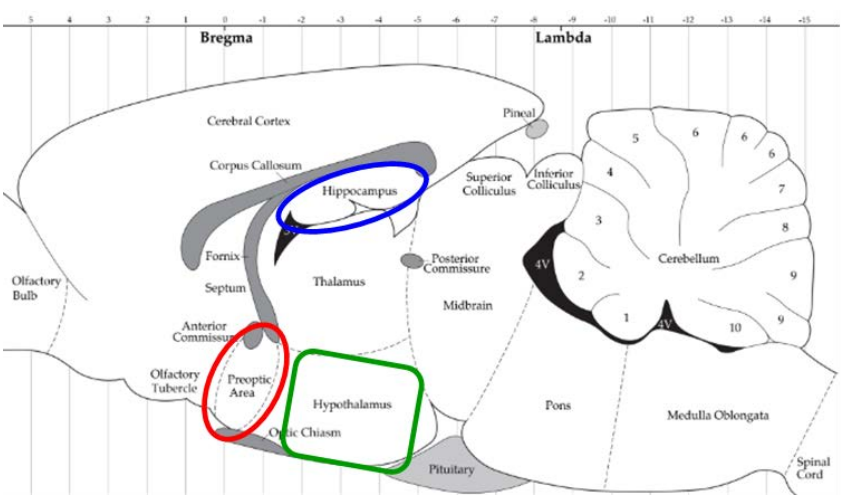

Figure 6.1. Lateral sagittal visual representation of brain sections excised. Red oval represents the mPOA. Green rectangle represents the hypothalamus (HTH). Blue oval represents the hippocampus (HC). Schematic modified from Paxinos and Watson (2007).

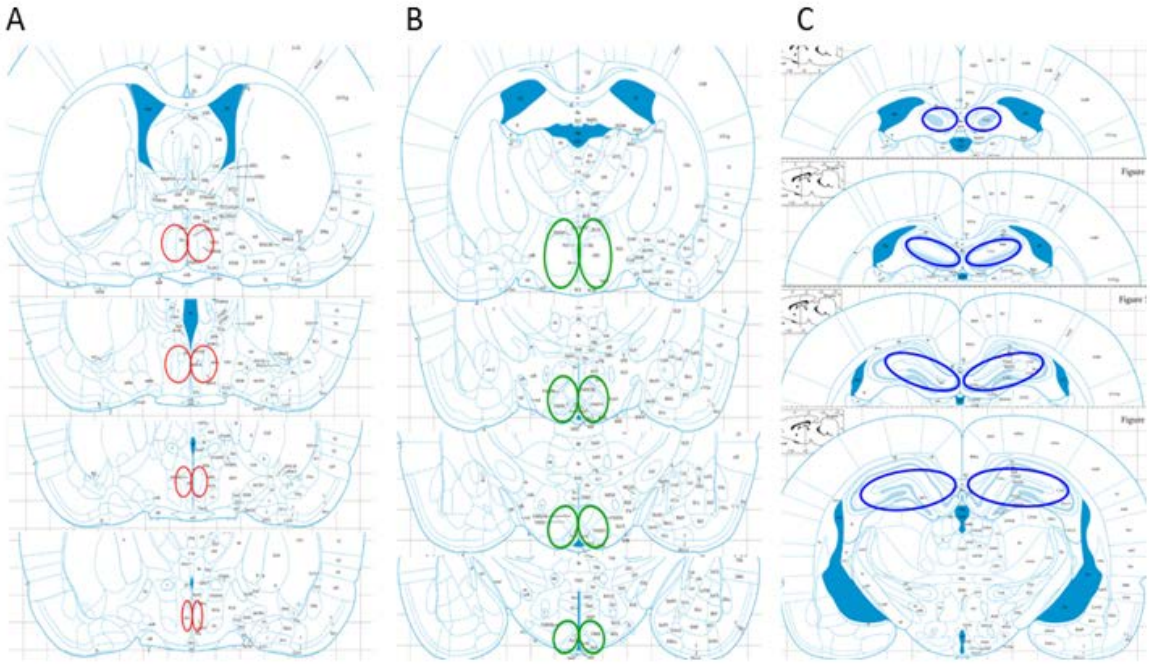

Figure 6.2. Coronal representation of brain sections excised. A) Red ovals represents the mPOA. B) Green ovals represents the hypothalamus (HTH). C) Blue ovals represents the hippocampus (HC). Schematic modified from Paxinos and Watson (2007).

6.2.3 RNA extraction, Reverse Transcription and qRT-PCR

RNA was extracted from homogenized brain tissue in Qiazol using an RNeasy mini kit (Qiagen) with added DNase treatment protocol (Invitrogen, CA, USA) in accordance with manufactures instructions. Nucleic acid purity and concentration was assessed in a 1µl volume by NanoDrop™ Spectrophotometer 2000c (Thermo Fisher Scientific, DE USA). In order to isolate sufficient quantities of mRNA, brain region samples that produced a low concentration due to tissue size were pooled within that specific treatment and region (maximum 2-3 animals per biological replicate). Reverse transcription polymerase chain reaction (RT-PCR) was performed using a SuperScript ® VILO cDNA synthesis kit (Life Technologies, Thermo Fisher Scientific) by combining the kit components to a total volume of 20µl per reaction according to the manufacturer's instructions, with sufficient extracted mRNA for 2000ng of final product. Quantitative RT-PCR was performed in 20µl reactions (10 µl of SYBR Green, 0.4 µl of each primer (forward and reverse), 4.6 µl nuclease free H_20, and 5 µl cDNA template (5ng/µl)) using SYBR Green reagents (Life Technologies, Thermo Fisher Scientific) and conducted on a 7500 RT-PCR Fast Instrument (Applied Biosystems, California, USA). All reactions were performed under the following optomised conditions; 95°c for 20s and 40 cycles of 95°c for 3s and 60°C for 30s. A melting curve was determined under the following conditions; 95°C for 15s, 60°C for 1 min, 95°C for 15s, and 60°C for 15s (as described in Sominsky et al., 2013a). All reactions were performed in triplicate accompanied by a RT-negative replicate as a negative control. Qualitative RT-PCR data were normalized to reference gene β-actin and Tubulin (Life Technologies, Australia) and analysed using the comparative C_T method equation $2^{-\Delta\Delta C(t)}$ (where C(t) is the threshold cycle at which fluorescence is first detected as statistically significant above background), and presented as a fold increase relative to the saline control group (Schmittgen & Livak, 2008). All qRT-PCR

was performed on at least six separate tissue isolations/biological replicates, with final gene expression changes presented as a normalised fold change relative to the nSAL/aNS control group. Primer sequences are supplied (Sigma Aldrich; Invitrogen, see table 2) and were optimized by qPCR both here and previously (Fuller et al., 2017; Meehan et al., 2017; Sominsky et al., 2013a; Sominsky et al., 2013b), with only primer pairs that presented efficiency between 90% and 110% used. The following mediators were examined*: IL-1β, IL-6, IL-6 receptor (R), TNFα, TNFα R, TLR4, mitogen-activated protein kinase 8/c-Jun N-terminal kinase1 (Mapk8/Jnk1), cyclooxygenase-2 (Cox2), Follicle stimulating hormone (FSH)R, Kisspeptin-1 (Kiss1), Kiss1R, Glucocorticoid (GC)R, mineralocorticoid (M)R, corticotrophin releasing hormone (CRH), CRHR1,* and *tyrosine hydroxylase (TH).*

Table 6.2 Primer forward and reverse sequence and efficiency for primer pairs.

Target Gene	Forward Primer Sequence	Reverse Primer Sequence	Primer Efficiency
β-actin	TCTGTGTGGATTGGTGGCTCTA	CTGCTTGCTGATCCACATCTG	94%
Tubulin	GAGGCCGAGAGCAACATGAA	CTTCCGACTCCTCGTCGTCA	95%
Mapk8/Jnk1	CGGAACACCTTGTCCTGAAT	GAGTCAGCTGGGAAAAGCAC	94%
Tlr4	ACTGGGTGAGAAACGAGCTG	CGGCTACTCAGAAACTGCCA	97%
Il-6	TGCCTTCCCTACTTCACAAG	CCATTGCACAACTCTTTTCTCA	103%
Il-6R	CGGAAGAACCCCCTTGTAAA	GGTGGTGTTGATTTTCTTTGC	106%
Tnfα	CGAGATGTGGAACTGGCAGA	CGATCACCCCGAAGTTCAGT	108%
TnfαR	AACCTCAAATGGAAACGTGA	CAGGATGCTACAAATGCGG	106%
Il-1β	AACATAAGCCAACAAGTGGT	TTCATCACACAGGACAGGTA	100%
Cox2	CAAGACAGATCAGAAGCGAG	TCCACCGATGACCTGATATT	107%
FSHR	TGGCTGTGTCATTGCTCTAA	TGAGCACAAACCTCAGTTCA	93%
Kiss1R	AATTTCTACATCGCTAACCTGG	TGCTGGATGTAGTTGACGAA	95%
Kiss1	AGCTGCTGCTTCTCCTCTGT	AGGCTTGCTCTCTGCATACC	98%
GR	CGTCAAAAGGGAAGGGAAC	TGTCTGGAAGCAGTAGGTAAG	98%
MR	CCAAATCACCCTCATCCAG	GCACAGTTCATACATGGCAG	98%
CRHR1	TGGAACCTCATCTCGGCTTT	CACTCGACCTGGTGTTTGGT	99%
CRH	CGCCCATCTCTCTGGATCTC	CGTTGTAAAGTAAAGGGCTATTAG	101%
TH	TGTGTCCGAGAGCTTCAATG	GCTGGATACGAGAGGCATAGTTC	101%

6.2.4 Data Analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Version 22 (SPSS Inc.). Data were analysed using appropriate factorial multivariate analysis of variance (MANOVA), with *neonatal treatment* and *adult stress* as independent variables, at a significance level of $p \leq 0.05$, using pairwise Bonferroni adjustments were appropriate. Oestrus phase were included as covariate if cycles deviated and reported where significant. Outliers present in the data that were more than ± two standard deviations away from the groups mean for that dependant variable were removed from analyses. All MANOVA assumptions were tested and violations reported if occurred. All statistics are presented in table format following each specific brain area reported.

6.3 Results

6.3.1 Hippocampus

Table 6.3 contains *F* statistics and *p*-values for all genes analysed in the hippocampus 24 hours following restraint stress. Significant *p* values reported in-text for clarity. A significant main effect of neonatal treatment existed for *TnfαR*, with mRNA upregulation demonstrated in nLPS treated animals ($p = .0001$) (Figure 6.3, B). A significant neonatal x adult interaction was observed for *Il-1β* ($p = .046$). Pairwise comparisons indicated a significant upregulation of *Il-1β* in nLPS/aNS females, compared to nLPS/aST females ($p = .018$) with no changes in other groups (Figure 6.3, E). A main effect of neonatal treatment existed for *Cox2* mRNA expression, with *Cox2* expression upregulated in nLPS females ($p = .011$), compared to nSAL. Notably, a differing pattern of *Cox2* expression was observed between neonatal groups, with adult stress upregulating expression in nSAL animals, but downregulating *Cox2* in nLPS treated females (Figure 6.3, H). A trend nearing significance for neonatal treatment was

observed for *Tlr4* (p = .064) (Figure 6.3, G). A significant effect of neonatal treatment was demonstrated for *GCR*, with nLPS females exhibiting increased expression compared to nSal animals (p = .021) (Figure 6.4, A). A significant main effect of neonatal treatment was observed for *Kiss1R* (p = .023), with upregulation demonstrated in nLPS females. Planned comparisons indicate that this difference was driven by greater upregulation in nLPS/aNS animals (p = .015) compared to controls (Figure 6.4, E). No significant neonatal treatment, adult treatment, or interaction effects existed for *Tnfα, Il-6R, MR, CRHR1,* or *CRH. FSH* was not detected in the hippocampus in any treatment groups at a CT threshold that could be confidently reported as specific signal and not background noise (see Table 6.3 for summary all F and p statistics; see Figure 6.3, A – H for inflammatory mediators expression & Figure 6.4, A – E for stress mediators & *Kiss1R* expression, p 226-228). Hippocampal Kiss1 immunoreactivity is lower than that of the hypothalamus with peptide expression below detection levels in hippocampal immunohistochemically studies (Arai, 2009; Brailoiu et al., 2005). Hence, only Kiss1R was examined in the hippocampus in the current study.

Table 6.3 ANOVA statistics of inflammatory and endocrine gene expression in the hippocampus.

	ANOVA Statistics		
Hippocampus	Neonatal Treatment	Adult Treatment	Neonatal x Adult
Tnfα	*F*(1, 20) = 2.166 *p* = .159	*F*(1, 20) = .014 *p* = .906	*F*(1, 20) = .075 *p* = .788
TnfαR*	*F*(1, 20) = 51.762 ***p* = .0001***	*F*(1, 20) = .966 *p* = .340	*F*(1, 20) = 1.118 *p* = .305
Il-6	*F*(1, 20) = .1.108 *p* = .307	*F*(1, 20) = 1.886 *p* = .187	*F*(1, 20) = .873 *p* = .363
Il-6R	*F*(1, 20) = 2.458 *p* = .135	*F*(1, 20) = .282 *p* = .602	*F*(1, 20) = 2.689 *p* = .119
Il-1β*	*F*(1, 20) = .005 *p* = .942	*F*(1, 20) = 2.04 *p* = .171	*F*(1, 20) = 4.621 ***p* = .046***
Cox2*	*F*(1, 20) = 8.099 ***p* = .011***	*F*(1, 20) = 2.751 *p* = .116	*F*(1, 20) = 1.037 *p* = .323
Mapk8/Jnk1	*F*(1, 20) = 2.519 *p* = .131	*F*(1, 20) = .657 *p* = .429	*F*(1, 20) = 1.454 *p* = .244
Tlr4	*F*(1, 20) = 3.930 *p* = .064†	*F*(1, 20) = .074 *p* = .788	*F*(1, 20) = .015 *p* = .905
Kiss1R*	*F*(1, 20) = 6.279 ***p* = .023***	*F*(1, 20) = 1.416 *p* = .250	*F*(1, 20) = 1.08 *p* = .313
FSHR	Nil detected		
GCR*	*F*(1, 20) = 6.426 ***p* = .021***	*F*(1, 20) = .885 *p* = .36	*F*(1, 20) = .018 *p* = .894
MR	*F*(1, 20) = .194 *p* = .665	*F*(1, 20) = 1.882 *p* = .188	*F*(1, 20) = 2.407 *p* = .139
CRHR1	*F*(1, 20) = .642 *p* = .434	*F*(1, 20) = .569 *p* = .461	*F*(1, 20) = .453 *p* = .510
CRH	*F*(1, 20) = .126 *p* = .727	*F*(1, 20) = 1.172 *p* = .294	*F*(1, 20) = .714 *p* = .41

*and bold denotes $p \leq .05$, † denotes $p \leq .08$

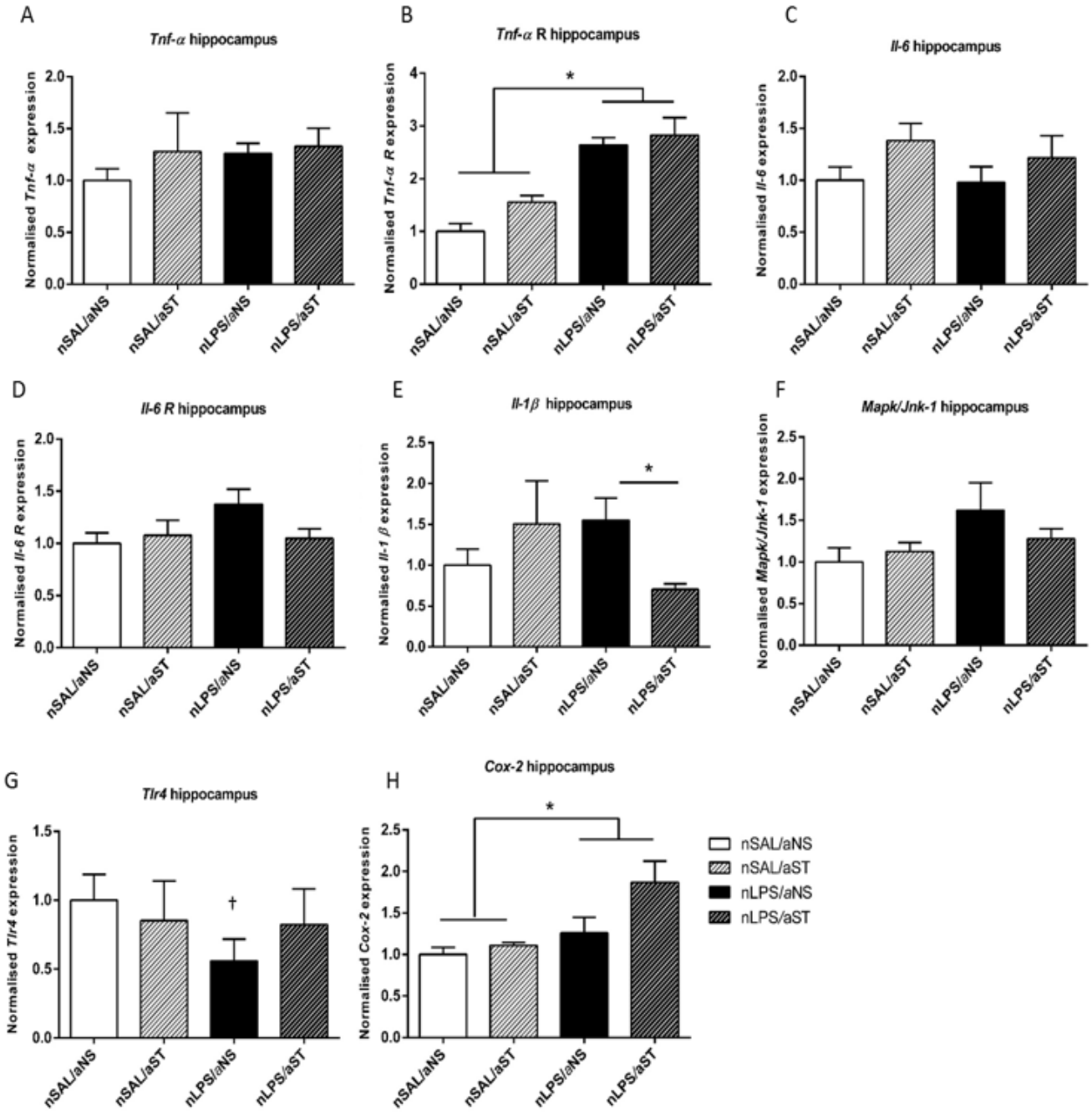

Figure 6.3. mRNA expression of inflammatory mediators in the hippocampus (HC) of female animals treated with either nSAL/aNS (hollow bars), nSAL/aST (light grey filled bars), nLPS/aNS (black filled in bars), and nLPS/aST (dark grey filled in bars). * indicates $p \leq .05$. † denotes trend nearing significance. Data expressed as normalised fold change compared to control (nSAL/aNS) ± SEM

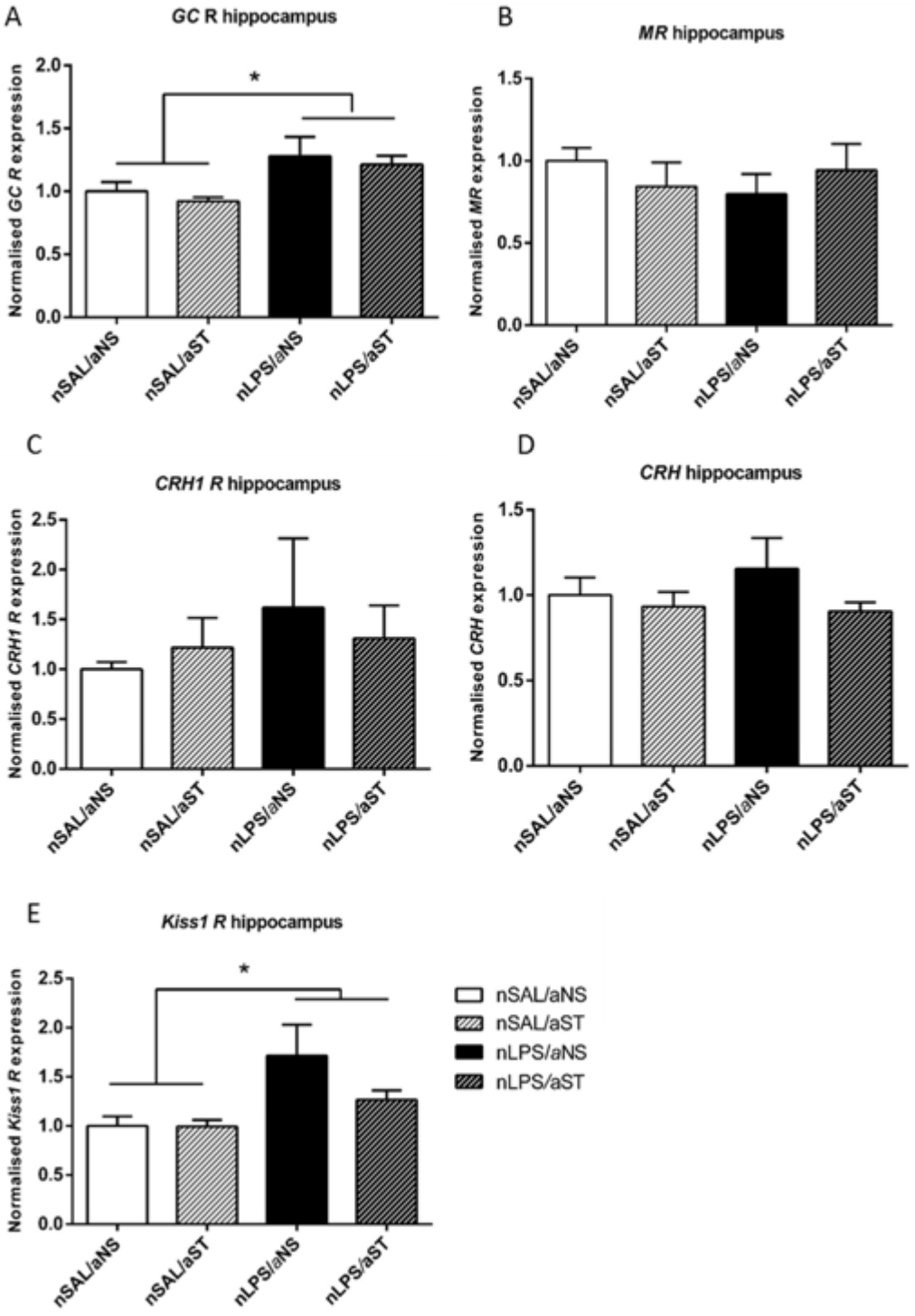

Figure 6.4. mRNA expression of stress and endocrine mediators, and Kiss1 receptor expression in the hippocampus (HC) of female animals treated with either nSal/aNS (hollow bars), nSal/aST (light grey filled bars), nLPS/aNS (black filled in bars), and nLPS/aST (dark grey filled in bars). * indicates $p \leq .05$. Data expressed as normalised fold change compared to control (nSAL/aNS) + SEM.

6.3.2 Hypothalamus

Table 6.4 contains *F* statistics and *p*-values for all genes analysed in the hypothalamus 24 hours after restraint stress. Significant *p* values reported in-text for clarity. A significant neonatal x adult interaction (p = .032) effect, main effect of neonatal treatment (p = .001), and main effect of adult treatment (p =.002) treatment existed for *TnfαR* expression. Pairwise comparisons indicate that nLPS/aNS demonstrated significantly upregulated gene expression of *TnfαR* compared to controls by ~ 3 fold ($p \leq .001$), as did nLPS/ST ($p \leq .001$), and nSAL/aST females (p = .001) (Figure 6.5, B). A main effect of neonatal treatment existed for *IL-1β* mRNA levels, with LPS treated females demonstrating significantly upregulated *IL-1β* expression compared to saline treated females (p = .015) (Figure 6.5, E). A significant main effect of adult stress was demonstrated for *Tlr4* expression, with stress upregulating *Tlr4* levels (p = .033). Additionally, pairwise comparisons indicates that the significant neonatal x adult interaction effect for *Tlr4* (p = .04) was driven predominantly by the increase in the nSAL/ST group compared to controls (p = .006) (Figure 6.5, G). A significant neonatal x adult treatment interaction effect was demonstrated for *Cox2* mRNA expression (p = .036). Pairwise comparisons indicate that nSAL/aST and nLPS/aNS demonstrated significantly increased levels of *Cox2* compared to the control group (p = .019 and p = .04 respectively) (Figure 6.5, H). A significant neonatal x stress interaction was demonstrated for *GCR* expression (p = .048), with nSAL/aST animals demonstrating significantly increased expression compared to controls (p = .009). As expected, a significant main effect of adult stress on *GCR* expression was demonstrated in the hypothalamus, with stress increasing expression levels in all ST groups (p = .044), driven by upregulation in nSAL/aST animals. A significant main effect of neonatal treatment was demonstrated for *MR* gene expression, with upregulated levels in nLPS animals compared to nSAL (p = .001) and a trend existed towards significance for a

neonatal x adult interaction effect, as such pairwise comparisons indicate that significant differences existed between nLPS/aST and nSAL/aST groups (p = .001), and nLPS/aNS and nLPS/aST groups (p = .018). No alterations in *Tnfα, Il-6, Il6R, Mapk8/Jnk1, Kiss1, Kiss1R, FSHR, CRH1R, CRH,* or *TH* existed (see Table 6.4; see Figure 6.5, A – H for inflammatory mediators expression & Figure 6.6, A – G for HPA axis mediators & *Kiss1R* expression. Table and figures continue p, 231-233).

