PERINATAL PROGRAMMING - INTEGRATION OF BRAIN, BEHAVIOUR AND IMMUNITY: IMPLICATIONS FOR REPRODUCTIVE FITNESS

Presented By

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Declaration

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List of publications included as part of the thesis

1. **Sominsky L.**, Walker A.K., Ong L.K., Tynan R.J., Walker F.R., Hodgson D.M. (2012) Increased microglial activation in the rat brain following neonatal exposure to a bacterial mimetic. *Behavioural Brain Research.* 226(1), 351-356.

2. **Sominsky L.,** Fuller E.A., Bondarenko E., Ong L.K., Averell L., Nalivaiko E., Dunkley P.R., Dickson P.W., Hodgson D.M. (2013) Functional programming of the autonomic nervous system by postnatal immune challenge: implications for anxiety. *PLOS ONE* 8(3): e57700. doi:10.1371/journal.pone.0057700

3. Walker A.K., Hiles, S.A., **Sominsky L.,** McLaughlin E.A., Hodgson D.M. (2011) Neonatal lipopolysaccharide exposure impairs sexual development and reproductive success in the Wistar rat. *Brain, Behaviour & Immunity,* 25 (4), 674-684

4. **Sominsky L.,** Meehan C.L., Walker A.K., Bobrovskaya L., McLaughlin E.A., Hodgson D.M. (2012) Neonatal immune challenge alters reproductive development in the female rat. *Hormones and Behavior*. 62(3), 345-355

5. **Sominsky L.,** Sobinoff A.P., Jobling M.S., Pye V., McLaughlin E.A., Hodgson D.M. (2013) Immune regulation of ovarian development: programming by neonatal immune challenge. *Frontiers of Neuroscience.* 7(100). doi: 10.3389/fnins.2013.00100

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Thesis abstract

Events occurring in early life can induce long-term physiological and behavioural changes through the process of perinatal programming. The concept of perinatal programming has an adaptive value, preparing the foetus for specific extra-uterine demands. As such, early life adversity is thought to enhance an immediate survival via physiological adaptation when the postnatal environment is similar to the prenatal environment. However, under conditions of discrepancy between the early and later life environment, this adaptation may prove disadvantageous, leading to physiological and psychological changes that may predispose the organism to poorer long term health outcomes. Early life adversity, elicited by changes in the nutritional environment, or due to an exposure to stressful and traumatic events, has received increasing recent attention. One model of early life adversity that has been useful in modelling developmental outcomes associated with the early life environment is the model of "neonatal immune challenge". Specifically, previous research has identified the early microbial environment as a critical factor in the development of mood and behaviour, with increased immune activation during neonatal life having been linked to an emergence of anxiety behaviours in adulthood. The primary aim of the current thesis was to investigate the immediate and long term effects of neonatal immune challenge on the neuroimmune and neuroendocrine pathways, which are proposed to underpin the altered behavioural phenotype. To achieve this aim the Wistar strain rat model was employed. To simulate an immune challenge, these animals were intraperitoneally administered lipopolysaccharide (LPS; *Salmonella enteri*ca, serotype *enteritidis*), on postnatal days (PNDs) 3 and 5 (birth = PND 1). Importantly, an established framework of an anxiety-like phenotype was expanded to encompass a wider range of behavioural changes. Thus, in addition to anxiety-like

behaviours, sexual behaviour was examined, along with the underlying regulatory mechanisms of reproductive development and function.

The first paper (Sominsky et al., 2012b) in this thesis reported that neonatal LPS exposure is associated with increased microglial activation in the adult brain, corresponding to an increase in anxiety-like behaviours. Given the mediating role of microglia in inflammation-induced psychopathology, the results of this study suggest a neuroimmune pathway which may underpin the long term behavioural changes observed in adulthood following neonatal LPS challenge. Moreover, the increase in microglial activation was specific to the hippocampal areas of the brain, suggesting a susceptibility of this primary HPA axis-regulatory region to neonatal immune challenge and thus supporting previous research which has demonstrated programming of the HPA axis activity by neonatal LPS exposure.

The second paper (Sominsky et al., 2013a) investigated the neurocircuitry of the anxiety observed in relation to early life exposure to LPS, specifically by examining the central gene expression in association with peripheral endocrine and autonomic activity. The data indicated that neonatal LPS induces an altered expression of the GABA-A receptor α2 subunit, CRH receptor type 1, CRH binding protein, and glucocorticoid receptor mRNA levels in the prefrontal cortex, hippocampus and hypothalamus of adult rats. These changes were associated with a persistent elevation of circulating corticosterone. Furthermore, the long term effects of neonatal LPS exposure were examined for the first time on autonomic function. The data indicate that neonatal LPS exposure results in increased autonomic arousal, as indicated by increased activity of tyrosine hydroxylase in the adrenal glands and increased respiratory rate in response to mild sensory stress. The findings of Paper 2 therefore suggest that neonatal immune challenge produces a prolonged alteration in both central and

peripheral measures of the HPA axis activity, associated with a persistent change in autonomic function, and potentially contributing to the anxiety-like phenotype.

Given the link between anxiety and reproductive outcomes a subsequent paper further characterised the behavioural and reproductive profile of neonatally treated rats. Sexual behaviour as well as reproductive capacity were assessed in Paper 3 (Walker et al., 2011). Outcomes of this study revealed that neonatally treated rats exhibited impaired mating behaviours, accompanied by persistent HPG suppression. In addition, morphological assessment of the male gonads revealed immediate and long term alterations in the testicular morphology of LPS-treated males. A follow-up Paper 4 (Sominsky et al., 2012a) continued to explore these outcomes with a focussed analysis of reproductive development in the female rat, including ovarian morphology. In addition to alterations in the timing of pubertal onset and endocrine function, diminished ovarian follicular reserve was observed in LPS-treated females when compared to non-treated animals. Taken together the findings of Papers 3 and 4 suggest that neonatally LPS-treated rats demonstrate a subfertile phenotype in adulthood, and this is mediated by functional and morphological changes to the gonads, indicating for the first time a specific susceptibility of the developing gonads to an immune challenge. Therefore the aim of the final Paper 5 (Sominsky et al., 2013b) was to assess whether neonatal LPS may have a direct impact on ovarian development via alteration of the ovarian immune milieu. The results of this paper indicated that neonatal LPS exposure induces activation of inflammatory signalling in the ovary, potentially mediated via increased expression of Toll-like receptor (TLR) 4. Given that common bacterial infections, such as *E.Coli* and *Chlamydia*, are associated with increased TLR4 expression in reproductive tissues, which is thought to result in impaired fertility, the findings presented in Paper 5 provide a valuable insight into the link between early life infection and reproductive fitness.

Taken together, the papers presented in this thesis demonstrate that neonatal immune challenge contributes to long term programming of physiology and behaviour, fundamentally influencing reproductive fitness and success. The novel insights presented in this thesis, particularly those related to programming of autonomic function and reproductive development, significantly contribute to the understanding of a critical role of the early microbial environment in determining the developmental trajectories of an organism and advance the current knowledge in the perinatal programming field. The observed effects of neonatal immune challenge may be placed into a wider perspective, integrating the continued interaction between the immune system, the brain, the gonads, and the behavioural outcomes of this interaction, reflective of phenotypic plasticity in response to the changing environment.

Introduction and literature review

1. Developmental Origins of Health and Disease: Implications for perinatal programming

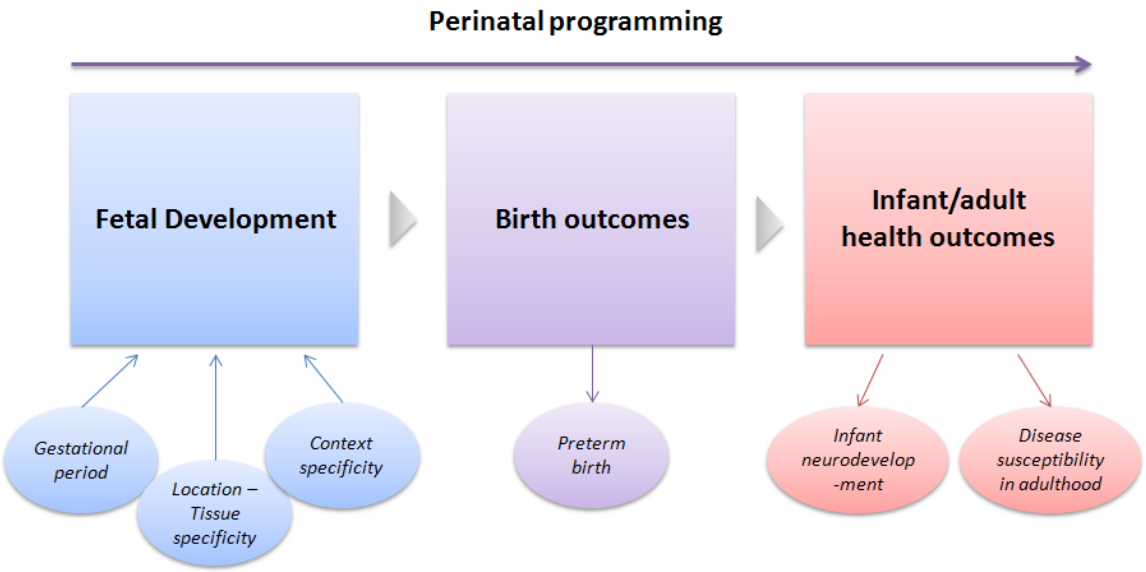
Parental and social environments are salient determinants of development, providing a set of values that help to establish who we become as a person. Biological determinants of development on the other hand often predict our physical appearance and health status. What is becoming increasingly apparent is the close interaction between the psychosocial and biological environments during early life, and its ability to influence development and function. This interaction between the environment and our biology is the focus of ever-expanding research, which has indicated that the degree of influence the environment holds over biological and psychological development, is dependent upon the timing of exposure and the nature of environmental events that we are exposed to. One period known to be particularly sensitive to environmental factors is the perinatal period, with many systems undergoing critical phases of development at this point in time. The area of research which focuses on the impact of environmental stimuli at or around the time of birth is known as *perinatal programming* (Hodgson and Coe, 2006).

The increased sensitivity of the organism to environmental inputs during the perinatal period is due to an elevated degree of developmental plasticity. Plasticity refers to the way the brain and other physiological systems can modify their phenotype in response to environmental pressure. While environmental regulation of the phenotype is a life-long process, development of neural and peripheral tissue occurs at a high rate during early life with each system demonstrating a critical period of enhanced sensitivity, after which the level of plasticity becomes more limited (Nelson, 2000). Hence, environmental factors can exert the greatest influence during these critical periods of development when plasticity is at its

highest. Once these critical periods have passed, reversal of the established phenotype becomes more difficult, explaining how the early life environment can induce persistent changes in morphology and function.

The enhanced plasticity of physiological systems during early development has evolutionary value in preparing the organism for the *ex-utero* environment, ensuring better adaptation and enhancing survival probabilities. However, any adversity experienced during this period may bring with it unwanted consequences and result in maladaptive programming, which interferes with the formation of essential physiological function and leading to negative health outcomes in later life. The epidemiologist Professor David Barker was the first person to formally present the potential for perinatal environmental factors to contribute to later life health and disease states. Barker proposed that adult disease states may have their origins in the early developmental period, and initially presented findings showing that death by stroke was more closely correlated to maternal mortality than any other cause of death, suggestive that maternal health was closely linked to the risk of disease in their offspring (Barker and Osmond, 1987). His subsequent studies demonstrated relationships between low birth weight and high placental weight with increased adult systolic blood pressure (Barker et al., 1990; Barker et al., 1989). Taken together, these data were the first to clearly connect the perinatal experience with later disease susceptibility, and eventually led to the establishment of *developmental origins of health and disease* (DOHaD) hypothesis. The DOHaD hypothesis provides a framework in which to understand human life-long development, stating that the long-term health or (susceptibility to) illness of an individual is strongly driven by the perinatal experiences of that individual (See Figure 1).

Figure 1: Perinatal programming of adult health and disease.



1.2 The impact of perinatal stress on adult health outcomes

Stressful or traumatic events experienced early in life have been demonstrated to facilitate the onset of later life pathologies and psychopathologies, supportive of the notion that environmental factors during critical periods of development can set the tone of physiological, cognitive and behavioural outcomes. Many lines of research have extended the DOHaD hypothesis, investigating how the different avenues of early growth and development may determine susceptibility not only to cardiovascular disease and type 2 diabetes, but also to other health conditions such as osteoporosis (Cooper et al., 1997), breast and ovarian cancer (Barker et al., 2009) and even predict longetivity of life (Shanley and Kirkwood, 2000). Moreover, low birth weight has been also associated with mental disorders, such as autism (Burd et al., 1999; Gardener et al., 2011), depression and suicide (Barker et al., 1996). Epidemiological and experimental evidence have indicated that discrepancies between the early and later life environments are not limited to nutritional factors, but extend to other potential impacts, such as immune and hormonal status, as well as mental states. As

such, the research has demonstrated how an exposure to physiological or psychological stressors during critical periods of foetal and neonatal development, involve vigorous activation of the neuroendocrine and immune response, disturb an internal milieu of the developing organism and thus are associated with increased risk of pathologies and psychopathologies. This evidence will be reviewed in the following sections and incorporated in the discussion of plasticity relating to neural, endocrine and immune systems during early development.

1.2.1 Perinatal programming of pathology

The impact of early life events is becoming increasingly apparent, as studies reveal how early development can shape long-term health. It is important to consider context-specificity, when interpreting the consequences of perinatal programming. In general, challenges experienced in early life have beneficial value, preparing an individual for life in a more hostile or challenging environment. The disease outcome of the programming event might not be induced by early life adversity per se, but by a mismatch between the early and later life environmental conditions, and by a more susceptible genetic predisposition of an individual. One of the most prominent examples of how an adaptive response may develop into later life adversity is the largely explored impact of reduced foetal growth. Apart from being a leading cause of perinatal morbidity and mortality, foetal growth retardation and consequential low birth weight has been linked to an increased risk of developing diseases in later life, including obesity, type 2 diabetes, hypertension and cardiovascular disease (Barker, 1998). Intrauterine growth restriction and low birth weight can be a result of both maternal high fat and unbalanced diet, as well as maternal malnutrition. This adverse intrauterine environment permanently alters the foetal metabolic and hormonal milieu, resulting in developmental adaptation for life in a sparse environment, to ensure survival. However, while

adjustments such as lower birth weight, earlier puberty, increased insulin resistance or increased capacity for fat storage allow for better adaptation and successful reproduction in an adverse environment, the same adjustments may become maladaptive when there is no lack of nutrients (Gluckman et al., 2009). Thus, when the *ex-utero* environment does not match previous metabolic experiences, an individual is more susceptible to metabolic dysfunction in later life. This predisposition is further exacerbated by adult lifestyle risk factors (e.g. smoking, alcohol consumption, lack of physical activity), increasing the risk of metabolic and cardiovascular disorders (Schlotz and Phillips, 2009). Therefore, consideration of the specific context (i.e. the match between the pre and post birth environment) is critical when investigating the relationship between early life stress and later life adversity.

This *mismatch* hypothesis is relevant not only to the physiological impact of changes in nutritional environment, but also to the effects of other stress factors on various health aspects, including behaviour (Schmidt, 2011). While subtle behavioural alterations, such as increased hypervigilance or avoidance, may protect an individual from potentially harmful situations in a dangerous environment, when these behavioural strategies are inappropriately or excessively utilised, they may develop into psychopathological behaviours, programming of which is briefly introduced below.

1.2.2 Perinatal programming of psychopathology

The foetal and neonatal brain is characterised by an extensive network of developing neuronal connections, and is especially vulnerable to the consequences of stress. Alterations in neural plasticity during this period can induce changes in cognitive function and behaviour, the nature and intensity of which appear to depend on the genetic vulnerability of the individual, the severity of perinatal stress and the timing of exposure (Belsky et al., 2009; Heim and Nemeroff, 2001; Heim et al., 2000).

Epidemiological evidence has indicated a link between maternal stress and increased risk for developing affective psychopathologies. Specifically it has been indicated that low birth weight and preterm birth, as a result of gestational stress, contribute to developmental impairments and motor dysfunction, common risk factors for schizophrenia (Weinstock, 2005). Other research has reported maternal stress during pregnancy to influence developmental delays, emotional status and learning skills in childhood (Brouwers et al., 2001; Kofman, 2002; Watson et al., 1999). These findings are however confounded by the continuing influence of maternal anxiety on the quality of maternal care, which can affect development and behaviour (Stevenson-Hinde et al., 2011). Retrospective studies reported a higher incidence of schizophrenia in adults born to mothers exposed to severe stress during pregnancy, such as the stress of war, famine, or a natural disaster (Susser and Lin, 1992; van Os and Selten, 1998; Watson et al., 1999). Moreover, postnatal abuse, maltreatment and neglect has been linked to a greater risk of psychiatric disorders in later life, including PTSD (Yehuda et al., 2005), anxiety disorders (Heim and Nemeroff, 1999), schizophrenia (van Os and Selten, 1998) and also mood disorders (Ackerman et al., 1998).

While data from human studies is able to provide only correlational links, animal models help to establish a causal relationship and allow in depth investigation of underlying mechanisms. Various animal models of prenatal and postnatal stress have been therefore utilised for the study of behavioural programming. In rats, maternal calorie restriction one week prior to conception has been demonstrated to result in increased anxiety-like behaviours in the male adult offspring (Levay et al., 2008). Similarly, increased anxiety-like behaviours were reported in animals subjected to acute foot shock stress *in utero* (Estanislau and Morato, 2005). Maternal immune activation, induced by administration of poly I:C, a viral mimetic, to pregnant dams on distinct gestational days has been repeatedly shown to modulate active avoidance learning, prepulse inhibition and latent inhibition in adult offspring, reflecting a

common endophenotype of schizophrenia. Prenatal immune stimulation increases amphetamine-induced locomotor activity in adulthood and produces histopathological abnormalities in the hippocampus and the entorhinal cortex, similar to the well-known mesolimbic dopaminergic and temporolimbic pathology in schizophrenic patients (Meyer et al., 2009; Smith et al., 2010; Zuckerman et al., 2003). Acute exposure to glucocorticoids during the early postnatal period in rodents has been reported to reduce brain weight and myelination, producing detrimental effects on brain development (Huang, 2011). Administration of dexamethasone (synthetic glucocorticoid) in neonatal rat pups has been shown to permanently alter brain function, by producing long-term alterations in anxiety-related behaviours and stress responsivity (Flagel et al., 2002).

Cumulatively, both prenatal and postnatal, human studies and animal models, of perinatal programming provide evidence that exposure to early life stress of various sources may permanently alter later life stress responsivity, leading to maladaptive stress responses and contributing to poor physical and mental health outcomes. Identifying the mechanisms implicated in the perinatal programming of stress responses is therefore paramount to increasing the understanding of affective disorders.

2. Mechanisms underpinning perinatal programming

The mechanism underpinning the DOHaD hypothesis can be referred to collectively as perinatal programming, whereby environmental factors predispose to later health outcomes via a shift in the functional 'tone' of physiological systems. Thus, the developing organism 'senses' the early life environment and uses this information to systematically establish homeostatic set points (Davies and Norman, 2002; Welberg and Seckl, 2001) . This involves the organisation of specific tissues and gene expression to optimise environmental adaptation (Meaney et al., 2007). Environmental factors, both intracellular and extracellular,

influence the development of whole organisms, systems, tissues, cells and molecules (Maes et al., 2009). Thus, programming persistently organises and imprints the development of bodily systems, consequently influencing physiology and behaviour.

The critical development of most physiological systems occurs in the early perinatal period. Among the major systems that are known to exhibit essential regulatory properties during early ontogeny are the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic pituitary gonadal (HPG) axis, the autonomic nervous system (ANS) and the immune system. The reciprocal neuroendocrine-immune relationship begins during the formation of these systems and regulates different stages of individual development. The establishment of this complex interaction has also an important role in programming physiological functioning in later life, hence any interference with the developmental trajectory of these systems and any disturbances in neuroendocrine-immune interactions at these early stages may lead to long-lasting predisposition to a variety of pathologies in adulthood.

Despite variability in the developmental timeline among different species, the functional activity of the neural, endocrine and immune systems is significantly lower in perinatal life than in adulthood. Therefore the early life period is crucial for the definitive development of an organism and disturbances in normal ontogeny of any of the physiological systems may lead to long term alterations in the functioning of other systems. The ability of environmental stimuli to produce robust programming effects is dependent on the developmental stage of the organism. While the same environmental stressor may cause no lasting effects in the mature organism, it can be detrimental if experienced during critical periods of development. The extent to which the trajectory of development of the HPA and HPG axes, the ANS and the immune system can be affected by environmental stimuli and

how these alterations can be manifested in physiological and behavioural abnormalities is described below.

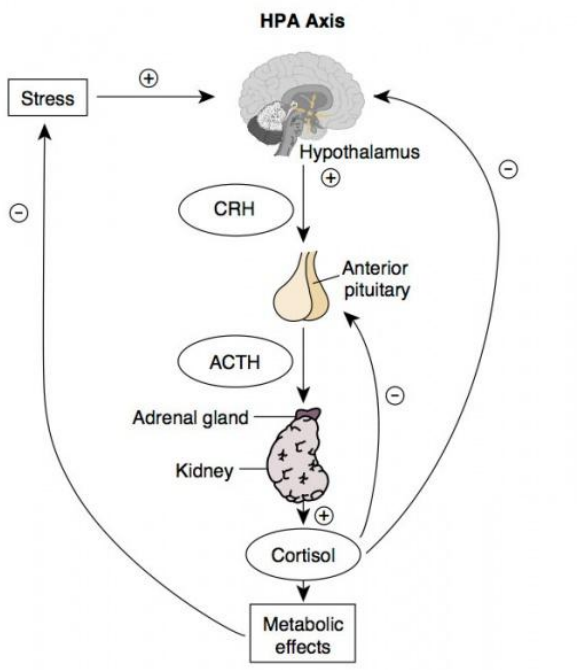
2.1 The Hypothalamic-Pituitary-Adrenal (HPA) Axis

The HPA axis is the major stress regulating system. The HPA axis cascade begins centrally with the hypothalamic secretion of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) from the anterior pituitary to activate the peripheral release of glucocorticoids (Tsigos and Chrousos, 2002) (See Figure 2). Specifically, cortisol is the primary glucocorticoid released in humans, and corticosterone is primarily released in rodents. Upon activation of the HPA axis, hypothalamic release of gonadal releasing hormone (GnRH) is decreased, and consequent secretion of pituitary gonadotropins is suppressed (Sapolsky et al., 2000). Glucocorticoids mobilise energy from stored nutrients primarily in the liver by gluconeogenesis and glycogenolysis, aiding in activation of energy mobilisation. Increased release of glucocorticoids also enhances cardiovascular tone, inhibits reproductive activity, reduces appetite and feeding behaviours and stimulates cognitive function (Darnaudéry and Maccari, 2008; Matthews, 2002; Sapolsky et al., 2000). Interestingly, the neuroendocrine response to stress is known to both stimulate and suppress immune function (Sapolsky et al., 2000).

Attenuation of the HPA axis response to stress occurs via the binding of glucocorticoids with glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) in the hippocampus, hypothalamus, and pituitary. This creates a negative feedback loop and reinstates homeostasis (De Kloet and Reul, 1987; Welberg and Seckl, 2001). In this way, the HPA axis is designed to provide a quick and appropriate mechanism for coping with stress by stimulating a relatively brief influx of glucocorticoids into the body. Moreover, this responsivity is in part determined by the relative affinity of glucocorticoids for MRs and GRs

(Lupien et al., 2009) and the balance in the expression of these receptors (Oitz et al., 1997). Specifically, an imbalance between MRs and GRs, which are highly expressed in limbic system structures, such as the amygdala and hippocampus, is proposed to disrupt the negative feedback loop, dysregulate HPA axis activity and increase susceptibility to stress-induced psychopathology such as anxiety and depression (Harris et al., 2012).

Figure 2: Activation of the HPA axis in response to stress and its negative feedback mechanisms (Hiller-Sturmhöfel and Bartke, 1998).



2.1.1 Programming of the HPA axis

Glucocorticoids are important for normal brain development during perinatal life, however exposure to increased levels of glucocorticoids may impair normal brain maturation and functioning (Lupien et al., 2009). The HPA axis is known to be particularly sensitive to perturbations in the early life environment and exposure to stress during the critical period of the HPA axis development may alter stress responsivity throughout life (Karrow, 2006).

While in humans the HPA axis completes its maturation during the last trimester of pregnancy, in rodents (i.e. rats and mice), the critical development of the HPA axis occurs postnatally, during the first week of life, and the first two postnatal weeks are characterized by a hyporesponsiveness to stress (Sapolsky and Meaney, 1986). Thus, programming of the HPA axis has been extensively studied using various rodent models of postnatal stress. For instance, neonatal immune challenge by administration of lipopolysaccharide (LPS) on postnatal days (PNDs) 3 and 5 in mice has been shown to increase mRNA levels of GRs, without concomitant changes in MRs, in the hippocampus at 2 weeks of age (Amath et al., 2012). In a similar model of neonatal LPS challenge in rats, contrasting findings were reported, whereby decreased GR binding and density was found in the hippocampus, hypothalamus and frontal cortex of adult rats, following adrenalectomy (Shanks et al., 1995). Increased CRH mRNA levels in the paraventricular nucleus (PVN) and increased median eminence levels of CRH and arginine vasopressin were also reported in this study (Shanks et al., 1995), indicating diminished negative feedback capacity and hence, increased HPA axis activity.

Alterations in maternal care during the first week of life in rats, via decreased pup licking and grooming and arched-back nursing, were found to be associated with hypermethylation within the exon 1_7 GR promoter and increased histone acetylation and transcription factor (NGFI-A) binding to the GR promoter in the hippocampus of adult offspring (Weaver et al., 2004), which is typically associated with reduced DNA binding and thereby reduced transcriptional activity (Razin, 1998). These effects were reversed by cross-fostering (Francis et al., 1999; Weaver et al., 2004), suggesting a causal link between differences in maternal care and programming of gene expression. Similarly, exposure to brief periods of handling in neonatal life has been demonstrated to lead to an increase in the

expression of GRs in the hippocampus of adult animals, resulting in increased feedback inhibition of CRH synthesis and reduced ACTH levels during stress, when compared to non-handled controls (Liu et al., 1997).

Peripheral release of glucocorticoids was also found to be differentially programmed using various perinatal stress paradigms. Generally, perinatal stress has been shown to result in impaired negative feedback and hypersecretion of glucocorticoids following stressful exposure in later life (Shanks et al., 1995; Welberg and Seckl, 2001; Witek-Janusek, 1988). However, this profile of peripheral glucocorticoid secretion has been found to be largely dependent on the strength, type and duration of stress exposure to not only the initial stressor but also a secondary stressor. Chronic stress exposures have typically been shown to produce blunted glucocorticoid output (Rich and Romero, 2005), which can become maladaptive when a strong glucocorticoid release is required. Neonatal LPS challenge on PNDs 3 and 5, followed by a subsequent later life stressor, has also been reported to result in a blunted corticosterone response (Walker et al., 2009).

The variable outcomes of perinatal stress on adult stress responsivity illustrate the complexity of HPA axis programming. The timing, the degree and the origin of stress exposure, all determine the long-term sensitivity and efficacy of the HPA axis in mediating an appropriate stress response. Ongoing research is still endeavouring to elucidate the critical determinants in programming of the HPA axis. In addition, there is a more recent focus on the impact of early life events on the ANS and its subsequent role in stress regulation.

2.2 The Autonomic Nervous System (ANS)

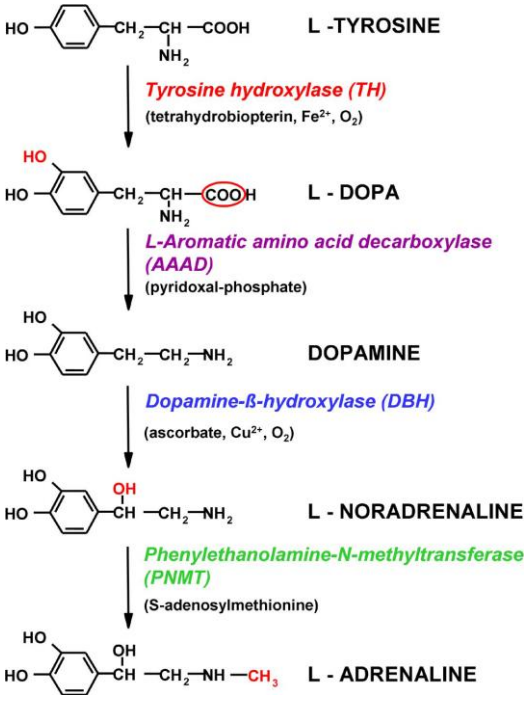
The ANS provides the most immediate response to stress, via innervation of end organs through its sympathetic and parasympathetic compartments. Importantly, the ANS regulates initiation and resolution of the ‘fight or flight’ response. Within seconds, activation

of the sympatho-adrenomedullary axis promotes the synthesis and release of catecholamines from the adrenal medulla and peripheral nerve endings. Sympathetic activity provokes numerous physiological responses, such as excitation of the cardiovascular system, resulting in increased heart rate and blood pressure (Ulrich-Lai and Herman, 2009). Accordingly with the rapid activation, the sympathetic responses resolve quickly through the counteracting effect of the parasympathetic nervous system, promoting the state of 'rest and digest' and limiting the augmentation of the acute stress response. As opposed to the relatively slower and protracted hormonal changes promoted by the activation of the HPA axis, the autonomic response consists of rapid physiological changes that can be assessed with high temporal resolution (Ulrich-Lai and Herman, 2009). Therefore, various autonomic measures, including cardiovascular parameters, thermoregulation, the galvanic skin response and changes in respiration are often assessed as indices of acute changes in arousal (Nalivaiko et al., 2011).

The principal neurotransmitters and hormones of the adrenomedullary, sympatho-neuronal and central catecholaminergic systems are the catecholamines: dopamine, noradrenaline and adrenaline. Increased release of catecholamines is an essential part of a stress response, which is in parallel accompanied by a compensatory increase in catecholamine biosynthesis. Catecholamines are synthesized from the amino acid precursor L-tyrosine (See Figure 3). Upon entry into adrenal chromaffin cells, sympathetic or central catecholaminergic nerve endings, tyrosine is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH), the first rate-limiting enzyme in catecholamine synthesis (Kvetnansky et al., 2009). Activation of TH is regulated through several mechanisms. In short term, TH activity is mediated via phosphorylation by a variety of kinases at several serine residues (such as Se19, Ser31 and Ser40) in the N-terminal domain. Specifically, phosphorylation at Ser40 increases the enzymatic activity directly and to a greater extent than

phosphorylation of the other sites (Bobrovskaya et al., 2007; Dunkley et al., 2004). Longer term mechanisms of TH regulation include transcriptional regulation, RNA stability and total protein levels (Kvetnansky et al., 2009). Both the acute and chronic mechanisms of TH activation are well established *in vitro* and *in vivo* (Bobrovskaya et al., 2010; Ong et al., 2011). Recently, a third mechanism for the control of TH activity has been introduced, and is referred to as the sustained phosphorylation phase (Bobrovskaya et al., 2007). The sustained phase of TH activation is mediated by phosphorylation, primarily at Ser40, within 1h to 24h and is then followed by TH protein synthesis, contributing to the further synthesis of catecholamines. The sustained phase of TH activation is distinguishable from the acute phase, as it only occurs in response to selected stimuli and is mediated by other protein kinases than those involved in the acute TH phosphorylation (Bobrovskaya et al., 2007) .

Figure 3: Pathway for catecholamine biosynthesis and its enzymatic steps. Adapted from Kvetnansky et al., 2009.



2.2.1 Programming of the ANS

While the majority of literature exploring the effects of early life stress focuses on programming of the HPA axis, the ANS is also susceptible to long term functional alterations by exposure to various stressors in early life. Similarly to other physiological systems, while certain environmental exposures may program beneficial adaption to the local environment, under some circumstances these changes may prove maladaptive in adulthood, and as such provide a basis for developmental origins of pathological states (Young, 2002b).

Due to the complexity of ANS structure and function, each subdivision is likely to respond to different environmental factors, generating differing programming outcomes. For instance thermoregulation, which is predominantly controlled by the SNS, is known to be susceptible to environmental modifications in early life. Exposure to a cold environment during early development improves adaptation to subsequent exposure to cold. Conversely, exposure to elevated temperature has been demonstrated to enhance tolerance to heat exposure in later life, in both animals and humans (Young, 2002b). Another important developmental factor that has been extensively studied is maternal and neonatal nutrition, which contributes to the development of the ANS anatomy and function. In animals, rearing in small litters results in a permanent increase in weight gain and body fat. Assessment of sympathetic function in animals reared in small litters revealed that while no consistent differences were observed in adrenergic innervation of peripheral organs, sucrose-induced activation of cardiac sympathetic activity was diminished in animals reared in small litters (Young, 2002a). Exposure to neonatal handling, which was shown to induce a long lasting reduction in the HPA responses to stress (Liu et al., 1997; Viau et al., 1993), induces a significant reduction in spleen and cardiac sympathetic activity, but an increased autonomic response (i.e. increased concentration of urine catecholamines) in response to fasting in

adulthood (Young, 2000). Exposure to prenatal stress on the other hand was shown to exaggerate stress responsivity in later life, as demonstrated by enhanced cardiovascular activity in response to restraint stress in adult rats (Igosheva et al., 2004).

Recently, a sustained phase of TH phosphorylation was demonstrated *in vivo* in a model of neonatal LPS challenge. Administration of LPS on PNDs 3 and 5 in rat pups resulted in increased TH phosphorylation of Ser40 at 4h and 24h in the adrenal medulla following the second LPS treatment. Increased phosphorylation of Ser40 corresponded with increased activity of the enzyme and was independent of TH protein synthesis, which was evident only at 48h after LPS exposure (Ong et al., 2012), indicating prolonged elevation in catecholamine biosynthesis in response to neonatal LPS exposure. Taken together, the previously noted data with that reported by Ong, et al., demonstrate the vulnerability of the sympatho-adrenolamedullary axis to early life inflammatory stress.

2.3 The Hypothalamic-Pituitary-Gonadal (HPG) axis

The HPG axis is a principal regulator of reproductive function in both males and females, via the production of hormones, released centrally and peripherally. Beyond its essential role in governing reproduction, the HPG axis influences the immune system as well as brain development, maturation and lifelong function, and has therefore been studied extensively. Due to the specific relevance of the current thesis to female reproductive development, HPG structure and function of the female is reviewed here to a larger extent than that of the male.

The effect of sex steroids on brain and behaviour can be divided into organisational and activational effects. During early development, organisational effects permanently impact on structural modelling of the nervous system. These effects are experienced again in

adolescence, when pubertal maturation of the HPG axis results in rewiring of the neural circuitry. Activational effects on the other hand are transient and occur throughout life. Activational responses to sex steroids in adulthood are largely programmed by the organisational influences during early development (Sisk and Zehr, 2005). The mature HPG hormonal cascade begins in the medial pre-optic area (MPOA) of the hypothalamus with the release of gonadotropin-releasing hormone (GnRH) from GnRH neurons. This collection of neurons, known as the GnRH pulse generator, releases GnRH in a synchronised pulsatile manner and is the central control mechanism for the reproductive cycle (Krsmanovic et al., 2009; Ohkura et al., 2009). The GnRH peptide is secreted from the nerve endings into the hypophyseal portal system, to stimulate the synthesis and release of gonadotropins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary (Millar, 2005). FSH and LH are also released in pulses into the blood stream to stimulate the gonadal release of sex steroids, which include oestrogen, progesterone, testosterone and inhibins that are primarily of gonadal origin, while activins and follistatin are produced in all tissues, including the gonads (Meethal and Atwood, 2005).

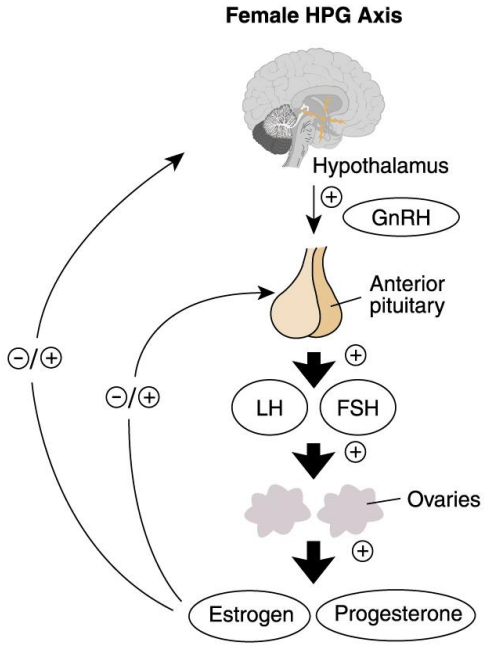
The levels of each of these hormones are regulated via a negative feedback loop. Activins, important members of the transforming growth factor-β (TGF-β) superfamily, released in the periphery stimulate the hypothalamic secretion of GnRH, which in turn stimulate the anterior pituitary to release the gonadotropins, FSH and LH, which then bind to their gonadal receptors and induce oogenesis in females and spermatogenesis in males, as well as the secretion of sex steroids and inhibins. The sex steroids and inhibins then exert a negative feedback inhibition at the level of the hypothalamus and the pituitary, resulting in a diminished gonadotropin secretion. Activin secretion is mediated via two different mechanisms, inhibin and follistatin. Inhibin binds in a competitive manner to activin receptors and inactivates its action. Follistatin on the other hand binds to activin, forming an

inactive complex and prevents activin from binding to its receptors (Meethal and Atwood, 2005).

The levels of the HPG hormones are altered with respect to the reproductive state of the organism, and therefore change dramatically throughout life. During the pre-pubertal period the frequency of GnRH pulses are generally low. With the onset of puberty in females, GnRH pulse frequency increases, inducing the increased production of FSH and LH, and initiating the beginning of the first reproductive cycle (Sisk and Foster, 2004). FSH then triggers maturation of an ovarian follicle, and a production of oestradiol as well as a small amount of progesterone. During the follicular maturation stage, oestradiol supresses the release of LH. The maturing follicle continues to increase oestradiol release which once in high concentrations reverses its negative feedback on LH to positive feedback resulting in the LH surge which triggers ovulation (release of the oocyte from the follicle). The ruptured follicle then develops into the corpus luteum which secretes high levels of progesterone along with lower levels of oestradiol, inhibiting GnRH, LH and FSH. As the corpus luteum degenerates, progesterone and oestradiol levels decrease, subsequently releasing the inhibition of GnRH, and gonadotropins, thus initiating a new cycle (Chrousos et al., 1998; Davies and Norman, 2002; Meethal and Atwood, 2005; Sisk and Foster, 2004) (See Figure 4).

Gonadotropins, and in particular FSH, have a critical role in the regulation of follicular maturation. Their impact is, however, exerted to a greater extent after the onset of puberty (McGee and Hsueh, 2000). The initial follicular growth and development is known to be largely coordinated by complex interactions between the oocyte, growth factors, cytokines and neurotrophins (Dissen et al., 2002; Schindler et al., 2010; Skinner, 2005), implicating a reciprocal role of the immune system in the initial gonadal development. This complex interaction will be discussed below.

Figure 4: The female HPG axis and the feedback mechanisms (Hiller-Sturmhöfel and Bartke, 1998).



2.3.1 Programming of the HPG axis

Experimental data in animals and some human observations indicate a critical role of early life events in programming the development and functioning of the HPG axis (Davies and Norman, 2002). During early development, the HPG axis is particularly sensitive to a variety of environmental steroids as well as endocrine-disrupting chemicals. One of the most commonly studied examples is the consequences of exposure to a synthetic oestrogen, diethylstilbestrol (DES) during pregnancy. During the mid-20th century, DES was prescribed in order to prevent pregnancy complications. While it was shown to be ineffective in its initial cause, exposure to DES *in utero* has been linked to a variety of adverse health outcomes. A link that was established between an exposure to DES *in utero* and an increased incidence of vaginal/cervical adenocarcinomas has led to the consideration of DES as a human carcinogen (Herbst et al., 1971). In addition to its carcinogenic properties, DES is also

considered a teratogen, as indicated by abnormalities of the reproductive tract, such as vaginal epithelial and uterine abnormalities, detected in DES-exposed daughters (Mittendorf, 1995).

Given the important role of oestrogen in sexual maturation, disruption of its normal levels during all stages of development may alter normal reproductive functioning. Animal studies have demonstrated that administration of exogenous oestrogens around the time of weaning in rats can advance the onset of puberty, while pre-pubertal ovariectomy prevents further maturation of the reproductive system (Goldman et al., 2000). Androgen exposure in early life may also disrupt normal reproductive function and appears to produce time-dependent effects. When investigated in female rats, androgen treatment during the first 3 weeks of life resulted in anovulation in adulthood, while similar treatment during foetal or late postnatal life only, had no such effect on adult reproductive function, emphasizing the critical issue of the timing of an insult (Tyndall et al., 2012).

Metabolic factors also play an important role in the regulation of sexual maturation. Altered onset of puberty, especially earlier pubarche and menarche, has been linked to foetal growth perturbation. Premature pubarche was shown to be more prevalent in girls born at a lower birth weight. Being small for gestational age was also found to be associated with hyperinsulinism, ovarian hyperandrogenism and ovarian hyporesponsiveness to FSH in adolescence (Ibanez et al., 1998). These are the prominent symptoms of polycystic ovary syndrome (PCOS) that emerges around puberty and is associated with high risk of infertility. As opposed to the low gestational and birth weight, which can lead to postnatal catch-up growth and earlier sexual maturation (Sloboda et al., 2007), malnutrition in childhood and around puberty has been associated with delayed menarche (Slyper, 1998). These findings amplify the importance of even minor developmental alterations in the origin of subsequent

risk of disease, as well as a particular significance of a timing of an insult in the specific pathological outcome.

The multi-directional relationships of the neural, endocrine and immune systems have been extensively implicated in the study of reproductive development. Along with the role of nutrition and metabolic changes, exposure to early life stress has been shown to program various aspects of HPG axis functioning. The HPA and HPG axes are known to co-regulate one another, and stress has been shown to influence reproduction (Rivier and Rivest, 1991; Tilbrook et al., 2000). Activation of the HPA axis produces an inhibitory effect on the female reproductive system, via CRH-mediated inhibition of GnRH release, and glucocorticoid-mediated inhibition of pituitary hormones and adrenal sex steroids. These inhibitory actions are accountable for the hypothalamic amenorrhea, which is typically associated with stress-related disorders, such as anxiety and depression, but also eating disorders and excessive exercise (Kalantaridou et al., 2004). At the gonadal level, the direct effect of glucocorticoids appear to be redundant, however it is the interaction with gonadotropins, such as LH and FSH that may affect pubertal maturation and therefore adult reproductive capacity (Rivier and Rivest, 1991; Tilbrook et al., 2000).

The impact of early life stress on reproductive fitness is still an emerging field, and its causal link has been investigated using animal perinatal models of stress. Intriguing findings were demonstrated by the Meaney laboratory, where prenatal stress induced by low quality maternal care was found to be associated with advanced onset of puberty, higher receptivity and increased fecundity. Moreover, these findings were associated with increased expression of oestrogen receptor α in brain regions associated with regulation of maternal behaviour and HPG function. Cross-fostering reversed the aforementioned effects, suggesting that varying reproductive strategies reflect environmental adaptation, whereby in some environments advanced reproductive maturation and enhanced reproductive success may be driven by

higher risk of mortality (Cameron, 2011). In addition, a direct relationship between the quality of maternal care received in early life and the expression of maternal care in adulthood has been established, whereby female offspring that were reared by dams exhibiting lower levels of licking and grooming, show similar maternal behaviours to their pups during lactation, and vice versa (Francis et al., 1999). These findings reflect phenotypic plasticity of behaviour as well as non-genomic inheritance of reproductive strategies, highlighting the importance of further investigation of the biological constructs underlying reproductive fitness and success.

2.4 The immune system

The immune system is a remarkable defence system that is capable of specifically recognising and eliminating a limitless variety of invading pathogens. As with other systems, the immune system follows an orderly developmental pathway following conception, continuing to develop after birth. Both developing and mature immune responses are generated through the close interaction with nervous, autonomic and endocrine systems, and are therefore of utmost significance for normal physiological functioning. The immune system integrates an extensive and dynamic network of cells and molecules, and its complexity rivals that of the nervous system. The response to pathogens is coordinated by the complex interactions and activities of the large number of diverse cell types involved in the immune response.

Immune responses can be divided into adaptive and innate immunity. *Adaptive immune responses* are driven by antigen-specific defence mechanisms, which may take days to develop, and are characterised by immunological memory for a lifetime, such that a second exposure to the same antigen results in an accelerated and specific response (Berczi, 1998). Adaptive responses are largely coordinated by lymphocytes, which originate in the bone

marrow and other hematopoietic tissues in mammals, but then migrate to and mature in either the thymus or the bone marrow. On this basis, they are classified as either T cells (thymus-derived) or B cells (bone marrow-derived). After maturation, the T and B cells circulate through the blood and lymph, and often reside in secondary immune organs such as the spleen and lymph nodes where they continuously search for pathogens.

Innate immune responses are the first line of defence. Innate immunity therefore refers to nonspecific resistance to pathogens, via the recognition of highly conserved homotopes on antigen surfaces. As such, LPS is the immunologically active cell wall component of gram-negative bacteria that is recognized by all macrophages and neutrophils, allowing the host to mount a rapid response to invading bacteria without dependence on prior exposure (Berczi, 1998). Innate immune responses are largely coordinated by macrophages, monocytes, neutrophils, and other phagocytic molecules.

Exposure to a peripheral immune challenge, such as bacteria, pathogens, or LPS generates an immediate acute phase reaction of the immune system, indicated by an increased release of proinflammatory cytokines, such as interleukin (IL)1β, IL-6, and tumour necrosis factor (TNF) α. These proinflammatory agents activate the entire network of the immune system, inducing additional release of proinflammatory cytokines, such as IL-12 and interferon-gamma (IFN-γ) by antigen presenting cells (APCs) and natural killer (NK) cells. Further release of these cytokines stimulates the differentiation of T-helper type 1 (Th1) cells, inducing the functional activity of macrophages, T-cytotoxic and natural killer (NK) cells, which are the major components of the cell-mediated immunity (CMI). Moreover, activation of the peripheral immune response is known to trigger cytokine-specific regulation of the HPA axis, cause neurotransmitter functional alterations, and induce sickness behaviour.

In response to an infection, IL-1β stimulates receptors expressed within the sensory neurons of the vagus, which signals the brain where the vagus primarily innervates the

nucleus of the tractus solitarius (NTS) and medulla. Noradrenergic activation then occurs, projecting towards the PVN and hippocampus where glial cells synthesize and release IL-1β and other cytokines. The dual action of noradrenaline and cytokine release works to activate the HPA axis (Goehler et al., 1999; Konsman et al., 2002; Watkins et al., 1995). Peripheral cytokines can directly cross the blood-brain barrier via specific cytokine transport mechanisms (Banks, 2005), and can directly enter the brain in areas where the BBB is absent, known as the circumventricular organs. These cytokines can stimulate the release of secondary messengers in blood vessel-associated cells, such as nitric oxide and cyclooxygenase 2 (COX-2) (Vitkovic et al., 2000), which can then propagate to nearby target sites involved in HPA axis and febrile regulation, including the PVN, and the catecholaminergic brainstem nuclei (Dantzer et al., 2008; Konsman et al., 2002; Watkins et al., 1995). Cytokines that reach the brain via these pathways disperse into the parenchyma via a process known as volume transmission, and thus activate all brain regions. Finally, the brain can induce cytokine synthesis itself for responding, regulating and initiating immune signalling.

The primary producers of cytokines within the brain are microglial cells. Microglia are the resident immune-competent cells of the brain. They monitor the brain for invading pathogens and immune insults and are capable of initiating an adaptive immune response (Garden and Moller, 2006). More recent evidence has also indicated that microglia are involved in synaptic pruning, therefore any alteration to these cells may have long-term implications on later life cognitive and behavioural development (Graeber, 2010). Microglia are derived from the bone marrow and migrate into the brain during early development, prior to the closure of the blood brain barrier. During their migration phase, microglia are known to exhibit what is referred to as an amoeboid morphology. Once the cells have migrated to their

final resting site within the brain they undergo ramification. Once the cells have achieved a fully ramified status they typically reside in a single location. These "surveillant" ramified microglia respond to activating stimuli with a rapid morphological transformation into "active" amoeboid microglia (Nakajima and Kohsaka, 2001). Activated microglia can be found in the brain under almost all pathological conditions, such as trauma, stroke, ischemia or infection, and are involved in tissue repair, initiation of local inflammatory processes, neurodegeneration and phagocytosis (Davalos et al., 2005). Microglia have close interactions with other surrounding cell types in the brain, including neurons, astrocytes, endothelial cells, and oligodendrocytes. Thus, microglial activation can have profound effects on the function of other cell types within the brain both during development and in adulthood (Schwarz and Bilbo, 2012).

2.4.1 Programming of the immune system

The development of the immune system is well established to be dependent on the immune, autonomic and endocrine signals that it receives early in life (Fagundes et al., 2012; Holladay and Smialowicz, 2000; Zakharova, 2009). This physiological programming of the immune system and its relationship to later life pathology has been associated with a number of long-term health outcomes mediated by inflammatory pathways including, a predisposition to asthma, allergies, autoimmune diseases, metabolic disorders, cardiovascular diseases, multiple sclerosis and more (Fagundes et al., 2012; Hodyl et al., 2008; Zakharova, 2009). One of the major disturbances in immune function related to early life factors is a predisposition to allergic diseases. Both the early onset and the escalating incidence of allergies, including asthma, rhinitis and atopic dermatitis, have raised a particular interest in

various environmental factors that may influence early immune development and are likely to play an important role in the propensity toward these diseases.

A classical allergic response has been characterised as an imbalance between Th1 and Th2 immune responses, with a shift towards Th2 immunity, associated with the release of anti-inflammatory cytokines (e.g. IL-4, IL-5) and production of IgE antibodies, leading to atopy. Although diminished Th1 function was initially proposed as an underlying factor, there is no conclusive evidence of impaired Th1 immunity and, at an individual level, there is accumulating evidence that atopy is associated with an increase in both Th1 and Th2 immune responses (Smart and Kemp, 2002). The combination of complex immunologic interactions that are required during pregnancy to maintain maternal tolerance for the foetus, and the immaturity of foetal, as well as neonatal immune system, imply an increased vulnerability to programming effects during the perinatal period. Maternal atopy, for example, is associated with a higher risk of allergic disease and relative immaturity of Th1 function in the offspring (Prescott et al., 2000), indicating that direct maternal immune interactions may have significant influences on the developing foetal immune system.

