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Hypothalamic mechanisms mediating inhibition of prolactin secretion following stress in early pregnant mice

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Declaration

This thesis has been composed by the candidate, Victoria Parker, and the experiments were performed by the author. It is clearly stated in the text where experiments involved collaboration with others. This work has not been submitted for any other degree or professional qualification except as specified.

Signed:

Date:

For Dominic

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Abstract

In early pregnancy prolonged exposure to stress is known to have profound adverse effects on reproduction and is associated with suppressed progesterone secretion and consequent disturbance of the pregnancy-protective cytokine milieu, thus threatening early pregnancy maintenance. Maternal neuroendocrine responses to stress in early pregnancy are poorly understood. Therefore, we designed experiments to (1) study the hypothalamo-pituitary-adrenal (HPA) axis responses to stress in early pregnant mice, to discover whether and how responses change and (2) to determine the effect of stress in early gestation on pregnancy hormones, with a particular focus on the secretion and regulation of prolactin. To establish the effects of stress in early pregnancy (day 5.5) two different ethologically relevant stressors were used: lipopolysaccharide (LPS) or 24h fast stress, to mimic situations that may potentially arise during pregnancy in women: infection or hunger. HPA axis secretory responses to immune stress in early-mid pregnancy were robust and comparable to that in virgins. Vasopressin rather than the usual CRH neurone responses play a key role in maintaining this. However, the mode of action of glucocorticoids in mediating pregnancy complications is not yet established. Prolactin, and its hypothalamic control mechanisms, is a key candidate to mediate brain-to-body responses to stress. Prolactin has important roles in progesterone secretion, pregnancy establishment and immune regulation. We hypothesised that stress would negatively affect prolactin and its neuroendocrine control systems. Prolactin is mainly under the inhibitory control of dopamine, released predominantly from the tuberoinfundibular dopamine (TIDA) neurones. Prolactin also negatively feeds back on itself via prolactin receptors on the TIDA neurones and janus kinase (JAK)2/signal transducer and activator of transcription (STAT)5 signalling. Both immune and fasting stressors strongly inhibited basal prolactin secretion in early pregnancy, accompanied by a mild increase in activation of TIDA as shown by elevated Fos expression, compared to virgins. In addition, pregnancy attenuated LPS-induced recruitment of parvo-cellular paraventricular nucleus neurones and increased activation of brainstem noradrenergic nuclei which could potentially contribute to altered control of the dopamine-prolactin system. Following either immune or fast stress in early pregnancy ovine prolactin was able to drive enhanced expression of phosphorylated (p)STAT5. However, stress alone did not alter pSTAT5 implying it is not exclusively responsible for the stress-reduced prolactin observed in early pregnancy and another stress-induced stimulus must be activating TIDA neurones in these mice. LPS did not alter dopamine activity the median eminence (DOPAC: dopamine ratio) suggesting dopamine does not underlie stress-reduced prolactin secretion and other mechanisms must be considered. Direct effects of LPS, or its associated cytokines, on pituitary lactotrophs to inhibit prolactin secretion is a possible candidate. To investigate the effect of proinflammatory cytokines on the prolactin system in early pregnancy, d5.5 mice were administered TNF-alpha (α) or interleukin (IL)-6. Both cytokines

increased TIDA activation, however, only TNF-α decreased plasma prolactin and progesterone, suggesting additional TNF-alpha action at the pituitary. As prolactin is anxiolytic we further proposed that stress would have a more profound effect on elevated plus maze performance in pregnant mice. However, early pregnant mice were generally more anxious vs. virgins regardless of LPS treatment. Taken together data show that stress in early pregnancy reduces prolactin and progesterone secretion, contributing to pregnancy complications/failure, but the neuroendocrine stress-related mechanism behind this suppression is yet to be determined.

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Abbreviations List

A1: catecholamine cell group
A2: catecholamine cell group
A6: catecholamine cell group
ACTH: adrenocorticotrophic hormone
ANS: autonomic nervous system
BBB: blood brain barrier
BSA: bovine serum albumin
CA: closed arm
CNS: central nervous system
COX: cyclo-oxygenase
cpm: counts per minute
CRH: corticotrophin releasing hormone
CVO: circumventricular organ
DA: dopamine antagonist
DAB: diaminobenzidine
ddH_2O: double distilled water
dH_2O: distilled water
DEPC: diethylpyrocarbonate
DNA: deoxyribonucleic acid
cDNA: complementary DNA
DOPAC: 3,4-dihydroxyphenylacetic acid
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
EPM: elevated plus maze
FBS; foetal bovine serum
FSH: follicle stimulating hormone
GABA: gamma-aminobutyric
GC: glucocorticoid
GH: growth hormone
h: hours(s)
20-αHSD: 20 alpha-hydroxysteroid dehydrogenase
11 βHSD: 11 beta-hydroxysteroid dehydrogenase
hCG: human chorionic gonadotrophin
HPA: hypothalamo-pituitary-adrenal axis
HPG: hypothalamo-pituitary-gonadal axis
hPL: human placental lactogen
HRP: horseradish peroxidase
ICC: immunocytochemistry
i.c.v: intracerebroventricular
IL: interleukin
i.p: intraperitoneal
ISH*: in situ* hybridisation
i.v: intravenous
IZ: inner zone
kb: kilobase
kDA: kilo-dalton
LC: locus coeruleus
LH: luteinizing hormone
LPS: lipopolysaccharide
ME: median eminence

min: minute(s)
mPVN: magnocellular division of PVN
mRNA: messenger RNA
NA: noradrenaline
NK: natural killer cell
NFκB: nuclear factor-kappa enhancer of activated B cells
ns: non-significant
NSB: non-specific binding
NSS: normal sheep serum
NTS: nucleus tractus solitarius
OA: open arm
OD: optical density
OFT: open field test
oPRL: ovine prolactin
OVX: ovarectomised animals
OZ: outer zone
PAF: paraformaldehyde
PB: phosphate buffer
PBS: phosphate buffered saline
PBT: PB with Triton X-100
PGs: prostaglandins
PL: placental lactogen
hPL: human placental lactogen
PLC: phospholipase C
PNS: parasympathetic nervous system
PVN: paraventricular nucleus
mPVN: magnocellular paraventricular nucleus
pPVN: parvocellular paraventricular nucleus
RIA: radioimmunoassay
RNA: ribonucleic acid
RVL: rostral ventrolateral medulla
s.c: subcutaneous
SCN: suprachiasmatic nucleus
SD: standard deviation
sec: second (s)
SEM: standard error of the mean
SNS: sympathetic nervous system
SOCS: suppressors of cytokine signalling
SON: supraoptic nucleus
SSC: standard sodium citrate buffer
STAT: signal transducers and activator of transcription
pSTAT: phosphorylated STAT
TdT: terminal deoxynucleotidyl transferase
TEA: triethanolamine
TIDA: tuberoinfundibular
TNF-α: tumour necrosis factor alpha
TRH: thyrotropin-releasing hormone
3V: third ventricle
V1a: vasopressin receptor subtype
VEH: vehicle
VIP: vasointestinal peptide
VMH: ventromedial hypothalamus

Chapter 1

General Introduction

Chapter 1: General Introduction

1.1 Background

Successful pregnancy is reliant on the interaction and co-ordination of many physiological processes. Pregnancy is highly susceptible to disruption, with spontaneous miscarriage occurring in around 15% of clinically recognised pregnancies. However, the total percentage of reproductive loss is likely to be much higher if the losses that occur prior to clinical recognition are included (Brown, 2008). There are many potential causes for pregnancy failure with the most common in early pregnancy being chromosomal abnormalities of the foetus. There are also a number of other factors including endocrine abnormalities and environmental factors which threaten pregnancy such as maternal stress, hunger and infection/the immune system response (Arck, 2001, Brown, 2008). Many of these encounters or stimuli can result in disruption of maternal physiological processes in an attempt to overcome or compensate for the problem e.g. to maintain homeostasis, conserve energy, etc.
The main physiological systems that may be disrupted and hence threaten pregnancy success are the hypothalamo-pituitary-gonadal (HPG) axis, the dopamine-prolactin system, the immune system and the hypothalamo-pituitary-adrenal (HPA) axis. The disturbance of any of these can risk pregnancy maintenance and induce foetal programming of physiology and behaviour.

1.2 Introduction to the major physiological systems involved in pregnancy success and failure

Pregnancy establishment and maintenance requires suitable activation and responses from a number of physiological systems for reproductive success including endocrine, neuroendocrine, neuronal, paracrine etc. responses. Disruption at any of these levels has adverse reproductive outcomes. This chapter will be limited to discussion on the HPG axis, the dopamine-prolactin system, the immune system (cytokine responses/balance) and the HPA axis, along with their interactions. Adaptations and responses in human pregnancy are outlined (where possible), along with key studies utilizing animal models (primarily rodents).

1.2.1 The hypothalamo-pituitary-gonadal (HPG) axis

The hormones of the HPG axis are the principle hormones responsible for regulating reproduction and for controlling the female reproductive cycles in rodents and humans. Briefly, gonadotrophin releasing hormone (GnRH) is secreted from the hypothalamus which stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH consequently bind to receptors in the gonads and stimulate release of sex steroids (follicular oestrogen and corpus luteum progesterone in females) (reviewed in (Vadakkadath Meethal and Atwood, 2005)). Disruption of the components of the HPG axis at any level can compromise reproduction and lead to infertility or pregnancy failure.

1.2.2 The dopamine-prolactin system

Prolactin is an essential reproductive hormone in women and rodents and facilitates a number of pregnancy-related processes, including uterine implantation and lactation. Prolactin is mainly under the inhibitory control of dopamine, originating from tuberoinfundibular (TIDA) neurones in the hypothalamus but it can also regulate its own secretion through a short loop negative feedback system on the TIDA neurones (Freeman et al., 2000). Prolactin levels rise throughout pregnancy in women and there is a profound change in the secretory rhythm of prolactin in rodents during early pregnancy. As pregnancy progresses the neuroendocrine control of prolactin release is altered to allow a state of hyperprolactinaemia to develop e.g. in late pregnancy prolactin negative feedback is absent in rodents (Grattan et al., 2008).

1.2.3 The immune system

The most important components of the cellular immunosystem in pregnancy are the helper T (Th) cells which produce and secrete a number of different pro- and anti-inflammatory cytokines (reviewed in (Guerin et al., 2009)). Successful pregnancy is dependent upon procuring an appropriate maternal cytokine balance, to provide an immune milieu that is suitable for implantation and foetal development. A number of endocrine factors influence the immune system, for example progesterone and prolactin. Progesterone is considered an immunosteroid and facilitates maternal tolerance to the foetus, whilst facilitating in providing a pregnancy-protective immune environment (Arck et al., 2007).

1.2.4 The hypothalamo-pituitary-adrenal (HPA) axis

Activation of the HPA axis by stress exposure triggers neurones in the paraventricular nucleus (PVN) of the hypothalamus to release corticotrophin-releasing hormone (CRH) and vasopressin. These two neuropeptides synergistically induce secretion of adrenocorticotrophic hormone (ACTH) from pituitary corticotrophs (Lamberts et al., 1984). ACTH subsequently stimulates the release of the glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. Glucocorticoids support a wide array of physiological systems and have important roles in immunity/inflammation, metabolism, reproduction and neural function (Sapolsky et al., 2000). Hormones of the HPA axis typically suppress the HPG axis and stress has profound detrimental effects on reproduction, particularly during pregnancy. Stress is implicated as a cause of pregnancy failure in women and rodents (Nakamura et al., 2008) and adverse programming of the health of the offspring in later life (Seckl and Meaney, 2004). In addition, multiple studies indicate a stress-induced immune-endocrine disequilibrium, with a number of mediators, such as cytokines, implicated to contribute to stress-induced pregnancy loss (Arck et al., 1995, Nakamura et al., 2008).

1.3 Role and adaptations in neuroendocrine systems in pregnancy

The maternal brain is a major force for driving crucial physiological changes during pregnancy, including being responsible for preparation of the uterine environment prior to pregnancy establishment. Drive from the brain to the reproductive organs comprises a number of key neuroendocrine systems which regulate vital reproductive hormones necessary for pregnancy establishment and maintenance. In addition, they signal mating and implantation, ensure a suitable hormone and immune environment for foetal development (avoiding foetal rejection) and prepare the mother/foetus for parturition and post-natal life (Douglas, 2010). A great number, possibly all, neuroendocrine hormones are involved in these complex processes and underlie adaptations of maternal physiological systems including fertility, appetite and metabolism, emotion, stress response and behaviours (Russell et al., 2003, Douglas et al., 2007, Brunton et al., 2008, Parker et al., 2011b). Maternal appetite and metabolism reset to support energy flow to the foetus and to store energy as adipose

tissue for milk production and lactation following parturition (Douglas et al., 2007). Immediately following birth the mothers exhibit a dramatic change in behaviour and start feeding, nurturing and defending their offspring. This maternal behaviour is a consequence of changes in the activity of neural circuits in late pregnancy, controlled by pregnancy hormones which also hold these behaviours until delivery. These changes alter emotionality and allow activation of maternal behaviours but the sudden withdrawal of pregnancy hormones can have adverse effects such as depression (Slattery and Neumann, 2008)

1.3.1 The hypothalamo-pituitary-gonadal axis

The HPG axis is vital for the development and regulation of many systems in the body, including reproductive and immune systems. The hormones of the HPG axis in women and rodents are the principle hormones responsible for regulating reproduction and control the female reproductive cycles (reviewed in (Vadakkadath Meethal and Atwood, 2005)). As mentioned previously hypothalamic GnRH stimulates the anterior pituitary to secrete LH and FSH and in turn LH and FSH induce the release of sex steroids. The HPG axis is regulated both at the hypothalamic and pituitary levels.

1.3.1.1 Regulation of the hypothalamo-pituitary-gonadal axis

1.3.1.1.1 Activin, follistatin and inhibin

Activin from the periphery can alter regulation of gonadotrophins at different levels. It selectively exerts effects on FSH at the pituitary level, stimulating FSH synthesis and secretion from gonadotrophs. It can also up-regulate gene expression of GnRH receptors, altering the synthesis and release of both FSH and LH in response to GnRH. Lastly, activin is able to induce GnRH secretion from GnRH neurones in the hypothalamus, again stimulating both FSH and LH secretion. In contrast, inhibin and follistatin, produced in the gonads, negatively regulate gonadotrophin release by preventing activin from binding to its receptors (Gregory and Kaiser, 2004) (see Figure 1.1).

1.3.1.1.2 Kisspeptin

Kisspeptin peptides have been recently identified as essential upstream regulators, integrating central and peripheral signals with GnRH secretion (d'Anglemont de Tassigny and Colledge, 2010). The distribution of kisspeptin neurones in the hypothalamus varies between species and gender. Utilizing immunocytochemistry and *in situ* hybridisation techniques, the distribution of these neurones in rodents have been localised to discrete regions in the hypothalamus. In the adult female mouse brain they have been found in the arcuate nucleus, as a periventricular continuum of cells within the rostral section of the third ventricle, including the anteroventral periventricular nucleus (AVPV), and also a low density of cells in the dorsomedial nucleus and posterior hypothalamus (Clarkson et al., 2009). Kisspeptin fibres have been identified in close proximity to GnRH neurone cell bodies in the preoptic area in adult female mice and rats and are suggested to modulate the preovulatory GnRH and LH surges. The AVPV is sexually dimorphic in rodents, with females displaying a greater number of kisspeptin neurones than males. In primates, kisspeptin fibres from the neurones originating the arcuate nucleus come into close contact with GnRH neurone axons in the median eminence and are proposed to regulate pulsatile GnRH release (d'Anglemont de Tassigny and Colledge, 2010). In humans exogenous kisspeptin treatment (intravenous; i.v) potently stimulates the HPG axis, increasing plasma LH in males and females (Dhillo et al., 2005, Dhillo et al., 2007).

1.3.1.1.3 Sex steroid feedback

Sex steroids provide feedback loops to regulate GnRH release from the hypothalamus (see Figure 1.1). This occurs indirectly as GnRH neurones do not express receptors for oestrogen (ERα) (or androgens). It is suggested that kisspeptin neurones are responsible for mediating sex steroid actions on GnRH neurones through sex steroid inhibition of kisspeptin expression in the hypothalamus. The majority of kisspeptin neurones express receptors for oestrogen receptor alpha in the female and male mouse brain (Smith et al., 2006) and also progesterone receptors, as shown in the ewe (Smith et al., 2007), consistent with a role for sex steroid feedback on the HPG axis. The idea of a simple negative feedback model on kisspeptin has been dissolved by the discovery that sex steroids induce kisspeptin mRNA in some

Figure 1.1

brain areas while suppressing it in others. In female rodents, sex steroids decrease expression of kisspeptin in the arcuate nucleus, and increase expression in the AVPV (Gottsch et al., 2006) (see Figure 1.2). The mechanism by which this occurs remains to be fully elucidated (d'Anglemont de Tassigny and Colledge, 2010). It has recently been reported that kisspeptin is vital for the oestrogen feedback-induced full preovulatory LH surge in sheep (Smith et al., 2011) and oestrogen induces rhythmic expression of kisspeptin-1 receptor in GnRH secreting cells in the hypothalamus (Tonsfeldt et al., 2011). In rodents, the surge regulation of GnRH is mediated by oestrogen feedback acting in an inhibitory manner until the afternoon of proestrous, at which point it switches to stimulate robust GnRH release. Tonsfeldt et al. (2011) propose oscillators in the GnRH neurones are modulated by oestrogen to cause the surge, possibly through up-regulation of kisspeptin receptors which was observed following high oestrogen levels *in vitro*.

1.3.2 Pregnancy establishment

In cycling females (non-pregnant) the HPG axis is responsible for signalling development of the ovarian follicle by triggering the secretion of the sex steroids from the ovarian follicle and driving oocyte maturation. LH-driven ovulation (release of the mature egg) then occurs followed by the conversion of the follicle to the corpus luteum. The corpus luteum secretes progesterone, under the control of LH, and prepares the endometrium for implantation of a developing blastocyst. If a fertilised oocyte does not implant the corpus luteum dies after around one week (or within a day of ovulation in rodents) (Douglas, 2010). Rodents, in order to maximise reproductive efficiency, have a shorter luteal phase and LH is not solely responsible for sustaining progesterone secretion for implantation.

1.3.3 The neurogenic reflex

In female rodents, copulation induces a neurogenic reflex that triggers the pituitary to release prolactin in a series of repeated peaks on a background of increasing basal prolactin secretion in early gestation (Larsen and Grattan, 2010). Both the neurogenic reflex, which also drives LH secretion, and the induced prolactin surges are pivotal for prolonged corpus luteum function and the secretion of progesterone (state known as pseudopregnancy). In this capacity LH and prolactin take on a similar role to

Figure 1.2

human chorionic gonadotrophin (hCG) in humans (Douglas, 2011). Prolactin surges in early pregnancy are associated with altered arcuate nucleus dopamine activity (Andrews et al., 2001). Dopamine normally inhibits prolactin secretion in virgins but it is intermittently inhibited itself by the prolactin releasing factor oxytocin which increases following copulation (Helena et al., 2009, Bertram et al., 2010).

1.3.4 Human chorionic gonadotrophin

Following implantation the conceptus, surrounded by trophoblast layers, produces and releases high levels of hCG which access the maternal circulation and signal to rescue the corpus luteum and sustain progesterone release which is essential for pregnancy establishment (Druckmann and Druckmann, 2005, Arck et al., 2007). hCG is also able to bind to LH receptors located on the endometrium due to possessing an identical α subunit to LH and, thus, can communicate with the maternal tissues to facilitate the implantation process. Recent evidence suggests that hCG influences the endometrial milieu, increasing receptivity and implantation success (Perrier d'Hauterive et al., 2007, Paiva et al., 2011) and something similar has also been observed in mice (Perrier d'Hauterive, personal communication, unpublished). It has been shown that hCG increases the production by human endometrial epithelial cells of growth factors and cytokines with known roles in receptivity and trophoblast function (interleukin-11), blastocyst migration and adhesion (CXCL10) and blastocyst development (granulocyte macrophage colony-stimulating factor) (Paiva et al., 2011). Angiogenic activity of hCG has also been reported through LH receptor activation in the endometrium and hCG promotes angiogenesis partly by stimulating vascular endometrium growth factor (VEGF) (Berndt et al., 2006).

1.3.5 Progesterone

Progesterone is a key pregnancy hormone and is crucial for both the establishment and maintenance of pregnancy. Progesterone is known to be essential for pregnancy maintenance as blocking progesterone binding sites results in abortion in a number of mammalian species, including humans (Szekeres-Bartho et al., 2001, Arck et al., 2007). Mice do not suffer from "abortions", however, the presence of resorption sites in the uterine horns indicate areas of foetal loss.

Progesterone supports a number of processes required for pregnancy, including ovulation and development of the uterine and mammary glands. The production of progesterone increases during gestation and the major source of progesterone is initially the corpus luteum in humans and rodents, however, progesterone production in pregnancy is eventually sustained by the placenta in women (Arck et al., 2007). In addition to its endocrine effects progesterone also facilitates establishment of a pregnancy protective immune environment for the developing foetus (Piccinni et al., 1995).

Two progesterone receptor isoforms (PR-A and PR-B; members of the nuclear receptor superfamily of transcription factors) are expressed in various reproduction tissues. The transcriptional activity of these receptors controls the uterine immune milieu, endometrial receptivity and decidualisation, as revealed from mice lacking progesterone receptors (Arck et al., 2007). Female mice with a null mutation for the progesterone receptor gene display a number of defects in all reproductive tissues. These include an inability to ovulate, uterine hyperplasia, extremely limited mammary gland development and an inability to display sexual behaviours (Lydon et al., 1995). Studies which target the two PR isoforms individually demonstrate that they are functionally distinct transcription factors. For example, in mice where PR-B was ablated, PR-A activation alone was sufficient for pregnancy establishment and maintenance but mice displayed reduced pregnancy-stimulated mammary-gland morphogenesis (Mulac-Jericevic et al., 2003). Mice where PR-A had been ablated were infertile (associated with defective implantation), demonstrating that PR-B activation alone is not adequate for pregnancy establishment (Mulac-Jericevic et al., 2000). Therefore, the expression of both these receptor isoforms seems to be crucial for appropriate and sufficient tissue responses to progesterone in pregnancy.

1.4 The prolactin system

Prolactin is an essential reproductive hormone in women and rodents and is required for lactation. However, prolactin is described as a ‘pleiotropic’ hormone (it has a single gene which controls several distinct and apparently unrelated phenotypic effects (Grattan and Kokay, 2008)) and is now recognised to have over 300 biological activities including not just reproductive but also homeostatic roles (Bole-Feysot et al., 1998). Not only does prolactin have multiple roles but it also has

multiple sources from which it is synthesised and secreted, although this predominantly takes place in the pituitary (Ben-Jonathan, 1996, Freeman et al., 2000). Prolactin has several unusual features among the pituitary hormones. Firstly, prolactin is mainly inhibited by the hypothalamus and is spontaneously secreted to high levels if this inhibition is disrupted. This is in contrast to normal secretion from all other pituitary hormones which require stimulation from the hypothalamus. Secondly, the classical hormone-mediated feedback system is absent for prolactin as the primary target for prolactin is not an endocrine organ. Prolactin is involved in regulating its own secretion through a short-loop feedback system where it feeds back on the hypothalamus (arcuate nucleus). Thirdly, prolactin acts on many different cell types over the body and, lastly, prolactin can be produced in the brain and function as a neuropeptide in addition to a neuroendocrine hormone (Freeman et al., 2000, Torner, 2002, Grattan and Kokay, 2008).

1.4.1 Pituitary synthesis of prolactin

The cells in the anterior pituitary gland that are responsible for synthesising and secreting prolactin are termed the lactotrophs (or mammotrophs). These cells comprise 20-50% of the cellular population in the anterior pituitary gland depending on the sex status of the animal and have been identified in the mouse, rat and human using species specific antibodies. In the rat prolactin-containing cells are sparsely distributed in the lateroventral region of the anterior pituitary lobe and as a band adjacent to the intermediate lobe (Freeman et al., 2000). Lactotrophs display morphological heterogeneity (e.g. they differ in shape, secretory granule size, and prolactin content) (Velkeniers et al., 1988, De Paul et al., 1997) as well as functional heterogeneity. For example, depending on regional distribution, lactotrophs from the outer zone of the anterior pituitary respond more to thyrotrophin releasing hormone than those in the inner zone. In contrast, this is reversed for dopamine-responsive lactotrophs which are more dense in the inner zone (Freeman et al., 2000).

1.4.2 Extra-pituitary prolactin synthesis, secretion and roles

1.4.2.1 The brain

Prolactin immunoreactivity has been reported in many brain regions including the hippocampus, amygdala and brain stem (Ben-Jonathan, 1996, Freeman et al., 2000).