Table 6.4 ANOVA statistics of inflammatory and stress mediator gene expression in the hypothalamus.

	ANOVA Statistics		
Hypothalamus	Neonatal Treatment	Adult Treatment	Neonatal x Adult
Tnfα	*F*(1, 20) = .2.32 *p* = .636	*F*(1, 20) = 1.142 *p* = .3	*F*(1, 20) = .393 *p* = .539
TnfαR*	*F*(1, 20) = 15.386 ***p* = .001***	*F*(1, 20) = 14.273 ***p* = .002***	*F*(1, 20) = 5.487 ***p* = .032***
Il-6	*F*(1, 20) = .053 *p* = .82	*F*(1, 20) = 2.471 *p* = .134	*F*(1, 20) = .002 *p* = .969
Il-6 R	*F*(1, 20) = .083 *p* = .777	*F*(1, 20) = 3.719 *p* = .071†	*F*(1, 20) = 1.407 *p* = .252
Il-1β*	*F*(1, 20) = 7.374 ***p* = .015**	*F*(1, 20) = .252 *p* = .622	*F*(1, 20) = .906 *p* = .355
Cox2*	*F*(1, 20) = .098 *p* = .758	*F*(1, 20) = 2.263 *p* = .151	*F*(1, 20) = 5.214 ***p* = .036***
Mapk8/Jnk1	*F*(1, 20) = 1.272 *p* = .275	*F*(1, 20) = 1.187 *p* = .291	*F*(1, 20) = .677 *p* = .422
Tlr4*	*F*(1, 20) = .385 *p* = .543	*F*(1, 20) = 5.411 ***p* = .033***	*F*(1, 20) = 4.955 ***p* = .04***
Kiss1R	*F*(1, 20) = .14 *p* = .713	*F*(1, 20) = 2.236 *p* = .153	*F*(1, 20) = .249 *p* = .624
Kiss1	*F*(1, 20) = .636 *p* = .436	*F*(1, 20) = .012 *p* = .916	*F*(1, 20) = .025 *p* = .876
FSHR	*F*(1, 20) = .877 *p* = .362	*F*(1, 20) = .493 *p* = .492	*F*(1, 20) = 2.229 *p* = .154
GCR*	*F*(1, 20) = .364 *p* = .554	*F*(1, 20) = 4.727 ***p* = .044***	*F*(1, 20) = 4.544 ***p* = .048***
MR*	*F*(1, 20) = 14.945 ***p* = .001***	*F*(1, 20) = 2.973 *p* = .103	*F*(1, 20) = 3.478 *p* = .08†
CRHR1	*F*(1, 20) = 1.53 *p* = .233	*F*(1, 20) = .343 *p* = .566	*F*(1, 20) = .313 *p* = .583
CRH	*F*(1, 20) = 2.664 *p* = .121	*F*(1, 20) = .328 *p* = .574	*F*(1, 20) = .862 *p* = .366
TH	*F*(1, 20) = .038 *p* = .847	*F*(1, 20) = .634 *p* = .437	*F*(1, 20) = 1.792 *p* = .198

*and bold denotes $p \leq .05$, † denotes $p \leq .08$

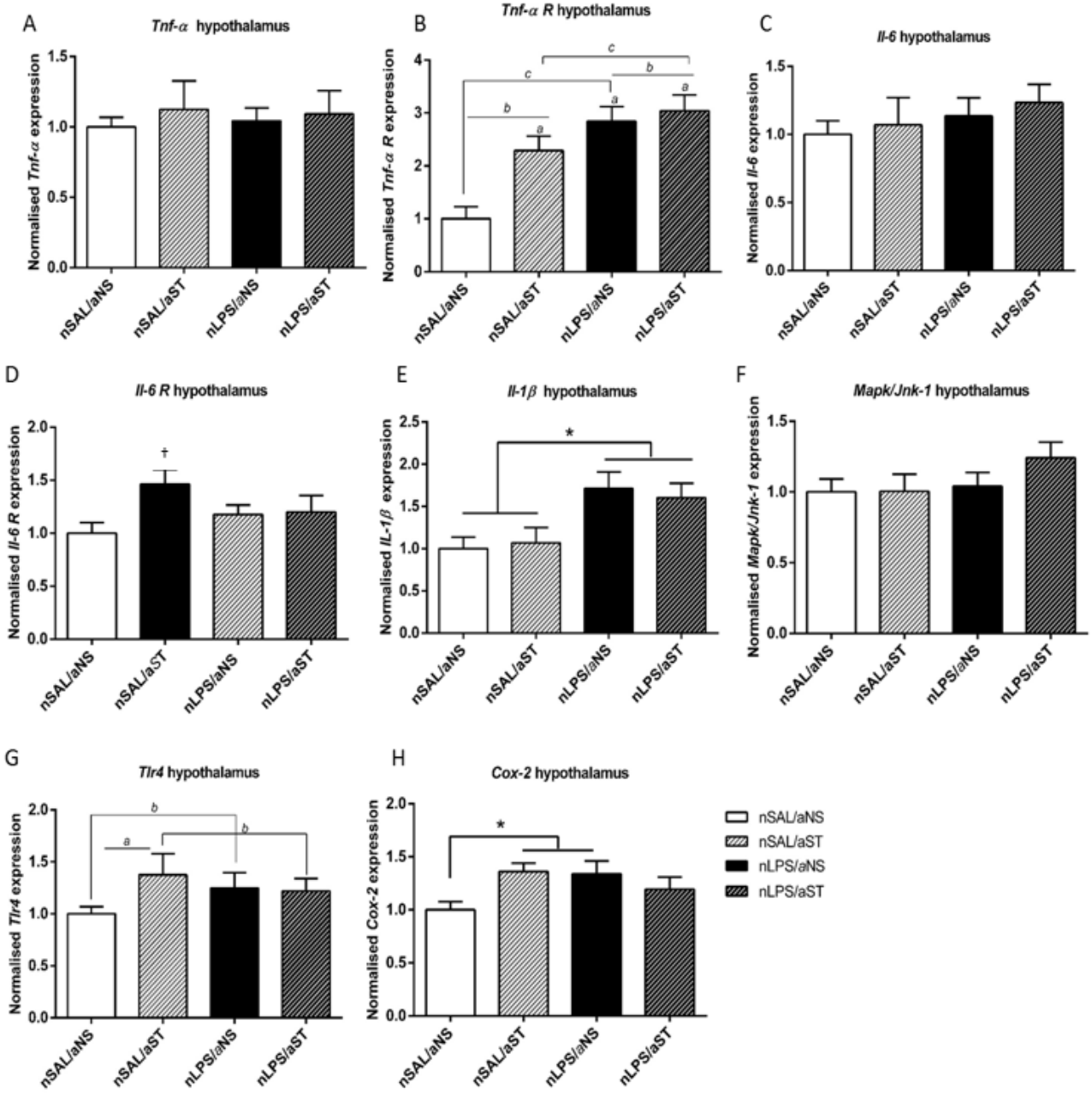

Figure 6.5. mRNA expression of inflammatory mediators in the hypothalamus of female animals treated with either nSAL/aNS (hollow bars), nSAL/aST (light grey filled bars), nLPS/aNS (black filled in bars), and nLPS/aST (dark grey filled in bars). Note differing order of *Il-6R* to represent adult treatment effect. * indicates $p \leq .05$. '*a*' indicates specific group is significantly different from control group, '*b*' indicates significant effect of neonatal treatment, '*c*' indicates significant effects of adult treatment (for *TnfαR* data only). Data expressed as normalised fold change compared to control (nSAL/aNS).

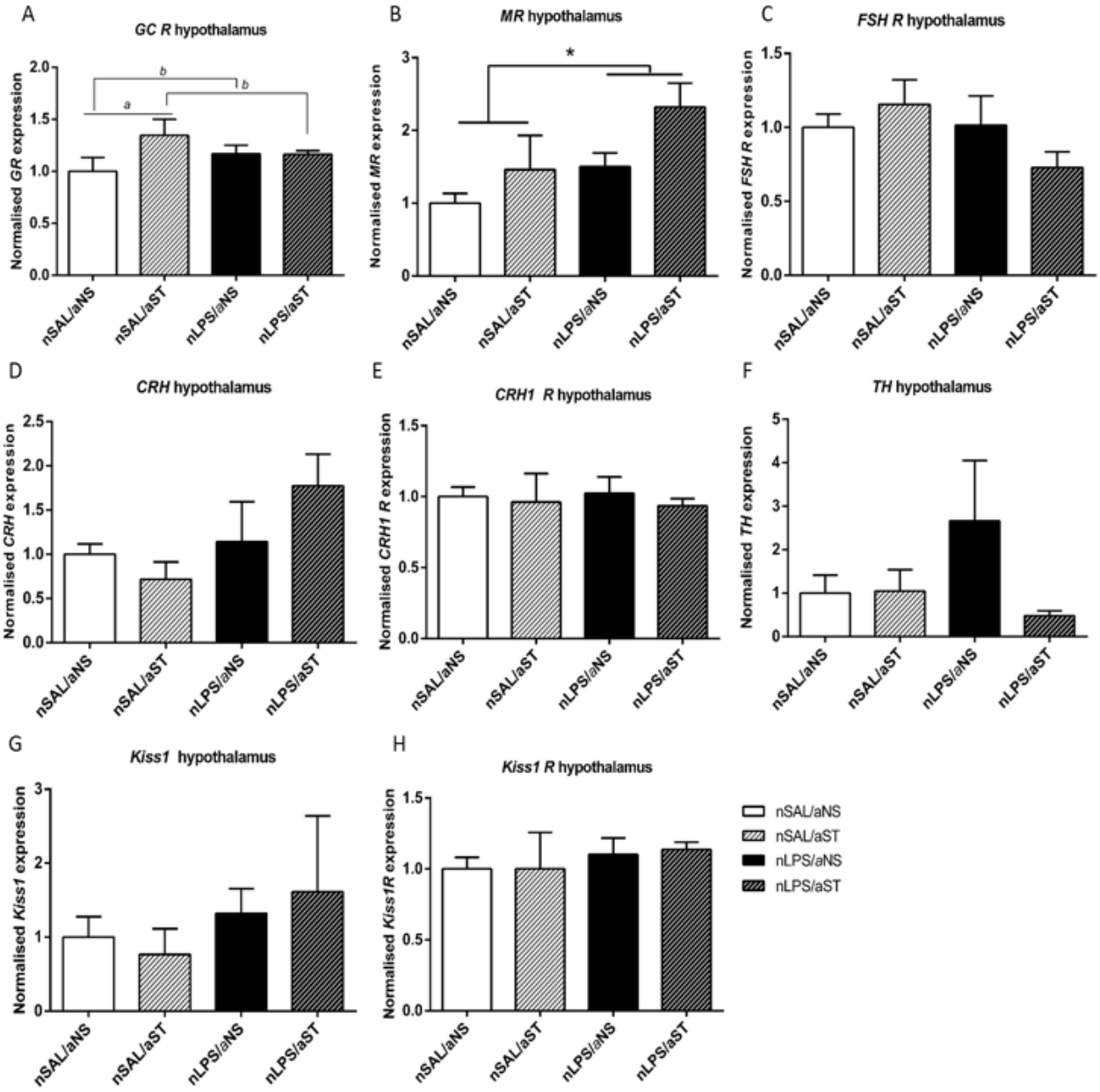

Figure 6.6. mRNA expression of stress and endocrine mediators, and Kiss1 receptor expression in the hypothalamus of female animals treated with either nSal/aNS (hollow bars), nSal/aST (light grey filled bars), nLPS/aNS (black filled in bars), and nLPS/aST (dark grey filled in bars). * indicates $p \leq .05$. '*a*' indicates specific group is significantly different from control group, '*b*' indicates significant effect of adult stress treatment (for *GCR* data only). Data expressed as normalised fold change + SEM compared to control (nSAL/aNS).

6.3.3 Medial Preoptic Area

Table 6.5 contains *F* statistics and *p*-values for all genes analysed in the mPOA 24 hours after restraint stress. Significant *p* values reported in-text for clarity. A significant main effect of neonatal treatment was demonstrated for *TnfαR*, with upregulation in nLPS females, regardless of adult procedure (p = .023) (Figure 6.7, B). A significant main effect neonatal treatment effect existed for *Il-1β*, with upregulation in nLPS treated females (p = .024) (Figure 6.7, E). A significant neonatal x adult interaction effect existed for *Cox2* mRNA expression (p = .034), with pairwise comparisons indicating that nLPS/aNS *Cox2* expression was significantly elevated compared to the nLPS/aST treatment group (p = .004). A significant neonatal x adult treatment effect existed for *TH* mRNA expression (p = .049), with pairwise comparisons indicating that significant increase in nLPS/aNS group compared to nSAL groups (p = .009). Additionally, nLPS/aNS was significantly upregulated compared to nLPS/aST group (p = .013) (Figure 6.8, D). No alterations in *Tnfα, Il-6, Il6R, Mapk8/Jnk1, Tlr4, Kiss1, Kiss1R, MR, GCR, CRH1R, or CRH* existed *CRH* and *FSH* gene expression were not analysed in the mPOA due to non-significant results in other areas and minimal cDNA quantities (see Table 6.5 for summary of all statistics; see Figure 6.7, A – H for inflammatory mediators expression and Figure 6.8, A – F for stress and neuronal expression).

Table 6.5 ANOVA statistics of inflammatory and endocrine gene expression in the medial pre optic area (mPOA).

	ANOVA Statistics		
mPOA	Neonatal Treatment	Adult Treatment	Neonatal x Adult
Tnfα	$F(1, 20) = 3.999$ $p = .06$†	$F(1, 20) = .230$ $p = .637$	$F(1, 20) = .955$ $p = .341$
TnfαR*	$F(1, 20) = 6.133$ **$p = .023$***	$F(1, 20) = .009$ $p = .924$	$F(1, 20) = .578$ $p = .456$
Il-6	$F(1, 20) = 2.433$ $p = .135$	$F(1, 20) = .001$ $p = .974$	$F(1, 20) = .809$ $p = .38$
Il-6 R	$F(1, 20) = .000$ $p = .996$	$F(1, 20) = 1.922$ $p = .182$	$F(1, 20) = .123$ $p = .73$
Il-1β*	$F(1, 20) = 6.006$ **$p = .024$***	$F(1, 20) = .709$ $p = .41$	$F(1, 20) = .253$ $p = .621$
Cox2*	$F(1, 19) = .16$ $p = .693$	$F(1, 19) = 4.696$ **$p = .043$***	$F(1, 19) = 5.211$ **$p = .034$***
MapK8/Jnk1	$F(1, 20) = 0.973$ $p = .336$	$F(1, 20) = .253$ $p = .621$	$F(1, 20) = .21$ $p = .652$
Tlr4	$F(1, 20) = .853$ $p = .367$	$F(1, 20) = 1.733$ $p = .203$	$F(1, 20) = .293$ $p = .594$
Kiss1R	$F(1, 20) = .643$ $p = .433$	$F(1, 20) = 2.522$ $p = .129$	$F(1, 20) = .182$ $p = .674$
Kiss1	$F(1, 20) = .858$ $p = .366$	$F(1, 20) = .089$ $p = .769$	$F(1, 20) = .441$ $p = .515$
GC R	$F(1, 20) = .055$ $p = .817$	$F(1, 20) = 2.768$ $p = .113$	$F(1, 20) = .02$ $p = .89$
MR	$F(1, 20) = 1.478$ $p = .239$	$F(1, 20) = .007$ $p = .936$	$F(1, 20) = .000$ $p = .998$
CRHR1	$F(1, 20) = .160$ $p = .693$	$F(1, 20) = .404$ $p = .533$	$F(1, 20) = .018$ $p = .894$
TH*	$F(1, 20) = 3.742$ $p = .068$†	$F(1, 20) = 2.844$ $p = .108$	$F(1, 20) = 4.415$ **$p = .049$***

*and bold denotes $p \leq .05$, † denotes $p \leq .08$

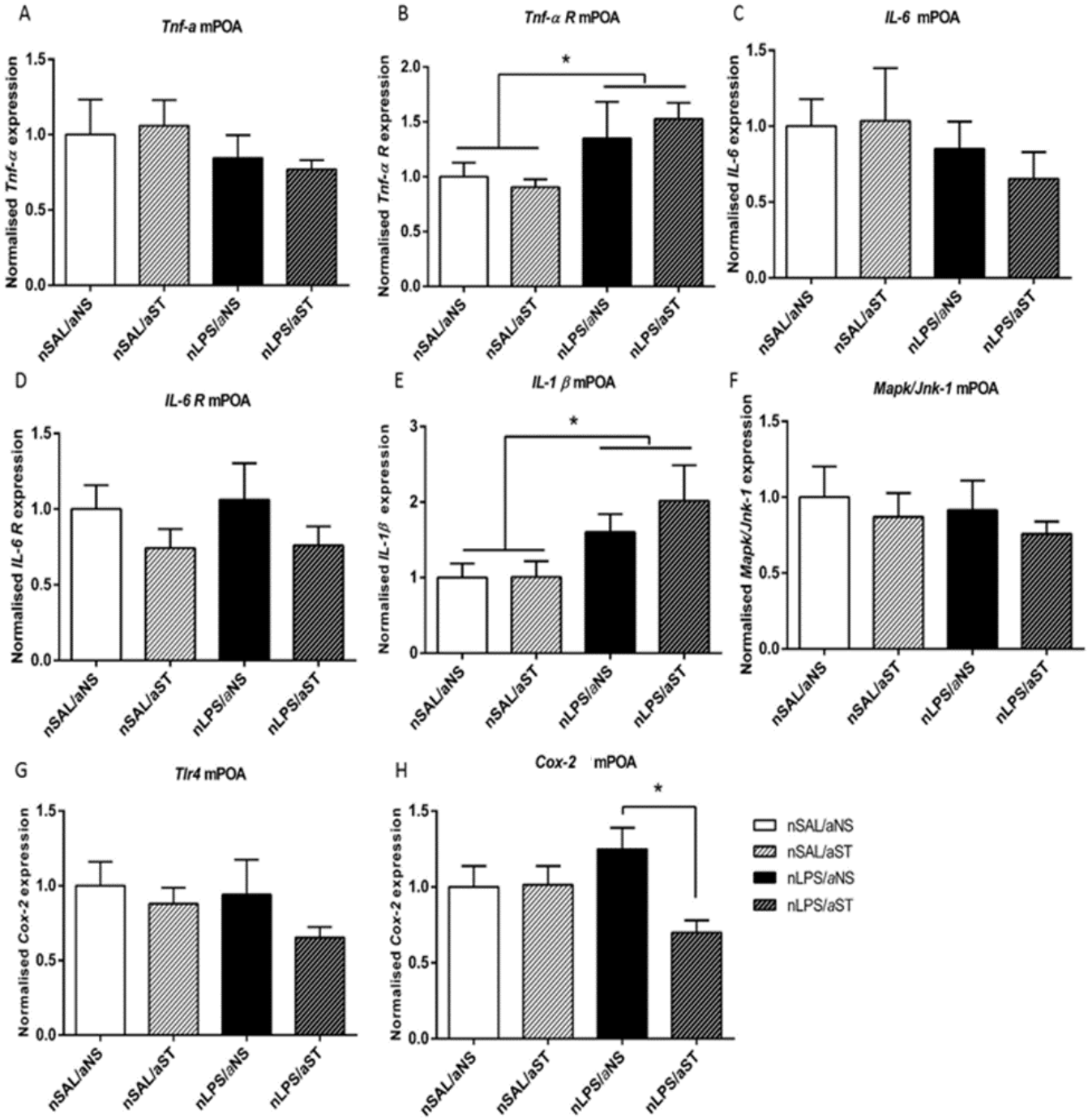

Figure 6.7. mRNA expression of inflammatory mediators in the mPOA of female animals treated with either nSAL/aNS (hollow bars), nSAL/aST (light grey filled bars), nLPS/aNS (black filled in bars), and nLPS/aST (dark grey filled in bars). * indicates $p \leq .05$. Data expressed as normalised fold change compared to control (nSAL/aNS) + SEM.

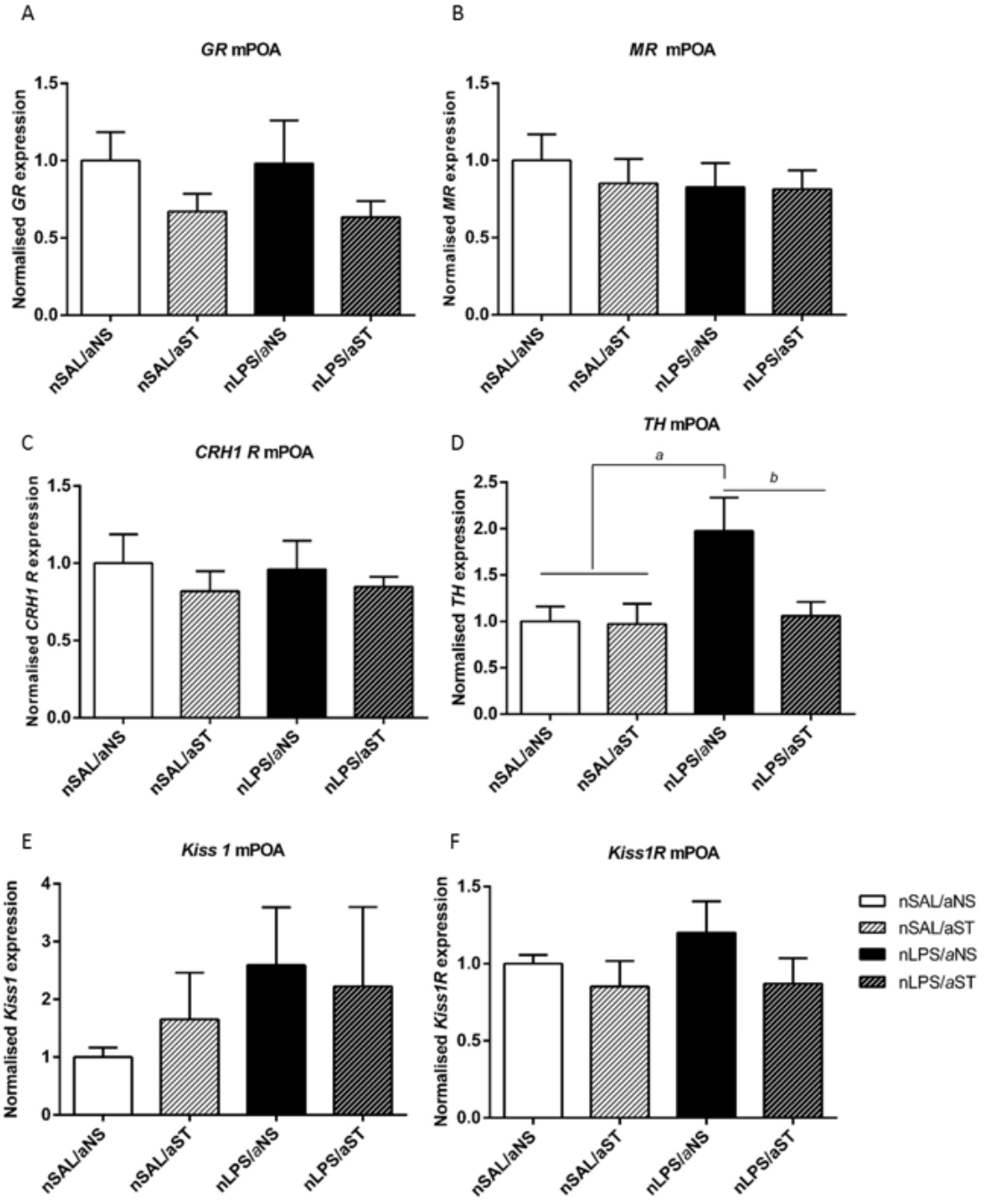

Figure 6.8. mRNA expression of stress mediators in the mPOA of female animals treated with either nSAL/aNS (hollow bars), nSAL/aST (light grey filled bars), nLPS/aNS (black filled in bars), and nLPS/aST (dark grey filled in bars). * indicates $p \leq .05$, '*a*' indicates specific group is significantly different from nSAL groups, '*b*' indicates significant difference between nLPS/aNS and nLPS/aST groups (for *TH* data only). Data expressed as normalised fold change compared to control (nSAL/aNS) + SEM.