Bacterial infections *in utero* and in early life are generally associated with an increased capacity for neonatal Th1 responses. This view is presented by the *hygiene hypothesis*, which suggests that a lack of exposure to certain microorganisms and bacteria may contribute to immaturity of the immune system and the inability to produce a sufficient inflammatory response (Strachan, 1989). In support of this hypothesis, exposure to higher levels of endotoxin from household dust in children has been associated with a decreased incidence of hay fever, asthma, and other allergic diseases (Ege et al., 2011). While perinatal exposure to subtle immune activation may be beneficial for developing a resistance to allergic diseases, inappropriate activation of the immune response via exposure to

immunotoxicants in early life has been linked to later life pathology (e.g., hypersensitivity disorders and autoimmune disease) (Holladay and Smialowicz, 2000). These opposing views emphasize the complexity of physiological development, suggesting different outcomes may depend on the timing and the extent of early life immunogenic exposure.

In response to immunogenic stimuli, the neonatal peripheral immune system generates a lower response when compared to that of the adult and thus is considered as functionally immature (Vosters et al., 2010). Immune factors in the developing brain however, have a very distinct expression. When compared to the adult brain, the expression of many cytokines in the developing brain is significantly increased, even in the absence of an immune challenge coinciding with the appearance of amoeboid microglia during early brain development (Schwarz and Bilbo, 2012). The expression of cytokines during the early developmental period has an important role in many neurodevelopmental processes, such as neurogenesis, neuronal and glial cell migration, proliferation, differentiation, and synaptic maturation and pruning. The distinct central cytokine profile and microglial morphology during development are therefore suggested to reflect the increased sensitivity of the developing brain to perinatal immune challenges, which may result in permanent alteration of major developmental processes and long-term programming of neuroimmune function.

2.4.2 Programming of the immune response via neural-endocrine-immune interactions

Multi-directional communication pathways between the neural, immune and various endocrine systems are established during the perinatal period, and these reciprocal interactions govern organismal development and maturation. Therefore, not only may perturbations to the development of individual systems affect later life functioning, but

disturbances to the interaction of these systems at critical periods of development are relevant to a variety of pathologies.

For instance, epidemiological evidence has indicated a link between early life stress and an enhanced inflammatory profile. Specifically, patients with childhood abuse-related PTSD displayed increased nuclear factor (NF)-κB pathway activity and decreased sensitivity of monocytes to glucocorticoids, indicative of increased inflammation (Pace et al., 2012). Similarly, depressed adults with a history of childhood maltreatment were found to exhibit elevated inflammation, as indicted by higher levels of C-reactive protein (Danese et al., 2008). Stressful early life events were also found to be associated with the reactivation of herpes virus, telomere shortening via increased T-cell proliferation, and with immune dysregulation of the tumour environment in different types of cancers (Fagundes et al., 2013).

In primates, prenatal stress and exposure to IL-1β in juvenility has led to a blunted inflammatory response with reduced plasma and cerebrospinal fluid levels of IL-6, and reduced febrile response to the IL-1β. This effect however, was evident only when prenatal stress was induced by administration of ACTH, but not by exposure to acoustic startle, a psychological stressor, suggesting that differences in prenatal environment may have a variable effect on adult physiology (Reyes and Coe, 1996). In a later study, exposure to an acute stressor (i.e. acoustic startle) in pregnancy resulted in diminished cellular cytokine response to an *in vitro* stimulation with LPS (Coe et al., 2002).

In rodents, prenatal restraint stress resulted in increased circulating T-cytotoxic and NK cells in adult offspring. These changes were associated with increased mRNA expression of IFN-γ, suggestive of long lasting pro-inflammatory consequences of prenatal stress in rats (Vanbesien-Mailliot et al., 2007). Prenatal stress induced by maternal separation in rats was

also shown to lead to impaired NK cytotoxicity and increased tumour colonization following adult restraint stress (Nakamura et al., 2011).

Programming of the immune system, with a particular emphasis on its association with the development and regulation of other physiological systems, has also been investigated using various models of perinatal immune activation. These models have utilized administration of cytokines, bacterial and viral agents including mimetics, (e.g. lipopolysaccharide (LPS) polyinosinic:polycytidylic acid (poly I:C)), live reagents (e.g. *Escherichia coli (E. coli)*), and other agents, such as toxins (i.e., endotoxins and exotoxins). The impact of postnatal immune challenge via administration of LPS has been the main focus of the work presented in this thesis and is therefore described in detail below.

3. An animal model of early life stress

Numerous experimental interventions have been used to simulate a range of stressful situations and evoke a physiological stress response in early life. Human studies are often limited in their ability to target mechanisms due to the ethical restraints of invasive procedures on humans. It is for this reason that preclinical experimentation on animals is required. Rodent models, which are most commonly utilised, allow researchers to examine biological mechanisms involved in this programming phenomenon using various examples of common experimental paradigms presented above, including maternal restraint, maternal separation, neonatal handling, prenatal and postnatal exposure to glucocorticoids or sex steroids. Numerous other stress paradigms are routinely employed in research investigating the impact of early life stress and many share overlapping effects on the stress response, immune system, neural development, behavioural outcomes and reproductive processes. The focus of the current thesis is to explore the impact of postnatal immune challenge mimicking

perinatal infection, which was induced by administration of LPS, on different aspects of neuroimmune, endocrine, autonomic and reproductive responses, reflected in an altered behavioural phenotype.

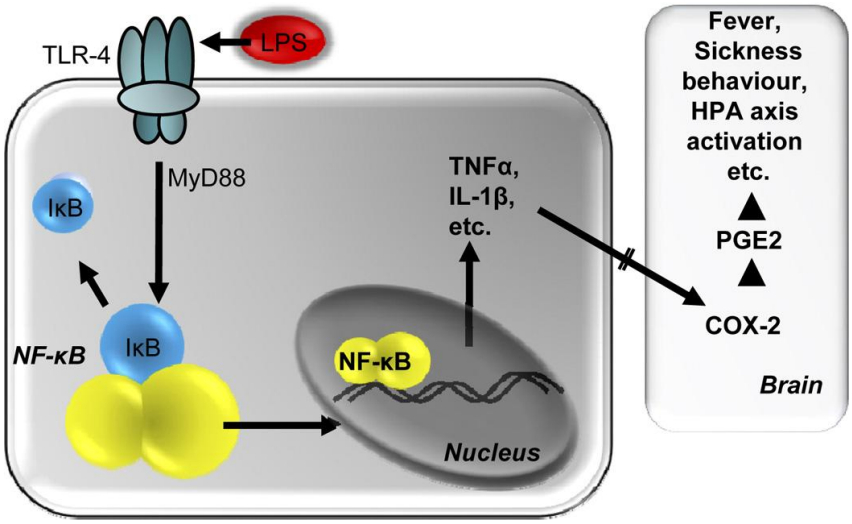
3.1 Lipopolysaccharide - An Immunological Stressor

An immune stimulus is typically recognised by pathogen-associated molecular pattern recognition receptors, such as Toll-like receptors (TLR) that are expressed by several cell types, including monocytes, macrophages, adipocytes and granulosa ovarian cells. A bacterial mimetic, LPS, comprising the cell wall of Gram-negative bacteria (usually derived from *Salmonella enteritidis* or *E. coli*), activates TLR4 (Erridge, 2010). LPS initiates an immune activation, and inflammation-induced behavioural symptomology in the host is largely identical to a live bacterial infection (Burrell, 1994; Rosenberger et al., 2000). Thus, LPS is considered to be a systemic immunological stressor (Beishuizen and Thijs, 2003). Administration of LPS is widely used due to its well-known advantages, the primary one being that as opposed to live bacteria, LPS does not replicate. This allows for tight control of dosage and limits the confounding nature of infection. LPS is therefore, commonly used to understand the complexities of the neuroimmune-neuroendocrine relationship (Bilbo et al., 2005a; Bilbo et al., 2005b; Boissé et al., 2004; Shanks et al., 1995; Walker et al., 2009).

Upon exposure to LPS, activation of TLR4 results in the activation of a series of phosphorylation steps, leading to activation of NF-κB, which then translocates to the nucleus where the transcription of several pro-inflammatory and anti-inflammatory cytokines is initiated. The release of these cytokines into the blood stream stimulates the synthesis of cyclooxygenase (COX)-2, the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandin (PG)E_2. PGE_2 action in the brain contributes to the initiation of the febrile

response. Additionally, signalling to the brain via stimulation of the vagus nerve results in activation of the HPA axis and increased glucocorticoid secretion, which help to control the magnitude of the inflammatory response (Spencer et al., 2011) (See Figure 5).

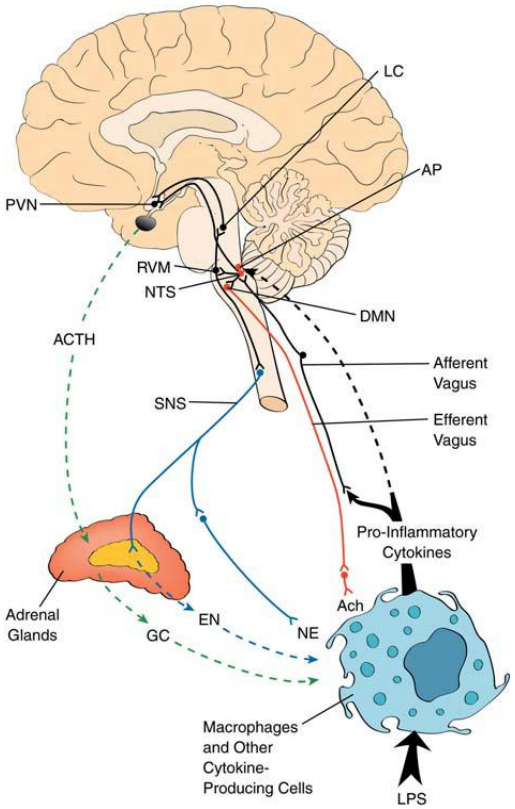
Figure 5: Central responses to an LPS-induced immune stimulation (Spencer et al., 2011).



The reciprocal nature of the neuroendocrine-neuroimmune interaction is such that whereas an initial short stimulation of the acute phase immune response by administration of LPS occurs in the presence of stress, slower wave effects of glucocorticoid secretion tend to dampen immune activity. These immunosuppressive effects of glucocorticoids have been recognised for decades, and have been documented to suppress proinflammatory cytokine and chemokine synthesis (Elenkov et al., 2005), reduce the proliferation of T (Ashwell et al., 2000) and B (Sapolsky et al., 2000) cells, inhibit dendritic cell maturation (Elftman et al., 2007), and produce a shift from a Th1 to a Th2 predominant immune response (Elenkov, 2004; Sorrells and Sapolsky, 2007).

The HPA axis is not, however, the only system involved in the regulation of the inflammatory response. As illustrated in Figure 6, administration of LPS in the periphery and a subsequent signalling to catecholaminergic brain regions results in the peripheral release of adrenaline and noradrenaline, from the adrenal medulla and peripheral nerve terminals, respectively. Therefore the HPA axis and the ANS work in a coordinated fashion to alleviate the stress response (Karrow, 2006; Pavlov and Tracey, 2004).

Figure 6: Neural and neuro-hormonal pathways of regulation of LPS-induced inflammation (Pavlov and Tracey, 2004).



3.2 Neonatal immune challenge by administration of LPS as a model of early life stress

Witek-Janusek (Witek-Janusek, 1988) was the first to demonstrate the sensitivity of developing rat neonates to endotoxic shock, and the sensitivity of the neonatal endocrine system to LPS. Following on from this work, a seminal paper by Shanks et al. (Shanks et al., 1995) demonstrated that LPS exposure in rats during the early postnatal period, PNDs 3 and 5, produced allostatic changes in the HPA axis. This resulted in a hyper-responsive HPA axis response to subsequent stress exposure in adulthood (day 85-90 of age). Several studies have since replicated this effect (Hodgson et al., 2001; Shanks et al., 2000; Walker et al., 2004b). While variation has been observed in rodents with regards to the amplitude and temporal response of circulating plasma ACTH and corticosterone to neonatal LPS exposure (Takemura et al., 1997), this has been attributed to variations in dosage and preparation of the LPS (Beishuizen and Thijs, 2003).

A body of evidence has characterised the effective dose for inducing long-term changes in HPA axis and stress-related behaviours. The evidence suggests that a single insult of LPS is most likely insufficient in producing long-term changes in stress responsivity. For instance, c-Fos m-RNA expression (a common marker of neuronal activity) has been shown to be prominent in catecholaminergic nuclei after a single LPS administration (Takemura et al., 1997). This is associated with upregulated expression of hippocampal GRs which inhibit glucocorticoid production. Thus, the HPA axis activity is inhibited too soon for any allostatic changes to occur. Alternatively, dual LPS exposure is thought to elicit a more generalised and longer lasting immune response, producing sufficient and increased activation of the HPA axis (Takemura et al., 1997). Therefore, neonatal LPS exposure administered on PNDs 3 and 5 remains a common and reliable model of perinatal immunological stress, which ultimately activates the HPA axis during its hyporesponsive period of development.

The timing of a perinatal insult is crucial in determining specific developmental outcomes; it is therefore not surprising that administration of LPS on different postnatal days produces a divergent impact on physiological and behavioural development. For instance, programming of adult febrile responses to inflammation is evident only in animals neonatally treated with LPS between PND 14 to PND 21, while no such effect was observed when LPS was administered on PND 7 or PND 28 (Spencer et al., 2006b). Moreover, while administration of LPS on PNDs 3 and 5 has been repeatedly reported to induce an anxiety-like phenotype in adulthood (Walker et al., 2012; Walker et al., 2009; Walker et al., 2008; Walker et al., 2004b), no such effect is observed when animals are subjected to a single LPS injection on PNDs 7, 14, 21 or 28 (Spencer et al., 2006b). The different lines of research discussed below encompass a variety of outcomes produced by the different models of LPS administration. These include the programming of metabolic and endocrine function, peripheral and central immunity, and finally the programming of behaviour, the potential for its transgenerational inheritance, and thus implications for reproductive success.

3.2.1 Impact of Neonatal LPS on Metabolic Function

Postnatal LPS exposure has been shown to alter metabolic activity in adulthood. Most notably, LPS administration to neonatal rodents leads to abnormal metabolic changes in body weight, food consumption, insulin sensitivity and serum leptin levels (Iwasa et al., 2010; Iwasa et al., 2009; Nilsson et al., 2002; Nilsson et al., 2001; Spencer et al., 2010; Walker et al., 2004b; Walker et al., 2006b). More specifically, both short-term and long-term changes in weight gain have been observed, however, inconsistencies remain with some studies demonstrating developmental weight loss (Spencer et al., 2010; Walker et al., 2004b) while others have shown increased weight gain (Iwasa et al., 2010; Iwasa et al., 2009) or no

statistical difference (Knox et al., 2009; Nilsson et al., 2002). These changes in weight gain have been suggested to be related to alterations in glucose uptake, insulin sensitivity and reduced pancreatic release of amylase (Jaworek et al., 2007). Walker et al. (2006) has reported that Fischer-344 rat pups exhibited reduced glucose tolerance following LPS exposure on PNDs 3 and 5, but this reduction in glucose tolerance was only observed during puberty. Similarly, increased food intake and serum leptin levels were found in Sprague-Dawley rats injected with LPS on PND 10 (Iwasa et al., 2010). In adulthood however, these studies demonstrated improved glucose tolerance and insulin sensitivity in the neonatally treated animals.

3.2.2 Impact of Neonatal LPS on Endocrine Function

Many studies have implicated the endocrine system in the programming of later life function following neonatal LPS, focusing on enzymes, hormones, and receptors (Boissé et al., 2004; Hodgson et al., 2001; Iwasa et al., 2009; Jaworek et al., 2007; Shanks et al., 1995; Spencer et al., 2010; Spencer et al., 2006a; Walker et al., 2009). Long-term HPA axis perturbations following LPS administration on PNDs 3 and 5 have been widely demonstrated with alterations to ACTH and corticosterone secretion during stress commonly reported (Hodgson et al., 2001; Shanks et al., 1995; Walker et al., 2009; Walker et al., 2004b). Additionally, reduced negative-feedback sensitivity has been observed in animals exposed to LPS during the neonatal period (Shanks et al., 1995). Other findings have demonstrated basal expression of urocortin 2 (UCN- 2) and CRH receptor (CRHR) 2 mRNA to be significantly higher in animals postnatally exposed to LPS (Iwasa et al., 2009). These neuropeptides are important in stress-induced suppression of the HPG axis. This hyper-responsive HPA axis is believed to cause increased neuronal cell loss in the hippocampus in postnatally LPS-treated

animals, and as such has been linked to impaired learning and memory, as well as increased anxiety-related behaviours (Kohman et al., 2008; Walker et al., 2009; Walker et al., 2004b).

3.2.3 Impact of Neonatal LPS on Immunity

Given the known neuroimmune pathways through which LPS exerts its effects, it is unsurprising that a number of studies have focused on determining the long-term impact of neonatal LPS exposure on immune function. Several studies have demonstrated neonatal LPS exposure to result in an attenuated febrile response and altered immune function when exposed to a secondary infection in later life (Boissé et al., 2004; Ellis et al., 2005; Mouihate et al., 2010; Spencer et al., 2010; Spencer et al., 2006a; Walker et al., 2006a). Fever is considered to be an essential component of the host response to infection, modifying cytokine expression, reducing bacterial loads, and limiting morbidity and mortality (Jiang et al., 1999; Kluger et al., 1998). Due to the increased survival value of fever, the hypothermic response observed in animals exposed to neonatal LPS has been interpreted as a maladaptive programming phenomenon. A number of potential mechanisms have been investigated to account for this change in febrile responsivity. Peripheral cytokine concentrations have provided largely inconsistent findings, with increased IL-6 levels being observed (Breivik et al., 2002) but no differences in circulating IL-1β or TNF-α being reported following LPS administration in later life (Walker et al., 2006a). Furthermore, administration of IL-1β in adulthood did not induce alterations in the febrile response of animals exposed to LPS during early life (Boissé et al., 2004; Spencer et al., 2006a). In a similar fashion, administration of the downstream mediator PGE-2 also produced no change in fever between neonatal treatment groups (Boissé et al., 2004). Despite these findings, there is some evidence to suggest peripheral cytokines may be associated with the attenuation of fever. For instance,

Ellis et al. (2005) found reduced levels of IL-1β, TNF-α and IL-6 following an immune challenge in adulthood, which reached significance for TNF- α and IL-6. In line with these findings a reduction in the activity of peripheral NF-kB, responsible for much of the LPS-induced cytokine production, has been observed (Ellis et al., 2005).

Centrally, neonatal LPS on PNDs 3 and 5 resulted in cytokine-specific increases with IL-1β and TNF-α protein concentrations being significantly greater in response to stress in adulthood. These central cytokine increases coincided with increased peak corticosterone responses, indicating neuroimmune regulation of the HPA axis (Walker et al., 2010). Other studies have reported increased limbic cytokines following neonatal immune activation (Bilbo et al., 2005b; Bilbo et al., 2007) which have been linked to alterations in cognition and behaviour (Dantzer et al., 2008), as well as endocrine function (Turnbull and Rivier, 1999).

Prostaglandins and their regulatory enzymes, such as cyclooxygenase-2 (COX-2), have similarly been targeted as likely candidates in the mediation of this attenuated febrile response primarily by the Pittman laboratory. Findings from this laboratory have demonstrated that an LPS-induced increase in hypothalamic COX-2 expression is evident only in animals neonatally treated with saline, whereas an attenuated COX-2 response was observed in animals treated with LPS during neonatal life (Boissé et al., 2004; Spencer et al., 2006a). While this evidence would seemingly implicate central COX-2 expression in the regulation of fever following neonatal LPS treatment, infusion of its downstream product, PGE2, brought about no change in fever intensity between neonatal treatment and control groups.

Neuroendocrine regulation of the immune response to infection has been implicated in the attenuation of fever observed in these animals (Mouihate et al., 2010). Corticosterone levels of animals exposed to LPS during both neonatal and adult life have been shown to be

significantly higher than relative controls. Subsequent administration of the corticosterone inhibitor (RU486) resulted in no significant difference in febrile responses between treatment and control groups (Ellis et al., 2005).

Finally, neonatal LPS exposure has been implicated in our laboratory to increase tumour susceptibility and lung metastases in response to stress in adulthood (Hodgson and Knott, 2002; Hodgson et al., 2001). Associated with these findings is reduced NK cell activity and increased neuroendocrine responsivity to stress in LPS-treated animals (Hodgson and Knott, 2002; Hodgson et al., 2001). Overall, the data presented above demonstrate the robust impact of subtle peripheral immune activation on various aspects of peripheral and central immune function, with implications for reduced resilience to adult disease.

3.2.4 Impact of Neonatal LPS on behaviour

Neonatal LPS exposure has been typically reported to increase anxiety-like behaviours in adulthood. Several studies from our and other laboratories have commonly reported LPS-treated animals exhibit reduced exploratory activity and increased avoidance of aversive areas and stimuli (Breivik et al., 2002; Walker et al., 2009; Walker et al., 2008; Walker et al., 2004b). These findings however, are less consistent when animals are tested during adolescence (Rico et al., 2010). A number of articles have indicated that the maternal care of LPS-treated neonates may be altered and hence amplify the stress-related behavioural effects observed in the offspring (Hood et al., 2003; Lucchina et al., 2010; Walker et al., 2004a). Although these studies seem to indicate that maternal care may interact with the immunostimulating effects of LPS, others have not observed this effect (Spencer et al., 2006b).

Neonatal immune challenge has also proven to impact significantly on cognition. While the majority of this research has employed rat models of live bacterial infections, such studies have been instrumental in indicating neuroimmune pathways through which cognition can be disrupted. Furthermore, these studies fit nicely with the LPS-based models investigating behavioural perturbations. Live bacterial exposure with *E. coli* to neonates has been demonstrated to impair learning and memory in adult rats (Bilbo et al., 2008; Bilbo et al., 2005a; Bilbo et al., 2005b; Bilbo et al., 2007). Such research has indicated the involvement of hippocampal glial cell reactivity, pro-inflammatory cytokines and brain-derived neurotrophic factor (BDNF) in producing these cognitive impairments (Bland et al., 2010a; Bland et al., 2010b) . Interestingly, the strongest data have occurred in the presence of a secondary immune challenge in adulthood (Bilbo et al., 2005b), indicative of the requirement of a "second hit" to produce observable perturbations. Importantly, there is evidence that a secondary stressful stimulus also amplifies the behavioural alterations, with increased anxiety-like behaviours being observed in the dual LPS exposure model, following a more chronic restraint stress in adulthood (Walker et al., 2009).

These studies clearly indicate that animal models of neonatal immune activation are effective tools in which to examine later life behavioural maladaptations. Importantly, they allow for greater examination of the underlying mechanisms involved in the programming of behavioural disorders. Recently, our laboratory has provided evidence of transgenerational inheritance of anxiety-related behaviours and neuroendocrine perturbations induced by neonatal LPS administration (Walker et al., 2012). The anxiety-like and neuroendocrine phenotype of rats neonatally subjected to LPS can be transmitted to a subsequent generation, despite not being directly exposed to the LPS itself. The transference of behavioural and endocrine phenotypes induced by neonatal LPS to the parental generation was shown to be

possible along both the maternal and paternal line. However, the mechanisms responsible for this transmittance are distinguishable depending on the source of transmission (i.e. maternal or paternal). Along the maternal line, assessment of maternal care determined that the mechanism for increased anxiety-like behaviour and HPA axis responses to stress in the second generation of females was a reduction in the quality of maternal care provided by the dams. Interestingly, cross-fostering induced a direct reversal of this phenotype. Offspring born to saline-treated mothers demonstrated the anxiety-like phenotype and hyper-responsive HPA axis response to stress when fostered to LPS-treated dams. While the offspring born to LPS-treated mothers, displayed reduced anxiety-like behaviours and corticosterone levels when fostered to saline-treated dams. Thus, the mechanism underlying the transgenerational anxiety-like and endocrine phenotype along the maternal line is the differential qualities of maternal care provided to their offspring. However, the transgenerational anxiety-like behavioural phenomenon, observed across the paternal line, appear to be mediated by germ line epigenetic imprinting. This is supported by the lack of differences in maternal care provided by the untreated females which were bred with the experimental males, to produce the second generation (Walker et al., 2012).

These observations unequivocally imply that reproductive mechanisms are involved, and possibly affected, in the LPS treated animals. The important role of reproductive success in all species and the critical importance of reproductive health to development are widely acknowledged. However, so far little is known regarding the impact of neonatal immune challenge on reproductive development and fitness.

3.2.5 Impact of Neonatal LPS on Reproduction

Many studies have implicated perinatal stress in the developmental trajectory of the reproductive system. However, only a few studies have investigated the impact of neonatal LPS exposure on reproductive fitness. Compelling findings have shown that reproductive hormones are sensitive to bacterial stimulation during postnatal life. Neonatal LPS-treated female rats exhibit a greater suppression of LH pulses when presented an immune challenge in adulthood (Li et al., 2007; Wu et al., 2011) as well as an extended oestrous cycle (Iwasa et al., 2009; Wu et al., 2011), and delayed puberty onset (Knox et al., 2009; Wu et al., 2011). Mechanisms suggested to be responsible for this change in HPG function include the HPG regulatory signals kisspeptin (Kiss1) and Kissr1 (its G protein-coupled receptor) via COX-1 and COX-2 (Iwasa et al., 2008) in the medial preoptic area and hypothalamic arcuate nucleus. These brain regions have demonstrated altered abundance of Kiss1 and Kissr1 following postnatal LPS administration (Knox et al., 2009).

Neonatal LPS exposure has also been shown to result in an increased expression of ovarian NGF receptor (p75NGFR) along with increased thickness of theca interna layer of the ovarian follicle, which is directly innervated by sympathetic nerves (Wu et al., 2011), suggesting a possible involvement of peripheral neuroimmune interactions in gonadal development and functioning. Moreover, these data coincided with a diminished follicular reserve in adult female rats, neonatally exposed to LPS (Wu et al., 2011).

Despite the previously documented alterations in the HPG axis activity in LPS treated animals, 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 largely governed and coordinated by complex interactions between the oocyte and peripheral immune factors, such as cytokines,

chemokines, growth factors, as well as neurotrophins, which are known to regulate certain immune functions (Dissen et al., 2002; Schindler et al., 2010; Skinner, 2005). Similarly in males, growth factors and cytokines are crucial in the regulation of testicular spermatogenesis and steroidogenesis (Barakat et al., 2012; Skinner, 1991). Moreover, leydig cells, which produce testosterone, are regulated by catecholamines, and display developmental plasticity such that earlier stages of development are associated with higher levels of testicular catecholamines and increased density of tyrosine hydroxylase (TH) fibers (Mayerhofer et al., 1996). Therefore, the impact of neonatal immune challenge on the early development of reproductive organs may be mediated via peripheral factors more so than via central mechanisms.

4. Aim and Rationale of Thesis

The aim of the current thesis was to examine the influence of early life immune challenge on the programming of neuroimmune-endocrine-gonadal communications, and the long-term impact they may have on behaviour and reproductive fitness. Using an animal model of neonatal LPS exposure we firstly aimed to determine the brain mechanisms associated with the anxiety-like phenotype that was previously established in this model. These included neuroimmune and neuroendocrine parameters. We then explored the long term programming effect of the early microbial environment on development and functioning of the autonomic nervous system, which is unequivocally involved in coordination of the LPS-induced stress response and therefore has an important role in regulation and control of anxiety-related behaviours. Our next aim was to examine whether the anxiety-like phenotype is associated with alterations to other behavioural aspects, such as reproductive behaviours, given that sexual deficits have been previously documented to coincide with increased anxiety (Barrot et al., 2005; Wallace et al., 2009). Finally, we examined an immediate and long term impact of neonatal immune challenge on gonadal development, HPG axis functioning and overall reproductive fitness. We were particularly interested in establishing peripheral inflammatory pathways and cellular mechanisms; the regulation of which may have significant implications for long-term fertility.

5. Overview of papers

The current thesis consists of five published papers. The first two papers examine the neuroimmune, neuroendocrine and autonomic mechanisms associated with anxiety-like behaviours. The next two papers examine the impact of neonatal LPS exposure on reproductive success and development, as an additional aspect of an anxiety-like phenotype. The final paper, explores in depth the molecular mechanisms of neonatal immune programming of gonadal development.

Paper 1

Sominsky L., Walker A.K., Ong L.K., Tynan R.J., Walker F.R., Hodgson D.M. (2012) Increased microglial activation in the rat brain following neonatal exposure to a bacterial mimetic. *Behavioural Brain Research.* 226(1), 351-356.

This first manuscript demonstrates that rats exposed to LPS during neonatal life exhibit increased hippocampal microglial activation in adulthood, along with an increase in anxiety-like behaviours. This paper has also demonstrated that rat pups treated with LPS respond with an immediate activation of the HPA axis and ANS, as indicated by increased circulating corticosterone and increased phosphorylation of TH in the adrenal medulla, respectively. These findings have provided insight into the complex interaction between the brain and immune system during early life, and suggest a neuroimmune pathway, which may underpin the long-term behavioural and neuroendocrine changes following neonatal immune challenge.

Paper 2

Sominsky L., Fuller E.A., Bondarenko E., Ong L.K., Averell L., Nalivaiko E., Dunkley P.R., Dickson P.W., Hodgson D.M. (2013) Functional programming of the autonomic nervous system by postnatal immune challenge: implications for anxiety. PLOS ONE 8(3): e57700. doi:10.1371/journal.pone.0057700

This second manuscript further examines the anxiety-like phenotype induced by neonatal LPS exposure; however, its primary focus is the long term effect of neonatal LPS treatment on ANS function and the associated neuroendocrine and behavioural changes. Neonatal LPS exposure produced an increase in TH phosphorylation and activity in the adrenal glands of adolescent and adult animals. Increased autonomic arousal of LPS-treated animals was also demonstrated via plethysmographic assessment of adult respiratory rate, when animals that were exposed to LPS as neonates r**e**sponded with enhanced respiratory rates to a lower threshold of sensory stimuli. These autonomic changes were associated with increases in anxiety-like behaviours and HPA axis activity. In addition, this paper reports altered expression of ANS and HPA axis regulatory genes in anxiety-related brain regions. These findings suggest that in addition to the commonly examined alterations in HPA axis activity, neonatal LPS challenge is associated with a persistent change in ANS functioning, associated with the anxiety-like phenotype.

Paper 3

Walker A.K., Hiles, S.A., Sominsky L., McLaughlin E.A., Hodgson D.M. (2011) Neonatal lipopolysaccharide exposure impairs sexual development and reproductive success in the Wistar rat. *Brain, Behaviour & Immunity,* 25(4): 674-684

This third manuscript aimed to extend the previously documented behavioural findings in the model of neonatal immune challenge, and explored the impact of LPS administered in early life on sexual, as well as anxiety-like behaviours. Additional aspects of

reproductive development were examined in this study and these included male and female HPG axis activity and male gonadal development. The data indicated that neonatal LPS exposure suppresses HPG function, which perturbs puberty onset and impairs mating behaviours independent to the anxiety-like behavioural phenotype in adulthood in both males and females. Gonocyte development in males appears delayed and morphological alterations to the testes are described. This finding provided an avenue to explore female gonadal morphology, as described in the following paper. Taken together, the data indicate that neonatal LPS exposure produces a long-term impact on reproductive success, given the alterations in pubertal onset and sexual behaviour. Associated changes in neuroendocrine measures suggest a possible mechanism through which a subfertile phenotype in both male and female animals may arise.

Paper 4

Sominsky L., Meehan C.L., Walker A.K., Bobrovskaya L., McLaughlin E.A., Hodgson D.M. (2012) Neonatal immune challenge alters reproductive development in the female rat. *Hormones and Behavior.* 62(3), 345-355

Given the pronounced alteration in sexual behaviour in LPS-treated animals, which was particularly apparent in females, the fourth manuscript further explored the impact of neonatal immune challenge on reproductive development in the female rat. Neonatal LPS exposure resulted in advanced onset of puberty and advanced reproductive senescence. These changes were associated with increased weight gain, elevated levels of circulating corticosterone and decreased HPG hormones. Importantly, diminished follicular reserve was evident in the ovaries of LPS-treated females. Some of these changes in reproductive development persisted into a subsequent generation of offspring born to LPS-treated mothers. These data indicate that neonatal immune challenge has a profound impact on the female

reproductive development, via the alteration of metabolic and neuroendocrine factors which regulate sexual maturation. Moreover, these data suggest increased susceptibility of females to the deleterious effects of neonatal immune stress and its possible inheritance to a subsequent generation.

Paper 5

Sominsky L., Sobinoff A.P., Jobling M.S., Pye V., McLaughlin E.A., Hodgson D.M. (2013) Immune regulation of ovarian development: programming by neonatal immune challenge. *Frontiers of Neuroscience.* 7(100). doi: 10.3389/fnins.2013.00100

The aim of the fifth and final manuscript was to provide a mechanistic insight into the previously documented changes in oestrous cyclicity and follicular pool of LPS-treated female rats. In this study, ovarian gene expression was assessed by a microarray analysis of neonatal ovaries. Microarray analysis revealed a significant upregulation in the expression of a substantial number of genes in the ovaries of LPS-treated rats. The altered genes were identified as components of several molecular networks, involved in inflammatory responses, immune signalling and reproductive development. These data indicate that neonatal immune challenge has a direct effect on the ovary during the sensitive period of follicular development. This further suggests that increased inflammatory signalling within the developing ovary may have significant implications for the programming of ovarian functioning and reproductive success.

Published Papers

Paper 1: Increased microglial activation in the rat brain following neonatal exposure to a bacterial mimetic

Luba Sominsky, Adam K. Walker, Lin K. Ong, Ross J. Tynan, F. Rohan Walker, Deborah M. Hodgson

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Statement of author contributions to manuscript

Author	Description of Contribution to Manuscript	Signature
Luba Sominsky	Designed and performed the experiments Analysed and interpreted the data Wrote the manuscript	
Adam K Walker	Designed and performed the experiments Analysed and interpreted the data Wrote the manuscript	
Lin Kooi Ong	Performed the analysis of tyrosine hydroxylase	
Ross J Tynan	Provided technical experimental assistance Assisted in data analysis and interpretation	
F. Rohan Walker	Assisted in the experimental design and data interpretation Provided intellectual contribution and critical input Contributed reagents / materials/ analysis tools Wrote and revised the manuscript	
Deborah M Hodgson	Assisted in the experimental design and data interpretation Provided intellectual contribution and critical input Contributed reagents / materials/ analysis tools Revised the manuscript	

* Luba Sominsky and Adam Walker contributed equally to this manuscript and share joint first authorship

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Short communication

Increased microglial activation in the rat brain following neonatal exposure to a bacterial mimetic

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ABSTRACT

Neonatal lipopolysaccharide (LPS) exposure increases anxiety-like behaviour in adulthood. Our current aim was to examine whether neonatal LPS exposure is associated with changes in microglial activation, and whether these alterations correspond with alterations in behaviour. In adulthood, LPS-treated animals exhibited significantly increased anxiety-like behaviour and hippocampal microglial activation. The efficacy of the LPS challenge was confirmed by increased neonatal plasma corticosterone and tyrosine hydroxylase (TH) phosphorylation in the adrenal medulla. These findings suggest a neuroimmune pathway which may underpin the long-term behavioural and neuroendocrine changes following neonatal infection.

Research over the last decade has shown that perinatal immune activation with lipopolysaccharide (LPS) can induce long term functional alterations in the metabolic [1], immune [2–5], behavioural [6–10], and neuroendocrine [3,11] systems. Our laboratory has been particularly interested in the increased levels of anxiety-like behaviours observed in adult rats exposed to LPS on postnatal days 3 and 5. We have consistently reported that this exposure paradigm increases anxiety-like behaviour in adulthood across a range of behavioural tests [8–10]. Recently, several studies have shown that glia, and in particular microglia, respond vigorously to LPS, and appear to be involved in modulating the expression of certain behaviours. Interestingly, Bilbo and colleagues, using a similar form of postnatal challenge, have shown that exposure to *Escherichia coli* on postnatal day 4 induces changes in microglia that persist until adolescence (28 days later). The same group later observed that adult rats challenged postnatally with *E. coli* and then with LPS produced higher levels of CD11b, a putative marker of microglial activation. Together, these findings suggest that exposure to Gram-negative bacteria, the source from which LPS is derived, causes persistent changes in microglial responsiveness. As yet, however, no studies have examined whether alterations in microglial activation may occur in rats challenged with LPS on postnatal days three and five, the most commonly used model of early life bacterial driven immune activation. The association between such increases in microglial activation and anxiety-like behaviour also remains to be determined. Here we tested postnatally challenged animals using the Elevated Plus Maze (EPM) and Holeboard tests. Immediately following testing, we collected the brains of these animals and assessed them for changes in microglial activation status using immunohistochemical labelling of the ionized calcium-binding adaptor molecule (Iba-1) protein. Iba-1 is recognised to be an effective method for identifying changes in microglial activation status [12–15]. Finally, we confirmed the efficacy of the LPS challenge in activating neonatal stress pathways

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by assessing plasma corticosterone concentrations and tyrosine hydroxylase (TH) phosphorylation (a rate-limiting enzyme crucial for catecholamine synthesis) in the adrenal medulla.

68 Wistar males deriving from 17 litters were used in this study. Animals were mated in the University of Newcastle Psychology vivarium. Litters were randomly allocated into either LPS (8 litters) or saline control conditions (9 litters) at birth (postnatal day [PND] 1). On PND 3 and PND 5, animals were briefly removed from their home cages, weighed, and administered intraperitoneally with either 0.05 mg/kg LPS (*Salmonella enterica*, serotype enteritidis; Sigma–Aldrich Chemical Co., USA) or an equivolume of non-pyrogenic 0.9% saline (Livingstone International, Australia) as described previously [10]. A subgroup of rats ($n = 56$ males, derived from 13 litters; 6 LPS-treated, 7 saline-treated) were euthanized 4 or 24 h following drug exposure on PND 5 to determine whether neonatal LPS administration was effective in activating the neonatal neuroendocrine and sympathetic nervous system stress responses. 4 or 24 h following drug exposure on PND 5 the immediate effects of LPS on plasma corticosterone, as well as TH protein and phosphorylation levels in adrenals were assessed. The remaining 4 litters were left with their dams until weaning (PND 22), at which time they were segregated into same-sex paired housing (41.5 cm × 28.0 cm × 22.0 cm cages; Mascot Wire Works, Sydney, Australia). Rats were left undisturbed from weaning until behavioural testing in adulthood (PND 85) apart from weights collected weekly. Housing conditions were identical to those previously reported [10]. All experimentation occurred in accordance with the 2004 NH&MRC Australian Code Of Practice For The Care And Use Of Animals For Scientific Practice.

All behavioural testing was conducted in adulthood (PND 85) in complete darkness under infrared lighting. Detailed protocols and conditions for the EPM and Holeboard have been previously described [9,10]. Anxiety-related variables assessed in the EPM included the percentage of time spent in the open arms and the number of closed and open arm entries. Distance and activity measures were recorded for indications of locomotor activity and freezing. Exploratory head dips, time spent in the central square, distance travelled, and activity were recorded to assess anxiety-like behaviours in the Holeboard. The order of the behavioural tests was counterbalanced across tasks and subjects.

Animals allocated for assessment of HPA axis activation following neonatal drug administration were rapidly decapitated 4 h and 24 h following drug administration on PND 5 and trunk blood was collected into EDTA-coated tubes (Livingstone International, Australia). Plasma corticosterone concentrations were assessed using a rat corticosterone ^{125}I radioimmunoassay kit following manufacturer's instructions (MP Biomedicals, USA)

Tyrosine hydroxylase protein and phosphorylation levels were analysed as previously described with some modifications [16]. Briefly, the adrenals were homogenised using a sonicator (Soniprep 150, MSE) in 200 μl homogenisation buffer (50 mM Tris–HCl, pH 7.5; 1 mM EGTA; 1 protease tablet; 1 mM sodium vanadate; 1 mM sodium pyrophosphate; 80 μM ammonium molybdate; 5 mM β-glycerophosphate; 2 μM microcystin). Samples were then centrifuged at 16,000 rpm for 20 min at 4 °C. The clear supernatants were collected and protein concentration was determined by a BCA assay according to the manufacturer's general protocol for protein analysis. Samples were diluted with homogenisation buffer to same concentration and mixed with sample buffer (1% SDS, 10% glycerol, 0.5% DTT and minimal bromophenolblue). 30 μg of each samples were subjected then to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose [17]. Membranes were then stained with Ponceau S (0.5% ponceau in 1% acetic acid) to assess the efficacy of the transfer. Membranes then were washed in Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris, 0.075% Tween-20, pH 7.5) and incubated with blocking solution (5% bovine serum albumin, 0.04% sodium azide in TBST) for 2 h at 25 °C. Membranes were washed in TBST and incubated with total or phosphor-specific TH antibodies for 1 h at 25 °C. The levels of total TH (tTH) protein, pSer40 and β-actin protein have previously been characterized [18]. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25 °C. Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using ECL plus detection reagents. The density of total TH, phospho-specific TH and β-actin bands were measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total TH protein levels were expressed as the ratio of TH protein to β-actin as β-actin levels are used as house-keeping proteins. Site-specific TH phosphorylation at Ser40 was expressed as the ratio to total TH protein to account for variability in total TH between samples.

Two hours following the conclusion of behavioural testing, animals were deeply anaesthetised with sodium pentobarbitone and perfused transcardially with heparinised phosphate buffered saline (PBS) followed by 4% formaldehyde (pH 9.5) in 0.1 M phosphate buffer (PB). Brains were then extracted and postfixed in a 15% sucrose solution containing the same fixative solution. After fixation, brains were transferred to a 15% sucrose solution in 0.1 M PBS for cryoprotection. Serial coronal sections (30 μm) were cut using a freezing (−25° C) microtome (Leica SM 2000R) and were divided into a one-in-six series, which was stored in an anti-freeze solution (4° C) until required for immunoperoxidase labelling. Sections were then processed using immunohistochemistry to assess microglia using ionized calcium-binding adaptor molecule (Iba-1) protein. The antibody is not expressed in neurons, astrocytes, or oligodendroglia [19–21], and is constitutively expressed and upregulated in activated microglia [21]. Previous research indicated that Iba-1 and Mac-1 (CD11b) antibodies overlap in their ability to label microglia [22]. However, Iba-1 and CD11b differ in the subcellular locations that they label. While CD11b is detected predominantly in the cytoplasm, with some labelling of the processes, Iba-1 labels the cytoplasm, nucleus and processes strongly. Accordingly, Iba-1 provides superior labelling, in particular for quantitative analysis.

For immunoperoxidase labelling, a series of sections from all animals in both treatment conditions was processed simultaneously. Sections were rinsed with 0.1 M PB and then endogenous peroxidases were destroyed in 0.1 M PB containing 3% hydrogen peroxide. Non-specific binding was blocked with 3% normal horse serum. The sections were then incubated with the primary antibody (anti-rabbit Iba-1, Wako, 1:10,000) in 0.1 M PB containing 1% horse serum, 0.1% bovine serum albumin (BSA) and 0.3% Triton-X for 48 h at 4 °C. Sections were then rinsed, incubated in the corresponding secondary antibody (Amersham donkey anti-rabbit, 1:300) in phosphate buffered horse serum for 2 h, rinsed, incubated in 0.1% extravidin peroxidise for 1 h, and then rinsed again. The reaction was detected under a microscope after applying 2% nickel sulphate in 0.1 M PB containing 0.05% 3,30-diaminobenzidine, and was stopped and rinsed with 0.1 M PB once optimal staining with minimal background labelling had been achieved. Sections were then mounted onto chrome alum coated slides, dehydrated using a series of graded alcohols (70%, 95%, 100%, absolute), cleared in xylene and coverslipped with ultramount (Fronine Laboratory Supplies, Australia).

Iba-1 immunolabelling data was analysed by an experimenter blind to the experimental conditions. Images from hippocampal and amygdala regions were taken using an Olympus BX51 microscope fitted with an Olympus DP71 camera and an Olympus UPlan-Fi objective (10×/0.30). The images were processed using DP Manager software (Version 3.1.1.208; Olympus Corporation) and stored at a resolution of 4086 × 3072 pixels (1 pixel = 0.429 μm^2

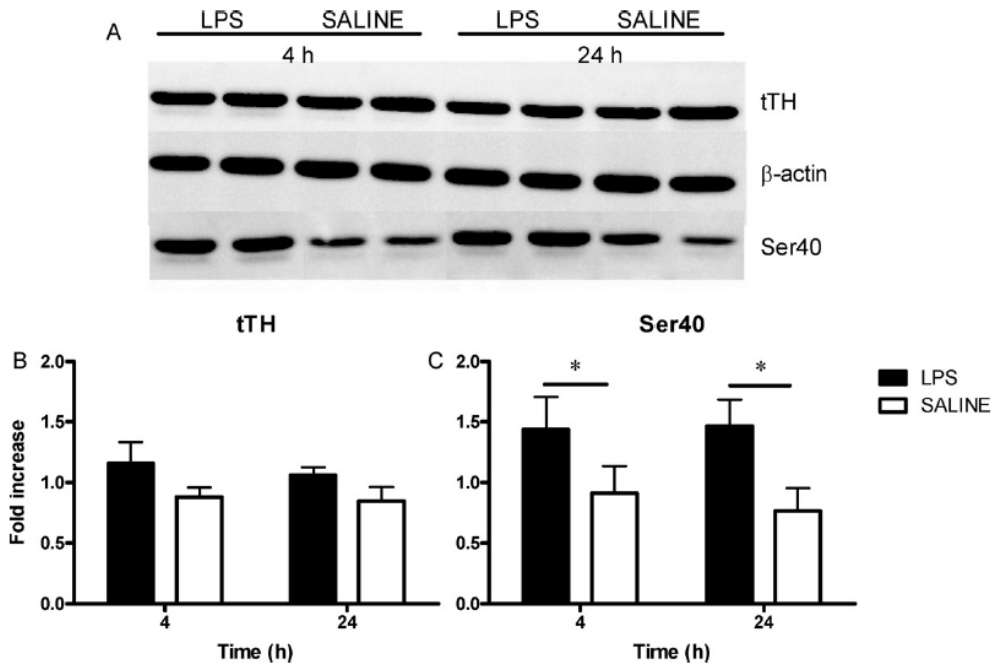

Fig. 1. Effect of neonatal LPS exposure on total tyrosine hydroxylase (TH) protein (±SEM) with respect to β-actin and the phosphorylation of serine (Ser) residue 40 of TH with respect to total TH in the adrenal gland 4 and 24 h after the administration of LPS or saline. (A) Representative immunoblots show the effect of LPS and saline. (B) Compared with saline, LPS had no effect on the TH protein at anytime point. (C) Phosphorylation of Ser40 occurred at 4 h and was sustained at 24 h. Filled bars represent neonatally challenged LPS males ($n = 6$ at each time point) and hollow bars represent neonatally challenged saline males ($n = 6$ at each time point), $^*p < .05$.

at 100× magnification). A rat brain atlas [23] was used to identify the anatomical location for each of the 3 regions of interest for Iba-1 immunolabelling, specifically, the CA1 region and the dentate gyrus of the hippocampus, and basolateral amygdala (CA1, DG and BLA, three sections, bregma −2.12 to −2.56 mm). In each of the regions, left and right hemispheres were recorded independently to assess inter-hemispheric asymmetries. The total density of immunoreactive material in the aforementioned regions for Iba-1 and Iba-1 density restricted to the soma region were determined using Metamorph software (Version 7.1.3.0; Molecular Devices). This program has been successfully employed to determine levels of Iba-1 immunolabelling previously [15].

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Volume 18 (SPSS Inc.). All data were analysed using analyses of covariance (ANCOVA) controlling for litter effects such as litter size and male-to-female

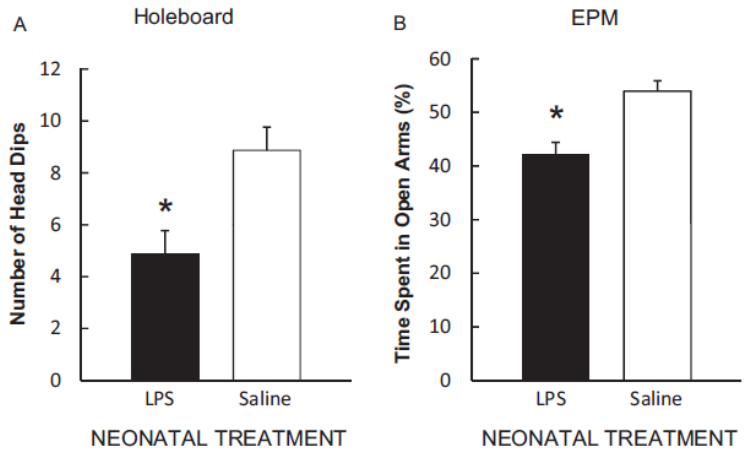

Fig. 2. Effect of neonatal LPS exposure on anxiety-related behaviour in adulthood. (A) LPS-treated males spent significantly less time (%; ±SEM) on the open arms of the EPM compared to saline-treated controls. The filled bar represents neonatally challenged LPS males ($n = 6$) and the hollow bar represents neonatally challenged saline males ($n = 5$), $^*p < .05$. (B) LPS-treated males exhibited significantly fewer exploratory head dips compared to saline-treated controls. The filled bar represents neonatally challenged LPS males ($n = 6$) and the hollow bar represents neonatally challenged saline males ($n = 6$), $^*p < .05$.