Multiple studies have been conducted to prove prolactin is synthesised locally in the brain (independent of the pituitary) rather than just accumulating in neurones from circulating sources. For example, some well-known pituitary prolactin stimulators (e.g. oestrogen) have been shown to induce hypothalamic prolactin production (Ben-Jonathan, 1996). Many putative functions have been attributed to prolactin in the brain including control of releasing/inhibiting factors and regulation of the sleep-wake cycle (Ben-Jonathan, 1996). Prolactin in the brain is involved in the regulation of several neuropeptides and neurotransmitters, the best known being the TIDA neurones (Grattan and Kokay, 2008).

1.4.2.2 The placenta and decidua

The placenta: The placenta has a number of endocrine roles in addition to its bi-directional foetal-maternal metabolic transport actions. It has a number of secretory products including the placental lactogen (PL) family which has been identified in many animals including rats (Colosi et al., 1988, Shiota et al., 1997), mice (Shida et al., 1992, Yamaguchi et al., 1994) and humans (Handwerger and Freeman, 2000). Human placental lactogen (hPL) is known to be involved in maternal metabolism during pregnancy and is also implicated in the regulation of foetal growth, along with placental growth hormone (Freemark, 2006).

As gestation progresses rodents, like women, also secrete PLs and at least two types have been identified in the rat. The rat placenta displays many prolactin-like molecules that are structurally similar to pituitary prolactin and act by activating prolactin receptors (Gertler and Djiane, 2002). Thus PLs are able to mediate similar effects to prolactin via prolactin receptors in other areas including the decidua and ovary/corpus luteum. At mid-gestation, when prolactin peaks diminish, PLs take over the role of prolactin in driving the secretion of progesterone from the corpus luteum. Simultaneously, PLs suppress prolactin secretion by triggering the prolactin negative feedback loop and increasing dopamine secretion (Lee and Voogt, 1999). PLs themselves are not subjected to negative feedback and thus they keep prolactin at relatively low levels at this time, until levels rise again at the end of gestation. This increase in prolactin occurs despite PL presence due to reduced dopamine secretion from TIDA neurones which become hyporesponsive to prolactin, allowing a large nocturnal surge the night before parturition (Grattan et al., 2008).

The decidua: Decidualisation is a critical step in the initiation and establishment of pregnancy in women. The main roles of the decidualised endometrium include providing a nutritive environment for the developing foetus throughout gestation and it also acts as a barrier protecting the foetus e.g. from the maternal immune system. A multitude of growth factors, cytokines and hormones, including prolactin, provide important signals for initiation and maintenance of decidualisation (Jabbour and Critchley, 2001). The human decidua, unlike the placenta, produces a prolactin-like molecule that is indistinguishable from pituitary prolactin in terms of immunological and biological criteria (Tomita et al., 1982, Freeman et al., 2000), except prolactin transcription is driven by an alternative promoter (Gerlo et al., 2006, Emera et al., 2011). The temporal expression pattern of decidual prolactin and prolactin receptors indicates a likely role in epithelial cell differentiation and implantation in humans (Jones et al., 1998). Other potential functions of decidual prolactin in early pregnancy include a role in trophoblast cell growth, angiogenesis and immune regulation (Jabbour and Critchley, 2001). In mice, decidual prolactin has been reported to silence expression of IL-6 and 20-α-hydroxysteroid dehydrogenase (HSD) genes, both of which are known to be detrimental to pregnancy maintenance: IL-6 for its pro-inflammatory properties and 20-α-HSD for its progesterone catabolism action (Bao et al., 2007).

1.4.2.3 The mammary glands and milk

Endogenous immunoreactive prolactin is detected in the epithelial cells of the lactating mammary gland of the rat and in the milk itself (Freeman et al., 2000). Although a portion of the prolactin in milk likely originates from the pituitary and travels to the mammary gland through the circulation, milk from both humans and rats has been found to contain more prolactin variants than those found in serum suggesting the mammary gland either modifies the prolactin sequestered and/or produces prolactin itself. Indeed, the mammary gland is the site where the 16-kDa prolactin variant is formed (prolactin un-cleaved is 23-kDa) (Ben-Jonathan, 1996).

1.4.2.4 The immune system

Prolactin is produced by a number of immune cells, for example, multiple lymphohemopoietic cells, such as peripheral blood lymphocytes (mainly T lymphocytes/cells) and monocytes. Prolactin plays an important role in regulating immune responses in animals and humans. As prolactin is involved in activating multiple immune responses it is implicated in a number of autoimmune diseases e.g. rheumatoid arthritis, celiac disease (De Bellis et al., 2005). Evidence suggests that lymphocytes are a source of prolactin in mice (Gala and Shevach, 1994), rats (Di Carlo et al., 1995) and humans. Immune cells from the thymus and spleen, in addition to peripheral lymphocytes contain prolactin mRNA and release bioactive prolactin (similar to pituitary prolactin) in rodents and humans (Freeman et al., 2000).

1.4.3 Prolactin secretion in non-pregnant animals

A circadian rhythm of prolactin secretion is observed in women (Freeman et al., 2000, Ben-Jonathan et al., 2008) and in rodents there is a change in rhythm at proestrous (Freeman et al., 2000). In humans, the maximum levels of plasma prolactin are reported to be during sleep and the lowest levels during the waking hours (Sassin et al., 1973, Parker et al., 1974, Caufriez et al., 2009). In rats, a preovulatory prolactin surge occurs during the afternoon of proestrous and this coincides with a surge of LH (Egli et al., 2010).

During pregnancy in women the levels of prolactin increase and this is reported in early pregnancy by 6 weeks (Ben-Jonathan et al., 2008), although it is not known whether the circadian rhythm is maintained. The secretory profile of rodents differs from women: as mentioned previously copulation induces the neurogenic reflex which generates a large nocturnal and a smaller diurnal prolactin surge on top of a steadily increasing basal level of prolactin through early-mid pregnancy (Grattan and Kokay, 2008, Larsen and Grattan, 2010).

1.4.4 Prolactin releasing factors

The search for a main prolactin-releasing factor/regulator has spanned decades. It is now considered that a single releasing factor does not exist and in fact multiple

factors contribute to suppressing the inhibitory action of dopamine on prolactin release.

1.4.4.1 The opioids

Opioids induce prolactin release by inhibiting dopamine, and are thus considered a prolactin releasing factor. The rat hypothalamus contains the soma of three classes of opioids: pro-opiomelanocortin, enkephalins and endorphins. These regulate TIDA neurones via kappa (κ) and mu (μ) receptors. In rats, during the prolactin surges at proestrous, during pregnancy, and during suckling, these interactions between opioids and the opioid receptors on TIDA neurones are especially extensive (Ben-Jonathan et al., 2008). The inhibitory effects on dopamine release may be mediated by dynorphin neurones in the arcuate nucleus which have terminals close to the TIDA neurones (Grattan et al., 2008).

1.4.4.2 Serotonin

Serotonin also stimulates prolactin secretion (Van de Kar et al., 1995, Ben-Jonathan et al., 2008). Serotonergic neurones originating in the raphe nucleus terminate in the arcuate nucleus, and lesions of the raphe nucleus prevent both sucking and stress-induced rise in prolactin in rats (Ben-Jonathan et al., 2008). However, there is conflicting data regarding the effect of serotonin agonists on the activity of TIDA neurones, and serotonin does not increase prolactin release *in vitro* (Freeman et al., 2000). It is possible serotonin exerts its effects on prolactin by stimulating other putative prolactin-releasing factors e.g. oxytocin (serotonin or serotonin agonist i.c.v treatment increases plasma oxytocin levels on rats (Jørgensen et al., 2003)).

1.4.4.3 Thyrotrophin-releasing hormone

Thyrotrophin-releasing hormone (TRH) is reputed to stimulate prolactin release and is known to increase in human pregnancy (Amino et al., 1981). TRH neurones are located in the PVN, have terminals in the median eminence and secrete TRH into the portal blood system, which is associated with increased pituitary prolactin secretion. TRH induces a rapid rise in cellular calcium, leading to elevated prolactin release, as well as induction of the prolactin gene (via protein kinase C and calcium-dependent activation of MAPK) (Ben-Jonathan et al., 2008). The prolactin-releasing action of

TRH was thought to take place in the pituitary where TRH receptors type 1 are located on the lactotrophs (Pfleger et al., 2004); however, a study in rats reported that TRH also works at the hypothalamic level, modulating the functional output of TIDA neurones (altering firing patterns) and consequently reducing its inhibitory effect on prolactin (Lyons et al., 2010, van den Pol, 2010).

1.4.4.4 Vasointestinal peptide

Vasointestinal peptide (VIP) is considered a less potent prolactin secretagogue than TRH. VIP neurones originate from the suprachiasmatic nucleus (SCN), with its fibres innervating the dopamine neurones in the arcuate nucleus (Egli et al., 2010). Lactotrophs produce and secrete VIP which maintains an elevated basal prolactin level by increasing intracellular cAMP and PKA activation. Although prolactin secretion is mildly increased by VIP in primary human pituitary cells, in humans, VIP is not considered a potent prolactin secretagogue. In addition, in VIP receptor deficient mice no modification in prolactin release is apparent (Ben-Jonathan et al., 2008).

1.4.4.5 Oxytocin

Oxytocin, produced by neurones in the PVN and SON, has been reported to be a crucial prolactin-releasing factor, playing a role in the regulation of prolactin secretion (McKee et al., 2007). The action of oxytocin on prolactin release is thought to be partly controlled by VIP. In support of this it has been found that fibres of VIP neurones also innervate oxytocin neurones in the PVN (Egli et al., 2010). Oxytocin is released at the times of heightened prolactin secretions e.g. afternoon of proestrous, following oestradiol administration and also during stress (immobilization in non-pregnant animals) (Ben-Jonathan et al., 2008). Indeed, administration of an oxytocin antagonist to rats blocks the proestrous prolactin surge (Freeman et al., 2000) and prevents suckling-induced prolactin secretion (Kennett et al., 2009).

Oxytocin is transported from the PVN via axons to the neural lobe where it is released from its terminals in the posterior pituitary (Freeman et al., 2000) and can travel to the lactotrophs. Oxytocin receptors have been identified on a sub-population of the lactotrophs (Breton et al., 1995). In rats, oxytocin is reported to be crucial for the bi-daily prolactin surges observed following cervical stimulation in

ovariectomized (OVX) rats. Administration of an oxytocin antagonist (that cannot cross the BBB) before cervical stimulation in OVX rats abolished prolactin surges (McKee et al., 2007).

1.4.4.6 Oestradiol

Prolactin release is also modulated by oestradiol, the circulating concentration of which rises between diestrous and proestrous phases of the oestrous cycle, and induces the prolactin surge on the afternoon of proestrous. It has been proposed that elevated oestrogen levels facilitate the oxytocin stimulatory actions on lactotrophs and increase prolactin release. The action/response to oxytocin at the lactotrophs is elevated during the afternoon of proestrous when oestrogen levels are high compared to diestrous. This is due to an increase in the number of oxytocin-responding lactotrophs and magnitude of their Ca^{2+}_{i} responses. An increase in oestrogen levels on proestrous may facilitate the action of oxytocin on lactotrophs, possibly through oestrogen sensitisizing the lactotrophs to oxytocin (Tabak et al., 2010). In addition, oestrogen also seems to contribute to prolactin release by inhibiting the activity of TIDA neurones, as observed by the reduced 3,4-dihydroxyphenylacetic acid (DOPAC; dopamine metabolite):dopamine ratio in the median eminence of oestrogen-treated OVX rats (DeMaria et al., 2000).

1.4.4.7 Kisspeptin

It has been recently reported that kisspeptin also regulates prolactin release (as well as LH) by inhibiting the hypothalamic dopamine neurones in female rats (Szawka et al., 2010). Kisspeptin treatment i.c.v induced prolactin release in a dose-dependent manner in oestrogen-treated OVX rats (no effect without oestrogen). Correlating with the increase in prolactin, Fos expression in tyrosine-hydroxylase-immunoreactive neurones in the arcuate (TIDA neurones) was reduced in oestrogen-treated OVX rats (no effect without oestrogen), as was DOPAC concentration and the DOPAC:dopamine ratio in the median eminence. In anterior pituitary cell cultures kisspeptin, with or without oestrogen, did not modify prolactin release. Thus, the data provide evidence for the role of kisspeptin in prolactin regulation through inhibition of dopamine neurones in the hypothalamus (Szawka et al., 2010).

1.4.5 Prolactin inhibitory factors

Pituitary derived prolactin is typically under the inhibitory control of dopamine which is released from neurosecretory hypothalamic neurones. Prolactin is thought to be regulated by three different dopaminergic systems: the TIDA, tuberohypophyseal (THDA), and the periventricular hypophyseal (PHDA) dopamine neurones (Grattan and Kokay, 2008). TIDA neurones, situated in the arcuate nucleus, provide the main source of dopamine which they release from their nerve terminals in the median eminence. Dopamine then diffuses into capillaries of the pituitary portal blood vessels and is transported to anterior pituitary where it inhibits the release of prolactin via activating its receptors on lactotrophs (Demarest et al., 1985). Dopamine activates D2 receptors located on the lactotrophs and can tonically inhibit secretion of prolactin (Freeman et al., 2000).

Thus an increase in the activity of TIDA neurones is associated with reduced prolactin secretion. Accordingly, the expression of Fos (neuronal marker of activation) in TIDA neurones has previously been reported to reflect changes in prolactin secretion, following an immune challenge (Hollis et al., 2005). Fos is not unequivocally considered a reliable predictor of basal TIDA activation (Hoffman et al., 1994) and other techniques to measure dopamine activity at TIDA neurones can also be employed. For example, the content in the median eminence of the metabolite of dopamine, DOPAC, is used to indicate TIDA/dopamine activity and an inverse relationship between prolactin levels and DOPAC levels in the median eminence has been demonstrated (Shieh and Pan, 2001). In addition, the DOPAC:dopamine ratio in the median eminence is also commonly used to measure dopamine activity (Andrews and Grattan, 2003).

1.4.6 Prolactin receptors and signal transduction

Mice express different isoforms of the prolactin receptor, termed the short and long forms. The long form is predominantly located in the hypothalamus. While both the long and short form of the prolactin receptor are expressed in the arcuate nucleus, the long-form is more highly expressed here (Pi and Grattan, 1998, Bakowska and Morrell, 2003). The short and long prolactin receptors display identical extracellular portions (thus both bind prolactin), but they differ in their ability to activate intracellular pathways (Grattan, 2002).

The intracellular domain in the long form of the prolactin receptor is constitutively associated with the tyrosine kinase, janus kinase 2 (JAK2). When prolactin binds the long form receptor it induces dimerization of the receptor molecules, followed by rapid phosphorylation of JAK2 (<1min) (Freeman et al., 2000) (see Figure 1.3). JAK2 then recruits and phosphorylates latent cytoplasmic proteins known as the signal transducer and activator of transcription (STATs). Phosphorylated (p) STAT molecules form homo- and hetero-dimers which translocate to the nucleus and bind to specific promoter sequences of target genes (Grattan, 2002). Prolactin signal transduction in numerous tissues is reported to involve several STATs, including STAT1, STAT3, STAT5a and STAT5b (Bole-Feysot et al., 1998).

The short form of the prolactin receptor does not activate the JAK-STAT pathway upon ligand binding; however, it mediates some of the actions of prolactin through mitogen-activated protein kinase (MAPK) pathways. These short form receptors are also expressed in the arcuate nucleus and could potentially have a role in regulating neuronal function (Grattan et al., 2008). In addition, prolactin is reported to regulate phosphorylation of tyrosine hydroxylase protein in TIDA neurones with multiple interacting signal transduction pathways implicated to be involved, such as protein kinases A and C, and the extracellular regulated kinases (ERK) 1 and 2 (Ma et al., 2005b, Grattan and Kokay, 2008).

Expression of prolactin receptors is remarkably low in the brain, except for in the choroid plexus which facilitates the active transport of prolactin into the brain. Prolactin receptor mRNA and binding sites are present in low levels in the rat hypothalamus, medial pre-optic area and in the median eminence (Ben-Jonathan, 1996). In the PVN, colocalisation of prolactin receptors with oxytocin and vasopressin neurones has been reported (Grattan et al., 2001a). Expression of the long form of the prolactin receptor on magnocellular oxytocin neurones in the SON and PVN is associated with prolactin regulation of oxytocin neurone activity. In addition, in non-pregnant rats i.c.v prolactin injection reduces oxytocin neurone firing (Kokay et al., 2006). In humans, high-affinity prolactin binding sites have also been reported in the choroid plexus, hypothalamus and hippocampus. As in the rat, the number of these sites are greater in hypothalamus of women compared to men (Di Carlo et al., 1992).

Figure 1.3

During pregnancy the expression of prolactin receptor mRNA in the choroid plexus increases by gestation day 7 and remains high throughout pregnancy and lactation with basal levels returning following weaning (Grattan et al., 2001a). This suggests increased access of prolactin to brain structures during gestation and lactation. Likewise, levels of prolactin receptor protein in the hypothalamus appear to increase during pregnancy and lactation with the most dramatic changes occurring in the SON and PVN magnocellular neurones. Expression of the long-form prolactin receptor on oxytocin neurones significantly increase during this period (Kokay et al., 2006). The mechanism controlling the increased expression of brain prolactin receptors during pregnancy and lactation is not clear. During pregnancy the high levels of oestrogen are reported to drive increased production of the short and predominantly the long forms of the prolactin receptor in chloroid plexus, aiding the entry of prolactin to the brain (Augustine et al., 2003, Pi et al., 2003). It may be that prolactin along with other pregnancy hormones (e.g. progesterone, oestrogen) all have a role in mediating the up-regulation of prolactin's own receptors during pregnancy (Grattan et al., 2001a).

1.4.7 Prolactin negative feedback

Prolactin activates a short-loop feedback system to regulate its own secretion by activating receptors on TIDA neurones (see Figure 1.4). The long form of the prolactin receptors are expressed on TIDA neurones (Kokay and Grattan, 2005) and ligand-induced activation stimulates expression of the tyrosine hydroxylase gene. Tyrosine hydroxylase is the rate-limiting enzyme involved in the biosynthesis of dopamine. Therefore, prolactin induces dopamine synthesis and indirectly suppresses its own synthesis(Grattan and Kokay, 2008). This is termed prolactin negative feedback (Ma et al., 2005a, Ma et al., 2005b). Activation of the TIDA neurones via prolactin receptor binding involves activation of the JAK2/ STAT5 signalling transduction pathway (Grattan et al., 2001b, Ma et al., 2005a). pSTAT5b is reported to specifically mediate prolactin negative feedback on the TIDA neurones. In rats, nuclear translocation of pSTAT5 is induced by ovine prolactin administration intraperitoneally (i.p) (Lerant et al., 2001). STAT5b deficient mice express elevated serum prolactin levels, although mRNA levels and immunoreactivity of tyrosine hydroxylase are low. This suggests signal transduction is impaired and demonstrates

Figure 1.4

that pSTAT5b is crucial for mediating prolactin negative feedback in TIDA neurones (Grattan et al., 2001b).

1.4.8 The prolactin system in pregnancy

Prolactin is essential to rodents and facilitatory in women to drive the secretion of progesterone. It acts as a luteotrophic hormone by maintaining the integrity of the corpus luteum following mating and is reported to be a potent survival factor for human granulosa cells. If prolactin levels are diminished the formation of the corpus luteum is compromised (Perks et al., 2003). In rodents prolactin drives progesterone secretion in two ways: it potentiates the steroidogenic effects of LH (through interaction with the LH receptors) and inhibits 20-α-HSD, an enzyme which inactivates progesterone (Freeman et al., 2000). The prolactin secretion in early pregnancy in rodents is thought to be essential for rescuing the corpus luteum and mice lacking prolactin receptors are unable to achieve pregnancy as a result of implantation failure due to decreased LH receptor expression in the corpus luteum, high 20-α HSD levels and inadequate progesterone levels (Bachelot et al., 2009). At mid-pregnancy, these prolactin peaks diminish and placental lactogens (PLs) take over the role of prolactin to drive progesterone secretion from the corpus luteum. Simultaneously, PLs trigger negative feedback on prolactin secretion and thus levels of prolactin decrease at this time until rising again at the end of gestation (Lee and Voogt, 1999). The secretory profile differs in women compared to rodents (see Figure 1.5). In women, increasing prolactin levels are reported in early pregnancy, from around gestation week 6, up to term (Ben-Jonathan et al., 2008). It has been reported plasma prolactin levels increase from around 6ng/ml (at week 8) to 210ng/ml near term (Biswas and Rodeck, 1976) and hPL is secreted after gestation week 6 in maternal and foetal circulations and also increases as pregnancy proceeds (Handwerger and Freeman, 2000).

1.4.8.1 Prolactin negative feedback during pregnancy

Prolactin negative feedback is maintained during early-pregnancy prolactin surges (Demarest et al., 1983, Grattan et al., 2008). Accordingly, TIDA neurones display a semicircadian pattern of activity, with reduced dopamine activity during the prolactin

Figure 1.5

surges and increased activity between them, thus allowing initiation and termination of the surges (McKay et al., 1982). However, during late pregnancy and lactation, there is an absence of short-loop negative feedback on TIDA neurones by prolactin (Grattan and Averill, 1995). TIDA neurones become less able to secrete dopamine in response to prolactin, resulting in a state of hyperprolactinemia. Several physiological changes in the regulatory pathways that control prolactin secretion occur during late pregnancy. The placenta produces placental lactogens in rodents (Lee and Voogt, 1999) and humans (Handwerger and Freeman, 2000). These bind to and activate prolactin receptors and are not subject to the normal inhibitory control by dopamine. Therefore, the short-loop feedback is bypassed in late pregnancy/lactation. This allows continued activation of the prolactin receptors, resulting in elevated progesterone secretion while simultaneously suppressing prolactin release itself (Grattan, 2002). TIDA neurones have also been shown to have a weakened capacity to respond to prolactin in late pregnancy, with reduced TIDA activation compared to early gestation (Grattan and Averill, 1995, Andrews et al., 2001). Prolactin secretion is able to resume at this time despite the continued presence of placental lactogens. Negative feedback is also impaired during lactation, providing the high prolactin levels required for milk production and maternal behaviour (Grattan et al., 2008). Suckling also stimulates prolactin release via a suckling-induced reduction in TIDA neurones activation (Selmanoff and Gregerson, 1985).

During late pregnancy and lactation, prolactin's ability to induce STAT5b phosphorylation and nuclear translocation is suppressed. This appears to be associated with a rise in suppressors of cytokine signalling (SOCS) mRNA which may underlie the reduced TIDA neurone dopamine output and high prolactin levels observed during this period (Anderson et al., 2006a). SOCS act as feedback inhibitors of signalling for a number of cytokines that utilize the JAK/STAT pathways and are involved in suppressing STAT phosphorylation and thus dampen signal transduction (Starr and Hilton, 1999).

1.5 Immune system in pregnancy

1.5.1 The cellular T system

The most important components of the cellular immunosystem in pregnancy are the helper T (Th) cells (reviewed in (Guerin et al., 2009)). Briefly, these cells can be differentiated into two polarised forms (Th1 and Th2) which display different effector responses. Th 1 cytokines (e.g. TNF-α) stimulate various cell-mediated cytotoxic and inflammatory responses. Th 2 cytokines (e.g. IL-10) are involved in B cell antibody production and are required for the secretion of hPL and hCG (Druckmann and Druckmann, 2005). The Th1:Th2 paradigm has recently been extended to include Th17 cells (producers of pro-inflammatory cytokine IL-17) and regulatory T (Treg) cells. Th17 cells are involved in induction of inflammation and the pathogenesis of rejection, while Treg contribute to immunoregulation and peripheral tolerance (Saito et al., 2010).

1.5.2 Immune balance in pregnancy

It has been proposed that normal pregnancy is characterised by a Th2 bias. High levels of Th2 cytokine production and release have been reported to be involved foetal allograft survival and pregnancy maintenance (Piccinni et al., 1995, Piccinni, 2006). Pregnant women with a history of successful pregnancies have significantly higher concentrations of Th2 cytokines in the first trimester compared to women undergoing unexplained recurrent spontaneous abortion who have significantly higher concentrations of Th1 cytokines compared to the women with successful pregnancies. Therefore, this study supports the theory that there is a Th2 bias in normal successful pregnancy (Raghupathy et al., 2000). However, evidence exists to the contrary with a study reporting a Th2 predominance in recurrent foetal loss (Saini et al., 2011). Together these studies indicate that early pregnancy maintenance is dependent upon procuring a suitable cytokine balance, with slightly higher levels of Th2 cytokines and lower levels of pregnancy-threatening Th1 cytokines.

1.5.3 Uterine dendritic cells

Uterine dendritic cells in humans and mice are proposed to control foetal tolerance and rejection (Kammerer et al., 2000, Gardner and Moffett, 2003, Blois et al., 2005a). Dendritic cells are the most potent antigen presenting cells involved in both

the innate immune response and maintenance of tolerance (Arck et al., 2007). Immature dendritic cells are characterised by low production of pro-inflammatory cytokines, high production of IL-10, and enhanced ability to induce regulatory T cells with suppressive actions, which all contribute to promoting pregnancy maintenance. These immature dendritic cells are found in early pregnancy decidua in women (Kammerer et al., 2000) and mice (Blois et al., 2005b).