6.4 Discussion

Converging evidence from both human and animal studies indicates that early-life immune activation induces a suite of physiological, psychological, and behavioural alterations that are related to alterations in immune signalling and activation status (Bay-Richter et al., 2011; Dantzer, 2009; Plata-Salaman et al., 1998; Spencer & Meyer, 2017; Turrin et al., 2001b; Vollmer-Conna et al., 2004). Chronic inflammation is a characteristic of both female reproductive disorders and psychopathologies that are associated with reduced fecundity in human females, such as depression and anxiety (Bradford & Meston, 2006; Kalmbach et al., 2014). As immune processes are essential to female reproductive development, including puberty, behaviour, and ovarian function; examining both central and peripheral alterations to immune process via NIA is critical (Avitsur & Yirmiya, 1999b; Bornstein et al., 2004; Simon & Polan, 1994). The present study demonstrates that female rats exposed to a neonatal LPS challenge on PND 3 and 5 exhibit altered mRNA levels of central immune mediators in the HC, the HTH and the mPOA, specifically, consistent dysregulation of *TnfαR*, *Il-1β* and *Cox2*. This was accompanied by increased gene expression of HPA-axis mediators in the HC and HTH, and the upregulation in *TH* gene expression, a marker of catecholamine synthesis, in the mPOA. Additionally, *Kiss1R* was upregulated in LPS treated females in the HC only. These findings suggest that NIA in female rats alters the long-term basal proinflammatory and stress-activation milieu in central regions associated with overall reproductive governance and stress mediation. This may be contributing to deficits in female sexual behaviours and reproductive parameters previously demonstrated (Walker et al., 2011; Chapter 3, Chapter 4, Chapter 5).

The current study indicates long-term alterations in *IL-1β* expression in the HTH, HC, and mPOA in adult female rats following neonatal exposure to LPS. This suggests that NIA has

a robust programming effect on this system in areas that contribute to the governance of reproductive function and behaviours, stress-mediation, and cognition. Cai et al. (2013) demonstrated that LPS on PND 5 upregulated IL-1β levels in males rodents at PND 70, however females were not examined. We have previously demonstrated that male rats exhibit increased basal hippocampal protein expression of IL-1β in adulthood following neonatal exposure to LPS, with females demonstrating increases in IL-1β only following a second hit of stress (Walker et al., 2010). Here, we demonstrate sustained significant increases in *IL-1β* gene expression in the mPOA and HTH due to a PND 3 and 5 LPS challenge, which was further exaggerated in the mPOA with the addition of adulthood restraint stress. Conversely, a differing pattern of *IL-1β* gene expression was observed in the HC of LPS treated females, where stress induced a significant downregulation of this gene compared to the nLPS/NS group. This may indicate that hippocampal *IL-1β* gene expression is more readily converted to protein following a stressor, whereas hypothalamic IL-1β gene expression may be prolonged and contribute to a more sustained inflammatory and stress response in these areas, potentially affecting behaviours. Additionally, hippocampal inflammation may need to be resolved at a faster rate in order to protect cognitive capacity, considering the known neurodegenerative action of this cytokine (Bland et al., 2010; Patel et al., 2003) and the high density of IL-1β receptors and microglia throughout this area (Farrar et al., 1987; Graeber & Streit, 1990). Interestingly, Jones et al. (2018) recently demonstrated that astrocytes are a predominant source of stress-induced hippocampal IL-1β. Astrocyte signalling has been implicated in stress activation by influencing synaptic activity and brain functioning, and are suggested to participate in anxiety, fear, and coping mechanisms (Lutz et al., 2015). Hence this downregulation of IL-1β seen here in the HC could be an adaptive protective mechanism and future studies are needed to investigate the GFAP and Iba-1 levels within these regions

to determine astrocyte and microglial activation status and the source of IL-1β production which may clarify functional relevance.

Behaviourally, IL-1β centrally modulates the sickness response (Rothwell, 1991) and plays a major role in coordinating the neuroimmunoendocrine response to stress and infection (Goshen & Yirmiya, 2009; Takao et al., 1995). Additionally, IL-1β participates in the regulation of female reproductive behaviours (Gerard et al., 2004; Ye et al., 2016; Yirmiya et al., 1995). Administration of IL-1β is associated with the rapid induction of sickness behaviours in rodents (Dantzer, 2009; Plata-Salaman et al., 1996; Yee & Prendergast, 2010). Yirmiya et al. (1995) established that IL-1β administration in adulthood significantly decreased female sexual behaviours, including proceptive behaviours and lordosis. Furthermore, Yirmiya and colleagues demonstrated that a LPS challenge elicited the same response in females, but neither treatment had an effect on male reproductive behaviour (Avitsur & Yirmiya, 1999b). Hence, the enhanced mRNA expression of IL-1β we observe in the mPOA and HTH of NIA animals may be a key factor participating in the sub-optimal sexual behaviours previously demonstrated in female rodents subjected to NIA (Chapter 3, Walker, et al, 2011). Avitsur and Yirmiya (1999b) suggest this may be in part due to a sickness-induced reduction in sexual motivation, however we have previously demonstrated that NIA females exhibit no differences in motivation in a paced mating paradigm during the receptive phase of their oestrus cycle compared to controls, nor do these rats exhibit motivational deficits in social interaction or sucrose preference testing (Chapter 3). Evolutionarily speaking however, these alterations may be a biologically adaptive strategy to decrease the likelihood of conception during illness to minimise gestational complications (Avitsur et al., 1997; Avitsur et al., 1999; Avitsur & Yirmiya, 1999a, 1999b; Yirmiya, 1996; Yirmiya et al., 1995). Importantly, the current findings suggest that neonatal exposure to infection may have long term consequences for

the perinatal programming of female reproductive behaviours as a result of chronic central inflammation, and these may be mediated specifically by IL-1β pathways and activation.

Importantly for female reproduction, IL-1β has been demonstrated as a potent inhibitor of gonadotropin-releasing hormone (GnRH) in rat models (Kalra et al., 1998; Rivest & Rivier, 1993). It has been demonstrated that IL-1β attenuates luteinizing hormone (LH) levels in female rats (Kalra et al., 1998; Kalra et al., 1990). Furthermore, IL-1β reduces spontaneous expression of immediate early gene c-Fos in GnRH nuclei when administered during the pre-ovulatory gonadotropin surge in female rats (Rivier & Vale, 1990). Female rodents centrally administered with IL-1β display an attenuation of hypothalamic GnRH secretion; with TNFα, but not IL-6, having the same effect (Rivier & Vale, 1990; Watanobe & Hayakawa, 2003). This is pertinent to the current results, where concomitant alterations in *Il-6* or *Il-6R* mRNA expression were not demonstrated. Speculatively speaking, the *Il-1β* increases in the mPOA and hypothalamus observed here may be directly influencing GnRH production to alter the hormonal aspects of female sexual behaviours during proestrus. Previous studies from our laboratory indicate decreased circulating levels of LH and FSH during adolescence, as well as reduced LH during mating in female NIA rats (Sominsky et al., 2012; Walker et al., 2011). However, no differences in oestrus cyclicity have been observed (Chapter 3; Chapter 5; Sominsky et al., 2012; Walker et al., 2011), whereas others have demonstrated oestrus deviations in this model (Iwasa et al., 2009; Wu et al., 2011). As such, perhaps the behavioural and endocrine alterations previously demonstrated may a consequence of chronic, low-grade inflammation in the female rat as a result of NIA.

In the current study, females neonatally challenged with LPS demonstrate significant increases in *TnfαR* expression across all three areas examined, not influenced by a second hit of psychological stress. However, *Tnfα* cytokine levels were not altered in any regions. These

findings are suggestive of a more global pattern of proinflammatory upregulation as a function of NIA in these females, driven by TNFα and IL-1β. TNFα signalling is critical for normal brain operation, and abnormalities in TNFα pathways has been associated with neuronal damage and disease pathogenesis (McCoy & Tansey, 2008). Additionally, TNFα signalling is a critical element mediating ovarian maturation, folliculogenesis, and oocyte apoptosis (Greenfeld et al., 2007; Marcinkiewicz et al., 2002; Terranova, 1997). In a *Tnfα*$^{-/-}$ mouse knockout model, female mice display increases in fertility, a larger follicle pool at birth and hyper-stimulation of ovarian follicle during ovulation (Cui et al., 2011). Interestingly, *TnfRα*$^{-/-}$ mice show an advanced day of vaginal opening, implicating this pathways in precocious pubertal onset (Roby et al., 1999). Hence central TNFα signalling may be potentiating greater systemic inflammation and negatively affecting ovarian function.

TnfαR null adult mice also demonstrate cognitive impairments, but a general decrease in anxiety-like behaviours was observed across numerous behavioural tests (Camara et al., 2013). Furthermore, these knockout mice exhibit reductions in depressive-like behaviours, and significantly lower levels of IL-1β expression in the HC compared to wild type mice, with no sex difference demonstrated in either study (Camara et al., 2015). These findings suggest that higher levels of TNFαR and IL-1β may be associated with a depressive/anxiety-like phenotype, however our female behavioural findings contradict this supposition, given that female NIA animals used for this thesis study do not demonstrate specific anxiogenic or anhedonic behaviours (Chapter 3; Walker et al., 2011). Bernardi et al. (2014) demonstrated corroborative results, with female animals exhibiting no differences in anxiety-like behaviours following NIA. As such, further examination of the behavioural phenotype associated with NIA in females is needed, however the results reported here and previously (Chapter 3) suggest that perhaps the effect of NIA may be context-specific to female mating behaviours. This is in

line with evidence suggesting the sexually dimorphic effects of both neonatal and adult LPS-stress in animal models (Bernardi et al., 2014; Cai et al., 2016; Curtis et al., 2006; Foley et al., 2014; Kokras et al., 2012; Spencer et al., 2006a; Tenk et al., 2007; Tenk et al., 2008).

It is established that glucocorticoids (GCs) exert an anti-inflammatory effect, which has a dampening effect on cytokine expression (Coutinho & Chapman, 2011). Conversely, It has also been demonstrated that cytokine receptor expression can be enhanced by GCs (Almawi et al., 1996; Wiegers & Reul, 1998b) and this enhancement correlates with an enhanced cytokine induction, potentiating effects (Wiegers & Reul, 1998a). This process may be contributing to the increased *TnfαR* signalling demonstrated in nLPS animals, particularly considering that nSAL/aST females are demonstrating a similar increase in hypothalamic *TnfαR* receptor levels. However, IL-6 receptor has been shown to increase following synthetic GC stimulation, which is not observed in the current study (Campos et al., 1993; Snyers et al., 1990).

NIA treatment increased *MR* gene expression in the HTH and *GCR* gene expression in the HC. Interestingly, nSAL animals displayed the greatest increase in hypothalamic *GCR* mRNA expression with the addition of stress. This may indicate a blunted stress response from LPS treated animals, in line with previous male data and suggestive of a post-traumatic-stress-disorder profile (Walker et al., 2009). The increase in *MR* demonstrated in the hypothalamus of the NIA treated female is interesting. Although no differences in this receptor were demonstrated in the hippocampus where dense populations of MR are known to exist, it may indicate alterations to the basal activity of the HPA axis (Berardelli et al., 2013). This supposition is corroborated by the findings in chapter 3 demonstrating generally increased CORT levels in NIA treated females. Furthermore, it has been suggested that MR signalling promotes neural integrity and regulates apoptotic GR signalling, therefore, increases

demonstrated in nLPS/aST animals may be a protective effect, promoting resilience (ter Heegde et al., 2015). Surprisingly, no differences were observed in hypothalamic *CRH*, *CRHR1,* or *FSHR* expression. These findings may point towards an immune-mediated behaviour effect in LPS treated female rats, where the *GCR* and *MR* alterations are perpetuated by inflammation, however further investigation is needed.

In regards to female reproductive development, both central and peripheral cytokine concentrations are suggested to play a vital role during puberty, including participating in the regulation body composition, bone growth and pubertal onset (Casazza et al., 2010). In a human study, Yu et al. (2014) demonstrated that precocious puberty onset in female children was associated with increased IL-1β and IL-6 levels as peak luteinizing hormone levels increased. Riis et al. (2014) demonstrated in a longitudinal study that younger girls (~11 years of age) had higher levels of proinflammatory cytokines, including IL-1β, levels which decreased with pubertal maturation (~17 years of age). Additionally, Ojeda et al. (2010) posits that astrocyte and glial activation, particularly in the HTH and in proximity of GnRH neurons, may also be contributing to female sexual precocity by participating in the control of GnRH secretion. Glial cells are known to mediate the central induction of IL-1β and TNFα (Srinivasan et al., 2004; Thornton et al., 2006). Hence, the increased basal levels of *IL-1β*, *TNFαR* and *Cox2* levels seen here in NIA treated females may be contributing to the advanced puberty onset we have previously demonstrated in this thesis (Chapter 3 and 5), as well as previously (Sominsky et al., 2012a; Walker et al., 2011). This is substantiated by others demonstrating that differential neonatal stressors, such as maternal separations, which are known to alter endocrine functioning, do not alter puberty onset (Rhees et al., 2001). Future studies could address this by examining the central expression of IL-1β at earlier developmental stages in the female rat with and without NIA, including at pre-puberty and day of vaginal opening.

Unexpectedly, no statistically significant difference in *Kiss1* or *Kiss1R* gene expression was demonstrated in the mPOA or HTH in NIA female rats, however a significant increase in Kiss1R was demonstrated in the HC of nLPS females. KISS1 and KISS1R has been localised in hypothalamic regions known to control gonadotropic secretion, including the mPOA, the arcuate nucleus, and the periventricular nucleus (Gottsch et al., 2004; Kinsey-Jones et al., 2009), as well as in the dentate gyrus (DG) of the HC (Arai, 2009). Knox et al. (2009) using the same model of NIA as the current study, demonstrated a significant decrease in *Kiss1* mRNA expression in the mPOA in prepubertal and adult female rats neonatally treated with LPS. Here, *Kiss1* gene expression in the mPOA follows the opposite pattern of expression, however is quite variable in LPS treated females and hence non-significant. Knox et al. (2009) also demonstrated a significant upregulation of *Kiss1R* mRNA expression in the mPOA of adult females who had been neonatally treated with LPS, a trend that was also observed in this study. Additionally, in the current study, *Kiss1* in the HTH and *Kiss1R* in the HC are slightly downregulated in LPS females with the addition of an adulthood stressor. A similar pattern of suppression was demonstrated by Kinsey-Jones et al. (2009), where restraint stress lead to a significant decrease in *Kiss1* mRNA in the mPOA. Further examining of KISS1 and KISS1R is needed within this model.

In the HC, a high density of KISS1R has been located in the granule cells of the DG, where *Kiss1* is suggested to act in an autocrine manner (Arai & Orwig, 2008). *Kiss1R* levels were significantly increased in the HC of NIA treated females in the current study. It has been suggested that the role and mode of action of KISS1 in the HC differs from that of the HTH, as it acts post-synaptically and does not affect membrane properties with activation (Arai, 2009; Arai & Orwig, 2008). In rats, the DG is implicated in anxiety-based behaviours, as well as the consolidation of spatial and odour information processing, social recognition, and reward

value decision making (Kesner, 2017; Weeden et al., 2014; Weeden et al., 2015). Patro et al. (2013) demonstrated that PND 3 and 5 LPS exposure prolonged microglial activation in the DG up to PND 30. Additionally, Schwarz and Bilbo (2011) demonstrated neonatal LPS exposure led to broad and robust inflammation in the hippocampal region of the neonatal rat, including upregulation of IL-1β, IL-6 and TNFα family members. These studies indicate that region-specific programming effects may be occurring, which may explain differences in activation patterns between the hippocampal and hypothalamic regions in the current study. Little is known regarding the exact role of KISS1 and its receptors in the HC, however KISS1 neurons have been located in the amygdala where they are thought to play a role in integrating social and olfactory information to coordinate appropriate behavioural output (Pineda et al., 2017). Considering the multitude of reciprocal pathways between the HC and the amygdala, as well as the limbic interconnectedness (Pitkanen et al., 2000), hippocampal *Kiss1R* expression and *Kiss1* activity may have similar roles.

In addition to female mating deficits, we previously demonstrated that adult female rats treated with neonatal LPS exhibited less facial/nose-to-nose sniffing behaviours during social interaction, a behaviour which is associated with the conveyance of social status and food preference in rats (Chapter 3). Interesting, higher *Kiss1R* expression was found to be associated with social status in a territorial fish model (Grone et al., 2010). Perhaps the hippocampal KISS1 system may be involved in modifying olfactory communication behaviour in the rat, considering its role in olfaction and the importance of this type of communication for reproduction and survival in the rat. A recent rat study by Doenni et al. (2017) indicated that NIA with LPS increases c-Fos expression in limbic circuitry that was particularly robust in female animals. Emerging evidence suggests that KISS1 and KISS1R is involved in sexual behaviours and reproductive processes, hence the KISS1 system should be a prospective

candidate for further investigation within limbic structures utilising the current model of NIA, considering its sensitivity to the effects of neonatal immune stress.

Neonatal LPS exposure significantly upregulated *Cox2* mRNA levels in the hippocampus and hypothalamus of NIA animals. COX2, the rate limiting enzyme that converts arachidonic acid (AA) to prostaglandin E_2 (PGE_2), is usually expressed at low basal levels (Eliopoulos et al., 2002), with activation mediating the effects of LPS exposure via TLR4 to moderate PG and central fever induction (Spencer et al., 2011). Cai et al. (2013) demonstrated that central COX2 expression was predominantly microglia-derived via MAPK pathways. In the current study, stress induced *Tlr4* increases were observed in the HTH, however these were not accompanied by *Makpk8/Jnk1* pathway regulation in any group. Previous studies have demonstrated increased COX2 expression following NIA in males but not females, or have observed attenuated level in females following a subsequent adult immune stressor (Kentner et al., 2010; Spencer et al., 2006a). Thus, the results demonstrated in the current study are amongst the first to demonstrate long-term alterations to *Cox2* mRNA expression in female rats following NIA. Avitsur et al. (1999) demonstrated that PG synthesis may mediate the IL-1 suppression of female sexual behaviours when overexpressed the HTH, hence these findings have implications for the sexual behavioural findings presented in chapter 3.

Prostaglandin synthesis is vital for female reproductive parameters both centrally and peripherally. $Cox2^{-/-}$ transgenic female mice demonstrate defective ovulation, abnormal ovarian follicular development, and infertility (Lim et al., 1997). Ojeda and Campbell (1982) demonstrated that prostaglandin synthesis within the hypothalamus is essential for the initial pre-ovulatory surge of GnRH during puberty, as chemical enhancement of PGE_2 synthesis in an animal model induced female puberty onset. The current study suggest that dysregulation

of the *Cox2* pathway as a result of NIA, particularly in the mPOA and HTH, may be a factor contributing to the precocious puberty onset demonstrated in our LPS treated females. As such, both COX2 and PGE_2 should be measured during puberty in the current model. The inhibition of COX2 has been demonstrated to decrease ovarian hyper-stimulation syndrome in a rat model, decrease inflammatory expression and lesions in patients with endometriosis, and reduce anxiety-like and depressive-like behaviour in rats (Ebert et al., 2005; Hermanson et al., 2013; Quintana et al., 2008). Further research may examine the specific inhibition of COX2 synthesis at number time points within this model, including in the prepubertal period prior to vaginal opening and prior to sexual behavioural testing, in order to ascertain the contribution of COX2 to the female behavioural and inflammatory phenotype current demonstrated.

Interestingly, in the current study female rats exposed to neonatal LPS exhibit significantly increased TH mRNA expression in the mPOA, suggesting upregulated catecholaminergic activity in this central area that is critical to female reproductive behaviours and processes (Damanhuri et al., 2012; Ong et al., 2014). Previous research from this laboratory has demonstrated peripheral increases in TH phosphorylation in the adrenals of PND 5 neonatal females, 4 hours following LPS exposure, yet long-term nor central expression was not measured (Sominsky et al., 2012a). Hence, the current TH findings are novel to the NIA model and are of great interest. It has been demonstrated that TH immunoreactivity cells are present in the mPOA and HTH in the female rat brain (Lonstein & Blaustein, 2004). Interestingly, we observed here no alterations in TH expression in nSAL animals. However, nLPS treated females demonstrated a reduction of TH in the mPOA to baseline levels, 24 hours following restraint. Gavrilovic et al. (2008) demonstrated that psychosocial stress lead to a significant decrease in TH mRNA expression, however protein

levels of TH were significantly increased, suggesting that transcription and translational modifications have taken place. This may explain the pattern of TH expression in nLPS/aST animals demonstrated here, and this postulation should be further confirmed by protein expression analysis. The findings presented here and previous studies from our laboratory suggest the involvement of the catecholaminergic system in the perinatal programming of female behaviour. Catecholamines are known to play an important role in ovarian process, including the direct sympathetic innervation of the ovary (Cruz et al., 2017; Gerendai et al., 2000), as well as partially govern female reproductive behaviours (Lorenz et al., 2015; Meston, 2000). Future research should aim to establish the extent to which these alterations stemming from NIA affect both male and female cohorts.

Although the exact mechanisms through which early life bacterial exposure modulates female reproductive behaviours, puberty onset and ovarian function remain to be explored, the data presented here suggests that the perinatal programming of immune dysregulation and chronic inflammation may contribute to the alterations in reproductive development, function and behaviours previously reported. This dysregulation has the potential to decrease fertility levels and reduce the female reproductive life span. Alterations to the immune milieu during critical periods of plasticity have important consequences for long-term central immune functioning and associated endocrine networks. The current study demonstrates novel findings regarding the basal and stress-potentiated central immune and stress status induced by NIA in a female rat, and aims to broaden the understanding of the central mechanisms involved in the phenotypic alterations observed in the NIA model that are specific to the female. Sexually dimorphic outcomes are known to arise from neonatal immune activation, and our results indicate that many parameters involved in the governance of inflammation, stress, and reproduction are altered as a result of a neonatal immune

challenge in the female rat. This is of particular importance given the critical links between proinflammation, reproductive dysfunction, psychopathology and disease. Thus, there is a need to understand the factors that are contributing to the female subfertility phenotype arising from early life immune stress, particularly given the increase in occurrence of idiopathic reproductive disorders and subfertility in increasingly younger women (Hernández-Angeles & Castelo-Branco, 2016; Kamath & Bhattacharya, 2012; Norman & Moran, 2015; Sominsky et al., 2015), and the increases in postnatal bacterial infections (Khan et al., 2017; Koumans et al., 2012; Lamagni et al., 2017; Lanari et al., 2007).

Chapter 7. Future Directions: The Kynurenine Pathway and the Catecholaminergic System.

7.1 Introduction

Complete elucidation of the mechanisms contributing to female reproductive dysfunction and subfertility remains to be established. This is in part due to the reciprocal nature of interconnected biological systems governing reproduction, and the pleiotropic and multifunctional nature of their mediators. The immune and endocrine systems are known to govern female reproduction and be sensitive to the effects of environmental stimuli during critical periods of development. Thus, these regulatory systems are often the focus of literature regarding the *perinatal programming* of female fertility outcomes. However this is an emerging topic of interest, and studies are beginning to examine the role of other systems involved in the early life determinants of female reproductive health and longevity. The previous studies throughout this thesis focus on immunoendocrine alterations arising from neonatal immune activation (NIA) in the female rat, and how this affects the immune milieu, establishment of the ovarian reserve, reproductive behaviours, and stress vulnerability. However, other pathways and mediators that are known to be sensitive to environmental influence and implicated in female reproduction should be examined in order to increase understanding of the impact of early life bacterial exposure in the female rat and its consequences for fertility.