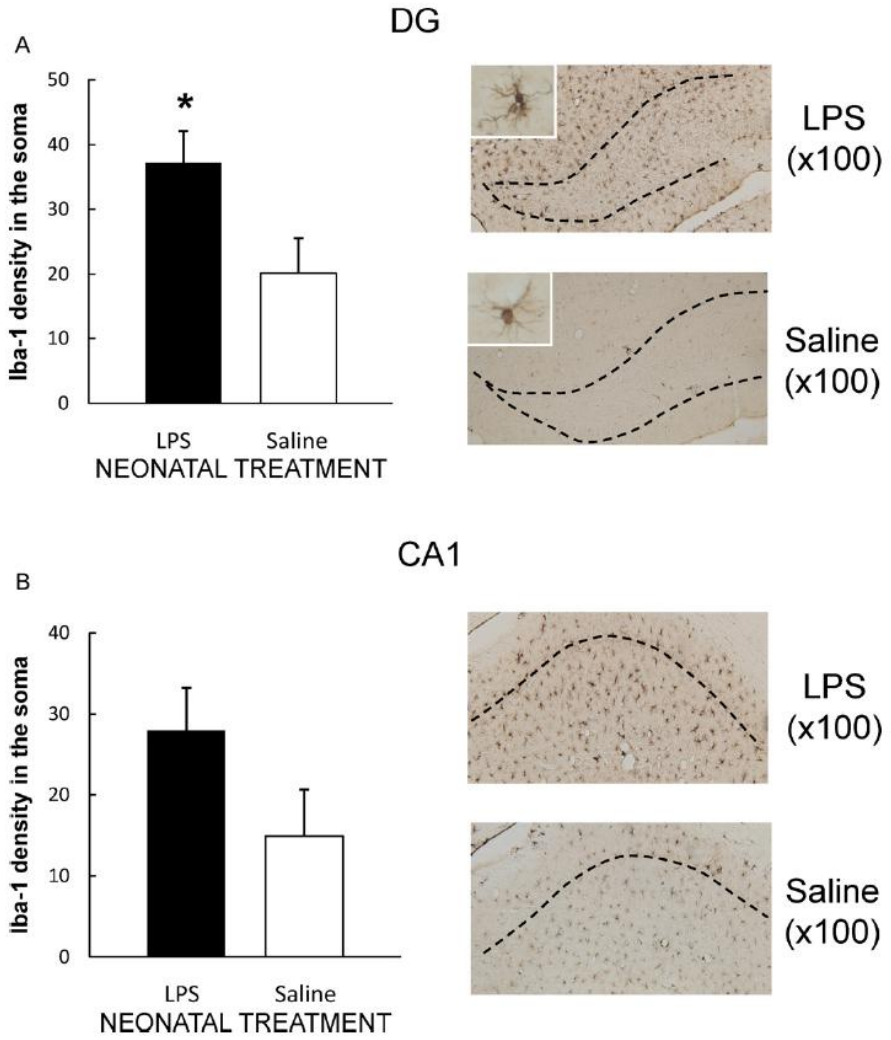

Fig. 3. Effect of neonatal LPS exposure on microglial activation in the hippocampus. (A and B) LPS-treated males ($n = 6$) exhibited higher Iba-1 immunolabelling in the dentate gyrus and CA1 compared to saline-treated ($n = 5$) controls, $^{*}p < .05$. Filled bars represent neonatally challenged LPS males and hollow bars represents neonatally challenged saline males. Representative photomicrographs exemplify greater Iba-1 immunolabelling in the dentate gyrus and CA1 of LPS-treated males compared to saline-treated controls.

ratio. The ANCOVA revealed that litter effects did not have an impact on any of the behavioural or physiological measures assessed.

LPS-treated males gained significantly more weight between PND 3 and PND 5 (M = 2.89 g, SEM = 0.08) compared to saline-treated controls (M = 2.55 g, SEM = 0.09), $F(14,70) = 9.17$, $p < .001$. No differences in weight gain were observed following weaning.

LPS-treated males exhibited significantly greater corticosterone concentrations 4 h following neonatal treatment on PND 5 (M = 19.84 ng/ml, SEM = 0.91) compared to saline-treated controls (M = 16.82 ng/ml, SEM = 1.12), $F(1,43) = 4.43$, $p < .05$.

While no differences were observed in regards to total TH protein levels, a significant and sustained increase in TH phosphorylation at Ser40 was evident in LPS-treated males 4 h and 24 h following injection on PND 5 compared to saline-treated controls, $F(1,21) = 6.19$, $p < .05$. See Fig. 1.

LPS-treated males spent a significantly lower proportion of time in the open arms of the EPM compared to saline-treated controls, $F(1,7) = 15.92$, $p < .05$. A main effect of neonatal treatment was observed in regards to exploratory head dips in the holeboard apparatus, $F(3,8) = 5.58$, $p < .05$. LPS-treated males exhibited significantly fewer head dips compared to saline-treated controls. See Fig. 2A and B.

A significant effect of neonatal treatment was observed for Iba-1 immunolabelling in the dentate gyrus, $F(1,9) = 5.28$, $p < .05$, whereby LPS-treated males exhibited significantly greater density of Iba-1 within the cell body of the microglia compared to saline-treated controls (Fig. 3A). Trends for Iba-1 immunolabelling of CA1 reflected that of the dentate gyrus, whereby LPS-treated males exhibited increased density of Iba-1 immunolabelling restricted to the soma region compared to saline-treated controls, however this failed to reach significance ($p = .1$; Fig. 3B). No difference in

total density of Iba-1 immunolabelling across the entire image was observed for either the dentate gyrus or CA1. No significant differences were observed in regards to Iba-1 immunolabelling within the soma region nor across the entire image in the basolateral amygdala.

Early life is exquisitely sensitive to environmental inputs. In particular, interaction with the early postnatal microbial environment is essential for setting the tone of both the endocrine and immune systems. Dual exposure to LPS during neonatal life is the most well characterized model of postnatal bacterial exposure, and has been extensively used to examine immune-neuroendocrine communication. We and others have previously shown that this dynamic interplay in early life determines the trajectory of a range of physiological and behavioural responses. Here, we have demonstrated that dual postnatal LPS exposure protocol produces an increase in anxiety-like behaviour that is co-incident with an increase in levels of microglial activation.

In the present study, we demonstrated that LPS challenge in the neonate robustly increased corticosterone release and TH phosphorylation in the adrenal medulla, the former measure indexing engagement of the hypothalamic-pituitary-adrenal (HPA) axis and the later the sympathetic nervous system. In adulthood, LPS challenged neonates exhibited increased anxiety-like behaviour on the EPM and Holeboard apparatus, evidenced by reduced time spent in the open arms of the EPM and a reduction in exploratory head dips in the Holeboard apparatus. Both the EPM and Holeboard are widely used to assess anxiety-like behaviour in rodents, and we have previously found that this dual LPS exposure protocol increases anxiety-like behaviour as demonstrated by these standard behavioural measures [8–10]. The particularly novel finding reported in the current study is the co-incident increase in microglial activation, as indicated by greater Iba-1 immunolabelling. Increase in microglial density following LPS challenge has been shown to be associated with prolonged activation and proliferation of microglia [24]. While our findings do not unequivocally implicate activated microglia in playing a central role in the enhanced anxiety observed, it is worthwhile to note that microglial activation can potently influence neuronal signalling [25].

Interestingly, changes in microglial activation were observed within the dentate gyrus but not as we had expected in the basolateral amygdala. Despite the absence of obvious changes in microglial activity within the BLA, functional alterations within the dentate gyrus have been linked with enhanced levels of anxiety [26–28]. While it is difficult to fully account for the regional differences in microglial activation observed, one possibility relates to the timing of the LPS challenge in relation to CNS development. In the current study, exposure to LPS occurred during the critical stress hyporesponsive period of development for the HPA axis, of which the hippocampus plays a fundamental role [29,30]. The increases in microglial activation in the hippocampus may reflect a pronounced susceptibility of primary HPA axis-related regions, of which the amygdala is involved but less influential. However, examination of microglial activation should be extended in the future to additional amygdaloid anxiety-related regions, such as the bed nucleus of the stria terminalis, in order to fully account for the discrepancies found in this study. Finally, it should be noted that while Iba-1 is a commonly used marker for microglia, it also stains for macrophages. However, it is generally accepted that microglia predominantly exist within the parenchyma, whereas macrophages are mostly considered to be a hematogenous cell type [31]. Thus, one can be relatively confident that the current findings in the hippocampus and BLA pertain almost solely to microglia.

The current study has provided insight into the complex interaction between the brain and immune system during early life of which microglia clearly play an interesting and intriguing role. Of importance are the seemingly divergent changes in microglial activation in regions of the hippocampus. These findings may represent potential mechanisms for the well-documented increases in anxiety-like behaviour in animals neonatally exposed to LPS. Certainly the evidence demonstrating that increased microglial activation in the hippocampus corresponds with induced anxiety and stress responses [32,33] lends credence to this assertion. However, it is clear that while both increased microglia and increased behavioural regulation in adulthood occur following neonatal LPS challenge, their contributions have not been directly linked to one another. Previous research investigating mechanisms in the generation of anxiety-like behaviour and microglial activation revealed potential pathways, by means of pharmacological intervention. Inhibition of IL-1β synthesis, in neonatally infected rats, prevented cognitive impairments induced by subsequent LPS challenge in adulthood, implicating the important role of IL-1β in the context of neonatal programming by immunological challenge [34]. Another study showed that increased anxiety-like behaviour, microglial activation and central levels of IL-1β induced by social defeat can be prevented by administration of propranolol – a β-adrenergic receptor antagonist. Moreover, IL-1 receptor type-1 deficient mice did not exhibit anxiety-like behaviour or microglial activation in the same context [35]. These latest findings indicate the changes induced by social defeat were dependent on activation of β-adrenergic and IL-1 receptors. Investigation of these and other pathways (such as GABA) in our model of postnatal LPS exposure can shed light on the mechanisms mediating behavioural changes in adulthood.

Conflict of interest

The authors declare there are no conflicts of interest.

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Paper 2: Functional programming of the autonomic nervous system by postnatal immune challenge: implications for anxiety

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Statement of author contributions to manuscript

Author	Description of Contribution to Manuscript	Signature
Luba Sominsky	Designed and performed the experiments Analysed and interpreted the data Wrote the manuscript	
Erin A. Fuller	Designed and performed the experiments Assisted in data analysis and manuscript preparation	
Evgeny Bondarenko	Assisted in the experimental design, data analysis and interpretation	
Lin Kooi Ong	Performed the analysis of tyrosine hydroxylase Revised the manuscript	
Lee Averell	Assisted in data analysis	
Eugene Nalivaiko	Contributed reagents/materials/analysis tools Provided intellectual contribution Revised the manuscript	
Peter R. Dunkley	Contributed reagents/materials/analysis tools Provided intellectual contribution Revised the manuscript	
Phillip W. Dickson	Contributed reagents/materials/analysis tools Provided intellectual contribution Revised the manuscript	
Deborah M Hodgson	Assisted in the experimental design and data interpretation Provided intellectual contribution and critical input Contributed reagents / materials/ analysis tools Revised the manuscript	

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Functional Programming of the Autonomic Nervous System by Early Life Immune Exposure: Implications for Anxiety

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Abstract

Neonatal exposure of rodents to an immune challenge alters a variety of behavioural and physiological parameters in adulthood. In particular, neonatal lipopolysaccharide (LPS; 0.05 mg/kg, i.p.) exposure produces robust increases in anxiety-like behaviour, accompanied by persistent changes in hypothalamic-pituitary-adrenal (HPA) axis functioning. Altered autonomic nervous system (ANS) activity is an important physiological contributor to the generation of anxiety. Here we examined the long term effects of neonatal LPS exposure on ANS function and the associated changes in neuroendocrine and behavioural indices. ANS function in Wistar rats, neonatally treated with LPS, was assessed via analysis of tyrosine hydroxylase (TH) in the adrenal glands on postnatal days (PNDs) 50 and 85, and via plethysmographic assessment of adult respiratory rate in response to mild stress (acoustic and light stimuli). Expression of genes implicated in regulation of autonomic and endocrine activity in the relevant brain areas was also examined. Neonatal LPS exposure produced an increase in TH phosphorylation and activity at both PNDs 50 and 85. In adulthood, LPS-treated rats responded with increased respiratory rates to the lower intensities of stimuli, indicative of increased autonomic arousal. These changes were associated with increases in anxiety-like behaviours and HPA axis activity, alongside altered expression of the GABA-A receptor $\alpha 2$ subunit, CRH receptor type 1, CRH binding protein, and glucocorticoid receptor mRNA levels in the prefrontal cortex, hippocampus and hypothalamus. The current findings suggest that in addition to the commonly reported alterations in HPA axis functioning, neonatal LPS challenge is associated with a persistent change in ANS activity, associated with, and potentially contributing to, the anxiety-like phenotype. The findings of this study reflect the importance of changes in the perinatal microbial environment on the ontogeny of physiological processes.

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Introduction

Activation of the immune system in early life is thought to play a role in predisposing to later life psychopathologies. Epidemiological evidence, for instance, indicates higher incidences of psychopathologies, including schizophrenia, negative emotionality and panic disorders in children exposed to viral or bacterial infections in-utero [1,2] or in early life [3]. Animal models that have been utilised to examine the impact of immune activation in early life have repeatedly demonstrated that exposure to bacterial or viral agents including mimetics (e.g. lipopolysaccharide (LPS); polyinosinic:polycytidylic acid (poly I:C)), live agents (e.g. *Escherichia coli (E. coli)*), and other agents, such as toxins (i.e., endotoxins and exotoxins) is associated with an increased likelihood of psychopathology in later life [4,5,6,7,8,9].

LPS exposure on postnatal days (PNDs) 3 and 5 is a well-documented rodent model used to examine the impact of "perinatal programming" on a variety of physiological and behavioural outcomes [4,5,6,10,11,12,13,14,15,16,17]. The concept of perinatal programming encompasses the role of the intrauterine and early postnatal environment in the onset of adult disease [18]. One of the most consistent observations is that exposure to LPS in early life results in offspring who, in adulthood, demonstrate increased anxiety-like behaviours [4,13,15]. The anxiety-like behaviours typically observed include more time spent in the closed arms and fewer entries to the open arms of an elevated plus maze (EPM), reduced exploratory behaviour in the holeboard apparatus and increased risk assessment behaviour in the open field apparatus [4,13,15]. Importantly, recent evidence indicates that these behavioural outcomes are not limited to the exposed animals alone, but have been shown to persist into a subsequent generation of offspring, born to either a maternal or paternal line of LPS-treated animals [6].

The system most commonly implicated in the development of anxiety-like behaviours, in the context of neonatal exposure to LPS, is the hypothalamic-pituitary-adrenal (HPA) axis. Alterations in circulating corticosterone have been reported in adult animals

treated as neonates with LPS. This is particularly apparent when these animals are subjected to an additional acute stressor in adulthood [4,11,12,16]. At the central level, neonatal exposure to LPS has been shown to be associated with increased corticotropin-releasing hormone (CRH) mRNA levels in the hypothalamus in male rats and decreased glucocorticoid receptor (GRs) density in the hypothalamus, hippocampus and frontal cortex of both male and female rats [11]. Exposure to live infection in early life was also reported to produce long term alterations in HPA axis activity, with increased plasma corticosterone levels in adult males and increased GR mRNA levels in the hippocampus of adult females, when compared to same sex controls [19]. Overall, it has been suggested that early life exposure to an inflammatory insult alters the later response of the HPA or stress axis. Whilst the HPA axis response to a peripheral inflammatory challenge is primarily mediated by the proinflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 [20], further evidence suggests a possible role of γ-aminobutyric acid (GABA) in LPS-induced activation of the HPA axis [21]. GABAergic innervation of the hypothalamus provides an inhibitory tone regulating the HPA axis activity [22]. Administration of GABA prior to LPS injection has been shown to reduce plasma corticosterone levels when compared to animals treated with LPS alone [21]. Nevertheless, increased activation of GABA-A receptors in neonatal rats increases hippocampal neuronal apoptosis, resulting in memory and learning impairments in later life [23]. Of particular relevance, neonatal LPS exposure on PNDs 7 and 9 has been recently demonstrated to result in a selective decrease of GABA containing interneurons in the hippocampal regions of adult rats [24], indicating altered GABA signalling induced by neonatal immune activation.

The HPA axis is not, however, the only system affected by neonatal exposure to an inflammatory insult. The HPA axis and the autonomic nervous system (ANS) work in a coordinated fashion to modulate the stress response. Recent evidence from our laboratory indicates that neonatal exposure of rodent pups to LPS results in an immediate and sustained response of the ANS as indicated by an increase in phosphorylation and activity of tyrosine hydroxylase (TH) in the adrenal glands [13,14,25]. Given the critical role of TH in catecholaminergic synthesis, this finding suggests that neonatal LPS treatment results in an increased activation of the sympathetic compartment of the ANS.

Exposure to LPS has been demonstrated to induce activation of the ANS during the acute-phase immune response. Specifically, parasympathetic nervous system signalling was evidenced via both the afferent and efferent vagal signals. Peripheral administration of LPS was reported to increase levels of circulating catecholamines and to cause a distinct activation of central catecholaminergic neurons that are likely to play a mediating role in the neuroendocrine responses to peripheral inflammation (reviewed in [26,27]). Moreover, whilst intraperitoneal administration of LPS led to an increase in noradrenergic, serotonergic and dopaminergic activity in a range of brain areas, intracerebroventricular (i.c.v.) administration of the bacterial mimetic did not have an effect on dopamine metabolism suggesting involvement of other factors induced by peripherally administered LPS [28].

ANS activation during the LPS-induced inflammatory response promotes HPA axis activity, facilitating physiological adaptation to the immunological challenge [27]. Given this evidence it is possible that exposure to neonatal LPS may alter the long term functioning of the ANS. To date, however, relatively little is known regarding the programming effect of early life exposure to LPS on ANS functioning. Our recent findings have indicated increased TH phosphorylation in the adrenal glands of LPS-treated neonates, in both males [13,25] and females [14]. The aim of the current study was to determine whether neonatal LPS exposure is able to induce long term alterations in autonomic activity. To assess this we utilised both a direct measure of autonomic activity (i.e., adrenal TH) and a behavioural measure of autonomic function (i.e. respiratory response as measured by plethysmography).

Respiratory rate is a sensitive and reliable index of autonomic arousal, independent of changes in cardiovascular parameters. Whilst cardiovascular measures are most commonly utilised, the use of plethysmography (a non-invasive technique able to measure respiration) facilitates the measurement of changes in respiration at thresholds far lower than that required to induce autonomically mediated changes in cardiovascular system [29,30]. Therefore, assessment of respiratory rate in rodents provides an index of the orienting response, constitutive of ANS arousal [30]. Moreover, in humans, anxiety states are often associated with respiratory dysregulation [31].

In the current study neonatal programming of the ANS was investigated via the assessment of TH activation in the adolescent and adult adrenal glands. Autonomic function was further validated by the measurement of respiratory responses to mild stressful stimuli in adult rats. To confirm the presence of an anxiety-like phenotype, adult anxiety-like behaviours were evaluated, and HPA axis activity at both the peripheral and central levels was assessed. Brain factors examined included mRNA levels of CRH, CRH binding protein (CRHBP), CRH receptor type 1 (CRHR1), GRs and mineralocorticoid receptors (MRs) as well as GABA-A receptor subunit alpha-2 (GABA-Arα2), due to its role in modulation of the stress response and anxiety states [32].

Methods

Experimental Procedures

Experimentally naive female Wistar rats were obtained from the University of Newcastle animal house and mated in the University of Newcastle Psychology vivarium, under conventional housing conditions. At birth (PND 1), pups were randomly allocated into either LPS or saline control conditions. Pups were briefly removed from their cages on PNDs 3 and 5, weighed and administered with LPS (Salmonella enterica, serotype enteritidis; Sigma-Aldrich Chemical Co., USA, dissolved in sterile pyrogen-free saline, 0.05 mg/kg) or an equivolume of saline (Livingstone International, Australia), intraperitoneally (i.p.). Neonatal drug administration protocols and housing conditions were previously reported [4,17]. Only male offspring were used in this study. A subgroup of 47 males (derived from four LPS and four saline-treated litters) was euthanized at 4 h, 24 h and 48 h post neonatal treatment on PND 5 to determine the efficacy of drug administration via assessment of plasma corticosterone levels (n = 6−10 per group, per time point). The remaining pups were housed with their dams until PND 22, at which point they were weaned and divided into same-sex housing (41.5 × 28.0 × 22.0 cm cages; Mascot Wire Works, Sydney Australia) and left undisturbed except for weekly weights and monitoring. In adolescence (PND 50) an additional subgroup of animals (16 males, derived from three LPS and two saline-treated litters; n = 6−10 per group) was sacrificed for analysis of plasma corticosterone and adrenal TH levels. In adulthood (PND 85), 10 LPS and 9 Saline treated male rats (derived from three LPS and two saline-treated litters) were subjected to respiratory and behavioural testing, as further described. Brains were collected from 13 animals (n = 5−7 per group) two weeks following the behavioural testing to determine expression of CRH, CRHR1, CRHBP, GABA-Arα2, GR and MR mRNA levels. An additional

group of 6 LPS and 6 saline-treated males (derived from two LPS and two saline-treated litters) were used for assessment of baseline plasma corticosterone and TH levels in adulthood. See the study design in Fig. 1a. For each experimental condition, animals were distributed as evenly as possible from all litters used per treatment, to avoid potential litter effects. All experiments were conducted in accordance with the 2004 NH&MRC Australian Code of Practice for the care and use of animals for scientific practice. This study was approved by The University of Newcastle Animal Care and Ethics Committee (ACEC 901).

Blood and Adrenals Collection

Neonatal time point. At 4 h, 24 h and 48 h following the last neonatal drug administration on PND 5, animals were rapidly decapitated and trunk blood was collected into EDTA-coated tubes (Livingstone International, Australia).

Adolescence. On PND 50 animals were deeply anesthetized with an overdose of Lethabarb (2 ml/kg i.p.; Virbac, Pty. Ltd, Milperra, Australia). Cardiac blood was obtained into EDTA-coated tubes. Adrenals glands were surgically removed, cleaned of any excess fat tissue, immediately frozen in liquid nitrogen and kept at −80°C until assayed.

Adulthood. On PND 85, immediately following the respiratory testing blood was collected via the saphenous vein. All blood samples were centrifuged at 1000 × g for 15 min at 4°C, and plasma stored at −20°C until assayed. Blood and adrenal glands were collected from an additional subset of animals on PND 85, after a lethal injection of Lethabarb (2 ml/kg), following the previously described methods.

Radioimmunoassay

Plasma corticosterone concentrations were assessed using a commercially available rat corticosterone 125I radioimmunoassay kit (MP Biomedicals, USA). The recovery of free corticosterone is 100%, with an inter- and intra-assay variability of 4.4% and 6.5%, respectively.

Behavioural Testing

Anxiety-like behaviours in adulthood were assessed on the elevated plus maze (EPM) and the holeboard apparatus, as per previously described protocols [4,6,16]. Animals were given 5 min to explore each of the apparatuses and the order of testing was counterbalanced. Behaviours assessed in the EPM included the percentage of time spent in the open arms, number of entries to the open and closed arms, and risk assessment. Anxiety-related variables examined in the holeboard apparatus included exploratory head dips and time spent in the centre square. Activity and distance travelled were assessed in both the EPM and the

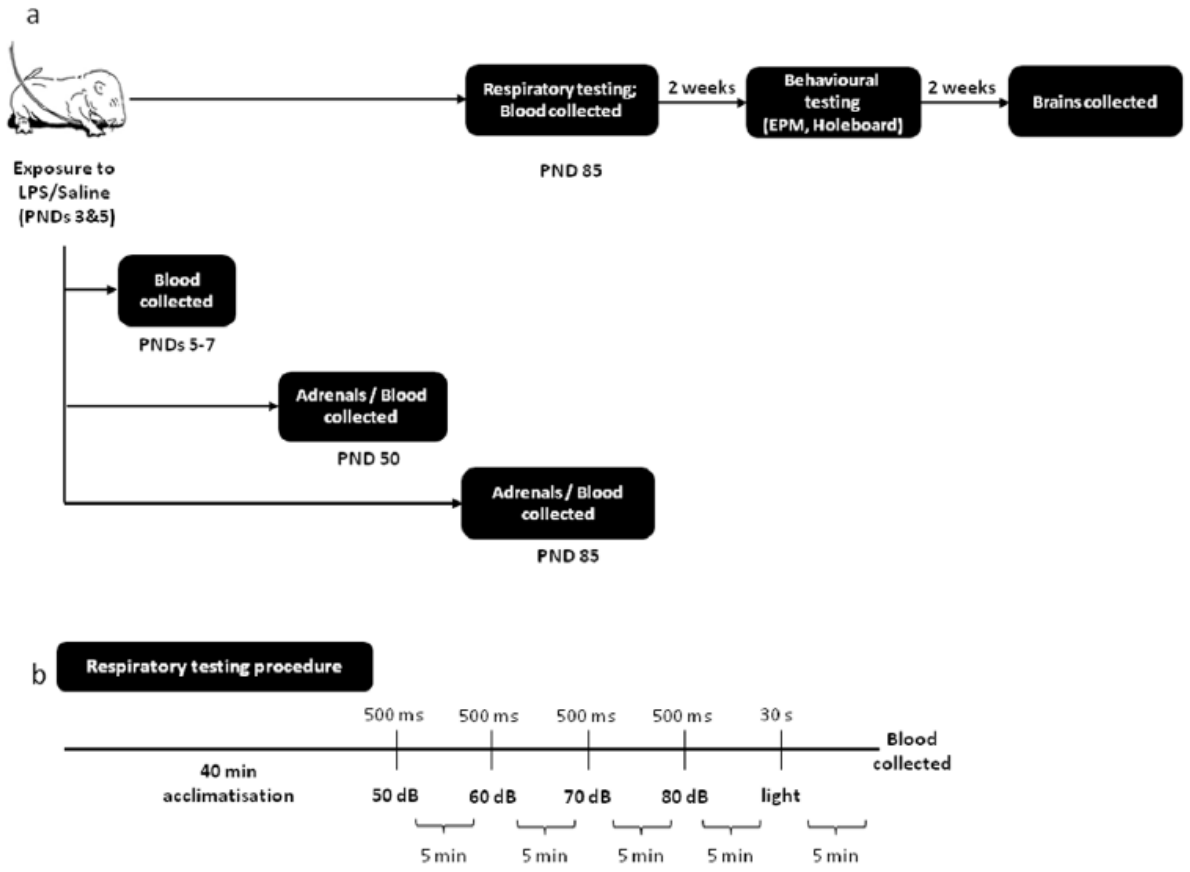

Figure 1. Experimental design. (A) A schematic timeline of the study design and experimental protocols. (B) A schematic representation of the respiratory testing procedure.
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holeboard, for indications of locomotor activity and freezing. Time and event-related data for the assessment of anxiety-like behaviours and locomotor activity were recorded using a computer-based automated behavioural tracking system (Motion Mensura Pty. Ltd., Australia). The computer software allows a definition of regions of interest and monitors entries to and time spent in these regions, using pixel intensity to determine the position of the animal and general locomotor activity.

Tissue Collections and Quantitative RT-PCR

Expression of CRH, CRHR1, CRHBP and GABA-Arα2 mRNA levels was determined by quantitative RT-PCR in the prefrontal cortex (PFC), hypothalamus and hippocampus of LPS and saline-treated animals. In addition, expression of GRs and MRs was determined in the hypothalamus and hippocampus. Two weeks following the respiratory testing, animals were deeply anesthetized with an overdose of Lethabarb (2 ml/kg i.p.; Virbac, Pty. Ltd, Milperra, Australia), brains were rapidly removed and areas of interest isolated (PFC, hypothalamus and hippocampus). Samples were then immersed in RNA*later* solution (Ambion, Austin, TX, USA), stored at 4°C overnight and then kept at −20°C until further analysis.

Total RNA extraction from brain tissue was carried out using RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometer, NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE USA). First-strand cDNA was synthesized from 2 µg of total RNA using a Super-Script® VILO™ cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA, USA), according to manufacturers' instructions. Real-time PCR was performed using SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) on a 7500 RT-PCR Fast instrument (Applied Biosystems, Foster City, CA, USA). Primer sequences are listed in Table 1. The 25 µl PCR mixture consisted of 12.5 µl SYBR Green PCR Master Mix, 9.5 µl water and 2 µl of each primer was added to 1 µl of the cDNA template (10 ng/ml). All reactions were performed in duplicate under the following conditions: 95°C for 20 s and 40 cycles of 95°C for 3 s and 60°C for 30 s. In addition, a melting curve was determined under the following conditions: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s. The data were normalized to an endogenous control, β-actin. A relative quantitative measure of the target gene expression compared with β-actin mRNA was obtained using the equation $2^{-\Delta\Delta C(t)}$, where $C(t)$ is the cycle at which fluorescence was first detected above background, and presented as a fold increase relative to the saline control.

Table 1. Real-time PCR primer details.

Target gene	Forward	Reverse
CRH	CGCCCATCTCTCTGGATCTC	CGTTGTAAAGTAAAGGGCTATTAG
CRHR1	TGGAACCTCATCTCGGCTTT	CACTCGACCTGGTGTTTGGT
CRHBP	GCGAAGGCGAGGGAAGAA	GTACCGACCGGAACACAGA
GABA-A-rα2	CAATGCACTTGGAGGACTTTCC	GGCTCCAGCACATTCGTATCG
GR	CGTCAAAAGGGAAGGGAAC	TGTCTGGAAGCAGTAGGTAAG
MR	CCAAATCACCCTCATCCAG	GCACAGTTCATACATGGCAG
β-actin	TCTGTGTGGATTGGTGGCTCTA	GACGAACGACTAGGTGTAGAC

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Tyrosine Hydroxylase Analysis

Tyrosine hydroxylase phosphorylation, protein and activity levels were analysed as previously described with some modification [25]. Briefly, the adrenals were homogenised using a sonicator (Soniprep 150, MSE) in 500µL homogenisation buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 protease tablet, 1 mM Sodium Vanadate, 1 mM Sodium Pyrophosphate, 80µM Ammonium Molybdate, 5 mM β-Glycerophosphate, 2 µM Microcystin). Samples were then centrifuged at 16,000 rpm for 20 min at 4°C. The clear supernatants were collected and protein concentration was determined by a BCA assay according to the manufacturer's general protocol for protein analysis. Samples were diluted with homogenisation buffer to same concentration (5 mg/mL).

Thirty µg of each sample was mixed with sample buffer (1% SDS, 10% glycerol, 0.5% DTT and minimal bromophenol blue) and subjected to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose. Membranes were incubated with blocking solution (5% bovine serum albumin) for 2 h at 25°C. Membranes were washed in Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris, 0.075% Tween-20, pH 7.5) and incubated with pSer19-, pSer31-, pSer40-, total-TH or β-actin-HRP antibodies for 1 h at 25°C. Membranes were washed in TBST and incubated with HRP-linked anti-IgG secondary specific antibodies for 1 h at 25°C. Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using Western HRP Substrate (Millipore). The densities of phospho-, total TH and β-actin bands were measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total TH protein levels were expressed as the ratio of TH protein to β-actin as β-actin levels are used as an endogenous control. pSer19, pSer31 and pSer40 were expressed as the ratio to total TH protein to account for variability in total TH between samples.

Fifty µg of each sample was then mixed with reaction mixture (36 µg catalase, 2 mM potassium phosphate pH 7.4, 0.008% β-mercaptoethanol, 24 µM L-tyrosine, 1 µCi 3,5-[^{3}H]-L-tyrosine, final volume 50 µL) and subjected to tritiated water release assay. The 50 µL 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 16,000 rpm 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 scintillation spectrometry for 10 min per sample. TH activity assays which were performed under these conditions were linear. The changes in TH activity were normalized to total TH protein levels and expressed as a fold increase relative to the saline control.

Respiratory Testing

Materials. Respiratory rate was recorded using a custom-built whole body plethysmograph, based on the procedure previously described [29,30]. Briefly, the apparatus consisted of a clear Perspex cylinder (inner diameter 95 mm, length 260 mm, volume 1.84 l, wall thickness 3 mm), flushed by a constant flow of compressed medical air (3 L/min). The output flow was separated into two lines by a plastic T-connector, with one line connected to a differential pressure amplifier (model 24PC01SMT, Honeywell Sensing and Control, GoldenValley,MN,USA) and the second line open to room air. Air pressure signal from the amplifier was digitised at 1 KHz and collected via a data acquisition system (PowerLab, Model 4SP, ADInstruments, Sydney, Australia).

Respiratory rate was computed online from the pressure signal using ChartPro 6.0 software (AD Instruments, Sydney, Australia). A piezoelectric pulse transducer (MLT1010/D, AD Instruments, Sydney, Australia) was located underneath the plethysmograph, to record gross motor activity.

Procedure. All recordings occurred in a dark chamber. As illustrated in Fig. 1b, following 40 min acclimatisation to the cylinder, animals were presented with four increasing intensities of acoustic stimuli in ascending order (50 dB, 60 dB, 70 dB, 80 dB). Each acoustic stimulus was presented for 500 ms with an inter-stimulus interval of 5 min. The light stimulus (30 Lux, 30 s duration) was presented 5 min after the last acoustic stimulus.

Analysis. Each baseline period was defined as ten respiratory cycles in duration with no motor activity preceding the presentation of the stimulus. In cases where motor activity was present, data was excluded from further analysis. Respiratory response to a stimulus was then determined as a maximal change in respiratory rate for a duration of at least two respiratory cycles. Hierarchical Bayesian analysis, based on Markov Chain Monte Carlo (MCMC) methods [33] was employed to evaluate changes in respiratory rate in response to increasing intensities of acoustic stimuli. The MCMC implementation was carried out in WinBUGS [34]. A Change Detection mathematical model was developed to assess the presence and magnitude of changes in respiratory rate in response to amplification of low-intensity acoustic stimuli among LPS and saline-treated animals. Thus, hierarchical modelling allowed us to determine whether the experimental groups vary in their propensity to physiological arousal, avoiding the bias associated with averaging data and or the noise associated with analysis of individual cases. Here we model the sequence of respiratory recordings as a normal distribution where each trial (recording) is estimated by the evaluation of the likelihood that the current posterior distribution and the proposed distribution are from the same sequence or not. When the latter is more likely, a new distribution is proposed and evaluated; iteratively the proposal distribution (prior) settles to a new posterior distribution at a new mean level, with equal variance assumed for each proposed distribution. The fixing of the variance parameter allows the mean estimates to be dependent upon the largest change in the sequence.

For each stimulus intensity (50 dB, 60 dB, 70 dB, 80 dB) changes in respiratory rate were presented as a difference (Δ values) attained by subtracting the baseline respiratory rate prior to the onset of stimulus from the peak respiratory response to the stimulus, as generated by the Change Detection model. Student t test analyses on Δ respiratory rate were performed and complemented by Bayesian t tests, presented as posterior odds of preference for either null (H_0) versus alternative hypotheses (H_1) (for additional information on Bayes factor analysis see [35]). Bayes factor for H_0 versus H_1 is presented as B_{01}.

Data Analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Version 18 (SPSS Inc.). All data, except for the changes in respiration in response to acoustic stimuli (as described above), were analysed using analyses of variances (ANOVA) design. A nested ANOVA design, whereby "litter" is nested into "treatment" and analyses of covariance (ANCOVA), whereby "male-to-female ratio" and "litter size" are covaried in the analyses, were also employed where appropriate and reported only when significantly contributing to the data. Planned comparisons were performed when significant interactions were observed using t test analyses adjusted for multiple comparisons. The significance level was set at $p \leq 0.05$.

Results

Developmental Weight Gain

Weight gain was recorded in all males born into the litters participating in this study. No differences in weight gain were observed between the LPS and saline-treated animals during the neonatal period. However, LPS-treated males gained significantly more weight, than saline-treated controls ($F_{(1,89)} = 26.925$, $p<.001$) following weaning (PND 22) to adolescence (PND 50). Significant weight gain in LPS treated animals, as compared to saline-treated controls, was also evident from adolescence (PND 50) to adulthood (PND 85) ($F_{(1,58)} = 17.623$, $p<.001$). See Table 2.

Plasma Corticosterone

Assessment of circulating corticosterone levels during the neonatal period revealed a significant effect of treatment ($F_{(8,26)} = 4.09$, $p<.005$), with LPS-treated males exhibiting significantly increased levels of circulating corticosterone ($M = 24.187$, $SE = 1.436$) than saline-treated controls ($M = 18.93$, $SE = 1.463$), and a significant interaction between the time point of blood collection and treatment ($F_{(10,26)} = 3.88$, $p<.005$). Planned comparisons revealed LPS-treated neonates had significantly increased corticosterone levels at 4 h following LPS challenge on PND 5 ($t_{(15)} = 2.89$, $p<.05$), with no differences at later time point, as shown in Fig. 2a.

LPS-treated males exhibited increased baseline corticosterone levels on PND 50 ($F_{(4,11)} = 4.69$, $p<.05$), and in adulthood, on PND 85 ($F_{(1,10)} = 8.89$, $p<.05$) as demonstrated in Fig. 2b and 2c. Significantly increased corticosterone levels were evident in LPS-treated males immediately following the respiratory testing ($F_{(1,13)} = 6.12$, $p = .05$), see Fig. 2d.

Anxiety-like Behaviours

Neonatal LPS treatment resulted in significantly increased percentage of time spent in closed arms of the EPM, compared to saline-treated controls ($F_{(4,14)} = 3.39$, $p = .05$), Fig. 3a. Fig. 3b demonstrates that LPS-treated animals engaged in more risk assessment behaviour (rearing events) in the holeboard apparatus ($F_{(1,15)} = 5.03$, $p = .05$). Trends, which approached significance ($p = .06$), indicated that LPS-treated males exhibited fewer exploratory head dips in the holeboard apparatus than their saline-treated counterparts, Fig. 3c. No significant differences were observed in other measures.

Gene Expression

Significantly decreased CRHR1 mRNA levels were evident in LPS-treated males in the PFC and hypothalamus ($F_{(1,10)} = 12.02$, $p<.05$; $F_{(1,10)} = 6.09$, $p<.05$, Fig. 4a and 4d, respectively). However, in the hippocampus trends indicated that neonatal LPS exposure resulted in increased CRHR1 mRNA expression ($p = .16$), Fig. 4f. Similarly, CRHBP mRNA levels were decreased in the PFC of LPS-treated animals, approaching significance

Table 2. Developmental Weight Gain.

Developmental timeframe	LPS	Saline
PND 22–50	213.52±5.23***	176.13±4.95
PND 50–85	241.55±11.99***	174.71±10.48

Values are mean±SEM.
***$p<.001$.
doi:10.1371/journal.pone.0057700.t002

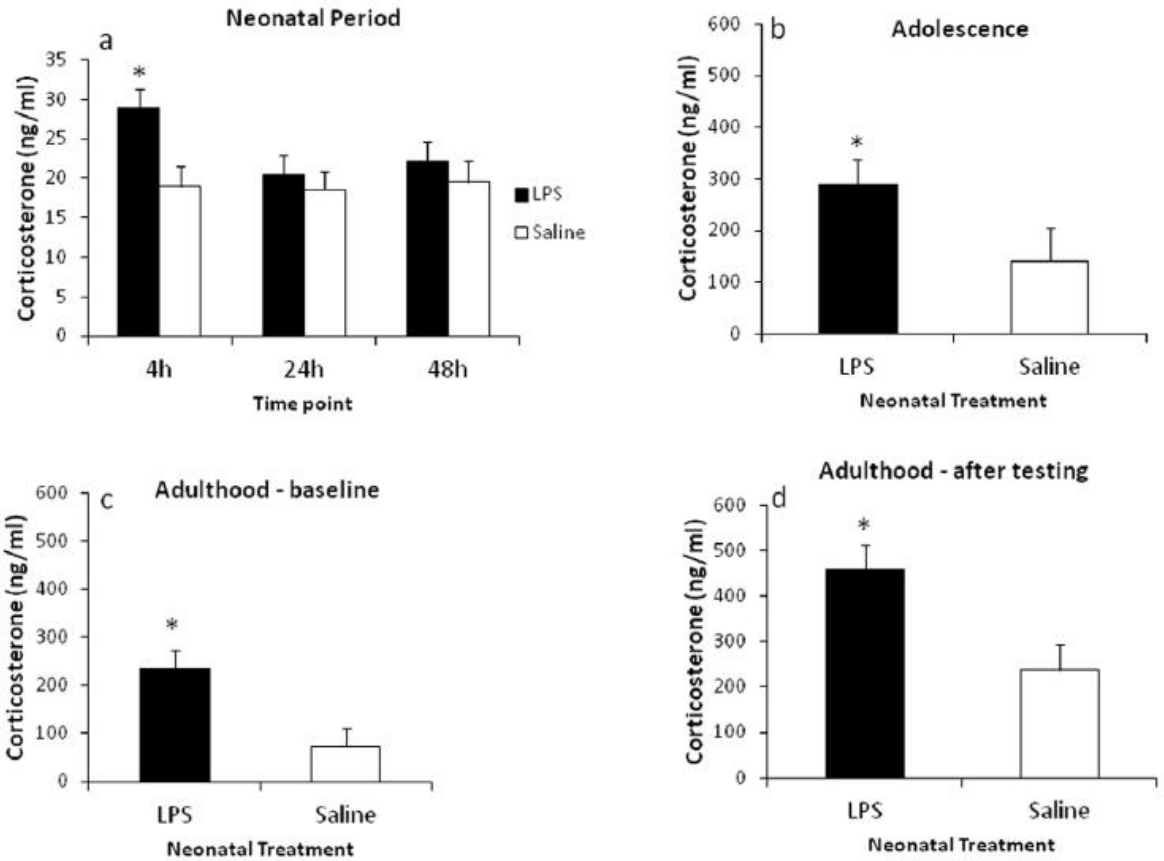

Figure 2. Effect of neonatal LPS exposure on plasma corticosterone levels. Corticosterone levels in neonatal period (PNDs 5-7) (A), in adolescence (PND 50) (B), and in adulthood (PND 85) (C). (D) Demonstrates changes in circulating corticosterone following the respiratory testing in adult rats (PND 85). Filled bars represent neonatally-treated LPS rats, hollow bars represent neonatally-treated saline controls. Values are mean±SEM. *$p<.05$.
doi:10.1371/journal.pone.0057700.g002

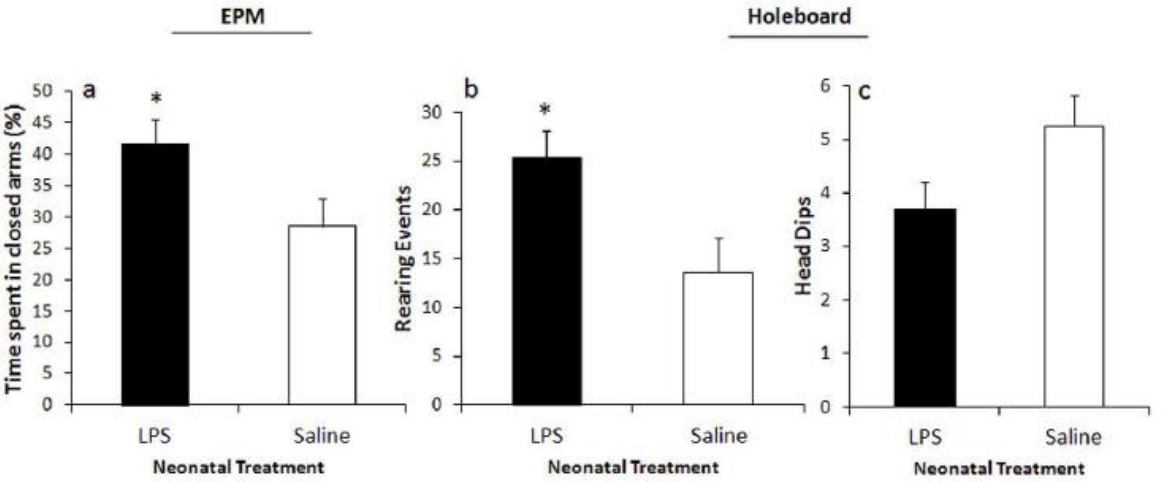

Figure 3. Effect of neonatal LPS exposure on anxiety-like behaviours in adulthood. Effect of neonatal LPS exposure on a percentage of time spent in the closed arms of the EPM (A), on a mean number of exploratory head dips (B) and risk assessment behaviours (C) in the holeboard apparatus. Filled bars represent neonatally-treated LPS rats, hollow bars represent neonatally-treated saline controls. Values are mean±SEM. *$p<.05$.
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($p = .09$), significantly decreased in the hypothalamus ($F_{(1,10)} = 19.24$, $p < .001$), but significantly increased in the hippocampal region ($F_{(1,10)} = 6.04$, $p < .05$), see Fig. 4b, 4e and 4g, respectively. Fig. 4c demonstrates that GABA-A-rα2 mRNA expression was significantly decreased in the PFC of LPS-treated males ($F_{(1,11)} = 5.54$, $p < .05$). Significantly increased GR mRNA levels were evident only in the hippocampus of LPS-treated animals ($F_{(1,11)} = 6.25$, $p < .05$), see Fig. 4h. No differences were observed in CRH and MR mRNA expression. Refer to Table 3 for the complete list of gene expression.

TH Analysis

A significant effect of treatment was observed in regards to TH phosphorylation at all 3 serine residues (pSer19, pSer31 and pSer40) and TH activity in adolescent ($F_{(1,12)} = 13.72$, $p < .005$; $F_{(1,12)} = 9.86$, $p < .05$; $F_{(1,12)} = 22.52$, $p < .0001$; $F_{(1,12)} = 5.36$, $p < .05$, respectively, see Fig. 5a) and adult animals ($F_{(1,10)} = 6.71$, $p < .05$; $F_{(1,10)} = 8.42$, $p < .05$; $F_{(1,10)} = 39.63$, $p < .001$; $F_{(1,10)} = 22.73$, $p = .001$, respectively, see Fig. 5b). No significant difference in total TH protein levels was evident at any of the time points.

Respiratory Testing

Analysis of changes in respiration by using a Change Detection model revealed that LPS-treated males responded with a tendency to increase respiration when introduced to the stimulus of lowest intensity (50 dB). Despite that *t* test analysis revealed no significant difference in the magnitude of response between the LPS and saline-treated animals ($t_{(12)} = 1.42$, $p = .18$), Bayes factor ($B_{01} = .97$) constitutes an approximately equal likelihood for the preference for either null or the alternative hypothesis. In response to the 60 dB stimulus, LPS-treated animals responded with a significantly increased respiratory rate ($t_{(15)} = 2.88$, $p < .05$; $B_{01} = .15$). This difference from saline-treated controls remained significantly increased when presented with the 70 dB stimulus ($t_{(15)} = 2.57$, $p < .05$; $B_{01} = .24$). No differences were observed at the highest intensity (80 dB) between the neonatally treated groups as reflected in both statistical approaches ($t_{(14)} = .005$, $p = .99$; $B_{01} = 2.18$). See Fig. 6b.

As demonstrated in Fig. 6c, in response to light stimulus LPS-treated males exhibited an increased respiratory rate as compared to saline-treated controls ($F_{(4,15)} = 3.61$, $p < .05$), with no differences in baseline respiratory rate obtained prior to the presentation of the stimulus. See Fig. 6a for a representation of raw data records.

Discussion

The primary aim of this study was to investigate the long term consequences of neonatal immune challenge on ANS functioning, and its association with behavioural and neuroendocrine indices in an anxiety-like phenotype. Here, we replicate and confirm this model by demonstrating increased anxiety-like behaviours and alterations to the HPA axis following neonatal LPS challenge. A significant outcome of this study is the demonstration that an exposure to LPS during the neonatal period is also associated with persistent alterations to ANS functioning. This is demonstrated using a behavioural index of ANS activity (via plethysmographic assessment of respiratory rate [29,30]), and supported by biochemical analysis of adrenal TH phosphorylation, activity and protein levels. These findings suggest for the first time, to our knowledge, that neonatal LPS results in persistent alterations to autonomic activity - a crucial component in the biobehavioural response to stress.

LPS challenge in the neonate induces an acute increase in TH phosphorylation and activity in neonatal adrenals [13,14,25]. The current study importantly demonstrates that this increased activity and phosphorylation persists into adulthood. TH is the first rate-limiting enzyme in catecholamine biosynthesis [36]. Increased release of catecholamines is an essential aspect of the stress response, including inflammatory stress. The acute phase response to an inflammatory insult typically includes an immediate activation of the ANS both at the level of the parasympathetic and the sympathetic nervous systems [27]. Increased catecholamine release is then accompanied by a compensatory increase in catecholamine synthesis, in which TH plays an essential role, catalysing the hydroxylation of L-tyrosine to DOPA (reviewed in [36]). Under these conditions TH activity is normally inhibited. In response to stress, TH phosphorylation at serine residues disinhibits the catalytic domain and initiates enzymatic activity. While phosphorylation at three serine residues (Ser40, Ser 31 and Ser19) in the N-terminal domain of TH contributes to its activity, phosphorylation at Ser40 increases the enzymatic activity directly and to a greater extent than phosphorylation of the other sites [36,37]. *In vitro* studies indicated that the acute phase response of TH activation occurs within minutes due to increased phosphorylation, and is then followed by a sustained phase response over several hours. During the chronic phase (over several days) this response is mediated by an increase in TH mRNA levels and a subsequent protein synthesis [37]. We confirmed this observation in *in vivo* studies through the demonstration that the sustained phase of TH activation in neonatal adrenal glands persists for up to 24 h after LPS administration [13,25]. These changes were manifested by increased phosphorylation and activity of the enzyme, without alterations in protein levels. Increases in TH protein were evident only after 48 h, without a concomitant change in TH activity [25].

In the current study, increased phosphorylation at all three serine residues (Ser19, Ser31 and Ser40) and increased enzymatic activity were observed independent of changes in TH protein, in animals treated with LPS as neonates, at both the adolescent and adult time points. Previous *in vivo* studies have reported a disassociation between alterations in TH phosphorylation and activity, and TH protein levels in response to an acute [38] or chronic [39,40] stressor, suggesting that sustained phosphorylation represents a distinct regulatory mechanism of catecholamine synthesis, independent of TH protein synthesis [37]. Therefore, our data suggests that neonatal exposure to LPS results in an initial and sustained phase of TH activation in the neonatal period, which is associated with a long lasting effect on TH activity, mediated by TH phosphorylation but not by protein synthesis.

Increased glucocorticoids, resulting from either exposure to stress or direct administration of synthetic glucocorticoids, increase TH activity in sites of catecholamine production, including the adrenal medulla [41]. A functional interdependence between the adrenal cortical and medullary systems [42] suggests that the increased TH activity evident in the current study could potentially be triggered by increased and prolonged exposure to glucocorticoids. However, since our previous data [13,25] indicate that in neonates TH activity is more extended than the corticosterone response to LPS treatment, it appears more likely that an immune challenge during the neonatal period independently activates the HPA axis and the ANS, resulting in developmental programming of both systems.