1.5.4 Progesterone as an immunosteroid

Alongside its endocrine effects progesterone acts as an 'immunosteroid' facilitating maternal tolerance to the fetal 'semi-allograft'. In this capacity, progesterone blocks very early T-cell lymphopoiesis (Tibbetts et al., 1999) and plays a role in providing a pregnancy protective immune milieu (Arck et al., 2007). It has been shown that progesterone favors development of Th cells which produce Th2 cytokines and an increase in Th2 cytokine production has been implicated to be involved in fetal allograft survival (Piccinni et al., 1995). Progesterone exerts an anti-abortive effect by stimulating progesterone-induced blocking factor (PIBF). It has been shown treatment with anti-PIBF IgG results in increased splenic natural killer (NK; lymphocytes which are a major component of the innate immune system) cell activity, and the rate of NK activity correlates with the rate of resorptions. Therefore, the antiabortive effect of PIBF in mice appears to be mediated by reducing NK activity (Szekeres-Bartho et al., 1997a) via interfering with arachidonic acid metabolism. It has been reported that neutralization of endogenous PIBF by a PIBF-specific antibody terminates pregnancy in mice (Szekeres-Bartho et al., 1997a, Szekeres-Bartho et al., 1997b). Lymphocytes and decidual $CD56^{+}$ cells synthesize PIBF and alter cytokine secretion. PIBF changes the cytokine profile of activated lymphocytes, favouring production of Th2 cytokines over Th1 cytokines. For example, PIBF increases interleukin (IL)-10 production and decreases IL-12 production (Szekeres-Bartho and Wegmann, 1996). However, a study in humans failed to demonstrate the expression of progesterone receptor mRNA in human decidual NK cells, thus, in women a direct role progesterone in attaining a suitable PIBF and cytokine environment is not clear (Henderson et al., 2003).

In addition, progesterone has been found to inhibit mature dendritic cells as well as dendritic cell-induced proliferation of T cells in a receptor-mediated manner,

indicating progesterone directly suppresses the ability of mature rat decidual cells to drive proinflammatory responses (Butts et al., 2007).

1.5.5 Prolactin regulation of cytokine balance

Prolactin, along with its wide variety of reproduction specific actions e.g. initiation and maintenance of lactation, is known to have a role in immunomodulation (Yu-Lee, 2002). Prolactin regulates the cytokine profile, and has been demonstrated to modulate production of IL-10 and IL-12 in a stimulus-specific fashion (Matalka and Ali, 2005). Prolactin is often referred to as a cytokine itself, with activation of its receptor driving intracellular JAK/STAT signaling. In addition, some features of cell responses common to prolactin are also associated with other cytokines, such as suppressors of cytokine signaling (SOCS) (Anderson et al., 2006a). Thus cross-talk between the responses can occur and have been reported in some tissues such as the mammary glands. Prolactin has been found to regulate SOCS3 expression and prolactin-induced SOCS3 acts as an anti-proliferative agent in breast cancer cells (Barclay et al., 2009).

In addition, prolactin has been shown to be protective against inflammation following severe trauma (Knoferl et al., 2000), as has oestrogen (Jarrar et al., 2000). Gender appears to play a role in trauma response with female patients having higher survival rates than males (Morris et al., 1990), supporting the idea female hormones may have a provide protection against hemorrhage and/or septic complications. It has been reported that male trauma patients have a higher susceptibility for infections, correlated with heightened serum proinflammatory cytokines such as IL-6 compared to females (Oberholzer et al., 2000). In rodent models, trauma-hemorrhage leads to depressed immune function and increased infection, morbidity and mortality. However, the administration of prolactin, as well as oestrogen, protected against trauma-hemorrhage, reducing plasma levels of IL-6 and corticosterone and subsequently improving survival to septic shock (Zellweger et al., 1996, Knoferl et al., 2000). These studies suggest that under stress conditions prolactin and oestrogen protect against inflammation and improve immune responses.

Prolactin and oestrogen also suppress IL-6 gene expression in female reproductive tissues (Deb et al., 1999a). During pregnancy decidual expression of IL-6 is suppressed by prolactin and oestrogen and it is known that elevated IL-6 levels are

associated with pregnancy failure. These two hormones down-regulate the expression of the gp130 component of the IL-6 receptor complex (Kurebayashi et al., 1997, Deb et al., 1999a). Decidual prolactin is also known to silence other genes thought to be detrimental to pregnancy, such as 20α-HSD (enzyme responsible for degrading progesterone) (Bao et al., 2007).

1.6 Effect of stress on the neuroendocrine system

As indicated earlier, environmental conditions and stress can disrupt the neuroendocrine systems in an attempt to overcome or compensate for the adverse stimuli e.g. to maintain homeostasis, conserve energy, etc.

1.6.1 Stressors

Stress can be experienced due to acute or sustained exposure to adverse conditions and are generally categorized as either psychological or physical. Different brain circuitries deliver information about these stress types to the hypothalamo-pituitary-adrenal (HPA) axis: stressors which pose an immediate physiological threat are primarily relayed directly from the brainstem, while psychological stressors are mainly processed through the limbic forebrain (Herman and Cullinan, 1997). Psychological stressors can be mild such as a novel environment or sustained noise, or it can be severe such as physical and mental abuse, or a repeated high pressure environment (e.g. high work demands/responsibility). Physical stressors include immune stress, hunger (including poor diet) or physical insult. Acute stressors appear abruptly over a relatively short period of time and lead to acute, transient symptoms (Douglas, 2010). Stress hormones and other mediators e.g. neurotransmitters, cytokines, are required for adaptation to stress (process termed “allostasis”) in order to maintain homeostasis. When released in response to stressors they promote adaptation and are generally beneficial often preventing further damage/effects (McEwen, 2004). In contrast, during chronic stress episodes which are of a longer duration and are recurring, these mediators are not turned off and are overused. This results in escalating changes that lead to wear-and-tear on the body/brain (termed “allostatic load/overload”) which can cause long-term health problems (McEwen, 1998). For example, allostatic overload can lead to impaired immunity, atherosclerosis (increasing risk of myocardial infarction), obesity and atrophy of

brain nerve cells. These are often observed in cases of depressive illnesses and chronic anxiety disorders (McEwen, 2004).

1.6.2 The stress system and hypothalamo-pituitary-adrenal axis

The stress system coordinates the adaptive responses of the individual to stressors and its activation results in behavioural and peripheral changes that allow the return to homeostasis and increase the chance of survival. The stress system encompasses the HPA axis, and the locus coeruleus–noradrenaline autonomic systems (Tsigos and Chrousos, 2002). Stress is defined as the activation of HPA axis and the consequent release of its hormones (Parker and Douglas, 2010). Exposure to stress triggers the increased secretion of CRH and vasopressin from hypothalamic PVN. During periods lacking stress both of these hormones are secreted in a circadian, pulsatile pattern (2-3 secretory pulses per hour) (Engler et al., 1989) and the amplitude of the pulses increases in the early morning (Horrocks et al., 1990). These two neuropeptides act synergistically to induce secretion of ACTH from the pituitary corticotrophs (Lamberts et al., 1984). During acute stress, the amplitude and synchronization of the CRH and AVP pulses into the hypophyseal portal system significantly increases, leading to a rise in ACTH secretion (Charmandari et al., 2005). ACTH subsequently stimulates the release of the glucocorticoids (cortisol in humans, corticosterone in rodents) from the in the cells of the zona fasciculata of the adrenal cortex (see Figure 1.6). Glucocorticoids are the final effectors of the HPA axis and they carry out their roles through ubiquitously distributed intracellular glucocorticoids receptors (Charmandari et al., 2005). Glucocorticoids are known to support a wide array of physiological systems and have important roles in cardiovascular tone, immunity/inflammation, metabolism, behaviour, reproduction and neural function (Sapolsky et al., 2000).

1.6.3 Glucocorticoid negative feedback

Glucocorticoids have an important role in regulating basal HPA axis activity and terminating the stress response and exert negative feedback effects on the HPA axis via extra-hypothalamic centres, including the hippocampus (Jacobson and Sapolsky, 1991, Furay et al., 2008), as well as the hypothalamus and the pituitary to suppress the release of CRH and ACTH (de Kloet et al., 1998) (see Figure 1.6). Negative

feedback limits the duration of tissue exposure to glucocorticoids, minimising the catabolic, immunosuppressive and anti-reproductive effects of these hormones (Charmandari et al., 2005) and it also prevents stress hormone depletion so as to maintain levels sufficient enough to mount successive stress responses (Sapolsky et al., 2000, Tasker and Herman, 2011). Glucocorticoid type II receptors are responsible for the negative feedback control of CRH and ACTH release (Charmandari et al., 2005). Chronic stress attenuates the negative feedback system by glucocorticoids, reportedly involving alterations in glucocorticoid receptor expression in the higher centres of the HPA axis: prefrontal cortex and hippocampus (Mizoguchi et al., 2003).

1.6.4 Mode of action of glucocorticoids

Glucocorticoids bind to and activate cytoplasmic glucocorticoid receptors resulting in their translocation to the nucleus. Here they interact as homodimers with the specific glucocorticoid responsive elements (GREs) in the promoter regions of hormone-responsive genes (e.g.lipocortin-1) and regulate the expression of glucocorticoid-responsive genes positively or negatively (Charmandari et al., 2005). However, the main anti-inflammatory effects of glucocorticoids appear to be predominantly due to interaction between the glucocorticoid receptor and transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which mediate the expression of pro-inflammatory genes (Hayashi et al., 2004).

1.6.5 The autonomic nervous system

The autonomic nervous system (ANS) rapidly responds to stressors and controls a wide variety of functions. The sympathetic and/or parasympathetic nervous systems (SNS, PNS) are involved in regulating a number of different areas including the cardiovascular, respiratory, gastrointestinal, renal, and endocrine systems. The parasympathetic system is able to aid or inhibit sympathetic functions through either by reducing or increasing its activity, respectively (Tsigos and Chrousos, 2002, Charmandari et al., 2005).

Both the neuroendocrine and the ANS stress responses converge on the adrenal glands, albeit on different timescales and with differing results. The neuroendocrine

Figure 1.6

response takes place over a slower timescale and via hypothalamic-pituitary hormones induces adrenal glucocorticoid secretion. The autonomic response is much faster and is triggered by sympathetic neural outputs (Tasker and Herman, 2011). Typically, stress rapidly and transiently increases adrenaline and noradrenaline secretion from the adrenal medulla and from sympathetic nerve endings (which innervate the smooth muscle of the vasculature, skeletal muscle and many organs including the heart, kidneys, etc.) (Tsigos and Chrousos, 2002). These catecholaminergic hormones carry out a more rapid stress response than glucocorticoids and supply an immediate energy source by mobilising glucose, and redirecting blood supply to from less essential processes to skeletal muscle. This allows 'flight' if necessary and facilitates stress coping mechanisms. In addition to peripheral responses, central ANS and SNS responses to stress induce activation of noradrenergic pathways from the brainstem to the limbic system and hypothalamus (Douglas et al., 2005, Douglas, 2010). Central adrenaline serves as an alarm system that reduces neurovegetative functions (e.g. eating, sleeping), accompanied by increases in endocrine/autonomic responses to stress (Tsigos and Chrousos, 2002).

1.6.6 Noradrenaline and the hypothalamo-pituitary-adrenal axis

A reciprocal neural connection exists between CRH and the LC-noradrenaline system (see Figure 1.7). CRH and noradrenaline stimulate each other, the latter principally through α1-adrenergic receptors (Tsigos and Chrousos, 2002). Noradrenaline from brainstem nuclei (A1, A2 and A6 cell groups in particular) is a major excitatory input to the PVN (Pacak et al., 1995a) and α1-adrenergic receptor agonists stimulate HPA axis activity (Itoi et al., 2004). However, noradrenaline has also been found to have inhibitory as well as stimulatory effects on CRH activity and HPA axis response to stress via α1-adrenergic receptors. In addition, central blockade of these receptors does not prevent restraint stress-induced increase in CRH mRNA expression in the PVN, thus, induction of HPA effects via noradrenergic inputs is stress specific and is utilised by most but not all stressors (Douglas, 2005a).

1.6.7 Stress-induced behavioural responses

Interactions of noradrenaline with various neuroendocrine systems leads to behavioural responses to stress such as fear and anxiety. Noradrenaline has been

shown in humans to mediate amygdala (principle brain region involved in fear conditioning) activity in humans when processing emotional stimuli (van Stegeren et al., 2005). Studies in rodents suggest that elevated noradrenaline levels/activity in the hypothalamus, amygdala and LC are involved in the provocation of anxiety and/or fear following stress exposure (Tanaka et al., 2000, Morilak et al., 2005). Thus, the central noradrenergic system is thought to be involved in a number of anxiety disorders, especially panic disorders and post-traumatic stress. This is supported by the finding that an α2-adrenergic agonist effectively alleviates anxiety symptoms while an α2-adrenergic antagonist intensifies them in patients with anxiety disorders, suggesting the central noradrenergic system is linked to emotional responses (Charney, 2003, Itoi and Sugimoto, 2010).

1.6.8 Effect of stress on the hypothalamo-pituitary-gonadal axis

Stress is well known to disrupt and suppress reproductive function in humans (Nakamura et al., 2008). For example, under-nutrition and intensive exercise in adolescents is associated with delayed menarche (Rogol et al., 2000), and a number of different stressors are known to reduce fertility (Nakamura et al., 2008, Ruder et al., 2009, Douglas, 2010). Limited studies in non-pregnant humans and primates suggest that CRH suppresses gonadotrophin secretion (Olster and Ferin, 1987, Barbarino et al., 1989). In contrast, it has been reported i.v. administration of human CRH had no inhibitory effect on gonadotrophin secretion in pre- or post-menopausal women (Fischer et al., 1992). However, there are many reports in rodents that acute stress disrupts gonadotrophin secretion, reduces LH pulsatility (Li et al., 2003) and elevates prolactin release (Gala, 1990, Poletini et al., 2006). Typically stress increases progesterone secretion in non-pregnant female mammals (Nakamura et al., 1997), which could be partly accounted for by the associated increase in prolactin secretion as prolactin is known to drive progesterone secretion.

1.6.8.1 Potential mechanisms mediating stress-suppression of the HPG axis

Mounting evidence suggests that stress suppresses the activity of the HPG axis, particularly its central regulator, the GnRH pulse generator. It is thought that inhibition occurs here by stress-induced activation of the CRH, sympathoadrenal

Figure 1.7

systems and the limbic brain (Li et al., 2010) via projections to the LC. The LC noradrenergic neurones project to the preoptic area where they have been demonstrated to inhibit GnRH neurones via gamma aminobutyric acid (GABA) secretion/activation (Mitchell et al., 2005).

Hypothalamic mRNA expression of kisspeptin and its receptor are down-regulated in the mPOA and arcuate nucleus following a variety of stressors (e.g. restraint, immune challenge) in female rats (Kinsey-Jones et al., 2009) and CRH administration i.c.v in female mice. In light of the role of kisspeptin signalling in regulating the HPG axis, this suggests that stress-reduced kisspeptin expression may contribute to stress-related suppression of LH release (Kinsey-Jones et al., 2009).

1.6.8.2 Stress-induced cytokines and the HPG axis

Stress-related cytokines can also affect the brain and pituitary, and subsequently the HPG axis. Cytokines are thought to potentially gain access to the brain via a number of different pathways, including the 'leaky' blood brain barrier regions (e.g. in the arcuate nucleus or via the brainstem). It has been shown that stress-induced cytokines can inhibit GnRH pulse generator activity. For example, LPS administration to rats has been reported to suppress the electrical activity of the GnRH pulse generator, reflected in reduced LH pulsatile secretion, which is at least partially mediated by TNF-α (Yoo et al., 1997).

1.6.9 Effect of stress on prolactin secretion

Acute stress typically increases prolactin secretion in non-pregnant rodents and humans (Nicoll et al., 1960, Armario et al., 1996, Freeman et al., 2000). Chronic sustained stress (around-the-clock intermittent foot shock) to both male and female (non-pregnant) rats increases anterior pituitary prolactin mRNA levels after 1 day and more so after 3 days. However, after 14 days there was no significant difference in prolactin mRNA compared to controls, with levels returning to pre-stress values (Dave et al., 2000). Various stressors have been demonstrated to induce prolactin secretion in rodents, such as immune challenge, restraint stress and ether exposure (Freeman et al., 2000). In humans, circulating prolactin levels have been shown to increase following the Trier social stress test (Kudielka and Wüst, 2010). However, in the male hamster social conflict and mild footshock decrease prolactin (Huhman et

al., 1995). Thus, the effect of different stressors on prolactin can be species/strain-specific e.g. restraint stress increases prolactin in C57BL/6 mice but not in BALB/cJ mice (Meerlo et al., 2001). It has been reported that the stress effects on prolactin are dependent on the pre-stress levels of prolactin: stress enhances prolactin release if pre-stress levels are low, and suppresses its release if pre-stress levels are high, for example, during the proestrous afternoon surge (Gala, 1990). However, in female rats it has been reported that the prolactin secretory response to stress is dependent upon the plasma levels of ovarian steroids rather than pre-stress levels (Poletini et al., 2006). Ether stress increased both low (e.g. morning of proestrous, OVX rats) and high (e.g. oestrogen-treated OVX rats) plasma prolactin levels. However, stress reduced the high prolactin levels observed on the afternoon of proestrous and also in OVX rats treated with oestrogen and progesterone. Thus data suggest oestrogen and progesterone modulate stress-induced prolactin release, regardless of pre-stress levels. Poletini et al. (2006) also investigated the role of the LC in the prolactin stress response but found that lesions to the LC did not affect the degree of the prolactin stress response. LC lesions did, however, suppress the prolactin surges on the afternoon of proestrous, and in oestrogen-treated and oestrogen and progesterone-treated OVX rats, indicating a specific role in ovarian steroid-induced prolactin surges.

1.7 Effects of stress on the neuroendocrine system during pregnancy

1.7.1 HPA axis

Typically human gestation is associated with increased HPA axis function as pregnancy progresses, leading to elevated circulating ACTH and cortisol. Total and free plasma cortisol levels rise in parallel across pregnancy, and elevated levels are observed as early as gestation week 11. The precise cause(s) of increased ACTH is currently unclear but may include placental synthesis and release of CRH and ACTH, pituitary desensitization to cortisol negative feedback or heightened response to corticotrophin releasing factors e.g. vasopressin and CRH (Lindsay and Nieman, 2005).

1.7.2 Foetal programming

The foetus is protected from the effects of maternal hypercortisolism by the placental enzyme 11-β hydroxysteroid dehydrogenase (11β-HSD2) in early gestation. Active glucocorticoids, cortisol and corticosterone, are converted by 11β-HSD2 in the placenta into their inactive metabolites (cortisone and 11-dehydrocorticosterone, respectively). This ensures foetal glucocorticoid levels are lower than maternal concentrations, which is critical as exogenous glucocorticoids have been associated with foetal growth retardation in both humans and experimental animals (Seckl et al., 2000). Epidemiological data suggests that a non-optimum foetal environment *in utero* has profound effects, permanently and adversely programming the physiology of the foetus (so called 'foetal programming'). Prenatal elevated levels of glucocorticoids, for example due to maternal stress, may lead to increased risk of cardiovascular, metabolic and neuroendocrine disorders in later life (Seckl and Meaney, 2004).

1.7.3 Early Pregnancy

1.7.3.1 HPA axis basal activity in early pregnancy

In rodents in early pregnancy, the secretion of ACTH and corticosterone is similar to virgins in the early light phase when HPA axis activity is at the circadian nadir. The expression of CRH and vasopressin genes in the parvocellular PVN, and glucocorticoid and mineralocorticoid receptor mRNA in the PVN and hippocampus are also unchanged in early pregnancy (reviewed in (Brunton et al., 2008)). However, the circadian pattern of basal HPA activity alters in very early pregnancy with peak circulating concentrations of ACTH and corticosterone attenuated in the late light phase compared to di-oestrous (Atkinson and Waddell, 1995). It has been reported that the circadian pattern continues to be suppressed as pregnancy progresses, with corticosterone levels decreasing until mid-pregnancy in the rat, when levels then begin to rise once more (Atkinson and Waddell, 1995). A similar reduced basal HPA axis profile is observed in women during early pregnancy. Typically, salivary cortisol levels are found to be markedly lower in early gestation compared to late pregnancy, with the difference between nadir and peak cortisol levels increasing as pregnancy proceeds (Obel et al., 2005, Harville et al., 2007).

1.7.3.2 HPA axis responses to stress in early pregnancy

Studies into the HPA axis responses to stress in women are limited and glucocorticoid release in response to stress in early pregnancy is only sparsely reported. Acute stress tests such as the Trier Social Stress Test (public speaking) at the start of the second trimester of pregnancy reveal that cortisol levels robustly increase (Nierop et al., 2008), however, it is not clear what the effects of prolonged stress are on cortisol secretion in early pregnancy. In a study investigating perceived stress levels in women during early pregnancy, no correlation was apparent between the degree of perceived stress and salivary cortisol (Obel et al., 2005). Unfortunately this study did not report pregnancy outcome and from the literature it is not possible to categorically conclude that high cortisol levels always result in pregnancy loss or programming (Nakamura et al., 2008). A study into the effects of stressful work on pregnancy found no correlation with pregnancy loss, although cortisol levels were not measured (Fenster et al., 1995).

In rodents, HPA axis responses to stress exposure in mid pregnancy are robust and do not differ from virgin animals (Arck et al., 1995, Brunton et al., 2008). Rodents that are exposed to stress at the peri-implantation period display increased secretion of ACTH, despite the risk to pregnancy due inhibition of progesterone. As in virgins, transient high levels of glucocorticoids prevail in early pregnancy following stress, suggesting this alone may not be acutely detrimental to pregnancy maintenance and homeostasis can be restored via rapid negative feedback (Douglas, 2010). However, in women the peri-implantation window may be particularly sensitive to stress and glucocorticoid action as elevated salivary cortisol levels are associated with foetal loss during this period (Nepomnaschy et al., 2006), while studies performed during the later stages of early pregnancy in women do not report this association (Douglas, 2010).

1.7.3.3 Reproductive hormones and stress in early pregnancy

1.7.3.3.1 hCG responses to stress in early pregnancy

The main gonadotrophin in early (first trimester) human pregnancy is hCG and reports on the effects of stress on hCG release are varied and conflicting. Stressful situations have been shown to reduce circulating hCG (low hCG is associated with miscarriage) (Gagnon et al., 2008), to not alter hCG levels at all (Milad et al., 1998),

or to increase circulating hCG levels (Kharfi Aris et al., 2007). Therefore, hCG is not considered a reliable first trimester marker or predictor of miscarriage.

1.7.3.3.2 LH and prolactin responses to stress in early pregnancy

The effects of stress on LH and prolactin secretion in pregnancy have not been extensively studied in humans and thus are not reported in the literature to my knowledge. Although maternal smoking is reported to decrease placental hormone levels generally (e.g. placental growth hormone, hCG, hPL) (Coutant et al., 2001, Varvarigou et al., 2009) it is not known or been reported what the responses to other stressors are. Rodent studies provide an insight into the effects of stress on pregnancy hormones which may alter gonadotrophin responses. In rodents LH secretion is typically suppressed by rising levels of exogenous pregnancy hormones progesterone and oestrogen via negative feedback mechanisms acting centrally and at the pituitary (Li et al., 2003, d'Anglemont de Tassigny and Colledge, 2010). Therefore, stress-disrupted progesterone in early pregnancy (see next section) may impact on LH release due to reduced negative feedback. This theory is supported by the finding that immune stress in early pregnant mice increases LH secretion (Erlebacher et al., 2004), however these mice also displayed attenuated ovarian expression of LH receptors which would decrease steroidogenesis.

1.7.3.3.3 Progesterone responses to stress in early pregnancy

Increasing evidence for a reduction in progesterone (and PIBF) following stress in early pregnancy is emerging in mice, which is in contrast to the elevated stress-induced progesterone levels in non-pregnant rodents (Nakamura et al., 1997). Various stressors, including 24h noise and immune stressors, have been reported to reduce circulating progesterone in early pregnancy (Joachim et al., 2003, Blois et al., 2004, Erlebacher et al., 2004, Parker and Douglas, 2010, Parker et al., 2011b). Noise stress on day 5 of pregnancy in an abortion-prone mouse model (DBA/2J-mated CBA/2J female mice) significantly decreases progesterone and PIBF levels and increases in the percentage of resorptions (Joachim et al., 2003). However, injection of the progesterone derivative Dydrogesterone, immediately prior to stress exposure, abrogated stress-induced resorptions in a dose-dependent manner. In addition, PIBF levels and the production of the pregnancy protective cytokine IL-4 significantly

increased with Dydrogesterone treatment (Joachim et al., 2003), highlighting the importance of progesterone for pregnancy maintenance. In women, low progesterone levels in early pregnancy have been shown to be predictive of later pregnancy failure (Elson et al., 2003, Arck et al., 2008).