The basic premise of this thesis has been that inflammation induced by LPS plays a critical role in altering female reproductive parameters. This chapter focuses on two separate pathways known to modulate inflammation and stress, including immune-mediated stress. The kynurenine pathway (KP), and the catecholaminergic system are known to be sensitive to environmental stimuli and contribute to the pathogenesis of disease and behavioural alterations. Importantly, both of these systems may influence female reproductive fitness as

they have been demonstrated to alter behaviour and neuroimmunoendocrine parameters in the presence of early life immune stress. Hence these pathways may contribute to both the behavioural alterations demonstrated in this model in both males and female rats, as well as contribute to functional alterations to biological systems, including the ovary, the immune system and the hypothalamic-pituitary-adrenal/gonadal (HPA/HPG) axes. The studies that are presented in this chapter use the current NIA model of bacterial exposure, however unlike previous chapters, utilises male rats. This is in order to develop, optimise, and standardise these methods for translation into experiments with female animal subjects to explore the subfertility phenotype.

The first study presented here will focus on the acute activation of the Kynurenine pathway in neonatal male rats immediately following post-natal day (PND) 3 and 5 LPS exposure. The functioning of this pathway, how it may be altered by early life immune stress, and its potential contribution to the perinatal programming of female biological and behavioural reproductive parameters is discussed. The second study presented in this chapter is the 3rd paper included in this thesis, demonstrating the long-term alterations in central catecholaminergic stimulatory pathways in male rats who were exposed to neonatal LPS. The perinatal programming of the catecholaminergic pathway is discussed, with a focus on its impact on female reproductive parameters. The implications for assessment of these pathways in the female rodent using the current model of NIA is discussed. The exploration of novel mechanisms within the LPS model of NIA in male animals aims to provide a platform to facilitate further research in this area and justify the examination of these mediators within a female animal model of early life immune activation.

7.2 Perinatal programming of the kynurenine pathway: Potential role in female NIA induced subfertility.

The Kynurenine pathway (KP) of tryptophan (Trp) metabolism and its associated metabolites are regulated by endocrine and immune activity. Of significance, the KP also has the ability to modify the functioning of these systems and therefore alter behaviours (Miura et al., 2008). Changes in the metabolism of Trp in the KP play an important role in neuro-endocrine-immune system communication. Indoleamine-pyrrole 2, 3-dioxygenase (IDO) is an intracellular enzyme induced by inflammatory mediators that initiates the rate-limiting first step of Trp metabolism along the KP, and contributes to numerous fundamental biological processes both centrally and peripherally (see Figure 7.1) (Wang et al., 2015). Tryptophan dioxygenase (TDO) is also a Trp metabolite, however induction of this enzyme is controlled via glucocorticoids (GCs), with expression abundant in the liver (Fatokun et al., 2013). IDO has been located in the spleen, lung, liver, kidney, thymus, skin, digestive tract and brain (Dai & Zhu, 2010) as well as throughout the female reproductive tract including the ovary and placenta (Ball et al., 2009; Sedlmayr et al., 2002) where it is secreted by microglia, astrocytes, macrophages, monocytes and dendritic cells depending on tissue specificity (Van der Leek et al., 2017).

Functionally, IDO plays a role in depleting Trp in enclosed, cellular microenvironments to prevent infection (both bacterial and viral), as well as contributing to the co-regulation of innate immune defence and control (King & Thomas, 2007). IDO is induced in response to inflammatory stimuli, and has been posited to play a role in pathology and psychopathology onset, including inflammatory disorders, psychiatric disorders, and cancers (Fatokun et al., 2013; Yeung et al., 2015). The principle inducer of IDO and Trp breakdown is proinflammatory cytokine Type II interferon (IFNγ) secreted by T cells, however IFN-β/α and other cytokines

including interleukin (IL)-6, IL-1 and tumour necrosis factor (TNF)α, as well as LPS exposure, have been demonstrated to induce and modulate IDO expression dependent on cell type and immune milieu (Campbell et al., 2014; King & Thomas, 2007; Miura et al., 2008; Wang et al., 2010a; Wirthgen et al., 2014; Yoshida & Hayaishi, 1978). Following peripheral inflammation, for example with LPS, IDO expression is significantly increased (Macchiarulo et al., 2009; Moreau et al., 2008), with peripheral kynurenine actively transported into the brain (Fukui et al., 1991). Neuro-inflammation however, leads to direct central kynurenine upregulation controlled by local enzyme activity (Kita et al., 2002). Central expression of IDO and downstream IDO activation products have been demonstrated to play dual roles contingent on metabolite pathway, with either neurotoxic or neuroprotective effects dependent on microglial (neurotoxic) or astrocyte (neuroprotective) facilitated metabolism, with TDO playing a lesser role in the brain (see Figure 7.1) (Campbell et al., 2014; Müller et al., 2009).

Inflammatory mediators play a critical role in the onset and perpetuation of disease and psychopathology. Chronic proinflammation and inflammation caused by viral or bacterial exposure promotes KP and IDO activation and potentiation, and thus may contribute to the negative effects of chronic Kyn upregulation and disease states. Recently, dysregulation and upregulation of the KP has been implicated in inflammatory and autoimmune disorders (Fatokun et al., 2013; Yeung et al., 2015), cancer (Routy et al., 2016), cardiovascular disease (Song et al., 2017), depression and anxiety (Dantzer, 2017; Dantzer et al., 2011; Salazar et al., 2012; Yong-Ku & Sang Won, 2017), and neurodegenerative and neurodevelopmental disorders (Erhardt et al., 2017; Lim et al., 2016; Lovelace et al., 2017; O'Farrell & Harkin, 2017). The breakdown of Trp is preferentially (>95%) channelled towards production of quinolinic acid (see Figure 7.1), branching towards the neurotoxic arm/N-methyl-D-aspartate (NMDA) agonistic of the KP (Bender & McCreanor, 1982), with accelerated Trp degradation and

upregulation of Kyn (aberrant Trp/Kyn ratio) associated with affective disorders and infectious diseases (Chen & Guillemin, 2009). Both IDO and TDO expression is relatively minimal in normal, non-pathological conditions, with TDO stability maintained by Trp expression (Won & Kim, 2016).

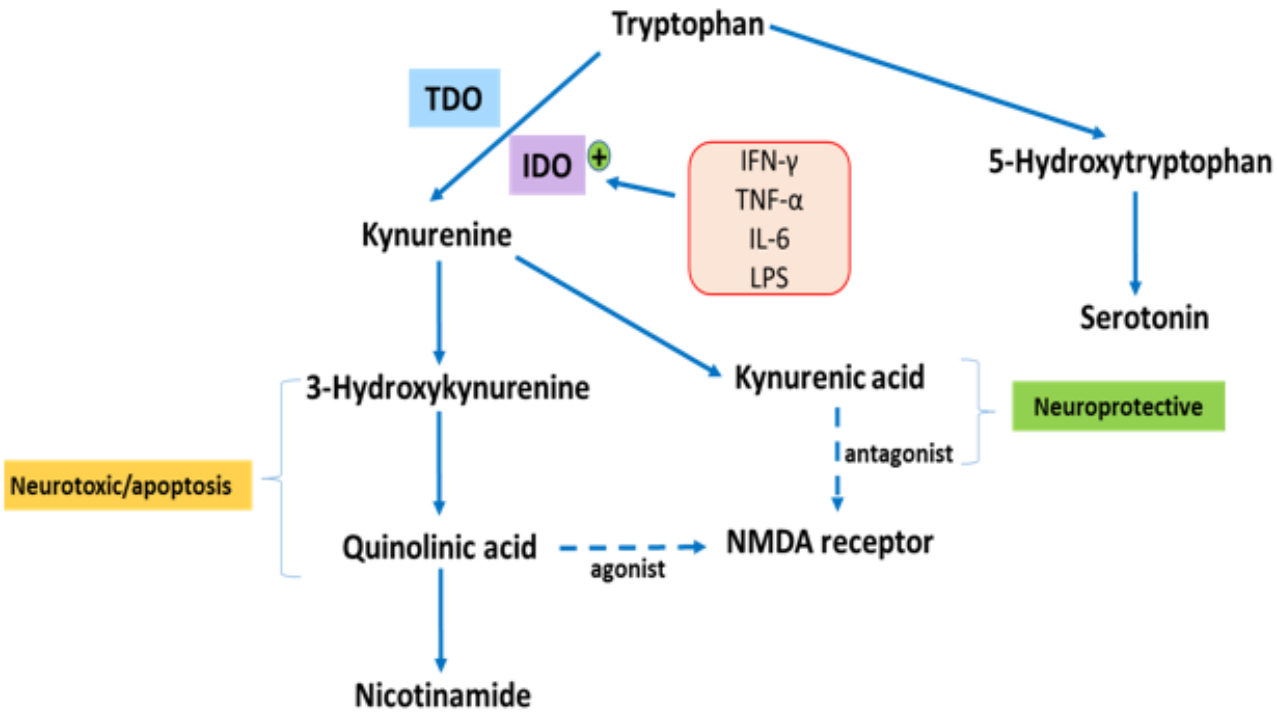

Figure 7.1 Simplified schematic of the kynurenine metabolic pathway. Metabolism of Trp occurs via the KP, with only a small portion of Trp designated to the synthesis of serotonin (5-HT) via 5-hydroxytryptophan (TPH). Trp is metabolised via stress-induced TDO, or via induction of immune-induced IDO. Kynurenine is degraded into both 3-hydroxykynurenine (3-HK) and quinolinic acid (QA), or kynurenic acid (KA), forming neurotoxic or neuroprotective catabolic branches. The metabolite of Trp, kynurenine, is readily transported across the blood-brain barrier via L-type amino acid transporter 1 (LAT-1) for degradation by central immune mediators including microglia (QA producing) and astrocytes (KA producing), with these products playing either an antagonist or agonist role on NMDA receptors. Due to this activity, the KP is implicated in inflammatory-associated psychopathologies and physiological pathologies (Maes et al., 2011; Plitman et al., 2017; Wichers & Maes, 2004). Schematic modified from Dantzer et al. (2008) and Müller et al. (2009).

Lipopolysaccharide exposure has been demonstrated to upregulate the KP centrally and peripherally following systemic administration (Connor et al., 2008). Although IDO activation is known to be regulated by IFN-γ, Wang et al. (2010b) demonstrated that LPS induced IDO expression via the MAPK/JNK pathway, as the inhibition of the LPS-induced MAPK/JNK pathway abrogated IDO expression in murine microglial cells. Additionally, TNF-α, IL-6 and IL-1 have been demonstrated to mediate IDO stimulation following LPS exposure (Connor et al., 2008; Fujigaki et al., 2006; Robinson et al., 2006). Importantly, we have previously demonstrated significant increases and alterations of these known cytokine mediators of IDO both centrally and peripherally in the female rat, following NIA with LPS (Chapter 5; Chapter 6; Fuller et al., 2017 [Chapter 4]). These known activation pathways have implications for the long-term activation and perhaps dysregulation of the KP and IDO expression, stemming from neonatal immune perturbation with LPS in both male and female rodents. Additionally, previous studies from our laboratory have demonstrated alteration to the HPA axis response in both male and female rats following neonatal LPS exposure (Walker et al., 2012; Walker et al., 2011; Walker et al., 2009; Walker et al., 2004a), which may be involved in TDO induction and Kyn upregulation via GC release. Due to this, examination of this pathway within our model is of particular interest and significance.

Emerging evidence examining this novel pathway demonstrates that KP dysregulation, particularly related to inflammatory IDO induction, is associated with immune-mediated behavioural alterations (Campbell et al., 2014; Dantzer, 2017; O'Farrell & Harkin, 2017; Wichers & Maes, 2004; Won & Kim, 2016; Yeung et al., 2015). Studies in healthy adult patients indicate that stimulation with IFN, the known inducer of IDO, increases cytokine and Kyn production and induces depressive symptoms (Eisenberger et al., 2010). Furthermore, higher plasma Trp/Kyn levels and ratios have been associated with patients experiencing major

depressive disorder (MDD), with these levels correlating to severity of behavioural symptomology (Gabbay et al., 2012; Miura et al., 2008). In systemic animal models of immune activation with a viral or bacterial mimetic, IDO-inducing cytokines have been demonstrated to be significantly centrally upregulated, along with increases in IDO expression, Trp, and 5-HT, with concomitant anxiety-and-depressive-like behaviours persisting up to 72 hours post exposure (Gibney et al., 2013; Moreau et al., 2008; O'Connor et al., 2008).

Alternate directions of KP dysfunction have been proposed for the behavioural and psychological alterations demonstrated in both human and animal models. Firstly, increases in IDO and TDO via inflammation or stress promote Trp breakdown, therefore depriving the 5-HT producing arm of the KP (Christmas et al., 2011; van Donkelaar et al., 2011). Secondly, the neuro-activity of Kyn and hence behavioural/physiological outcome is dependent on metabolite direction (neurotoxic versus neuroprotective) (Van Gool et al., 2008; Wichers et al., 2005). Literature concerning the perinatal programming of the KP is limited, however, there is evidence to suggest that this system may be skewed by early life environmental challenges. The KP is regulated by immune activation and stress activation, including central astrocytes and microglia, peripheral immune activation, and GCs; which are known to be vulnerable to early life immune stress (Galic et al., 2009; Kita et al., 2002; Riazi et al., 2008; Spencer et al., 2011). Studies in adult humans and in experimental rodent models indicates that inflammation induces KP activation and its downstream metabolites are responsible for altered behavioural and immune outcomes (Christmas et al., 2011; Dantzer et al., 2011; Liu et al., 2014; O'Connor et al., 2009). Additionally, the KP is regulated by proinflammatory cytokines, upregulation of which is strongly linked to early life immune challenges (Chapter 5; Chapter 6;Ortega et al., 2011; Spencer et al., 2006; Walker et al., 2010).

Kynurenine pathway metabolites have an essential role in neurodevelopment (Notarangelo & Pocivavsek, 2017). Higher central levels have been demonstrated during prenatal development in animal models, which decreases throughout the perinatal period and lower levels still throughout adolescence and adulthood (Ceresoli-Borroni et al., 2007; Walker et al., 1999). The limited information available regarding the perinatal programming of this pathway indicates that it is sensitive to early life stressors, however long-term repercussions have not been fully examined. Maternal stress has recently been demonstrated to impact on KP metabolites. In rats, maternal restraint stress in the late gestational period increased Trp, Kyn and kynurenic acid (KA) levels in maternal plasma and the foetal brain, as well increased GC mediated TDO activity in the maternal liver (Notarangelo & Schwarcz, 2016). In a recent study using a rabbit model of maternal inflammation, endotoxin administration significantly upregulated placental and foetal brain levels of IDO and decreased 5-HT precursor 5-hydroxyindole acetic acid (Williams et al., 2017). Mice exposed to systemic *neurotropic influenza A virus* in the early neonatal period (PND 3 or PND 4) demonstrated central upregulation of IDO, but not TDO, as well as elevated metabolites following viral clearance on PND 7 - 13 (Holtze et al., 2008). Furthermore, Zavitsanou et al. (2014) demonstrated in male rats that prenatal exposure to Poly I:C in a maternal immune activation (MIA) model increased KP metabolites in the preadolescent offspring, skewing the Trp/Kyn ratio in the neurotoxic direction.

Regardless of this evidence and to our knowledge, the direct implication of NIA with LPS on both acute and sustained Trp/Kyn dysfunction remains to be examined. The majority of research in this area is in regard to neurodevelopmental disorders such as schizophrenia and autism due to the involvement of glutamatergic dysregulation demonstrated in these disorders (Javitt, 2010) and the direct influence the KP has on this receptor (Stone et al., 2013).

However, as glutamatergic signalling is an important aspect governing female reproduction in both the ovary and the brain (Christensen et al., 2012; Gill et al., 2008), this also has implications for the programming of female fertility. As LPS and cytokine products are known inducers of the immune-regulated IDO pathway, the perturbation of this system during critical periods of development where Kyn expression is naturally increased has the potential for long-term detrimental consequences. As such, the KP and associated IDO/TDO mechanisms offer a potential and highly valid pathway for further analysis in a model of early life immune activation using LPS, a known stimulator of the KP and IDO. Increases in production of Kyn via either GC-mediated TDO or inflammatory-mediated control of IDO during conditions of inflammation and stress may be occurring, and contributing to maladaptive behavioural alterations.

The current study aimed to examine the immediate peripheral and central inflammatory status of neonatal male rats following NIA with LPS and determine the associated acute activation state of IDO and TDO. We propose that neonatal LPS may induce IDO and TDO via both immune and HPA axis mechanisms. Therefore, this study aims to establish if the Trp/Kyn pathway and its metabolites are activated in early life with exposure to NIA, as a first step in establishing if this mechanism may be perinatally programmed via a neonatal immune challenge, a thus provide a platform for further examination in female animals.

7.3 Methods

7.3.1 Animals and neonatal treatment.

Animals were housed and bred as previously described (Chapter 2). For this initial study, male animals only were utilised and were derived from 5 litters (2 x saline treated; 3 x LPS treated) resulting in 10 animals per treatment group. Whole litters were exposed to either

LPS injection (0.05mg/kg LPS, *Salmonella enterica, serotype Enteritidis*; Sigma-Aldrich Chemical Co., USA in sterile pyrogen-free saline) or equivolume saline (Livingstone International, Australia) on PND 3 and again on PND 5 via intraperitoneal injection (IP). Animals were then returned to their home cage and left undisturbed until tissue collection 6 hours following PND 5 injections.

7.3.2 Tissue collection

PND 5 animals were euthanised via IP microinjection of pentobarbital. On confirmation of euthanasia, transcardial perfusion was performed using ~100mL chilled, sterile phosphate buffered saline (PBS) delivered via a 26 gauge needle. After blood was visibly cleared from tissue, the spleen, liver and brain were excised, rinsed in molecular grade PBS and snap frozen in dry ice. Tissue was stored at -80°C until assessment.

7.3.3 RNA extraction, reverse transcription and qRT-PCR.

Total ribonucleic acid (RNA) extraction was performed on thawed spleen, liver and brain tissue. Tissue was homogenised using a TissueLyzer® (Qiagen, Netherlands; 4 min at 20 Hz) in 1 mL TRIzol reagent (Life Technologies, Carlsbad, CA) using 5 mm diameter stainless steel beads (Qiagen, Netherlands) prior to being prepared for RNA extraction and PCR. RNA was extracted from homogenized tissue using an RNeasy mini kit (Qiagen) with added DNase treatment (Invitrogen, CA, USA) protocol in accordance with manufactures instructions. For brain tissue, analyses were conducted on entire half brains cut down the midline in order to include a representation of all brain regions. Nucleic acid purity and concentration was assessed in a 1µl volume by NanoDrop™ Spectrophotometer 2000c (Thermo Fisher Scientific, DE USA). All reverse transcription reactions were performed in a PTC Programmable Thermal Controller (MJ Res, Fitchburg, MA), using an Ambion PureLink RNA Mini reverse transcriptase kit (Life Technologies; catalogue number: 12183018A),

according to manufacturer's instructions, with random decamer primers for each reaction (as per Walker et al., 2013). Real-time (RT)-PCR was carried out on an Applied Biosystems Prism7900 using TaqMan gene expression assays (Thermo Fisher Scientific; see Table 7.1) for *IL-1ϐ, IL-6, IL-10, TNF-α,TDO, IDO, mineralocorticoid receptor (MR)*, *glucocorticoid receptor (GR)*, and *glial fibrillary acidic protein (GFAP)*, depending on tissue specificity. Reactions were performed in triplicate accompanied by a RT- control, with a minimum of six biological replicates. Relative quantitative measurement of target gene levels was performed using the ΔΔCt method, where Ct is the threshold concentration normalised to the control group. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous housekeeping control.

Table 7.1 Real-time PCR gene targets and gene assay IDs.

Target	TaqMan Assay ID
Il-1β	Rn00580432_m1
IL-6	Rn01410330_m1
IL-10	Rn01483988_g1
TNF-α	Rn01525859_g1
TDO	Rn00574499_m1
IDO	Rn01482210_m1
MR	Rn00565562_m1
GR	Rn00561369_m1
GFAP	Rn01253033_m1

7.3.4 Data Analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Version 22 (SPSS Inc.) using a series of independent sample t-tests. Outliers present in the data that were more than ± two standard deviations away from the group mean for that particular measure were removed from all analyses. All assumptions were tested and, if occurring, violations reported.

7.4 Results

7.4.1 Neonatal weight

No significant differences existed in weights between treatment groups on PND 5 (t(1.944) = 18, p = .069) (see Figure 7.2).

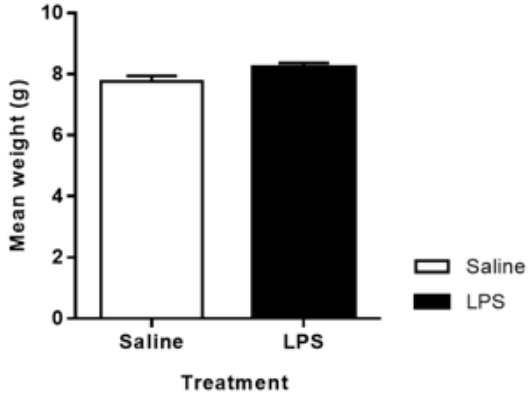

Figure 7.2. Mean weights for neonates on PND 5. Hollow bars = saline, filled bars = LPS, mean + SEM graphed, *= p < .05.

7.4.2 Peripheral tissue examination

In the spleen, LPS exposure significantly upregulated *IL-1β* mRNA expression in the 6 hours following last injection (t(12) = 8.66, p = .01) (Figure 7.3, A). No significant differences existed for *IL-6*, however there was a trend for upregulated *IL-10* mRNA expression (t(12) = 4.04, p = .067) (Figure 7.3, C). In the liver, LPS significantly upregulated *IL-1β* (t(12 = 6.24, p = .03) (Figure 7.3, D). A trend existed for higher *IL-6* levels in the liver of LPS treated animals (t(11) = 4.19, p = .07) (Figure 7.3, E). No significant differences existed in *IL-10* or *TNF-α* mRNA levels, regardless of observed upregulation. Additionally, expression of *TDO* in the liver was significantly downregulated in LPS treated animals (t(11) = 7.31, p = .02), compared to controls (Figure 7.3, H). *IDO* levels were activated in the liver, although non-significantly, in response

to LPS treatment only (mean CT = 36.06). *IDO* levels were undetectable in saline treated controls (see Figure 7.3, A – H).

7.4.3 Central tissue examination

Following LPS stimulation, central levels of *IL-1β* mRNA were significantly upregulated in LPS treated animals ($t(12) = 5.28$, $p = .04$) (figure 4 A). There were no significant alterations in *IL-6, TNF-α, IL-10, MR, GR, TDO* or *IDO* mRNA levels demonstrated at this time. A trend existed for increased *GFAP* levels in LPS treated rats ($t(3.80)$, $p = .075$) (figure 4 D), however this was non-significant (see figure 7.4, A – I).