Given that the TH analysis has indicated long term alterations in autonomic activity, we aimed to validate this observation using a behavioural paradigm that specifically targets autonomic function. Using a recently established model of respiratory testing,

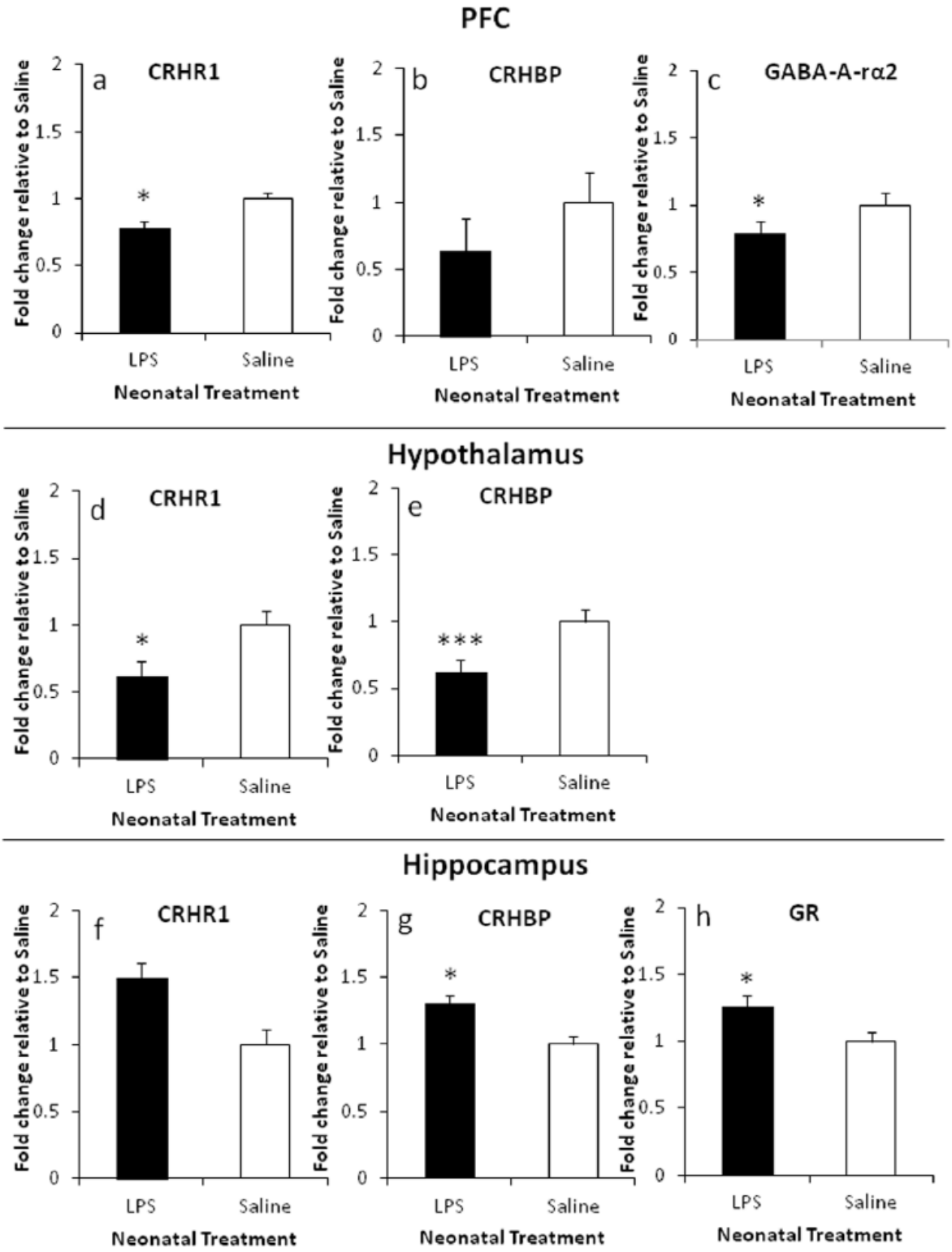

Figure 4. Gene expression in the PFC, the hypothalamus and the hippocampus, presented as a fold change relative to the saline control. (A–C) Represent changes in CRHR1, CRHBP and GABA-Arα2 mRNA levels in the PFC. (D–E) Represent changes in CRHR1 and CRHBP mRNA

levels in the hypothalamus. (F–H) Represent changes in CRHR1, CRHBP and GR mRNA levels in the hippocampus. Values are mean±SEM. *$p<.05$, ***$p<.001$.
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which assesses respiratory responses to mild sensory stimuli, the current study assessed respiration in rats as a measure of arousal. Previous studies have demonstrated that this behavioural measure is a valid, sensitive and reliable index of rapid changes in autonomic activity [29,30]. A recent study using a similar respiratory testing procedure indicated that systemic administration of an anxiolytic drug substantially reduces respiratory responses to acoustic stimuli and restraint stress [43], suggesting that assessment of respiratory rate in rodents represents a distinct measure of anxiety states. Moreover, due to the rapidity of change, respiratory responses are argued to be more sensitive than changes in cardiovascular parameters, providing an efficient temporal resolution, similar to EEG desynchronization, for measuring ANS activity [30]. As such, a simultaneous assessment of heart rate and respiratory parameters in response to short-lasting acoustic stimuli (60 dB to 90 dB) revealed that while respiratory responses typically show intensity-dependent increases, heart rate responses to the same intensities of the stimuli are less consistent and demonstrate no obvious trend [29,30]. The lack of a significant increase in heart rate responses in these previous studies was attributed to the relatively low intensity of the stimuli, suggesting an insensitivity of heart rate to subtle changes in arousal [29]. In the current study LPS-treated rats, when tested in adulthood, exhibited significantly increased respiratory rates at acoustic intensities that were several fold lower than those generating the same magnitude of response in saline-treated controls. This finding was replicated in response to bright light, and as such indicated that LPS-treated animals, when tested in adulthood, responded more intensely in terms of respiratory rate when exposed to visual and auditory stimuli.

In the current study, alterations in respiratory responses exhibited by the LPS-treated animals were associated with increases in anxiety-like behaviours and HPA axis activity. This is consistent with previous reports from this laboratory [4,6,13,15]. Therefore to extend these findings we investigated the expression of genes implicated in the aetiology of anxiety in the limbic regions, involved in the regulation of endocrine and autonomic stress-related responses, and responsible for the anxiety-related behavioural changes [22]. A significant reduction in GABA-Arα2 mRNA levels was evident in the PFC of LPS-treated animals. Given the major role of the α2 GABA-A receptor subtype in mediating anxiolytic effects of benzodiazepines, this is consistent with the reported anxiety-like phenotype in the current and other studies [4,6,13,15]. Moreover, GABAergic innervation of the PVN provides an inhibitory tone, modulating the HPA axis and autonomic activity. Within the PFC, GABAergic interneurons regulate the activity of glutamatergic neurons [44]. Reduced GABA-mediated inhibition, achieved by blockade of cortical GABA-A receptors, has been previously reported to result in cognitive deficits in schizophrenia models [45,46]. Although forebrain limbic structures do not directly innervate the PVN, inhibitory input from these limbic areas is received via intermediate synapses, inhibiting the HPA axis and autonomic responses to stress (reviewed in [22]). Whilst only one subunit of the GABA-A receptor system was examined in this study, this finding suggests a possible reduction in GABAergic content in the PFC of LPS-treated males, which may subsequently result in reduced GABA-mediated inhibition of cortical glutamatergic pyramidal neurons and lead to increased HPA axis activity and increased sympathetic response. In addition, further distinct regional assessment of GABA functioning is required. Nevertheless, this data is of a particular importance, given that neonatal LPS stimulus coincided with a critical period of functional maturation of the GABAergic synapses. Specifically, during the first week of life GABAergic plasticity in the neonatal rat hippocampus can be induced by postsynaptic activity of pyramidal cells [47]. Whether the peripheral immune challenge results in synaptic activity, which may induce long-term plasticity at inhibitory synapses, is yet to be elucidated.

Table 3. Gene expression in the PFC, hypothalamus and hippocampus.

Target gene	PFC	Hypothalamus	Hippocampus
CRH	1.229 (±.166)	1.114 (+.214)	1.188 (+.119)
CRHR1	**−1.287 (±.049)***	**−1.605 (+.108)***	**1.496 (+.231)**
CRHBP	**−1.567 (±.144)**	**−1.602 (+.061)*****	**1.304 (+.087)***
GABA-A-rα2	**−1.269 (±.066)***	−1.067 (+.137)	−1.025 (+.042)
GR	—	1.098 (+.064)	**1.262 (+.077)***
MR	—	1.003 (+.115)	1.033 (+.078)

Data presented as a fold change relative to the saline control (mean±SEM).
*$p<.05$,
***$p<.001$. See Fig. 4 for graphic representation of the highlighted values.
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Examination of CRHR1 and CRHBP mRNA levels revealed substantial downregulation of gene expression in the PFC and the hypothalamus, but upregulation in the hippocampus of LPS-treated animals. The CRH system has a regulatory role in HPA and ANS functioning [22]. While CRH exerts its actions via two receptors subtypes (CRHR1 and CRHR2), it binds with higher affinity to CRHR1 [48], and CRHBP regulates availability of CRH to its receptors [49]. Regional-specific expression and signalling capacity of CRH and its receptors has implications for anxiety and depression disorders [49]. While CRH deficiency has been reported to result in impaired HPA axis activity with no behavioural effects [50], CRHR1 depletion in mice has been associated with a decrease in anxiety-like behaviours [51], suggesting a differential importance of the receptor and its ligand in the regulation of stress-induced activity. A selective model of postnatal deletion of CRHR1 in the forebrain, introduced by Müller and colleagues, revealed a distinct role of CRH/CRHR1 neuronal pathways involved in behavioural regulation from those regulating HPA axis activity [52], with deletion of CRHR1 in limbic structures leading to aberration of anxiety-like behaviours independent of the HPA axis functioning. It implies, therefore, that CRHR1 is essential in mediating the behavioural responses to stress.

CRHBP, together with CRH receptors, mediates the activity of CRH in the brain and the pituitary, presumably acting as a negative regulator of CRH. CRHBP expression can be modulated by glucocorticoids and is cell-dependent, with increased pituitary levels in response to stress, suggesting its role in a negative feedback on the HPA axis [53]. Models of CRHBP overexpression and deficiency demonstrated an intricacy of homeostatic mechanisms regulating the HPA axis activity, with no changes in basal and adrenocorticotropic hormone (ACTH) levels in these models, but with a blunted ACTH response to the LPS stimulus in mice overexpressing CRHBP centrally and

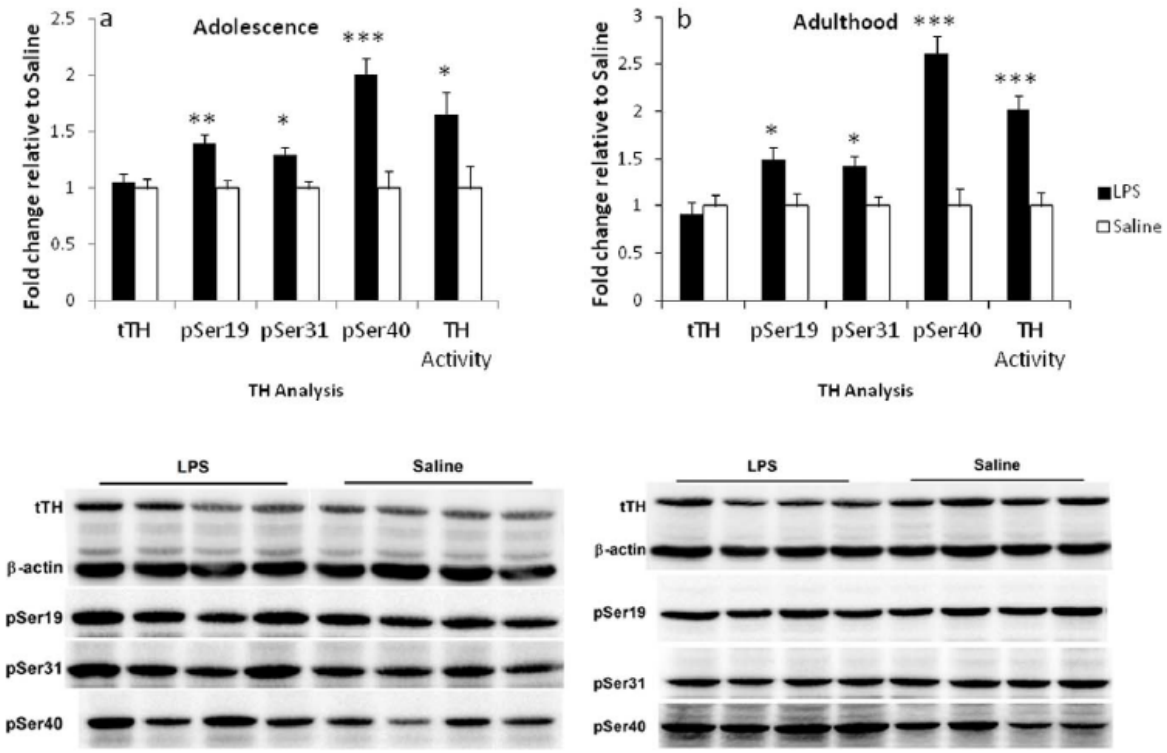

Figure 5. Effect of neonatal LPS exposure on TH protein, phosphorylation at Ser19, Ser31, Ser40 and TH activity levels. Changes in TH are expressed as a fold change relative to the saline control in the adrenal glands in adolescence (PND 50) (A) and adulthood (B). Representative immunoblots demonstrate the effect of LPS and saline treatments. Filled bars represent neonatally-treated LPS rats, hollow bars represent neonatally-treated saline controls. Values are mean±SEM. *$p<.05$, **$p<.01$, ***$p<.001$.
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peripherally, suggesting a compensatory increase or decrease of CRH may occur in response to alterations in CRHBP levels (reviewed in [53]).

A model of HPA axis dysfunction in depression and anxiety, proposed by Reul and Holsboer (2002) [54], suggests that the HPA hyperactivity may derive from the decreased negative inhibitory feedback, normally exerted by the hippocampal structures on the hypothalamus, but an increased effect of the central nucleus of amygdala, stimulating further activation of the HPA axis. Therefore the regional variability in the expression of both CRHR1 and CRHBP in the current study may be a result of prolonged exposure to circulating glucocorticoids, leading to diminished ability of the hippocampus to exert an inhibitory tone on the hypothalamic parvocellular neurons and potentially a compensatory alteration of CRHR1 and CRHBP mRNA levels in the PFC and the hypothalamus. Neonatal LPS exposure was previously found to result in increased mRNA expression of CRH in the PVN as well as increased median eminence levels of CRH and arginine vasopressin in adult animals neonatally treated with LPS [11]. In addition, increase in CRH mRNA content in the PVN was reported in 1-month-old mice, neonatally treated with LPS [55]. While this effect was not evident in the current study, we have assessed the mRNA content of the whole hypothalamic and hippocampal extracts. Therefore, examination of subregions may provide an insight on the regional-specific gene expression. Nevertheless, here we demonstrate the programming effect of neonatal LPS challenge on other components of the CRH system, which play an unequivocal role in regulation of the ANS, HPA axis activity and anxiety-like behaviours.

Increased expression of GR mRNA content was found in the hippocampus of LPS-treated animals. Previously, neonatal LPS treatment has been reported to decrease GR binding and density in the hippocampus, hypothalamus and frontal cortex of adult animals, adrenalectomized prior to brain collection [11]. The discrepancy between our findings and the latter may reflect differences in the functional aspects of GRs measured, since the current study assessed mRNA levels in animals with intact adrenals. In support of the current trend, increased hippocampal expression of GR mRNA, and no changes in MR mRNA levels, have been previously demonstrated in a similar model of neonatal LPS exposure in mice, when assessed during the second week of life [55]. In addition, contradictory findings have been reported, whereby increased glucocorticoid levels have been associated with either increased [56,57], decreased [58,59], or unchanged [60] GR binding or mRNA levels in the hippocampus. It is also plausible that elevated levels of circulating corticosterone, in conjunction with increased expression of GR in the hippocampus may constitute a state of glucocorticoid resistance, commonly present in inflammatory and neuropsychiatric disorders [61], accompanied by an altered balance of GR and MR expression, contributing to the increased emotional arousal [62].

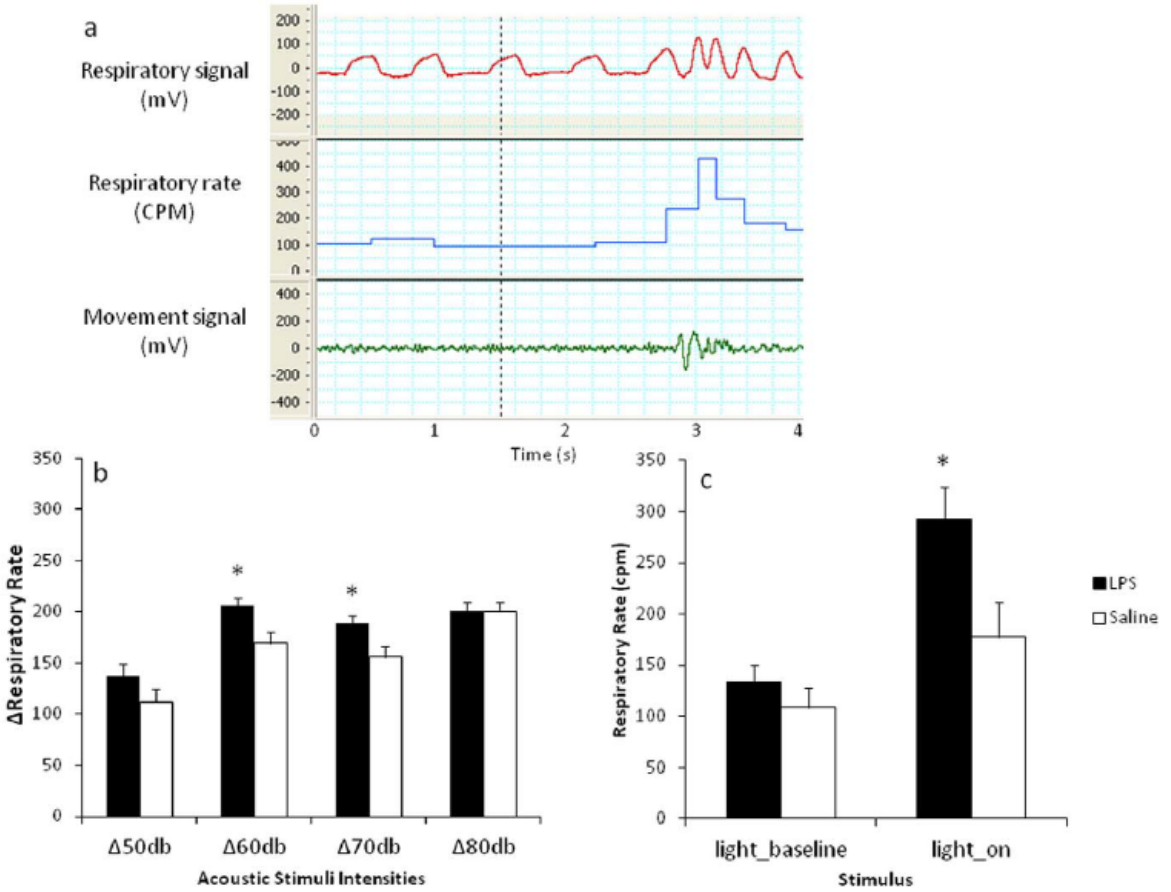

Figure 6. Effect of neonatal LPS exposure on respiratory rate in adulthood. (A) Representative raw data records of respiratory signal (mV), respiratory rate (cycles per minute (CPM)) and movement signal (mV) prior and following a sensory stimulus (broken vertical line). (B) Represents Δ changes from baseline (±SEM) in respiratory rate in response to acoustic stimuli as generated by the Change Detection model. (C) Represents changes (CPM±SEM) in respiratory rate prior and in response to light stimulus. Filled bars represent neonatally-treated LPS rats, hollow bars represent neonatally-treated saline controls. *$p<.05$.
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Conclusions

Our findings suggest that exposure to an immune challenge in the early postnatal period of life drives long term alterations in the neuroendocrine and autonomic systems, and is associated with alterations in behavioural functioning, overall indicative of an anxiety phenotype. While programming of the HPA axis activity and anxiety-like behaviours by neonatal LPS exposure has been previously demonstrated, this study provides novel insights into the effects of an early life immune challenge on ANS functioning. Since prolonged exposure to increased endocrine and autonomic activity is a known epidemiological risk factor for development of metabolic disorders, such as visceral obesity and insulin resistance, as well as cardiovascular diseases [63], our study further points to the significance of alterations in the neonatal microbial environment on long term physiological outcomes. Whether the functional changes in the ANS are induced directly by the neonatal LPS exposure or mediated via the hyperactive HPA axis is yet to be determined, however, alterations in specific gene expression in the brain in mutual control regions suggests concurrent programming of physiological systems may exist.

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Author Contributions

Provided intellectual contribution and critical input: EN PD DH. Conceived and designed the experiments: LS EB DH. Performed the experiments: LS EF LKO. Analyzed the data: LS EB LA. Contributed reagents/materials/analysis tools: LA EN PRD PWD DH. Wrote the paper: LS DH.

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Paper 3: Neonatal lipopolysaccharide exposure impairs sexual development and reproductive success in the Wistar rat

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Statement of author contributions to manuscript

Author	Description of Contribution to Manuscript	Signature
Adam K. Walker	Designed and performed the experiments Analysed and interpreted the data Wrote the manuscript	
Sarah A. Hiles	Performed the experiments Assisted in data analysis and interpretation Assisted in manuscript preparation	
Luba Sominsky	Assisted in data analysis and manuscript preparation	
Eileen A. McLaughlin	Assisted in the experimental design and data interpretation Provided intellectual contribution and critical input Revised the manuscript	
Deborah M Hodgson	Assisted in the experimental design and data interpretation Contributed reagents / materials/ analysis tools Provided intellectual contribution and critical input Revised the manuscript	

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Neonatal lipopolysaccharide exposure impairs sexual development and reproductive success in the Wistar rat

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ABSTRACT

We investigated, in rats, whether neonatal exposure to bacterial lipopolysaccharide (LPS) impairs sexual development, sexual decline, and reproductive behaviour in later life. Rats were administered either LPS (*Salmonella enterica*, serotype enteritidis, 0.05 mg/kg, ip) or saline (equivolume) on days 3 and 5 postpartum. The immediate and long-term effect of treatment on HPA and HPG hormones, testicular morphology, and mating behaviour was assessed. Neonatal LPS exposure induced a significant increase in corticosterone compared to controls, as well as reduced testosterone and LH in males and LH in females immediately following neonatal drug exposure. Neonatal LPS exposure disrupted the normal weight-to-age ratio of puberty onset in males and females, and impaired sexual performance in adulthood. Reproductive function was reflected in significantly diminished sperm presence in rats that had received neonatal LPS. LPS-treated females exhibited LH suppression during puberty, and males demonstrated testosterone suppression in late adulthood. Testosterone and LH surges during mating were significantly reduced in adult offspring treated with LPS as neonates. Furthermore, animals exposed to neonatal LPS and subsequent stress in adulthood, exhibited significantly blunted corticosterone responses. Morphometric assessment of testes taken from neonates revealed reduced gonocyte genesis immediately following LPS exposure and increased seminiferous disorganisation of the epithelium in these animals in adulthood. This research demonstrates the long-term impact of neonatal bacterial exposure on reproductive success given that early life exposure to bacteria disrupted puberty onset and sexual performance. Associated changes in neuroendocrine functioning suggest a possible mechanism through which a subfertile phenotype may arise.

1. Introduction

Reproductive dysfunction is a commonly occurring phenomenon (Davies and Norman, 2002), and often the underlying cause of such dysfunction is unknown (Hull et al., 1985). While it is accepted that teratogenic insults affect fertility, the ever-growing increase in unexplained infertility and subfertility has led to suggestions that perhaps subtle, developmental factors may contribute to limiting the reproductive success of some individuals. Identifying potential perinatal events, which may shift the functional outcome of bodily systems, and hence contribute to later life health has been referred to as *perinatal programming* (Hodgson and Coe, 2006).

Research investigating this phenomenon has demonstrated long-term changes in immune (Shanks et al., 1995; Hodgson et al., 2001), metabolic (Lindsay et al., 1996; Walker et al., 2006), and neuroendocrine function (Matthews, 2002; Walker et al., 2008, 2010), as well as behaviour (Breivik et al., 2002; Walker et al., 2004, 2009) following perinatal stress. Such findings suggest that these programming outcomes may, in some cases, increase disease susceptibility (Hodgson and Coe, 2006). Indeed, various perinatal stressors have been shown to increase susceptibility to diabetes (Freimanis et al., 2003; McPherson et al., 2009), cardiovascular disease (Gluckman et al., 2008), infection (Breivik et al., 2002; Boisse et al., 2004), tumour metastasis (Hodgson et al., 2001), and cognitive and behavioural disturbances (Widom, 1999; Walker et al., 2004, 2009; Gibb et al., 2007; Bilbo, 2010).

Given the body of research indicating that exposure to perinatal environmental insults may result in a 'suboptimal' phenotype predisposing to increased health risks, it is meaningful to view such susceptibilities within an evolutionary setting. An understanding of how environmental insults during this period may alter

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reproductive fitness is particularly important, and assists the emerging field of perinatal programming by elucidating how long-term changes in physiology and behaviour may affect genetic succession to subsequent generations. Thus particular programmed phenotypes may become more or less robust within a population. Of interest in the current study is the way in which neonatal exposure to a bacterial mimetic, lipopolysaccharide (LPS), can alter sexual development and reproductive fitness throughout the lifespan.

To date, few studies have examined the influence of postnatal immune activation on hypothalamic–pituitary–gonadal (HPG) outcomes (Li et al., 2007; Iwasa et al., 2009a,b) and none have examined sexual behaviour concurrently. The scarcity of such research is surprising given that bacterial infection is a common occurrence during early postnatal life, and HPG activity is strongly upregulated during this period (Main et al., 2000). Furthermore, given the trend for couples to delay their child-bearing in recent years (Lutz et al., 2003), environmental factors, such as bacterial exposures, that may increase subfertility, and thus alter puberty onset and HPG function have become increasingly relevant for conception in the modern backdrop. Hence, understanding the role that the early microbial environment plays in influencing reproductive development may elucidate potential pathways through which subfertility can arise. The current study, therefore, utilised a rodent model to understand how neonatal exposure to bacteria may alter HPG outcomes, including sexual behaviour and puberty onset. HPG endocrine function was assessed throughout development and sexual behaviour, including receptive and rejection behaviours, was monitored in adulthood. Estrous cyclicity and success of mating were similarly determined.

Importantly, the timing of the bacterial exposure in our model coincides with the early postnatal surge of LH, follicle stimulating hormone (FSH), oestrogens and androgens responsible for the differentiation of Leydig cells and spermatogenesis in the testes (Sharpe et al., 2003). Therefore, we also examined testicular morphology during neonatal life and in adulthood to determine whether hormonal perturbations brought about by neonatal LPS exposure coincided with changes in testicular formation.

2. Materials and methods

2.1. Animals and neonatal drug administration

Twelve experimentally naïve female Wistar rats obtained from the University of Newcastle animal house were bred in the University of Newcastle Psychology vivarium resulting in a total of 130 (65 males, 65 females) offspring, which were used in this study. At birth (postnatal day [PND] 1), litters were randomly allocated into either LPS or saline control conditions. No significant difference in litter size was observed between litters allocated to LPS ($M = 11.5$ pups, $SD = 3.12$) or saline ($M = 13.3$ pups, $SD = 3.21$). No difference in the percentage of males and females per litter were observed (all litters $\approx$ 50% males). On PND 3 and 5, animals were administered 0.05 mg/kg LPS (*Salmonella enterica*, serotype enteritidis; Sigma–Aldrich Chemical Co., USA, dissolved in sterile pyrogen-free saline) or an equivolume of non-pyrogenic saline (Livingstone International, Australia) via intraperitoneal microinjection. This dosage and timing of LPS exposure has previously been found to be critical in producing long-term reproductive changes (Knox et al., 2009). Drug administration procedures and housing conditions were identical to those previously described (Walker et al., 2009). Animals were randomly allocated to either (1) the developmental study ($n = 25$ for each sex) or (2) the adult sexual behaviour study ($n = 40$ for each sex). Apart from a subset of animals sacrificed to assess the immediate effect of treatment on HPA and HPG function, animals underwent daily assessment of pubertal markers from weaning. Animals were maintained under a 12 h light/dark schedule (lights on 06:00 h for animals in the developmental study, and lights on 02:00 h for animals in the sex behaviour study as copulation typically occurs nocturnally). Weekly weights were collected from weaning (i.e. PND 22, 29, 36, 43, 50, 57, 64, 71, 78, 85). All experimentation occurred in accordance with the 2004 NH&MRC Australian Code of Practice for the care and use of animals for scientific practice.

2.2. Neonatal blood and testicle collection

A subgroup of rats was sacrificed during neonatal life in order to assess plasma corticosterone, testosterone and LH responses to treatment, as well as for morphometric assessment of testes. Four hours following injection on PND 5, trunk blood and testes were collected. This time point was chosen as we have previously demonstrated neuroendocrine activation 4 h following neonatal LPS administration (Walker et al., 2009, 2010). Collection of trunk blood occurred via rapid decapitation into EDTA-coated tubes (Livingstone International, Australia) and testes were excised, excess connective tissue was removed, and placed immediately into Bouin's Solution (Sigma–Aldrich, Australia) for fixation.

2.3. Sexual developmental cohort

Animals were monitored daily following weaning for physical markers of puberty, such as preputial separation for males and vaginal opening for females. Once vaginal opening occurred, estrous cyclicity was monitored using a Rat Vaginal Impedance Checker (Muromachi Kikai, Tokyo, Osaka; described in Walker et al., 2010) according to the manufacturer's instructions, and proestrous was confirmed using vaginal smears.

Blood was collected via the saphenous vein into EDTA-coated tubes (Livingstone International, Australia) over three time points during adolescence to assess LH and testosterone in males (PND 43, 46, 49), and to assess LH in females (PND 33, 36, 39). These time points were chosen given that the hormonal surges necessary for the attainment of puberty have been shown to typically occur during these time periods (Goldman et al., 2000; Zapatero-Caballero et al., 2003). These hormones were again assessed in late adulthood to determine sexual decline. At 9, 10, 11, and 12 months of age blood was collected via the saphenous vein from males and weights were taken. Females were monitored for cyclicity at these ages and blood was collected during proestrus.

2.4. Behavioural testing in adulthood

2.4.1. Three-day stress protocol

Prior to sexual behaviour testing, half of the animals underwent an additional "restraint and isolation stress" protocol as previously described (Walker et al., 2009) given that the impact of early life LPS exposure has been shown to be amplified in the presence of a subsequent stressor in adulthood (Walker et al., 2009, 2010). Animals allocated to sexual behaviour testing in adulthood (PND 85) were randomly divided into either a 3-day "stress", or "no stress" condition, following which animals were exposed to sexual behaviour testing.

2.4.2. Behavioural procedures

All behavioural testing was conducted in adulthood (PND 85) under infrared light between 16:00 h and 18:00 h during the dark phase. During the 15 min provided for experimental animals to habituate to the mating arena prior to the introduction of the untreated conspecific, anxiety-like behaviours were assessed using the arena as an adapted Hide Box/Open Field. A description of

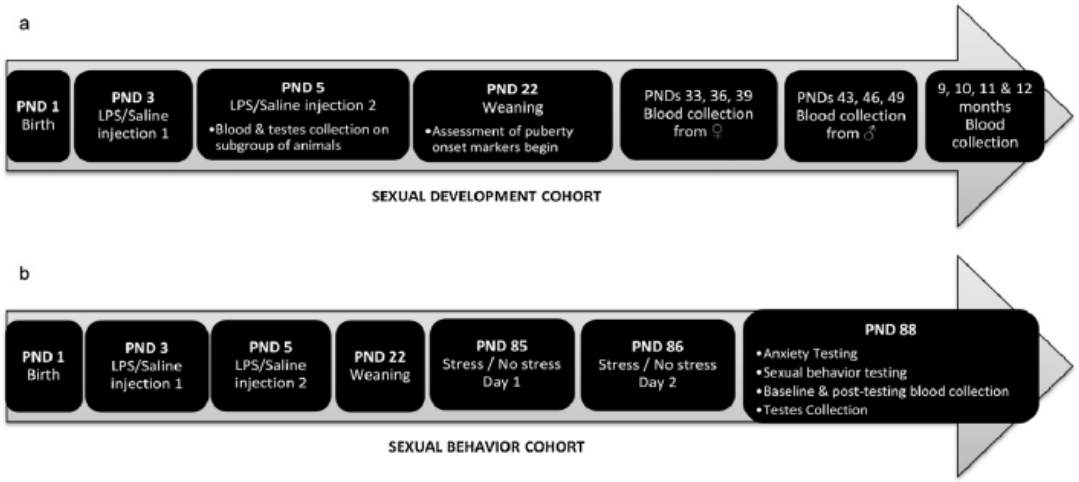

Fig. 1. A timeline schematic of experimental procedures conducted. (a) Represents the particular protocols conducted and the age of animals (postnatal day [PND]) when protocols were conducted for animals allocated to the sexual development cohort. (b) Represents the particular protocols conducted and the age of animals (postnatal day [PND]) when protocols were conducted for animals allocated to the sexual behaviour cohort.

the Hide Box/Open Field and behavioural measures recorded has been previously published (Walker et al., 2009). Sexual behaviours, consistent with those previously reported (Agmo, 1997; Hull and Dominguez, 2007), were scored by an experimenter blind to conditions using video monitoring equipment.

2.4.3. Assessment of anxiety-like behaviour

The mating/open field arena consisted of a 61.0 cm × 61.0 cm square field enclosed by a continuous 30-cm high wall constructed of plywood material. A 25.0 cm (length) × 20.0 cm (width) × 15.0 cm (height) hide box, with a 7.0 cm circular entry/exit hole at the front, was positioned alternately on the southern and eastern side of the open field (counterbalanced between sessions). See Walker et al. (2009).

2.4.4. Sexual behaviour testing

Following Hide Box/Open Field testing, the hide box was removed and an untreated conspecific of the opposite sex (4 males, 34 females) was introduced on the centre square. The untreated males were proven studs and were reused on non-consecutive days. Untreated females were sexually naïve virgins and used only once to reduce any confounds that may arise from multiple matings such as pregnancy or practice effects. All females were tested during proestrous and were ovary–intact. Pairs were recorded for 30 min. The testing arena was sanitised and deodorised after each animal with 10% ethanol solution. Following sexual behaviour testing, a vaginal smear was taken from all females to determine sperm presence as a confirmatory measure of our behavioural observation of a successful mount and ejaculation. A subgroup of males exposed to "neonatal treatment" but not "adult stress" was euthanized via ip injection with 1 ml of lethabarb (Virbac Pty. Ltd., Australia) and testes collected for histological morphometric assessment.

2.5. Blood sampling and radioimmunoassay procedures

In addition to blood collection during neonatal life, adolescence and late adulthood, saphenous bleeds were also taken prior to and immediately following behavioural testing. Levels of circulating testosterone, LH, and corticosterone were quantified using ^{125}I radioimmunoassay kits (MP Biomedicals) according to manufacturer's instructions. Samples were centrifuged at 1000*g* for 20 min at 4 °C, and plasma stored at −20 °C until assayed.

2.6. Morphometric study of testes

Testes were collected from neonates 4 h following LPS or saline treatment on PND 5 and from a randomly chosen subgroup of males in adulthood. For fixation, the testes were placed in Bouin's solution. Neonatal testes remained in Bouin's solution for 4 h while adult testes were fixed for 24 h, and then washed four times in 70% ethanol. Fixed tissues were dehydrated, embedded in paraffin, and sectioned at 4 μm. Three non-serial and four non-serial slices were hematoxylin and eosin (H&E) stained for morphometric analysis in neonates and adults, respectively. Neonatal tissue was assessed for structural differences including proliferation of seminiferous tubules and gonocyte cell number. Assessment of adult tissue included proliferation of seminiferous tubules, seminiferous tubule area, stage of seminiferous tubules based on spermatogenic cycle, and epithelial disorganisation and detached germ cells.

2.7. Data analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Volume 18 (SPSS Inc.). Behaviours and circulating hormones, weight gain and pubertal markers were analysed using analyses of covariance (ANCOVA), controlling for pre-existing weight differences, litter size and male-to-female ratio (reported only when significantly contributing to the data). Litter effects were further controlled by nesting litter into treatment for each ANCOVA model. Loglinear modelling was used to analyse estrous cyclicity and sperm presence following sexual encounter. Planned comparisons between experimental conditions were performed using Bonferroni's α correction to 0.05 and *t* test analyses adjusted for multiple comparisons where significant interactions were observed. A schematic and timeline of the protocols involved in the animal testing is represented in Fig. 1.

3. Results

3.1. Impact of neonatal LPS exposure on developmental weight gain

LPS-treated animals gained significantly more weight (M = 3.56 g, SEM = .10) than saline-treated controls (M = 2.94 g, SEM = .11), $F(17, 128) = 4.81$, $p < .001$. However, litter size ($F(17, 128) = 11.11$, $p < .001$) and weight at PND 3 ($F(17, 128) = 27.36$, $p < .001$) were significant covariates. No sex differences were observed.

Weight gain analysis from weaning (PND 22) to adolescence (PND 50) and from adolescence to adulthood (PND 85) indicated a significant main effect of "sex" for both periods, $F(1, 92) = 69.91$, $p < .001$ and $F(1, 92) = 115.2$, $p < .001$, respectively. Males gained significantly more weight than females from weaning to adolescence (males: M = 224.36 g, SEM = 5.58; females: M = 150.94 g, SEM = 6.31), and from adolescence to adulthood (males: M = 180.82 g, SEM = 5.98; females: M = 82.87 g, SEM = 6.73). No treatment effects were observed.

3.2. Impact of neonatal LPS on pubertal onset

3.2.1. Preputial separation in males

Given that weight has been shown to impact greatly on pubertal timing, weight at PND 36 (just prior to preputial separation) was included as a covariate in the analysis of preputial separation, and was found to be a significant covariate ($F(1, 47) = 41.83$, $p < .001$). A regression analysis was subsequently performed between weight at PND 36 and age at preputial separation. As expected, Pearson's correlation revealed a strong, negative relationship between weight at PND 36 and preputial separation ($r(26) = -.92$, $p < .01$) for saline-treated controls, indicating that males that were heavier at PND 36 had earlier preputial separation. Linear regression indicated weight significantly predicted day of separation, $\beta = -.92$, $b = -0.10$, $a = 56.22$, $r^2 = .84$, $p < .01$. However, no such relationship was observed for LPS-treated males ($r(20) = -.15$, $p = .50$, $r^2 = .02$). See Fig. 2a.

3.2.2. Vaginal opening in females

As with preputial separation in males, weight at PND 29 (just prior to vaginal opening) was covaried with age of vaginal opening in females, and was again observed to be a significant covariate ($F(1, 39) = 8.02$, $p < .01$). Pearson's correlations revealed a significant strong and negative relationship between weight at PND 29 and vaginal opening for saline-treated animals ($r(17) = -.79$, $p < .01$), indicating that females higher in weight at PND 29 recorded earlier vaginal opening. Regression confirmed that weight at PND 29 significantly predicted vaginal opening, $\beta = .79$, $b = -0.10$, $a = 43.11$, $r^2 = .61$, $p < .01$. However, no significant relationship was found for LPS-treated females, $r(21) = -.25$, $p = .25$, $r^2 = .06$. See Fig. 2b.

Counts of regular estrous cyclicity prior to PND 60 for neonatal treatment were analysed with backward loglinear modelling (probability of removal .05). The final model indicated a main effect of "estrous regularity" but not "neonatal treatment", and was not significantly different from the saturated model, $\chi^2(2) = 1.39$, $p = .50$. Females were 2.42 times more likely to record a regular than a non-regular estrous cycle.

3.3. Impact of neonatal LPS exposure on behaviour in adulthood

3.3.1. Anxiety-related behaviour

Under conditions optimised for testing sexual behaviour in rats, "neonatal treatment" was found to only alter the percentage of time spent in the central square ($F(1, 56) = 12.30$, $p < .05$), with

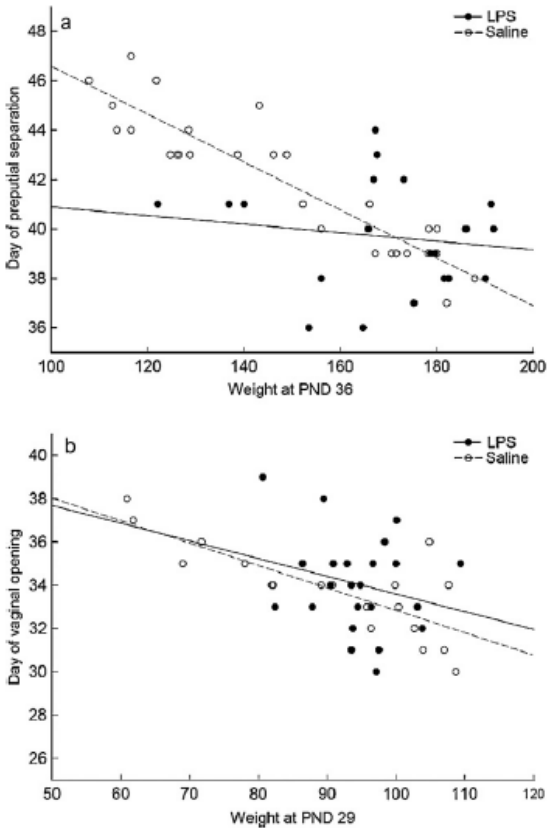

Fig. 2. (a) Linear relationship between weight at PND 36 and day of preputial separation for neonatally challenged LPS males (n = 22) and neonatally challenged saline males (n = 28). The trendline for saline males is dotted. (b) Linear relationship between weight at PND 29 and day of vaginal opening for neonatally challenged LPS females (n = 23) and neonatally challenged saline females (n = 19). The trendline for saline females is dotted.

LPS-treated animals spending less time in the central square (M = 11.80%, SEM = .68) compared to saline-treated controls (M = 15.22%, SEM = .70). "Neonatal treatment" did not impact on any other of the anxiety-related behaviours observed.

Unsurprisingly, stress in adulthood significantly increased anxiety-like behaviours, such as time spent in the hide box ($F(1, 56) = 7.78$, $p = .01$) and time spent engaged in vigilance behaviour operationalised as scanning the open field arena by remaining in the hide box ($F(1, 60) = 9.15$, $p < .01$) for males but not females. Planned comparisons revealed that males exposed to stress in adulthood spent a significantly higher proportion of time (M = 21.30%, SEM = 2.97) in the hide box compared to their female counterparts (M = 11.76%, SEM = 1.03), $t(29) = 3.52$, $p < .05$. Finally, males exposed to stress in adulthood spent significantly more time engaged in hypervigilance (M = 3.32%, SEM = .86) compared to all other groups: male stress vs. male no stress (M = 13.86%, SEM = 1.85; $t_{.05}(31) = 3.15$, $p < .05$); male stress vs. female stress (M = 11.76%, SEM = 1.03; $t_{.05}(30) = 3.87$, $p < .05$); male stress vs. female no stress (M = 14.86%, SEM = 1.34; $t_{.05}(33) = 2.92$, $p < .05$).

3.3.2. Sexual behaviour

Of the initiatory behaviours, both neonatal LPS exposure and stress in adulthood resulted in significantly fewer total mounts for both males and females compared to their respective controls

Table 1
Male and female sexual behaviour.

Behavior	LPS-treated animals	Saline-treated animals	p
Female			
Number of kicks to untreated stud	27.15 (10.45)	1.18 (3.31)	<.05
Number of hops of experimental female	71.73 (9.18)	22.85 (9.64)	<.01
Interaction time between experimental female and untreated male	813.68s (63.27)	607.13s (41.18)	<.05
Latency of untreated male to mount experimental female	1018.95s (254.32)	71.73s (34.11)	<.01
Number of attempted mounts by untreated to experimental female	2.08 (0.78)	0.45 (0.22)	<.05
Number of mounts by untreated male to experimental female	8.69 (3.63)	34.45 (2.71)	<.01
Latency to ejaculation of untreated males paired with experimental females	1663.52s (79.78)	906.9s (124.67)	<.01
Number of ejaculations of untreated males paired with experimental female	0.23 (0.13)	1.36 (0.21)	<.01
Male			
Number of times kicked by untreated female	18.90 (2.62)	14.47 (6.29)	n.s.
Number of hops by untreated female	8.90 (1.42)	37.20 (11.34)	n.s.
Interaction time between experimental male and untreated female	967s (114.64)	792.40s (86.44)	n.s.
Latency of experimental male to mount untreated female	1238.30s (258.34)	747.89 (237.80)	n.s.
Number of attempted mounts by experimental male to untreated female	3.3 (1.24)	2.73 (1.54)	n.s.
Number of mounts by experimental male to untreated female	3.70 (1.89)	14.13 (3.56)	<.05
Latency to ejaculation of experimental male paired with untreated female	1624.49 (176.05)	1436.97 (111.95)	n.s.
Number of ejaculations of experimental male paired with untreated female	0.30 (0.07)	0.57 (0.17)	n.s.

Mean scores (±SEM) for receptive behaviours of LPS-treated ($n = 13$ for all) and saline-treated ($n = 11$ for all) females paired with untreated studs, and sexual behaviours of LPS-treated ($n = 9$ for all) and saline-treated ($n = 14$ for all) males paired with untreated females. Significant differences between neonatal treatment groups were observed on all behavioural measures for females but only total number of mounts was found to be significantly different for males.

($F(1, 21) = 5.17$, $p < .05$, and $F(1, 21) = 5.21$, $p < .05$), although no interaction was observed. Significant neonatal treatment effects were observed on all parameters measured in females. Regarding receptive behaviours, LPS-treated females exhibited significantly fewer hops ($F(1, 20) = 12.57$, $p < .01$), significantly more kicks to their mating partner ($F(1, 20) = 4.92$, $p < .05$), and significantly greater interaction times ($F(1, 20) = 5.83$, $p < .05$.) with their mating partner compared to controls.

Initiatory behaviours of the partners of experimental females also demonstrated significant effects despite the random assignment of the untreated studs. Despite the partners of LPS-treated females making a significantly greater number of attempted mounts compared to partners of saline-treated females ($F(1, 20) = 4.77$, $p < .05$), LPS-treated females were actually mounted significantly fewer times than saline-treated females ($F(1, 20) = 31.88$, $p < .01$). Furthermore, the latency to mount for the partners of LPS-treated females was significantly greater than their saline counterparts, $F(1, 20) = 10.93$, $p < .01$. In line with these findings, LPS-treated females experienced significantly fewer *successful* mounts, resulting in fewer recorded ejaculations compared to saline-treated females, $F(1, 20) = 28.68$, $p < .01$. Finally, latency to ejaculation was significantly longer for LPS-treated females, $F(1, 20) = 32.71$, $p < .01$. See Table 1.

3.3.3. Sperm presence

Backward loglinear modelling (probability of removal .05) was used to assess sperm presence in experimental females and the recipients of experimental males. For males, the final model indicated a main effect of neonatal treatment, not significantly different from the saturated model, $\chi^2(4) = 1.07$, $p = .90$. The partners of LPS-treated males were 4.59 times less likely for sperm presence to be observed compared to the partners of saline-treated males. For females, the final model revealed a main effect of neonatal treatment, not significantly different from the saturated model, $\chi^2(4) = 2.42$, $p = .66$. LPS-treated females were 11.27 times less likely than saline-treated females to record positive sperm presence.

3.4. Assessment of HPG and HPA hormones

3.4.1. Neonatal HPA and HPG response to treatment

LPS-treated males exhibited significantly lower LH concentrations ($F(3, 12) = 4.01$, $p < .05$) 4 h following drug exposure on PND 5 compared to saline controls. Trends reflected this finding for plasma testosterone concentrations, but only approached significance ($p = .06$). LPS-treated females similarly exhibited significantly lower LH concentrations compared to saline controls at this time point, $F(1, 16) = 2.97$, $p < .05$. Plasma corticosterone responses to treatment were significantly greater for animals exposed to LPS compared to controls ($F(1, 39) = 2.44$, $p < .05$) but did not differ by sex. See Fig. 3.

3.4.2. Luteinising hormone and testosterone during puberty

No significant effect on testosterone or LH was observed in males across adolescence, possibly due to the unexpected spontaneity of pubertal onset in LPS-treated animals. In females, LPS treatment significantly reduced plasma LH concentrations across all three time points compared to controls, $F(1, 18) = 12.52$, $p < .01$. See Fig. 4.

3.4.3. HPA and HPG responses during mating in adulthood

The surge in testosterone levels during mating was significantly reduced in males exposed to neonatal LPS ($F(1, 24) = 5.987$, $p < .05$) as well as males exposed to stress in adulthood ($F(1, 24) = 7.60$, $p < .05$) compared to their respective controls, after accounting for the significant covariate of litter size ($F(1, 24) = 5.14$, $p < .05$). This was also evident for LH in males during mating ($F(1, 27) = 8.38$, $p < .01$). See Fig. 5.

As with males, both neonatal LPS treatment ($F(1, 28) = 96.67$, $p < .05$), and stress in adulthood ($F(1, 28) = 83.43$, $p < .05$) were found to independently and significantly reduce the LH surge in females during mating, See Fig. 6.

An interaction was observed between "neonatal treatment", "adult treatment" and "sex" in regards to plasma corticosterone differences from baseline to post-sexual behaviour testing, $F(1, 31) = 4.90$, $p < .01$.While LPS-treated animals exposed to restraint and isolation stress all showed blunted corticosterone responses to mating, planned comparisons revealed this to only reach significance for males ($t_5(16) = 2.60$, $p < .05$). Neonatal treatment groups exposed to "no stress" in adulthood did not differ for either males or females. See Fig. 7.