1.7.4 Late pregnancy

1.7.4.1 HPA axis basal activity in late pregnancy

It is difficult to assess the maternal HPA axis in late pregnant women due to the fact that the placenta also produces and secretes HPA axis peptides/steroids (Chen et al., 1986, Sasaki et al., 1988). This is not the case in rodents where very late pregnancy and birth is associated with attenuated basal activity of the HPA axis in the rat (Brunton et al., 2008). For example, expression of CRH and vasopressin mRNA in the pPVN are reduced (Johnstone et al., 2000), associated with decreased CRH content in the median eminence (Ma et al., 2005c) and reduced mRNA expression of POMC (the ACTH precursor) (Brunton et al., 2008). It is possible that heightened glucocorticoid negative feedback may underlie the reduced basal HPA activity, supported by reports of elevated glucocorticoid receptor mRNA levels in the hippocampus during late pregnancy. However, functional studies in late pregnant rats do not confirm an active increase in negative feedback and therefore reduction in the forward drive, rather than increased negative feedback, may underlie hyporesponsiveness of the HPA axis to stress in late pregnancy (Johnstone et al., 2000).

As pregnancy progresses circulating corticosterone levels rise from mid-pregnancy until term in the rat (Atkinson and Waddell, 1995). In women plasma cortisol levels have been reported to increase progressively from gestation week 12 to week 26, at which point they stabilise until parturition (Carr et al., 1981). Throughout gestation the metabolic clearance of corticosterone is sustained at pre-pregnancy levels in rodents, although free corticosterone levels decline due to an increase in corticosterone binding globulin (CBG) (Douglas et al., 2003, Brunton et al., 2008).

1.7.4.2 HPA axis responses to stress in late pregnancy

Stress-induced HPA axis activity is strongly reduced by late pregnancy in women (Kammerer et al., 2002) and rodents (Douglas et al., 2003, Russell et al., 2008) and presumably protects the foetus from the adverse programming effects of glucocorticoids. In women, exogenously administered CRH does not evoke an increased circulating ACTH or cortisol response in late pregnancy (Schulte et al., 1990) and they also become insensitive to cold stress with suppressed salivary cortisol levels (Kammerer et al., 2002). In rats, HPA axis responses to various psychological stressors (e.g. noise stress, forced swimming (Neumann et al., 1998)) and physical stressors (e.g. immune challenge (Brunton and Russell, 2008)) decline in the last week of pregnancy, reflected as decreased ACTH and corticosterone secretion. HPA axis hyporesponsiveness continues through parturition (Neumann et al., 2003) and lactation until weaning takes place (Windle et al., 1997).

1.8 Stress and the immune system in pregnancy

Mounting evidence indicates an immune-endocrine disequilibrium in response to stress, with a number of mediators (including cytokines) implicated to contribute to stress-induced pregnancy loss (Arck et al., 1995, Nakamura et al., 2008). Reciprocal regulation exists between the central nervous system (CNS) and the immune system, whereby immune factors/cytokines crosstalk with the neuroendocrine-endocrine systems. The CNS signals to the immune system via the endocrine stress responses previously mentioned, and the immune system signals to the CNS via cytokines. Cytokines are considered important in alerting the CNS to immune challenge and during inflammation they are produced and released from the affected area and immune cells and signal to the brain, causing fever and sickness symptoms (Webster et al., 2002). Dysregulation of the delicate communication system threatens pregnancy maintenance.

1.8.1 HPA regulation of the immune system

The release of stress-induced HPA axis hormones, including CRH, ACTH and glucocorticoids, may strongly influence maternal immune balance and affect pregnancy outcome (Nepomnaschy et al., 2007). For example, central CRH is thought to be involved in stress-induced (heat stress) immune suppression (e.g.

reduced splenic NK cell activity) in rats. Administration of a CRH receptor antagonist i.c.v to day 16 pregnant rats antagonised heat-induced immunosuppression, reversing NK cell activity. This suggests immunosuppression produced by heat stress is mediated by the central CRH system (Nakamura H, 2001). Circulating glucocorticoids modulate the transcription of multiple cytokines and upregulate transcription of anti-inflammatory cytokines IL-4 and IL-10, while suppressing pro-inflammatory/pregnancy-disturbing cytokines such as IL-1, IL-2, IL-6 and TNF-α (Arck, 2001, Webster et al., 2002). The anti-inflammatory properties of glucocorticoid action are primarily due to inhibition of transcription factors nuclear factor kappa enhancer of activated B cells (NFκB) and/or activator protein 1 (AP1) which typically induce proinflammatory gene expression (De Bosscher et al., 2000). Cortisol has also been reported to suppress NK activity, which is thought to be beneficial to pregnancy success since induction of NK activity threatens foetal survival (Arck, 2001), and reduce circulating dendritic cells (Suda et al., 2003). Therefore, it seems strange that stress-induced glucocorticoid, in light of its protective immune effects, does not prevent foetal loss. It may be that physiological levels of glucocorticoids are immunomodudulatory but stress-induced levels are immunosuppressive (Webster et al., 2002) and disrupt the maternal immune balance threatening pregnancy.

1.8.2 Effect of immune system and peripheral cytokines on the brain and pituitary

Peripheral sources of cytokines are primarily the immune cells which respond to bacteria or other infections. Other sources include cells in the reproductive system such as in the ovary, uterus/decidua, placenta, and the pituitary, which secrete cytokines in responsive to infective factors and stressors (Parker et al., 2011a). Cytokine release following infection/inflammation can directly or indirectly impact on reproductive organs, as well as the brain. Cytokines are released into the blood system and can thus travel throughout the body. For example, LPS administration induces secretion of a number of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β into the circulation. TNF-α is reported to alter ovarian follicular responses to gonadotrophins, reducing progesterone secretion and LH receptor mRNA (Erlebacher et al., 2004). The pituitary corticotrophs, gonadotrophs and lactotrophs also express receptors for a number of cytokines e.g. IL-6 and TNF-α (John and

Buckingham, 2003, Zaldivar et al., 2011) and may impact on secretion of hormones such as ACTH, LH and prolactin (indirectly reducing progesterone).

1.8.2.1 Mechanisms of peripheral cytokines gaining access to the brain

Circulating peripheral cytokines can profoundly affect the brain. There appears to be two main communication pathways to do this: (i) a neural route via the primary afferent neurones innervating the site of infection, resulting in cytokine production by microglial cells; and (ii) a humeral route involving proinflammatory cytokine production/release by phagocytic cells in the circumventricular organs (CVOs) and choroid plexus, leading to production of central cytokines that can diffuse into the brain parenchyma (Dantzer, 2009). Alternatively they could utilize the specific but limited active transport system into the cerebrospinal fluid to gain direct access to neurones with cytokine receptors (Ericsson et al., 1995, John and Buckingham, 2003). In addition, LPS and cytokines may act on the brain by inducing COX2 expression and thus driving local prostaglandin synthesis in the cerebral microvasculature (such as in the brainstem) which drives neuronal activation (Rivest et al., 2000, Rivest, 2001).

1.8.2.2 Effect of immune system and peripheral cytokines on the HPA axis

Immune signals from LPS and cytokines (including TNF-α and IL-1) are able to activate the HPA axis. They drive CRH and vasopressin gene expression in the hypothalamus, stimulating ACTH secretion from the pituitary and glucocorticoid release from the adrenals, thus are considered to be stressors (Brunton and Russell, 2008). The responses of the brainstem and forebrain to TNF-α and IL-1β are similar to LPS, however, IL-6 does not induce activation (Rivest, 2001).

1.8.2.3 Effect of immune system and peripheral cytokines on the dopamine-prolactin system

In the brain and pituitary it is not only the HPA axis that is affected by immune signals. In male mice LPS administration i.p reduced prolactin secretion, associated with increased activation of noradrenergic brain stem (A1, A2 and A6) and TIDA neurones (Hollis et al., 2005). LPS and TNF-α have been shown to increase

dopamine turnover in the hypothalamic-pituitary axis, along with decreasing prolactin secretion, in male mice (de Laurentiis A, 2002).

1.8.3 Expression and roles of central cytokines

In addition to peripheral cytokines entering the brain, some cytokines are constitutively expressed in brain itself (e.g. IL-1, IL-6 and TNF-α) and can therefore exert their effects there directly. Cytokines and their receptors are produced in a number of cell types in the brain including glia, neurones and macrophages, and in several different brain regions including the hypothalamus, hippocampus and brainstem nuclei (Schiepers et al., 2005). In contrast to the periphery, genes encoding anti-inflammatory cytokines (e.g.IL-10) are expressed at much lower levels than pro-inflammatory cytokines (e.g. TNF-α) (Szelényi, 2001).

The exact role of central cytokines is yet to be fully determined, although it is thought they have key roles in neuronal cell death and survival (Webster et al., 2002), neuronal development and plasticity, learning and memory (Schiepers et al., 2005). High levels of proinflammatory cytokines can have detrimental effects on brain cell function. For example, following CNS insults IL-1 promotes neuronal damage and exacerbates inflammatory processes in the brain by promoting production/ release of other proinflammatory cytokines (Anisman and Merali, 2002).

1.8.4 Effect of stress on central cytokines

In addition to peripheral cytokines, central cytokines are also involved in the interaction of the immune system with the HPA axis (see Figure 1.8). Exposure to acute stressors increases expression of proinflammatory cytokines both peripherally and centrally. LPS peripheral immune stimulation induces expression and immunoreactivity of IL-1β protein in the hypothalamus, hippocampus and prefrontal cortex in rats (Nguyen et al., 1998) and also rapidly increases TNF-α levels in the hypothalamus (Sacoccio C, 1998). In contrast, chronic psychosocial stress reduces IL-1β and TNF-α mRNA in the hippocampus in mice (Bartolomucci et al., 2003). Thus, the effects of stress on central cytokine expression and activity are dependent upon the nature of stressor employed. Stress-induced changes in central cytokines are also both region and cytokine specific. For example, only IL-1β, and not TNF-α, was induced in the hippocampus, hypothalamus and pituitary of rats subjected to

inescapable shock (O'Connor et al., 2003). However, regarding pregnancy, it is not yet established whether pregnancy alters brain cytokine expression or activity in response to stress, or whether central cytokines negatively impact directly on pregnancy maintenance.

Consistent with stress-induced alteration in proinflammatory cytokine production, evidence indicates that central pro-inflammatory cytokines are involved in stress-induced physiological and behavioural changes. Proinflammatory cytokines produced in response to a pathogen (mainly IL-1, IL-1β, IL-6 and TNF-α) trigger 'sickness behaviour' (Dantzer, 2009) which is often associated with fever, fatigue, reduced appetite and depressed mood. The hypothalamus mediates the majority of these responses which seem to be due as a result of reprioritizing central factors to cope with the sickness (Schiepers et al., 2005). Both systemic and centrally administered cytokines are able to trigger 'sickness behaviour' response, indicating their causal involvement. Mounting evidence also indicates an association between proinflammatory cytokines and stress-related disorders e.g. pro-inflammatory cytokines administered both centrally and peripherally lead to behavioural alterations similar to depression (Capuron and Dantzer, 2003).

Once again, although cytokines profoundly respond to stress, and stress is a source of pregnancy-related anxiety and depression, substantial evidence to provide a physiological link between them is yet to be reported.

Figure 1.8

1.9 Thesis aims and hypotheses

The experiments in this thesis were designed to investigate the effects of stress in early pregnant mice on prolactin regulation/activity and the maternal HPA axis. Due to the differing secretory profiles of women versus mice we aimed to focus on the effect of stress on basal prolactin levels in mice (as women also display steadily increasing levels of circulating prolactin during pregnancy but do not experience bidaily prolactin surges). Mice were used as a model to investigate the effects of stress in early pregnancy, in order to build upon previous studies in this area (e.g. (Arck et al., 1995, Joachim et al., 2003). Day 5.5 of pregnancy was selected as a suitable time to study, as it represents very early pregnancy but is 1 day after implantation, therefore, it does not interfere with this process. Several approaches/experiments were conducted in order to investigate the effects of stress in early pregnant mice and the specific aims were:

1. to investigate the effects of stress on prolactin secretion, TIDA neurone activation and dopamine activity in early pregnant mice.
2. to measure the maternal HPA axis responses to stress in early pregnancy.
3. to determine the effect of stress in early pregnancy on foetal loss/survival.
4. to determine the effects of stress in early pregnancy on prolactin negative feedback to the TIDA neurones.
5. to investigate the impact of pregnancy-threatening pro-inflammatory cytokines (TNF-α and IL-6) on circulating prolactin and TIDA neurone activation in early pregnancy.
6. to determine the effect of stress on anxiety in early pregnancy.

Hypotheses:

1. stress negatively impacts on prolactin secretion during early pregnancy and is associated with increased TIDA neurone activation and dopamine activity.
2. HPA axis responses are heightened in early pregnancy vs. virgins.
3. stress exposure in early pregnancy increases pregnancy failure.
4. stress enhances prolactin negative feedback in early pregnancy.
5. TNF-α and IL-6 reduce prolactin secretion during early pregnancy and increase TIDA neurone activation.
6. stress enhances anxiety-related behaviour in early pregnancy vs. virgins.

Chapter 2

General Materials and Methods

Chapter 2: General Materials and Methods

2.1 Animals

Female C57BL/6J mice, weighing between 18-24g (8-10 weeks old), were used in all experiments. Mice were purchased from Harlan (Harlan Laboratories UK Ltd., Leicestershire, UK) or they were obtained from an onsite colony derived from Harlan-purchased mice. Mice were housed in groups of 5-6 and maintained under standard laboratory conditions (temperature, 19-21^{o}C; humidity, 50-55%) with food and water available *ad libitum*. The mice were under a 12:12hr light/dark cycle (lights on from 7am until 7pm). In order to obtain pregnant mice for studies, virgin female mice were mated overnight with C57BL/6J stud males. Female mice were examined the following morning for evidence of mating and the appearance of a vaginal plug was taken as day 0.5 of pregnancy. Mice identified as being plugged on the same day were housed together. All experiments were carried out in the early-mid light phase of the circadian day (9.00-13.00h) in order to avoid the prolactin surge on the afternoon of proestrous in virgins and the bi-daily prolactin peaks in early pregnant mice. Virgin, day (d)5.5 pregnant (model of post-implantation early pregnancy) and d10.5 pregnant (model of mid pregnancy) mice were age-matched and selected at random for treatment groups in all experiments. Following the experiments and tissue/blood collection, pregnancy was confirmed by laparotomy and inspection of the uterine horns for the evidence of implantation sites. Animal care and experimental procedures were followed according to institutional guidelines and conformed to current requirements of UK Home Office regulations. All procedures were approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

2.2 Anaesthesia (non-recovery)

Prior to transcardial perfuse fixation and collection of blood by cardiac puncture, mice were deeply anaesthetised with an overdose of sodium pentobarbitone (short to intermediate acting barbiturate) i.p (0.3ml, 54.7mg/ml; Centravet, 22106 Didan, France). The needle was carefully inserted into the abdominal cavity, taking care to avoid puncturing any major organs.

2.3 Injections

2.3.1 Subcutaneous (s.c) injections

Subcutaneous injections were performed on the flank of the mouse. Mice were gently restrained in a holding bag (disposable icing bag; Lakeland, Cumbria, UK) and an injection (25G needle) made into the fold of loose skin on the flank.

2.3.2 Intraperitoneal (i.p) injection

Mice were gently restrained in a holding bag and the ventral side exposed. The needle (25G) was inserted into the lower abdomen of the mice.

2.4 Stressors

2.4.1 Immune stress protocol

Virgin and pregnant (d5.5, d10.5) mice were gently restrained in a holding bag and injected with lipopolysaccharide (LPS; 10 or 12.5µg/100µl saline, i.p; *E.coli* E055:B5, Sigma, Poole, Dorset, UK), controls were given vehicle (isotonic saline, 100µl, i.p). Mice were identified using marker pen scores on their tail. Mice were then returned to their home cage until sample collection or further behavioural analysis before sample collection.

2.4.2 24h fasting protocol

Virgin and d5.5 pregnant were fasted for 24h, with food removed from their home cage at 10am (3h after lights on), controls were allowed to feed normally. After 24h mice were either sacrificed and tissues/blood collected as required or they were used for further behavioural analysis before sample collection.

2.5 Blood collection

2.5.1 Cardiac puncture

Once mice were deeply anaesthetised and showing no signs of breathing (no chest movement) and loss of sensory/reflex response (no reaction to toe pinch), the abdominal cavity was opened up to expose the still beating heart. A 25G needle was inserted into the pointed tip of the heart and ~0.2ml blood extracted and added to a microcentrifuge tube (Greiner Bio-One Ltd, Gloucestershire, UK) containing ice-cold ethylenediaminetetraacetic acid (EDTA; an anticoagulant, Sigma-Aldrich Corp.,

Poole, UK; 5% solution, 15µl/100µl blood) and aprotinin (a protease inhibitor, Sigma-Aldrich Corp., Poole, UK; 0.04 trypsin inhibitor units in 10µl/tube).

2.5.2 Trunk blood

Mice were gently restrained in a holding bag and quickly decapitated using sharp surgical scissors. Trunk blood was collected from the site of decapitation in a 1.5ml microcentrifuge tube containing ice-cold EDTA (5% solution, 15µl/100µl blood) and aprotinin (0.04 trypsin inhibitor units in 5µl/tube).

2.6 Radioimmunoassay (RIA)

2.6.1 Progesterone RIA - Kit 1

A commercial progesterone RIA kit was purchased (DSL-3900, Diagnostic Systems laboratories, Texas, USA) for measuring progesterone in mouse plasma. This kit was used for the majority of the experiments. However, in 2010 it became unavailable and therefore another kit had to be sourced and validated (kit 2) for use in the final experiment that required progesterone analysis.

2.6.1.1 General principles

This assay allows accurate measurement of plasma progesterone concentration (assay sensitivity 0.12ng/ml) by following the basic principles of RIA where there is competition between the radioactively-labelled antigen (I-125 progesterone) and the non-radioactively labelled antigen in the plasma, for antibody binding sites. Decanting the contents of the anti-progesterone-coated tubes allows separation of the bound from the free antigens and thus the amount of the radioactively-labelled antigen can be measured using a gamma counter. This is inversely proportional to the concentration of non-radioactive antigen that is present in the plasma sample.

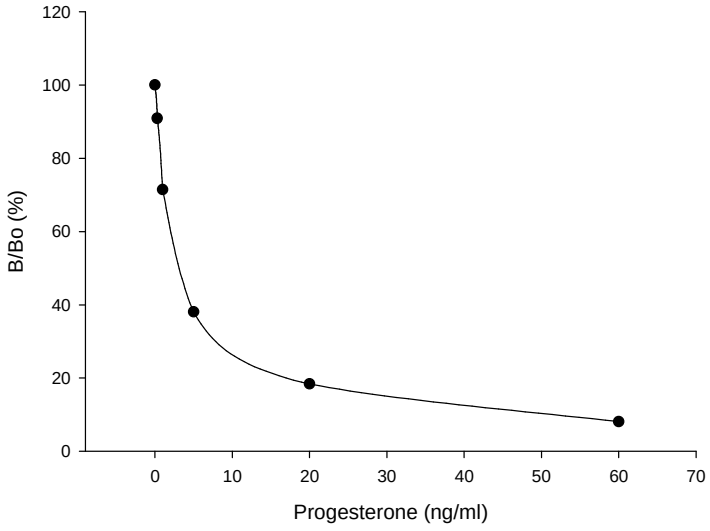
2.6.1.2 Method

Prior to starting the assay, the kit contents were brought to room temperature and mixed gently by inversion. Controls and standards were reconstituted in 0.5ml deionised water (except 0ng/ml standard which required 1ml deionised water) and were included in each assay run. Values obtained from standards were used to create a standard curve from which the concentrations of the unknown plasma samples

could be derived (example of standard curve is shown in Figure 2.1). Controls were included to confirm the validity of the standard curve generated.

Two uncoated tubes were labelled for total counts and progesterone-coated tubes were labelled for standards, controls and the unknown samples (in duplicate). To the appropriate tube, 25µl of standards (0-60ng/ml), controls or samples were pipetted to the base. Immediately after this, 500 µl of progesterone I-125 reagent was added in the same manner to all tubes and the rack gently shaken to mix the tube contents. Following 70 min incubation in a waterbath at 37 ^{o}C, all tubes (except total counts) were decanted by simultaneous inversion in a foam rack. Tubes were struck sharply on absorbent paper and then left to drain for 2 min. Tubes were blotted to remove droplets and then counted using a gamma counter (Wizard 1470 Automatic Gamma Counter, Perkin Elmer) for one minute.

Figure 2.1: Example of standard curve for progesterone RIA (kit 1).



Values used are taken from the data given by the manufacturer (Diagnostics Systems Laboratories) as typical active progesterone standard curve data (DSL-3900). The standard curve was generated by plotting % of zero bound (B/Bo) for each standard (B/Bo = mean absorbance for standard/mean absorbance for zero standard) against concentration and a simple spline curve/line was fitted using SigmaPlot.

2.6.2 Progesterone RIA - Kit 2

A second progesterone kit (Progesterone ImmunChem Double Antibody RIA kit; MP Biomedicals, LLC, Orangeburg, USA) was purchased as the original kit (kit 1) was discontinued. This kit, therefore, required validation. In order to do this controls from the original kit, plasma samples previously measured with original kit and 'new' progesterone controls (Control set I, MP Biomedicals, LLC, Orangeburg, USA) were included in a test assay.

2.6.2.1 General principles

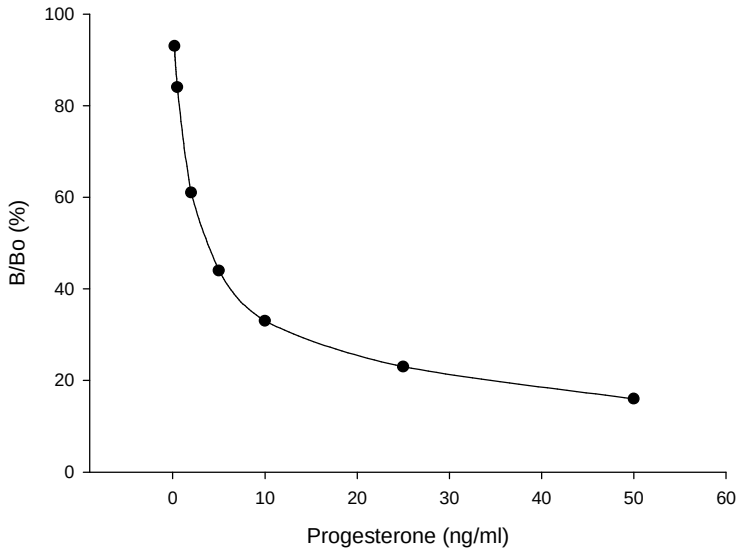
This assay works along the same basic RIA principles as kit 1 (see section 2.5.1.1) and allows accurate measurement of plasma progesterone concentration (assay sensitivity 0.11ng/ml).

2.6.2.2 Method

Prior to starting the assay, the kit contents were brought to room temperature and tubes labelled appropriately in duplicate. 'Kit 2' progesterone standards (Control set I, MP Biomedicals) were reconstituted in 5ml distilled water (aliquoted and frozen at -20 ^{o}C) and 'kit 1' controls were reconstituted in 0.5ml deionised water. Plasma samples were diluted 1 in 10 with steroid diluent, provided in the kit. Values obtained from standards were used to create a standard curve from which the concentrations of the unknown plasma samples could be derived (example of standard curve is shown in Figure 2.2). Controls were included to confirm the validity of the standard curve generated.

To two tubes, 500µl of steroid diluent and 100µl of the 0ng/ml standard were pipetted to the base (to check for non-specific binding, NSB). 100µl of standards (0-50ng/ml), controls and unknown samples were then added to the appropriate tubes. To all tubes (excluding ones for total counts and NSB) 500µl of anti-progesterone was added. To all tubes 200µl of progesterone I-125 was added and the tubes vortexed before incubating for 60 min in a waterbath at 37 ^{o}C. Following incubation 500µl of precipitant solution was added to all tubes (excluding total counts). Tubes were vortexed thoroughly and centrifuged at 1000 x g for 15 min at 4 ^{o}C. Tubes were then aspirated before precipitate was counted in a gamma counter (Wizard 1470 Automatic Gamma Counter, Perkin Elmer).

Figure 2.2: Example of standard curve for progesterone RIA (kit 2).



Values used are taken from the data given by the manufacturer (MP Biomedicals) as typical active progesterone standard curve data. The standard curve was generated by plotting % of zero bound (B/Bo) for each standard (B/Bo = mean absorbance for standard/mean absorbance for zero standard) against concentration and a simple spline curve/line was fitted using SigmaPlot.

All control measurements from both ‘kit 1’ and ‘kit 2’ were within the limits expected for each control. The previously measured plasma samples (measured using kit 1) were <7% different from the original measurements (see Table 2.1)

Table 2.1: Comparison of measured progesterone controls and plasma samples with the expected control ranges and previously measured progesterone concentrations.