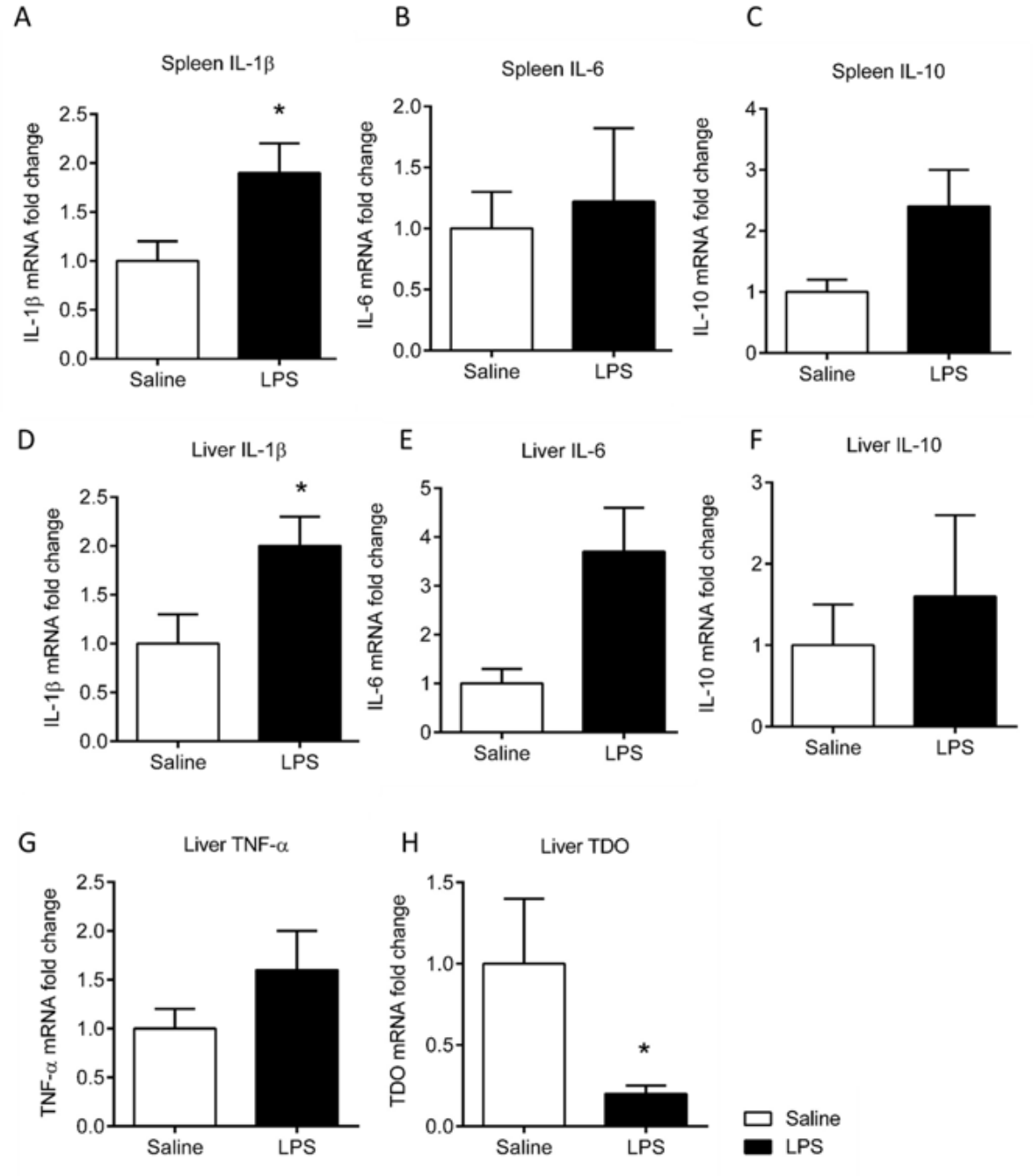

Figure 7.3 Normalised fold change mRNA expression examining the induction of a systemic proinflammatory response of cytokines and KP enzymes 6 hours following LPS exposure neonates. A – C; pro and anti-inflammatory mediators in spleen tissue. D – H; pro and anti-inflammatory mediators in the spleen. * represents significance of $p \leq .05$

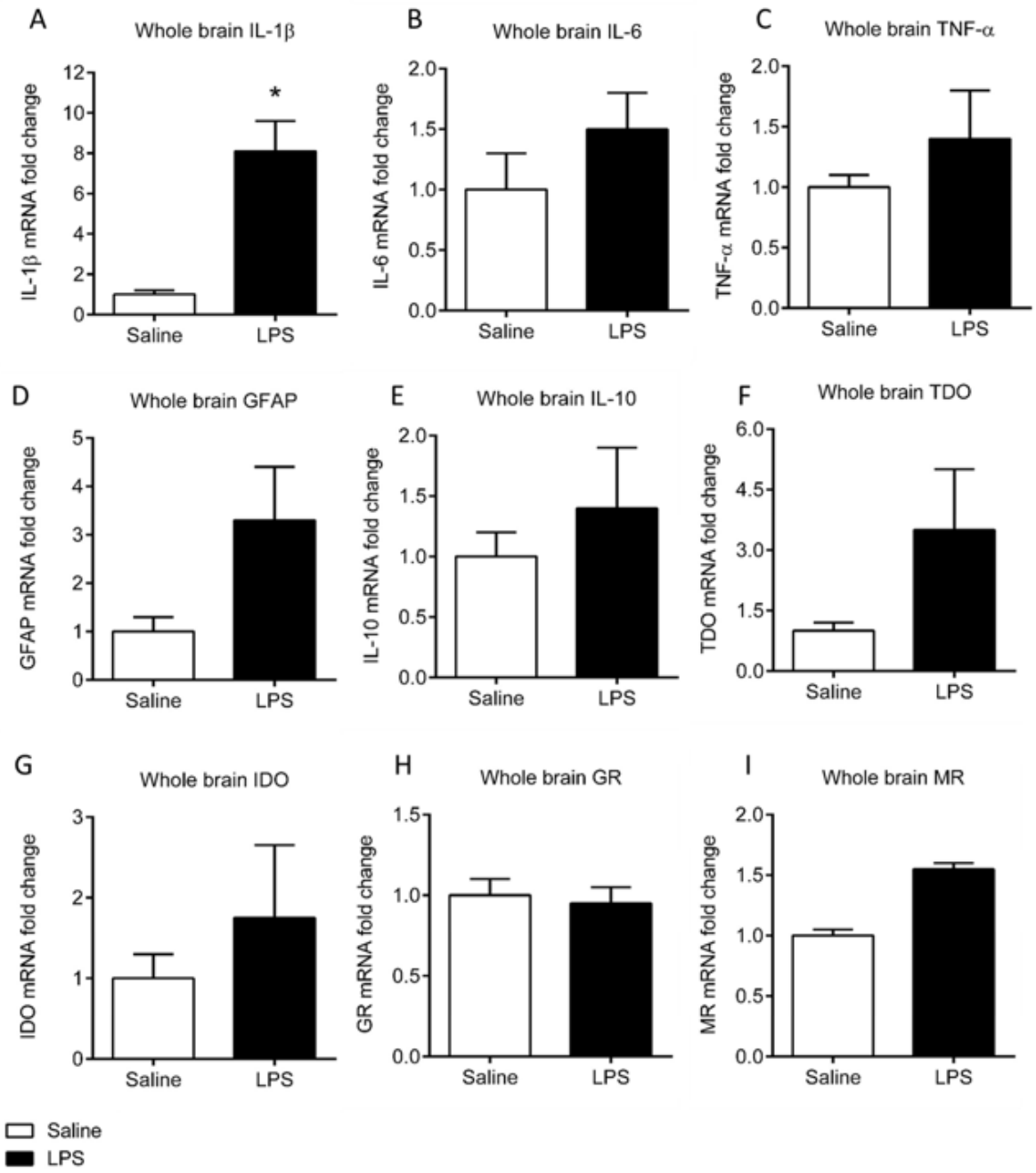

Figure 7.4 Normalised fold change RT-PCR data examining the induction of central proinflammation, KP enzymes, and HPA-axis activation 6 hours following LPS exposure in PND 5 neonates. A – D; pro and anti-inflammatory central mediators including cytokines and microglial activation. F –G; Kynurenine pathway enzymes TDO and IDO. G – H; HPA-axis glucocorticoid and mineralocorticoid receptor expression. * represents significance of $p \leq .05$.

7.5 Discussion

The complex interaction between the early-life environment and developing systems is highlighted by the continued study of the developmental origins of health and disease. It is becoming clearer that the biological mechanisms responsible for the perinatal programming of later life health outcomes are varied, intricately connected, and multidimensional. During critical periods of development, the immune and endocrine systems are exquisitely sensitive to the effects of early life immune activation. The current study assessed the immediate peripheral and central inflammatory profile of neonatal rats exposed to LPS in order to determine the activation status of IDO/TDO in the KP. Specifically, neonatal LPS on PND 3 and 5 significantly increased the mRNA expression of *IL-1β* in the spleen and the liver, 6 hours following LPS exposure. *TDO* expression in the liver of LPS treated neonates was significantly downregulated compared to controls, with *IDO* gene activation only detectable in the liver of LPS treated neonates. Centrally, whole brain levels of *IL-1β* mRNA were significantly increased in LPS treated animals, with *GFAP* upregulation indicating microglial activation nearing significance. This initial study is amongst the first examining the acute perinatal programming of the KP with neonatal LPS immune activation. Importantly, this study contributes to determining the primary alterations in KP function during development which may be responsible for long-term behaviour and immunoendocrine alterations demonstrated in this model.

In the current study, neonates treated with LPS demonstrate upregulation in *IL-1β* both centrally and peripherally 6 hours following administration. IL-1β contributes to normal brain maturation, development and central and peripheral immune activation (Giulian et al., 1988). Hence altered levels of IL-1 may perturb growth trajectories and alter long-term astrocyte and microglia function, especially as the early neonatal period is a critical time point

for central immune maturation and plasticity in the rat (Reemst et al., 2016). Previous studies from our laboratory have indicated long-term upregulation of central inflammatory mediators in the male rat, including hippocampal IL-1β, TNF-α and microglial activation, following LPS NIA (Sominsky et al., 2012b; Walker et al., 2010). Furthermore, the current studies present in this thesis indicate sustained altered cytokine profiles in the female rat following NIA. Increases in central IL-1β levels have been demonstrated to negatively impact neurogenesis and behaviour, and induce the synthesis of neurotoxic Kyn metabolites (Farooq et al., 2017; Montkowski et al., 1997; Zunszain et al., 2011). Speculatively, NIA with LPS may skew immune and Kyn function to favour the proinflammatory, neurotoxic arm of the pathway, which may have a particularly detrimental effect during the perinatal period where Kyn is needed for growth and development. Furthermore, this may produce a vulnerable glial-neuronal network that may contribute to the development of maladaptive behavioural alterations previously demonstrated males and females using the NIA model (Chapter3; Walker et al., 2011; Walker et al., 2009; Walker et al., 2004b).

Unexpectedly, *IDO* gene expression was not significantly upregulated, although it was only determinable in the liver of LPS treated animals. The KP is downstream of IL-1β production, and this cytokine and others have been demonstrated to lead to both IDO and TDO activation (Urata et al., 2014). This suggests that 6 hours following immune activation may not be the optimal time to capture peak IDO expression in this model. Conversely, there was a significant downregulation of *TDO* gene expression in the liver of LPS treated animals. TDO is highly sensitive to GC stimulation, and this downregulation suggests that the inflammatory dampening occurring due to the HPA axis activation may not yet be induced. Neonatal alterations to this TDO/IDO balance may be occurring due to NIA. Interestingly, IDO and TDO were not significantly upregulated centrally, although an increase in these mediators

was observed. Previous studies demonstrating central upregulation in IDO and KP mediators in neonates typically use a live virus, hence, the LPS dose used here most probably has a differing time frame of activation. Of note here, TDO knockout mice demonstrate increased levels of Trp, Kyn, 5-hydroxyindoleacetic acid, and 5-HT; exhibit an anxiolytic effect on behaviours in the elevated plus maze (EPM) and open-field tests, and enhanced neurogenesis (Kanai et al., 2009). This indicates that the downregulation of TDO seen here may also serve as a protective effect for neurogenesis at this critical time of central and immune-endocrine development in the rat.

Interleukin-10 gene examination was included in the current study as it is a potent anti-inflammatory cytokine produced by immune cells, including toll-like receptor 4 (TLR4) activated macrophages. The data demonstrates upregulation of this anti-inflammatory cytokine in both central and peripheral tissue, suggesting the beginnings of inflammatory dampening. Lipopolysaccharide mediated IL-10 induction is activated by IFNs via Janus kinase signal transducers and activators of transcription (JAK/STAT) pathways (Iyer et al., 2010). JAK/STAT pathways are associated with developmental signalling and homeostasis maintenance, as well as being the main signalling mechanism for proinflammatory cytokines and growth factors (Rawlings et al., 2004). IDO has also been demonstrated to be induced via JAK/STAT pathways mediated by IFNγ (Campbell et al., 2014), with IL-10 treatments demonstrated to significantly enhance IFNγ induction of IDO (Yanagawa et al., 2009). Additionally, IDO knockout mice demonstrate enhanced levels of anti-inflammatory IL-10 and decreased proinflammatory cytokines following LPS administration (Jung et al., 2009). As IDO induction and IL-10 share common activation pathways, IL-10 here may also serve as a positive control, and future studies should also include the examination of IFNγ and the expression of anti-inflammatory cytokines, such as IL-27, IL-4 and IFNα expression following LPS activation.

In regards to female reproduction, IDO is expressed in the female reproductive tract where it participates in innate immune function (Sedlmayr et al., 2002). The ovary also has a resident population of macrophages and immune cells (Nash et al., 1999; Wu et al., 2004), and these cells are known to secrete IDO. Furthermore, IDO expression has been localised in the placenta where it facilitates the regulation of foetal-maternal tolerance and protection against intra-and-extracellular pathogens (Sedlmayr et al., 2002). In clinical populations of females experiencing endometrial, ovarian and vulva cancers, plasma serum concentration of Kyn are demonstrated to be significantly higher, and these patients also exhibit higher Trp/Kyn ratios, indicative of KP dysregulation (de Jong et al., 2011; Sperner-Unterweger et al., 2011; Turkoglu et al., 2016). Additionally, increased expression of both IDO and TDO have been reported in ovarian cancer patients, along with greater IL-1β, TNF-α, IL-6, TLR4), MAPK and NF-κβ level (Charbonneau et al., 2013), all of which are essential to LPS-mediated inflammation. Early life stress has been implicated in cancer development and tumour progression, including ovarian cancers (Cramer, 1990). Moreover, early menopause is a risk factor for the development of ovarian cancer, the causes of which have been suggested to be determined in early life during formation of the ovarian reserve (Aiken et al., 2015; Ruth et al., 2016). To this effect, we have previously demonstrated that NIA with LPS in a female rat model depletes the ovarian follicular pool, a major factor contributing to reproductive decline, and brought female puberty and senescence forward, altering the reproductive lifespan (Chapter 5; Fuller et al., 2017; Sominsky et al., 2012a).

Kynurenine pathway dysregulation is also implicated in the pathogenesis of depression and inflammation, both of which are associated with and often present comorbidly with female reproductive disorders including polycystic ovarian syndrome (PCOS) and endometriosis (Deeks et al., 2010; Dokras, 2012; Duleba & Dokras, 2012; Jiang et al., 2016;

Siedentopf et al., 2008). Interestingly, IL-1β and TDO upregulation have been linked to immune-modulated endometriosis development (Urata et al., 2014), and other female reproductive disorders (PCOS, premature ovarian insufficiency/failure [POI/F]) are consistently linked to chronic proinflammation and stress dysregulation (Benson et al., 2009; Boots & Jungheim, 2015; Duleba & Dokras, 2012; Dumesic et al., 2007). As female reproductive dysfunction is associated with increases in both stress mediators and inflammatory parameters, which are known regulators of KP activation, further investigation into the deregulation of the KP following early life immune stress is warranted when exploring a female subfertility phenotype.

Kynurenine pathways dysregulation may also be associated with alterations to female rat mating behaviour. Tryptophan degradation is associated with the production of 5-HT. In chapter three of this thesis, we demonstrated that NIA decreased female reproductive behaviours, namely decreasing lordosis and altered mating cues given by LPS treated females. Interesting, increases in 5-HT is well known for its inhibitory effects on lordosis behaviour. Animal studies utilising pharmacological manipulations of 5-HT have been linked with decreases in lordosis behaviours in female rats and a general decrease in female sexual behaviour (Mendelson, 1992; Snoeren et al., 2014; Uphouse, 2014). Serotonin receptor antagonists block this inhibition of female sexual behaviour (Guptarak et al., 2010). Furthermore, Trp and 5-HT expression has been detected in human ovarian tissue, indicating that these mediators are synthesised in the ovary (Itoh et al., 1999) and play a role in early ovarian follicular development and oocyte-granulosa cross-talk (Amireault & Dube, 2005; Dube & Amireault, 2007). Regardless of these links, investigation of the KP in female reproduction remains limited and largely unexplored. Considering the importance of Trp as the substrate of both Kyn and 5-HT, perhaps fundamental alterations to this pathway,

particularly relating to the IDO/TDO balance of the KP, are occurring via early life immune stress. Hypothetically, the downregulation of IDO and/or TDO enzyme activation would perhaps increase levels of Trp synthesis via the Trp hydroxylase, thus increasing 5-HT and affecting lordosis behaviour. Further investigation in the NIA model is needed to substantiate this hypothesis.

The evidence presented here indicates that the KP is a valid novel mechanism to examine within the PND 3 and 5 LPS model. This includes the investigation of short and long-term dysregulation to this pathway in both male and female animals. Perinatally programmed alterations to these systems may be additional influences contributing to the sexual behaviour and ovarian alterations we have previously demonstrated in this thesis and elsewhere (Sominsky et al., 2012a; Sominsky et al., 2013b; Walker et al., 2011), especially considering the critical role the KP plays in inflammatory resolution and the expression of KP enzymes throughout the female reproductive tract, ovary, and placenta. Further examination and elucidation of these mechanisms will hopefully lead to a greater understanding of the way in which early life infection impacts upon development and the role this plays in female reproductive parameters.

7.7 The Catecholaminergic System and Early Life Stress

The two division of the autonomic nervous system (ANS), the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) are vital to stress and inflammatory modulation. Stress, including immune stress, activates the HPA axis, the sympathoadrenomedullary system, and the immune system concomitantly (Karrow, 2006; Lukewich et al., 2014; Zhou & Jones, 1993b). Inflammatory responses evoke cytokines, release of which are known to be modulated by epinephrine (EPI) and norepinephrine (NE) (Hasko & Szabo, 1998; Won & Kim, 2016). Endotoxin administration has been shown to activate

catecholamine synthesis both centrally and peripherally (Dunn, 1992; Xia & Krukoff, 2003; Zhou & Jones, 1993a). Additionally, early life LPS immune activation is implicated in the programming of this system. Zavitsanou et al. (2013) demonstrated long-term alterations to dopamine (DA) binding in the hippocampus (HC) and hypothalamus (HTH) in male rats treated with LPS on PND 3 and 5, implicating central catecholamine synthesis alterations are associated with NIA. We have also previously demonstrated that male pups show an increase in acute adrenal tyrosine hydroxylase (TH) phosphorylation activity at Ser40 following LPS exposure (Sominsky et al., 2013a). TH is the initial rate limiting enzyme involved in all catecholamines synthesis including DA, EPI and NE, with the regulation of TH activity occurring at phosphorylation sites Ser19, Ser31, and Ser40 (Damanhuri et al., 2012; Ong et al., 2014). What remains to be established in the current model of NIA, is if sympathetic activation and catecholamine-related dysregulation may be contributing to the female subfertility phenotype present in the NIA animal model, particularly through central alterations to catecholaminergic systems.

7.7.1 The catecholaminergic system, early life stress, and female reproduction

The ovary is known to be innervated by vagal parasympathetic and sympathetic nerves, including both afferent and efferent innervation (Aguado & Ojeda, 1984; Burden et al., 1983; Robbins et al., 1992; Uchida, 2015). These multi-synaptic neural pathways provides a link between the ovary and the central nervous system (CNS), reaching the ovary via the ovarian nerve plexus following the ovarian artery and the superior ovarian nerve (Aguado, 2002; Morales et al., 1998). It is suggested that ovarian vagal innervation moderates ovarian function, including partial control of the ovarian reserve and sex hormone release (Gerendai et al., 2000; Morales et al., 2007). Additionally, nociceptive information from the ovaries, such as that associated with gonadal inflammatory events including ovulation, ovarian cyst rupture

and cancer is conveyed via afferent fibres leading to autonomic responses including pain, hypotension and anorexia (Uchida et al., 2015). Manipulations to ovarian-vagal nerve connections via vagotomy results in hypothalamic pituitary gonadal (HPG) hormonal alterations, delays in puberty onset, and negatively affects ovarian function, weight, and follicular development (Magoffin, 2002; Morales et al., 2007; Ojeda et al., 1983; Uchida, 2015).

Catecholamines and other important mediators of the ANS have been demonstrated to play a key role in ovarian follicular development and steroidogenesis (Aguado, 2002; Deady & Sun, 2015; Lara et al., 1990; Ricu et al., 2008). All three major catecholamines transmitted by sympathetic neurons are present in the ovary, where they facilitate hormonal ovarian steroidogenesis and promote follicular development (Mayerhofer et al., 1998). Ricu et al. (2008) describes that sympathetic innervation and responses to β-adrenergic stimulation in the mammalian ovary is present prior to birth, and further develops as the ovary reaches reproductive maturity, indicating that changes to the early life environment may potentially impact this developmental process. Ovarian follicular levels of NE in the prepubertal period co-regulates the follicular response to gonadotropins and facilitate initial ovulation, with increases in sympathetic activity disrupting rat oestrus cyclicity and perturbing follicular maturation (Morales-Ledesma et al., 2015). Morales-Ledesma et al. (2015) also demonstrated that chemical and physical sympathetic denervation on post-natal day (PND) 3, 4, or 7 in female rats delayed puberty onset and compromised fertility, providing evidence of the early neonatal period being critical to long-term sympathetic ovarian function in concert with endocrine and immune control. An animal model of chronic stress using both cold and restraint stressors has been demonstrated to lead to systemic increases in sympathetic activity, including within the ovary (Dorfman et al., 2003; Paredes et al., 1998). What's more, chronic stress and increases in sympathetic tone in younger female animals has been linked

to the development of ovarian cysts and androgen dysregulation, similar to that seen during the aging process (Acuna et al., 2009; Cruz et al., 2017; Greiner et al., 2005; Paredes et al., 1998). As such, sympathetic activation in the ovary has been linked to ovarian disorders including PCOS morphology and syndrome both in humans and animal models, as well as premature ovarian follicle depletion (Dissen et al., 2009; Garcia-Rudaz et al., 2011; Ricu et al., 2008).

In our laboratory, Sominsky et al. (2012a) demonstrated that LPS exposure on PND 3 and 5 increased phosphorylation at Ser31 in the adrenals of PND 5 neonatal females, differing from the Ser40 phosphorylation site demonstrated in males at the same time and providing evidence for sexually dimorphic ANS pathway activation following NIA. Using a similar model, neonatal LPS exposure in female rats has been demonstrated to lead to long-term increases in ovarian sympathetic nerve activity measured via levels of nerve growth factor (NGF) receptor p75NGFR, which is presented on the cell surface of sympathetic neurons (Chao, 1994; Wu et al., 2011). Wu et al. (2011) also demonstrated that NIA decreased the ovarian follicle reserve, in line with our own results (Chapter 5; Fuller et al., 2017 [Chapter4]), signifying that increases in ovarian sympathetic tone may be occurring via early life immune stress (Dorfman et al., 2003). The evidence outlined above implicates the SNS and catecholaminergic pathways as a potential co-mechanism regulating ovarian function and hence female reproductive parameters, along with immuno-endocrine mediators, which may also contribute to reproductive behavioural alterations previously demonstrated. Regardless of the known role the ANS plays in stress/immune activation, behaviour, and the endotoxin response; the long-term effects of early-life endotoxin exposure on the development and long-term functioning of both peripheral and central catecholaminergic pathways remains relatively limited, particularly within a female cohort.

The publication presented at the end of this chapter examines the long-term alteration in central catecholamine and immune activity in male animals following NIA with LPS on PND 3 and 5, as a first step in understanding the way in this pathway may be altered by the early life microbial environment. This paper elaborates on the previous stress-related behavioural alterations and sustained peripheral sympathoadrenomedullary increases seen in our NIA model (Sominsky et al., 2013a; Sominsky et al., 2012a) and acts as a starting point for examination of this pathway in female animals. The main findings of this publication indicates that NIA produces long-term activation of catecholaminergic phosphorylation and protein levels in central regions including the locus coeruleus (LC), the substantia nigra (SN) and the ventral tegmental area (VTA) in male animals. These changes were associated with variations in GFAP and ionized calcium-binding adapter molecule 1 (Iba-1), markers for astrocyte and microglial activity in the LC and SN. This suggests that neonatal immune upregulation may generate long-term alterations in central immune and TH activation status, impacting on chronic immune and stress profiles. Importantly, these sustained alterations were demonstrated without further manipulation, indicating a direct effect of neonatal LPS on long-term centrally mediated catecholaminergic development and chronic low-grade inflammation. Furthermore, these findings are in line with those reported in chapter 6 of this thesis, providing promising evidence for further investigation.