3.4.4. HPG concentrations in late adulthood

LPS-treated males were found to have significantly lower testosterone levels ($M = .07$ ng/ml, $SEM = .19$) from 9 to 12 months compared to saline controls ($M = 1.31$ ng/ml, $SEM = .16$;

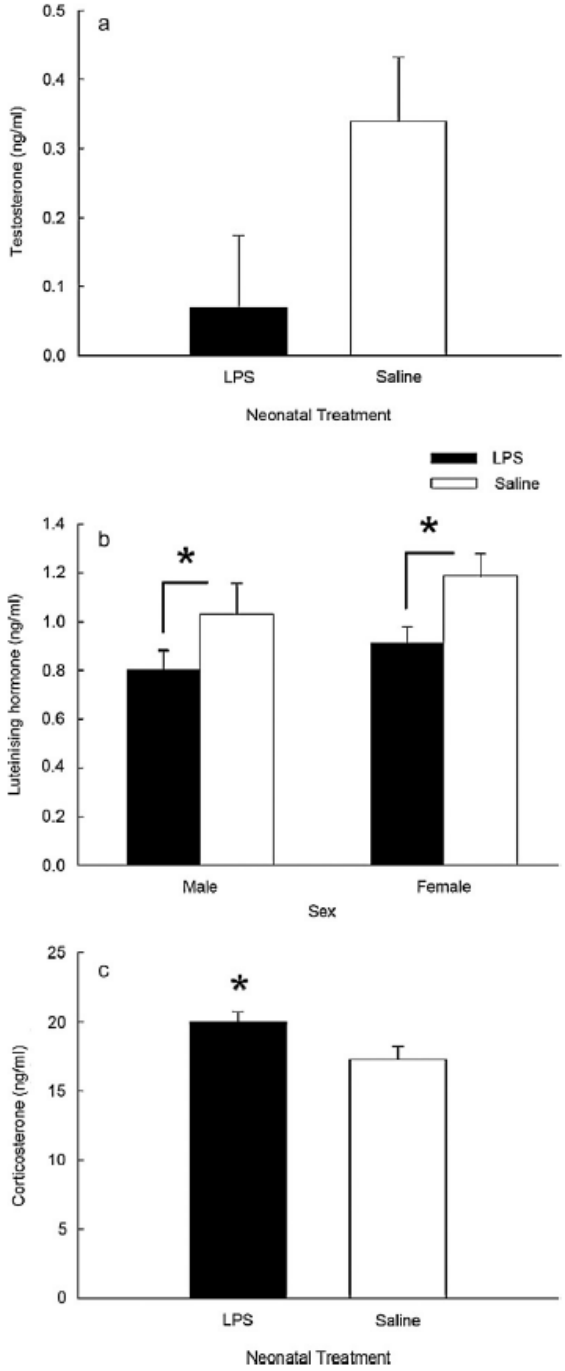

Fig. 3. Immediate effect of neonatal LPS exposure on plasma hormone concentrations. (a) Male plasma testosterone concentrations (ng/ml; ±SEM). The filled bar represents neonatally challenged LPS males ($n = 13$) and the hollow bar represents neonatal challenged saline males ($n = 17$). (b) Male and female plasma LH (ng/ml; ±SEM). The filled bars represent neonatally challenged LPS males ($n = 7$) and females ($n = 9$) and the hollow bars represent neonatal challenged saline males ($n = 9$) and females ($n = 13$). $^*p < .05$. (c) Plasma corticosterone concentrations collapsed across sex (ng/ml; ±SEM). The filled bar represents neonatally challenged LPS animals ($n = 30$) and the hollow bar represents neonatal challenged saline animals ($n = 25$). $^*p < .05$.

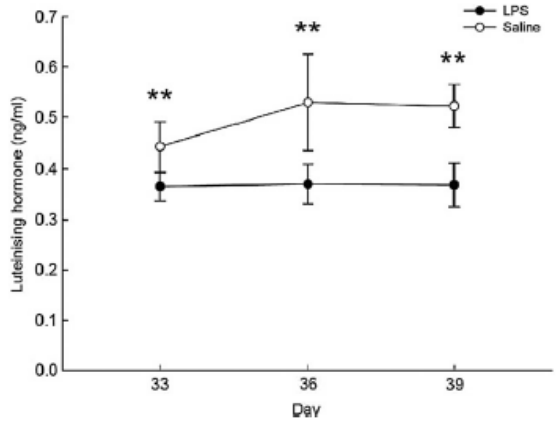

Fig. 4. Effect of neonatal LPS on plasma LH across adolescence in females (ng/ml; ±SEM). The filled circles represent neonatally challenged LPS animals ($n = 10$) and the hollow circles represent neonatal challenged saline animals ($n = 10$). $^{**}p < .01$.

$F(1, 13) = 6.00$, $p < .05$), after the significant covariate of male to female ratio ($F(1, 13) = 5.92$, $p < .05$) was accounted for. No differences in LH concentrations were observed for either sex.

3.5. Morphometric study of testes

Assessment of neonatal testis tissue following LPS exposure indicated no difference in seminiferous tubule number per mm^2, nor in the mean tubule area. However, a significant difference in the mean number of gonocytes per tubule was observed, $F(1, 11) = 7.98$, $p < .05$. LPS-treated males exhibited significantly fewer gonocytes per tubule ($M = 1.29$, $SEM = .09$) compared to saline-treated controls ($M = 1.67$, $SEM = .1$). Furthermore, LPS-treated neonates were found to exhibit a significantly greater percentage of tubules containing no gonocytes ($M = 28.33\%$, $SEM = 3.25$) compared to controls ($M = 14.07\%$, $SEM = 3.25$), $F(1, 10) = 9.63$, $p < .05$. Histomorphological analysis also suggested that tubules of LPS-treated animals were developmentally delayed compared to controls. Gonocytes had not migrated to the basement membrane and Sertoli cell cytoplasms were much larger in saline-treated animals.

No differences in seminiferous tubule number per mm^2 nor seminiferous tubule area were observed in adulthood. However, the percentage of tubules demonstrating structural irregularity significantly differed between neonatal treatment conditions, whereby a greater percentage of tubules indicated seminiferous germinal epithelial disorganisation for LPS-treated animals ($M = 6.83\%$, $SEM = .76$) compared to saline controls ($M = 3.29\%$, $SEM = .71$), $F(3, 7) = 6.44$, $p < .05$. Examination of seminiferous tubule stage showed a strong trend for LPS-treated animals to have significantly lower proportions of tubules in stages IX–XIV ($M = 41.61\%$, $SEM = 7.14$) as compared to saline-treated animals ($M = 69.79\%$, $SEM = 6.73$), which approached significance after accounting for litter effects ($p = .08$). Fig. 8 provides photomicrographs of the major histological findings.

4. Discussion

The current study demonstrated that postnatal exposure to low-level LPS is sufficient to disrupt development of HPG-related functions, including puberty onset, sexual behaviour, hormone regulation and reproductive success. Furthermore, such perturbations appear to be relatively independent of peripheral HPA axis activa-

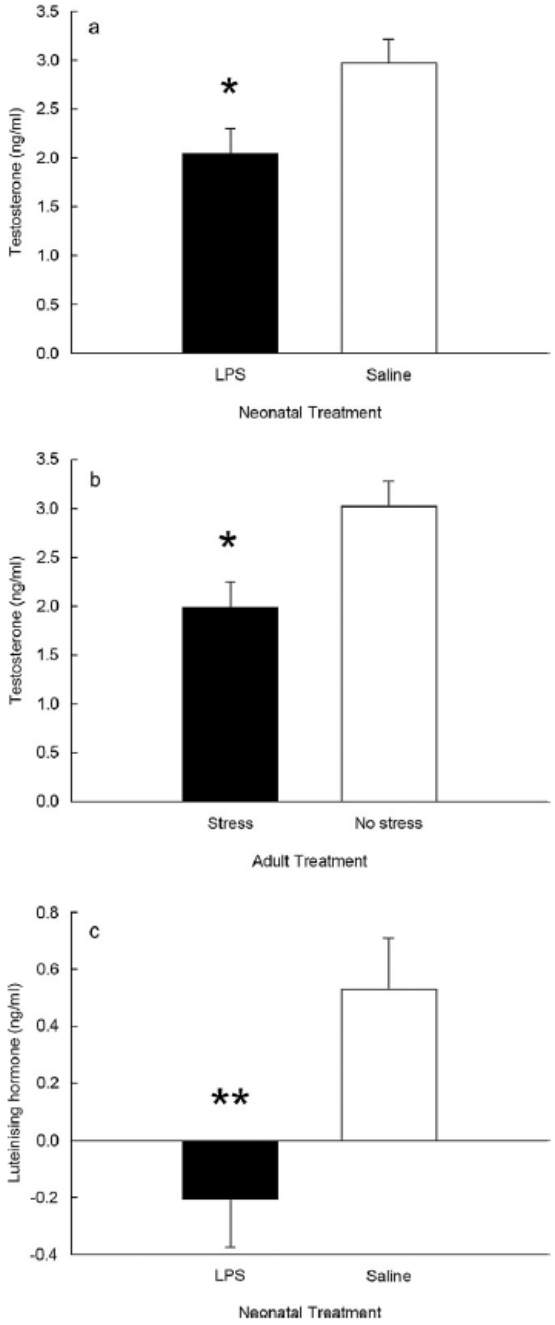

Fig. 5. Effects of neonatal LPS and adult stress on plasma hormonal surges during mating for males. (a) Male plasma testosterone surges (ng/ml; ±SEM) for neonatal treatment groups. The filled bar represents neonatally challenged LPS males ($n = 15$) and the hollow bar represents neonatally challenged saline males ($n = 14$), $^{*}p < .05$. (b) Male plasma testosterone surges (ng/ml; ±SEM) for adult treatment groups. The filled bar represents males exposed to stress in adulthood ($n = 15$) and the hollow bar represents males exposed to no stress in adulthood ($n = 14$), $^{*}p < .05$. (c) Male plasma LH surges for neonatal treatment groups (ng/ml; ±SEM). The filled bar represents neonatally challenged LPS males ($n = 16$) and the hollow bar represents neonatally challenged saline males ($n = 15$), $^{**}p < .01$.

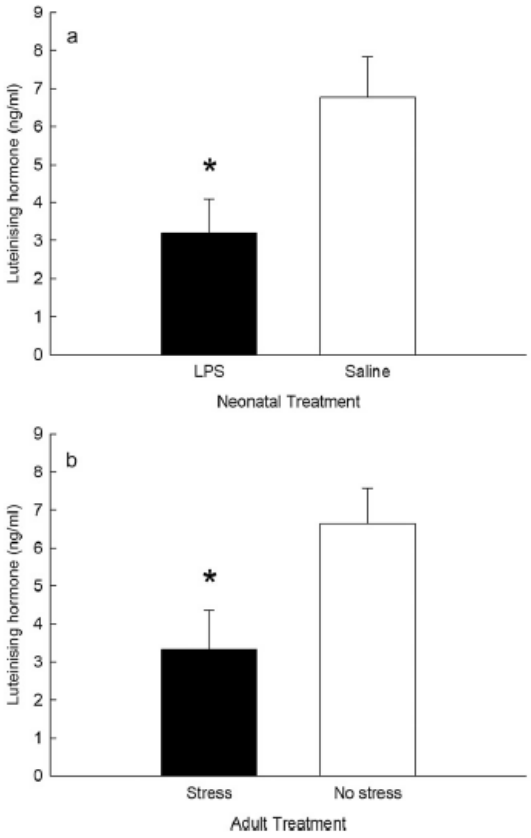

Fig. 6. Effects of neonatal LPS and adult stress on plasma LH surges during mating for females. (a) Female plasma LH surges (ng/ml; ±SEM) for neonatal treatment groups. The filled bar represents neonatally challenged LPS females ($n = 19$) and the hollow bar represents neonatally challenged saline females ($n = 13$), $^{*}p < .05$. (b) Female plasma LH surges (ng/ml; ±SEM) for adult treatment groups. The filled bar represents females exposed to stress in adulthood ($n = 14$) and the hollow bar represents females exposed to no stress in adulthood ($n = 18$), $^{*}p < .05$.

tion also observed during the neonatal period. Examination of testis morphology indicates structural perturbations and gonocyte loss which may be ultimately responsible for the reduced reproductive success observed.

4.1. Impact of neonatal LPS on weight gain

Weight gain was monitored to determine if any spurious effects observed were attributable to growth disturbances as a result of nutritional deficiency, which is known to alter puberty onset (Engelbregt et al., 2000). There was no evidence of nutritional deficiency as LPS-treated animals gained more weight than controls between days 3 and 5 of drug exposure. This is further supported by the fact that no neonatal treatment differences were observed in regards to weight gain following weaning, consistent with previously reported findings (Knox et al., 2009).

4.2. Impact of neonatal LPS on the onset of puberty

Neonatal LPS exposure altered the onset of puberty in both males and females. It was expected that neonatal drug treatment

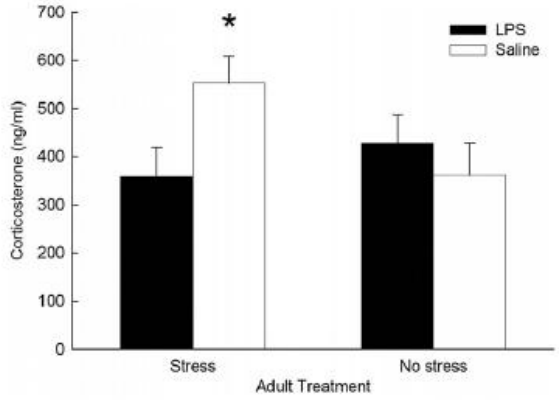

Fig. 7. Effect of neonatal LPS combined with adult stress on plasma corticosterone surges during mating for males (ng/ml; ±SEM). The filled bars represent neonatally challenged LPS males exposed to either stress ($n = 8$) or no stress ($n = 8$) in adulthood. The hollow bars represent neonatally challenged saline males exposed to either stress ($n = 10$) or no stress ($n = 7$) in adulthood, $^*p < .05$. Identical trends were observed for females.

would differentially accelerate or delay puberty onset as previously reported with other perinatal stressors (Biagini and Pich, 2002; Zambrano et al., 2005, respectively). However, we observed that the response to neonatal LPS does not adjust the timing of puberty in a clear forward or backward direction. When we investigated the weekly weights collected prior to the onset of puberty of the first male and female it was clear that neonatal LPS exposure disrupts the linear relationship between body weight and puberty onset, typical of normal development (Odum et al., 2004). Thus our data would indicate that the HPG axis failed to interact in the characteristic manner with the metabolic signals which initiate puberty onset (Hall, 2003). This is consistent with previous observations of neonatally stressed animals (Engelbregt et al., 2000; Lau et al., 1996). However, no studies to our knowledge have correlated body weight with age of puberty onset, which may underlie the inconsistency reported previously in regards to whether puberty onset is delayed (Engelbregt et al., 2000; Knox et al., 2009; Kinsey-Jones et al., 2010), accelerated (Biagini and Pich, 2002) or unchanged (Lau et al., 1996) following perinatal stress.

4.3. Impact of neonatal LPS on sexual behaviour in adulthood

Neonatal LPS administration affected sexual behaviour in both sexes but the strongest effects were observed among females. Whereas only the total number of mounts was significantly reduced in males, all behavioural parameters measured in females neonatally treated with LPS were affected. This is important because the female controls sexual behaviour in rodents (Agmo, 1997). Females treated with LPS as neonates showed clear alterations in sexual behaviour cues, as indicated by the increase in

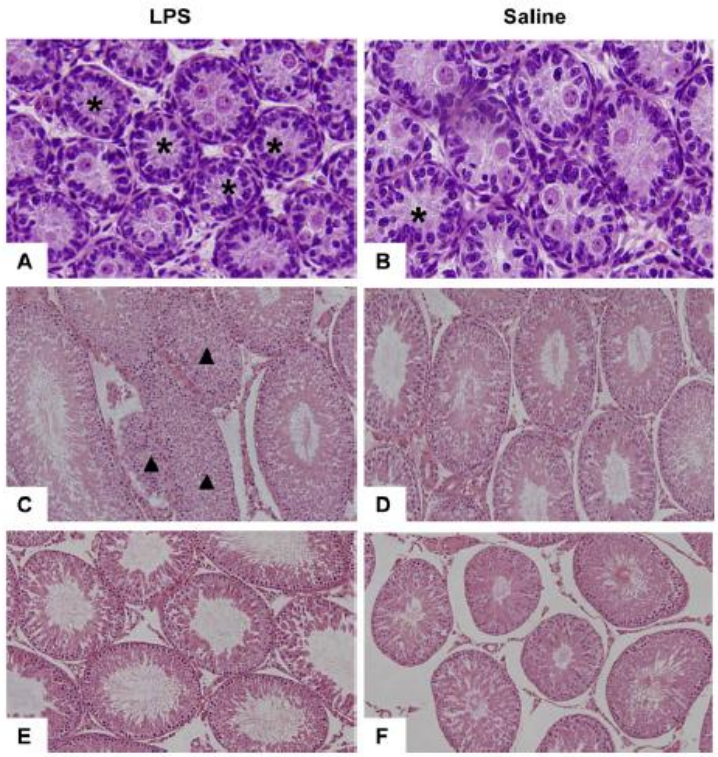

Fig. 8. Photomicrographs of seminiferous tubules of rats from neonatally LPS-treated (A, C and E) and neonatally saline-treated (B, D and F) males stained by H&E. (A and B) In the seminiferous tubules of neonates LPS-treated males display greater numbers of tubules containing no germ cells (asterisks). (C and D) LPS-treated rats exhibit greater numbers of tubules displaying epithelial disorganisation and detached germ cells filling the tubular lumen in adulthood (arrowheads). (E and F) A greater proportion of seminiferous tubules of LPS-treated rats are at earlier stages compared to saline-treated controls based on the spermatogenic cycle.

rejection behaviours such as kicks, and decrease in receptive signals such as hops. This resulted in a diminished performance by the studs, evidenced by fewer attempted mounts, actual mounts, and increased mount latency. Consequently, increased latency to ejaculation, and hence, decreased frequency of ejaculations was observed. It should be noted that future examination of lordosis would further strengthen our understanding of female receptivity in this model.

A key indicator of the altered behavioural signalling of LPS-treated females was that the amount of time spent interacting with their male partners was significantly greater compared to controls. This finding is indicative of two things: (1) LPS-treated females were not disinterested in the male stud and (2) may reflect the reduced time spent in the post-ejaculatory refractory interval which is characterised by low interaction. Therefore, while females were clearly interested in their mating partners, they were unable to provide the appropriate behavioural cues for initiation of mating. Such diminished reproductive success suggested by these behavioural outcomes was confirmed with the significantly reduced sperm presence for LPS-treated males and females.

Importantly, the observed perturbations in copulatory behaviour of LPS-treated animals do not appear to be influenced by the potential confounding effect of anxiety-like behaviour as only one of the six anxiety-related measures reached significance for LPS-treated animals.

4.4. Impact of neonatal LPS on HPG and HPA hormones

Changes in the sexual behaviour of animals neonatally treated with LPS were found to be associated with both short and long-term changes in HPG and HPA axis hormones. Neonatal LPS administration suppressed HPG hormones immediately following drug exposure in both males and females. LPS is known to suppress HPG activity (Matsuwaki et al., 2006), however, this is the first study to our knowledge to identify such hormonal suppression as a consequence of bacterial exposure during the early postnatal period, and indicates this period to be one of vulnerability for sexual development. Evidence indicates that inflammatory LPS challenges affect HPG regulatory signals kisspeptin (Kiss1) and Kissr1 (its G protein-coupled receptor) via Cox-1 and Cox-2 (Iwasa et al., 2008), suggestive of a neuroimmune pathway through which LPS may be influencing neonatal HPG control.

In adolescence, LPS-treated females demonstrated lower concentrations of LH across all three time points measured, however, no hormone differences were observed in males, which may indicate sexually dimorphic vulnerabilities during this time period.

In adulthood, both neonatal LPS exposure and stress in adulthood independently suppressed the surge in HPG hormones during mating for males and females. Stress in adulthood is well known to suppress HPG hormones (Tilbrook et al., 2000), however, the impact of bacterial exposure during the early postnatal period alone on HPG suppressive responses is relatively novel with only one study that we are aware of showing HPG regulatory systems being perturbed in adulthood (Knox et al., 2009). This indicates that neonatal immune activation alone can profoundly impede the long-term sexual functioning of peripheral HPG hormones during mating. LPS-treated males exhibited significantly lower levels of testosterone in late adulthood compared to saline controls. No effect of LH for females was observed in regards to hormonal decline in late adulthood, however, by 12 months of age a greater percentage of LPS-treated females appeared to have finished cycling.

Importantly, we concurrently examined corticosterone responses with HPG responses to treatment during neonatal life as well as in adulthood. Consistent with previous findings (Walker et al., 2009, 2010), neonatal LPS exposure increased neonatal plasma corticosterone concentrations, demonstrative of the ability of LPS to activate the HPA axis. It is, therefore, possible that the increase in corticosterone immediately following exposure to the mimetic is responsible for the suppression of HPG hormones occurring simultaneously. Glucocorticoids are known to suppress testosterone and LH (Tilbrook et al., 2000), however, given that the LPS exposure occurred during the stress hyporesponsive period, resulting in a relatively small increase in corticosterone, it is unlikely that the increased HPA axis activity is the sole mediator for this downregulated HPG function. Furthermore, the corticosterone response to sexual behaviour testing in adulthood was blunted for LPS-treated animals exposed to stress in adulthood, and ACTH has been shown not to be altered with this model (see Walker et al., 2009). These findings indicate that the impact of neonatal treatment on HPG function is not mediated by peripheral HPA dysfunction. However, the potential for central HPA axis circuitry to suppress gonadotropin-releasing hormone (GnRH) action is high. Studies have demonstrated CRH to reduce the frequency of LH pulses (Barbarino et al., 1989; Kinsey-Jones et al., 2010) with CRH type 2 receptors being fundamental to LPS-induced suppression of GnRH pulse generation (Li et al., 2006). The neural circuitry which has been strongly implicated in stress-induced HPG suppression include the hypothalamic paraventricular nucleus, medial preoptic area and hypothalamic arcuate nucleus (Kinsey-Jones et al., 2010; Knox et al., 2009). Importantly, the latter regions have both demonstrated altered abundance of Kiss1 and Kissr1, believed to be central in mediating HPG function, following 0.05 mg/kg *Escherichia* coli LPS exposure on PND 3 and 5 (Knox et al., 2009). Given the similarity in timing and dosage between Knox et al.'s (2009) study and the current paper, it is likely that central regulatory signals of CRH and Kiss1 are influencing GnRH release, and thus suppressing testosterone and LH in LPS-treated animals. The involvement of these central mechanisms in driving HPG function following early life inflammation is reviewed in Kentner and Pittman (2010).

4.5. Impact of neonatal LPS on testicular morphology

Examination of testis structure in early and later life revealed distinctive changes in testicular development of animals exposed to neonatal LPS. Most notably, examination of neonatal testes indicated LPS-treated animals displayed reduced gonocyte presence and delayed development even during early neonatal life. Contrastly, most tubules in adulthood showed prolific spermatogenesis, indicating that perhaps the immediate exposure of LPS works to delay germ cell proliferation in the tubule but does not completely impede it. This potential explanation of delay is further supported by the seminiferous tubules of adult LPS-treated animals to be primarily between stages I and VIII, whereas those of saline-treated animals were largely between stages IX and XIV suggesting an altered spermatogenic cycle, as well as the reduced ejaculated sperm in recipients following sexual behavioural testing in adulthood for LPS-treated animals.

The increased adulthood epithelial disorganisation of the seminiferous tubules of LPS-treated males similarly suggests long-term developmental alterations following neonatal immune activation. Several studies have demonstrated that various interventions lead to disorganisation of the seminiferous epithelium in adulthood (Hamid et al., 2010; Caneguim et al., 2009), however, this is the first paper to our knowledge, demonstrating such disorganisation in adult testicular morphology following bacterial challenge in early life. Of importance is the fact that no differences in the mean tubule number per mm^2 were observed during neonatal or adult life, suggestive that differences in the number of interstitial Leydig cells, which secrete testosterone, are unlikely. Therefore, the testosterone suppression observed appears to represent a functional

perturbation in line with our other findings suggesting a centralised mechanistic change in the hypothalamus.

5. Conclusions

The current study indicates that neonatal exposure to LPS alters HPG and HPA axis regulation, which has implications for both responding to metabolic triggers of puberty and optimal sexual behaviour. Changes to testicular morphology and gonocyte proliferation indicate potential mechanistic alterations for the reduced reproductive success of LPS-treated animals, which appear independent to HPA axis programming, at least peripherally. The current findings hold promise for explaining some causes of reproductive dysfunction, and have significantly quantified the influence of perinatal infection on subfertility outcomes. The effects on impaired sexual performance are obvious, and the fact that these behaviours are largely driven by HPG hormones suggests a causal link between endocrine and behavioural function. Critically, the results highlight the importance of appropriate monitoring of infection when it occurs in the early postnatal period, as if untreated may have deleterious effects in both health and reproductive potential.

Conflicts of interest

The authors declare there are no conflicts of interest.

Acknowledgments

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Paper 4: Neonatal immune challenge alters reproductive development in the female rat

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Statement of author contributions to manuscript

Author	Description of Contribution to Manuscript	Signature
Luba Sominsky	Designed and performed the experiments Analysed and interpreted the data Wrote the manuscript	
Crystal L. Meehan	Performed the experiments Assisted in data analysis and interpretation Assisted in manuscript preparation	
Adam K. Walker	Assisted in the experimental design and data interpretation Provided intellectual contribution and critical input Revised the manuscript	
Larisa Bobrovskaya	Performed the analysis of tyrosine hydroxylase and provided intellectual contribution	
Eileen A. McLaughlin	Assisted in the experimental design and data interpretation Contributed reagents / materials/ analysis tools Provided intellectual contribution and critical input Revised the manuscript	
Deborah M Hodgson	Assisted in the experimental design and data interpretation Contributed reagents / materials/ analysis tools Provided intellectual contribution and critical input Revised the manuscript	

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Neonatal immune challenge alters reproductive development in the female rat

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ABSTRACT

This article is part of a Special Issue "Neuroendocrine-Immune Axis in Health and Disease."

Neonatal lipopolysaccharide (LPS) exposure alters neuroendocrine, immune and behavioural responses in adult rats. Recent findings indicate that neonatal LPS treatment may have a more pronounced effect on the mating behaviours of females compared to males. The current study further explored the impact of neonatal inflammation on reproductive development in the female rat. Wistar rats were administered LPS (0.05 mg/kg, i.p.) or saline (equivolume) on postnatal days (PNDs) 3 and 5. The immediate effect of treatment was assessed on plasma corticosterone and tyrosine hydroxylase (TH) phosphorylation in the adrenal medulla. Weight gain and vaginal opening were recorded, and oestrous cyclicity was monitored post-puberty and in late adulthood. Blood and ovaries were collected throughout development to assess HPA and HPG hormones and to examine ovarian morphology. Reproductive success in the first (F1) generation and reproductive development in the second (F2) generation were also assessed. Neonatal LPS exposure resulted in increased TH phosphorylation in the neonatal adrenals. LPS treatment increased the corticosterone concentrations of females as juveniles, adolescents and adults, and reduced FSH in adolescence. Increased catch-up growth was evident in LPS-treated females, prompting earlier onset of puberty. Diminished follicular reserve was observed in neonatally LPS-treated females along with the advanced reproductive senescence. While fertility rates were not compromised, higher mortality and morbidity were observed in litters born to LPS-treated mothers. Female offspring of LPS-treated mothers displayed increased corticosterone on PND 14, increased catch-up growth and delayed emergence of the first oestrous cycle. No differences in any of the parameters assessed were observed in F2 males. These data suggest that neonatal immunological challenge has a profound impact on the female reproductive development, via the alteration of metabolic and neuroendocrine factors which regulate sexual maturation. Evidence of altered development in the female, but not male offspring of LPS-treated dams suggests increased susceptibility of females to the deleterious effects of neonatal immunological stress and its possible transferability to a subsequent generation.

Introduction

Extensive evidence has indicated sexual dimorphism with regard to human disease prevalence and susceptibility. Men are known to be more susceptible to cardiovascular and infectious diseases (Schroder et al., 1998), while women demonstrate higher rates of autoimmune and affective disorders (Bale, 2009; Whitacre, 2001). Several mechanisms have been suggested to underpin these sex-related differences. The primary hypothesis is based on differences in sex hormones, initiated via their organising effects in early life, and continuing into activating effects which influence the development of physiological and behavioural aspects of adult life (Van Goozen et al., 1995). In addition to the role played by gonadotrophin-releasing hormones (GnRH) and gonadal steroids in sexual differentiation and reproduction, sex hormones are also involved in immune system modulation and development (Tanriverdi et al., 2003; Verthelyi, 2001), as well as playing an important role in brain ontogeny (Harris, 1964). Sexual dimorphism in immune and neuroendocrine responses, driven by reproductive hormonal fluctuations, has been investigated in a number of human and animal studies (Grossman, 1985; Naor et al., 2009). Oestrogen has been shown to attenuate, and testosterone to exacerbate, inflammatory responses in rats (Razmara et al., 2005). In humans, oestrogen has been suggested to have an immunoprotective role, while testosterone has been found to be immunosuppressive (Schroder et al., 1998).

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Given that major hormonal changes occur in adolescence, it is not surprising that sex differences in disease prevalence typically emerge during this period (Goel and Bale, 2009; McCormick and Mathews, 2007). However, the foundation for such susceptibilities is thought to be laid down earlier in life. The impact of the perinatal environment on development and predisposition to pathology has been gaining increasing attention in the last few decades. The developmental origins of health and disease (DOHaD) hypothesis proposes that exposure to adverse events during the perinatal period alters long term health outcomes (Barker and Osmond, 1987). Epidemiological data, for instance, have demonstrated an association between malnutrition experienced in early life and increased risk of cardiovascular disease, stroke and diabetes in later life (Barker et al., 1989; Barker, 1994, 2006). More recently the DOHaD hypothesis has been extended to explain the impact of a variety of stressors during the perinatal period. Typically stress during the perinatal period is associated with altered stress responsivity in the offspring, leading to an increased risk of health complications, including psychopathologies, later in life. A common occurrence during the perinatal period is exposure to infection, which activates the host immune response. Early life immune activation is known to impact normal development in both animals and humans resulting in long-term metabolic (Dimock et al., 2011; Walker et al., 2006), immune (Galic et al., 2009; Hodgson et al., 2001; Martinez et al., 1998; Walker et al., 2010), neuroendocrine (Matthews, 2002; Shanks et al., 2000) and behavioural (Meyer et al., 2006; Walker et al., 2009b) alterations. Sexual dimorphism in response to a neonatal immune challenge has also been recently shown to predict differences in adult immune and behavioural responses to inflammation (Kentner et al., 2010).

A growing body of evidence suggests that reproductive health and sexual behaviour might be affected by bacterial exposure occurring early in life (Knox et al., 2009; Kentner and Pittman, 2010; Wu et al., 2011). A recent study in our laboratory by Walker et al. (2011) has demonstrated that immune activation during early life compromises reproductive fitness in adulthood. Specifically, the study reported that male and female rats exposed to a bacterial mimetic (lipopolysaccharide: LPS) on days 3 and 5 postpartum exhibited altered puberty onset, and diminished hypothalamic–pituitary–gonadal (HPG) hormonal function. Interestingly, in this study males but not females exhibited increased anxiety-like behaviours in adulthood. When sexual behaviour was examined in adulthood, both sexes had impaired sexual performance, however, a greater number of behaviours were affected in females compared to males treated neonatally with LPS. Walker et al. (2011) also reported neonatal exposure to an immune challenge to induce long-term changes in testicular development and spermatogenesis. To date the impact of early life exposure to an immunological challenge on the development of female reproductive morphology and function has not been examined.

Given the previous findings indicating that neonatal immune activation has a robust effect on female sexual behaviour (Walker et al., 2011), and given the important role of the female rodent in the regulation of mating behaviour (Agmo, 1997), the aim of this study was to further explore the impact of neonatal immune activation on reproductive development in the female rat. The current study examined the effect of exposure to a neonatal immune challenge on the onset of puberty, female gonadal development by examining ovarian morphology at several critical time points in reproductive development and determined the onset of reproductive senescence. Finally, we assessed the impact of neonatal LPS exposure on reproductive success in the first (F1) generation (in terms of mating and offspring outcomes) and on reproductive development in the second (F2) generation. The important question being addressed in this component of the study is whether the reproductive history of the mother is transmitted to her offspring.

In an attempt to understand the mechanisms mediating alterations to reproductive fitness, this study also assessed the role of the sympathetic nervous system (SNS). Sympathetic innervation of the ovary is known to affect reproductive function and can be altered by systemic sympathetic activity (Greiner et al., 2005). Catecholamines, and in particular norepinephrine, play a significant role in the control of ovarian steroidogenesis and follicular development at puberty (Aguado et al., 1982; Aguado and Ojeda, 1984). Moreover, loss of sympathetic innervation in neonatal rats has been shown to result in the delayed onset of puberty and disruption of oestrous cyclicity (Lara et al., 1990). Catecholaminergic assessment is particularly relevant to this study given that we have recently reported that male rats exposed to LPS during the neonatal period exhibit an immediate and sustained activation of the sympatho-adrenomedullary system, by increasing phosphorylation of tyrosine hydroxylase (TH) (Sominsky et al., 2012). TH is a rate-limiting enzyme in catecholamine synthesis (Dunkley et al., 2004). It is unknown at this point whether the same activation occurs in female rats in response to neonatal LPS.

Methods

Experiment 1 — First generation (F1) study

Animals

Naïve female Wistar rats (8–10 weeks of age) obtained from the University of Newcastle animal house were mated in the University of Newcastle Psychology vivarium. 28 litters were randomly and evenly allocated into either LPS or saline control conditions at birth (postnatal day [PND] 1). No significant differences in litter size and male-to-female ratios were observed between LPS and saline-treated litters. On PND 3 and PND 5, pups were briefly removed from their home cages, weighed, and administered intraperitoneally (i.p.) with either 0.05 mg/kg LPS (Salmonella enterica, serotype enteritidis; Sigma-Aldrich Chemical Co., USA, dissolved in sterile pyrogen-free saline) or an equivolume of non-pyrogenic saline (Livingstone International, Australia). Drug administration procedures and housing conditions were identical to those previously described in A.K. Walker et al. (2009, 2010). A subgroup of animals (26 females: 12 LPS, derived from 3 litters; 13 Saline, derived from 4 litters) was sacrificed at 4 h and 24 h following neonatal drug administration on PND 5 when trunk blood and adrenal glands were collected to assess the immediate effect of treatment on HPA axis and SNS function. All male rats were treated identically to females and remained within the litters until weaning. The remaining female rats were randomly allocated for assessment at five developmental groups: (1) PND 14 (9 LPS, derived from 6 litters; 8 Saline, derived from 5 litters); (2) puberty onset (~PND 33) (40 LPS, derived from 8 litters; 33 Saline, derived from 8 litters); (3) adolescence (~PNDs 45–50) (20 LPS, derived from 6 litters; 22 Saline, derived from 7 litters); (4) adulthood (~PND 85) (7 LPS, derived from 5 litters; 7 Saline, derived from 5 litters); and (5) oestrous cyclicity decline in late adulthood (9–12 months) (11 LPS, derived from 3 litters; 8 Saline, derived from 2 litters). A final group of animals was used for mating purposes (4 LPS, derived from 3 litters; 4 Saline, derived from 3 litters) in order to assess the potential for transgenerational changes in sexual development in the second (F2) generation. Figs. 1(a and b) provides a schematic timeline of the protocols. Apart from those sacrificed on PND 5 and PND 14, animals were left undisturbed until weaning (PND 22) when they were separated into same-sex pair housing (41.5 cm × 28.0 cm × 22.0 cm cages; Mascot Wire Works, Sydney). Following weaning, female rats underwent daily assessment of pubertal markers. Animals were maintained under a normal 12 h light/dark schedule (lights on 06:00 h); temperature (21 ± 1 °C), with food and water available ad libitum. All experimentation occurred in accordance with the 2004 NH&MRC Australian Code of Practice for the care and use of animals for scientific practice.

Neonatal blood and adrenal gland collection

Four hours following injection on PND 5 blood and tissues were obtained for assessment of plasma corticosterone responses to

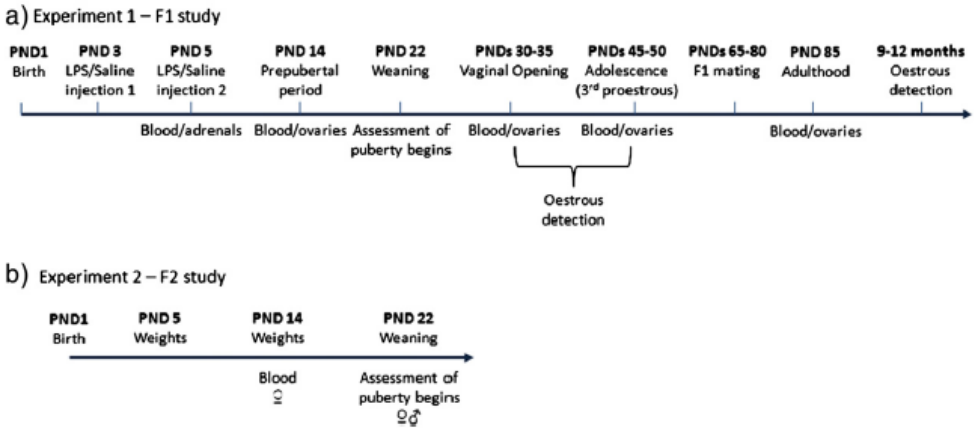

Fig. 1. (a) A schematic timeline of the experimental protocols conducted for the F1 generation. (b) A schematic timeline of the experimental protocols conducted for the F2 generation.

treatment and TH protein and phosphorylation levels in the adrenals. Rats were rapidly decapitated and trunk blood was collected into EDTA-coated tubes (Livingstone International, Australia). At the same time, adrenals were surgically collected from the same animals, excess fat tissue was removed, and glands were immediately frozen on dry ice and kept at −80 °C until assayed.

Assessment of puberty onset and oestrous cyclicity

Following weaning, female rats were monitored daily for vaginal opening, a physical marker of puberty onset. Once vaginal opening occurred, vaginal smears were taken daily, between 1 and 3 pm, to determine oestrous cyclicity until proestrous of the third consecutive normal cycle was detected, and again from PND 80 until the conclusion of the experiment. The phases of the oestrous cycle were identified according to the predominant cell population in the vaginal smears under light microscope. A normal oestrous cycle was defined as a four or five day cycle with two or three days of diestrous. Animals were weighed weekly until the conclusion of the experiment, as well as on the day of vaginal opening. Animals allocated to group 3 (adolescence) ended the experimental procedure on the third regular proestrous corresponding to the adolescent period. Animals allocated to group 4 (adulthood) were euthanized in adulthood during proestrous as soon as one regular cycle was identified. Appearance of normal oestrous cyclicity was determined in females allocated to group 5 at months 9–12 of age.

Blood sampling and hormonal assessment

At the time of tissue collection, all rats were deeply anaesthetized via i.p. injection with 15–20 mg/100 g Lethabarb (Virbac, Pty. Ltd, Milperra, Australia). Rats were allocated for tissue collection on PND 14, in puberty, adolescence and adulthood. At each of these time points, apart from PND 14, animals were euthanized immediately after the vaginal smear procedure. Cardiac blood was withdrawn and placed into EDTA-coated tubes. Samples were centrifuged at 1000 *g* for 15 min at 4 °C, and the plasma was stored at −20 °C until assayed. Levels of plasma corticosterone were assessed using a rat corticosterone 125I radioimmunoassay kit (MP Biomedicals, CA, USA). The recovery of free corticosterone was 100%, with a mean inter- and intra-assay variability of 4.4 and 6.5%, respectively. Circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were quantified using rat LH and rat FSH ELISA kits (Shibayagi, Co., LTD, Gunma, Japan). The recoveries are 97.7–103% for LH and 94–102% for FSH, with a mean inter- and intra-assay variability of <5% and <3%, respectively. All assays were performed according to the manufacturer's instructions.

Tyrosine hydroxylase analysis

Reagents. Mouse anti-total TH antibody (catalogue # T1299) and mouse β-actin antibody (catalogue # A3854) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Nitrocellulose membrane (Hybond ECL) and ECL Plus kit were from GE Health Care (Little Chalfont, UK). Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA). PageRuler Prestained Protein Ladder and secondary antibodies (rabbit anti-sheep IgG (H + L) peroxidase conjugated (catalogue # 31480); goat anti-rabbit IgG (H + L) peroxidase conjugated (catalogue # 31460) and goat anti-mouse IgG (H + L) peroxidase conjugated (catalogue # 31430)) were from Thermo Fisher Scientific. Phospho-specific TH antibodies (pSer19TH, pSer31TH and pSer40TH) were generated and were tested for specificity as described (Gordon et al., 2009).

Procedure. TH phosphorylation and protein levels were analysed as previously described (Ong et al., 2011a,b). Briefly, the adrenals were homogenised using a sonicator (Sonics & Materials Inc., model VCX 130, USA) in homogenisation buffer (2% SDS, 2 mM EDTA, 50 mM Tris, pH 6.8) with ratio 1 ml of buffer per 50 mg of tissue. Samples were then boiled for 5 min and centrifuged at 15,000 rpm for 20 min. The clear supernatants were collected and protein concentrations were determined by Pierce micro BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Samples were aliquoted and were kept frozen at −80 °C for further analysis. Western blotting was performed as previously described with some modifications (Ong et al., 2011a,b). 30 μg of each sample was subjected to SDS-PAGE gel electrophoresis and then transferred to nitrocellulose membranes (Hybond ECL, GE Health care). Nitrocellulose membranes were stained with Ponceau S (0.5% Ponceau in 1% acetic acid) to assess the efficacy of the transfer. Membranes were incubated with total TH, phospho-specific TH or β-actin antibodies for 1 h at room temperature followed by secondary antibodies for 1 h at room temperature. Membranes were visualized on ImageQuant LAS-4000 imaging system (GE Health care) using detection reagents (Amersham ECL Plus Western Blotting Detection Reagents, GE Health care). The density of the bands was analysed using ImageQuant TL software (GE Health care). Site-specific TH phosphorylation was expressed as the ratio of TH phosphorylation at Ser19, Ser31 or

Ser40 to total TH protein, to account for variability in total TH protein between samples. TH protein was expressed as the ratio of TH to β-actin to account for variability in protein loading.

Ovarian morphology assessment

Ovarian tissue was obtained on PND 14, day of vaginal opening, adolescence and adulthood. Animals were euthanized with Lethabarb (15–20 mg/100 g, i.p.) and both ovaries were surgically excised and weighed. One ovary from each animal was fixed in Bouin's solution (Hirshfield and Midgley, 1978) overnight (counterbalanced between left and right ovaries). Ovaries were then washed four times in 70% ethanol. Fixed ovaries were dehydrated, embedded in paraffin and sectioned at 4 μm with every 4th section mounted and haematoxylin–eosin (H&E) stained for morphometric analysis. The total number of follicles was quantified by an experimenter blind to the experimental conditions. The follicles were categorized according to the following classification: (a) primordial follicles: follicles with oocytes surrounded by one layer of flattened pregranulosa cells; (b) primary follicles: follicles with oocytes surrounded by no more than one layer of cuboidal granulosa cells; (c) preantral follicles: follicles without any antral cavity and with two or more layers of cuboidal granulosa cells; (d) antral follicles: follicles with antral cavity and with two or more layers of cuboidal granulosa cells (Lara et al., 2000; Myers et al., 2004). The total number of primordial and primary follicles was counted across the third section on every second and fourth H&E stained slide. Given the small diameter of primordial and primary follicles (~25 μm), the total number of these follicles per ovary was adjusted based on the follicle and ovarian size at each time point. Preantral and antral follicles were quantified across the first and third sections of all five H&E stained slides per animal. Given the large diameter of these follicles (0.2–0.4 mm), the quantification procedure resulted in all follicles within the ovary being counted, without additional adjustment required. Only follicles with a visible nucleus of the oocytes were quantified (Wu et al., 2011).

Experiment 2 — Second generation (F2) study

Mating of generation 1 (F1) females

4 LPS and 4 saline-treated female rats were randomly selected to produce F2 generation animals. At ~70 days of age, these animals were monitored for oestrous cyclicity and once proestrous was determined, females were pair-housed with another female from the opposite neonatal treatment condition (LPS-treated female pair housed with saline-treated control) and an experimentally naïve male stud obtained from the University of Newcastle Central Animal House. After two weeks, the male stud was removed and the females were housed individually.

Second generation monitoring

Neonatal weights of the F2 generation were obtained on PND 5 and PND 14. Blood was collected from a subgroup of females (5 from F1 LPS, 5 from F1 Saline) on PND 14, to assess plasma corticosterone levels, identically to the method described above. On PND 22 all animals were weaned and separated into same-sex pair housing. Male rats were culled to five animals per litter. Following weaning, F2 females were monitored in the same manner as F1 animals, which included assessment of puberty onset and oestrous cyclicity. F2 males were monitored for puberty onset identified by preputial separation, as previously described (Walker et al., 2011). Weekly weights and weights on the day of puberty onset were obtained. Monitoring concluded in females when at least 3 consecutive oestrous cycles were determined and in males at the onset of puberty.

Data analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Volume 18 (SPSS Inc.). Data were analysed using a nested analyses of variances (ANOVA) design to control for potential litter effects, whereby "litter" was nested into "treatment". Analyses of covariance (ANCOVA), whereby weight and litter size were covaried into the analyses, were also employed where appropriate (reported only when significantly contributing to the data). Planned comparisons were performed between experimental conditions using *t* test analyses adjusted for multiple comparisons where significant interactions were observed. Significance level was set at $p \leq 0.05$.

Results

Experiment 1 — F1 study

Developmental weight gain

Neonatal weight gain. LPS-treated females gained significantly less weight between PND 3 and PND 5 ($M = 3.019$ g, $SEM = 0.74$) than saline-treated controls ($M = 3.272$ g, $SEM = .81$), $F_{(15,78)} = 7.639$, $p < .001$. On PND 14, LPS-treated females weighed significantly more ($M = 33.551$ g, $SEM = .473$) than saline-treated controls ($M = 27.586$ g, $SEM = .524$), ($F_{(10,6)} = 41.270$, $p < 0.001$).

Weight gain from weaning to adulthood. A significant effect of treatment was observed for weight gain from weaning (PND 22) to adolescence (PND 50), with LPS treated females gaining more weight ($M = 130.232$ g, $SEM = 2.379$) than saline-treated controls ($M = 125.519$ g, $SEM = 2.335$), ($F_{(9,21)} = 4.099$, $p < .005$). No significant changes in weight gain were evident between adolescence and adulthood.

Pubertal onset and body weight

Vaginal opening. LPS-treated females were significantly lighter ($M = 120.110$ g, $SEM = 2.101$) on the day of vaginal opening compared to saline-treated controls ($M = 127.421$, $SEM = 2.315$), ($F_{(1,69)} = 2.234$, $p < .05$), after accounting for the significant covariate of litter size ($F_{(1,69)} = 7.578$, $p < .05$). However, this effect appears to have been driven by the significantly earlier onset of vaginal opening in LPS-treated rats ($F_{(1,69)} = 17.848$, $p < .001$), as shown in Fig. 2a. The weekly weight gain profile of LPS-treated rats from weaning to immediately prior to the first onset of vaginal opening in our sample (PND30) indicated that LPS-treated females had significantly increased weight on both PND 22 ($F_{(12,50)} = 37.302$, $p < .001$) and PND 29 ($F_{(12,50)} = 15.411$, $p < .001$; Fig. 2b).

Oestrous cyclicity. As illustrated in Fig. 2c, the first proestrous that was followed by a regular oestrous cycle appeared significantly earlier in LPS treated females then in saline controls ($F_{(1,38)} = 5.823$, $p < .05$) (LPS: $M = 39.446$, $SEM = .538$; Saline; $M = 41.276$, $SEM = .512$). Cycle regularity did not differ between LPS-treated or saline-treated females at any time point. However, between 9 and 12 months of age there was a significant decline in oestrous cycling in LPS-treated females ($\chi^2 = 6.115$, $df = 1$, $p < .05$) at 11 and 12 months of age not apparent in saline-treated animals. Fig. 2d demonstrates that the majority of LPS-treated rats (90.9%) did not show a regular cycling pattern in these months, whereas only 37.5% of saline controls ceased regular cycling during this time.

TH phosphorylation in neonatal adrenals

While no significant differences were observed in regards to total TH protein levels or TH phosphorylation at Ser19 and Ser40 at 4 h and 24 h following LPS treatment on PND 5, there was a significant increase in TH phosphorylation in LPS-treated females at Ser31 ($F_{(4,14)} = 2.991$, $p < .05$) along with a significant interaction between time and treatment ($F_{(4,14)} = 6.334$, $p < .01$), as illustrated in Fig. 3.

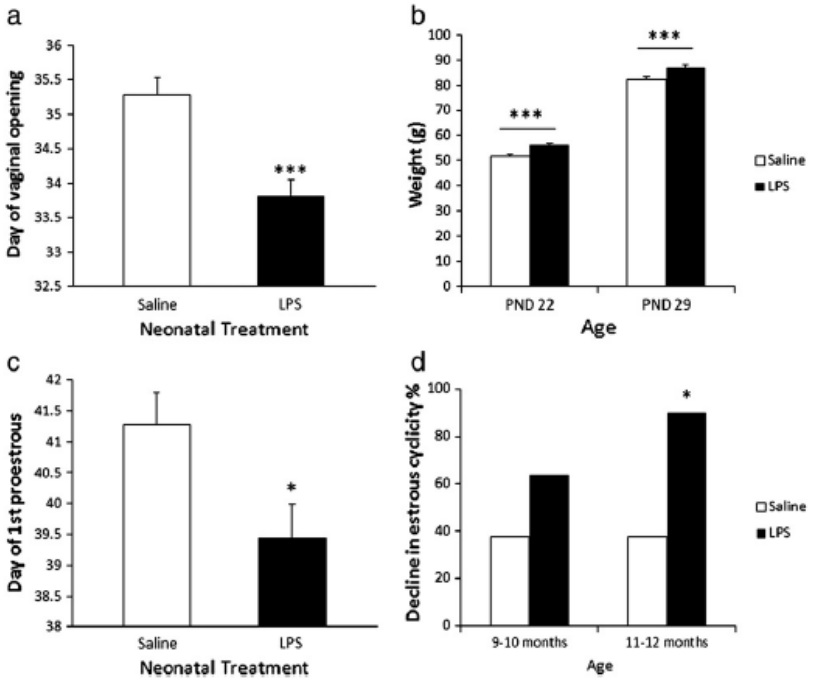

Fig. 2. (a) Effect of neonatal LPS treatment on the day of vaginal opening (n = 33–40 per group). (b) Weekly weight gain from weaning (PND22) to immediately prior to the first onset of vaginal opening (PND29) (n = 31–32 per group at each time point). (c) Effect of neonatal LPS exposure on the emergence of the first proestrous, followed by a regular oestrous cycle (n = 20–22 per group). (d) The percentage of decline in oestrous cyclicity at 9–10 and 11–12 months of age (n = 8–11 per group at each time point). The hollow bars represent neonatally treated saline females and the filled bars represent neonatally treated LPS females. *p<.05, ***p<.001.