Sample		measured [progesterone] ng/ml	control ranges/ previously measured [progesterone] ng/ml
old kit	Control I	0.782	1+/- 0.3
	Control II	9.346	10+/-3
new kit	Control A	0.636	0.48-1.0
	Control B	11.56	8.0-12.4
	Control C	21.637	19.7-28.9
Plasma	mouse 1	17.73	16.8
	mouse 2	21.3	22.9

Controls from kits 1 and 2 were measured using the new assay (kit 2). The values of all the controls (two from kit 1, three from kit 2) were within the expected control ranges, verifying the kit was accurately measuring samples. As an extra check, two plasma samples that had been measured using the original kit were also analysed. The newly read values were <7% different from the previously measured values.

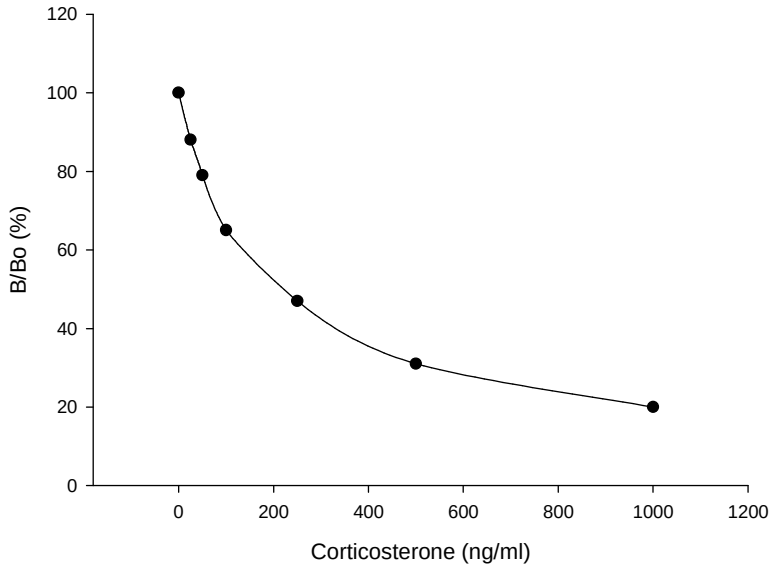
2.6.3 Corticosterone RIA

2.6.3.1 General Principles

This assay (Corticosterone ImmunChem Double Antibody RIA kit; MP Biomedicals, LLC, Orangeburg, USA) allows accurate measurement of plasma corticosterone concentration (assay sensitivity of 10ng/ml). In this assay, a limited amount of antibody is reacted with radioactively-labelled corticosterone. In the presence of a sample containing corticosterone a corresponding decrease in radioactively-labelled corticosterone bound to the antibody is observed, following the basic principle of RIA where there is competition between the radioactively labelled antigen (I-125 corticosterone) and the non-radioactively labelled antigen in the plasma, for antibody binding sites. Decanting the contents of the tubes allows separation of the bound from the free antigens and thus the amount of radioactively labelled antigen can be measured using a gamma counter (Wizard 1470 Automatic Gamma Counter, Perkin Elmer) by reading off the standard curve generated (see Figure 2.3 for example

standard curve). This is inversely proportional to the concentration of non-radioactive antigen that is present in the plasma sample.

Figure 2.3: Example of standard curve for corticosterone RIA



Values used are taken from the data given by the manufacturer (MP Biomedicals) as typical active corticosterone standard curve data. The standard curve was generated by plotting % of zero bound (B/Bo) for each standard (B/Bo = mean absorbance for standard/mean absorbance for zero standard) against concentration and a simple spline curve/line was fitted using SigmaPlot.

2.6.3.2 Method

Prior to starting the assay the kit components were brought to room temperature and tubes labelled as required. Controls (provided in the kit) were reconstituted with 2ml distilled water and allowed to stand at room temperature for at least 30 min before use. Plasma samples were diluted 1:200 with steroid diluent. 300µl of steroid diluent was added to two tubes (for NSB) and 100µl of steroid diluent added to a further two tubes (for 0ng/ml standard). 100µl of corticosterone standards (25-1000ng/ml), controls and diluted plasma samples were pipetted into the appropriate labelled tubes. 200µl of corticosterone I-125 was added to all tubes (excluding two tubes for total counts). 200µl of anti-corticosterone was added to all tubes (excluding tubes for total counts and NSB). Tubes were vortexed before incubation at room temperature

for 2 h. Following incubation, 500µl of precipitant solution was added to all tubes (excluding total count tubes), and tubes vortexed then centrifuged at 1000 x g for 15 min. Tubes were aspirated and rims blotted, before the precipitate was counted in a gamma counter (Wizard 1470 Automatic Gamma Counter, Perkin Elmer).

2.7 ELISA

2.7.1 General principles

Enzyme-linked immunosorbent assay (ELISA) is a technique employed to detect the presence of either an antibody or an antigen in a sample. The basic principle is that an unknown amount of antigen is fixed to a surface (usually a 96 well plate) and a specific antibody is then added which binds to the antigen. This antibody is linked to an enzyme so that when a 'substance' is added the enzyme converts it to a detectable signal, typically a colour change.

The two ELISAs used for measuring mouse prolactin and interleukin (IL)-6 (both R&D Systems, Inc., Minneapolis, US) were examples of "sandwich" ELISAs (see Figure 2.4 for diagram of assay steps).

2.7.2 Prolactin ELISA

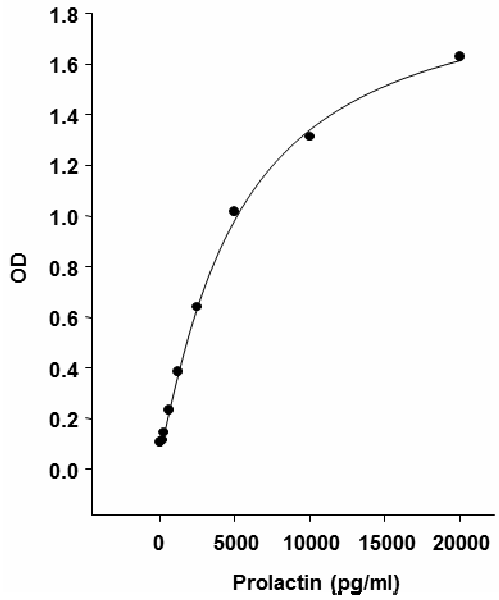
2.7.2.1 Development and validation

In order to measure prolactin in mouse plasma a DuoSet ELISA kit was purchased (DY1445, R&D Systems, Inc., Minneapolis, US). The mouse prolactin ELISA was originally designed for the analysis of prolactin in cell culture supernates, thus it had to be adapted and developed for use with plasma. In order to do this a diluent for plasma was required as the concentrations of prolactin in plasma exceed the standard curve produced from the kit. To find and validate a suitable diluent standard curves were generated using phosphate buffered saline (PBS) supplemented with varying concentrations (10-50%) of heat-inactivated foetal bovine serum (FBS; Qualified, Heat-inactivated FBS; Invitrogen, California, USA) as suggested by R&D Systems. Standard curve samples for each diluent were all measured in triplicate on the same 96-well plate (Costar EIA plate, DY990, R&D Systems) and construction of standard curves (using a 4-parameter logistic curve fit) revealed PBS supplemented with 10% FBS gave the best curve fit: r^2 was 0.9950 with 10% FBS, 0.9888 with 25% FBS and 0.9924 with 50% FBS over the range of prolactin concentrations that were expected

Figure 2.4

to be encountered experimentally (see Figure 2.5 for 10% FBS standard curve and Figure 2.6). The 10% FBS diluent generated a standard curve with an r^2 closest to 1 (i.e. it fits the recommended standard curve best), therefore this dilution was selected for use with in all subsequent assays for standard curves and for diluting experimental mouse plasma.

Figure 2.5: Example of standard curve for prolactin ELISA



Standard curve generated using 10% FBS diluent. 4-parametric curve fitted as instructed by the manufacturer (R&D Systems) with optical density (OD) plotted against the concentration of prolactin.

To determine whether the ELISA reported accurate readings for unknown plasma samples, spike and linearity experiments were performed. Plasma for validating the kit was obtained by sacrificing virgin female C57BL/6J mice (by conscious decapitation) and collecting trunk blood (0.4ml) into tubes containing ice-cold EDTA (Sigma-Aldrich Corp., Poole, UK; 5% solution, 15µl/100µl blood) and aprotinin (Sigma-Aldrich Corp., Poole, UK; 0.04 trypsin inhibitor units in 10µl/tube). Blood samples were centrifuged at 1000 x g for 5 min to separate the plasma from the other blood components, and the plasma collected and stored at -20^{o}C until use in the ELISA. Samples were created for the spike assays by "spiking" the plasma

samples with 100ng/ml prolactin (supplied in kit) to test against the standard curve generated. In addition, spiked samples were also serially diluted (1 in 25, 1 in 50 and 1 in 100) to test for linearity (Figure 2.6, Table 2.2). r^2 was found to be 0.9925 indicating a linear relationship with dilution.

Table 2.2. Comparison of expected and measured prolactin concentrations at three different dilutions using the adapted prolactin ELISA

Expected prolactin (pg/ml)	Measured prolactin (pg/ml)	Dilution Factor (DF)	DF x measured prolactin (pg/ml)	DF x measured prolactin (ng/ml)	% of Expected
4000.00	4185.44	25	104635.92	104.64	104.64
2000.00	1803.08	50	90154.12	90.15	90.15
1000.00	994.06	100	99405.93	99.41	99.41

Mouse plasma was spiked with 100ng/ml prolactin and subsequently diluted by factors of 25, 50 or 100, in order to confirm that parallel values are achieved with serial dilution. Measured values were found to be within 10% of the expected prolactin concentration (100ng/ml).

Plasma samples collected from all subsequent experiments were typically diluted 25-fold; if data provided for samples were not read from the linear part of the curve (falling lower on the standard curve) samples were reassayed at a dilution of 10-fold. Sensitivity for this assay was 1.57pg/ml. Inter-assay standards were included on every experimental plate to check assay consistency; the coefficient of variation between assays was 5.2%. For the 'spiked' control, plasma from control virgin mice were spiked with 100ng/ml prolactin, diluted x 25 and aliquoted and frozen (-20ºC) for use in future assays.

As a final check of the ELISA, assay samples were collected from mice expected to have high plasma prolactin concentrations, a positive control. Virgin female C57BL/6J mice were injected i.p. with vehicle (isotonic saline; 100µl; n = 6) or a dopamine antagonist (metoclopramide, Sigma Aldrich Poole UK; 1µg/g bodyweight;

Figure 2.6

n = 6) and were sacrificed by conscious decapitation 30 min later. Trunk blood was collected to provide plasma (as described above) and an assay carried out using the parameters determined previously for the ELISA kit. Metoclopramide significantly increased plasma prolactin concentration, as predicted, compared to vehicle (Student's t-test, $p<0.01$; Figure 2.7).

2.7.2.2 Method

Prior to carrying out the assay all components were brought to room temperature. Firstly, a 96-well plate was prepared for use in the assay by coating it with capture antibody. The capture antibody (144 µg of goat anti-mouse prolactin) was reconstituted in 1ml PBS to form a stock solution (137mM NaCl, 2.7mM KCl, 8.1mM Na 2HPO4, 1.5mM KH 2PO4; pH 7.2 - 7.4; 0.2 mm filtered) and left for at least 15 min before use. Stock was then diluted to a working dilution of 0.8µg/ml in PBS, or aliquoted and frozen (-20ºC) for future assays. 100µl of diluted capture antibody was pipetted (using a multichannel pipette) into each well of the plate, plates then sealed (ELISA plate sealers, DY992, R&D Systems) and left to incubate at room temperature overnight (~18 h).

The following day, well contents were decanted by inverting the plate abruptly and each well was then washed 3 times with around 0.4ml wash buffer (WA126, Wash Buffer Concentrate x25; solution of buffered surfactant with preservatives, 0.05% Tween 20 in PBS; R&D Systems; diluted in deionised water) using a squirt bottle. Following the last wash the plate was inverted and struck onto absorbent paper to remove any remaining wash buffer. The plate was then blocked by adding 300µl of Reagent Diluent (DY995, 1% bovine serum albumin (BSA) in PBS, 0.2µl filtered; R&D Systems) to each well and the plate incubated at room temperature for approximately 1 h 15 min. During this time the prolactin standard was reconstituted with 0.5ml Reagent Diluent (1390 ng/ml prolactin) and allowed to stand for a minimum of 15 min with gentle agitation prior to make dilutions (or aliquoting and freezing at -70ºC). A nine-point standard curve was created by using a 2-fold serial dilution (diluted in 10% FBS diluent as described above) starting with a top high standard of 20,000pg/ml. Plasma samples were diluted typically 25-fold using the 10% FBS diluent also. Following the incubation with Reagent Diluent, the decanting/washing step was repeated again. 100µl of standards, samples and a

Figure 2.7

"spiked" control were pipetted into the appropriate wells of the plate in duplicate, plates were sealed and left to incubate at room temperature for 2 h. The decanting/washing step was then repeated, before incubation for a further 2 h with 100µl/well of Detection Antibody (plates sealed). The detection antibody (36 µg biotinylated goat anti-mouse prolactin) was reconstituted with 1.0 ml of Reagent Diluent and left for at least 15 min before use. Stock was then diluted to a working dilution of 200ng/ml in Reagent Diluent, or aliquoted and frozen (-20ºC) for future assays. Decanting/wash step was repeated as before. 100µl of streptavidin-horseradish peroxidase (HRP) was added to each well and plates covered to avoid direct light for 20 min at room temperature. Streptavidin-HRP was diluted to a working concentration as instructed on the bottle, typically a 200-fold dilution, with Reagent Diluent. The aspiration and wash step was repeated for a final time before incubation for a further 20 min with 100µl/well of Substrate Solution (1:1 combination of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine); DY999, R&D Systems), again avoiding direct light (colour change from clear to blue). In order to terminate the assay, 50µl of 2N H_2SO_4 was added to each well (colour change from blue to yellow) and the plate read immediately to determine the optical density using a microplate reader (PowerWave XS, BioTek Instruments, Winooski, Vermont, USA) and software (KC junior, BioTek Instruments, Winooski, Vermont, USA) set to a primary wavelength 450nm and reference wavelength of 540nm.

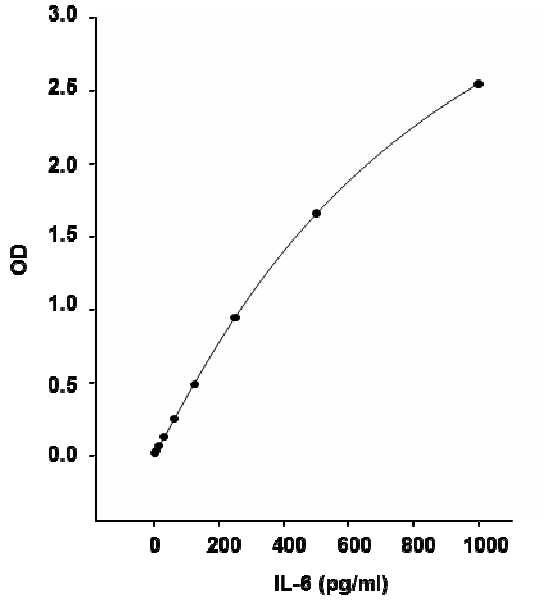
2.7.3 Interleukin (IL)-6 ELISA

2.7.3.1 Development and validation

The IL-6 DuoSet ELISA kit (DY406, R&D Systems, Inc., Minneapolis, US) was also designed for the analysis in cell culture supernates and thus was developed and validated in the same manner as the prolactin ELISA (see section 2.6.2.1). To find and validate a suitable diluent, standard curves were generated using phosphate buffered saline (PBS) supplemented with varying concentrations (10-50%) of heat-inactivated fetal bovine serum (FBS; Qualified, Heat-inactivated FBS; Invitrogen, California, USA) as suggested by R&D Systems. Standard curve samples for each diluent were all measured in triplicate on the same 96-well plate (Costar EIA plate, DY990, R&D Systems) and construction of standard curves (using a 4-parameter

logistic curve fit) revealed PBS supplemented with 10% FBS gave the best curve fit: r^2 was 0.9999 with 10% FBS, 0.9977 with 25% FBS and 0.9996 with 50% FBS over the range of IL-6 concentrations that were expected to be encountered experimentally (see Figure 2.8 for 10% FBS standard curve and Figure 2.9). The 10% FBS diluent generated a standard curve with an r^2 closest to 1, thus, this dilution was selected for use with in all subsequent assays for standard curves and for diluting experimental mouse plasma.

Figure 2.8: Example of standard curve for IL-6 ELISA



Standard curve generated using 10% FBS diluent. 4-parametric curve fitted as instructed by the manufacturer (R&D Systems) with optical density (OD) plotted against IL-6 concentration.

To determine whether the ELISA reported accurate readings for unknown plasma samples, spike and linearity experiments were perform as with the prolactin ELISA. Samples were created for the spike assays by "spiking" the plasma samples with 2500pg/ml IL-6 (supplied in kit) to test against the standard curve generated. Spiked samples were also serially diluted (1 in 25, 1 in 50 and 1 in 100) to test for linearity (Figure 2.9, Table 2.3). r^2 was found to be 0.9976 indicating a linear relationship with dilution.

Table 2.3. Comparison of expected and measured IL-6 concentrations at three different dilutions using the adapted IL-6 ELISA

Expected IL-6 (pg/ml)	Measured IL-6 (pg/ml)	Dilution Factor (DF)	DF x measured IL-6 (pg/ml)	% of Expected
100.00	95.57	25	2389.21	95.57
50.00	46.13	50	2306.31	92.25
25.00	26.06	100	2605.50	104.22

Mouse plasma was spiked with 2500pg/ml IL-6 and subsequently diluted by factors of 25, 50 or 100, in order to confirm that parallel values are achieved with serial dilution. Measured values were found to be within 10% of the expected IL-6 concentration (2500pg/ml).

Plasma samples collected from all subsequent experiments were diluted between 10 and 50-fold depending on the treatment group. Plasma from LPS-treated mice were typically diluted 50-fold (due to high IL-6 levels), while plasma from vehicle and fasting/control mice were diluted 10-fold (due to lower IL-6 levels). Sensitivity for this assay was 7.81pg/ml. Inter-assay standards were included on every experimental plate to check assay consistency; the coefficient of variation between assays was 4.2%. For the 'spiked' control, plasma from control virgin mice was mixed with 2500ng/ml IL-6, diluted x 25, aliquoted and frozen (-20ºC) for use in future assays. As an additional check of the ELISA, assay samples were collected from mice expected to have high plasma IL-6 concentrations, as a positive control. Spare plasma from pseudopregnant mice was used to test the ELISA. These mice had been injected with LPS (12.5µg/100µl saline, i.p;*E.coli* E055:B5, Sigma, Poole, Dorset, UK; n = 6) or vehicle (isotonic saline; 100µl; n = 7) and were sacrificed by conscious decapitation after 60, 120, or 240 min. Trunk blood was collected to provide plasma (as described above) and an assay carried out using the parameters determined previously for the ELISA kit. LPS treatment significantly increased plasma IL-6 concentration, as predicted, compared to vehicle (pooled time point data; Student's t-test, $p<0.001$; Figure 2.10).

Figure 2.9

Figure 2.10

2.7.3.2 Methods

This assay was carried out in the same manner as the prolactin ELISA. Prior to carrying out the assay all components were brought to room temperature. A 96-well plate was prepared for use in the assay by coating it with capture antibody. The capture antibody (360 µg of rat anti-mouse IL-6) was reconstituted in 1ml PBS to form a stock solution (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.2 - 7.4; 0.2 mm filtered) and left for at least 15 min before use. Stock was then diluted to a working dilution of 2µg/ml in PBS, or aliquoted and frozen (-20ºC) for future assays. 100µl of diluted capture antibody was pipetted (using a multichannel pipette) into each well of the plate, plates then sealed (ELISA plate sealers, DY992, R&D Systems) and left to incubate at room temperature overnight (~18 h).

The following day, well contents were decanted by inverting the plate abruptly and each well then washed 3 times with around 0.4ml wash buffer (WA126, R&D Systems) using a squirt bottle. Following the last wash the plate was inverted and struck onto absorbent paper to remove any remaining wash buffer. The plate was then blocked by adding 300µl of Reagent Diluent (DY995, R&D Systems) to each well and the plate incubated at room temperature for approximately 1 h 15 min. During this time the IL-6 standard was reconstituted with 0.5ml Reagent Diluent (90ng/ml IL-6) and allowed to stand for a minimum of 15 min with gentle agitation prior to make dilutions (or aliquoting and freezing at -70ºC). A nine-point standard curve was created by using a 2-fold serial dilution (diluted in 10% FBS diluent as described above) starting with a top high standard of 1,000pg/ml. Plasma samples were diluted typically 10 to 50-fold using the 10% FBS diluent also. Following the incubation with Reagent Diluent, the decanting/washing step was repeated again. 100µl of standards, samples and a "spiked" control were pipetted into the appropriate wells of the plate in duplicate, plates were sealed and left to incubate at room temperature for 2 h. The decanting/washing step was then repeated, before incubation for a further 2 h with 100µl/well of Detection Antibody (plates sealed). The detection antibody (36 µg biotinylated goat anti-mouse IL-6) was reconstituted with 1.0 ml of Reagent Diluent and left for at least 15 min before use. Stock was then diluted to a working dilution of 200ng/ml in Reagent Diluent, or aliquoted and frozen

(-20ºC) for future assays. Decanting/wash step was repeated as before. 100µl of streptavidin-HRP was added to each well and plates covered to avoid direct light for 20 min at room temperature. Streptavidin-HRP was diluted to a working concentration as instructed on the bottle, typically a 200-fold dilution, with Reagent Diluent. The aspiration and wash step was repeated for a final time before incubation for a further 20 min with 100µl/well of Substrate Solution (1:1 combination of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine); DY999, R&D Systems), again avoiding direct light (colour change from clear to blue). In order to terminate the assay, 50µl of 2N H_2SO_4 was added to each well (colour change from blue to yellow) and the plate read immediately to determine the optical density using a microplate reader (PowerWave XS, BioTek Instruments, Winooski, Vermont, USA) and software (KC junior, BioTek Instruments) set to a primary wavelength of 450nm and a reference wavelength of 540nm (according to manufacturer's instructions).

2.8 BD Cytometric bead array (CBA)

CBAs were carried out by Evelin Hagen (Charité, Berlin, Germany; Petra Arck lab group) using a commercial CBA mouse Th1/Th2 cytokine kit (Catalogue No. 551287; BD Biosciences, San Jose, CA, USA) for measuring tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) in mouse plasma.

2.8.1 General principles

This assay provides a sensitive and precise method of simultaneously detecting multiple cytokines from a single sample by capturing them with beads of known size/fluorescence (assay sensitivity/limit of detection is 4.5pg/ml for TNF-α and IFN-γ, up to a maximum of 5,000pg/ml). This makes it possible to detect and measure several cytokines from a sample using flow cytometry. Each individual capture bead has been previously conjugated to an antibody specific for each cytokine. In addition, detection reagents (made up of phycoerythin (PE)-conjugated antibodies) are added to the unknown samples/standards, providing a specific fluorescent signal proportional to the amount of each bound cytokine (see Figure 2.11). Thus, the sandwich complexes formed (bead+cytokine+detection antibody) can be measured by flow cytometry to reveal particles with fluorescent characteristic of the specific

Figure 2.11

bead and the detection antibody. The concentration of each cytokine can then be determined by reading off standard curves generated.

2.8.2 Method

Firstly, the cytokine standards were prepared by reconstituting one vial of lyophilised mouse standards with 2ml assay diluent (provided in kit). This top standard was allowed to incubate for at least 15min before a nine-point standard curve was created by using a 2-fold serial dilution (diluted in assay diluent). Capture beads were mixed in equal proportion for each cytokine to be tested, allowing enough for 10µl of each per assay tube (including standards and control). Mixed capture beads were then thoroughly vortexed and transferred to assay tubes. 50µl of standards and unknown samples were added to the relevant tubes. In addition, 50µl of assay diluent was added to one tube as a negative control. 50µl of PE detection reagent was applied to all reaction tubes, which were incubated at room temperature for 2 h, protected from light. Following incubation, 1ml of wash buffer was added to each tube and these were then centrifuged for 5 min at 200 x g. Tubes were aspirated and the supernatant discarded. The pellets were resuspended in 300µl wash buffer. These samples were then passed through a flow cytometer and analysed using FCAP Array software according to manufacturer's instructions (BD Biosciences).

2.9 Brain tissue processing

2.9.1 Transcardial perfusion for fixation (for processing by immunocytochemistry)

Transcardial perfuse fixation is a technique used to preserve tissue and prevent decay. The mouse was administered a lethal dose of sodium pentobarbital (0.3ml i.p; Centravet, 22106 Didan, France). Once the mouse was deeply anaesthetised (exhibited no signs of breathing, non-responsive to toe pinch) the heart was exposed. The mouse was perfused with heparinised isotonic saline (129mg/L; ~100ml) and then fixed with 4% paraformaldehyde (~100ml) using a peristaltic pump.