Of the central regions examined in this paper, the most robust alterations were demonstrated within the LC where TH protein levels were 4 fold those of controls. Female rats are known to have a larger LC region and greater number of NE-containing neurons compared to males (Curtis et al., 2006; Pinos et al., 2001). Interesting, LC lesions have been linked to the development of PCOS-like symptomology in a female rat model (Zafari Zangeneh et al., 2012) and the LC is also known to participate in the regulation of ovarian hormone

secretion and HPG axis function (Pau et al., 2000; Szawka et al., 2009). Furthermore, noradrenergic activity is modulated by 5-HT which has inhibitory effects on sexual behaviours and innervates the LC (Leger & Descarries, 1978). Considering this and the LC alterations we demonstrate in NIA treated males in the adjoined paper (Ong et al., 2017), this region may be of particular interest in females within our model. Hence, examining the central catecholaminergic regions can increase our knowledge regarding the female sexual behaviour alterations we have previously demonstrated (Chapter 3; Chapter 5; Walker et al., 2011).

Greater understanding of the impact of the early life microbial environment in a female animal model is of importance due to the known immune-regulatory and stress-modulatory role of the central catecholaminergic system and the known sympathetic actions involved in female reproductive functioning. Therefore, the current publication aims to provide an interesting and novel springboard for further examination of sympathetic parameters within our model of NIA in a female cohort and lends itself to a greater understanding of female reproductive behaviours and ovarian functioning following early life bacterial exposure. The paper included in this chapter hopes to establish and facilitate further studies in this area.

Paper 3

Early life peripheral lipopolysaccharide challenge reprograms catecholaminergic neurons

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Statement of author contributions to the publication.

Author	**Description of contribution to manuscript**	**Signature**
Lin Kooi Ong	Designed and performed experiments Analysed and interpreted the data Wrote and revised the manuscript	
Erin A Fuller	Designed and performed experiments Assisted in data interpretation Provided intellectual contribution and critical input Wrote and revised the manuscript	
Luba Sominsky	Assisted in experimental design and data interpretation Provided intellectual contribution and critical input Revised the manuscript	
Deborah M Hodgson	Assisted in experimental design Contributed reagents/materials/analysis tools Provided intellectual contribution and critical input Revised the manuscript	
Peter R Dunkley	Assisted in experimental design Contributed reagents/materials/analysis tools Provided intellectual contribution and critical input Revised the manuscript	
Phillip W Dickson	Assisted in experimental design Contributed reagents/materials/analysis tools Provided intellectual contribution and critical input Revised the manuscript	

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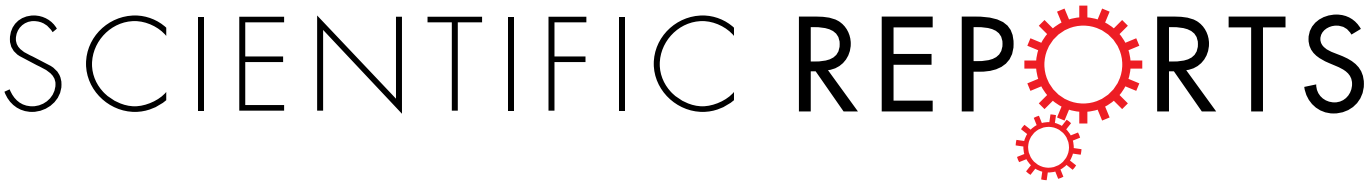

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Early life peripheral lipopolysaccharide challenge reprograms catecholaminergic neurons

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Neonatal immune challenge with the bacterial mimetic lipopolysaccharide has the capacity to generate long-term changes in the brain. Neonatal rats were intraperitoneally injected with lipopolysaccharide (0.05 mg/kg) on postnatal day (PND) 3 and again on PND 5. The activation state of tyrosine hydroxylase (TH) was measured in the locus coeruleus, ventral tegmental area and substantia nigra on PND 85. In the locus coeruleus there was an approximately four-fold increase in TH activity. This was accompanied by a significant increase in TH protein together with increased phosphorylation of all three serine residues in the N-terminal region of TH. In the ventral tegmental area, a significant increase in TH activity and increased phosphorylation of the serine 40 residue was seen. Neonatal lipopolysaccharide had no effect on TH activation in the substantia nigra. These results indicate the capacity of a neonatal immune challenge to generate long-term changes in the activation state of TH, in particular in the locus coeruleus. Overall, the current results demonstrate the enduring outcomes of a neonatal immune challenge on specific brain catecholaminergic regions associated with catecholamine synthesis. This highlights a novel mechanism for long-term physiological and behavioural alterations induced by this model.

Early life stress events can exert long lasting programming effects that manifest in adulthood. The bacterial mimetic lipopolysaccharide (LPS) has been used extensively to document the long-lasting effects of a neonatal immune challenge on a variety of physiological and behavioural effects in the adult animal[1]. LPS exposure on postnatal days (PND) 3 and 5 is a well-documented rodent model used to examine the impact of "perinatal programming" on autonomic and hypothalamic-pituitary-adrenal (HPA) stress response systems, the immune system, and the associated long-term behavioural consequences[2–7]. Neonatal inflammatory challenges can activate the sympathoadrenomedullary system leading to release of catecholamines from the adrenal medulla and HPA axis activation resulting in release of glucocorticoids from the adrenal cortex[8].

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthetic pathways for catecholamine synthesis[9]. TH is regulated acutely by phosphorylation at three serine residues (Ser19, Ser31 and Ser40) in the N-terminal regulatory region of TH, and chronically by changes in TH protein synthesis[10]. We previously investigated the effect of neonatal immune challenge at PND 3 and 5 on the sympathoadrenomedullary activation by examining the activation state of TH in the adrenal medulla. Neonatally LPS-treated animals showed significant increases in TH phosphorylation and TH activity up to 24 hours after LPS administration[11,12]. Remarkably, this increase in TH phosphorylation and TH activity was maintained into adolescence and adulthood despite there being no further intervention[7]. Such a long-term sustained activation of TH has not been seen in the other stress models that do not involve development. Therefore, this indicates that PND 3 and 5 LPS exposure has a unique capacity to generate long-term changes in the activation state of the catecholamine producing chromaffin cells in the adrenal medulla *in vivo*.

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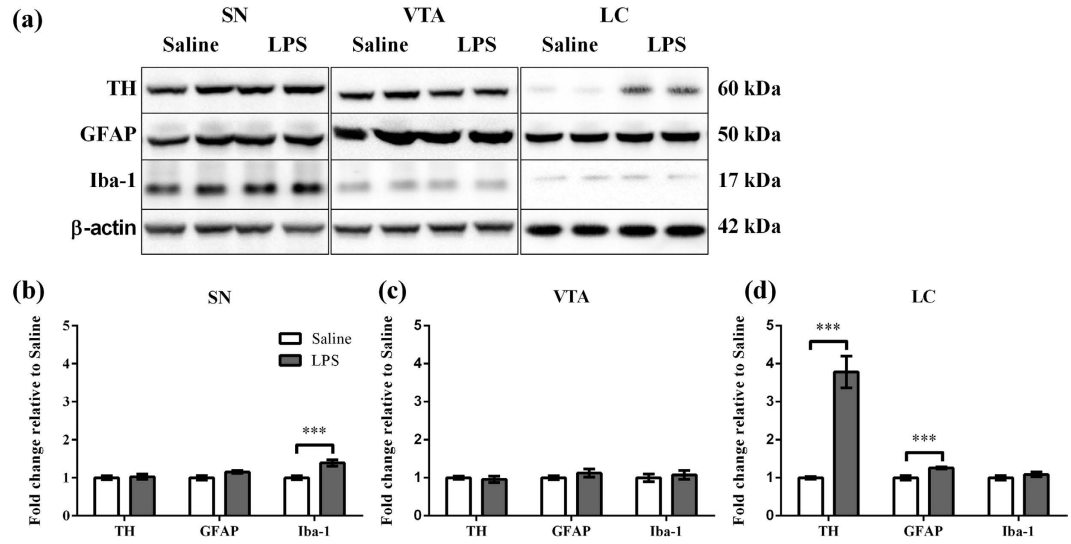

Figure 1. Effect of TH, GFAP and Iba-1 levels in the SN, VTA and LC following neonatal peripheral LPS challenge (Saline, n = 12 and LPS, n = 15). (**a**) Representative immunoblots for TH, GFAP, Iba-1 and β-actin from the SN, VTA and LC comparing the effects of Saline and LPS treatment are shown for two different animals for each treatment. The results of TH, GFAP and Iba-1 levels were calculated relative to β-actin levels from the (**b**) SN, (**c**) VTA and (**d**) LC. Values for Saline and LPS groups were expressed ad fold increase of the mean ± SEM realative to the mean of the Saline group. ***$p < 0.001$.

LPS can alter brain catecholamine levels[13] and the functioning of central immune mediators[1]. It is therefore likely that challenges with inflammatory molecules such as LPS can induce changes in this critical developmental period that are not seen in the adult animals. Given our previous findings regarding peripheral sympathoadrenomedullary and HPA axis hyperactivity, central immunological alterations and long-term behavioural alterations, the current study aimed to investigate the effect of neonatal LPS challenge on the long-term effect on catecholaminergic systems in the brain. We hypothesised that neonatal LPS challenge would induce a long-term activation of TH in the main catecholaminergic nuclei in the brain, the substantia nigra (SN), the ventral tegmental area (VTA) and the locus coeruleus (LC). The current data show that neonatal LPS challenge can produce profound long-term activation of TH in brain catecholaminergic nuclei and that there are major differences in the response of different nuclei suggesting that they are each reprogramed to a different extent by early life LPS challenge.

Results

Neonatal peripheral LPS challenge induced long-term alterations in TH, GFAP and Iba-1 protein levels. The long-term effect of neonatal peripheral LPS challenge in the substantia nigra (SN), ventral tegmental area (VTA) and locus coeruleus (LC) was determined for TH, GFAP (an astrocyte specific cytoskeletal protein marker)[14] and Iba-1 (microglia calcium homeostasis protein marker)[15]. These parameters were examined at PND 85. TH, GFAP and Iba-1 and each appeared as a single band corresponding to molecular masses of 60, 50 and 17 kDa respectively (Fig. 1a). TH, GFAP and Iba-1 levels were calculated relative to β-actin levels (Fig. 1b–d). In the SN, LPS treatment caused a significant increase in Iba-1 levels (1.4 fold, $p < 0.001$), but not in TH and GFAP levels relative to Saline treatment (Fig. 1b). In the VTA, there was no effect of LPS treatment on TH, GFAP or Iba-1 levels (Fig. 1c). In the LC, LPS treatment caused a significant increase in TH protein levels (3.8 fold, $p < 0.001$) and GFAP levels (1.3 fold, $p < 0.001$), but not in Iba-1 levels relative to Saline treatment (Fig. 1d).

Neonatal peripheral LPS challenge induced long-term alterations in TH activity and TH phosphorylation. The effect of neonatal LPS challenge on the TH activation parameters was examined on PND 85 (Fig. 2). As the level of TH protein changed in different brain regions, TH activity levels were calculated as total TH activity by correcting for changes in β-actin levels (Fig. 2b–d). There was a significant increase in total TH activity in the VTA (2.2 fold, $p < 0.01$) and LC (4.6 fold, $p < 0.001$), but not in the SN when LPS treatment was compared to Saline. A major mechanism for control of TH activity is the phosphorylation of serine residues in the N-terminal region of TH[10]. We therefore determined the phosphorylation levels of Ser19, Ser31 and Ser40. Again, as the level of TH protein changed in different brain regions, the phosphorylation of the three sites was calculated relative to β-actin levels. Representative immunoblots are shown in Fig. 2a for phospho-TH (pSer19, pSer31 and pSer40) in the SN, VTA and LC after Saline or LPS treatment. In the SN, there was no effect of LPS treatment on phospho-TH levels (Fig. 2b). In the VTA, LPS treatment caused a significant increase in pSer40 levels (1.4 fold, $p < 0.05$), but not in pSer19 and pSer31 levels relative to Saline treatment (Fig. 2c). In the LC, LPS treatment caused a significant increase in pSer19 (8.6 fold, $p < 0.001$), pSer31 (2.5 fold, $p < 0.001$) and pSer40 levels (4.8 fold, $p < 0.001$) relative to Saline treatment (Fig. 2d).

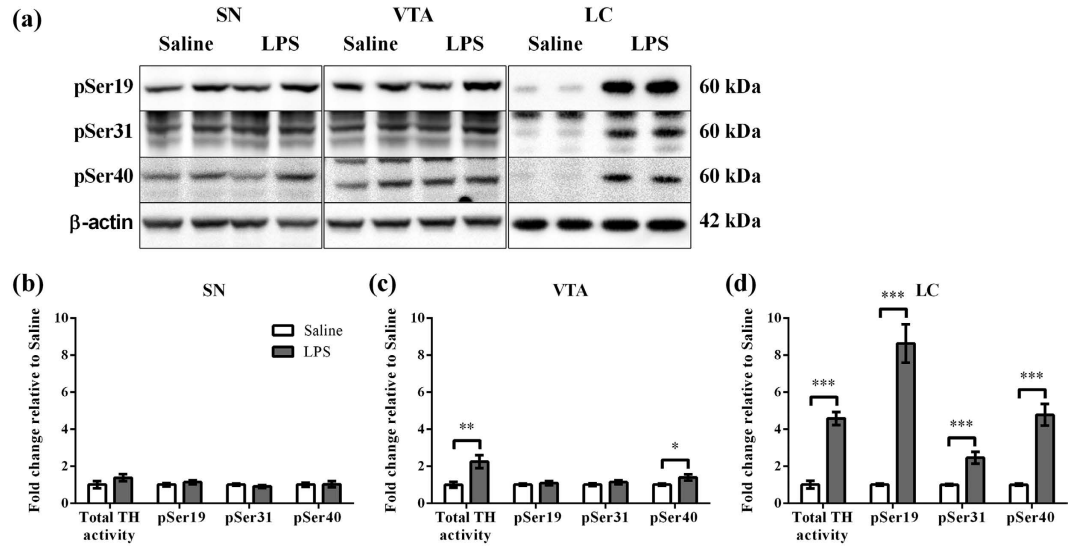

Figure 2. Effect of phospho-TH (pSer19, pSer31 and pSer40) levels in the SN, VTA and LC following neonatal peripheral LPS challenge. (**a**) Representative immunoblots for pSer19, pSer31, pSer40 and β-actin from the SN, VTA and LC comparing the effects of Saline and LPS treatment are shown for two different animals for each treatment. The results of pSer19, pSer31 and pSer40 levels were calculated relative to β-actin levels from the (**b**) SN, (**c**) VTA and (**d**) LC. Values for Saline and LPS groups were expressed as fold increase of the mean ± SEM relative to the mean of the Saline group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

The major aim of this study was to investigate the long-term consequences of neonatal immune challenge on three different central catecholaminergic nuclei, by examining the activation status of TH and associated glial markers. Our findings indicate that neonatal LPS challenge generates long-term changes in TH activation and glial marker levels in the SN, VTA and LC of adult rats. The changes that we have determined are likely to be due to a direct effect of LPS challenge in inducing a low level inflammation. It is possible though that the sickness of the pups may influence the care given by the mother, however no significant differences existed in weight gain between treatment groups (data not shown), suggesting similar nursing and maternal care between treatment groups. Although we took steps to minimise these issues by having all pups in a litter challenged by LPS or saline (see Animal Protocols), a potential role of differences in maternal care for the LPS challenged pups in generating some of the effects seen cannot be ruled out.

The results show that there was no evidence of long-term changes of TH activation in the SN. This was interesting as the SN was the only brain region that showed a significant sustained increase in Iba-1, a microglial cytoskeletal marker. We have previously reported that neonatal LPS challenge induced increased microglia activation in the hippocampus of adult rats[12]. In a study using a neonatal LPS challenge model but with a much higher LPS dose of 2 mg/kg, Cai *et al.*[16] demonstrated increases in the microglia activation marker OX42+, a significant decreased expression of TH in the SN, as well as evidence of decreased viability of dopaminergic neurons[16]. Such a high dose of LPS is utilised in adult Parkinson's disease inflammatory models and results in loss of dopaminergic neurons is the SN[17]. This indicates the potential of LPS to generate long-term responses in the dopaminergic neurons of the SN, but suggests that the LPS dose utilised (0.05 mg/kg) in this study may be too low to produce effects on the TH activation parameters of the SN even though it caused changes in the microglia cytoskeletal marker.

Neonatal LPS challenged animals displayed a significant increase in TH activity in the VTA, which could be due to the significant increase in Ser40 phosphorylation. Ser40 phosphorylation dissociates the bound inhibitory catecholamines and activates TH[9]. Interestingly, these TH alterations were evident without change in the levels of the microglia and astrocyte markers, particularly as microglia and astrocytes are known to be responsive to catecholaminergic stress activation, and the immunoregulatory role of the central dopaminergic system[18–20]. In contrast, there was no change in the level of the TH protein in the VTA. The nature of the changes seen here in the VTA with respect to the mechanism of TH activation is similar to that which we have previously determined in the adrenal gland of adult animals treated with neonatal LPS challenge[7], that is a chronic increase in TH phosphorylation without any alteration in TH protein levels. With regards to the adrenal gland, there were increases in the phosphorylation of Ser19, Ser31 and Ser40 sites, but the major increase was in the phosphorylation of Ser40. Therefore, the increased activation of TH that we report in the current study could be indicative of an increase in activation of the VTA in response to the neonatal LPS challenge. Consistent with this, we have previously shown that neonatal LPS challenge leads to increased dopamine D2 receptor binding in the nucleus accumbens, a target region of the VTA[21].

The LC demonstrated significantly pronounced alteration in the TH protein expression, TH phosphorylation of all three sites, as well as TH activation. The increased level of TH protein was accompanied by increased phosphorylation of Ser31 and Ser40, two sites that have been shown to be directly associated with TH activation *in vivo*[7,11,22–26]. This would explain the very significant increase in the LC TH activity. Ser19 phosphorylation

is associated with protein binding rather than activation of TH[27]. Ser19 phosphorylation showed the greatest increase in response to neonatal LPS challenge of the three sites, more than double the fold increase in the level of TH protein. We have shown under basal conditions that the stoichiometry of phosphorylation of Ser19 in the LC is 0.35 mol pTH/ mol total TH[23]. This indicates that in the LC of neonatal LPS challenged animals, around 80% of the TH subunits are phosphorylated at Ser19. This has the potential to significantly alter the nature of protein-protein interactions of TH under these conditions. Of all three brain regions studied, increased levels of GFAP were only found in the LC suggesting increased reactive astrocytes in this region[28], that may have an immunomodulatory effect on catecholaminergic pathway activity, potentially altering behavioural and sympathoadrenomedullary parameters previously demonstrated[7].

The changes in the LC of neonatal LPS challenged animals can be compared to other *in vivo* stress models. In short-term *in vivo* stress models (less than 1 hour) there were increases in Ser31 and Ser40 phosphorylation in response to social defeat and footshock[23,29] and increases in Ser31 phosphorylation alone in response to restraint, hypotension and glucoprivation[22,30]. In contrast to short-term stressor results, the effect of the neonatal LPS challenge on the LC produced much more robust responses in relation to Ser40 phosphorylation and produced a dramatic change in Ser19 phosphorylation that was not seen in the other models. Short-term stress responses are an adaption to an immediate threat but prolonged or repeated stress can be maladaptive. The changes seen in the TH protein levels in the LC in response to the neonatal immune challenge (4 fold) can be put in context by the fact that they are similar to the changes seen in the LC in response to repeated restraint stress over 2 or 6 days (4 to 6 fold)[31]. Therefore, the current findings indicate that the neonatal LPS challenge can produce pronounced long-term changes in the LC that are similar in magnitude to that obtained immediately after what is one of the strongest rodent stress protocols.

The increased activation of the LC can in part explain the activation of the other catecholaminergic cell groups that we have examined. There are both direct and indirect anatomical connections between the LC and the VTA and LC activation can elicit burst firing in the VTA[32]. The LC can activate the pre-ganglionic sympathetic fibres which in turn innervate the adrenal medulla chromaffin cells and activation of the pre-ganglionic sympathetic fibres leads to release of epinephrine and norepinephrine and subsequent requirement for activation of TH[33]. Moreover, the LC sends projections to most brain regions with the exception of the basal ganglia[32]. Therefore the effect of the neonatal LPS challenge in programming the LC to a more activated state has potential to impact on many different brain functions. The findings of the study indicate that neonatal LPS challenge may program central catecholaminergic pathways that are associated with the modulation of endocrine and sympathetic nervous system stress responses. These current outcomes refine and substantiate our previously demonstrated long-term HPA axis, autonomic, and anxiety-like behaviour outcomes using the neonatal LPS challenge model[2,7,11,12]. Importantly, this study suggests a novel mechanism of central catecholaminergic and immunoregulatory pathways mediating the perinatal programming of anxiety-like behaviours and associated pathologies, specifically implicating the catecholaminergic pathways of the LC.

Materials and Methods

Antibodies. Total-TH antibody (tTH) and phospho-specific TH antibodies (pSer19, pSer31 and pSer40) were generated and were tested for specificity as described[34]. GFAP antibody (#3670) was purchased from Cell Signaling Technology (Danvers, MA, USA). Iba-1 antibody (AB5076) and anti-goat immunoglobulin (horseradish peroxidase-linked) were purchased from Abcam (Cambridge, UK). β-actin horseradish peroxidase-linked antibody (A3854) were purchased from Sigma-Aldrich. (MO, USA). Anti-sheep antibody (horseradish peroxidise-linked) were purchased from Thermo Fisher Scientific (MA, USA). Anti-rabbit- and anti-mouse-immunoglobulin (horseradish peroxidase-linked) were purchased from Bio-Rad Laboratories (CA, USA). MagicMark™ XP Western Protein Standard was purchased from ThermoFisher Scientific (NSW, Australia).

Animal Protocols. All animal protocols were approved by the University of Newcastle Animal Care and Ethics Committee and performed in accordance with the New South Wales Animal Research Act and the "Australian code of practice and use of animals for scientific purposes". Animals were treated as described[7,11]. Briefly, Wistar rats were mated at the University of Newcastle. Male neonatal rats were allocated into either saline control (n = 12, derived from 3 litters) or LPS (n = 15, derived from 5 litters) conditions at birth PND 1, with a maximum of 4 pups used per litter. On PND 3 and PND 5, rats were removed from their home cages, weighed and administered intraperitoneally with either 0.05 mg/kg LPS (*Salmonella enterica, serotype Enteritidis*: Sigma-Aldrich, USA in non-pyrogenic saline) or an equal volume of non-pyrogenic 0.9% saline (Livingstone International, Australia). In order to minimise effects of maternal care, pup weights were taken on treatment days, and animals were monitored twice daily for 10 days post-treatment for abnormalities including vocalisation, litter proximity, and nursing. No differences were observed between groups (data not shown). Rats were housed with their dams until PND 22, at which point they were weaned and divided into housing and left undisturbed except for monitoring. Rats were euthanized on PND 85 with a lethal injection of sodium pentobarbital (200 mg/kg, Virbac, Pty. Ltd, Milperra, Australia).

The SN, VTA and LC were dissected from the coronal sections as previously described which will separate out the different brain sections such that they only have the ascribed catecholaminergic nuclei[22,23]. The SN, VTA and LC samples were separately processed as previously described[23]. Brain samples were sonicated in homogenizing buffer (2 mM potassium phosphate buffer, 1 mM EGTA, 1 mM EDTA, 1 protease inhibitor cocktail tablet, 1 PhosStop tablet, 1 mM DTT, 80 μM ammonium molybdate, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 5 mM β-glycerolphosphate, 2 μM microcystin, pH 7.4) with a microsonicator (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany). Samples were centrifuged at 14 000 g for 20 min at 4 °C. The clear supernatants were collected and protein concentrations were determined. Samples were aliquoted into two equal volumes. One aliquot of each sample was mixed with sample glycerol buffer (2% sodium dodecyl sulfate, 50 mM Tris,

10% glycerol, 1% DTT, 0.1% bromophenol blue, pH 6.8) and this was used for western blot. The second aliquot from the same sample was used for tritiated water release assay.