Planned comparisons revealed this increase to be significant at 4 h following drug exposure on PND 5 ($t_{(11)} = 3.7$, p<.01).

Plasma corticosterone

No significant effect of neonatal treatment was evident for neonatal plasma corticosterone levels on PND 5. However, as illustrated in Figs. 4(a and b), LPS treatment significantly increased corticosterone levels on PND 14 ($F_{(1,10)} = 5.31$, p<.05) and on the day of vaginal opening ($F_{(9,5)} = 7.208$, p<.05). A significant main effect of treatment on corticosterone levels was also evident in adolescence and adulthood ($F_{(10,12)} = 3.562$, p<.05), see Fig. 4c.

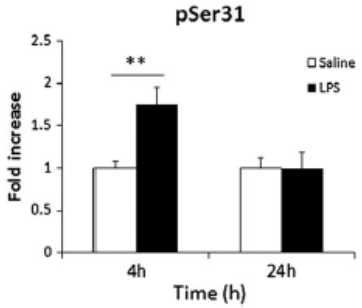

Fig. 3. Effect of neonatal LPS exposure on tyrosine hydroxylase (TH) phosphorylation at Ser31 at 4 h and 24 h following LPS exposure on PND5. Hollow bars represent neonatally treated saline females (n = 7 at each time point), and filled bars represent neonatally challenged LPS females (n = 6 at each time point). **p<.01.

Plasma HPG hormones

Assessment of LH and FSH on the day of vaginal opening and in adolescence (on the day of 3rd proestrous) revealed no significant effect of treatment for either of the time points for LH, see Fig. 5a. A significant interaction between time and treatment was observed in regards to plasma FSH levels ($F_{(1,25)} = 6.949$, p<.05). As illustrated in Fig. 5b, planned comparisons revealed that LPS-treated females had significantly reduced FSH levels compared to controls in adolescence but not on the day of vaginal opening ($t_{(11)} = 2.767$, p<.05).

Ovarian weights and morphology

A significant effect of neonatal treatment was observed on PND 14, with regard to ovarian weight as a percentage of body weight ($F_{(1,14)} = 5.895$, p<.05) with LPS-treated females having smaller ovaries at this time point (LPS: M = .031, SEM = .005; saline: M = .049, SEM = .005).

Analysis of follicular population conducted for each follicular type across time and treatment revealed a significant interaction between time and treatment ($F_{(8,15)} = 2.81$, p<.05) for the number of primordial follicles. As shown in Fig. 6a, planned comparisons demonstrated that LPS-treated females had significantly reduced numbers of primordial follicles on PND 14 ($t_{(6)} = -3.742$, p<.01). Photomicrographs demonstrating differences in the number of primordial follicles on PND 14 are shown in Fig. 6b.

A significant effect of treatment was observed for the number of primary follicles ($F_{(8,15)} = 2.981$, p<.05), with fewer primary follicles evident in the ovaries of LPS-treated females (M = 176.867, SEM = 14.091) than their saline-treated counterparts (M = 225.167, SEM = 15.401). With regard to preantral and antral follicles, there was a significant effect of time ($F_{(3,15)} = 4.399$, p<.05; $F_{(3,15)} = 3.334$, p<.05, respectively), but not treatment. In respect of the total number

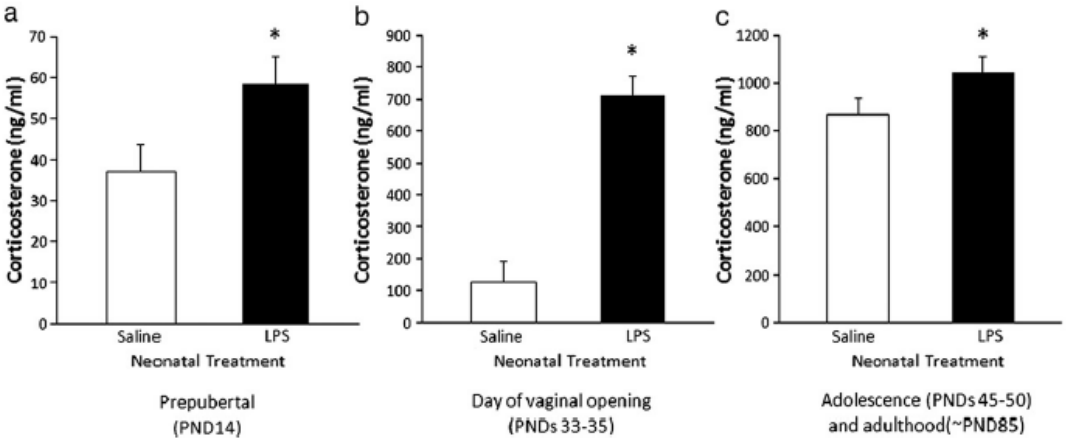

Fig. 4. Represents the effect of neonatal LPS exposure on plasma corticosterone levels (ng/ml) on PND 14 (n = 6 per group) (a), on the day of vaginal opening (PNDs 33–35) (n = 7–8 per group) (b), and during adolescence (PNDs 45–50) and adulthood (~PND85) (n = 7 per group at each time point) (c). Hollow bars represent neonatally challenged saline females, and filled bars represent neonatally challenged LPS females. *$p<.05$.

of follicles, there was a significant effect of treatment ($F_{(5,15)} = 4.051$, $p<.05$), with fewer total number of follicles evident in LPS-treated females ($M = 662.583$, $SEM = 38.608$) compared to saline-treated controls ($M = 741.452$, $SEM = 42.198$).

Experiment 2 — F2 study

Litter effects

Mating of all F1 females resulted in live birth with no significant difference in the date of delivery between LPS and saline-treated females. LPS-treated females had significantly larger litter sizes (LPS-treated: $M = 13.924$, $SEM = .122$; saline-treated: $M = 12.692$, $SEM = .106$; $F_{(4,90)} = 85.337$, $p<.001$) and a significantly higher ratio of male to female pups (LPS-treated: $M = .612$, $SEM = .010$; saline-treated: $M = .508$, $SEM = .008$; $F_{(4,90)} = 49.802$, $p<.001$). Mortality rates were significantly higher for pups born to LPS-treated dams ($\chi^2 = 13.477$, $df = 1$, $p<.001$). Over 23% of pups exhibited physical signs of malnourishment on PND 5 (weight ≤ 8 g) and were therefore euthanized according to the University of Newcastle animal ethics guidelines and excluded from further analysis. No such cases were observed in pups born to saline controls nor was such an effect observed in F1 generation animals.

Weight gain

Weight analysis on PND 5 for F2 offspring indicated a significant main effect of "F1 treatment" ($F_{(7,79)} = 7.877$, $p<.0001$) and a significant effect of sex ($F_{(7,79)} = 4.785$, $p<.05$), with F2 pups born to LPS-treated dams exhibited significantly lower body weights ($M = 10.706$, $SEM = .144$) compared to their matched controls ($M = 11.233$, $SEM = .122$). Male offspring also, as expected, weighed significantly more than females (males: $M = 11.176$, $SEM = .128$; females: $M = 10.764$, $SEM = .139$). This difference in body weight between treatment groups persisted until PND 14 ($F_{(5,61)} = 28.371$, $p<.0001$), with animals born to LPS-treated mothers gaining less weight ($M = 27.131$, $SEM = .427$) than those born to saline controls ($M = 31.555$, $SEM = .377$).

Analysis of weekly weights from weaning (PND 22) to adolescence (PND 50) revealed a significant interaction between sex and treatment ($F_{(7,52)} = 2.701$, $p<.05$). Planned comparisons confirmed, that while no significant difference was observed in weight gain for

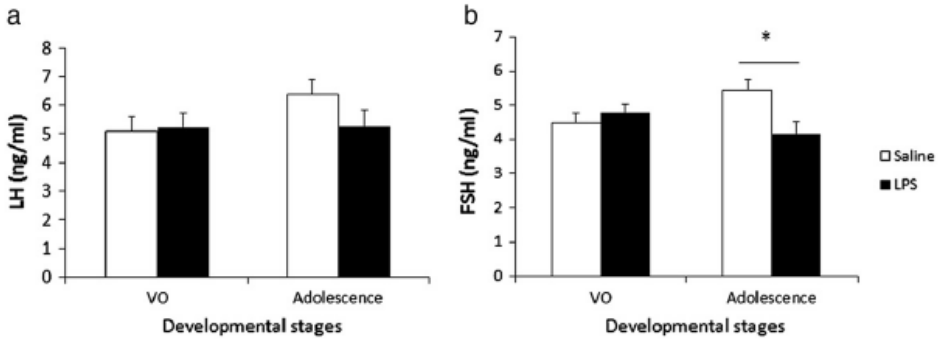

Fig. 5. Effect of neonatal exposure to LPS on plasma HPG hormones on the day of vaginal opening (VO) (PNDs 33–35) and in adolescence (PNDs 45–50). (a) Represents plasma LH levels (ng/ml). (b) Represents plasma FSH levels (ng/ml). Hollow bars represent neonatally treated saline females, and filled bars represent neonatally challenged LPS females (n = 6–9 per group at each time point, for both (a) and (b)). *$p<.05$.

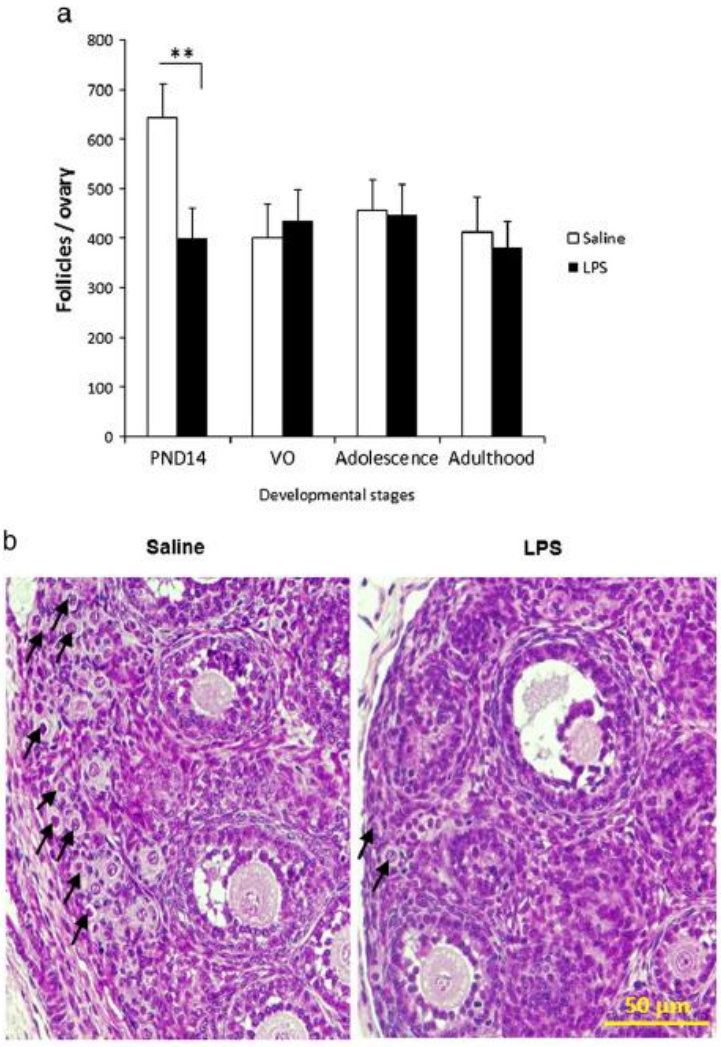

Fig. 6. (a) Represents the effect of neonatal LPS treatment on the number of primordial follicles on PND 14, on the day of vaginal opening (PNDs 33–35), in adolescence (PNDs 45–50) and in adulthood (~PND85), (n = 5–6 per group at each time point). Hollow bars represent neonatally challenged saline females, and filled bars represent neonatally challenged LPS females. $^{**}p<.01$. (b) Representative photomicrographs demonstrating differences in the number of primordial follicles on PND 14 in saline and LPS-treated females. Arrows indicate primordial follicles. Bar = 50 µm.

males, females born to LPS-treated dams exhibited increased weight gain at this time point ($t_{(29)} = 2.224$, $p<.05$).

Plasma corticosterone

As demonstrated in Fig. 7, on PND 14, F2 females born to LPS-treated dams exhibited significantly increased corticosterone levels than offspring of saline-treated mothers ($F_{(5,10)} = 34.744$, $p<.01$).

Puberty markers

In female offspring, there was no effect of F1 treatment in regards to puberty onset (i.e. the day of vaginal opening). However, as shown in Fig. 8a, the day of 1st proestrous, and the difference between the day of vaginal opening and the 1st proestrous (Fig. 8b) were significantly delayed for females born to LPS-treated dams ($F_{(7,22)} = 3.154$, and $F_{(7,22)} = 1.518$; $p<.05$ for both). No significant effect of F1 treatment was found in males in regards to the day of preputial separation.

Discussion

Our laboratory has previously demonstrated in rats, that neonatal immune challenge alters sexual behaviour to a greater extent in females than males (Walker et al., 2011). The current study expands this research to demonstrate that neonatal LPS exposure alters a number of physiological parameters which play a cumulative role in reproductive development and functioning, including catch-up growth, puberty onset, oestrous cyclicity, SNS, HPA and HPG activity. The diminished primordial follicle pool observed in prepubertal rats exposed to an immune challenge, suggests accelerated exhaustion

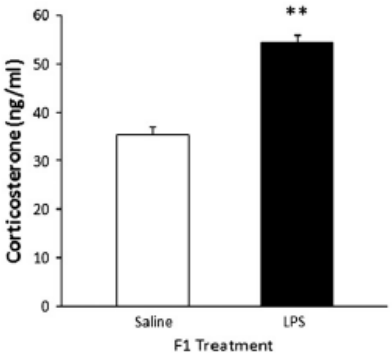

Fig. 7. Effect of F1 treatment on plasma corticosterone levels in F2 females on PND 14 (ng/ml). Hollow bars represent F2 females born to saline-treated mothers, and filled bars represent F2 females born to LPS-treated mothers (n = 5 per group). **p < .01.

of the follicular reserve, possibly due to direct ovotoxic effects (Sobinoff et al., 2010, 2011). Moreover, delayed onset of oestrous cycling in females born to LPS-treated mothers indicates the possible transmission of altered reproductive parameters, expanding upon our recent findings which have demonstrated transgenerational inheritance of an anxiety phenotype induced by neonatal exposure to LPS in male and female rats (Walker et al., 2012).

Developmental weight gain

Neonatal LPS exposure resulted in decreased weight gain between PND 3 and PND 5, confirming an LPS-induced sickness response, consistent with previous findings (Walker et al., 2004, 2010). After recovery from LPS, LPS-treated females gained significantly more weight from PND 14 into adolescence. Previous research using an alternative animal model of infection, has demonstrated that exposure to *Escherichia coli* in early life results in an immediate weight loss but increased catch-up growth in infected rats, compared to controls (Samuels and Baracos, 1992). Increased catch-up growth is well documented in epidemiological studies as a risk factor for development of diseases in later life, such as obesity, type-2 diabetes and cardiovascular diseases, in particular when growth above normal rates occurs following neonatal growth retardation (Barker et al., 2005; Huxley et al., 2000; Ong et al., 2000). These metabolic alterations are pertinent to the current study as body weight greatly contributes to pubertal maturation in both humans and animals (Frisch and Revelle, 1970; Goldman et al., 2000), most importantly, by prompting ovulation (Frisch and McArthur, 1974).

Pubertal onset

Neonatal immune challenge resulted in the precocious onset of puberty, as indicated by advanced vaginal opening and emergence of proestrous. Others, using a similar model of neonatal LPS exposure, have reported delayed pubertal onset (Knox et al., 2009; Wu et al., 2011). Several factors can account for this difference, most notably, the studies by Knox et al. (2009), and Wu et al. (2011) were conducted in Sprague–Dawley rats, whereas our previous and current findings were demonstrated in Wistar rats (Walker et al., 2011). Wistar rats have a bimodal distribution in the age at vaginal opening, with one peak occurring on PND 34 and the second on PND 39 (Rivest, 1991). Thus, variability in the timing of pubertal onset is to be expected.

In addition to advanced vaginal opening, the first regular oestrous cycle occurred earlier in LPS-treated females. The first proestrous is typically observed 5–7 days following vaginal opening, which is primarily dependent on growth rates rather than specific age. Accelerated growth is known to lead to earlier vaginal opening and first proestrous (Maeda et al., 2000). Therefore, metabolic cues resulting from the elevated weight gain observed throughout the juvenile and prepubescent period are most likely responsible for the advanced sexual maturation in LPS-treated females.

Findings from human studies indicate that earlier menarche can be related to smaller size at birth and increased catch up growth (Dunger et al., 2006). Moreover, lower age at menarche has implications for the development of metabolic disease in adulthood (Chen et al., 2011) and is associated with earlier mortality (Giles et al., 2010).

SNS, HPA and HPG hormones

Neonatal TH phosphorylation

Female pups exhibited increased TH phosphorylation at Ser31 residue 4 h following LPS challenge on PND 5. TH, the rate-limiting enzyme involved in biosynthesis of catecholamines (Nagatsu et al., 1964), is known to be regulated acutely by phosphorylation at its serine residues (i.e. Ser19, Ser31 and Ser40) (Kumer and Vrana, 1996). Analysis of TH phosphorylation at Ser19, Ser31 and Ser40 over time is a sensitive index in the assessment of the effects of different stressors on catecholamine-producing cells (Ong et al., 2011a). Previously published data have shown increased and sustained phosphorylation of TH at Ser40 in LPS-treated male pups (Sominsky et al., 2012). This is the first time that TH phosphorylation in the neonatal adrenals following an immune challenge has been assessed in female rats. Catecholamines play an important role in ovarian follicular development and steroidogenesis (Lara et al., 1990; Aguado et al.,

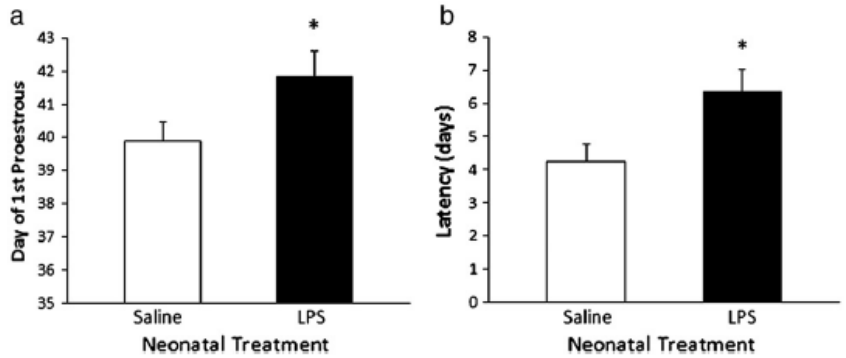

Fig. 8. Effect of F1 treatment on the emergence of first proestrous (a) and the latency between the day of vaginal opening and the first proestrous (b). Hollow bars represent F2 females born to saline-treated mothers, and filled bars represent F2 females born to LPS-treated mothers (n = 12–18 per group). *p < .05.

1982). Prepubertal increase in the follicular norepinephrine content regulates follicular response to gonadotrophins and ovulation (Aguado and Ojeda, 1984; Morales et al., 1998), while denervation of the ovary results in a delay of follicular development and puberty onset (Lara et al., 1990). Increased sympathetic innervation of the ovary has been shown to disrupt oestrous cyclicity and impair follicular maturation (Dissen et al., 2000; Dorfman et al., 2009). Sympathetic activity in the ovary, indicated by increased expression of nerve growth factor (NGF) and TH has implications in the origin of polycystic ovary (PCO) syndrome in animal models (Lara et al., 2000). Data from humans also implicate increased sympathetic activity in the ovary in the development of the syndrome (Greiner et al., 2005). Neonatal immune activation results in an increased expression of ovarian NGF receptor (p75NGFR) along with increased thickness of theca interna layer, which is directly innervated by sympathetic nerves, in adult rats (Wu et al., 2011). The current data indicate increased peripheral sympathetic activity immediately following LPS exposure, potentially contributing to the long-term changes in sexual maturation. It is of interest that in the present study we did not find any changes in TH phosphorylation at Ser40 in female rats, but found significant changes in TH phosphorylation at Ser31. Ser40 and Ser31 residues on TH are phosphorylated by specific protein kinases due to activation of different signalling pathways (Dunkley et al., 2004). Our results suggest that LPS causes activation of distinct signalling pathways in the adrenal gland of male and female rats.

HPA axis activity throughout development

Increased TH phosphorylation in the neonatal adrenals was not associated with HPA axis activity as no differences were found in plasma corticosterone levels on PND 5. We have previously shown an immediate effect of neonatal treatment on circulating corticosterone levels in males (Sominsky et al., 2012) or in both male and female rat pups (F.R. Walker et al., 2004; A.K. Walker et al., 2009, 2010, 2011). In rats, the HPA axis completes its maturation postnatally during the first week of life when circulating corticosterone levels are very low (Vázquez, 1998), which may account for the lack of significant differences at this time point. Nevertheless, increased basal levels of corticosterone were evident in LPS-treated females on PND 14, during adolescence, and in adulthood, indicative of a prolonged effect of neonatal immune challenge on the HPA axis functioning. The elevation in circulating corticosterone is most likely a consequence of impaired negative feedback due to glucocorticoid receptor downregulation, which has been reported to be associated with this model (Shanks et al., 1995).

The HPA and HPG axes co-regulate one another, and stress is known to influence reproduction (Rivier and Rivest, 1991; Tilbrook et al., 2000). Stress-induced alterations in the HPG axis is mediated primarily by the hypothalamus (affecting the release of GnRH) and the pituitary (affecting the release of gonadotrophins), while effects at the gonadal level are considered less influential (Tilbrook et al., 2000). However, adrenalectomy in prepubertal rats has been shown to delay the occurrence of vaginal opening and first oestrous while sham laparotomy produces opposite effects (Gorski-Firlit and Lawton, 1974). The mechanism considered responsible for these phenomena are sex steroids produced by the adrenal cortex (i.e., progesterone and oestrogens). Administration of corticosterone was also found to advance pubertal maturation in male rats (Biagini and Pich, 2002). As such, it has been proposed that while corticosterone may not have a direct effect on reproductive development, its interactions with gonadotrophins, such as LH and FSH are likely to facilitate early sexual maturation (Rivest, 1991).

HPG hormones

Neonatal LPS administration resulted in reduced FSH levels in adolescence, with no significant differences observed on the day of vaginal opening or in LH levels, although we have previously demonstrated reduced plasma LH concentrations in this model (Walker et al., 2011). Of significance is the finding that neonatal LPS exposure reduces FSH levels. FSH plays a particularly important role in follicular maturation. Pulsatile elevations in preovulatory circulating FSH levels promote cyclic recruitment and survival of antral follicles which exhibit enhanced FSH sensitivity. This leads to eventual ovulation of the selected dominant follicles (McGee and Hsueh, 2000). Reduced FSH levels during proestrous therefore places LPS-treated rats at risk of having decreased ovulatory capacity.

Ovarian development

We have previously reported that neonatal LPS challenge induces immediate and long-term morphological changes in the male gonads (Walker et al., 2011). In this study, assessment of ovarian morphology throughout development revealed morphological alterations in the ovary, indicated by a reduction in follicular numbers in females treated neonatally with LPS. Of importance is the reduction in primordial follicles on PND 14, accompanied by the decreased ovarian weight at this time. The finite pool of primordial follicles originates during foetal development in humans (Reynaud and Driancourt, 2000) and postnatally until PND 3 in rats (Rajah et al., 1992, Skinner, 2005). Following their formation, a small proportion of primordial follicles will initiate growth every day and develop into primary, preantral and then antral follicles (i.e. initial recruitment) (McLaughlin and McIver, 2009). While most undergo atretic degeneration, some of the follicles will develop into preovulatory follicles following puberty onset via stimulation of pulsatile release of gonadotrophins (McGee and Hsueh, 2000).

While gonadotrophins, and in particular FSH, have a critical role in the regulation of follicular maturation, their impact is likely to be exerted to a greater extent after the onset of puberty (McGee and Hsueh, 2000). The initial follicular growth and development in earlier stages are known to be largely coordinated by complex interactions between the oocyte, growth factors, cytokines and neurotrophins (Dissen et al., 2002; Skinner, 2005; Schindler et al., 2010). Exposure to LPS in this study coincided with the critical stage of primordial follicle formation and maturation. Administration of LPS was responsible for an immediate activation of the SNS, indicated by adrenal TH phosphorylation. Moreover, LPS exposure in neonates was previously shown to increase circulating levels of proinflammatory cytokines (F.R. Walker, 2009). Given the number of parameters affected by neonatal immune challenge, it is likely that the insult interfered with primordial follicle assembly and with their further development, evidenced by the reduction in primary and total number of follicles evident in LPS-treated females.

However, a more direct mechanism of action is possible. LPS is recognised by toll-like receptor 4 (TLR4) present on peripheral immune and endothelial cells, initiating cascade of responses by these cells (Elenkov et al., 2000; Johnson et al., 2008). TLR4 receptors are also expressed in ovarian granulosa cells. Recent findings based on in-vitro studies indicate that exposure to LPS can impair follicular growth and function, by alterations in follicular steroidogenesis (Herath et al., 2007). In addition, LPS-induced inflammation has been shown to directly impair oocyte meiotic competence (Bromfield and Sheldon, 2011). Therefore, although the impact of peripheral LPS on TLR4 expression in the ovary was not examined in this study, it is possible that such a direct effect on ovarian development exists.

In addition to a reduction in follicular numbers, a greater percentage of LPS-treated females exhibited a pattern of reproductive senescence by 12 months of age. Given that the size of the initial pool of primordial follicles is one of the factors determining the reproductive lifespan (Hirshfield, 1994), this finding further confirms the potentially deleterious effects of neonatal exposure to LPS on reproductive capacity in the female rat.

F2 generation study

Given the observed changes in pubertal development and gonadal morphology in neonatally LPS-treated females, we determined whether neonatal immune challenge compromises fertility. LPS treatment in the F1 generation did not affect fertility rates, but did appear to have increased rates of pups exhibiting extremely low weight and failure to thrive. All pups born to LPS-treated mothers had a reduced body weight on PND 5, and increased catch-up growth was observed in female offspring born to LPS-treated mothers, similar to the first generation of animals. F2 generation females of LPS-treated mothers also had increased prepubertal basal corticosterone levels on PND 14.

Our recent study provided evidence of transgenerational inheritance of anxiety-related behaviours and neuroendocrine perturbations induced by neonatal LPS administration (Walker et al., 2012). Moreover, it appeared that neonatal exposure to LPS altered maternal care and this effect was reversed by cross-fostering. Studies by Cameron et al. (2008a,b) have demonstrated the programming effects of maternal care on female reproductive development and functioning. Lower levels of maternal care were found to be associated with earlier onset of puberty, higher sexual receptivity and increased fecundity. Cross-fostering reversed this phenotypic variation, suggesting that differences in reproductive strategies reflect adaptation to a given environment, whereby in some environments rapid reproductive development and increased reproductive success may be driven by higher risk of mortality. Although maternal care was not assessed in the current study, it is plausible that poor maternal care may account for the impaired growth and development of the pups along with increased corticosterone levels in the female offspring. Interestingly, our previous findings indicate that neuroendocrine perturbations are transmitted along the maternal, but not paternal line which is consistent with the current findings (Walker et al., 2012).

Observation of pubertal markers in both male and female offspring revealed again a difference in females only, indicated by delayed emergence of the oestrous cycle. Although the pattern of alteration of pubertal onset is reversed in the F2 females, it is clearly altered, and it is yet to be determined whether this change is mediated through the reduction in maternal care, or via other pathways of non-genomic inheritance originated by neonatal LPS exposure in the first generation.

Conclusions

The current study indicates a profound and long-lasting effect of neonatal immune challenge on reproductive development in the female rat. Together with our previous findings, a broad picture of physiological and behavioural alterations arises. Neonatal LPS exposure alters metabolic, neuroendocrine and behavioural parameters throughout the lifespan, strongly affecting and determining reproductive development, in both male and female rats. However, sexually dimorphic outcomes are apparent in regards to the relative degree of the alterations observed, whereby wider-ranging effects occur in females exposed to LPS during neonatal life or when born to LPS-treated mothers. This is of particular importance given the major role of the female in governing reproduction (Beach, 1976) and given the association between advanced puberty and increased risk of adult disease (Chen et al., 2011; Giles et al., 2010). Finally, given the decline in fertility rates and the tendency to delay childbearing, there is a critical need to understand the factors, such as inflammation, contributing to human infertility.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Paper 5: Immune regulation of ovarian development: programming by neonatal immune challenge.

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Mathew S. Jobling	Assisted in experimental procedures	
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Deborah M Hodgson	Assisted in the experimental design and data interpretation Contributed reagents / materials/ analysis tools Provided intellectual contribution and critical input Revised the manuscript	

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Immune regulation of ovarian development: programming by neonatal immune challenge

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Neonatal immune challenge by administration of lipopolysaccharide (LPS) produces enduring alterations in the development and activity of neuroendocrine, immune and other physiological systems. We have recently reported that neonatal exposure to an immune challenge by administration of LPS results in altered reproductive development in the female Wistar rat. Specifically, LPS-treated animals exhibited diminished ovarian reserve and altered reproductive lifespan. In the current study, we examined the cellular mechanisms that lead to the previously documented impaired ovulation and reduced follicular pool. Rats were administered intraperitoneally either 0.05 mg/kg of LPS (*Salmonella Enteritidis*) or an equivalent volume of non-pyrogenic saline on postnatal days (PNDs) 3 and 5, and ovaries were obtained on PND 7. Microarray analysis revealed a significant upregulation in transcript expression (2-fold change; $p < 0.05$) for a substantial number of genes in the ovaries of LPS-treated animals, implicated in immune cell signaling, inflammatory responses, reproductive system development and disease. Several canonical pathways involved in immune recognition were affected by LPS treatment, such as nuclear factor-κB (NF-κB) activation and LPS-stimulated mitogen-activated protein kinase (MAPK) signaling. Quantitative Real-time PCR analysis supported the microarray results. Protein expression analysis of several components of the MAPK signaling pathway revealed a significant upregulation in the expression of Toll-like receptor 4 (TLR4) in the neonatal ovary of LPS-treated animals. These results indicate that neonatal immune challenge by administration of LPS has a direct effect on the ovary during the sensitive period of follicular formation. Given the pivotal role of inflammatory processes in the regulation of reproductive health, our findings suggest that early life immune activation via TLR signaling may have significant implications for the programming of ovarian development and fertility.

Keywords: LPS, TLR4, MAPK, neonatal, immune challenge, reproductive development

INTRODUCTION

Worldwide there is a trend for declining fertility. This is particularly apparent for the female population, whereby 10.9% of women suffer from impaired fertility and 6% are infertile (Martinez et al., 2012). Decreased female fecundity has been related to advanced maternal age but the increasing representation of young women in this category (Martinez et al., 2012), suggests a more complex epidemiology. Accumulating evidence points to the critical role of the early life environment in the establishment of reproductive health. Substantial epidemiological and experimental evidence indicates that adult physiological function and health status may have their origins in the early developmental period (Barker and Osmond, 1986; Barker et al., 1989; Barker, 1990). Plasticity during the perinatal period allows adequate adaptation of an organism to given environmental conditions and predicts later life functioning. While physiological plasticity holds a beneficial value for the developing organism, exposure to adverse environmental conditions during critical maturational periods, may result in alterations to the normal developmental trajectory, increasing susceptibility to physiological malfunction and pathology in later life. The process by which early life environment can have permanent effect on physiological systems has been described as *perinatal programming* (Welberg and Seckl, 2001; Davies and Norman, 2002).

One of the major physiological systems that undergoes development and maturation during foetal and early postnatal life is the immune system. Due to functional immaturity of the neonatal immune system in both animals and humans, there is increased susceptibility to infections and a lower response to immunogenic stimuli when compared to that of the adult (Vosters et al., 2010). The impaired immune responses, upon exposure to infectious agents, during the perinatal period have been associated with the decreased capacity of the neonate to develop mature protective T helper type 1 immune responses (De Wit et al., 2003). As such, low levels of proinflammatory cytokines have been detected in response to immune stimulation

by LPS and *Staphylococcus epidermidis* as documented in neonatal rats and mice, as well in human cord blood (Angelone et al., 2006; Hartel et al., 2008; Hodyl et al., 2008). The development of the immune system is well-established to be dependent on the immune, autonomic and endocrine signals that it receives early in life (Holladay and Smialowicz, 2000; Zakharova, 2009; Fagundes et al., 2012). This physiological programming of the immune system and its relationship to later life pathology has been associated with a number of long-term health outcomes mediated by inflammatory pathways including, a predisposition to asthma, allergies, autoimmune diseases, metabolic disorders, cardiovascular diseases, multiple sclerosis, and more (Zakharova, 2009; Fagundes et al., 2012), depending on the timing and the extent of early life immunogenic exposure.

Programming of the immune system has been investigated using various animal models. Experimentally-induced early life immune activation is commonly achieved by administration of lipopolysaccharide (LPS) [derived from *Salmonella Enteritidis* or *Escherichia (E.) coli*], the cell wall of Gram-negative bacteria, in rodents. LPS is recognized by the Toll-like receptor 4 (TLR4) that is expressed by several cell types, such as monocytes, macrophages, adipocytes as well as gonadal supporting cells (i.e., ovarian granulosa and testicular sertoli cells) (Medzhitov, 2001; Richards et al., 2008). The impact of neonatal LPS exposure has been examined on a variety of physiological and behavioral outcomes, such as adult immune responses (Boisse et al., 2004; Spencer et al., 2006; Walker et al., 2009b; Mouihate et al., 2010), metabolism (Walker et al., 2006; Iwasa et al., 2009a), brain morphology (Bilbo et al., 2008; Amath et al., 2012; Sominsky et al., 2012b), stress responsivity, and anxiety-like behaviors (Walker et al., 2009a, 2012; Sominsky et al., 2013).

The reciprocal relationship between the hypothalamic-pituitary-gonadal (HPG) axis and the immune system has an extensive impact on development and functioning of both systems, as well as on health outcomes. Oestrogen and androgen receptors are expressed on immune cells (Tanriverdi et al., 2003), and these in turn have been shown to populate all reproductive organs (Seamark et al., 1992). Recently, increasing attention has focused on the effect of neonatal immune challenge on reproductive development and functioning. Activation of the immune system during neonatal life has long-term consequences for reproductive functioning (Marchetti et al., 2000; Morale et al., 2003). Experimental evidence has indicated altered puberty onset, diminished HPG axis activity and impaired sexual behavior in both male and female rodents neonatally exposed to bacterial endotoxin, LPS, in particular on PNDs 3 and 5 (Iwasa et al., 2009b,c; Knox et al., 2009; Walker et al., 2011; Wu et al., 2011; Sominsky et al., 2012a), suggesting that these changes may be attributed to the critical window of reproductive development. Supporting this assertion, the critical period for programming of pubertal onset in the female rat has been determined to be before 7 days of age. LPS treatment on PNDs 3 and 5 has been shown to result in a significant delay in puberty, while LPS administered on PNDs 7 and 9 or PNDs 14 and 16 produced no such effect (Knox et al., 2009). Furthermore, neonatal LPS exposure has been demonstrated to aggravate LPS-induced suppression of LH pulse frequency in adulthood, when compared to neonatal saline-treated group (Li et al., 2007). Similarly, neonatal LPS-treated female rats exhibited prolonged oestrous cycle in response to LPS injection in adulthood, while no such response was observed in neonatal saline-treated animals (Iwasa et al., 2009c).

Neonatal LPS exposure has also been reported to produce alterations in gonadal morphology. Reduced gonocyte populations in neonatal testes, as well as increased epithelial disorganization and delayed spermatogenesis in adulthood were demonstrated in male rats neonatally exposed to LPS (Walker et al., 2011). Robust alterations in gonadal morphology in response to neonatal LPS challenge have also been documented in female animals. Diminished follicular reserve has been detected in the ovaries of LPS-treated female rats (Wu et al., 2011), with reduced population of primordial follicles being evident at 2 weeks of age, after LPS exposure on PNDs 3 and 5 (Sominsky et al., 2012a). This later finding corresponded with advanced senescence and poor pregnancy outcomes in LPS-treated females (Sominsky et al., 2012a), suggesting a prolonged and more robust effect of an immune challenge on female reproductive lifespan. Of particular relevance to the model of neonatal immune challenge, is that the initial follicular assembly and maturation are known to be dependent on local inflammatory agents, such as growth factors and cytokines (Dissen et al., 2002; Skinner, 2005; Schindler et al., 2010). Even after puberty, when ovarian function is largely governed by the HPG hormones (McGee and Hsueh, 2000), ovulation resembles an inflammatory process, as constituted by increased vasodilation and hyperaemia of ovarian follicles, and their ability to produce cytokines, chemokines, and prostaglandins (Espey, 1980; Richards et al., 2002). This concept is further strengthened by the abundance of macrophages, principal innate immune cells, in the ovary, contributing to the regulation of ovarian function (Wu et al., 2004). Furthermore, ovarian granulosa and cumulus cells exert several immune-related functions, including the expression of TLR4, which during ovulation respond to endogenous ligands, resulting in the release of pro-inflammatory cytokines, such as IL-6 (Richards et al., 2008).

While immune-regulation of ovarian function is crucial for normal fertility, any alteration in this delicate relationship may lead to ovarian dysfunction and subfertility. Common ovarian diseases, such as polycystic ovarian syndrome (PCOS) and endometriosis, have been associated with increased levels of follicular and serum pro-inflammatory cytokines, constitutive of chronic low grade inflammation (Wu et al., 2004). The direct impact of LPS exposure on ovarian function has been previously assessed *in-vitro*, and was reported to result in diminished follicular function (Herath et al., 2007) and impaired oocyte meiotic competence (Bromfield and Sheldon, 2011). No studies have investigated *in vivo* the mechanisms that underpin the effect of peripheral LPS exposure on ovarian development during the neonatal period. In the current study, we aimed to identify the ovarian pathways that lead to the previously documented impaired ovulation and reduced oocyte development, in the model of dual LPS exposure on PNDs 3 and 5 (Knox et al., 2009; Wu et al., 2011; Sominsky et al., 2012a). In order to characterize the cellular mechanisms that underpin the immediate effect of peripheral LPS exposure on PNDs 3 and 5 on ovarian

development, we examined its effect on the ovarian transcriptome on PND 7. Given that in the rat, the assembly of primordial follicles is not established until PND 3 (Rajah et al., 1992; Skinner, 2005), we propose that an immune challenge by administration of LPS at this time point may directly intervene with the formation and establishment of the finite follicular pool via activation of inflammatory pathways.

METHODS

ANIMALS AND NEONATAL TREATMENT

All animal experimental procedures were conducted with the approval of the University of Newcastle Animal Care and Ethics Committee (ACEC). Seven experimentally naive female Wistar rats were obtained from the University of Newcastle animal house and mated in the University of Newcastle Psychology vivarium. Animals were maintained under normal housing conditions at 21–22°C, under a 12 h light/dark regime. At birth (PND 1), whole litters were randomly allocated into either LPS (4 litters; litter size $M = 13$, $SD = 1.8$) or saline-treated conditions (3 litters; litter size $M = 15$, $SD = 0$). As documented previously (Walker et al., 2004, 2006, 2009a,b, 2010; Sominsky et al., 2012a,b), on PNDs 3 and 5 pups were briefly removed from their home cages, weighed and administered intraperitoneally with either LPS (Salmonella enterica, serotype enteritidis; Sigma-Aldrich Chemical Co., USA, dissolved in sterile pyrogen-free saline, 0.05 mg/kg) or an equivolume of saline (Livingstone International, Australia). No significant differences in neonatal weight were observed when assessed on PND 3, PND 5, and PND 7 in the female offspring. We have previously reported neonatal LPS treatment to produce variable effects in regards to the neonatal weight gain, inducing weight loss (Walker et al., 2004; Sominsky et al., 2012a), weight gain (Walker et al., 2011) or no significant change (Walker et al., 2009a). Litter size and male-to-female ratio were not significant covariates to this analysis, confirming that these factors did not contribute to the possible developmental effects of litter.

On PND 7, female pups were euthanized by rapid decapitation. Ovaries were collected, frozen on dry ice and stored at −80°C until further analysis. For microarray and qRT-PCR analyses, ovaries were obtained from 5 animals per treatment group (derived from 2 LPS and 2 saline-treated litters). For western blotting analysis, ovaries were obtained from 4 animals per treatment group (derived from 2 LPS and 1 saline-treated litters). An additional subset of ovarian samples was used for the exploratory immunohistochemical analysis. Ovaries were obtained from 3 LPS and 3 saline-treated animals (derived from 2 LPS and 2 saline-treated litters) on PND14. This later time point was chosen to ensure a complete structural representation of the neonatal ovary, as at this age the ovary has been shown to contain antral follicles, which are completely absent prior to 12 days of age (Carson and Smith, 1986).

RNA EXTRACTION

RNA was isolated from ovaries using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions, with slight modifications. Briefly, ovaries were homogenized in lysis buffer (RNeasy buffer RLT) using a basic plastic homogenizer. The homogenized RNA was then passed five times through a 20 gauge needle. Lysate was centrifuged at 13,000 rpm for 3 min at 25°C. The supernatant was collected, 350 µl 70% ETOH added and mixed. The solution was then transferred to an RNeasy spin column and placed in a 2 ml collection tube. Following 20 s centrifugation at 10,000 rpm the flow through was discarded. Seven hundred micro liters of wash buffer (RNeasy buffer RW1) was added to the RNeasy spin column, centrifuged for 20 s at 10,000 rpm, following which the flow through was discarded. This step was repeated twice with 500 µl of RNeasy buffer RPE. RNeasy spin column was placed in a new 1.5 ml collection tube, add 30 µl RNase-free H_2O were added directly to the column membrane and centrifuged for 1 min at 10,000 rpm to collect RNA. RNA concentrations were determined using spectrophotometer, NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE USA) (refer to Table S1 for RNA quality information). The extracted RNA was used for microarray analysis and for verification by qRT-PCR.

MICROARRAY ANALYSIS

Total RNA obtained from neonatal ovaries (1–2 µg) was submitted to the Australian Genome Research Facility (AGRF). Microarray analysis was performed using Agilent SurePrint G3 Rat GE 8x60K Array platform. Total RNA obtained from neonatal ovaries was quality ascertained on the Agilent Bioanalyser 2100 using the NanoChip protocol (Table S1). A total of 1 µg was labeled using the Ambion Total Prep RNA amplification kit (Applied Biosystems—AMIL1791). The quantity of amplified product was ascertained using the Agilent Bioanalyser 2100 using the NanoChip protocol. For the Agilent 8x60k chip format, 600 ng of amplified cRNA was used for the Cy3 coupling process using a ULS labeling kit (Kreatech EA-006). After coupling the dye, the reaction was cleaned using the KREApure columns in the kit. The Cy3 labeled cRNA samples were then fragmented using the Ambion fragmentation kit (Applied Biosystems—AM8740). The fragmented cRNA was quality checked on the Agilent Bioanalyser 2100 using the NanoChip protocol. The fragmented Cy3 labeled cRNA was then prepared for hybridization to the Agilent 8x60k Rat array using the GX hybridization kit (Agilent—5188-5242) where each hybridization reaction had a final volume of 50 µl. The Agilent hybridization chambers were prepared according to manufacturer's instructions. Each sample was loaded onto the Agilent hybridization gaskets slide which is placed into a hybridization chamber. The 8x60k Rat array was carefully lowered onto the gasket to create a sealed array for each sample. The hybridization chambers were then placed in a rotating hybridization oven at 65°C for 17 h. After hybridization, the chip was washed using the appropriate protocols as outlined in the Agilent manual using the GX washing buffers (Agilent—5188-5327). Upon completion of the washing, the chips were then scanned in the Agilent DNA Microarray Scanner (High Resolution). The scanner operating software, Agilent Scan Control, converts the signal on the array into a TIFF file which can be used for subsequent analysis. The experiment was conducted in three biological replicates, which consisted of ovarian tissue obtained from five animals per treatment group. Differentially expressed genes were determined by a twofold difference and a significance level of $p < 0.05$. Ingenuity Pathways Analysis (Ingenuity Systems,

Redwood City, CA) software was used for further analysis to identify canonical signaling pathways affected by LPS treatment. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE46436 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46436).

REVERSE TRANSCRIPTION AND QUANTITATIVE REAL-TIME PCR

Reverse transcription was performed with 1 μg of total RNA using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA, USA), according to manufacturers' instructions, by combining the components [5× VILO reaction mix (4 μL), 10× SuperScript enzyme mix (2 μL), RNA sample (up to 1 μg), diethyl pyrocarbonate (DEPEC) treated water (to 20 μl)]. Tube contents were gently mixed, incubated at 25°C for 10 min, and then incubated at 42°C for 60 min. The reaction was terminated at 85°C at 5 min. qRT-PCR was performed using SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) on a 7500 RT-PCR Fast instrument (Applied Biosystems, Foster City, CA, USA). Primer sequences were designed using the Primer3 software (http://frodo.wi.mit.edu). Sequence specificity was tested using the Basic Local Alignment Search Tool at NCBI (Altschul et al., 1997) and primer pairs were obtained from Invitrogen (custom DNA oligonucleotide synthesis service). PCR efficiency for each pair of primers was determined by a standard curve method, where $C(t)$ values for serial dilutions are related to the logarithm of the dilution factor, and the slope is a measure for reaction efficiency. Information regarding primer sequences, including product size and PCR efficiency, is listed in Table S2. The 25 μl PCR mixture consisted of 12.5 μl SYBR Green PCR Master Mix, 9.5 μl water and 2 μl of each primer was added to 1 μl of the cDNA template (10 ng/ml). All reactions were performed in duplicate under the following conditions: 95°C for 20 s and 40 cycles of 95°C for 3 s and 60°C for 30 s. Melting curve was determined under the following conditions: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s. The data were normalized to an endogenous control, β-actin. A relative quantitative measure of the target gene expression compared with β-actin mRNA was analysed using the 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.

PROTEIN EXTRACTION AND WESTERN BLOTTING

Protein was extracted using RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8, 1% Triton X-100), supplemented with a protease inhibitor cocktail (ProteCEASE, G-Biosciences St. Louis, MO, USA). Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Quantified aliquots were separated by electrophoresis and transferred onto a nitrocellulose Hybond C-Extra membrane (Amersham) prior to blocking for 1 h in 5% skim milk powder in TBST (0.1% Tween-20), as previously described (Sobinoff et al., 2010). After blocking, the membranes were incubated overnight at 4°C with goat polyclonal TLR4 (L-14) (diluted 1:200; Santa Cruz, sc-16240); goat polyclonal PKCβ1 (C-16) (diluted 1:500; Santa Cruz, sc-209); or rabbit polyclonal anti-JNK1 (diluted 1:2000; Abcam, ab10664) primary antibodies. Following washing and incubation with horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz, sc-2020) at a 1:3000 dilution or goat anti-rabbit secondary antibody (Millipore DC03L) at a 1:2000 dilution for 1 h at room temperature, proteins were visualized using an ECL Detection Kit (GE Healthcare Life Sciences) according to manufacturer's instructions. The membranes were then stripped of primary and secondary antibodies in Western Re-Probe (G-Biosciences) according to the manufacturer's instructions at room temperature for 1 h and reprobed using a mouse monoclonal anti-α-tubulin (Sigma, T5168) as a loading control. The densities of TLR4, PKCβ1 and JNK1 bands were visualized using ImageQuant LAS 4000 imager (GE Healthcare Life Sciences). The protein bands were measured using MultiGauge V3.0 (Fuji, Stamford, CT, USA) software and were expressed as the ratio to α-tubulin.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was used to localize TLR4, PKCβ1, and JNK1 proteins in the neonatal ovaries and for proliferating cell nuclear antigen (PCNA) detection of primordial follicle activation and growth. Ovaries were obtained from LPS and saline-treated animals on PND14. Tissue was fixed in Bouin's fixative solution for 4 h, washed four times in 70% ethanol dehydrated, embedded in paraffin and sectioned at 4 μm. Slides were dewaxed in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 3 × 3 min in Na Citrate buffer (10 mM Sodium Citrate, pH 6) for TLR4, PKCβ1, and PCNA, and in Tris buffer (50 mM, pH 10.6) for JNK1. Slides were allowed to cool before being blocked in 3% BSA/Tris-buffered saline (TBS) for 1 h at room temperature. Sections were incubated overnight at 4°C with anti-TLR4 (diluted 1:100; Santa Cruz, sc-16240); anti-PKCβ1 (1:200, Santa Cruz, sc-209) and mouse monoclonal anti-PCNA (diluted 1:200; Merck KGaA, NA03T). For detection of anti-JNK1 (diluted 1:500, Abcam, ab10664) sections were incubated for 2 h at room temperature. The above primary antibodies were used for Western blotting prior to their application for the immunohistochemical analysis, ascertaining antibodies specificity (Kurien et al., 2011). Slides were then washed in TBS containing 0.1% Triton X-100, and incubated with the appropriate fluorescent-conjugated secondary antibodies (Alexa Fluor 594 donkey anti-goat IgG; Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG; Invitrogen; 1:200 dilution). Sections were then counterstained with 4#-6-diamidino-2-phenylindole (DAPI) for 2 min, mounted in Mowiol (4-88, Sigma), viewed using an Axio Imager A1 fluorescent microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) under fluorescent optics and pictures taken using an Olympus DP70 microscope camera (Olympus America, Center Valley, PA).

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE dUTP NICK END LABELING (TUNEL) ANALYSIS

Bouin's fixed sections were dewaxed and rehydrated as mentioned above. Slides were then boiled in Tris buffer (50 mM, pH 10.6) for 20 min and treated with 20 μg/ml Proteinase K for 15 min in

a humidified chamber. TUNEL analysis was then performed as previously described (Sobinoff et al., 2010, 2011, 2012) using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics Pty Ltd), according to the manufacturer's instructions. Slides were then counterstained with DAPI for 5 min, mounted in Mowiol, and viewed using an Axio Imager A1 fluorescent microscope (Carl Zeiss) under fluorescent optics and pictures taken using an Olympus DP70 microscope camera (Olympus).