The brain was removed carefully from the skull using surgical scissors and transferred to a bijou vial containing a 4% paraformaldehyde and 15 % sucrose solution. The brain was then stored at 4ºC overnight (or until the brain had sunk to the base of the vial). The solution was then exchanged for a 30% sucrose solution in 0.1M phosphate buffer and the brain left overnight again at 4ºC (until brain had

sunk). Following this, the brain was removed, rolled in absorbent paper to remove excess solution, frozen on foil in dry ice and wrapped in foil before storage in small labelled bag at -70ºC.

2.9.2 Flash freezing (for processing by in situ hybridisation)

Flash freezing is a technique used to freeze samples quickly so as to avoid the formation of ice crystals that can damage the tissue. Following brain removal, the brain (or other tissues) was placed onto foil sat upon dry ice. The brain was then quickly covered in powdered dry ice. Once frozen the brain was wrapped in foil, placed in a small labelled bag and stored at -70ºC until use.

2.9.3 Brain sectioning

2.9.3.1 Microtome sectioning

Brains that had previously been perfused fixed were sectioned using a freezing microtome (Leica CM 1325, Leica; set at -20 ºC). Coronal brain sections (containing paraventricular nucleus (PVN), arcuate nucleus, ventromedial hypothalamic nucleus (VMH), and supraoptic nucleus (SON); see Figure 2.12) and brainstem sections (containing the locus coeruleus (LC), nucleus tractus solitarius (NTS), and rostral ventrolateral medulla (RVLM); see Figure 2.13) were cut at 48µm and divided equally (with alternate sections) into 2 pots containing 0.1M phosphate buffer. If the sections were to be used straight away in immunocytochemistry they remained in 0.1M phosphate buffer, however, if they were for use at a later date they were stored in cryoprotectant and kept at -70ºC until use.

2.9.3.2 Cryostat sectioning

Fresh frozen brains required for *in situ* hybridisation (ISH) were serially sectioned using a cryostat (at -16 ºC). Coronal brain sections (15µm) containing the hypothalamic PVN (see Figure 2.12 A) were mounted on Polysine microscope slides (coated with a permanent adhesive; VWR International, Leicestershire, UK) with 4 sections per slide. Once 4 sections had been mounted on the Polysine slide the slides were placed into a slide box (VWR) along with a silica bag (to reduce condensation ice damage). When the box was full of slides it was sealed with electrical tape and stored at -70ºC until slides were required for use in *in situ* hybridisation. The

Figure 2.12

Figure 2.13

locations of areas of interest were confirmed with marker sections collected on gelatinised microscope slides (1 marker section for every 8 sections collected). Marker slides were dipped in Acetic Alcohol Fixative (AAF) for 10 seconds, rinsed with water, stained with 1% toluidine blue for 10 seconds, and rinsed with water before examination once dry under a light microscope.

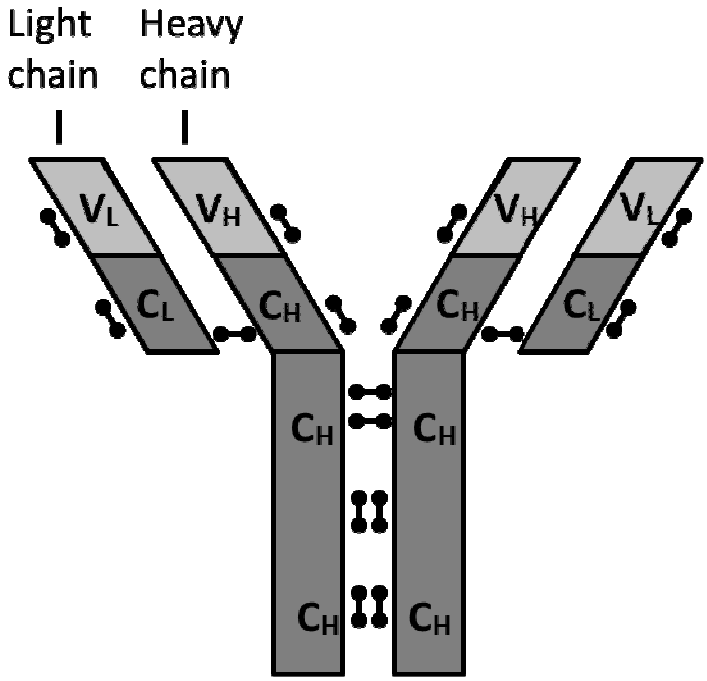
2.10 Immunocytochemistry

2.10.1 General principles

Immunocytochemistry (ICC) was first described by Coons et al. (1941) and is still used widely in research today. It is a technique used to neuro-anatomically identify a given antigen present in a tissue sample (or cytological preparation) by the binding to an antibody specific for that antigen. It can be used to identify neuronal phenotype (e.g. tyrosine hydroxylase ICC identifies dopaminergic neurones, see section below) and can detect activity of neurones (e.g. Fos and pSTAT5 ICC, see sections below). Binding of the antibody to its corresponding antigen is followed by a labelling reaction to visualise the antigen-antibody complex, for example, by an enzymatic or fluorescently-labelled detection method (Renshaw, 2007). The quality of antibody reagents used is of fundamental importance for successful and reproducible staining. Antibodies are members of the immunoglobulin (Ig) family of proteins which play a central role in the adaptive immune response. Antibody molecules are fundamentally Y-shaped in structure, composed of two identical heavy polypeptide chains and two identical light polypeptide chains, supported by disulphide bonds (see Figure 2.14 for a schematic diagram). Both chain types contain variable and constant domains with the variable regions containing most amino acid sequence variation and are the site of antigen binding.

The antibodies may be monoclonal (have an epitope and single antigen binding site) or polyclonal (have multiple epitopes and binding sites). The advantage of monoclonal antibodies are that they are highly specific for an antigen, however, they are difficult to generate successfully and low levels of labelling often occur due to low affinity or the fact that the antibody binds to an infrequent epitope on proteins. The use of polyclonal antibodies has the advantage of giving high levels of labelling for a single antigen due to it containing multiple clones of antibodies to different epitopes on the same antigen. The disadvantage of polyclonal though is that they can

Figure 2.14: Schematic of the immunoglobulin structure



The basic Y-shaped antibody molecule is made up of four polypeptide chains (two heavy (H) and two light (L)), stabilised by disulphide bonds (indicated by the black circles joined together). The chains contain a number of immunoglobulin domains referred to as constant (C) and variable (V). The constant domain is responsible for dictating the antibody isotype and contains a conserved amino acid sequence. The variable domain contains sequence variation allowing specific antigen recognition (adapted from Renshaw, 2007).

be less specific as shared epitopes on different proteins (not the antigen of interest) may be labelled (Burry, 2010). Thus, it is important to have a suitable antibody that has sufficient affinity and avidity (to prevent antibody being washed off the sections) properties.

Visualisation

There are a number of methods for visualisation of the antigen-antibody complex such as the avidin-biotin peroxidase complex (ABC) technique (see Figure 2.15). Following exposure to the primary antibody, tissue sections are incubated with a biotinylated secondary antibody, followed by an avidin-biotin complex conjugated to an enzyme (e.g. horseradish peroxidase). This enzyme then converts the chromogen (e.g. diaminobenzidine-tetrahydrochloride (DAB)-nickel) to a coloured precipitate (black/dark brown for DAB) at the location of the enzyme, and thus, the antibody-antigen complex, which can be visualised by light microscopy (Renshaw, 2007). Any endogenous peroxidase in the sample needs to be blocked in order to reduce non-specific background staining (since endogenous peroxidase will also react with the chromogen). H_2O_2 is therefore added to suppress endogenous peroxidase activity, without damaging the epitopes present in the tissue.

When double labelling is required the second primary antibody is visualised using a DAB reaction minus the nickel ammonium sulphate. This results in the formation of a brown precipitate that can be distinguished from the original black DAB nickel ammonium sulphate precipitant (generated for the first primary antibody).

Fos

c-fos is an immediate early gene and has been extensively used to identify activated neurosecretory neurones. It is generally thought that c-fos and its protein product Fos are good markers for identifying activated neurons that respond to a number of physiological and stress challenges (Kovács, 2008).

Fos expression in experimental mouse brains and brainstems were typically evaluated 90 min after treatment as this is the reported time for peak neuronal Fos expression after stimulation (Sharp et al., 1991). By double labelling with tyrosine hydroxylase (see below) activation of tuberoinfundibular dopamine (TIDA) neurones in the arcuate nucleus could be determined.

Figure 2.15

Tyrosine hydroxylase

Tyrosine hydroxylase is the rate-limiting enzyme in dopamine biosynthesis (Anderson et al., 2006a). It is responsible for converting L-tyrosine to dihydroxyphenylalanine, which is the precursor for dopamine. Tyrosine hydroxylase can be used as a marker for dopaminergic neurones and thus for TIDA neurones, which are located in the dorsomedial and ventrolateral regions of the arcuate nucleus (Grattan and Kokay, 2008). It can also be used as a marker of noradrenergic neurones.

pSTAT5

Prolactin receptor-mediated signal transduction in TIDA neurons appears to exclusively use the transcription factor Signal Transducer and Activator of Transcription Proteins 5b (STAT5b) (Grattan et al., 2001b), to stimulate transcription of genes such as tyrosine hydroxylase (Ma et al., 2005a). Prolactin binding to the long form of its receptor leads to the phosphorylation of STAT5. Phosphorylated (p)STAT5 can be used as a marker for prolactin signalling (negative feedback) in the TIDA neurons, when double labelled with tyrosine hydroxylase (Grattan, 2002).

2.10.2 Fos immunocytochemistry protocol

Brains were sectioned using a microtome (as described in section 2.9.3.1) at 48µm, typically stored in cryoprotectant at -20ºC and processed for Fos ICC (as previously described in our lab (Brunton et al., 2006)). For ICC, sections were transferred to small baskets and placed in petri dishes containing the solution required for each step. These were placed on a shaker at 70 rpm for washes. For incubations (e.g. with antibodies) sections were transferred to bijou vials and placed on the shaker at 80-100 rpm. To start, sections were washed in 0.1M phosphate buffer (PB) with 0.3% Triton X-100 (PBT; 2 x 5 min, 2 x 10 min PBT) and once in 0.1M PB (5 min). Endogenous peroxidase was blocked with 0.3% H_20_2 in 0.1M PB for 15 min. Sections were washed in 0.1M PBT (3 x 10 min) and 0.1M PB (1 x 5 min) before blocking for non-specific immunoreactivity with 3% normal sheep serum (NSS) in 0.1M PBT, for 1 h at room temperature. Sections were then incubated in primary antibody (anti-Fos Rabbit Polyclonal; 1:1000, Santa Cruz Biotechnology, Inc., Santa

Cruz, CA; sc-52)(Cai et al., 2010) in 3% NSS in 0.1M PBT (1ml/vial) for 20 h at 4ºC.

After ~20 h, sections were washed in 0.1M PBT (4 x 5 min, 2 x 10 min) before incubation in biotinylated anti-rabbit immunoglobulin (1:100) and normal goat serum (3:100) in 0.1M PBT (Vectastain ABC Elite Kit PK6101; Vector Laboratories, UK) for 1 h at room temperature. Sections were washed in 0.1M PBT (3 x 5mins) and incubated for a further 1 h in an Avidin DH and biotinylated horseradish peroxidase complex (1:50; Vector Laboratories; made up at least 30 min before use) in 0.1M PBT. Sections were washed in 0.1M PBT (2 x 10 min) and 0.1M sodium acetate (5 min) before visualisation using diaminobenzidine (DAB; 0.25mg/ml), nickel II sulphate (0.025g/ml) and 0.3% H_2O_2 for approximately 2 min, resulting in black labelling of the cell nuclei. The reaction was terminated with 0.1M sodium acetate (5 min) and the sections washed with 0.1M PB (4 x 5 min, 1 x 10 min) to reduce background staining. Sections were serially mounted onto gelatinised microscope slides and left to dry overnight. Slides were then passed through a series of ethanols (70%, 90%, 95%, 100% and 100%; 5 min each) followed by clearing in xylene (2 x 5 min). Slides were covered with the mounting medium DPX (Merck-BDH, Lutterworth, UK) and glass coverslips (25mmx60mm; VWR).

2.10.3 Fos and tyrosine hydroxylase immunocytochemistry

Brains were sectioned and processed for Fos immunocytochemistry as described above (see section 2.10.2) and tyrosine hydroxylase (Meddle et al., 2000). Following the Fos visualisation step, sections were washed in 0.1M PB (6 x 5 min) and 0.1M PBT (2 x 5 min). Unreacted peroxidase was blocked with 0.3% H_2O_2 (15 min) and sections washed in 0.1M PBT (2 x 10 min) before incubation with tyrosine hydroxylase primary antibody (anti-tyrosine hydroxylase Rabbit Polyclonal IgG, 1:4000; AB152; Millipore, CA, USA) in 3% NSS in 0.1M PBT (1ml/vial) for 40h at 4ºC.

Following incubation sections were washed in 0.1M PBT (3 x 10 min) and then incubated in biotinylated anti-rabbit immunoglobulin (1:2000; Vector Laboratories, Bucks, UK) and normal goat serum (3:2000) in 0.1M PBT for 2 h at room temperature. After washes in 0.1M PBT (3 x 10 min), sections were incubated for a further 2 h in Avidin DH and biotinylated horseradish peroxidase complex (1:200;

Vector Laboratories; made up at least 30 min before use) in 0.1M PBT. Sections were washed in 0.1M PBT (2 x 10 min) and 0.1M PB (5 min) before visualisation using DAB (0.25mg/ml) and 0.3% H_2O_2 for approximately 2 min, resulting in brown cytoplasmic labelling. The reaction was terminated with 0.1M PB (5 min) and the sections washed with 0.1M PB (6 x 5 min) to reduce background staining. Sections were serially mounted onto gelatinised microscope slides, left to dry overnight, and passed through a series of alcohols and xylene. Slides were covered with DPX mountant (Merck-BDH, Lutterworth, UK) and glass coverslips (25mmx60mm; VWR).

Positive technical controls: spare sections from mice which had previously had other sections processed through the ICC protocol were included for individual Fos and tyrosine hydroxylase antibody staining and also for double staining.

Negative technical controls: Omission of the primary Fos antibody resulted in no nuclear immunolabelling. Likewise, omission of tyrosine hydroxylase antibody led to no detection of cytoplasmic labelling (see Figure 2.16).

2.10.4 pSTAT5 immunocytochemistry

A protocol for pSTAT5 (phosphorylated Signal Transducer and Activator of Transcription 5) ICC was kindly supplied by Rosemary Brown (Grattan Lab, University of Otago, NZ)(Brown et al., 2011). Brains were sectioned using the microtome (as described in section 2.9.2) at 48µm and typically stored in cryoprotectant at -20ºC. Sections were washed in 0.05M Tris-buffered saline (TBS; 3 x 10 min). Antigen retrieval was carried out by incubating the sections in 0.01M Tris-HCL (pH 10) for 5 min at 90ºC (in a waterbath). Sections were left to cool for 5 min at room temperature before washing for 10 min in 'incubation solution' (0.05M TBS supplemented with 0.3% Triton X and 0.25% bovine serum albumin (BSA; Sigma-Aldrich)). Sections were then washed with TBS (3 x 10 min) before blocking endogenous peroxidase with 40% methanol, 9% H_2O_2 in 0.1M TBS. Sections were then washed in 0.1M TBS (3 x 10 min) before incubation with primary antibody diluted in 'incubation solution' and 2% normal goat serum (1:400, Rabbit Polyclonal phosphor-STAT5 (Tyr 694); Cell Signalling Technology, Massachusetts, USA). Sections were incubated for 72 h on an orbital shaker at 4ºC.

Figure 2.16

Following incubation, sections were washed 3 times in 0.1M TBS (10 min) and incubated in biotinylated anti-rabbit immunoglobulin (1:200; Vector Laboratories; diluted in 'incubation solution') for 90 min. Sections were washed again in 0.1M TBS (3 x 10 min) and 0.1M sodium acetate (5 min) before visualisation using DAB (0.25mg/ml), nickel II sulphate (0.025g/ml) and 0.3% H_2O_2 for approximately 2 min, resulting in black labelling in the cell nuclei. Reaction was terminated with 0.1M sodium acetate (5 min) and sections washed in 0.1M TBS (3 x 10 min) before mounting, and drying. Slides were then covered with DPX mountant (Merck-BDH, Lutterworth, UK) and glass coverslips (25mmx60mm; VWR).

2.10.5 pSTAT5 and tyrosine hydroxylase immunocytochemistry

Brains were sectioned and processed for pSTAT5 ICC as described above (see section 2.10.4). Following the visualisation step, the reaction was terminated with 0.1M sodium acetate (5 min) and sections washed in 0.1M TBS (3 x 10 min) as normal. Following this, sections were washed in 0.1M PB (4 x 5 min, 2 x 60 min) and 0.1M PBT (2x 5 min). Tyrosine hydroxylase ICC was then carried out as shown in section 2.10.3, starting with blocking of un-reacted peroxidase activity with 0.3% H_2O_2.

Positive technical controls: sections of tissue previously processed through the ICC protocol were included for individual pSTAT5 and tyrosine hydroxylase antibody staining and also for double staining (if possible).

Negative technical controls: Omission of the primary pSTAT5 antibody resulted in no nuclear immunolabelling. Likewise, omission of tyrosine hydroxylase antibody led to no detection of cytoplasmic labelling (see Figure 2.17).

2.10.6 Immuno-labelled cell quantification

Slides were coded so the experimenter was blind to treatment and examined under a light microscope (20x or 40x objective). Typically 4 sections were analysed per mouse for each area of interest (defined using (Franklin K, 1997)). For Fos or pSTAT5 single labelling, black or dark grey nuclei were counted as positively labelled cells. For double ICC with tyrosine hydroxylase, all cells with brown cytoplasmic staining (tyrosine hydroxylase-positive) were counted (both those with

Figure 2.17

and without a black/dark grey nucleus). The percentage of doubled labelled tyrosine hydroxylase-positive neurons were then calculated (see Figures 2.16 and 2.17).

2.11 HPLC analysis of DOPAC and dopamine content

2.11.1 General Principles

High-performance liquid chromatography (HPLC) is a powerful tool for analysis. It is used to separate compounds e.g. monoamines from a mixture to identify, quantify and purify specific components. This method can be utilized to determine dopamine activity by measuring the DOPAC and dopamine content of the median eminence (Shieh and Pan, 2001, Andrews and Grattan, 2003).

2.11.2 Method

Tissue preparation

A coronal section (~1mm thick) was cut rostrally from the optic chiasm using a mouse brain matrix for each mouse. The median eminence was then extracted from the section and immersed in 100µl of ice-cold 0.2 M perchloric acid. The tissue sample was homogenised using a polytetrafluoroethylene pestle and a cooled glass tissue grinder. This was then placed on ice for 30 min to denature, followed by centrifugation at 30,000xg for 15 min at 4^{o}C. 75 µl of supernatant was adjusted to ~pH 3 with 10 µl of 1M sodium acetate and frozen (-70°C) until the day of analysis. After centrifugation, pellets were resuspended in 30µl of distilled water and assayed in duplicate for total protein using a Coomassie protein assay kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). (This monoamine work was carried out by Dr John Menzies, Centre for Integrative Physiology, The University of Edinburgh).

HPLC analysis of DOPAC/Dopamine

Samples were thawed and then diluted 1 in 2 with 0.02M acetic acid containing 10µM EDTA. 40ng isoproterenol was added to each standard and unknown sample as an internal control. An Eicom HTEC-500 stand-alone HPLC system linked to an Eicom Insight autosampler (Eicom Europe, Ireland) was used to measure the monoamine content of each sample. Dopamine and DOPAC were separated using a reverse-phase Eicompak SC-3ODS 3x100mm analytical column in series with a 3x4mm pre-column packed with AC-ODS (Eicom Europe, Ireland) and were eluted

at a flow rate of 400µl/min in a mobile phase composed of 80% 0.1M citrate-acetate buffer (pH 3.5) and 20% methanol containing 220mg/l sodium octane sulphonate (TCI Europe, Zwijndrecht, Belgium) and 5mg/l EDTA. An electrochemical detector was used to detect voltage offset by applying a +750mV potential through a graphite WE-3G electrode at 25^{o}C. Dopamine and DOPAC were eluted within 10min of injection and the eluted peaks separated by using mixed monoamine standards (dopamine, Tocris, UK; DOPAC, Sigma, UK). A four-point standard curve was run daily before and after sample injection as a control. PowerChrom chromatography software (eDAQ, Poland) was used to record and integrate the signal from the detector, which was manually compared to retention times for each monoamine standard and converted to a concentration value (pg/ml). Finally, DOPAC:dopamine concentration ratios were also calculated. Intra-assay coefficient of variation was <10%. (The monoamine analysis was carried out by Dr John Menzies, Centre for Integrative Physiology, The University of Edinburgh).

2.12 In situ hybridisation (ISH)

2.12.1 General principles

In situ hybridisation (ISH) delivers invaluable information concerning the localisation of gene expression in morphologically preserved tissue. It is a very sensitive technique and thus allows maximal use of tissues that may be in short supply (Wilcox, 1993). DNA is a double-stranded nucleic acid chain, with two complementary strands consisting of four deoxyneucleotides, linked by phosphodiester bonds. The four bases in DNA are adenine, cytosine, guanine and thymidine and these are linked via hydrogen bonds to bind the two strands together (adenosine binds to thymidine, cytosine binds to guanine). RNA is a single strand nucleotide with thymidine substituted for uradine.

The principle of *in situ* hybridisation is specific annealing (hybridisation) of a labelled probe to complementary target DNA or RNA sequences present in tissue. A number of different types of probes can be used for example riboprobes or synthetic oligonucleotide probes. Riboprobes are prepared by transcription *in vitro* using complementary DNA (cDNA) sequences as a template. Subcloning is used to insert the cDNA into a transcription vector and is flanked by two different RNA polymerase initiation sites, enabling either sense- or antisense-strands to be

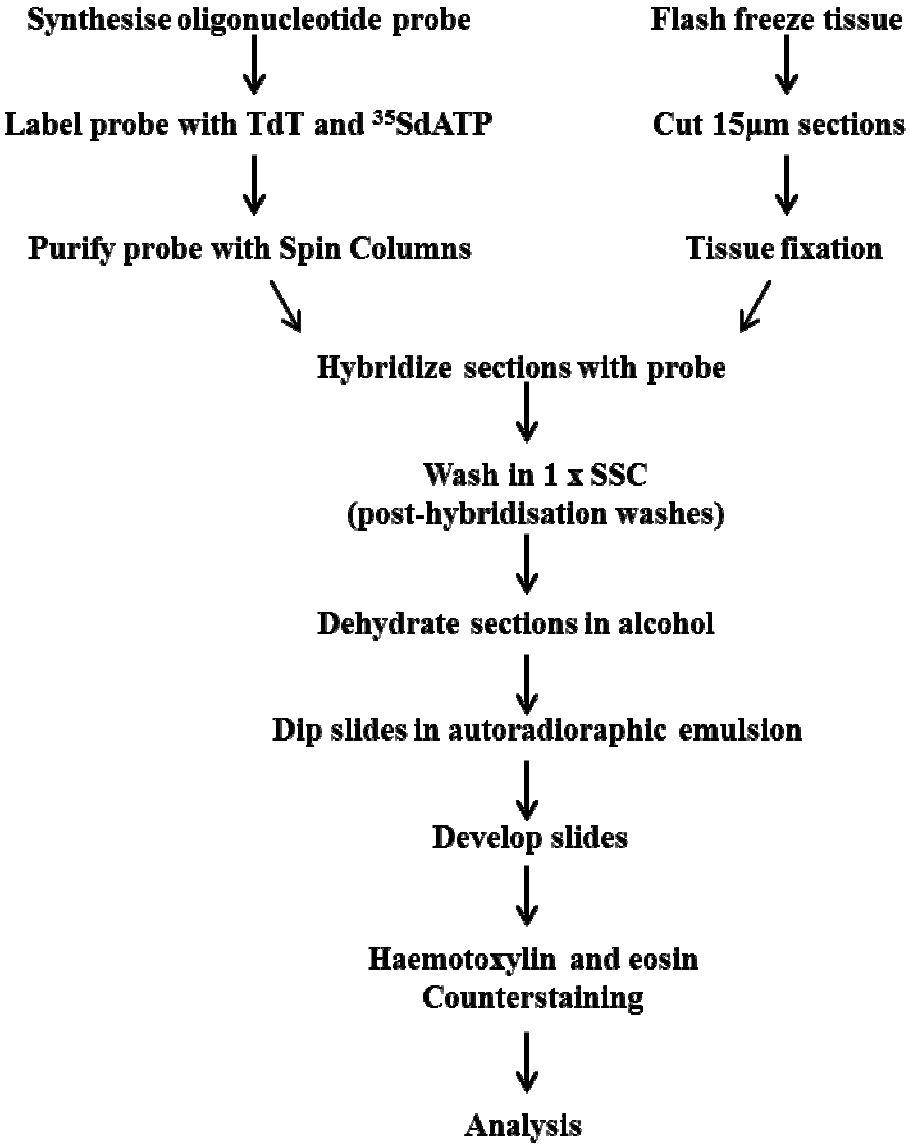
generated. Oligonucleotide probes are single strands of DNA (20-50 bases) that are synthesised then labelled. They are less sensitive than longer riboprobes and thus are typically used for detection of relatively abundant gene expression e.g. hormone mRNAs. To detect CRH, nur77 and vasopressin, oligonucleotide probes were used that were radioactively labelled (with ^{35}S). The stability of an oligonucleotide:RNA hybrid is dependent on length, composition (% guanosine:cytosine pairs vs. adenosine:thymidine pairs), temperature, salt concentration and the number of base-pair mismatches. The strength of the hybrid is more destabilised with shorter probe lengths, lower % guanosine:cytosine content (as guanosine:cytosine pairs have 3 hydrogen bonds compared to adenosine:thymidine which have 2 hydrogen bonds), higher temperatures (can dissociate (melt) probes depending of their stability), and increased numbers or mismatched base-pairs (Lewis ME, 1985).
Following hybridisation with the probe, sections are washed at a temperature relevant to the length of the probe (usually 20ºC below the melting temperature of the probe used). Detection and visualisation of the nucleic acid hybrids is then carried out (see Figures 2.18 and 2.19). The hybrids can be detected by autoradiographic emulsion (for radioactively labelled probes) or by histochemical chromogen means (for non-isotopically labelled probes) (Jin and Lloyd, 1997).