Western blot. Western blot was performed as previously described with some modifications[23]. Samples (30 μg of total tissue protein), protein standard and TH specific positive controls were subjected to NuPAGE Novex 4–12% Bis-Tris Midi Gels. Gels were transferred to nitrocellulose membranes by western blotting in boric acid transfer buffer (50 mM boric acid, 2 mM EDTA, pH 8.9). Nitrocellulose membranes were washed in Tris-buffered saline with tween (TBST) (150 mM NaCl, 10 mM Tris, 0.075% Tween-20, pH 7.5) and blocked in 5% skim milk powder (SMP) in TBST for 1 h at 25 °C. Membranes were incubated with primary antibodies (tTH; 1:5000 in 1% SMP, GFAP; 1:5000 in 5% SMP, Iba-1; 1:1000, pSer19; 1:2000 in 1% SMP, pSer31; 1:500 in 1% SMP, pSer40; 1:1000 in 1% SMP for overnight at 4 °C or β-actin horseradish peroxidase-linked antibody; 1:50,000 for 1 h at 25 °C) and horseradish peroxidase-linked anti-IgG secondary specific antibodies (anti-rabbit; 1:7500, anti-mouse; 1:10000, anti-sheep; 1:7500, anti-goat; 1:5000) for 1 h at 25 °C. In between each incubation step, membranes were washed in TBST. Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji Film, CT, USA) using Luminata Classico detection reagents. The density of the bands was measured using MultiGauge V3.0 (Fuji Film). TH, GFAP, Iba-1 (Fig. 1b–d) and phospho-TH (pSer19, pSer31 and pSer40) (Fig. 2b–d) levels were normalized to β-actin levels and expressed as fold change relative to the Saline samples. Full-length blots were presented in Sup Fig. 1 (for SN), Sup Fig. 2 (for VTA) and Sup Fig. 3 (for LC).

Tritiated water release assay. Tritiated water release assay was performed as previously described (Ong *et al.*[23]). Samples (50 μg of total tissue protein) were mixed in reaction mixture (2 mM potassium phosphate, 36 μg catalase, 0.008% β-mercaptoethanol, 24 μM L-tyrosine, 1 μCi 3, 5-[3 H]-L-tyrosine, pH 7.4). The reactions were initiated with the addition of 100 μM tetrahydrobiopterin in 5 mM HCl. Control representing background reactions were added with 5 mM HCl but did not contain tetrahydrobiopterin. Assays were performed for 20 min at 30 °C and were stopped by addition of 700 μL charcoal slurry (7.5% activated charcoal in 1 M HCl). Mixtures were vortexed for 1 min and were centrifuged at 14 000 g for 10 min at 30 °C. 350 μL supernatants were added to 3 mL scintillation cocktail and were vortexed for 10 s. Mixtures were assayed by Liquid Scintillation Analyzer (Tri-Carb 2810 TR, PerkinElmer) for 10 min per sample. The assessment of TH protein, phospho-TH and TH activity was conducted on the same sample. Therefore, the changes in total TH activity levels (Fig. 2b–d) were normalized to β-actin levels and expressed as fold change relative to the Saline samples.

Statistical analysis. The data for Saline and LPS groups were expressed as a fold change of the mean ± SEM to the mean of the Saline group. These data were analysed by using Prism 6 for Windows (Version 6.01, GraphPad Software, Inc., La Jolla, CA, USA). The data were analysed using two-tailed unpaired parametric Student's *t*-test. All differences were considered to be significant at $p < 0.05$.

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Author Contributions

L.K.O. contributed to the design of the experiments, carried out the experiments and wrote the manuscript. E.F. and L.S. carried out the experiments and revised the manuscript. P.W.D., P.R.D. and D.H. contributed to the design of the experiments, supervised the experiments and revised the manuscript.

Additional Information

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8. General Discussion

8.1 Introduction

During early life, developing systems adapt to their environment. This *plasticity* ensures that environmental cues can be used as a strategy to ensure survival and efficacy of biological function for longevity and reproductive success. The biological and behavioural outcomes become maladaptive however, when the environmental influence is adverse or a mismatch in predicted environments occurs (Gluckman et al., 2010; Langley-Evans et al., 2012). Hence, the *perinatal programming* of physiological systems can have sustained detrimental effects due to the stability of tissue-and timing-specific alterations (Hodgson & Coe, 2006). In the rat, the neonatal period is a critical window of plasticity for the developing immune system, endocrine system, and associated brain circuitry (Kentner & Pittman, 2010; Nesterenko & Aly, 2009; Spencer et al., 2011). It is also a critical period for gonadal development (Rivest, 1991; Skinner, 2005; Zambrano et al., 2014), particularly in the female rat (Grive & Freiman, 2015; Knox et al., 2009). Development during this time is a tightly controlled and delicate process, governed by environmentally-vulnerable systems. As such, stressful environmental disturbances that occur during these sensitive windows have wide-ranging implications for developmental trajectories and physiological systems. Evidence indicates that even subtle alterations to immature systems can skew functioning, create stress vulnerabilities, and have manifold short and long-term effects due to the reciprocity of systems involved.

This thesis is primarily concerned with examining the influence of the early life environment on reproductive development, behaviour, and ovarian functioning in the female rat. This is in order to gain a greater understanding of the female subfertility phenotype that

manifests behaviourally in neonatally LPS treated female rats. These studies focus on inflammatory activation and immune-driven alterations due to the known role inflammatory mediators play in female behaviour and reproductive development and function/dysfunction (Boots & Jungheim, 2015; Derry et al., 2015; Simon & Polan, 1994). The chapters that comprise this thesis employ a model of early life immunological stress using the dual exposure of 0.05mg/kg lipopolysaccharide (LPS) given intraperitoneally on postnatal day (PND) 3 and 5. This model is well established in our laboratory and others to reliably elicit both an immune and neuroendocrine response during a critical period of development for these systems (Hodgson et al., 2001; Shanks et al., 1995; Shanks & Meaney, 1994; Sominsky et al., 2012a; Walker et al., 2012; Walker et al., 2011; Walker et al., 2009; Walker et al., 2010; Walker et al., 2004a; Walker et al., 2008).

The data presented in this thesis provide evidence that this model of postnatal bacterial infection is associated with a specific behavioural phenotype in the female rat, and a range of biological alterations pertaining to reproductive fitness. These studies identify a link between perinatally programmed immune dysregulation and both acute and sustained changes to peripheral and central inflammation and ovarian morphology, having implications for overall reproductive fitness and health. The results presented within this thesis supports the overarching hypothesis that early life bacterial exposure disturbs the delicate process of immune driven ovarian and reproductive development, and alters the peripheral and central immune milieu to a proinflammatory bias. Moreover, it suggests inflammation is involved in the modulation of associated pathways contributing to female reproductive behavioural deficits.

8.2 Defining the Female Behavioural Phenotype

The first aim of this thesis was to examine and refine the long-term female behavioural phenotype that presents following neonatal LPS exposure. Previous studies from our laboratory indicate that male animals display a robust anxiety-like phenotype (Sominsky et al., 2013a; Sominsky et al., 2012b; Walker et al., 2011; Walker et al., 2009; Walker et al., 2008; Walker et al., 2004b), however this phenotype was inconsistent in female rats, presenting mildly only following a *second hit* of stress (Walker et al., 2012; Walker et al., 2011). Furthermore, Walker et al. (2010) demonstrated that both male and female LPS treated rats spent a significantly decreased amount of time resisting restraint stress compared to controls, indicating a stress vulnerability that is associated with learned helplessness and depressive-like behaviour (Harris, 1989). These sexually dichotomous results have been reported by others, with Pohl et al. (2007) demonstrating that female rats exhibit a depressive-like phenotype following adolescent stress, whereas male rats showed exaggerated anxiety-like responses. These results call into question the role early life immune stress plays in the behavioural phenotype of the female rat. This is of relevance, as anxiety and depression are highly comorbid in human populations and their aetiology is complex (Blanco et al., 2014; Kircanski et al., 2017). What is more, both animal models and human literature demonstrate similar mechanistic alterations between the two disorders, namely, chronic proinflammation and hypothalamic-pituitary-adrenal (HPA) axis dysfunction.

Interestingly, female rats treated with neonatal LPS have previously demonstrated impairments in sexual behaviour when tested in an open-field setting, an alteration not observed in such a robust manner in LPS-treated male counterparts (Walker et al., 2011). Sexual dysfunction has been reported as a component of depression, as well as anxiety,

relating to anhedonia (Bradford & Meston, 2006; Kalmbach et al., 2014). However, this open-field assay does not allow for motivational aspects of anhedonia, and hence female mating behaviour to be assessed. Nor does it take into consideration that naturalistically, sexual interactions in the wild are initiated and paced by the female rat through patterns of soliciting, including proceptive and receptive behaviours (Erskine, 1989). The open-field method of testing often obscures the initiative and appetitive aspects of female rat sexual behaviour due to active male pursuit (Kondo & Sakuma, 2005). Therefore, in order to confidently infer a suboptimal sexual behavioural phenotype in the female, confirmation of previously results in a more 'naturalistic' laboratory setting was needed.

The current thesis addressed this in the first experimental chapter (Chapter 3), using a paced mating paradigm, where female rats control copulation. Thus, giving a truer reflection of sexual motivation whilst allowing for the clear measurement of other proceptive and receptive female mating behaviours (Martínez & Paredes, 2001; Paredes & Vazquez, 1999; Zipse et al., 2000). Furthermore, this behavioural study addresses the questions surrounding the anxiety versus depressive-like phenotype in female animals arising from neonatal LPS treatment, focusing on assessable motivational and anhedonic aspects of female rat behaviour in the paced mating test (PMT), the social interaction test (SIT) and the sucrose preference test (SPT). These assays were previously unexamined in our laboratory using the current model of neonatal immune activation (NIA) in the female, therefore allowing for the refinement of the female behavioural phenotype.

The results from this study confirmed that female rats treated with LPS exhibited impairments in proceptive mating behaviours, with less hops and darts compared to saline treated animals. Furthermore, the naïve male stud attempted more mounts on LPS treated

females, with these females exhibiting a greater frequency of aggressive behaviours towards the male. Biologically speaking, impairments in female rat mating communication may be leading to unwanted male advances, in this sense, the increased mounts and increases in female aggression are compatible. These findings were echoed in a recent study by Sylvia and Demas (2017), demonstrating increased aggression and communication impairments following neonatal immune stress in the female Siberian hamster. An important aspect of this study which builds on previous work by Walker et al. (2011) is the examination of lordosis behaviour in the female as a measurement of female receptivity to the male. LPS treated females demonstrated a diminished lordosis frequency compared to saline treated females, however, this did not lead to differences in sperm presence following testing. Lordosis behaviours have been associated with increased serotonin (5-HT) levels. As such, pathways associated with the synthesis should be addressed in future studies, such as the tryptophan (Trp)/kynurenine (Kyn) pathway. Preliminary data indicating alterations to this pathway from neonatal LPS in a male rat cohort is presented in Chapter 7, and examination of this pathway in female animals provides a promising direction for future research due to its shared immune-and-stress modulatory properties.

Social interaction testing allowed for the examination of communicative and motivational behaviours directed towards a naïve rat of the same sex. This enabled further delineation of the female phenotype, to analyse whether these communication deficits were strictly mating contextual, or rather part of a broader communication impairment. We report in Chapter 3 that LPS treated females did not demonstrate any impairments in social behaviour when analysed/observed in the environmental context with a naïve rat of the same sex. However, differences were demonstrated between treatments groups in facial sniffing behaviours, with less none-to-nose sniffing carried out by LPS treated females. These effects

were subtle, however may be interpreted as alterations in communication, considering the known importance of nose-to-nose sniffing in the transmission of information regarding social hierarchy, food and nutrition, and health status.

Motivationally, LPS treated females did not demonstrate deficits in time spent with the male in the PMT, nor in any interactive behaviours in the SIT. Female animals visited the male more frequently that saline treated animals, indicating a preference for the male chamber but an inability to efficiently direct copulatory advances, hence more attempts. Female animals directed similar social behaviours toward same sex counterparts in the SIT, regardless of neonatal treatment group. Nor did LPS treated females display anhedonic behaviours in the SPT. Taken together, the behavioural findings presented in Chapter 3 indicate LPS treated females do not display a depressive-like phenotype, rather one that is context-specific to suboptimal reproductive behaviours and perhaps impairments in communication. Thus, the impairments in mating behaviours do not seem to be a result of an anhedonic effect or as a result of a depressive-like phenotype, as originally hypothesised. As such, the following chapters focused on the contribution of the ovary, as bidirectional cross-talk between the gonads and the brain is known to facilitate reproductive success and behaviour (Marchetti et al., 1990). It is important to note here, that LPS treated females are not infertile, as Walker et al. (2012) and Sominsky et al. (2012a) demonstrated the successful generation of F2 offspring, however mortality was increased in these litters. Hence, NIA females are displaying reproductive behavioural deficits that can be considered suboptimal to the ultimate evolutionary goal of procreation and proliferation.

Converse to the original hypothesis of anhedonia due to NIA and confirming the suggestion that it is not a depressive-like phenotype, LPS treated females consumed more

sucrose per body weight compared to controls, both during the habituation period and test phase of the SPT. This possibly suggests a sensitisation of reward pathways following NIA, such as altered catecholaminergic signalling involved in the mediation of rewarding stimuli that may be driving this behaviour (Baik, 2013; Berridge & Robinson, 1998). Dopamine (DA), norepinephrine (NE) and tyrosine hydroxylase (TH) are present in the rat brain approximately three days prior to birth, and this catecholaminergic system continues to develop rapidly in the neonatal period (Breese & Traylor, 1972; Coyle, 1973). Hence, the current NIA model coincides with a critical period of sensitively for this system. Chapter 7 of this thesis presented a paper utilising male animals which examined the NIA programming of catecholaminergic neurons. Findings from this paper suggest that NIA has a long-term stimulatory impact on these neural pathways. Taken with the behavioural findings from Chapter 3, these results suggest that this pathway may be modified by NIA in females, and as such, provides a novel focus of future behavioural studies.

Neuroendocrine influence must be taken into account when discerning the female subfertility behavioural phenotype. Although this thesis has a strong focus on inflammatory mediators due to the nature of the stress model utilised and the known importance of immune mediators to reproductive fitness and psychopathology, it needs to be mentioned that the hormonal contribution to female reproductive behaviour is not discounted. The endocrine system and the immune system are inextricably linked, hence perturbations to one will affect the other. It is highly probable that hormonal alterations have occurred due to the known effects of NIA on the perinatal programming of neuroendocrine system. LPS treated females in this study demonstrated generally increased circulating corticosterone (CORT) levels in blood taken pre and post the SIT. This stress increase is reflected in the amplification of rearing behaviour displayed in LPS treated females in both the SIT and the PMT when taken

as a quotient of anxiety-like behaviour. However, both saline and LPS treated rats displayed similar increase in CORT levels following this mildly stressful test, which may suggest that this rearing is exploratory in nature. When paired with sniffing alterations, perhaps chemoinvestigation is another communication parameter altered by NIA in the female rat. Furthermore, although alterations to luteinising hormone (LH) and follicle stimulation hormone (FSH) were demonstrated, there was no significant difference between groups, results that differ from previous reports from our laboratory indicating dampening of the LH surge. Regardless, the investigation of neuroendocrine mediators needs to be included in future research regarding female reproductive behaviours, including the investigation of mediators such as leptin, gonadotropin releasing hormone (GnRH), oestrogen and progesterone and other hypothalamic-pituitary-gonadal (HPG) axis products (Christensen et al., 2012; Garcia-Juarez et al., 2013).

The behavioural studies conducted for this thesis completed their aim of defining and confirming the female behaviour phenotype following NIA. From the findings reported in this thesis, it may be derived that the behavioural impairments demonstrated in NIA treated female rats are associated with the inadequate behavioural signalling of precopulatory behaviours and of behavioural cues which are contextually specific to mating behaviours. The suggestion that these impairments are motivationally or anhedonically driven was not supported, rejecting the hypothesis that NIA may result in a depressive-like phenotype in female rats. Furthermore, the current thesis indicates that an anxiety-like behavioural phenotype does not robustly manifest in NIA treated females in a social context, extending on previous reports from our laboratory corroborating these results in the female rat using traditional tests of anxiety-like behaviour. Furthermore, this study allowed for the narrowing of potential mechanisms involved in the perinatal programming of female reproductive

behaviours, and implicated ovarian parameters and central inflammation which may be driving these behavioural alterations (as examined in Chapters 4, 5 and 6).

8.3 Perinatal Programming of Reproductive Development: Puberty Onset and First Oestrus

This thesis examined a marker of reproductive development in the female rat, namely pubertal onset marked by day of vaginal opening (DVO) and emergence of first proestrus. Female DVO and first proestrus was examined across two different studies derived from a combined 17 litters (10 LPS, 7 Saline; Chapter 3 and Chapter 5). Both studies demonstrated an earlier onset of puberty in LPS treated females compared to saline controls. These results corroborate previous findings from our laboratory (Sominsky et al., 2012a; Walker et al., 2011). However, they are contradictory to others who demonstrate delays in puberty onset in female rats following neonatal LPS. Knox et al. (2009) demonstrated that PND 3 and 5 LPS exposure delayed DVO in Sprague-Dawley rats, results that were echoed by Wu et al. (2011b) and Wang et al. (2017) in the same rat species. The differences demonstrated in this thesis may be due to methodological differences from the studies mentioned above, including litter manipulations, LPS strain, and rat species. Wistar rats typically exhibit a bimodal distribution of age at vaginal opening occurring at PND 34 and PND 39, accounting for some variability (Rivest, 1991).

Biologically speaking, it makes sense for animals to delay puberty or oestrus by dampening the HPG axis if animals are sick, therefore diminishing risk of reproductive complications (Avitsur & Yirmiya, 1999b). The Wistar rats used in the current thesis study demonstrate no overt signs of sickness, nor did they demonstrate sickness-behaviours in behavioural tests, as the female rats exposed to adult LPS in Avitsur and Yirmiya (1999) did. Chapters 4, 5 and 6 of this thesis however, do demonstrate proinflammatory alterations both

centrally and peripherally, which may be skewing puberty onset as discussed within Chapter 6. Inflammation has been demonstrated to influence reproductive parameters, conjointly mediated via the neuroendocrine hormones and neuropeptides (Kentner et al., 2010). This includes HPG axis stimulation and suppression by the actions of Kisspeptin (KISS1) signalling and corticotropin releasing hormone (CRH), the long-term expression of which was examined in Chapter 6 of this thesis.

The size of the ovarian follicle pool is a major determinant of reproductive longevity in the female (Banerjee et al., 2014; Monniaux et al., 2014). Chapters 4 and 5 of this thesis demonstrated that NIA leads to both the acute and sustained depletion of the ovarian reserve, as discussed below. Previously in our laboratory, Sominsky et al. (2012) demonstrated that LPS treated females exhibited advancement of senescence. In an evolutionary sense, the current results and those demonstrated by Sominsky indicate a developmental trajectory shift, bringing the reproductive lifespan forward in order to maximise reproductive capacity during a period of optimal health, particularly as untimely menopause is associated with a multitude of health complications (De Vos et al., 2011; Hernández-Angeles & Castelo-Branco, 2016). In keeping with the same argument, neonatal treatment with LPS also resulted in advanced emergence of 1st proestrus however cycle regularity did not significantly differ between treatment groups, similar to previous findings from our laboratory (Sominsky et al. 2012a). These findings differ from others (Iwasa et al., 2009; Knox et al., 2009; Wu et al., 2011b) who demonstrate delayed first proestrus and altered normal cyclicity following neonatal LPS exposure. Nilsson et al. (2002), using the same dose, LPS strain, and rat species as the current study, echoed the rat oestrus cyclicity findings of this thesis.

In all, these pubertal timing alterations add a further dimension to the female subfertile phenotype. Precocious pubertal onset in female children has been demonstrated to have developmental origins and be linked to the adult onset of disease (Anderson, 2003; Ibáñez et al., 1998; Wierson et al., 1993). Furthermore, earlier onset of puberty is associated with small birth weight and catch-up growth (Dunger et al., 2006; Sloboda et al., 2007). Previous literature indicates a strong link between metabolic status and pubertal onset, and although the female rats utilised throughout this thesis do not demonstrate a heavier bodyweight at puberty, periods of catch-up growth are demonstrated between PND 22-43, prior to DVO (Chapter 3; Chapter 5), implicating metabolic factors. As outlined in Sloboda et al. (2007), accelerated post-natal growth has been linked to metabolic disturbances and disease onset, the onset of reproductive disorders such as polycystic ovarian syndrome (PCOS), and endocrine dysregulation which may compromise female heath and reproductive fitness.

8.4 Perinatal Programming of Peripheral Inflammation and Immune Vulnerability

An additional later-life psychological stressor was including in the experimental design in Chapter 5 and 6 based on previous studies from this laboratory and others demonstrating that a second hit is often required in revealing a physiological vulnerability induced by early life immune stress in the female rat (Li et al., 2007; Shalev & Belsky, 2016; Walker et al., 2012; Walker et al., 2011; Walker et al., 2010). In response to a restraint stress protocol, female animals treated with LPS displayed an exaggerated immune response indicated by upregulated circulating interleukin (IL)-6 expression. Of note, LPS treated animals are displaying a classic response, as this attenuated response pattern was also typical of saline animals treated with stress. Additionally, LPS animals treated with LPS displayed alternate

patterns of IL-2 expression both in response to LPS and a double hit of stress. As IL-2 is a known inducer of T-cell differentiation (Price-Troska et al., 2016) this may suggest dysregulation of T cell propagation, a profile that is reported in female patients with idiopathic infertility (Lukassen et al., 2003). Together, these findings suggest that NIA treated females have a particular immune susceptibility to later life psychological stress, leading to exaggerated inflammatory responses. Although an alternative adulthood stressor is utilised in this thesis, the current findings are in line with studies demonstrating neonatal LPS paired with adult LPS exposure leads to immune dysregulation (Boisse et al., 2004; Ellis et al., 2006; Spencer et al., 2006; Spencer et al., 2011). The current results indicate a specific immune vulnerability to a number of commonplace stressors, including bacterial exposure and psychological stress, which may be detrimental both on their own, but have an additive effect when paired. What is more, dysregulation in these circulating mediators is involved with ovarian disorders, including ovarian cancer, PCOS and endometriosis, and therefore may be contributing to a subfertile phenotype.

8.5 Perinatal Programming of the Ovarian Reserve

8.5.1 Acute impact of neonatal immune activation

The current model of neonatal LPS exposure coincides with a sensitive window of immune-mediated ovarian development in the rat (Pepling, 2006, 2012; Pepling & Spradling, 2001). In humans, this process begins around week 16 (Motta et al., 1997), and consist of the final breakdown of germ cell clusters to create primordial follicles. The number of primordial ovarian follicles represents the entire ovarian reserve that a female woman or rat will possess over the reproductive lifespan, and thus is a tightly mediated and controlled event. In chapter 4 of this thesis (Fuller et al., 2017), we examined the immediate effects of LPS on the

morphology of the neonatal ovary in order to gain a greater understanding of the mechanisms within the ovary which may be influenced by immune stimulation and to establish if follicle depletion was present in the early neonatal period. In this study, it was demonstrated that NIA leads to a depletion of the primordial follicle pool on PND 5, following LPS treatment. Additionally it was demonstrated that LPS exposure lead to a significant increase in the activation state of primordial follicles. This is of importance during this initial stage of ovarian development, as premature activation of otherwise quiescent follicles leads to apoptosis. It is important to note here, that this period of ovarian development in the rat is characterised by a normal wave of follicular atresia, however this is an intricate event dictated by controlled checkpoints, which ensures that only the highest quality of follicles proceed through to later stages of development (Grive & Freiman, 2015). Changes in the immune milieu of the ovary may alter the process of these specific checkpoints. No changes in primary follicle depletion was demonstrated in PND 5 animals (Fuller et al., 2017), suggesting that this may not be the optimal time to observe diminishment of this follicle type, particularly as depletion was observed in the long term both in this thesis and previously (Chapter 5; Sominsky et al., 2012).

In Chapter 4, the diminishment of the ovarian reserve coincided with upregulation of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) expression in LPS treated females, indicating increased apoptosis was occurring (Fuller et al., 2017). Hence, this premature activation of the follicle pool by LPS stimulation may not only lead to further spontaneous depletion of the ovarian reserve, but also decrease the quality of the remaining follicles that are to be chosen for later selection and ovulation following puberty onset. Future studies may examine the oocyte quality of LPS treated females using both cellular and molecular predictors including morphology of the polar body and meiotic spindle, and mitochondrial status, however this was outside the scope of this thesis. Therefore, LPS

treatment in this model possibly compromises both the fidelity and stability of the ovarian reserve via immune mediation as discussed in subsequent sections. Additionally, alterations to the fundamentals of reproductive functioning within the ovary may disrupt cytokine and chemokine mediated oocyte-granulosa crosstalk and ovarian-brain communication, contributing to behavioural alterations and culminating in a subfertile phenotype.