DATA ANALYSIS

Statistical analyses were conducted using the Statistical Package for the Social Sciences for Windows, Version 18 (SPSS Inc.). Data were analysed using analyses of variances (ANOVA) design. The significance level was set at $p \leq 0.05$.

RESULTS

IMPACT OF LPS TREATMENT ON THE NEONATAL OVARIAN TRANSCRIPTOME

Administration of LPS on PNDs 3 and 5 caused a significant change in ovarian gene expression for 712 genes, representing 2.4% of the total number of genes present on the array, with significant upregulation of 710 genes (**Figure 1**; Table S3). Further functional analysis using Ingenuity Pathway Analysis software identified these significantly altered genes as components of several molecular networks implicated in cell signaling, immune cell trafficking, inflammatory response as well as reproductive development and disease (see **Table 1**). These results suggest a robust impact of peripheral immune challenge on a variety of molecular mechanisms regulating ovarian development and function. No distinct function has been assigned to the two significantly downregulated genes, *SBK2* and *TEDDM1*.

CANONICAL PATHWAYS SIGNIFICANTLY UPREGULATED BY NEONATAL LPS EXPOSURE

Further functional assessment of differentially regulated genes revealed several canonical pathways involved primarily in immune recognition and inflammation were activated in response to neonatal treatment [e.g., virus entry via endocytic pathways; nuclear factor-kappaB (NF-κB) activation; mitogen-activated protein kinase (MAPK) signaling; pattern-recognition receptors signaling; MSP-RON signaling, whereby macrophage-stimulating protein (MSP), acts through the RON receptor tyrosine kinase, and plays a regulatory role in the inflammatory response], suggesting dysregulation of inflammatory processes in response to peripheral LPS exposure may occur also locally, in the ovary (**Figure 2**; Table S4). **Figures A1–A7** provide a graphical representation of the significantly altered genes in the top canonical pathways.

qRT-PCR CONFIRMATION OF MICROARRAY ANALYSIS

LPS-stimulated MAPK signaling pathway was chosen for validation of microarray results, due to its specific relevance to the current model of LPS challenge. qRT-PCR analysis confirmed significant upregulation of 8 out of 9 genes that were identified as

Table 1 | Functional categorization of genes that were upregulated by neonatal LPS exposure.

Molecular and cellular function	Upregulated genes
Inflammatory response	50
Immune cell trafficking	29
Inflammatory disease	54
Organismal development	38
Developmental disorder	39
Reproductive system development and function	29
Reproductive system disease	28
DNA replication, recombination, and repair	34
Cell morphology	32

Significantly upregulated genes were analysed using Ingenuity Pathway Analysis software for molecular and cellular functions. Only those genes exhibiting a greater than two-fold change in expression were analysed ($p < 0.05$). Some genes are listed in multiple functional groups.

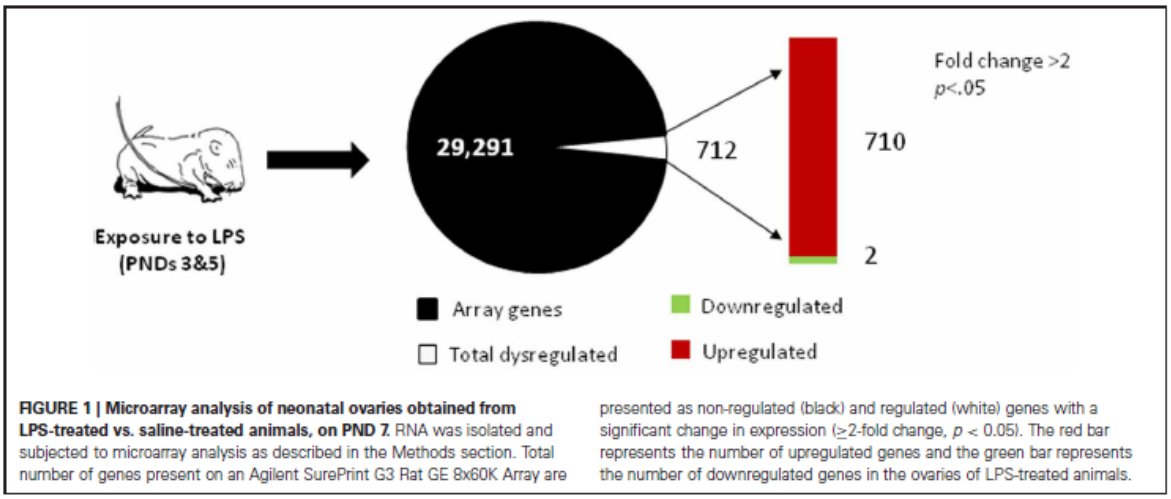

FIGURE 1 | Microarray analysis of neonatal ovaries obtained from LPS-treated vs. saline-treated animals, on PND 7. RNA was isolated and subjected to microarray analysis as described in the Methods section. Total number of genes present on an Agilent SurePrint G3 Rat GE 8x60K Array are presented as non-regulated (black) and regulated (white) genes with a significant change in expression (≥2-fold change, $p < 0.05$). The red bar represents the number of upregulated genes and the green bar represents the number of downregulated genes in the ovaries of LPS-treated animals.

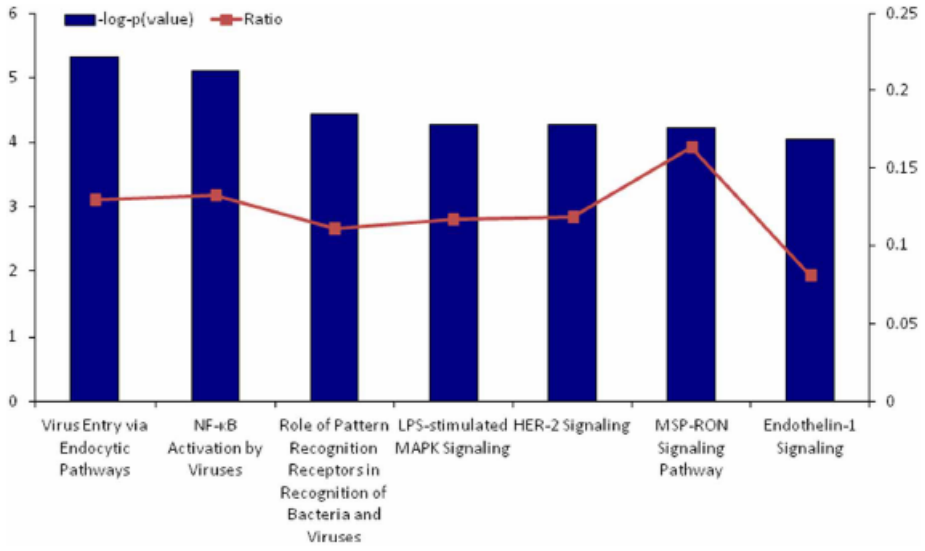

FIGURE 2 | Top canonical pathways that were significantly upregulated by neonatal LPS treatment, as identified by Ingenuity Pathway Analysis. The significance of association between upregulated genes and the canonical pathway was assessed using a right-tailed Fisher's exact test to calculate a *p*-value determining the probability that the association is explained by chance alone (blue bars, y-axis). Ratios defining the proportion of upregulating genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (line graph, z-axis).

components of this pathway, in the ovaries of LPS-treated animals (**Table 2**). Of these genes, upregulation of *MAPK8/JNK1* has been previously associated with oocyte dysfunction (Sobinoff et al., 2010); *PKC*β has been reported to be involved in oocyte activation (Carbone and Tatone, 2009); *PIK3C2A* has been implicated in proliferation of ovarian theca-interstitial cells (Kwintkiewicz et al., 2006); *PIK3R1* and *PKCZ* have been associated with the incidence of PCOS (Diamanti-Kandarakis, 2008; Kim et al., 2009; Rivero et al., 2012); *PKD3* has been implicated in organogenesis (Ellwanger et al., 2008); *RELA* and *RRAS2* have been associated with ovarian tumorigenesis (Chan et al., 1994; Niesporek et al., 2007; Fan and Richards, 2010); and finally, increased expression of *TLR4* (receptor for LPS) has been demonstrated to underpin impaired follicular growth and function (Herath et al., 2007).

PROTEIN ANALYSIS BY WESTERN BLOTTING

To further validate the microarray results, immunoblotting analysis was performed to examine protein expression of three significantly upregulated genes (*TLR4*; *PKC*β; MAPK8/JNK1) in the LPS-stimulated MAPK signaling pathway. Densitometry analysis of protein expression in the ovaries of LPS-treated rats, as compared to α-tubulin, revealed a significant increase of two protein bands associated with TLR4 expression [$F_{(1,\ 5)} = 10.2$; $F_{(1,\ 5)} = 17.06$, $p < 0.05$ for both]. No significant differences were evident in the expression of PKCβ1, however, a trend was observed in increased expression of JNK1 protein in the ovaries of LPS-treated animals [$F_{(1,\ 5)} = 4.12$, $p = 0.09$; see **Figure 3B**]. Representative immunoblots are presented in **Figure 3A**.

IMMUNOHISTOCHEMICAL ANALYSIS

To detect localization of TLR4, PKCβ1 JNK1, in the neonatal ovary, observational immunohistochemical staining was performed in the ovaries of LPS and saline-treated animals, obtained on PND14. As demonstrated in **Figures 4A,B**, TLR4 immunolabeling was detected in the oocytes. In the ovaries of LPS-treated animals TLR4 was also detected in ovarian blood vessels, suggesting activation of local immune cells. PKCβ1 staining was evident in the ovarian blood vessels, to a greater extent in the ovaries of LPS-treated animals (**Figures 4C,D**). JNK1 immunolabeling was detected in theca cells, in both treated and control samples (**Figures 4E,F**).

LPS and saline-treated ovaries were also probed for markers of follicular activation and cell death. PCNA staining was equally detected in the granulosa cells and the oocytes of primordial, primary and secondary follicles in both treatment and saline control group. TUNEL staining did not reveal any significant apoptosis in either of the experimental groups (data not shown).

DISCUSSION

In this study we assessed, for the first time, the effect of immune challenge by peripheral administration of LPS on the ovarian transcriptome in neonatal rats. Microarray analysis revealed specific activation of immune-related pathways, such as virus entry via endocytosis, NF-κB activation by viruses, role of pattern recognition receptors in recognition of bacteria and viruses, LPS-stimulated MAPK pathway and MSP-RON signaling pathway. Exposure to LPS upregulated expression of genes implicated in cell signaling, immune cell trafficking, inflammatory response as

Table 2 | qRT-PCR confirmation of microarray results for the components of LPS-stimulated MAPK signaling pathway, upregulated in the ovaries of LPS-treated animals.

Gene symbol	Gene name	Summary of function	Fold change
MAPK8/JNK1	Mitogen-activated protein kinase/c-Jun N-Terminal Protein Kinase 1	Increased expression is associated with xenobiotic-induced oocyte dysfunction (Sobinoff et al., 2010)	2.99 ± 0.04*
PIK3C2A	Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit 2A	Involved in modulation of proliferation of ovarian mesenchyme (Kwintkiewicz et al., 2006)	2.74 ± 0.04*
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1	Involved in the insulin receptor signaling pathway; associated in the pathogenesis of PCOS (Kim et al., 2009)	3.32 + 0.04*
PKCβ	Protein kinase C, beta	Involved in oocyte activation. Reduced expression of *PKCβ1* is associated with ageing (Carbone and Tatone, 2009)	3.71 ± 0.04*
PKCZ	Protein Kinase C, Zeta	Involved in the insulin pathway; decreased expression in PCOS patients (Diamanti-Kandarakis, 2008; Rivero et al., 2012)	−1.61 ± 0.06
PKD3	Protein kinase, D3	Implicated in organogenesis (Ellwanger et al., 2008)	5.11 ± 0.04*
RELA	v-rel reticuloendotheliosis viral oncogene homolog A	Downregulated in women with preeclampsia (Hansson et al., 2006). Upregulated in ovarian carcinoma, leading to overexpression of COX-2 (Niesporek et al., 2007)	3.72 ± 0.04*
RRAS2	Related RAS viral oncogene homolog 2	Oncogene, defects in RRAS2 increase susceptibility to ovarian cancer (Chan et al., 1994; Fan and Richards, 2010)	7.45 ± 0.07*
TLR4	Toll-like receptor 4	Expressed by granulosa cells, mediating the effect of bacterial infection on impaired ovarian follicle growth and function (Herath et al., 2007)	2.35 ± 0.1*

*Fold changes are reported as mean ± SEM. *$p < 0.05$.*

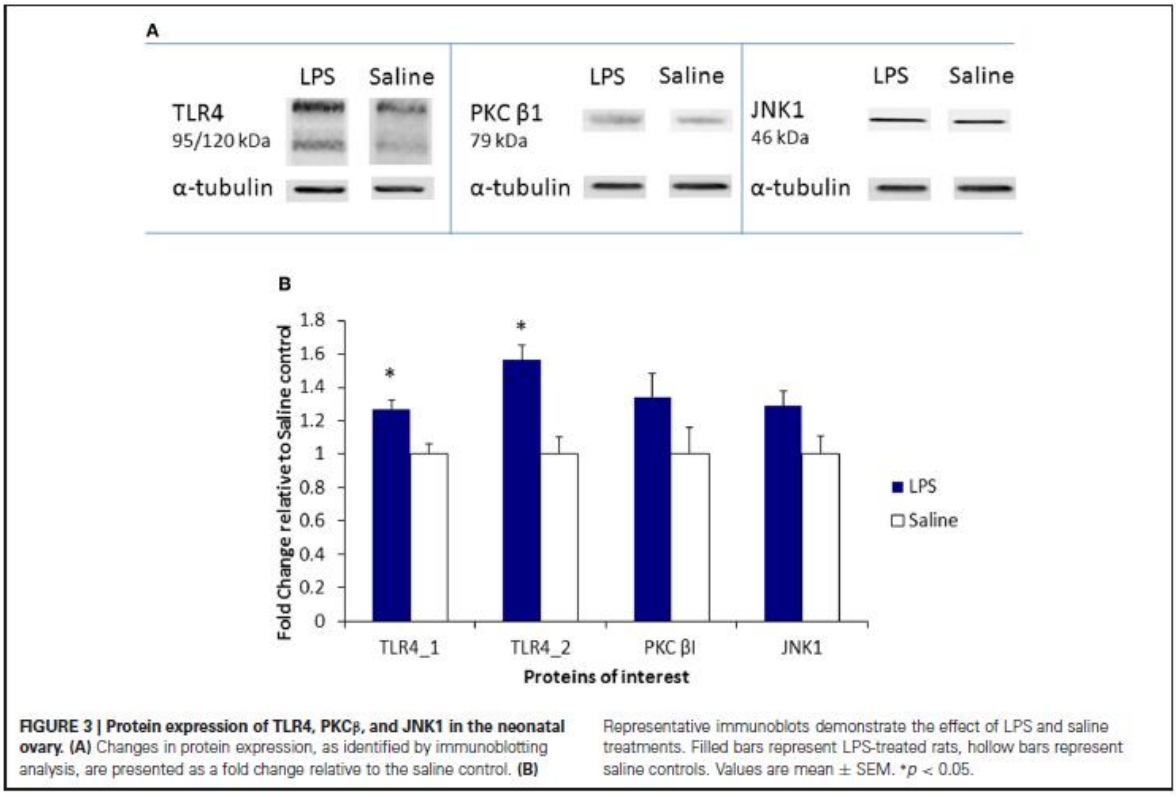

FIGURE 3 | Protein expression of TLR4, PKCβ, and JNK1 in the neonatal ovary. (A) Changes in protein expression, as identified by immunoblotting analysis, are presented as a fold change relative to the saline control. **(B)** Representative immunoblots demonstrate the effect of LPS and saline treatments. Filled bars represent LPS-treated rats, hollow bars represent saline controls. Values are mean ± SEM. *$p < 0.05$.

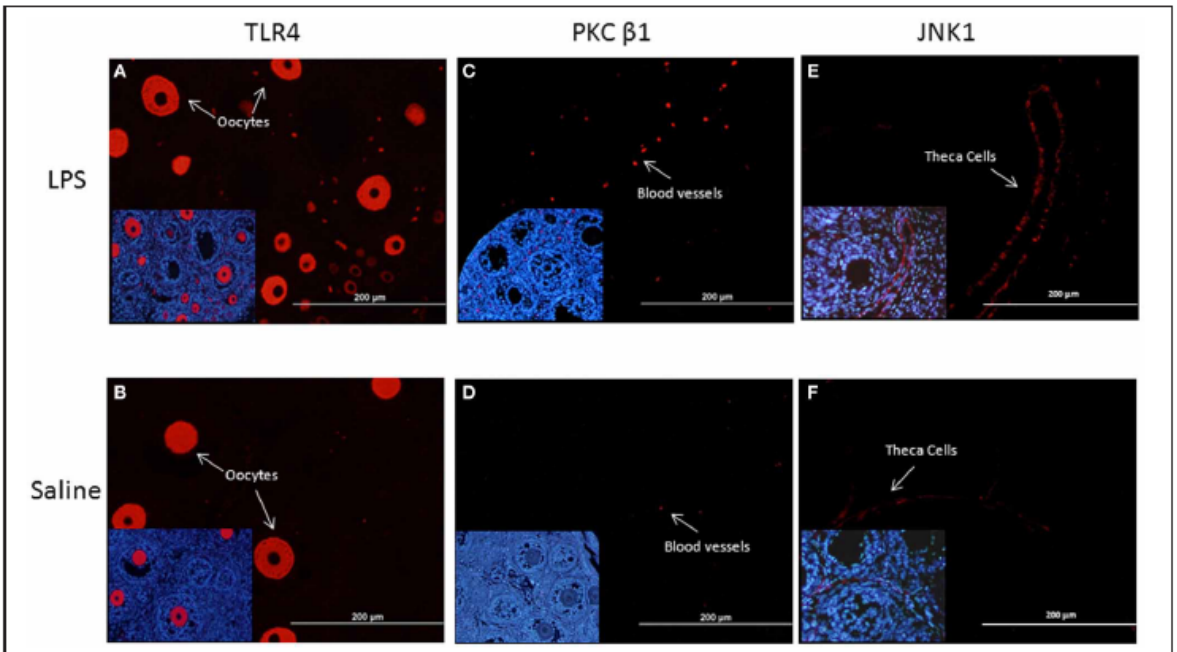

FIGURE 4 | Fluorescent immunolocalization of TLR4, PKCβ, and JNK1 protein in the ovaries of LPS and saline-treated animals. (A–B) Represent TLR4 immunolabeling detected in the oocytes; **(C–D)** Represent PKCβ1 immunolabeling in ovarian blood vessels; **(E–F)** Represent JNK1 immunolabeling in theca cells. Insert at higher magnification is located at the bottom left corner of each representative micrograph. Blue staining (DAPI) represents nuclear staining; red staining represents specific staining for the protein of interest.

well as reproductive development and disease, suggesting dysregulation of inflammatory processes in response to peripheral LPS exposure may occur also locally in the ovary, contributing directly to programming of reproductive health.

Given the specific relevance of LPS-stimulated MAPK pathway to the model of neonatal LPS exposure, microarray results were confirmed and elaborated on the altered components of this pathway. Activation of MAPK signaling by LPS binding of TLR4 results in transcriptional regulation and synthesis of inflammatory agents, such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β) and IL-6, as well as nitric oxide synthase-2 (NOS2) and cyclooxygenase-2 (COX2) (Nagano et al., 2002; Kawai and Akira, 2007). Other downstream targets include phosphoinositide-3-kinase (PI3K), which stimulation enhances activity of protein kinase C (PKC) (Frey et al., 2006). In the current study, several subunits of these genes, identified with the MAPK pathway, were upregulated in the ovaries of LPS-treated animals.

PI3K has been reported to induce activation of the serine/threonine kinase Akt, also known as protein kinase B (PKB), which in turn stimulates the mammalian target of rapamycin (mTOR) protein. Overactivation of PI3K/Akt/mTOR signaling pathway, induced by exposure to carcinogens and xenobiotics, has been implicated in premature activation of primordial follicles, leading to early follicular atresia (McLaughlin and Sobinoff, 2010; Sobinoff et al., 2010, 2011). Activation of PI3K/Akt has also been associated with the development of PCOS (Lima et al., 2006). While in the current study immunohistological assessment for markers of follicular activation and atresia revealed no significant alterations were induced by neonatal LPS exposure, our previous findings have indicated diminished population of primordial follicles in the late neonatal period in the ovaries of LPS-treated females (Sominsky et al., 2012a). These data suggest that apoptotic processes may occur early on, during the acute response to the LPS immune challenge, hindering follicular development.

The current model of neonatal LPS administration induces an acute pro-inflammatory response which involves the release of pro-inflammatory cytokines (i.e., TNF-α, IL-6, IL-1β), and this response typically resolves within 3–6 h from the time of drug administration, followed then by an anti-inflammatory release of glucocorticoids (Walker et al., 2004, 2009b). This brief activation of the immune response has been recently shown to impact on the development of the immune, reproductive, endocrine, metabolic, nervous and other physiological systems (Walker et al., 2010; Wu et al., 2011; Amath et al., 2012; Sominsky et al., 2012a, 2013). In the rat, the primordial follicle pool undergoes its final stages of formation and assembly postnatally, continuing till approximately day 3 after birth (Rajah et al., 1992; Skinner, 2005). This process is considered to be gonadotropin independent, supported and guided by growth factors and cytokines, one of which is the proinflammatory cytokine TNF-α. Previous research has demonstrated that ovarian TNF-α in the neonatal rat has an important role in determining the finite size of the primordial follicle pool.

It has been demonstrated *in vitro* that TNF-α can decrease the number of oocytes and primordial follicles, via induction of apoptosis (Morrison and Marcinkiewicz, 2002). The formation of primordial follicles is then followed by the initial event of follicular development—transition to the primary follicle stage (Fortune et al., 2000; Skinner, 2005). As opposed to the later stages of follicular development, the transition from primordial to primary stage is hormone independent (Skinner, 2005). Exposure to LPS in adulthood has been also shown to induce follicular atresia *in vivo* in mice (Bromfield and Sheldon, 2013) and rats (Besnard et al., 2001) and *in vitro* in cattle (Bromfield and Sheldon, 2013). Interestingly, LPS-induced atresia is particularly detrimental to the population of primordial follicles (Bromfield and Sheldon, 2013), indicating that depletion of the primordial follicle pool during early development would subsequently impact the ovarian reserve and result in impaired fertility in later life. To our knowledge, no studies have assessed the gene expression of ovarian inflammatory pathways which were examined in the current study. While it is likely that similar changes would be evident in response to adult LPS exposure, the impact of such changes during early development, when the ovary has not yet morphologically and functionally developed, has significantly stronger implications for the growing follicle populations. It is therefore plausible to suggest that the neonatal LPS treatment instantly interferes with the delicate process of primordial follicle pool assembly and the subsequent activation of follicular development, through stimulation of an acute pro-inflammatory response. While this might be a transient perturbation, it ultimately leads to long lasting alterations in the size of the follicular pool, as observed in previous studies (Wu et al., 2011; Sominsky et al., 2012a). Even though no evidence of primordial follicle activation or apoptosis was evidenced in the current study, these processes might have occurred at an earlier time-point. Therefore, further investigation is required to clarify the time-course of apoptotic processes leading to the diminished follicular reserve reported in later life following neonatal exposure to LPS.

Activation and growth of primordial follicles are stimulated by several signaling pathways, including the MAPK and PKC (Jin et al., 2005; Du et al., 2012). The expression of several PKC isoforms (PKC α, β, δ, and ζ) was previously identified in the rat ovary (Cutler et al., 1994). In the neonatal rat ovary, MAPK, and PKC signaling cascade is involved in maturation of primordial follicles (Du et al., 2012). In the current study increased gene expression of MAPK8 and PKCβ in the ovaries of LPS-treated animals was determined by the microarray analysis and further confirmed by qRT-PCR. No significant changes in the expression of MAPK8 and PKCβ1 proteins were evident at the same time-point. However, significant increase in protein expression of TLR4 was found in the LPS-treated ovaries, and was associated with the increased mRNA levels of this gene.

TLR4, pathogen-associated molecular pattern recognition receptor, is expressed by innate immune and tissue specific cells, and recognizes bacterial molecules (e.g., LPS) (Medzhitov, 2001). While TLR4 activation is critical for generation of both innate and adaptive immune responses, its inappropriate activation can be harmful (Peroval et al., 2013). TLR4s are present in the ovary and expressed by ovarian surface epithelial cells, granulosa/cumulus cells (Herath et al., 2007; Liu et al., 2008; Richards et al., 2008), as well as by ovarian macrophages (Zhou et al., 2009), and play an essential role in regulation of fertility, through the support of ovulation and sperm capacitation (Liu et al., 2008; Shimada et al., 2008). Ovarian TLR4 signaling is normally induced by endogenous ligands, one of such is hyaluronan (HA)-rich matrix (Richards et al., 2008). Its synthesis during the ovulatory process is initiated by the surge of luteinising hormone (LH). HA-rich matrix is then recognized by cumulus cells, inducing inflammation and expression of innate immune-related genes, leading to the release of prostaglandins, TNF-α, IL-6 and other cytokines, and chemokines (Liu et al., 2008; Richards et al., 2008). These inflammatory agents activate chemokine receptors present on sperm, inducing sperm capacitation and motility (Shimada et al., 2008). Even though regulated production of cytokines is essential for ovulation and successful fertilization, dysregulation of cytokine production can impair fertility (Richards et al., 2008). In human patients, the existence of endometriosis was found to be associated with increased levels of IL-6 in circulation and in the follicular fluid, and is proposed to be related to the impaired follicular development and decreased oestradiol production, which results in infertility (Garrido et al., 2000; Pellicer et al., 2000). Activation of ovarian TLR4 by LPS has been studied in various animal and *in vitro* models. For instance, impaired antral follicle growth and function, as well as suppressed oestradiol production were reported in response to uterine *E. coli* infection, intravenous infusion with LPS and following exposure of granulosa cells to LPS *in vitro*, in cattle and sheep (Battaglia et al., 2000; Herath et al., 2007; Sheldon et al., 2009). A recent study in adult mice has demonstrated that LPS-induced atresia of primordial follicles is mediated via TLR4, since no follicle atresia was evident in *TLR4*-deficient mice (Bromfield and Sheldon, 2013).

The long-term effect of neonatal LPS exposure on TLR4 expression has been previously assessed in adult rats. Increased TLR4 mRNA expression was evident in the spleen and liver, two primary organs responsible for the synthesis of pro-inflammatory cytokines (Tracey, 2002), of neonatally-treated animals (Mouihate et al., 2010). In addition, increased expression of COX-2, the principal target of TLR4 activation, in the liver of LPS-treated animals was associated with a transient rise in circulating prostaglandin and increased activation of the HPA axis, in response to adult LPS exposure (Mouihate et al., 2010). These outcomes suggest that early life exposure to LPS has a persistent programming effect on the neuroimmune axis and this occurs via activation of TLR4. The current findings report that postnatal LPS challenge results in increased mRNA and protein expression of TLR4 in the neonatal ovary. In addition to its role in the LPS-stimulated MAPK pathway, TLR4 was associated with a number of inflammatory pathways activated in response to the neonatal treatment, as revealed by the canonical pathways analysis (i.e., role of pattern recognition receptors, MSP-RON and NF-κB signaling pathways). Given the important regulatory role of TLR4 in reproductive function, activation of the receptor and the subsequent pro-inflammatory cascade in early life are most likely to have significant effects on ovarian development. Imbalance in the internal ovarian milieu may potentially interrupt the delicate process of follicular formation and growth,

compromising reproductive capacity. Importantly, the process of primordial follicle assembly and development is highly conserved between mammals, and similar regulation of this process is considered to occur in different species, including humans (Skinner, 2005). Thus, while further studies are required to elucidate the immune mechanisms involved in the impaired ovarian development and functioning, our results importantly suggest that exposure to an immune challenge in early life, resulting in activation of TLR-related inflammatory pathways, may have significant consequences for programming of reproductive health in later life. Moreover, given that alterations in TLR4 expression are associated with pathological outcomes of common bacterial infections, such as *Escherichia coli* and *Chlamydia trachomatis*, including impaired fertility (Herath et al., 2009; Laisk et al., 2010), the current findings provide a valuable insight into the link between early life infection and fertility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Neuroendocrine_Science/10.3389/fnins.2013.00100/abstract

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APPENDIX

A graphical representation of the top canonical pathways that were significantly upregulated by neonatal LPS treatment, as identified by Ingenuity Pathway Analysis. Red shading indicates up-regulation of the gene. No color indicates genes that form the pathway and have no change.

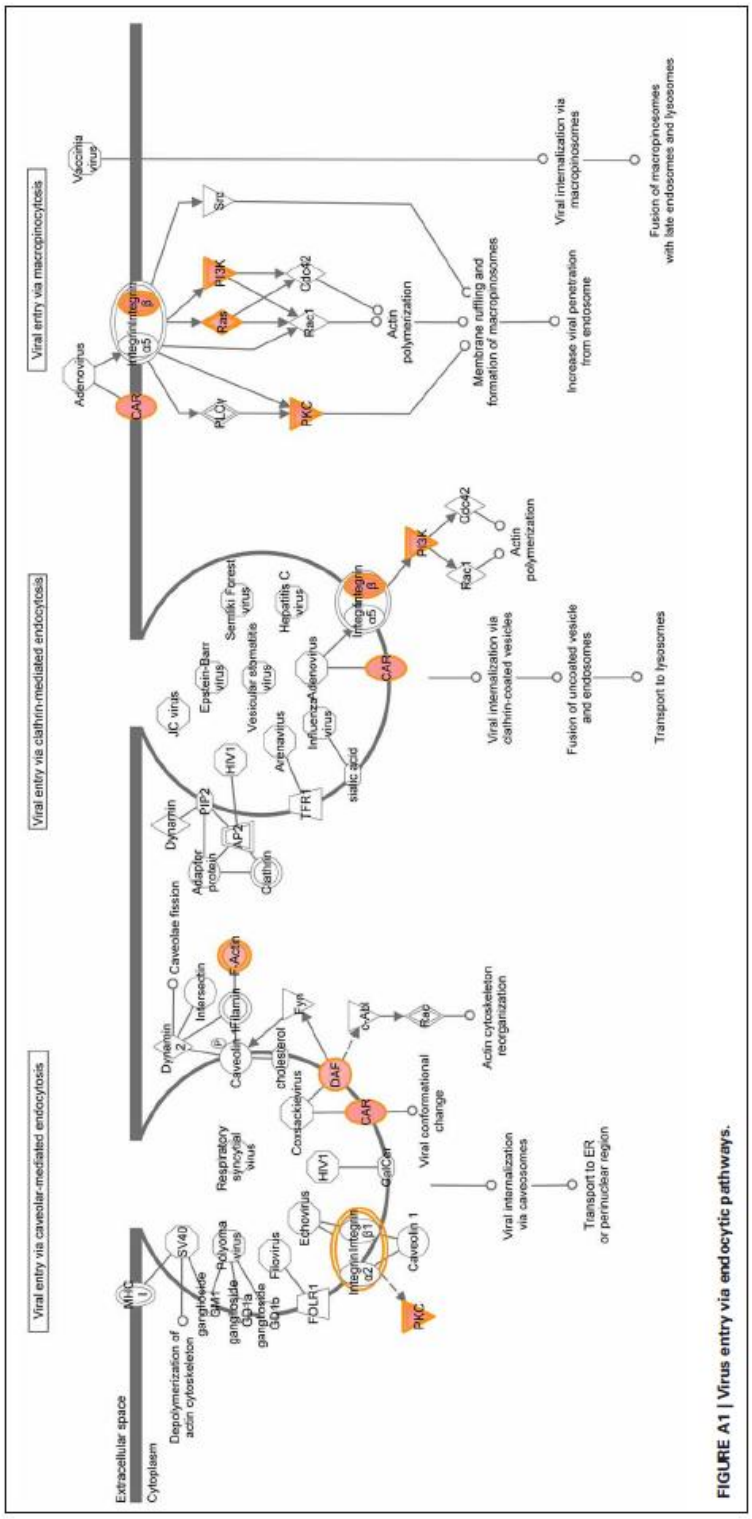


FIGURE A1 | Virus entry via endocytic pathways.

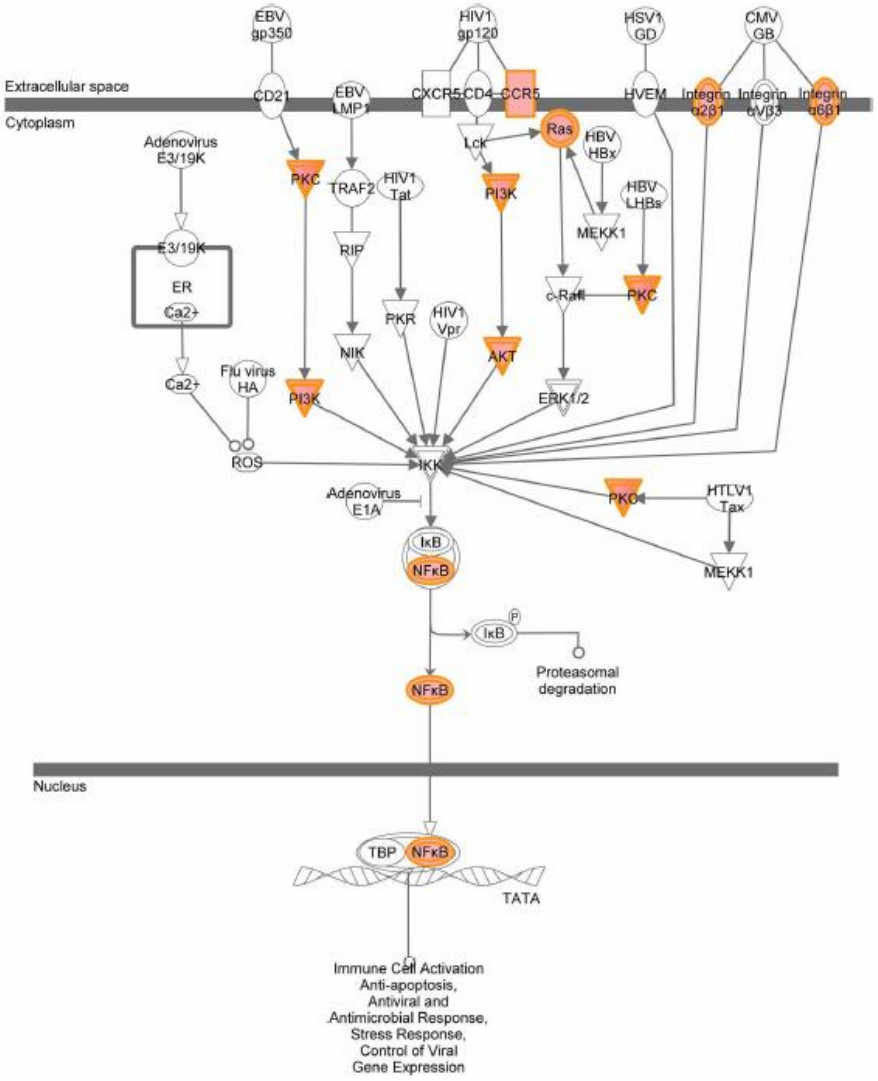


FIGURE A2 | NF-κB activation by viruses.

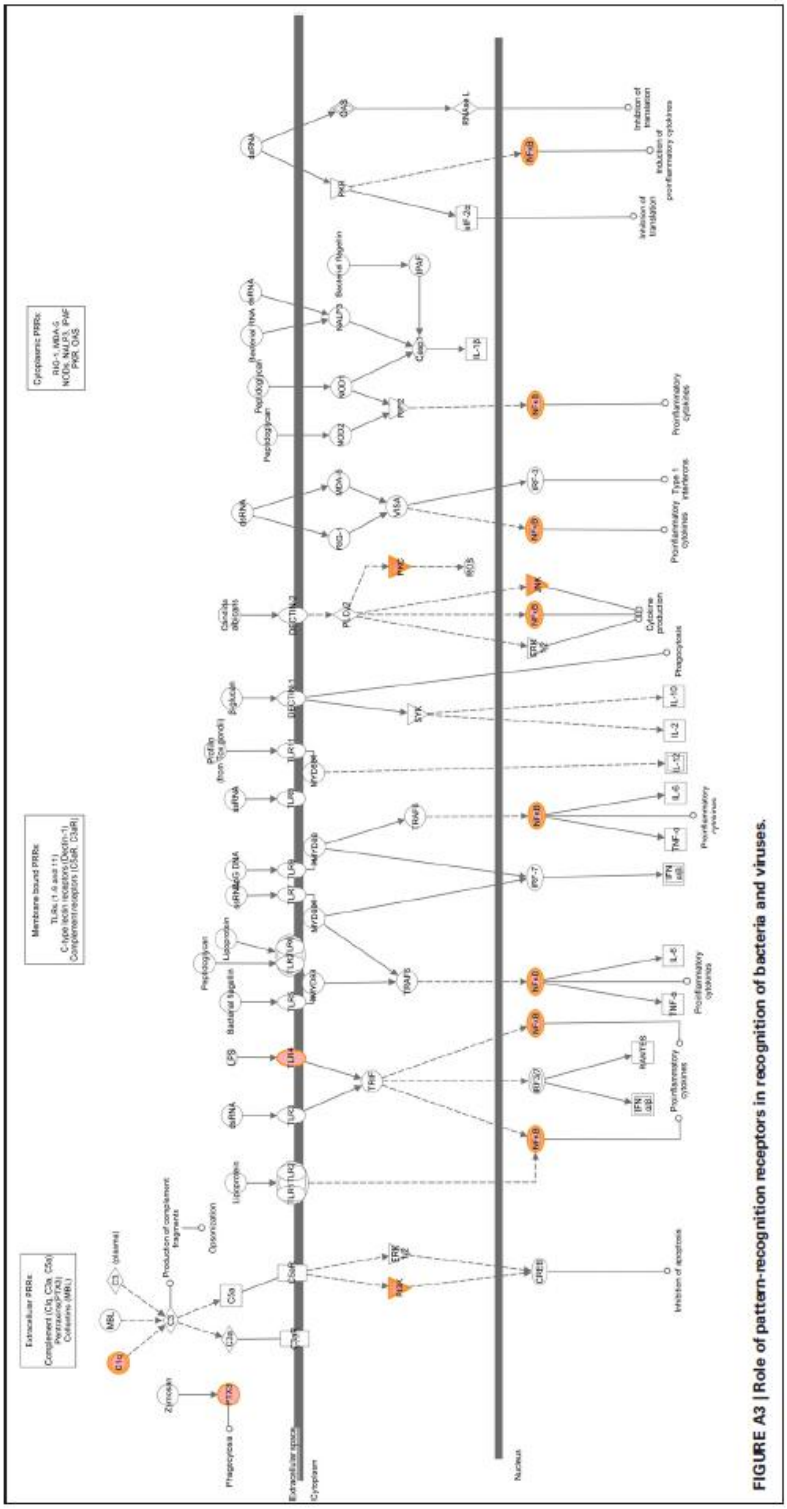


FIGURE A3 | Role of pattern-recognition receptors in recognition of bacteria and viruses.

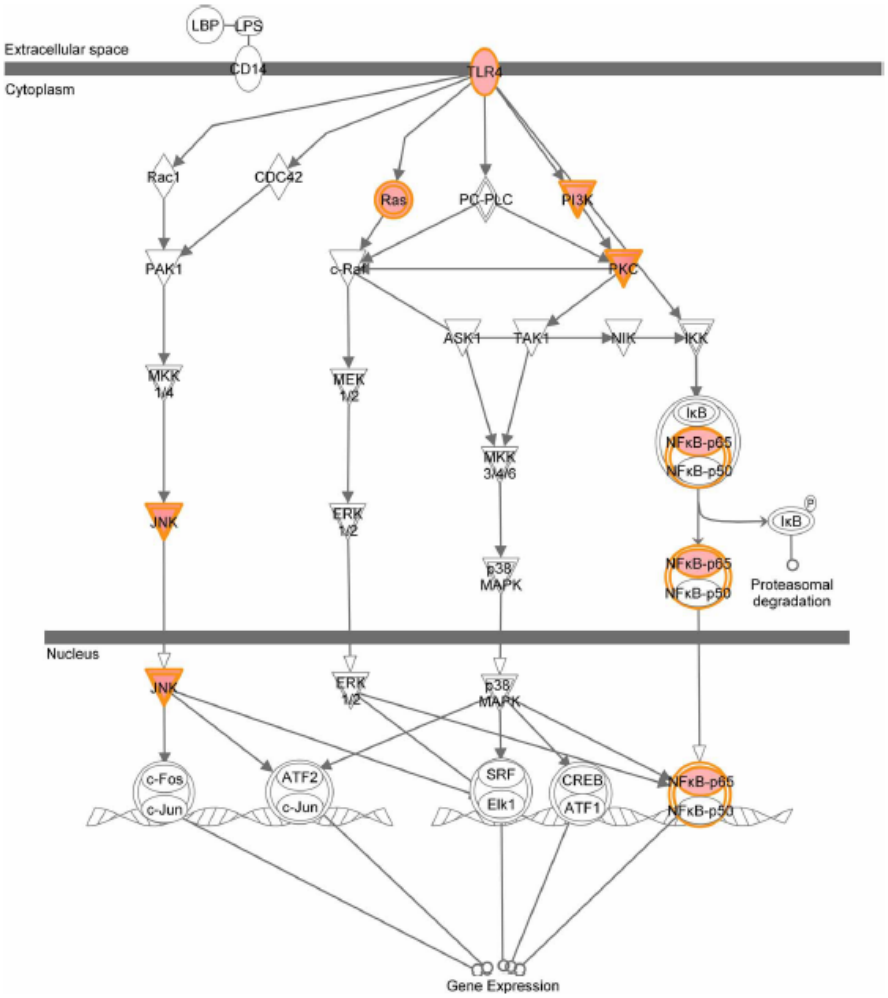


FIGURE A4 | LPS-stimulated MAPK signaling.

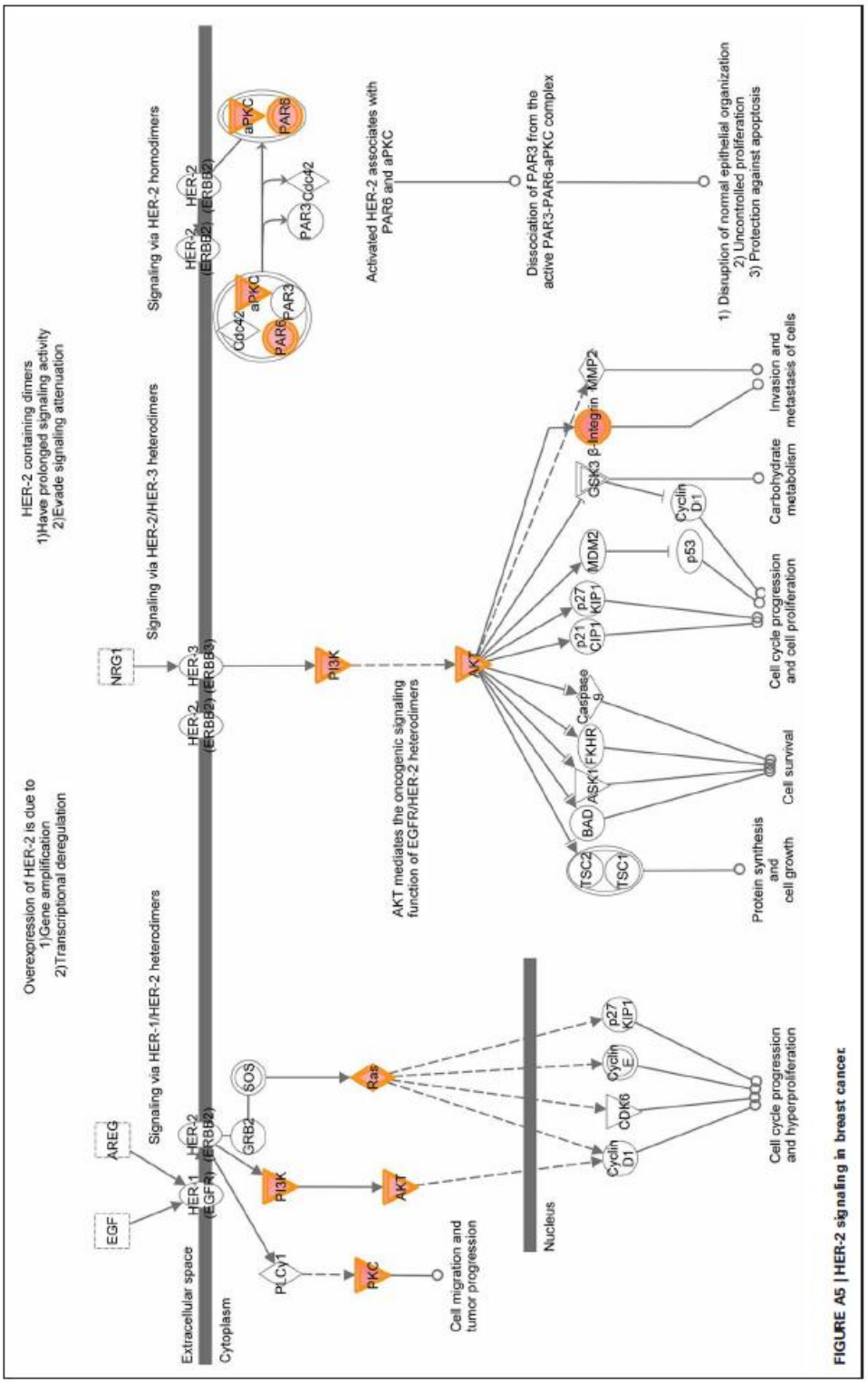


FIGURE A5 | HER-2 signaling in breast cancer.

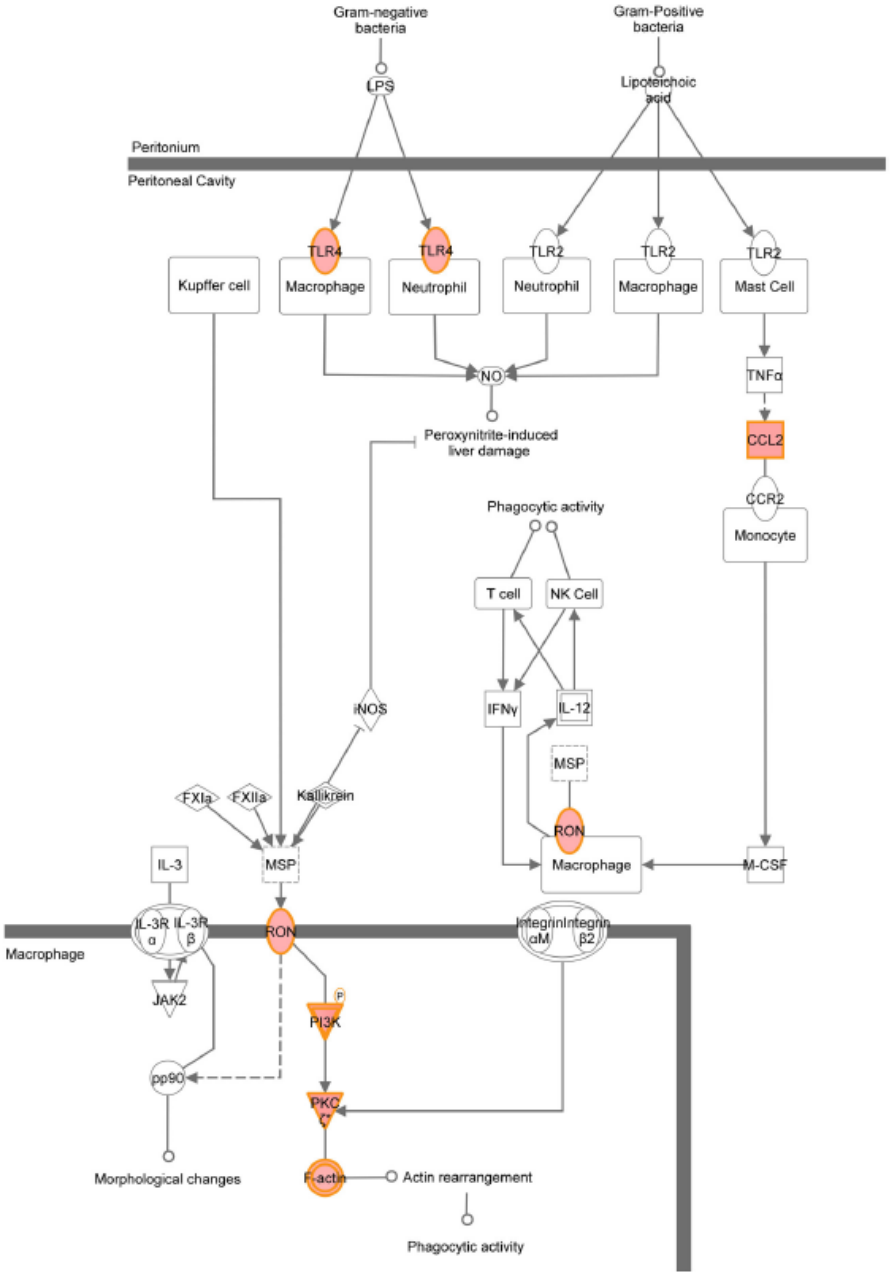


FIGURE A6 | MSP-RON signaling pathway.