2.12.2 Method

In situ hybridisation was carried out (as described in (Douglas et al., 2003, Pincus et al., 2010)) to visualise and semi quantify mRNA for the immediate early gene nur77, corticotrophin releasing hormone (CRH) and vasopressin. The protocol below was used for all three of these, with the temperature of post-hybridisation washes and exposure time to autoradiographic emulsion changing depending on which probe was being used.

Figure 2.18:

Figure 2.19: Outline of oligonucleotide *in situ* hybridisation steps



Outline of *in situ* hybridisation steps using a labelled oligonucleotide probe.

Making stock and working solutions of unlabelled oligonucleotide probe

Oligonucleotide probes were synthesised (MWG Biotech AG, Ebersberg, Germany) corresponding to mouse CRH mRNA, nur77 mRNA and vasopressin mRNA. The probe sequences were complementary to: mouse CRH mRNA (bases 496-537) 5'- CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC -3'; mouse nur77 (bases 201-236) 5'- GTC TCG GGG CTG GCC AGG TCC ATG GTA GGC TTG CCG -3'; and vasopressin mRNA (bases 466-501) 5'- GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT -3'.

The lyophilised oligonucleotide probes was diluted to 100pmolμl^{-1}using sterile H_2O (autoclave ddH_2O) with stock solution and stored in 10µl aliquots at -20ºC until required. On the day of labelling, the stock solution was diluted to give a working solution of 10pmolμl^{-1} with ddH_2O.

3' end labelling of oligonucleotide probe

The 3' end of oligonucleotide probes (MWG Biotech AG, Ebersberg, Germany) were labelled with ^{35}S-dATP (NEN, PerkinElmer Life Sciences (UK) Ltd, Cambridge, UK) using terminal deoxynucleotidyl transferase (TdT; Promega, Southampton, UK; 25 units per 20pmole of probe). TdT is an enzyme which catalyses the addition of nucleotides to the 3' terminus of a DNA strand without requiring a template and was used to label the oligonucleotide probes with radioactivity labelled nucleotides.

The following were put into a sterile microcentrifuge tube, vortexed and incubated for 2 h at 37ºC (waterbath):

35µl sterile ddH_2O
10µl buffer
2 µl ^{35}S dATP
2 µl probe
1µl TdT enzyme (added last to start the reaction)

After 1 h a further 1 µl ^{35}S dATP and1µl TdT were added. Once the incubation was complete the reaction was stopped by placing the tubes on ice.

Purification Using Spin Columns

A QIAGEN quick nucleotide removal kit was used to 'clean-up' the radioactive probe by removing unincorporated nucleotides, salts, and other contaminants and oligonucleotides (>17 nt) and DNA are purified using a bind-wash-elute procedure. 1µl of each labelling reaction was removed before purification and put it into a scintillation tube together with a 3.5ml of scintillation fluid (Ultima Gold) to be counted using a β-counter (Packard Tri-Carb, 2500 TR liquid scintillation analyzer). Buffers provided in the QIAGEN quick nucleotide removal kit was used purify the probes. 500µl of buffer PN was added to the reaction sample (50µl) and the contents transferred into a spin column. The sample was centrifuged for 1 min at 6000rpm and the spin column placed into a new collection tube (radioactive flow-through discarded). 500µl of buffer PE (containing ethanol) was added to the column and centrifuged for 1 min at 6000rpm (radioactive flow-through discarded). This wash was repeated with another 500µl buffer PE and the tube (empty) centrifuged for an additional 1 min at13,000 rpm. The spin column was placed into a clean 1.5ml microcentrifuge tube and DNA eluted by adding 50µl buffer EB (elution buffer = 10mM TrisCl, pH 8.5) or sterile ddH_2O to the centre of the spin column. This was left to stand for 1 min before centrifuging for 1 min at13,000 rpm, which eluted the labelled probe into the micro-centrifuge tube. Another 1µl sample was removed and put it into a scintillation tube together with a 3.5ml of scintillation fluid (Ultima Gold). Using scintillation fluid as a blank, both the before and after purification samples were counted using the β-counter to calculate the percentage incorporation of the radiation. The tubes containing labelled probes with the highest percentage incorporation were used for hybridisation later (stored at -20ºC).

Tissue Fixation (section preparation for hybridisation)

Slides were removed from freezer and allowed to warm to room temperature before slide selection (with the aid of marker slides). Slides were transferred to slide racks and placed in 4% paraformaldehyde in 0.1M PBS for 10 min and then washed twice in 1x PBS for 5 min. Slides were then incubated in Triethanolamine (TEA; Sigma) and acetic anhydride (Sigma) for a further 10 min to help reduce background. Slides were then rinsed in ddH_2O for 2 min before being dehydrated through a series of

alcohols: 70% ethanol, 95% ethanol, 100% ethanol, 100% chloroform, 100% ethanol and 95% ethanol, all for 3 min. Slides were then thoroughly air dried.

RNase predigestion controls

Two RNase-treated slides were included to act as negative controls for the ISH. RNase (ribonuclease) is an enzyme that catalyses the breakdown and degradation of RNA. 2µl of RNase A (Sigma-Aldrich, UK) was added to 2.9ml RNase buffer (10mM Tris, 0.5M NaCl, 1mM EDTA). 40µl of the solution was spread onto each section (160µl/slide) and slides were incubated for 1 h at 37ºC in a humid chamber. Humid incubation chambers were prepared by lining a plastic box (labelled RNase only) with moist (ddH_2O) filter paper. Slides were arranged on glass platforms. Following incubation, the solution was tipped off the slides and they were placed in a coplin jar filled with ddH_2O for 5 min, before drying.

No probe controls

As another negative control two slides were included that were not it contact with the radioactively-labelled probe (no probe controls).

Hybridisation

Hybridisation solution was prepared with 45µl required for each slide (based on four mouse sections/slide). The sections were hybridised using hybridisation solution (hybridisation buffer + formamide + diothioreitol) containing the labelled oligonucleotide probe (210,000cpm per slide; 45µl per 4 sections).

The required volume of hybridisation buffer (containing formamide) was added to a sterile tube. 15µl of DTT per 1ml of Hybridisation Solution was added along with the necessary volume of probe. Specific radioactivity: for nur77 = 923017cpm/pmole probe (26.5fmole/µl hybridisation buffer); CRH = 983395cpm/pmole probe (29.7fmole/µl hybridisation buffer); and vasopressin = 914412cpm/pmole probe (31.6fmole/µl hybridisation buffer).

Probe Calculations:

- Total volume of hybridisation mixture required = 45 µl x no. of slides = X µl
- Hybridisation mixture requires 4615 cpm/µl, therefore,
  Total radioactivity (cpm) required = total volume (X) x 4615 = Y cpm
- Volume of probe required = Y/labelled probe count = Z µl of probe in X µl of hybridisation mixture.

Using a pipette 45µl of hybridisation mixture was added to one end of each slide and evenly distributed across the sections using a Nescofilm 'coverslip'. Two no probe slides were coated with hybridisation buffer/DTT only. Slides were then placed into the moist chambers and left to hybridise at 37ºC overnight (20hours).

Post-Hybridisation Washes

Slides were removed from the oven and 'swept' back and forth through 1 x SSC to remove the Nescofilm coverslip, at room temperature. The slides were then ran through 2 more rinses (at room temperature) with 1 x SSC before being placed into slide racks. A water bath was heated to the appropriate temperature: 58ºC for CRH probe and 55ºC for vasopressin and nur77 probes. 1 x SSC was heated and the slides in racks rinsed for 4 x 15 min. After the warm washes slides were rinsed in room temperature 1 x SSC for 2 x 30 min washes and finally in ddH_2O before slides were left to dry.

Dipping slides in autoradiographic emulsion

Emulsion (Kodak NTB-3) was removed from the fridge and allowed to warm to room temperature (~2 h). In a dark room (under light-safe conditions) the emulsion bottle was placed into a black plastic pot in a waterbath (40ºC) to melt (~1.5 h). Once melted, the emulsion was diluted (3 emulsion: 1 sterile ddH_2O) and transferred to a coplin jar in the water bath (under safe-light conditions). Slides were arranged in plastic grips and dipped in the emulsion (each for the same length of time). Slides were allowed to drip on tissue before transfer to light-tight drying boxes (left overnight). Once the emulsion was dry, slides were transferred into slide boxes (under safe-light conditions) containing silica bags. Boxes were then sealed with electrical tape, wrapped in tin foil and sealed in a plastic bag. These were then stored

either at room temperature (CRH and nur77) or at 4ºC (vasopressin) for the desired exposure time (CRH about 6 weeks, nur77 about 4 weeks and vasopressin about 7 days).

Developing emulsion dipped slides

Developer (Kodak D19; 157g added to 500ml ddH_2O) and fixer (Ilford Hypam rapid; diluted 100ml in 400ml ddH_2O) were prepared. In the dark room under safe-light conditions slides were transferred to plastic racks and placed in fresh developer for 5 min. Slides were then dipped in ddH_2O for 2 sec and placed in fixer (2 x 5min), followed by ddH_2O (2 x 5 min). Slides were allowed to dry thoroughly before counter-staining

Haematoxylin and eosin counterstaining

Slides were placed in 0.4% haematoxylin (filtered) for 2 min and rinsed in water. They were then dipped in acid alcohol, rinsed in water then placed in Scott's Tap Water Substitute for 3 min. Slides were washed in water for 3 min, placed in 1% Eosin (filtered) for 2.5 min and rinsed again in water before dipping in potassium alum for 2 min. Slides were then rinsed in water and passed through graded alcohols (70%, 80%, 95%, 100%, 100% ethanol) for 5 min each before clearing with xylene (2 x 5 min). Slides were covered with DPX mountant (Merck-BDH, Lutterworth, UK) and glass coverslips (25mmx60mm; VWR).

Negative Controls

RNase treated and no probe controls (see Figure 2.20 for example of vasopressin controls) showed very little background labelling and no positive cells.

Positive cell counting

Slides were coded so the experimenter was blind to treatment group. The number of positive cells in the area of interest were counted under a light microscope (40x objective). A positive cell was typically identified as one that contained more than three times the number of silver grains than the background (area with low signal close to the area of interest). The number of positive cells was counted in the area of interest (e.g. PVN) in each section (~4 sections per mouse) and mean number of cells in each section calculated.

Figure 2.20

Grain Area Analysis

The grain area of cells identified as positive were measured using a computer image analysis system Open Lab (Improvision Inc, Lexington, MA, USA). The grain area of 20 positive cells (or fewer if <20 positive cells present) per section was measured, along with 4 background cells. The average background measurements were subtracted from the average positive cell measurements for each mouse to calculate the true mean grain density of a positive cell.

2.13 Behavioural testing

In order to test for anxiety behaviour, two commonly used behavioural tests were carried out: the elevated plus maze and the open field test. These both exploit the natural aversion of rodents to open areas.

2.13.1 Elevated plus maze

The elevated plus maze (EPM) is commonly used as an assay for anxiety-related behaviour in rodents. The maze is made up of 2 open arms and 2 closed arms (enclosed by a wall). Behaviour in this test reflects the conflict between the preference for enclosed protected areas (closed arm) and the motivation to explore novel environments (open arm). In contrast to other behavioural anxiety tests which rely on noxious stimuli (e.g. electric shock) and normally produce a conditioned response, the maze relies on the rodent's penchant for dark and enclosed spaces and an unconditioned fear of open spaces and heights (Walf and Frye, 2007). Anti-anxiety behaviour can be observed by increased time/activity in the open arm. Motor activity can also be determined by recording the number of entries to open and closed arms. Discrepancies often arise between conclusions made from studies using the EPM, likely reflecting differences in analysis parameters. Therefore, in an attempt to better characterise the anxiety behaviour a number different criteria can be used including classic temporal and spatial parameters as well as other ethological parameters, such behaviours aimed at assessing the presence of danger, risk assessment behaviours (e.g. stretched attended posture) (Carola et al., 2002).

An EPM was constructed for use with mice. It comprised of four arms (2 open, 2 closed) coming out from a central area (5x5cm square) on a 1m high cross-stand. The closed arms were enclosed by walls (15cm high x 30cm long x 5cm wide), while the open arms were completely open (30 cm long x 5cm wide) (Carola et al., 2002)(see

Figure 2.21). Mice were placed into the centre square (neutral area) of EPM and allowed to freely move whilst being video recorded from above for 5 min (experimenter switched on video and left the behavioural room for 5 min). It has been previously demonstrated by Montgomery (1955) that rats display a more robust avoidance response in the first 5 min of the test. The behavioural recording was later analysed manually for typical anxiety behaviour criteria (Carola et al., 2002), the latency to first open arm entry, number of open arm entries, total entries (to open and closed arms), percentage time spent in each arm (open arm, closed arm), and the number of stretch attends performed. Stretch attends represent risk assessment involving the mouse stretching its body, from a more protected area (closed arm/neutral zone) towards a less protected area (open arm).

2.13.2 Open field test

The open field test (OFT) is frequently used to measure anxiety-related behaviour in rodents and consists of an empty and bright square/arena, surrounded by walls. The OFT task reflects the conflict between the rodent's innate fear of the central area (open, unknown and potentially dangerous) versus their desire to explore novel environments. When anxious, the natural tendency of the rodent is to stay in close proximately to the walls of the arena (thigmotaxis). In this context, anxiety-related behaviour is measured by the degree to which the rodent avoids the central area (inner zone) of the arena (Choleris et al., 2001).

The open field test is carried out on an arena: board divided into 25 squares (5 x 5 squares; 10cm^2) with a 15cm high wall (Kimura et al., 2009); see Figure 2.22). The central 9 squares are considered to be the inner zone (more anxiogenic) and the outer 16 squares the outer zones (less anxiogenic). The area was made out of thick cardboard and coated in sticky-back plastic to allow cleaning between each mouse tested. The mouse was placed onto the central square (in the inner zone) and allowed to move freely over the arena whilst being video recorded for 5 min (experimenter switched on video and left the behavioural room for 5 min). The behavioural recording was later analysed manually for percentage of time spent in the inner zone, number of inner zone entries, the number of crossings over the grid lines in the inner zone, total crossings (to give indication of locomoter activity) and the number wall rears (vertical exploration).

Figure 2.21

Figure 2.22

2.14 General statistics

All data is presented as means ± SEM. The statistical tests used are mentioned in each chapter. These were typically 1-way ANOVAs, 2-way ANOVAs, and 3-way ANOVAs, along with Holm-Sidak post hoc tests. Unpaired Students t-tests or Chi-squared tests were also used in some cases. P values <0.05 were considered significant. Statistical tests were carried out using the programme SigmaPlot/SigmaStat (Jandel Scientific, Germany).

Chapter 3

Effects of stress on the hypothalamo-pituitary-adrenal axis and the prolactin system in early pregnancy

Chapter 3: Effects of stress on the HPA axis and the prolactin system in early pregnancy

3.1 Introduction

3.1.1 Control of prolactin secretion

Prolactin, produced predominantly in the anterior pituitary, is normally under the inhibitory control of dopamine, released from neurosecretory hypothalamic neurones. TIDA neurones provide the major source of dopamine and are located in the arcuate nucleus. They release dopamine from their nerve terminals in the median eminence, which diffuses into capillaries of the pituitary portal blood vessels where it is then transported to anterior pituitary. Here dopamine inhibits the release of prolactin via activating its receptors on lactotrophs (Demarest et al., 1985). Dopamine activates its D2 receptors located here to tonically inhibit secretion of prolactin (Freeman et al., 2000). A circadian rhythm is apparent in both women (Freeman et al., 2000, Ben-Jonathan et al., 2008) and rodents at proestrous (Freeman et al., 2000). A preovulatory prolactin surge occurs during the afternoon of proestrous in rodents, coinciding with the luteinising hormone (LH) surge (Egli et al., 2010). Rodents have a different secretory profile to women: during early pregnancy in mice it has been shown that serum prolactin increases in a pattern of bi-daily prolactin surges. Mating in rats and mice induces a neurogenic reflex consisting a large nocturnal and a smaller diurnal a prolactin surge (Grattan and Kokay, 2008, Larsen and Grattan, 2010), although studies have reported only a single diurnal surge in pseudopregnant mice (Yang et al., 2009).

As secretory prolactin profiles are known to alter in early pregnancy, we aimed to determine if the prolactin response to dopamine differed from virgins by administering a D2 dopamine antagonist to reduce activation of D2 receptors. We hypothesised that the prolactin response to the antagonist would be more exaggerated in early pregnancy, showing an alteration to the prolactin control system enabling the elevated levels of prolactin observed in early pregnancy. In addition, blood samples from extra mice administered a dopamine antagonist were also collected to allow further validation of the prolactin ELISA adapted for use with plasma.

3.1.2 Effect of stress in early-mid pregnancy on the HPA

Prolonged exposure to stress in early pregnancy is known to have profound adverse effects on reproduction and has been associated with pregnancy failure (Nakamura et al., 2008, Parker and Douglas, 2010) and foetal programming (Seckl and Meaney, 2004). Mice do not experience "abortions", however, the presence of resorption sites in the uterine horns indicate foetal loss. High stress situations cause the secretion of corticotrophin-releasing hormone (CRH) and vasopressin (AVP) from the hypothalamus. These two neuropeptides then act synergistically to stimulate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary corticotrophs and consequently the release of the stress hormone cortisol (corticosterone in rodents) from the adrenal cortex. It is known that the HPA response to stress is attenuated in late pregnancy (Brunton and Russell, 2008, Brunton et al., 2008), however, responses are poorly understood in early pregnancy.

We aimed to investigate the HPA responses in early-mid gestation and to do this we used two ethologically relevant stressors, chosen to mimic situations that may potentially be experienced by pregnant women: infection/fever or hunger possibly arising from malnutrition or hyperemesis. Thus, if experienced during gestation they may have detrimental effects on pregnancy maintenance, similar to other stressors (e.g. psychological or noise stress (Joachim et al., 2003)). In order to determine the effects of stress on the HPA axis in early pregnancy, mice were injected with lipopolysaccharide (LPS; immune stress) and mRNA expression was measured for hypothalamic CRH, nur77 (immediate early gene and regulator of CRH) and vasopressin in the paraventricular nucleus, along with plasma corticosterone and progesterone. Progesterone was measured to investigate the acute effects of stress in early pregnancy on the secretory function of the corpus luteum.

3.1.3 Effect of stress in early pregnancy on the prolactin system

A major candidate to mediate brain-to-body responses to stress is prolactin and its hypothalamic control mechanisms. Prolactin is essential for rodents and facilitatory in women to enhance the secretion of progesterone, acting as a luteotrophic hormone by maintaining the integrity of the corpus luteum following mating. It has been reported to be a potent survival factor for human granulosa cells, while reduced prolactin has been shown to compromises corpus luteum formation (Perks et al.,

2003). Prolactin, is involved in a number of other important processes including initiation and maintenance of lactation, reproductive behaviour and immune modulation (Freeman et al., 2000).

Typically stress increases prolactin secretion in virgin or male rodents (Freeman et al., 2000). However, it has been proposed that stress stimulates prolactin release if pre-stress levels are low, but inhibits its secretion if pre-stress levels are high, for example, during the proestrous afternoon surge (Gala, 1990). However, Poletini et al. (Poletini et al., 2006) have shown that the pattern of prolactin secretion in response to stress in female rats depends upon the plasma levels of ovarian steroids rather than prestress levels. From the literature it seems that very little work has been carried out investigating the effects of stress on the prolactin system in early pregnancy.

The aim of this study was to determine whether immune stress and/or 24h fast in mice decreases prolactin concentration in early pregnancy, corresponding with reported stress-reduced progesterone secretion (Joachim et al., 2003), and furthermore, whether stress-reduced prolactin is associated with an increase in TIDA neurone activation. Stress-reduced prolactin in early pregnancy may represent an exciting new target for potential prolactin therapy in early pregnant women displaying low prolactin concentrations as this has been linked with an increased incidence of miscarriage in women (Douglas, 2010).

Immunocytochemistry for nuclear Fos, the protein product of the immediate early gene c-fos, has long been used to measure neuronal activation (Sagar et al., 1988). For the neuroendocrine systems it has provided vital information regarding when and where stimuli are processed (Hoffman and Lyo, 2002). By double-labelling with another protein marker it allows activation of specific neuronal cell types to be measured. Tyrosine hydroxylase (rate-limiting enzyme in dopamine biosynthesis) can be used as a marker for TIDA neurones in the arcuate nucleus (Anderson et al., 2006a), therefore, Fos-positive tyrosine hydroxylase stained cells indicate neuronal activation of these TIDA neurones in the arcuate nucleus. We aimed to use double labelling with tyrosine hydroxylase and Fos to determine whether there was an increase in activation of TIDA neurones following stress (LPS and 24h fast) in early pregnancy, associated with reduced prolactin. Despite Fos expression in the TIDA neurones after immune challenge being observed to reflect alterations in prolactin

secretion (Hollis et al., 2005), Fos is reported to not always provide a good prediction of TIDA activation (Hoffman et al., 1994). Therefore, we also measured the dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC; dopamine metabolite) content in the median eminence and used these and the DOPAC:Dopamine ratio to provide more insight into dopamine activity.

3.1.4 Aims and Hypotheses

Aims:

1. To determine if the prolactin response to dopamine is altered in early pregnancy compared to virgins, demonstrating a change in the prolactin control system to permit the increased prolactin concentrations typically observed in early pregnancy.
2. To investigate the HPA axis responses to stress (immune stress) in early-mid pregnancy and compare these to virgin mice.
3. To determine the effect of immune or 24h fast stress on circulating prolactin levels in early pregnancy.

Hypotheses:

1. The prolactin response to dopamine is attenuated in early pregnancy vs. virgins.
2. The HPA axis responses to stress will be elevated in early pregnancy compared to virgin mice.
3. Exposure to stress will cause a reduction in prolactin secretion in early pregnant mice compared to control and this will be associated with reduced progesterone levels and an increase in activation of TIDA neurones.
4. Exposure to stress in early pregnancy will lead to a reduction in pregnancy maintenance/rate.

3.2 Methods

3.2.1 Control of prolactin in early pregnancy

3.2.1.1 Treatment

A. time responses. Preliminary trials were carried out on virgin mice to test the time-course of prolactin response to the dopamine antagonist metoclopramide. Metoclopramide was dissolved in 0.9% saline (25µg/100µl saline) and a dose of 1µg/g body weight (or 100µl saline) was administered subcutaneously (s.c.) This dose has been reported to significantly increased plasma prolactin in mice (Knoferl et al., 2000). Mice were returned to their home cage and then sacrificed by conscious decapitation after 10, 30, 60 or 120 min (n = 4/group). Trunk blood was collected, processed for plasma and stored at -20ºC until assay using the prolactin ELISA.

B. dose responses. Dose-response curves (prolactin response) were generated for virgin and d5.5 pregnant mice. Virgin and d5.5 mice were administered various doses of metoclopramide (0.1, 0.5, 1, 5, or 15µg/g body weight; s.c; n = 6) and returned to their home cages. After 10 min mice were quickly moved to an adjacent room and sacrificed by conscious decapitation (10 min time-point found to generate the largest prolactin peak in the preliminary trial above). Trunk blood was collected, processed for plasma and stored at -20ºC until assay with the prolactin ELISA. Following blood collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

3.2.1.2 Prolactin ELISA

Plasma samples were diluted 1 in 25 and assayed in duplicate using an adapted protocol (see general methods section 2.7.2.2) for the mouse prolactin DuoSet ELISA (DY1445, R&D Systems, Inc., Minneapolis, US).