8.5.2 Sustained impact of neonatal immune activation

Chapter 5 of this thesis aimed to garner an understanding of the long term ramifications of NIA on the finite ovarian reserve, and determine whether ovarian follicle numbers are susceptible to a second hit of adulthood stress. Previous studies from our laboratory report that ovarian primordial follicle loss was demonstrated in the late neonatal period, following LPS exposure (PND 14) (Sominsky et al., 2012a). This thesis extends this by demonstrating acute depletion in Chapter 4 (Fuller et al., 2017). Chapter 5 develops this further, reporting that primordial follicle depletion in LPS treated females is sustained through to adulthood, a novel finding in this laboratory. The premature loss of the ovarian reserve has detrimental consequences for reproductive fitness and longevity, but also health complications arising from deficiency and dysregulation of ovarian-produced steroids, including PCOS, osteoporosis, metabolic disorders and autoimmune disorders.

Neonatal LPS treatment also lead to the significant increase in activated follicles in adulthood, as demonstrated in Chapter 5, which is a novel finding. The addition of restraint stress did not alter the number of follicles in there activated morphological state LPS treated animals, but there was an increase in activation (demonstrated by granulosa number and shape) demonstrated in saline/stress treated females. This indicates that the ovary is indeed sensitive to adulthood psychological stress under normal conditions, which may result in

follicular loss if it is particularly severe or chronic. This is likely, as it is known that stress has a deleterious effect on female reproduction by suppression of the HPG axis, however it may also be effecting the ovary directly, perhaps via immune regulation as demonstrated throughout this thesis (Dobson & Smith, 2000; Joseph & Whirledge, 2017; Nepomnaschy et al., 2007; Wingfield & Sapolsky, 2003). Interestingly, the activated follicle number did not increase as a function of stress past those levels already elevated by NIA. Perhaps the early-life compromised ovary deploys a perinatally programmed compensatory mechanism in order to protect the follicle pool from further depletion.

Importantly, a decrease in primary follicles was apparent following the combination of neonatal LPS and adulthood stress. This indicates that more mature follicles may be vulnerable to later life stressors following NIA, and/or that activated follicles are not reaching this developmental stage due to early apoptosis. No significant differences existed for larger follicle types in adult animals. Of note, antral follicle populations were observed to be increased in those animals that were exposed to stressors, compared to the control group. This may indicate a premature increase in cohort numbers destined for follicle selection and therefore atresia, however, may also be indicative of the ovary needing to sample from a larger pool to find competent dominant follicles in the rat. The loss of ovarian reserve has been demonstrated by others using similar LPS models (Wu et al., 2011a; Wu et al., 2011b), as well as in differing model of early life stress, including under and over nutrition (Chan et al., 2015; Sominsky et al., 2016), indicating the vulnerability of the ovarian reserve to early life environmental perturbation.

8.6 Mediators of Acute and Sustained Ovarian Follicle Depletion

The mechanisms governing initial follicle dynamics are elusive in nature and a matter of continued investigation. However, current evidence indicates that complex interactions between cytokines, chemokines, growth factors, transcription factors, neuroendocrine mediators and catecholamines coordinate the activation of quiescence of the ovarian reserve (Grive & Freiman, 2015; Kim, 2012; Reddy et al., 2010). Disruptions to these delicate processes can lead to dysfunction. As such, Chapter 4 (Fuller et al., 2017) and 5 of this thesis aimed to elucidate potential short and long-term mediators within the ovary that may be deregulated by NIA and contribute to follicular depletion and behavioural change.

Investigation of apoptosis was needed in order to confirm follicle depletion as a consequence of NIA. This was established in the paper presented as Chapter 4. Caspase 3 (CASP3) staining was increased in the ovaries of LPS treated animals, indicating the beginnings of apoptosis stimulated pathways on PND 5. Apoptosis was increased in LPS treated neonatal females, with the significant upregulation of TUNEL positive staining in the ovary, most probably due to PND 3 exposure. Additionally, gamma H2AX staining indicated that this DNA damage was localised to the oocyte which would affect signalling to the granulosa cells. These data extend on previous research from our laboratory as changes in proliferation and apoptosis were not demonstrated (Sominsky et al., 2013b). These current data would be further strengthened by the additional assessment of markers of autophagy, including Beclin1 and microtubule-associated protein light chain 3 (LC3) in order to determine more specific pathways of follicular loss. However, as they stand, these findings indicate cell and timing specific apoptosis which may regulate the ovarian reserve and dictate the reproductive lifespan.

Expanding on previous findings, Chapter 4 explored protein expression in the PND 5 ovary by mass spectrometry. This indicated that 29 proteins were differentially expressed in the ovaries of LPS animals. On analysis, these were associated with acute LPS-mediated inflammatory signalling, apoptotic pathway activation, and ovarian steroidogenesis. Ingenuity pathway analysis indicated the top protein signalling pathways in the ovary of LPS treated females was associated with the acute phase response and cholesterol synthesis. These findings highlight specific molecular pathways and mediators of novel interest for examination within the NIA model which may be contributing to follicular depletion and possible ovarian impairments, however point towards immune mediation of follicular apoptosis.

Chapter 4 also aimed to supplement previous work by examining the genetic expression of growth factors, which were previously unexplored in the NIA model yet are crucial to oocyte-granulosa crosstalk and follicular maturation and maintenance. Growth differentiation factor (GDF()-9 and forkhead box O3 (FOXO3)-a are known to govern the maintenance and development of the ovarian reserve and be influenced by inflammatory stimulation (Greene et al., 2014; Liu et al., 2009; Saatcioglu et al., 2016; Smith et al., 2014). In this chapter, we demonstrated that Gdf-9 gene expression, however not Foxo3-a, was upregulated in the ovaries of LPS treated female pups on PND 5. Gdf-9 has a stimulatory effect of primordial growth, whereas Foxo-3a is implicated in the maintenance of the follicular pool. Hence, enhanced expression of Gdf-9 stimulated by immune pathways, may be prematurely initiating activation of the primordial reserve and contributing to follicle loss. Aberrant Gdf-9 signalling also has detrimental long term consequences, as it is involved in ovarian hormonal processes and implicated in PCOS and premature ovarian failure (POF), as discussed in Chapter 4. Unexpectedly, Foxo-3a remained unchanged indicating that this transcription

factor may be not be involved in LPS induced defence of the ovary at this time. Further investigation at differing time points is needed, along with additional growth and transcription factor expression analysis, including kit ligand. However, as the mechanisms governing follicle processes remain to be fully elucidated, these findings provide insight into the processes stimulating premature follicle loss from early life bacterial exposure.

We suggest that early life immune perturbation alters immune mediated ovarian development and consequently sets a long term proinflammatory tone within the ovary. As such, proinflammatory cytokines and pathways were analysed within the neonatal and adult ovary in order to test this supposition and pinpoint pathways of dysregulation. Acutely, tumour necrosis factor (TNF)α and mitogen activated protein kinase 8/Jun N-terminal kinase 1 (MAPK8/JNK1) gene expression was upregulated in the neonatal ovary. Importantly, this upregulation was sustained into adulthood in unstimulated NIA treated females, as described in Chapter 5. Additionally, sustained changes to ovarian IL-6 and IL-6 receptor gene expression was demonstrated in the ovaries of adult females treated with NIA and an additional stressor. These findings suggest that key proinflammatory mediators are dysregulated within the ovary, and importantly, are vulnerable to psychological stress. These cytokines are known to stimulate and potentiate inflammation and the stress response, and are critical mediators of ovarian processes, both in early development and throughout the reproductive lifespan (Boots & Jungheim, 2015; Bornstein et al., 2004; Eddie et al., 2012; Tingen et al., 2009; Wu et al., 2004). As such, changes to the immune balance within the ovary may also contribute to hormonal alterations and behavioural deficits.

Previous reports from our laboratory suggest that toll-like receptor (TLR) 4 signalling within the ovary may be responsible for increases in long-term ovarian inflammation. In order

to test this hypothesis, we examined both the short and long term gene expression of TLR4 in the ovary. TLR4 is reported to be present on ovarian granulosa cells (Bromfield & Sheldon, 2013; Herath et al., 2007). Previous studies indicate an upregulation of TLR4 gene expression on PND7 (Sominsky et al., 2013b). In the current thesis, no changes to the protein expression of TLR4 were demonstrated in the neonatal ovary following LPS stimulation. Additionally, a trend for TLR4 gene downregulation was observed on PND 5, which is not surprising given the timing of LPS stimulation on PND 3 and 5 which may be creating a habituation effect.

Additionally, we examined the long term expression of TLR4 in adult ovaries. No changes were demonstrated across any treatment groups, indicating that not only does there seem to be prolonged alterations to this receptor within the ovary, but that it also was not susceptible to a psychological stressor. Together, these findings indicate that TLR4 expression does not mediate increased cytokine production within the ovary in the long term. This isn't to suppose that changes would not be seen with a different mode of second hit. Perhaps immune stimulation, or a more chronic stressor in later life may be needed to demonstrate a perinatal programmed vulnerability to this receptor. Others have shown TLR4 mRNA increases after chronic stress and social stress in rats and mice (Bailey et al., 2007; Gu et al., 2009; Wang et al., 2011), however the ovary may be resistant to such changes. Perhaps other immune receptors that are known to be expressed on ovarian macrophages may be responsible for the sustained inflammation demonstrated in this thesis, for example, proinflammatory cytokine binding. This is possible, considering the known susceptibility and plasticity demonstrated by macrophages in response to early life inflammation (Mosser & Edwards, 2008) and the macrophage and cytokine contribution to ovarian function (Figueroa et al., 2015; Wu et al., 2004). Additionally, the ovary is known to produce local cytokines expression in response to infection, but also as part of normal processes, including follicular

selection and ovulation. This idea is lent credence by the findings presented in this thesis demonstrating consistent upregulation of proinflammatory cytokines both in the neonatal period and in adulthood within the ovary and in central regions.

8.7 Perinatal Programming of Central Mediators: Contribution to Behaviour

Chapter 6 of this thesis aimed to investigate the long term gene expression of central inflammatory and stress mediators in female rats following NIA and a second hit of stress. Both immune and endocrine parameters were measured, as was the neuropeptide KISS1 and its receptor, and tyrosine hydroxylase gene expression. Early life immune stress is known to lead alterations in central immune activation, however this has not be established in the context of female subfertility. Furthermore, the additional of a subsequent later life stressor allowed for the examination of central vulnerability if these mediators to stress. Three regions were investigated in the female adult brain that are known to be involved in the control of reproductive and stress related behaviours, and that have previously been demonstrated to yield robust changes in response to NIA and adulthood stress.

In Chapter 6 we establish that early life stress upregulates central inflammation in the hypothalamus, the hippocampus and the medial preoptic area. All three areas exhibited consistent changes in mRNA expression of TNFα receptor, IL-1β, and cyclooxygenase (COX)2, changes similar to that in the ovary demonstrated in Chapter 5. These cytokines are expressed by activated microglia and other central immune cells. Additionally, they share a similar activation pathways, namely the canonical nuclear factor (NF)-Kappa β (Kβ) pathway and the MAPK/JNK pathway (Lawrence, 2009). Activation to these pathways and their mediators are involved with pathological neurodegenerative diseases as well as the modulation of behaviours. Chapter 6 demonstrated subtle alterations to the central MAPK pathway,

however NF-Kβ was not examined. However, the robust and multiregional changes demonstrated in central IL-1β expression indicated the alterations to this pathway are likely. NF-Kβ is activated via IL-1β on the cell membrane. The lack of significant change in MAPK/JNK suggests that this may be mediated via IL-1β receptor 1, working through MyD88/TRAF facilitated interleukin-1 receptor–activated protein kinase (IRAK) pathway. Downstream regulators of this IL-1β activated NF-Kβ pathway should be explored. This argument is further strengthened by the alterations in gene expression of COX2, a known IL-1β/ NF-Kβ responsive gene (Weber et al., 2010).

Importantly, IL-1β is also a potent modulator of female sexual behaviour, acting synergistically with TNFα. Avitsur and Yirmiya (1999b) demonstrated that both LPS and IL-1β exposure in adulthood supressed all aspects of female, but not male, mating behaviour. Additionally, it was demonstrated by Avitsur and Yirmiya that this was context specific to sexual behaviour, and the effect was inhibited with an IL-1 receptor antagonist. As such, these aforementioned reports and the current data presented in Chapter 6 provides strong evidence for the hypothesis that IL-1β and TNFα are driving the female mating impairments induced by LPS exposure in early life. Additionally, Avitsur and Yirmiya (1999a) demonstrated that inhibition of TNFα prevented lordosis suppression in female rats following adult LPS exposure, indicating that this cytokine is essential for the lordosis response. Chapter 3 demonstrated a reduced lordosis in neonatally treated LPS females. When paired with the findings in Chapter 6, strong evidence emerges indicating that NIA is mediating the behavioural changes demonstrated via chronic upregulation of IL-1β and TNFα signalling, which may be further mediated by COX2 and prostaglandin (PG) signalling, as described by (Avitsur et al., 1999; Avitsur & Yirmiya, 1999a; Yirmiya et al., 1995). Hence, the findings of this thesis suggest that the effects of neonatal LPS exposure on female reproductive behaviours

are mediated by perinatally programmed inflammatory dysregulation, of both central and peripheral origin.

This central immune dysregulation may be modulated by a number of factors. Firstly, the findings from Chapter 6 indicate central alterations to TLR4 gene expression within the hypothalamus (HTH), which may be altering stress responsivity. When paired with the peripheral findings presented in Chapter 5, this suggests that TLR4 expression is differentially altered as a result of NIA within the brain and the ovary. Secondly, alteration to HPA axis components were also observed in LPS treated females, including glucocorticoid receptor (GR) gene expression in the hippocampus (HC) and both GR and mineralocorticoid (MR) expression in the HTH. Results presented in Chapter 3 demonstrate a hyperactive CORT response in NIA treated females yet no significant changes to LH and FSH. Regardless, neuroendocrine involvement is likely mediating this inflammation but perhaps only subtly contributing to HPG axis processes. Of note here, significant changes in CRH and CRHR1 were not demonstrated in Chapter 6 of this study, however they are known to contribute to suppression of central GnRH pulsality. Thirdly, NIA increased KISS1R gene expression in the hippocampal region. A key regulator of HPG signalling, this neuropeptide may contribute to alterations in pubertal onset demonstrated in this thesis. Others have shown downregulation of KISS1 in the hypothalamus following neonatal LPS exposure, results which were not corroborated in these studies. However, the novel findings of KISSR1 alterations in the hippocampus demonstrates that KISS1 signalling may contribute to limbic governance of sexual behaviour and ovarian functioning, with region specific alterations occurring via neonatal LPS exposure. Fourthly, significant alterations in TH were observed in the medal preoptic area reported in Chapter 6, a region directly involved in mating and female reproductive behaviours. This indicates the involvement of catecholaminergic signalling.

Sustained NIA induced alterations to this central signalling pathway were demonstrated in male rats (Chapter 7; Ong et al., 2017). When paired with data from Chapter 6, this provides a compelling indication that catecholamine activation may be involved in the programming of the female subfertility phenotype and is a novel pathway of interest. Lastly, Chapter 7 suggests a second novel pathway for examination of the female subfertility phenotype, the kynurenine pathway (KP). Preliminary data provides evidence of NIA sensitivity to this pathway and its mediators, including indolamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO). Immune and stress mediators activate branches of the KP, and the hyperactivity of both systems was demonstrated in female animals challenged with NIA throughout this thesis. Additionally, emerging evidence links this pathway to behaviour alterations. As such, examination of this pathway within a female subfertility context is warranted.

8.8 Conclusions, Future directions, and Implications

8.8.1 General Summary

The chapters presented within this thesis provides evidence for the short term impact of neonatal bacterial exposure and the long term perinatal programming of a specific female subfertile phenotype. Furthermore, data presented here indicates that behavioural and biological alterations following NIA have a proinflammatory basis, set from early life experience. Chapter 3 of this thesis establishes the subfertility behavioural phenotype in the female rat including precious puberty onset, and hones the factors that may be driving these alterations. Paper 2, presented as Chapter 4, examines the short term impact of neonatal immune activation on the ovary in order to gain a better understanding of how fundamental ovarian development may be contributing to the subfertile phenotype. The findings from this

paper indicate that neonatal LPS has a direct influence of the inflammatory status of the neonatal ovary, and immune dysregulation is involved in the acute depletion of the ovarian follicular pool. Chapter 5 built on the previous chapter by examining the long term peripheral consequences of NIA, both with and without an additional stressor. Here, it was demonstrated that NIA leads to an immune vulnerability to later life stress in the female rat. Furthermore, it confirmed earlier onset of puberty and provided evidence for sustained changes to the ovarian inflammatory environment which may be sensitive to exogenous stressors. Lastly, this chapter demonstrated that the acute depletion of the ovarian reserve was carried through to adulthood, which has implications for female reproductive fitness, longevity and health. The following Chapter 6 examined the long term central alterations in three regions that control reproductive behaviours and are associated with psychopathologies. This chapter identified sustained changes in the gene expression of key inflammatory mediators that were stable through the three regions, implicating these in the perinatal programming of female sexual behaviours. Hence, this thesis creates a more defined picture of the female subfertile phenotype that arises from an early life neonatal LPS challenge.

8.8.2 Future Directions

Implications for further research are discussed throughout this thesis. Specific novel pathways of interest, including the perinatal programming of central catecholaminergic and KP activation, are outlined in Chapter 7 and discussed throughout this chapter. Multiple avenues may be considered for future direction within this NIA model, with some having implications for both female and male animals. These include both additional behavioural and molecular testing.

Behaviourally, the findings from this thesis would be strengthened with the addition of a second measure of depressive-like behaviours, such as the forced swim test in order to rule out depressive-like behaviours following NIA without doubt. Furthermore, behavioural tests relating to reward pathway sensitisation should be examined in order to establish the contribution of catecholamine synthesis in the female as well as the male rat, such as progressive ratio testing or amphetamine locomotion for dopamine sensitisation. Additionally, greater examination of the neuroendocrine contribution to the female subfertility phenotype would be of benefit. This thesis examined circulating CORT, LH, FSH and gene expression of FSH receptor in the ovary and brain, however other hormonal mediators and their receptors, including progesterone and oestrogen would facilitate a greater understanding. Additionally, to further test the subfertility phenotype, subsequent F1 and F2 generations may be bred to examine the impact on fertility of these parental generation LPS treated females. Previous investigations from our laboratory (Sominsky et al., 2012a) demonstrated increased no alterations in F1 fertility rate, however mortality, morbidity, and corticosterone levels were increased in females born to neontally treated LPS mothers. This suggest alterations in pathways which mediate reproductive parameters.

In regards to ovarian and central inflammation, inhibition of cytokines IL-1β and TNFα may demonstrate the direct consequence of these factors on female sexual behaviours and ovarian development from NIA. This may be done a number of ways, including prior to neonatal LPS stimulation, or prior to sexual behavioural testing following NIA. Furthermore, given the role of macrophages, microglia and astrocytes to central and peripheral inflammation, these are good targets for future evaluation. Pinpointing the origin of sustained inflammation stemming from neonatal LPS exposure will inform on functional significance. A number of molecular pathways remain to be analysed within the ovary in order to understand

the inflammatory contribution to the acute and sustained loss of the ovarian reserve, including the contribution of growth and transcription factors, and the specific inflammatory pathways that may contribute to dysregulation including NF-Kβ. Furthermore, ovarian follicular quality, and the quality of communication between the oocyte-granulosa complex following NIA, should also be assessed both acutely and long-term. Investigation of these parameters would further elucidate on the mechanisms and system interactions involved in the female subfertile phenotype stemming from neonatal LPS exposure.

8.8.3 Implications

This thesis raises important lines of questioning regarding the role of the early life environment in female reproductive development. Firstly, in a contemporary setting, does the ecological validity and outcomes of an early life bacterial stressor hold? And secondly, how do these findings translate into human female populations in order to broaden our understanding of long term reproductive health and fitness. Furthermore, a distinction should be made between 'infertility' and 'subfertility' within the context of this thesis. This thesis demonstrates that seemingly innocuous alterations in immune and endocrine functioning at a critical period of development may subtly alter the long-term tone of these integrated systems. For the rat, these alterations may manifest as changes in female reproductive parameters that do not affect fertility rates but lead to a suboptimal reproductive phenotype. Hence, the rat phenotype explored here is one of subfertility, rather than infertility. However, in more complex biological systems such as humans, these affects may become more pronounced, especially when considering the many stressors that exist for women in the current climate and the interaction of these within everyday environments.

In an age where immunisations and increased hygiene awareness have dramatically decreased the incidence of bacterial and viral exposure in early life, it may seem that bacterial exposure as a perinatal stressor may be redundant. However, emerging evidence indicates a resurgence of bacterial-born infections, antibiotic-resistant strains of bacteria, and increases in sexually transmitted infections (Wells & Piddock, 2017). Recent literature indicates an increase in scarlet fever (a strep-family bacteria) outbreak in the UK and Asia, predominantly infecting newborns and those in nurseries and preschool, which has implications for the prepubertal ovarian reserve and the developing infant immune system (Lamagni et al., 2017). Sexually transmitted infections, such as chlamydia, contribute to pelvic inflammatory disease and result in infertility. Increased instances of unchecked maternal chlamydia, the most commonly reported sexually transmitted infection (STI) in Australia, the US, and Europe, leads to pneumonia and conjunctivitis infection in newborns, with approximately 15% of newborns infected at birth (Darville, 2005). These high infection rates demonstrated are presumably due to the non-symptomatic nature of this infection and the normalisation of unprotected sex. Additionally, novel investigations examining interactions between infectious diseases and climate change in human populations are only now beginning to emerge, these include increases in bacterial cholera and tuberculosis outbreaks and parasitic outbreaks such as leishmaniasis and Zika (The Lancet Editorial, 2017; Liang & Gong, 2017; Wu et al., 2016). As such, bacterial exposure and inflammation modelling not only maintains validity as an appropriate environmental stressor, but has increased significance as a naturalistic laboratory model.

In order to answer these questions in full, a brief examination of the perinatal stressor employed in this model is needed. LPS is used to model bacterial exposure and immune activation, where it leads to the upregulation of inflammatory pathways and mediators via

TLR4 binding, as well as activating the HPA axis. Hence, this stressor has implications for interconnected systems that are known to be susceptible to environmental influence, and thus vast repercussion for numerous tissues and cells (Bluthé et al., 1994; Ellis et al., 2006; Kentner & Pittman, 2010; Spencer et al., 2011). As such, the mechanisms of LPS as a stressor may be transposed to the broad concept of 'inflammation' and 'chronic inflammation'. Current evidence indicates that inflammatory induction results from a number of factors and sources, including pollutants, toxins and teratogens, nutritional status, fatty acids, oxidative stress, and stress. Additionally, there is cross over between activation pathways, toll-like receptors and pathogen-associated molecular patterns. Hence, inflammatory activation via a bacterial mimetic has wide-ranging implications for a number of states that may induce similar immune activation in early life and contribute to long-term chronic inflammtion.

This notion of inflammatory stress is of particular importance when considering the increase in obesity worldwide. A hallmark of obesity is chronic proinflammation, which is associated with other metabolic and reproductive disorders including glucose intolerance, diabetes, cardiovascular disorder, PCOS and endometriosis. Obesity is a current epidemic that is increasing in prevalence in younger children, as well as young women of reproductive age. As such, this has transgenerational implications. Furthermore, body mass and body fat index is associated with advanced pubarche and menarche in female children, a known risk factor of adulthood disease. Additionally, the important role nutritionally induced inflammation plays on the developmental origins of female reproduction and ovarian function are fully emerging (Newnham et al., 2002; Sloboda et al., 2011). Thus, evidence points towards the importance of the early life environment in adult health and disease, and the need for continued investigation of this field.

In conclusion, this thesis demonstrates that the early neonatal period in the female rat is a critical window of sensitivity for the immune programming of female reproductive physiology and behaviour. The development of the finite ovarian follicular pool, the brain, and the immune and endocrine system is dependent on homeostatic processes, which if disrupted, may lead to sustained alterations to ovarian physiology. This timing equates to the 3rd trimester in human development where immune activation and bacterial exposure is commonplace. Thus inflammatory activation experienced during this time in the rat has implications for stress and inflammatory exposure experienced during the perinatal period in human females. The findings presented within this thesis are of particular importance due to a number of factors including; the increases of idiopathic subfertility and reproductive disorders in younger women, the advanced age of childbearing and the complications this may incur if the ovary is compromised, and the constant emerging evidence in support of a developmental origin for female reproductive health and longevity (Gur et al., 2015; Hernández-Angeles & Castelo-Branco, 2016; Ho et al., 2017; Isaksson & Tiitinen, 2004; Kamath & Bhattacharya, 2012; Maheshwari et al., 2008; Sloboda et al., 2011).

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