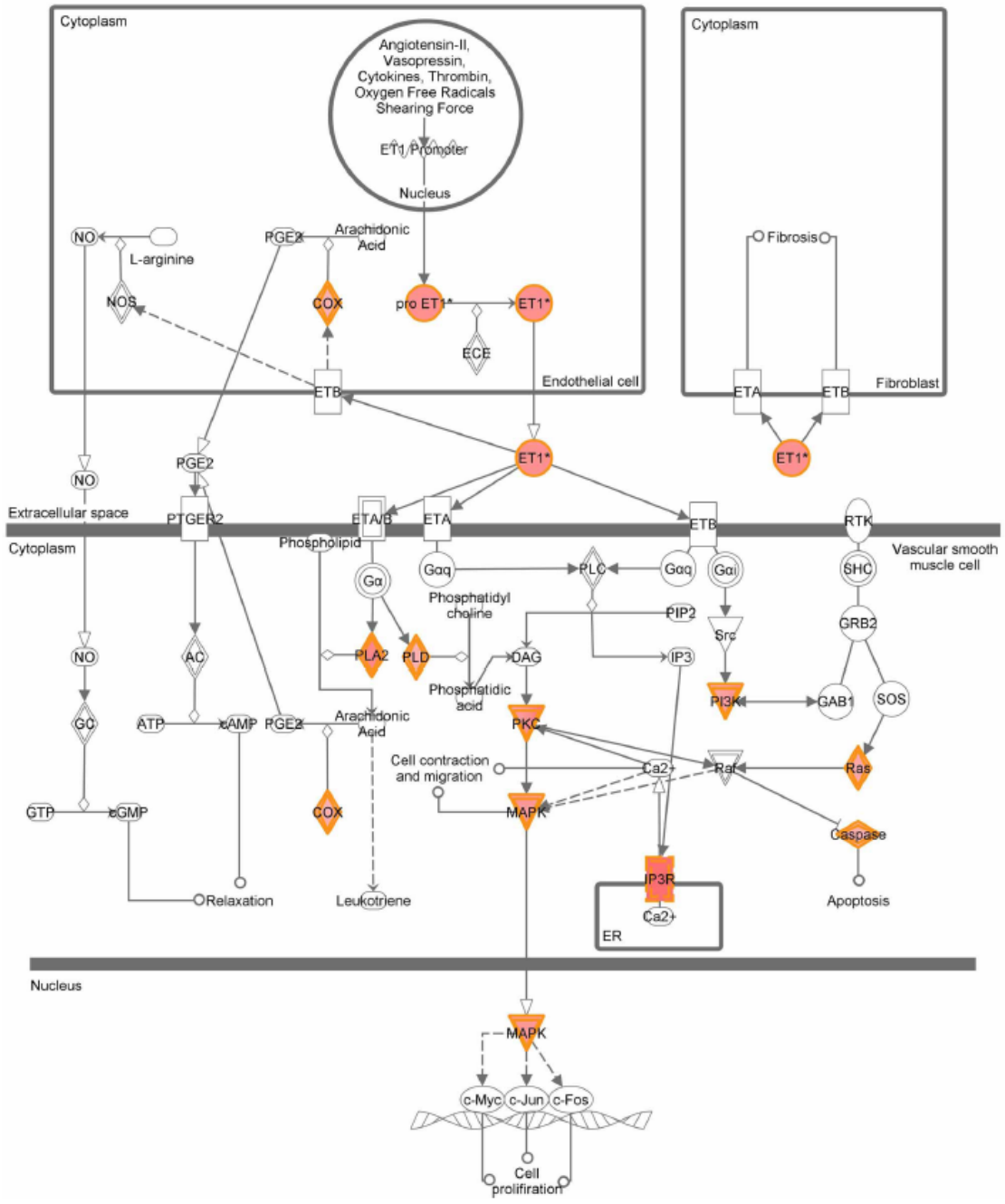


FIGURE A7 | Endothelin-1 signaling.

Discussion

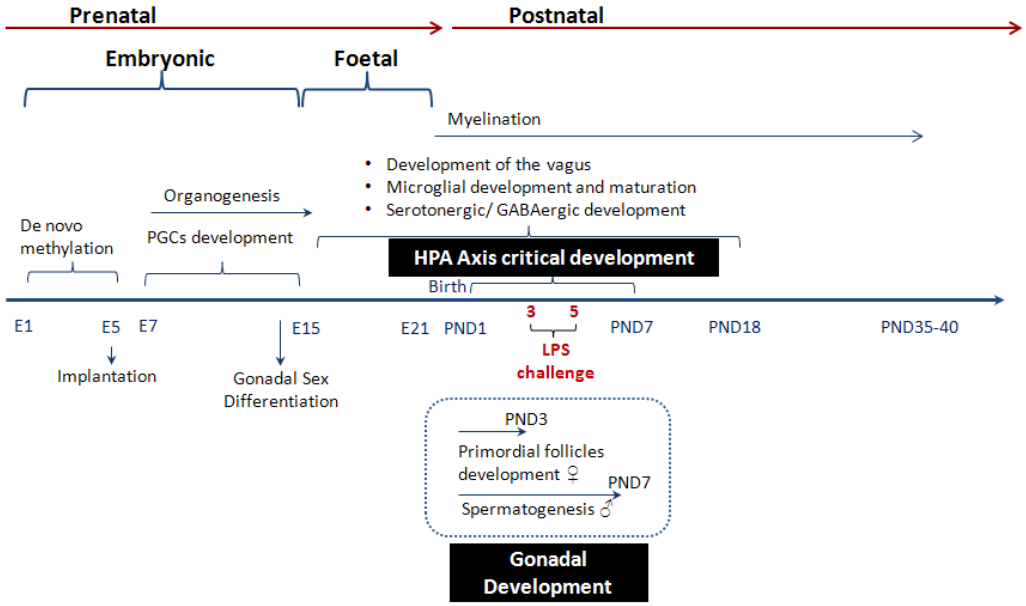
1. General Discussion

The neonatal period is a critical window, during which the developing organism is acutely sensitive to environmental impacts. This increased susceptibility is due to the higher degree of developmental plasticity which occurs during this period. Environmental stressors experienced in early life vary in their ability to produce different functional outcomes according to the developmental stage of the organism at the time of exposure. The cause for this variability is the stage-specific order of critical periods of development for different physiological systems. Moreover, the timing of critical periods of development relative to birth is species-dependent; nevertheless, the sequence of these periods is highly conserved and comparable across species. In the rat, early postnatal life is the critical period of development for many brain regions including those that regulate emotionality and behaviour, as well as neuroendocrine activity (Clancy et al., 2001; Clancy et al., 2007; Edwards and Burnham, 2001). It is also a sensitive period of gonadal development (Barakat et al., 2012; Skinner, 2005). Thus, the impact of environmental perturbations during this time can have wide-ranging implications for the developmental trajectory of these systems. Even subtle alterations to the functional set points of developing systems may initiate a cascade of effects which may not be detected immediately but lead to greater propensities towards pathology later in life.

This thesis is primarily concerned with the influence that the early life microbial environment exerts on the developing organism. The papers which comprise this thesis have all employed a dual LPS exposure model in the Wistar rat, whereby animals were administered 0.05mg/kg LPS intraperitoneally on PNDs 3 and 5. This model has been used by other laboratories to study the programming effects of neonatal immune challenge on

neuroendocrine and behavioural indices. Given that the particular time point at which LPS exposure occurs is critical to the development and maturation of the HPA axis (M Vázquez, 1998) (see Figure 7), our laboratory and others have most consistently demonstrated that neonatal LPS exposure is associated with altered stress responsivity and a predisposition to anxiety-like behaviours. This thesis further explores the anxiety-like phenotype, by examining neural pathways underlying this phenomenon. In addition, the data presented in this thesis adds a novel insight into the long term effects of neonatal LPS administration on the development of other systems, such as the ANS, as well as on gonadal physiology. As illustrated in Figure 7, the timing of LPS exposure coincides not only with a critical period of the HPA axis development, but also with important stages of gonadal development in both male and female rats. As reviewed in the introduction of this thesis, initial stages of ovarian as well as testicular development are known to be gonadotropin-independent and largely regulated by immune factors (i.e cytokines and growth factors), expression of which is typically altered following an immune challenge with LPS. We have therefore hypothesized that exposure to an early life immune challenge may be an important predictor of gonadal development and function in later life. This chapter considers the theoretical implications of these findings and their significance in an evolutionary context.

Figure 7: The prenatal and postnatal development of the rat. LPS challenge on PNDs 3 and 5 coincides with a critical period of the HPA axis development. It is also an important stage of gonadal development. In the rat ovary, primordial follicles are formed by PND3. In the testes, gonocytes resume mitosis and differentiate into spermatogonia during the first week of life.



2. Activation of neural pathways by neonatal LPS challenge

The first two papers presented in this thesis (Sominsky et al., 2013a; Sominsky et al., 2012b) explored the primary neuroimmune and neuroendocrine mechanisms believed to contribute to the long term changes in behaviour following neonatal LPS exposure, as previously reported (Walker et al., 2009; Walker et al., 2008; Walker et al., 2004b). The link between central immune regulation and behavioural and neuroendocrine output has become increasingly accepted over recent years. Bidirectional communication between the immune system and the brain allows the activated immune cells to produce not only physiological, but also cognitive and behavioural changes that serve to alleviate inflammation, and promote restoration of homeostasis and recovery from the acute inflammatory response (Maier and Watkins, 1998). Collectively these changes are often classified as *sickness behaviours*, which typically are organised strategies, critical to host survival (Dantzer and Kelley, 2007). It has

been hypothesised however, that inappropriate continuation of sickness behaviour may pose a risk to mental health, inducing certain psychopathological consequences (Dantzer et al., 2008). This is particularly pertinent to individuals with increased stress vulnerability, which can be a consequence of early life adverse experiences (Brent and Silverstein, 2013). Increasing evidence has indicated that infectious/inflammatory exposures during perinatal life are not only a significant contributor to acute brain injury in both neonates and adults, but also affects brain development in the long term (Hagberg et al., 2012). For this reason, implications of neonatal immune activation for the developing organism, and in particular brain mechanisms responsible for activation and resolution of sickness responses have been the focus of recent interest.

Microglia, the immune cells of the brain, which are in constant communication with the peripheral immune system and play an important role in neural circuitry formation during perinatal development, have drawn increasing attention. In the foetal and neonatal brain, microglia perform functions critical to neural development, such as processes associated with neuronal proliferation and differentiation, as well as phagocytosis of apoptotic neurons or cellular debris, contributing to synaptic pruning (Harry and Kraft, 2013). A growing body of evidence indicates excessive microglia activation in the developing brain of patients with neurodevelopmental disorders, such as autism spectrum disorders (Morgan et al., 2010; Suzuki et al., 2013), suggesting that developmental neuroinflammation actively contributes to the disease process. The effects of systemic inflammation on microglial expression in the developing brain and the subsequent behavioural outcomes have been studied using animal models. For instance, systemic exposure to live infection with *E. coli* on PND4 in rats has been shown to induce a strong microglial response, which persisted into adulthood (Bland et al., 2010b), and was associated with impairments in memory in learning (Bilbo et al., 2005a; Bilbo et al., 2005b). Moreover, administration of *E. coli* in neonatal mice has been shown to

result in hyperactive behaviour and increased reactivity of microglia following a secondary immune challenge by administration of LPS (Lieblein et al., 2012). Treatment with live *E. coli* infection or LPS are two common models of neonatal immune activation. These models however, produce specific inflammatory profiles within the neonatal brain. While neonatal infection with *E. coli* on PND4 in the rat induces a robust activation of neonatal immune system within the hippocampus via an IL-1-mediated pathway, administration of LPS at this time point results in a significantly broader immune response within the same region of the brain (Schwarz and Bilbo, 2011). Moreover, previous findings from our laboratory indicate increased predisposition to anxiety-related behaviours observed in adult rats neonatally exposed to LPS (Walker et al., 2009; Walker et al., 2008; Walker et al., 2004b). Since the first week of life in the rat is characterised by a marked increase in the number of activated microglia (Dalmau et al., 1998), we were interested to investigate the microglial profile in the model of dual neonatal LPS administration. Importantly, changes in microglial activity in adult animals were studied in association with anxiety-like behaviours, given the implication of microglial dysregulation in affective disorders. Thus, the first paper in this thesis (Sominsky et al., 2012b) examined the long-term effect of neonatal immune challenge with LPS on hippocampal and amygdaloid microglial activation. Increased microglial immunostaining was observed in the CA1 and the dentate gyrus areas of the hippocampus in adult animals, neonatally treated with LPS. This increase corresponded with increased anxiety-like behaviours, demonstrated in the same animals. No such differences were observed in the amygdala, suggestive of site-specific susceptibility in the limbic regions to microglial activation via neonatal LPS exposure.

The observed regional specificity in microglial activation may highlight the differences in the timing of the LPS challenge in relation to CNS development. In the current study, exposure to LPS occurred during the critical period of development for the HPA axis,

of which the hippocampus plays a fundamental role (Anisman et al., 1998), and early life LPS challenge is known to induce enhanced HPA axis responsivity (Mouihate et al., 2010). The long term effect of neonatal LPS exposure on hippocampal microglial activation may therefore reflect a greater susceptibility of this major HPA axis regulatory region. Moreover, our laboratory has previously demonstrated that neonatal LPS challenge results in elevated proinflammatory cytokine levels in the hippocampus of adult animals (Walker et al., 2010). Microglia cells are the primary producers of cytokines in the brain and increasing evidence suggests strong association between microglia-driven immune dysregulation and the pathophysiology of anxiety. Therefore, our findings of increased activation of microglia and a concurrent increase in anxiety-like behaviours, as presented in Paper 1 (Sominsky et al., 2012b), alongside the previously reported increased central cytokine levels (Walker et al., 2010) provide potential support for neuroimmune regulation of the HPA axis and stress-related behaviours.

A growing body of evidence suggests that the role of microglia in behavioural regulation is mediated via neuroendocrine factors, which interact with microglia to produce inflammation-dependent neuropsychiatric conditions. Specifically, rat microglia have been shown to express functional CRHR1 (Wang et al., 2002), and CRH binding to CRHR1 resulted in proliferation of microglia and release of the proinflammatory cytokine TNF-α (Wang et al., 2003). Moreover, the stress-induced elevation of glucocorticoids has been demonstrated to activate microglia in rats (Sugama et al., 2013; Tynan et al., 2010). Thus increased stress responsivity, which ultimately results in increased release of CRH and glucocorticoids may modulate microglial activity, inducing a release of proinflammatory mediators, and leading to behavioural dysregulation and a range of psychopathological symptoms, such as anxiety, fear and depression (Kato et al., 2013). Therefore, in order to elucidate the central neuroendocrine-related mechanisms in our model of neonatal LPS

exposure, the second paper in this thesis (Sominsky et al., 2013a) examined the expression of gene transcripts involved in the regulation of endocrine and autonomic stress responsivity, such as CRH, CRHR1, CRH binding protein (CRHBP), GR, MR as well as GABA-A receptor subunit alpha-2 (GABA-Arα2). These genes, which are also implicated in the aetiology of anxiety, were examined in the limbic regions. Regional-specific variability in the expression of CRH regulatory genes (CRHR1 and CRHBP) was observed, with downregulation of these transcripts in the prefrontal cortex (PFC) and the hypothalamus, but upregulation in the hippocampus of LPS-treated animals, emphasising once again potential site-specific susceptibility of limbic structures to the impacts of neonatal immune activation. Supporting the behavioural anxiety-like phenotype of adult animals neonatally exposed to LPS, a significant reduction in mRNA levels of GABA-A receptor subunit alpha-2 (GABA-Arα2), receptor subtype that is primarily responsible for the anxiolytic effects of benzodiazepines, was found in the PFC of LPS-treated animals. In addition, increased expression of GR mRNA levels was evident in the hippocampus of LPS-treated rats. Neonatal LPS treatment has been previously reported to decrease GR density and binding in limbic regions attenuating the negative-feedback effects of glucocorticoids on the HPA axis (Shanks et al., 1995), while our data suggests an opposite trend. It should be noted however, that the current findings reported in Paper 2 in this thesis (Sominsky et al., 2013a) present a different property of GR levels (e.g. mRNA levels). Additionally, consistent with these current findings, increased GR mRNA levels and no changes in MR mRNA were also found in the hippocampus of juvenile mice, neonatally exposed to LPS on PNDs 3 and 5 (Amath et al., 2012), suggesting an altered GR/MR balance, rather than a change in GR expression per se, might be responsible for the elevated emotional arousal experienced by animals neonatally treated with LPS.

Collectively, Paper 1 (Sominsky et al., 2012b) and Paper 2 (Sominsky et al., 2013a) demonstrate that neonatal LPS exposure produces long term alterations in microglial activity as well as in the expression of neuroendocrine-regulatory genes. Importantly, these neural changes that were detected in the limbic regions (i.e. hippocampus, hypothalamus and PFC) coincided with elevated anxiety-like behaviours, thus providing supportive evidence for perinatal programming of neuroimmune-neuroendocrine interactions by neonatal immune challenge and the potential for altered behavioural outcomes that could be indicative of psychopathology, such as anxiety.

3. Programming of the HPA axis and ANS by neonatal LPS challenge

Given our findings showing neonatal LPS exposure programs long term alterations of gene expression in the limbic structures, which are involved in regulating peripheral endocrine and autonomic homeostasis, the associated lifelong changes in peripheral HPA axis activity were investigated in males in Paper 2 (Sominsky et al., 2013a) and in females in Paper 4 (Sominsky et al., 2012a). The impact of neonatal LPS on ANS functioning was introduced in Paper 1 (Sominsky et al., 2012b) and further explored in Paper 2 (Sominsky et al., 2013a).

Early life exposure to an inflammatory insult, induced by administration of LPS, has been repeatedly demonstrated to alter HPA axis function in later life (Ellis et al., 2005; Mouihate et al., 2010; Shanks et al., 1995; Shanks et al., 2000). This programming effect has been related to the timing of LPS exposure, coinciding with the critical period of HPA axis development (Levine, 1994). Our laboratory has previously reported that dual LPS exposure on PNDs 3 and 5 leads to immediate and long term implications for HPA axis functioning, resulting in a significant elevation in circulating corticosterone in response to a subsequent stressor in adulthood (Hodgson et al., 2001; Walker et al., 2012; Walker et al., 2004a; Walker

et al., 2006a). The current findings, presented in Paper 2 (Sominsky et al., 2013a) and Paper 4 (Sominsky et al., 2012a) in this thesis, provide evidence for a continuous elevation in circulating corticosterone throughout lifetime, reporting for the first time that these differences persist from the neonatal period, into adolescence and ultimately into adulthood in male and female animals exposed to neonatal LPS treatment alone.

Programming of the HPA axis by early life stress has been relatively well established and researched. One of the ways in which this thesis has expanded on this research and provided novel findings and insights into the field of perinatal programming is the investigation regarding programming of the ANS. In the first paper in this thesis (Sominsky et al., 2012b) we reported that neonatal LPS challenge induced sustained increase in TH phosphorylation in neonatal adrenal glands, indicative of increased catecholaminergic synthesis. These data were replicated in a later study in this laboratory, which is not included in this thesis, where increased TH phosphorylation in neonatal adrenals following LPS exposure was accompanied by a concomitant increase in TH activity, providing evidence for the first time that sustained phase of TH activation occurs *in vivo* (Ong et al., 2012).

In Paper 2 in this thesis (Sominsky et al., 2013a), the investigation of TH activation was extended into later life and additional time points were examined. In this paper we have demonstrated that neonatal LPS treatment resulted in increased phosphorylation of TH and increased activity of the enzyme in adolescence and in adulthood. Importantly, increased TH activity was observed independent of changes in total protein levels. These data suggest that neonatal LPS exposure results in an initial and sustained phase of TH activation in the neonatal period, which is associated with a long lasting effect on TH activity, mediated by TH phosphorylation but not by protein synthesis. Significantly increased levels of circulating corticosterone were evident in the same LPS-treated animals. It is important to note, that these animals were undisturbed until the tissue collection, apart from weekly observation of

their weight and minimal handling. Therefore the data reported in this thesis in Paper 2 (Sominsky et al., 2013a) present intriguing findings, indicating an abnormal and constant TH activity under baseline conditions, suggesting that increased catecholamine synthesis occurs in animals neonatally exposed to LPS throughout their lifetime, corresponding to the hypersecretion of corticosterone at the same timepoints. Increased circulating glucocorticoids have been shown to induce an increase in TH mRNA levels and activity in sites of catecholamine production, including the adrenal medulla (Stachowiak et al., 1988). Therefore, it is plausible to suggest that the increase in adult TH activity evident in Paper 2 (Sominsky et al., 2013a) was triggered by an increased and prolonged exposure to glucocorticoids. However, as indicated in Paper 1 (Sominsky et al., 2012b), neonatal LPS also has the capacity to produce an immediate and concurrent effect on the activity of both, the HPA axis and TH, resulting in a more extended increase in TH activity in LPS-treated neonates for as long as 24h post treatment, than an increase in circulating corticosterone, which lasts up to 4h following LPS administration. Thus it is likely that a neonatal immune challenge independently activates the HPA axis and the ANS, resulting in long term programming of both systems.

To explore the significance of increased TH activity as a marker of autonomic function from a behavioural perspective, experiments reported in Paper 2 (Sominsky et al., 2013a) utilised a novel behavioural index of ANS function, respiratory plethysmography. Plethysmographic assessment of respiratory rate was employed as an innovative measure of autonomic arousal. Changes in respiratory rate are independent of changes in cardiovascular parameters and can be assessed at thresholds significantly lower than those required to induce ANS-mediated changes in cardiovascular system (Kabir et al., 2010; Nalivaiko et al., 2011). Therefore, assessment of respiratory rate provides a measure of ANS arousal (Nalivaiko et al., 2011). The findings presented in Paper 2 (Sominsky et al., 2013a) indicate that adult

animals that were treated with LPS as neonates respond with increased respiratory rate to lower thresholds of sensory stimuli, indicative of an increased arousal state. Taken together, these data indicate that neonatal immune challenge with LPS induces persistent changes in both endocrine and autonomic functions. The translational outcomes of these are most likely reflected in the increased anxiety-like behaviours we have consistently reported (Walker et al., 2009; Walker et al., 2008; Walker et al., 2004b).

4. Anxiety-like phenotype: a broad behavioural spectrum

Increased anxiety-like behaviours are typically induced in adult animals, subjected to neonatal LPS treatment. This phenomenon has been demonstrated in our laboratory (Walker et al., 2012; Walker et al., 2009; Walker et al., 2008; Walker et al., 2004b) and by others (Breivik et al., 2002; Shanks et al., 2000). The behavioural changes are particularly robust when animals are subjected to an acute stressor prior to behavioural testing (Walker et al., 2012; Walker et al., 2009). In Paper 1 (Sominsky et al., 2012b) and Paper 2 (Sominsky et al., 2013a) presented in this thesis, anxiety-like behaviours were induced in neonatally treated animals even when an additional stressor was not applied. This indicates that the LPS administration which occurred during critical periods of limbic and neuroendocrine development in the neonatal rat, induces pronounced behavioural alterations later in life. The traditional measures of anxiety-like behaviours (e.g. Elevated Plus Maze (EPM) and the Holeboard apparatus) were expanded, and a novel respiratory testing paradigm of ANS arousal was introduced in Paper 2 (Sominsky et al., 2013a). The findings presented in this latter paper imply that behavioural changes, induced by a neonatal immune challenge, comprise a broad framework of an anxiety-related phenotype, suggesting that manifestation of this phenotype may not be limited to the traditionally measured anxiety-like behaviours,

but may be also evident in other behavioural domains, such as the alterations in respiratory responses.

In Paper 3 in this thesis (Walker et al., 2011), in addition to anxiety-related behaviours, mating behaviour of male and female animals was examined. 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 (Barrot et al., 2005; Wallace et al., 2009). Therefore, to assess the possibility that similar deficits in sexual behaviour will coincide with increased anxiety-like behaviours in the model of neonatal LPS exposure, mating behaviours in sexually naïve male and female neonatally-treated rats were assessed in adulthood. While neonatal LPS treatment impaired sexual behaviour in both sexes, the strongest effects were observed in female animals, with all behavioural parameters that were measured being significantly reduced. While in males only one behavioural parameter (i.e. the number of mounts) was significantly affected, all sexual behavioural parameters that were measured in LPS-treated females were altered. It appears that females treated with LPS as neonates exhibited decreased receptivity and alteration in sexual behaviour cues, which subsequently led to a diminished performance by the naïve males. LPS-treated females were not disinterested in the male stud, but rather required more time for successful mating due to their inability to provide appropriate behavioural cues for the initiation of mating, corresponding with the previous literature where similar effects were observed in animals subjected to social isolation stress, and in which increased anxiety-like behaviours coincided with the delayed latency to initiate sexual behaviour (Barrot et al., 2005; Wallace et al., 2009).

It is important to note that animals neonatally treated with LPS are not infertile, given that a subsequent generation of offspring was successfully created when neonatally LPS-treated females were mated with naïve counterparts, as demonstrated in Paper 4 in this thesis

(Sominsky et al., 2012a). Therefore, the impairment in mating strategies presented in Paper 3 (Walker et al., 2011) characterises a subfertile, rather than infertile phenotype. A later study conducted in our laboratory, but not included in this thesis (Walker et al., 2012) has demonstrated that a second generation of offspring, born to animals of which only one parent was neonatally treated with LPS, via both maternal and paternal lines, exhibit similarly increased anxiety-like behaviours when they are subjected to an acute psychological stressor in adulthood, without being exposed however, to a neonatal immune challenge themselves. In addition, female animals that were neonatally exposed to LPS (first generation), exhibited significantly reduced maternal care towards their offspring, demonstrating again that an anxiety-like phenotype incorporates a wide range of behavioural alterations that are typically induced under potentially distressing situations. Interestingly, the offspring of females exposed to LPS as neonates exhibited not only behavioural characteristics of anxiety, but also potentiated corticosterone response to stress, while offspring of neonatally-treated males exhibited behavioural response only. These effects were completely reversed by cross-fostering. These findings suggest that the stress caused by receiving low quality maternal care sets up increased vulnerability to later life trauma. Importantly, these findings correspond with those of Michael Meaney's laboratory, which has demonstrated that reduced maternal care results in increased stress-related behaviours and neuroendocrine responses, which are reversible in the presence of fostering to dams known to provide higher quality maternal care to young (Francis et al., 1999; Liu et al., 1997; Weaver et al., 2004). Extending beyond Meaney's work, our laboratory have demonstrated that neonatal LPS exposure is sufficient to not only induce anxiety-like phenotype, but also impair maternal care behaviour provided to the next generation, directly regulating stress responsivity of the second generation (Walker et al., 2012). Given that maternal behaviours have been shown to be learned in rats (Francis et al., 1999), 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 reproductive fitness of the offspring (Cameron, 2011; Parent et al., 2012). As such, we were interested to examine whether exposure to neonatal LPS has the capacity to not only induce behavioural changes such as those associated with increased anxiety-like behaviours, impaired mating and maternal care strategies, but also to directly influence reproductive functionality, increasing the potential for transmission of alternative reproductive phenotypes.

5. Programming of reproductive development by neonatal LPS challenge

Previous research has indicated that inflammation has a direct effect on reproductive behaviours. This influence is thought to be mediated by the sex hormones and neuropeptides that control mating (Kentner and Pittman, 2010). Given the critical role of early life stress in programming the development of neuroendocrine function, we aimed to examine the developmental trajectory of the reproductive system following neonatal LPS exposure. As demonstrated in Papers 3 and 4 in this thesis (Sominsky et al., 2012a; Walker et al., 2011), significant impairments in mating behaviours and other strategies in animals neonatally exposed to LPS were accompanied by various alterations in reproductive development. These included altered weight gain, changes in age at puberty, with earlier onset of puberty being evident in males and females, along with the advance of reproductive senescence as exhibited by the LPS-treated females. Moreover, given the important role of sex hormones in the control of sexual maturation and behaviour, Papers 3 and 4 (Sominsky et al., 2012a; Walker et al., 2011) investigated the impact of neonatal LPS exposure on HPG axis functioning and reported that neonatal immune activation resulted in long term suppression of the HPG axis. Immediate HPG responses to LPS administration were observed in the neonatal period

whereby circulating testosterone and LH in males and LH in females were decreased. Such suppression of HPG activity was shown to persist throughout the life span. LH and FSH suppression was observed in puberty for females, and suppression of testosterone and LH surges was observed for both sexes during mating. In males, testosterone levels were suppressed in late adulthood. This suppression of HPG hormones coincided with increased corticosterone levels consistent with known stress-induced suppression of HPG function (Baker et al., 2006; Li et al., 2006). As mentioned above, these endocrine perturbations resulted in a disruption to puberty onset and impairment in sexual behaviour, in both male and female animals.

These peripheral endocrine findings support previously published work demonstrating central limbic circuitry to be responsible for HPG function, pubertal timing and sexual behaviour (Meethal and Atwood, 2005; Sisk and Foster, 2004). Studies have shown CRH expression in the PVN to be increased in rats neonatally exposed to LPS (Shanks et al., 1995), which diminishes LH secretion (Barbarino et al., 1989; Kinsey-Jones et al., 2010). As demonstrated in Paper 2 in this thesis (Sominsky et al., 2013a), neonatal LPS challenge also exerts programming effects on other components of the CRH system. Furthermore, HPG regulatory signals Kiss1 and Kissr1 in the hypothalamus have been reported to be affected following LPS challenge via Cox-1 and Cox-2 (Iwasa et al., 2008). In female rats, dual LPS administration on PNDs 3 and 5 has also been demonstrated to result in a downregulation of Kiss1 mRNA levels in the medial preoptic area at the time of puberty, but a subsequent upregulation in adulthood (Knox et al., 2009). No such effects occurred when LPS was administered after PND7, pointing towards a critical issue of timing of a perinatal insult (Kentner and Pittman, 2010).

Taken together, these findings indicate that neonatal neuroimmune-neuroendocrine interactions are particularly important in determining limbic development and driving long-

term physiological and behavioural trajectories. Specifically, the major limbic structures, the hypothalamus and hippocampus, involved in regulating peripheral endocrine homeostasis, emotionality and behaviour, including reproductive-related behaviours, appear to be directly susceptible to LPS exposure during the neonatal period, leading to the observed increase in anxiety-like behaviours, and specifically impaired sexual behaviours. While the neural circuitry undoubtedly governs behaviour, the control of reproduction is not limited to the central nervous system, but is also influenced by gonadal function and sex steroids. Thus, in order to more fully evaluate the impact of neonatal LPS exposure on reproductive development and behaviour, assessment of gonadal physiology is essential. The results of various analyses of gonadal morphology and function were reported in Papers, 3, 4, and 5 in this thesis (Sominsky et al., 2012a; Sominsky et al., 2013b; Walker et al., 2011) and these outcomes are discussed below.

6. Long term alterations in gonadal physiology: an emphasis on ovarian function

The timing of LPS exposure in our model occurs during an important period in functional development of the gonads. In Papers 3 and 4 in this thesis (Sominsky et al., 2012a; Walker et al., 2011) we demonstrated that neonatal LPS challenge results in morphological alterations in the developing male and female gonads, which have profound effects on gonadal function in later life. Fewer gonocytes were present in the neonatal testes of LPS-treated males, this was followed by increased epithelial disorganisation and spermatogenic delay in the testes of these males in adulthood. These findings have an additional significance, given that the timing of LPS exposure occurred during early mitotic division of the gametes, a period surrounding their global epigenetic erasure and imprinting (Skinner and Guerrero-Bosagna, 2008; Surani, 2001). Therefore, the anxiety-like behaviours of the second generation of offspring via the paternal lineage, reported in later work from this

laboratory, but not included in this thesis (Walker et al., 2012), may potentially represent heritable epigenetic programming, given that unlike the maternal line, it does not require direct environmental exposures for the subsequent generations to also exhibit the phenotype.

In females, the timing of LPS exposure coincided with the final processes in the formation and establishment of the primordial follicle pool (Skinner, 2005). As shown in Paper 4 in this thesis (Sominsky et al., 2012a), a reduction in follicular numbers was induced in females treated neonatally with LPS. Importantly, there was a reduction in primordial follicles on PND 14, along with the decreased ovarian weight. Given that the initial follicular growth is known to be strongly regulated by peripheral immune factors and is considered to be gonadotropin-independent (Dissen et al., 2002; Schindler et al., 2010; Skinner, 2005), we were interested to examine the possibility that peripherally administered LPS challenge directly interferes with ovarian development and function. As illustrated in Figure 8, several mechanisms of actions may co-exist. Long term programming of the HPA and HPG axes, as well as the ANS, which were demonstrated in this thesis, may affect ovarian function. For instance, increased sympathetic innervation of the ovary has been shown to disrupt oestrous cyclicity and impair follicular maturation (Dissen et al., 2002; Dorfman et al., 2009). As mentioned in the previous section, the HPA and HPG axes co-regulate one another, and stress is known to impair reproductive success (Rivier and Rivest, 1991; Tilbrook et al., 2000). While corticosterone itself may not have a direct effect on reproductive development, its interactions with gonadotropins, such as LH and FSH are likely to alter sexual maturation (Rivest, 1991). These pathways however, are likely to influence ovarian function during the later period of development, while in the immediate timeframe when LPS is administered its actions are likely to be exerted via circulating and local immune factors. Neonatal LPS challenge is known to induce an acute rise in proinflammatory cytokines (i.e. TNF-α, IL-6, IL-1β) and these are known to play role in the primary stages of follicular development (Ben-

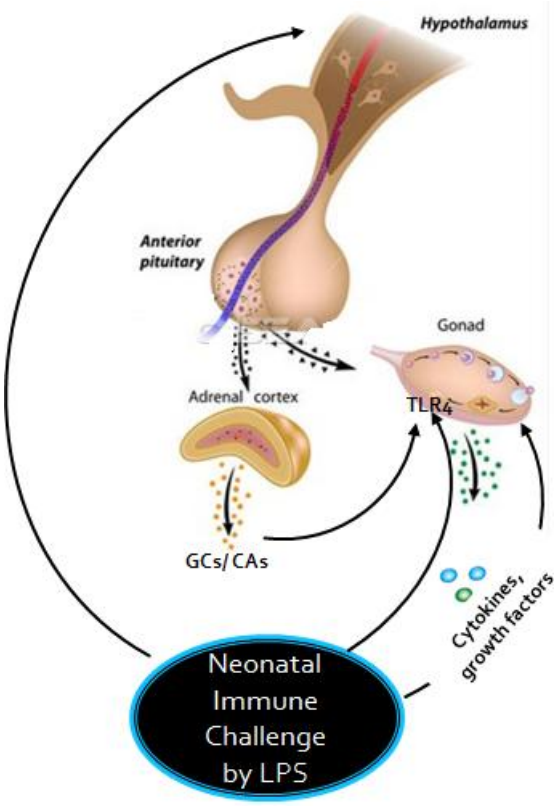
Rafael and Orvieto, 1992; Morrison and Marcinkiewicz, 2002), the formation of primordial follicles and transition to the primary stage, which are hormone independent (Skinner, 2005). It is therefore reasonable to suggest that neonatal LPS treatment instantly interferes with the delicate process of primordial follicle pool assembly and the subsequent activation of follicular development, through stimulation of an acute proinflammatory response. While this might be a transient perturbation, it ultimately leads to long lasting alterations in the size of the follicular pool, as observed in Paper 4 (Sominsky et al., 2012a) and by others (Wu et al., 2011).

Additionally, a more direct pathway through which LPS may exert its effects on the ovary is possible. The receptor for LPS, TLR4, is present in the ovary and expressed by ovarian epithelial cells, granulosa/cumulus cells and ovarian macrophages (Herath et al., 2007; Liu et al., 2008; Richards et al., 2008; Zhou et al., 2009). In the ovary these immune receptors regulate fertility, by supporting ovulation and sperm capacitation (Liu et al., 2008; Shimada et al., 2008). Previous research has indicated that in response to LPS, ovarian granulosa cells respond acutely, with rapid phosphorylation of TLR 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 (Bromfield and Sheldon, 2011; Price et al., 2013). Exposure to LPS *in vivo* in adult animals or *in vitro* have been shown to result in impaired follicular growth and function, inducing follicular atresia in cattle (Bromfield and Sheldon, 2013; Herath et al., 2007) and in rodents (Besnard et al., 2001; Bromfield and Sheldon, 2013). To test this proposal Paper 5 in this thesis (Sominsky et al., 2013b) examined the potential cellular mechanisms that may have led to the impaired ovulation and diminished follicular pool, as documented in Paper 4 (Sominsky et al., 2012a). We have conducted a microarray analysis to study global gene expression in the neonatal ovaries obtained on PND 7. Microarray analysis indicated substantial upregulation in a significant number of

inflammatory genes. The top canonical pathways as indicated by the analysis were involved in immune function, of which LPS-stimulated mitogen-activated protein kinase (MAPK) signalling pathway was chosen for further examination. The expression of TLR4 transcript, a major component of this pathway, was significantly upregulated as indicated by the microarray and qRT-PCR analyses. TLR4 protein expression was also significantly increased in the ovaries of LPS-treated animals. These data therefore indicate that peripheral administration of LPS during the early neonatal period in the rat results in activation of ovarian TLR4 signalling. Given that in the rat, the formation of primordial follicles is not established until PND 3 (Rajah et al., 1992; Skinner, 2005), it is plausible to suggest that an immune challenge at this time point may directly intervene with the formation and establishment of the finite primordial follicle pool via activation of inflammatory pathways.

In summary, the data presented in Paper 5 in this thesis (Sominsky et al., 2013b) have provided evidence that neonatal exposure to LPS not only induces general peripheral inflammation, but that this proinflammatory response is also expressed locally in the ovaries. These findings suggest that neonatal LPS-induced ovarian inflammation is mediated through an upregulation of TLR4 signalling, which may be sustained into later life. Bacterial infections, such as *E. Coli* and *Chlamydia trachomatis*, are associated with alterations in TLR4 expression and pathological outcomes such as impaired fertility (Herath et al., 2009; Laisk et al., 2010). Additionally, common ovarian diseases such as polycystic ovarian syndrome and endometriosis are associated with chronic increases in inflammatory mediators (Wu et al., 2004). Together, the data presented in Papers 3, 4 and 5 in this thesis (Sominsky et al., 2012a; Sominsky et al., 2013b; Walker et al., 2011) provide a valuable insight into the link between early life infection and reproductive fitness.

Figure 8: Several direct and indirect pathways through which neonatal LPS exposure may alter ovarian development and function. These include the programmed HPA and HPG axis, increased sympathetic activity as well as induced proinflammatory response and finally via binding to ovarian TLR4.



7. Conclusions

7.1 Summary

The papers presented in this thesis provide an evidence for the long term programming effect of a neonatal immune challenge on later life physiological and behavioural functioning. This included changes in the limbic neural circuitry, examined in Papers 1 and 2 (Sominsky et al., 2013a; Sominsky et al., 2012b) followed by the programming of peripheral endocrine and autonomic function, presented in Papers 2, 3 and 4 (Sominsky et al., 2013a; Sominsky et al., 2012a; Walker et al., 2011), which were proposed to underlie the behavioural changes, such as the increased anxiety-like behaviours, reported in Papers 1, 2 and 3 (Sominsky et al., 2013a; Sominsky et al., 2012b; Walker et al., 2011),

and the impaired sexual behaviours, presented in Paper 3 (Walker et al., 2011). Finally, a novel and important development by this thesis, is the observation related to programming of reproductive development in the female rat, revealed in Papers 4 and 5 (Sominsky et al., 2012a; Sominsky et al., 2013b), given the crucial role of reproductive health in fertility.

7.2 A new perspective: Brain-Immune-Gonadal (BIG) axis

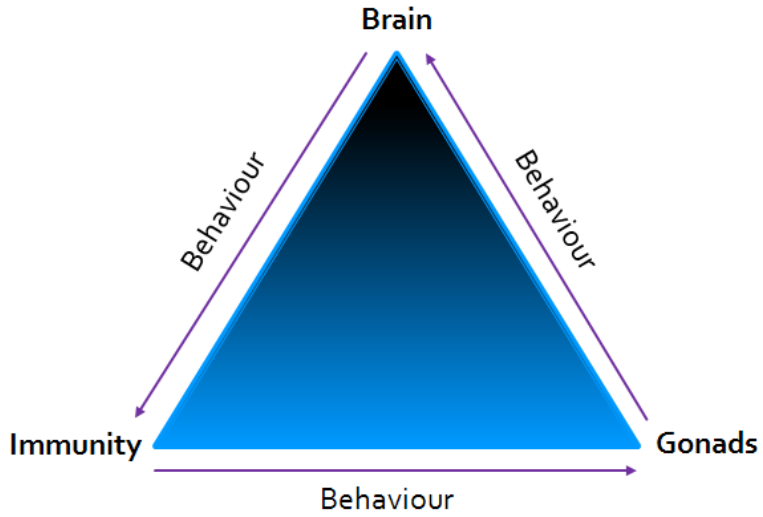
The findings of the papers in this thesis build a novel unifying approach to programming of physiological function that ultimately influences reproductive health and fitness. The neonatal immune stimulant induces lifelong programming of neuroimmune-neuroendocrine interactions, resulting in increased stress responsivity and therefore in permanent behavioural alterations, constitutive of an anxiety-like phenotype. Firstly, peripheral immune activation to the neonate provokes an acute inflammatory response, initiating a release of peripheral pro-inflammatory cytokines such as IL-1β and TNF-α (Hawiger, 2001; Turrin et al., 2001). These cytokines subsequently traffic to the brain via different pathways such as circumventricular organs, as well as through vagal nerve stimulation (Borovikova et al., 2000). This influx of cytokines to the brain then stimulates microglial activation and activates the HPA axis, either sequentially or simultaneously. Given that the immune challenge is presented during a critical period of limbic development, hippocampal, hypothalamic, amygdaloid and associated cortical regions adjust their neural circuitry to accommodate the anticipated robust HPA axis stress response that will be required throughout life. In later life this programming effect is demonstrated in enhanced HPA axis activity, stronger immunoresponsivity of microglia, as well as increased autonomic activity. The behavioural outcome of such physiological alterations is an increased tendency to exhibit a variety of anxiety-related behaviours, which under certain environmental conditions may prove maladaptive.

In line with the programming of neuroimmune-neuroendocrine cross-talk, exposure to LPS induces lifelong suppression of the HPG axis. This suppression may result from the upregulated neuroimmune response or activation of central CRH pathways, which are known to influence central HPG activity (Iwasa et al., 2008; Li et al., 2006). Consistent with the diminished HPG function, LPS-treated animals exhibit altered reproductive lifespan, along with impaired mating performance, which in female animals is also associated with poor maternal care. These behaviours comprise a subfertile phenotype which is comorbid with the anxiety phenotype.

Alteration of gonadal morphology and function also occurs in animals neonatally exposed to LPS. Delayed gonocyte development and other testicular structural changes are observed throughout the lifetime in males, along with the diminished follicular reserve observed in females. Ovarian mechanisms of LPS action that were identified in Paper 5 (Sominsky et al., 2013b) point towards a local inflammatory response induced during the sensitive time of ovarian follicular maturation and potentially responsible for the reduction in follicular counts as evidenced in Paper 4 (Sominsky et al., 2012a). Moreover, we propose that in the long term, this imbalance in the ovarian milieu is augmented through interactions with the dysregulated HPA and HPG axes, and enhanced autonomic activity.

Thus, while further studies are required to elucidate the endocrine-immune mechanisms involved in the impaired ovarian development and functioning, and while this link is yet to be completely established in males, we propose to incorporate our findings in a holistic framework, where the continuous interaction between the immune system, the brain and the gonads is mediated via the behavioural changes, reflective of an altered phenotype (See Figure 9).

Figure 9: The Brain-Immunity-Gonadal (BIG) axis. The interaction between the brain, the immune system and the gonads is mediated via behaviour.



8. Implications

The impact of the neonatal microbial environment on later life development described in this thesis raises important questions. Most pertinent is how this phenomenon, designed to enhance adaptedness and which has been laid down early in evolution, holds ecological relevance in contemporary populations. For instance, if infections are a common occurrence during such a vulnerable period of life, what makes some of us more susceptible than others to experience behavioural and physiological alterations in response to adversity later in life. Is this phenotypic variability necessarily maladaptive? And why is it still important to consider this risk given the advances in public health, such as immunization programs, and antibiotics? Finally, does exposure to environmental stressors, such as infection, produce deleterious outcomes or rather aim to produce adaptive changes relevant to the present context?

In order to answer these questions, the programming consequences need to be viewed in a context-specific manner. Early life developmental plasticity allows an organism to shape

its unique phenotypic characteristics in response to given environmental conditions. Hence it is important to view perinatal programming initially as a regulatory mechanism, the goal of which being to increase adaptation and enhance survival. Correspondence between early and later life environments, whether favourable or adverse, promotes physiological and behavioural adaptation. However, when a mismatch occurs and the programmed function is no longer beneficial in a given environment – that is when the risk for adverse consequences increases (Gluckman et al., 2005).

Contextual differences need to be considered also on a broader perspective of time. Behavioural and physiological functions that were necessary for survival in the past may not be beneficial in modern society. Given that the traditional threats to survival in the modern society have altered compared to early subsistence conditions, behavioural outcomes of perinatal stress and anxiety, such as increased vigilance or easily distracted attention may not serve the same purpose for an individual these days as in our ancestral environment.

However, we need also to acknowledge this phenomenon at a population-based level. Early life adversity may elicit expression of divergent genotypes and thus have different consequences for individuals in later life, while increasing phenotypic variability in a general population. This way, developmental plasticity of an organism allows for variability in responses to the same environmental condition. From an evolutionary perspective, individual variability has always had a beneficial impact on the survival of the group. Thus increased susceptibility of some individuals to diseases and psychopathologies, or in particular – increased susceptibility to the effects of programming, may be a disadvantage from an individual perspective, but can have a favourable effect for the group. For example, increased hyperactivity and impulsiveness of some individuals can lead to engagement in dangerous activities, with negative outcomes, which will serve as a warning to others. On the other

hand, increased hypervigilance and decreased novelty seeking may prevent falling into potentially harmful situations. Therefore transgenerational transmission of these diverse phenotypes has evolutionary significance in preserving living organisms in a constantly changing environment, while also providing phenotypic variability in case of habitat change.

Reproductive fitness serves a regulatory role in this interplay between heredity and environment. The findings from our laboratory have demonstrated transgenerational transmission of anxiety-related behaviours, induced by a neonatal immune challenge (Walker et al., 2012). As mentioned above, while transmission of the anxiety-like phenotype through the maternal line was directly influenced by changes in maternal behaviour, transmission via the paternal line suggests the potential for epigenetic germ line modifications. The diverse strategies and pathways that are involved in transmission of this potentially maladaptive phenotype, suggests the importance in its preservation across generations. This notion is supported by the findings documented in Paper 3 (Walker et al., 2011) showing that rats treated neonatally with LPS exhibit a subfertile phenotype. This allows for the potentially deleterious phenotype to be limited within the population but also maintained in case the environmental conditions are altered such that elevated caution and vigilance becomes adaptive. If this were to happen, then evolutionary principles of survival of the fittest would dictate that more of the non-treated rats would not survive to reproductive age, while the LPS-treated line would increase. Thus, the general proportion of the population would change, but the species would survive.

Therefore, perinatal programming and its transgenerational potential allows for increased plasticity and a more flexible phenotypic outcome for a population as a whole. This in turn, increases the chance of survival for the species in times of environmental change. It is important to note that although improved public health and hygiene, including immunization

and the use of antibiotics, may have significantly decreased the impact of early life infections, other sources of inflammation, are of increasing concern worldwide. One highly prevalent inflammatory source is consumption of a high fat diet and consequent obesity. It is increasingly recognised that obesity is characterized by a chronic state of inflammation and that inflammatory signalling pathways in obesity are causally linked to insulin resistance (Wellen and Hotamisligil, 2005). Of specific relevance to the current thesis is the role of TLR4 in obesity-induced inflammation. Recent research indicates that saturated fatty acids activate predominantly TLR4 signalling in adipocytes and macrophages inducing increased cytokine expression, and the capacity of fatty acids to induce an inflammatory response is diminished in the absence of TLR4 (Shi et al., 2006; Tsukumo et al., 2007). TLR4 is also a major target for fatty acids in the hypothalamus, mediating activation of an inflammatory response, predominantly in the medium eminence and arcuate nucleus, determining the resistance to anorexigenic stimuli (Milanski et al., 2009).

Obesity is the greatest public health challenge worldwide and is currently increasing in prevalence especially in children and women of reproductive age, influencing future generations. Health risks associated with overweight and obesity include type 2 diabetes mellitus, hypertension, cardiovascular disease and several types of cancer, primarily breast cancer (Eliassen et al., 2006; Kopelman, 2007). Importantly, obesity is strongly associated with poor female reproductive function, including an increased risk of miscarriage, PCOS and infertility. Nutrition has an important impact on reproductive maturation and functioning, in particularly nutritional status in early life. A recent review by Sloboda et al., (2011) indicates the critical role of both pre- and postnatal nutritional environments in reproductive health and disease. As highlighted in this review, increased body fat and childhood obesity may directly advance age at menarche, which is known to be associated with a range of adult

pathologies, including increased risk of cardiovascular disease, insulin resistance, increased susceptibility of developing breast cancer and premature reproductive senescence (Sloboda et al., 2011). Evidence from animal studies further points to the direct impact of the perinatal nutritional environment on reproductive development and ovarian function (Sloboda et al., 2009). While both maternal caloric restriction and maternal high fat diet in rats have been found to result in accelerated onset of puberty, different developmental pathways were suggested to be involved. Interestingly, postweaning high fat diet alone was sufficient to advance the onset of puberty, and when preceded by perinatal caloric restriction it appeared to further accelerate the age at puberty. However, exposure to maternal high fat diet had a predominant effect on reproductive maturation and was not influenced by subsequent nutrition (Sloboda et al., 2009). While the link between diet-induced inflammation and the acceleration of pubertal maturation resulting in diminished reproductive success is yet to be fully established, the findings reported in this thesis, in particularly those presented in Papers 4 and 5 (Sominsky et al., 2012a; Sominsky et al., 2013b), suggest common mechanisms may exist. Neonatal LPS exposure similarly led to advanced pubertal onset, which was associated with increased postnatal growth, subsequently resulting in advanced reproductive senescence. Importantly, these changes were accompanied by a diminished follicle reserve (Sominsky et al., 2012a). Activation of ovarian TLR4 signalling in the neonatal ovary has been then proposed to underlie this reduction in follicular numbers (Sominsky et al., 2013b). Given the major function of TLR4 in mediating the immune responses to saturated fatty acids, its role in reproductive-related changes induced by high fat diet is an important area of future investigation.

Exposure to a high fat diet has also been implicated in the onset of psychopathology, with overweight and obese individuals being at an increased risk of developing anxiety and

depression (Scott et al., 2008). The impact of prenatal high fat diet on behavioural disturbances in later life, such as anxiety, has been suggested to be mediated by prolonged microglial activation and thus persistent neuroinflammation (Bilbo and Tsang, 2010; Sullivan et al., 2012), which at least in part has been proposed to be mediated via TLR4 signalling within the hippocampus (Bilbo and Tsang, 2010). In conclusion, increased TLR4 signalling appears to be a crucial component of both LPS-induced and high fat diet-induced inflammation, promoting significant health consequences, in particularly when experienced during critical periods of perinatal development. This evidence thus points to the important role of the early microbial environment in predisposing to health or disease in later life. Ongoing studies will be important in understanding this contribution.

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