3.2.2 Effect of immune stress on plasma hormones, IL-6 and neuronal activation in early pregnancy

3.2.2.1 Treatment

Virgin and d5.5 pregnant mice were gently restrained in a holding bag and injected with lipopolysaccharide (LPS; 12.5µg/100µl saline, i.p; *E.coli* E055:B5, Sigma, Poole, Dorset, UK; n = 6) (Hollis et al., 2005) or vehicle (100µl saline, i.p; n = 6, 8

respectively) and mice returned to their home cages (these mice were used for hypothalamic neuronal activation analysis). A second cohort of mice (virgin and d5.5 pregnant) were treated in exactly the same way to provide plasma and brainstems for hormone assays and neuronal activation analysis in the brainstem (these samples were not collected from the original set of mice; n = 6). A third cohort of mice (virgin and d5.5 pregnant) were treated as before with LPS (n = 8) or vehicle (n = 8, 9 respectively) and were used to provide median eminence samples for measuring the content of the monoamines dopamine and its metabolite DOPAC. The DOPAC:dopamine ratio was used as an additional measure of dopamine activity (Andrews and Grattan, 2003).

3.2.2.2 Blood/tissue collection and perfuse fixation

After 90 min, mice (from the first two cohorts) were quickly moved to an adjacent room and injected with an overdose of sodium pentobarbitone (0.3ml i.p; Centravet, 22106 Didan, France). Once the mouse was deeply anaesthetised the heart was exposed. Blood was removed by cardiac puncture before the mouse was perfused with heparinised saline, then fixed with 4% paraformaldehyde (see General Methods section 2.9.1) The brain and brainstems were carefully removed and stored overnight (4ºC) in 4% paraformaldehyde and 15 % sucrose solution. They were then cryoprotected in a 30% sucrose solution at 4ºC overnight and frozen (-70°C). Blood was processed for plasma and frozen (-20°C) before assays. Mice from the final cohort were killed after 90 min by conscious decapitation and the brains removed and processed for HPLC analysis of DOPAC and dopamine content in the median eminence (see General Methods section 2.11; monoamine work was carried out by Dr John Menzies, Centre for Integrative Physiology, The University of Edinburgh). Following blood/tissue collection in the above experiments pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

3.2.2.3 Prolactin ELISA

Plasma samples were diluted 1 in 25 and assayed in duplicate using an adapted protocol (see General Methods section 2.7.2.2) for the mouse prolactin DuoSet ELISA kit (DY1445, R&D Systems, Inc., Minneapolis, US).

3.2.2.4 Progesterone RIA

Plasma samples (undiluted) were assayed in duplicate using a progesterone RIA kit (DSL-3900; kit 1; see General Methods section 2.6.1.2).

3.2.2.5 IL-6 ELISA

Plasma samples were diluted 1 in 50 and assayed in duplicate using an adapted protocol (see General Methods section 2.7.3.2) for the mouse IL-6 DuoSet ELISA kit (DY406, R&D Systems, Inc., Minneapolis, US).

3.2.2.6 Corticosterone RIA

Plasma samples were diluted 1 in 200 and assayed in duplicate using a cortocosterone RIA kit (Corticosterone ImmunChem Double Antibody RIA kit; MP Biomedicals) (see General Methods section 2.6.3.2).

3.2.2.7 Brain sectioning, tyrosine hydroxylase/Fos ICC and analysis

Brains and brainstems (from first two cohorts) were sectioned using a freezing microtome (48µm). Coronal brain sections containing paraventricular nucleus (PVN), arcuate nucleus, ventromedial hypothalamic nucleus (VMH), supraoptic nucleus (SON), and brainstem sections containing the locus coeruleus (LC), nucleus tractus solitarius (NTS), rostral ventrolateral medulla (RVLM) were stored in cryoprotectant before double immunocytochemistry (ICC) was carried out for Fos and tyrosine hydroxylase (TH) (see General Methods section 2.10.3).

Using a light microscope (20x or 40x objective) on coded slides, Fos-positive cells (dark grey/black nucleus) were counted in roughly 4 sections per mouse for each area of interest (defined using (Franklin K, 1997)). For the PVN, Fos-positive cells were quantified for the magnocellular (m) and parvocellular (p) PVN separately. In addition to Fos only counts, the arcuate nucleus and the brainstem, were analysed for the number of tyrosine hydroxylase-positive cells colocalized with Fos (expressed as a percentage of the total number of tyrosine hydroxylase cells).

3.2.2.8 HPLC analysis of DOPAC/Dopamine

The DOPAC and dopamine content of the median eminence was measured using HPLC (see General Methods section 2.11; monoamine analysis was carried out by Dr John Menzies, Centre for Integrative Physiology, The University of Edinburgh).

3.2.3 Effect of immune stress in early and mid-pregnancy on HPA axis and pro-inflammatory cytokine activity

3.2.3.1 Treatment

Virgin and pregnant (d5.5, d10.5) mice were gently restrained and injected i.p. with either LPS (10µg/100µl saline; *E.coli* E055:B5, Sigma; n = 9,6,5, respectively) or vehicle (100µl saline; n = 9,7,4 respectively) and mice were replaced into their home cage. Following 90 min mice were moved to an adjacent room and were immediately decapitated (<1 min after removal from home cage). Trunk blood was collected, processed for plasma and frozen (-20 ^{o}C). The brains were collected, quickly frozen in crushed dry ice and stored at -70^{o}C. Another cohort of virgin and day 5.5 pregnant mice were injected i.p with LPS (12.5µg/100µl) (n = 7, 5 respectively) or vehicle (saline; n = 5, 5 respectively) and sacrificed 4h later. Brains were collected as before. Following sample collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

3.2.3.2 Plasma hormone levels

Plasma from the 90 min experiment was assayed for corticosterone and progesterone, using the same dilutions as described above (see General Methods section 3.2.2).

3.2.3.3 Proinflammatory cytokine levels

Plasma from the 90 min experiment was assayed for TNF-α and IFN-γ by cytometric bead array (see General Methods section 2.8.2).

3.2.3.4 In situ hybridisation and analysis

Brains were sectioned (15µm) on a cryostat prior to *in situ* hybridisation for quantitative analysis of CRH and nur77 (stress-induced immediate early gene) mRNA expression in the pPVN (see General Methods section 2.12.2). The second group of mice were processed for vasopressin mRNA expression in the pPVN.

Vasopression drives ACTH secretion synergistically with CRH and its response to stress is typically seen in vasopressin mRNA after 4h. Coded slides were analysed for the number of positive cells and the grain cell area. The total grain area (μm^2) was calculated by multiplying the mean number of positive cells by the mean grain area.

3.2.4 Effect of immune stress in early pregnancy on pregnancy rate

3.2.4.1 Treatment

d5.5 'pregnant' mice (mice where a plug was found on d0.5) were gently restrained and injected with either LPS (12.5µg/100µl saline, i.p; n = 11) or vehicle (100µl saline, i.p; n = 10). Mice were returned to their home cages. On gestation day 13.5 mice were quickly moved to an adjacent room and killed by conscious decapitation. Trunk blood was collected, processed for plasma and frozen (-20ºC) until use in hormones assays.

3.2.4.2 Pregnancy status analysis

The pregnancy status was established on d13.5 by laparotomy and inspection of the uterine horns for healthy foetal development and signs of resorption. Mice were considered 'pregnant' if they had ≥4 developing foetuses and 'not pregnant' if they had ≤3 developing foetuses and signs of resorption (see Figure 3.1).

3.2.4.3 Plasma hormone levels

Plasma samples were measured for prolactin and progesterone using the same dilutions as described previously (section 3.2.2).

3.2.5 Effect of 24h fast stress on plasma hormones, IL-6 and neuronal activation in early pregnancy

3.2.5.1 Treatment

Virgin and d5.5 pregnant mice were fasted for 24h (n = 6, 8 respectively), with food removed from their home cage at 10am (lights on at 7am), controls were allowed to feed normally (n = 6, 8 respectively). After 24h mice were quickly moved to an adjacent room and were sacrificed by conscious decapitation (to provide trunk blood for assays). A second cohort of mice were treated exactly the same but were perfused

Figure 3.1

fixed following 24h treatment to provide tissue for analysis of neuronal activation (virgin fast and control, n = 6; d5.5 fast and control n = 5, 6 respectively).

3.2.5.2 Blood/tissue collection and perfuse fixation

For blood collection one cohort of mice were killed by decapitation and their trunk blood collected, processed for plasma and frozen (-20ºC) until use in assays. For brain collection the second cohort of mice were perfused fixed as described in section 3.2.2.2 and brains were frozen before sectioning on a microtome. Following blood/tissue collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

3.2.5.3 Plasma hormone and IL-6 levels.

Plasma samples were measured for prolactin, progesterone and corticosterone as previously described (section 3.2.2). Samples were also diluted 1 in 10 and assayed in duplicate for IL-6 using the adapted mouse IL-6 DuoSet ELISA (DY406, R&D Systems, Inc., Minneapolis, US; see General Methods section 2.7.3.2).

3.2.5.4 Brain sectioning, tyrosine hydroxylase/Fos ICC and analysis

As described in section 3.2.2, tyrosine hydroxylase and Fos ICC was carried out on coronal brain sections containing the PVN, arcuate nucleus, VMH, and SON, and the expression of Fos and tyrosine hydroxylase was analysed.

3.2.6 Effect of 24h fast stress in early pregnancy on pregnancy rate

3.2.6.1 Treatment

d5.5 pregnant mice were fasted for 24h (n = 11), with food removed from their home cage at 10am (lights on at 7am), controls were allowed to feed normally (n = 10). On gestation day 13.5 mice were quickly moved to an adjacent room and killed by conscious decapitation. Trunk blood was collected, processed for plasma and frozen (-20ºC) until use in hormones assays and the pregnancy status was established.

3.2.6.2 Pregnancy status analysis

The pregnancy status was established by laparotomy and inspection of the uterine horns for healthy foetal development and signs of resorption. Mice were considered

‘pregnant’ if they had ≥4 developing foetuses and ‘not pregnant’ if they had <3 developing foetuses and signs of resorption.

3.2.6.3 Plasma hormone levels

Plasma samples were assayed for prolactin and progesterone as previously described, using the same dilutions (section 3.2.2).

3.2.7 Statistics

Statistical evaluation of the mean hormone concentrations (prolactin, progesterone and corticosterone), IL-6 concentration, neuronal activation and mRNA gene expression for each group were typically determined by 2-way ANOVAs with a Holm-Sidak post-hoc test. Data for DOPAC and dopamine content in the median eminence were analysed by 2-way ANOVAs on rank-transformed data, as the unranked data failed normaility testing. Prolactin and progesterone data from d13.5 LPS and vehicle treated mice could not be analysed using a 2-way ANOVA (as there were no mice in the ‘pregnant’ LPS group) and Student t-tests were carried out on the remaining groups. Pregnancy rate was evaluated using a chi square test. $p<0.05$ was considered significant. Data are presented as group means±SEM (excluding outliers: mean±3*SEM).

3.3 Results

3.3.1 Control of prolactin secretion in early pregnancy

3.3.1.1 Effect of metoclopramide on prolactin concentration

Basal prolactin concentrations did not differ over time after injection of saline. Metoclopramide treatment significantly increased plasma prolactin and this varied depending on how long after administration mice were sacrificed (2-way ANOVA, interaction treatment x group $p= 0.025$). Mice sacrificed 10 min after metoclopramide administration had plasma prolactin levels that were significantly higher than the vehicle treated group at the same time (Holm-Sidak post hoc test, $p<0.05$ vs. vehicle) (Figure 3.2 A).

3.3.1.2 Effect of pregnancy on dopaminergic inhibition of prolactin secretion

Administration of increasing doses of metoclopramide elevated plasma prolactin concentrations in both virgin and d5.5 pregnant mice (2-way ANOVA, $p<0.001$ across dose (treatment), $p=0.002$ across group; Figure 3.2 B). Prolactin secretion was significantly higher in d5.5 pregnant mice, compared to virgins, following metoclopramide treatment at the 0.5 and 1.0μg/g doses (Holm-Sidak post hoc test, $p<0.05$ vs. vehicle). Therefore, the dose-response curve was shifted to the left in pregnant mice.

3.3.2 Effect of immune stress on plasma hormones, IL-6 and neuronal activation in early pregnancy

3.3.2.1 Prolactin concentration

Basal prolactin concentration was significantly higher in d5.5 pregnant vs. virgin mice (vehicle-treated). LPS treatment had no significant effect on plasma prolactin concentration in virgin mice but significantly decreased it in d5.5 pregnant mice (2-way ANOVA, interaction treatment x group $p<0.001$; Figure 3.3 A).

3.3.2.2 Progesterone concentration

Basal plasma progesterone was significantly greater in pregnant vs. virgin mice. LPS significantly increased plasma progesterone in the virgin group compared to control. In contrast, LPS treatment in d5.5 pregnant mice significantly decreased plasma progesterone (2-way ANOVA**,** interaction treatment x group $p<0.001$; Figure 3.3 B).

Figure 3.2

3.3.2.3 IL-6 concentration

Basal levels of IL-6 in the plasma were low and similar in both virgin and early pregnant mice. LPS treatment significantly increased plasma IL-6 in both groups (2-way ANOVA, interaction treatment x group $p<0.001$) although to a higher degree in early pregnancy (Holm-Sidak post hoc test, $p<0.05$ vs. virgin; Figure 3.3 C).

3.3.2.4 Corticosterone concentration

In both virgin and early pregnant mice the basal corticosterone concentrations were similar. LPS treatment significantly elevated plasma corticosterone compared to vehicle-treated mice and there was no difference between virgin and pregnant mice (2-way ANOVA, $p<0.001$ across treatment; Figure 3.3 D).

3.3.2.5 Activation of TIDA neurones

TIDA neurones are located in dorsomedial and ventrolateral regions of the arcuate nucleus (see Figures 3.4A and 3.4B). The basal number of Fos-positive neurones per arcuate profile was similar in both virgin and pregnant mice and was not significantly altered following LPS treatment (2-way ANOVA, ns; Figure 3.4C). The basal level of double labelled neurones (presented as % Fos-positive tyrosine hydroxylase neurones) in the arcuate nucleus (TIDA neurones) were also similar in both virgin and d5.5 pregnant mice (Figure 3.4D). However, TIDA activation (indicated by double-labelling) was significantly increased after LPS in d5.5 pregnant mice only compared to control groups (2-way ANOVA, interaction treatment x group $p=0.017$).

3.3.2.6 Median eminence monoamine content

DOPAC: The basal levels of normalised DOPAC content (i.e. pg/ml/μg protein) in the median eminence appeared to be higher in virgin mice vs. pregnant but this was not significant (2-way ANOVA on rank transformed data, ns; Figure 3.5 A). LPS treatment appeared to reduce DOPAC content in virgin mice but not significantly so.
Dopamine: The basal levels of normalised dopamine content in the median eminence appeared to be slightly higher in virgin mice vs. pregnant but this was not significant (2-way ANOVA on rank transformed data, ns; Figure 3.5 B). LPS treatment had no effect on dopamine levels in either virgin or early pregnant mice.

Figure 3.3

Figure 3.4

Figure 3.4 cont’d

DOPAC:dopamine ratio: The basal DOPAC:dopamine ratio in virgin vehicle-treated mice tended to be lower than in early pregnancy, but this was not significant (2-way ANOVA on rank transformed data, ns, Figure 3.5 C). LPS treatment seemed to reduce the ratio in virgin mice vs. vehicle (not significant) but it remained unchanged in early pregnant mice.

3.3.2.7. Activation of other hypothalamic cell groups

Supraoptic nucleus (SON): In the SON basal expression of Fos was similar in virgin and pregnant mice. LPS treatment significantly increased the number of Fos-positive neurones in both virgin and d5.5 pregnant mice with no difference between the two groups (2-way ANOVA, $p<0.01$ across treatment; Figure 3.6).

Paraventricular nucleus (PVN): Basal Fos expression in the mPVN was higher in pregnant vs. virgin mice. LPS treatment elevated Fos expression in virgin and pregnant mice but the increase was not significant in pregnancy (2-way ANOVA, $p<0.05$ interaction treatment x group; Figure 3.7). Basal Fos expression in the pPVN was increased in pregnant vs. virgin mice also. LPS increased the number of Fos-positive neurones in both groups, with no significant difference between virgin and pregnant mice (2-way ANOVA, $p<0.01$ interaction treatment x group; Figure 3.7).

Ventromedial hypothalamus (VMH): Basal Fos expression in the VMH was similar in both virgin and early pregnant mice. Following LPS treatment Fos expression was significantly increased in virgin mice but not in d5.5 pregnant mice, although there being no significant difference between LPS-treated virgin and pregnant mice (2-way ANOVA, $p<0.05$ across treatment; Figure 3.8).

Figure 3.5

Figure 3.6

Figure 3.7

Figure 3.7 Cont’d

Figure 3.8

Figure 3.8 cont’d

3.3.2.8. Activation of tyrosine hydroxylase-positive cell groups in the brainstem

Locus coeruleus (LC): Basal expression of Fos-positive nuclei was similar in both virgin and early pregnant mice. LPS treatment increased the number of Fos-positive cells in both groups but was only significant in the virgin mice vs. vehicle (2-way ANOVA, $p<0.001$ across treatment; Figure 3.9). Basal expression of Fos-positive tyrosine hydroxylase (TH) neurones (%) in the LC was similar between virgin and pregnant mice. Following LPS treatment expression was significantly increased in both groups (2-way ANOVA, $p<0.001$ across treatment) with no significant difference between the two groups.

Nucleus tractus solitarius (NTS): In the NTS, basal expression of Fos was similar in both virgin and pregnant mice. LPS treatment significantly increased the number of Fos-positive cells similarly in virgin and early pregnant mice (2-way ANOVA, $p<0.001$ across treatment; Figure 3.10). Basal expression of Fos-positive TH neurones (%) was not significantly different between pregnant and virgin mice. After LPS, expression was increased in both groups with the response greater in pregnant mice vs. virgins (2-way ANOVA, $p<0.05$ across group, $p<0.001$ across treatment).

Rostral ventrolateral medulla (RVLM): In the RVLM, basal expression of Fos was similar in both virgin and pregnant mice. LPS treatment significantly increased the number of Fos-positive cells similarly in both virgin and pregnant mice (2-way ANOVA, $p<0.001$ across treatment; Figure 3.11). Basal expression of Fos-positive TH neurones (%) was also not significantly different between pregnant and virgin mice. After LPS, expression was elevated in both groups with a greater response in pregnant mice vs. virgins (2-way ANOVA, $p<0.05$ across group, $p<0.001$ across treatment).

Figure 3.9

Figure 3.9 cont’d

Figure 3.10

Figure 3.10 cont’d

Figure 3.11

Figure 3.11 cont’d

3.3.3 Effects of stress in early and mid-pregnancy on HPA axis and proinflammatory cytokine activity

3.3.3.1 Corticosterone concentration

At 90 min basal corticosterone concentrations were similar in virgin and pregnant groups and LPS-injected mice exhibited significantly increased corticosterone concentration vs. vehicle controls in all groups (2-way ANOVA, $p<0.001$ across treatment; Holm-Sidak post hoc, $p<0.05$ vs. virgin and d5.5 LPS; Figure 3.12 A).

3.3.3.2 Progesterone concentration

Basal plasma progesterone concentration was significantly greater in pregnant groups (d5.5 and d10.5) vs. virgin. Plasma progesterone levels were significantly increased by LPS treatment in virgin mice but significantly decreased by LPS in pregnant mice compared to vehicle (2-way ANOVA, interaction treatment x group $p<0.01$; Figure 3.12 B). There was not a significant difference between LPS-treated groups.

3.3.3.3 TNF-α and IFN-γ concentration

At 90 min basal TNF-α levels in the plasma were similar between virgin and pregnant groups. LPS-injected mice exhibited significantly increased TNF-α levels vs. vehicle in virgin and pregnant groups, although the stress-induced levels were higher in virgin compared to both pregnant groups (2-way ANOVA, interaction treatment x group $p<0.001$; Holm-Sidak post hoc, $p<0.05$ vs. virgin; Figure 3.12 C). Basal interferon-gamma (IFN-γ) levels were similar between all groups and LPS treatment had no significant effect on circulating IFN-γ concentrations (2-way ANOVA, ns; Figure 3.12 D).

3.3.3.4 PVN gene expression

nur77: In the pPVN, the basal number of nur77-positive cells (Figure 3.13 A-D) was similar in virgin and pregnant mice and LPS significantly increased the number of nur77-positive cells in all groups (2-way ANOVA, $p<0.001$ across treatment; Figure 3.13 E). Grain area per cell was higher in virgin vs. pregnant vehicle treated mice and was increased by LPS treatment in all groups, however, this response was attenuated in pregnancy compared to virgins (2-way ANOVA $p<0.001$ across group,

Figure 3.12

$p<0.001$ across treatment; Holm-Sidak post hoc test, $p<0.05$ vs. virgin LPS; Figure 3.13 F). Basal nur77 mRNA total expression was slightly higher in vehicle-treated virgins compared to pregnant (d5.5 and d10.5) mice. LPS significantly increased expression in all groups compared to vehicle, however, the response in pregnancy was attenuated compared to virgins (2-way ANOVA $p<0.05$ across group, $p<0.001$ across treatment; post hoc $p<0.05$ vs. virgin LPS ; Figure 3.13 G), primarily due to attenuated grain area per cell.

CRH: In the same group of mice, following vehicle treatment, the number of CRH-positive cells and grain cell area in the pPVN (Figure 3.14 A-D) were similar between virgin and pregnant mice. LPS treatment did not significantly alter the number of CRH-positive cells in the pPVN of any group (2-way ANOVA, $p=0.056$ across treatment; Figure 3.14 E) or the grain area per positive cell (2-way ANOVA, ns; Figure 3.14 F). Basal CRH mRNA total expression in the pPVN did not differ between pregnant and virgin groups. However, LPS significantly elevated the total CRH mRNA expression in virgins whilst having no significant effect on expression in early-mid pregnancy. Therefore, LPS-induced CRH expression in early-mid pregnancy (like nur77) was significantly attenuated compared to virgin mice (2-way ANOVA, $p<0.05$ across treatment, $p<0.05$ across group; Figure 3.14 G).

Vasopressin: In mice from the 4h study, basal levels of vasopressin-positive cells and grain area (Figure 3.15 A-B) were similar between virgin and d5.5 pregnant mice. LPS significantly increased the number of vasopressin-positive cells in the pPVN of both virgin and d5.5 pregnant mice (2-way ANOVA, $p<0.001$ across treatment; Figure 3.15 C). The grain area per positive cell was also increased following LPS in both groups, although the response was attenuated in pregnant compared to virgin mice (2-way ANOVA, $p<0.001$ across treatment; Figure 3.15 D). The basal total vasopressin mRNA in the pPVN was similar between vehicle-treated d5.5 pregnant mice and virgin mice. LPS significantly elevated vasopressin mRNA expression in virgin and pregnant mice (2-way ANOVA, $p<0.001$ across treatment; Holm-Sidak post hoc, $p<0.05$ vs. virgin LPS, Figure 3.15 E).

Figure 3.13

Figure 3.13 cont’d

Figure 3.14

Figure 3.14 cont’d

Figure 3.15

Figure 3.15 cont’d

3.3.4 Effect of LPS stress in early pregnancy on pregnancy rate

3.3.4.1 Pregnancy rate analysis on d13.5

LPS treatment significantly decreased the pregnancy rate from 70% (vehicle-treated) to 0 % by gestation day 13.5 (Chi-square $p<0.01$ vs. vehicle; Figure 3.16A).

3.3.4.2 Prolactin concentration on d13.5

As there were no mice in the pregnant LPS group (due to occult foetal loss) a 2-way ANOVA was not carried out. Student t-tests showed that there was no significant difference in plasma prolactin levels between the vehicle-treated pregnant and 'non-pregnant' mice (identified as plugged but have ≤3 developing foetuses at d13.5) and no difference with treatment in the 'non-pregnant' mice at d13.5 (Figure 3.16 B).

3.3.4.3 Progesterone concentration on d13.5

Vehicle-treated mice had significantly reduced progesterone in non-pregnant mice vs. pregnant ($p=0.001$, students t-test). There was no significant difference with treatment in the non-pregnant at d13.5 (Figure 3.16 C).

3.3.5 Effect of 24h fast stress on plasma hormones and neuronal activation in early pregnancy

3.3.5.1 Prolactin concentration

Basal prolactin concentration was significantly higher in d5.5 pregnant vs. virgin mice (controls). 24h fast treatment had no significant effect on plasma prolactin levels in virgin mice but it significantly decreased prolactin secretion in d5.5 pregnant mice (2-way ANOVA, interaction treatment x group $p<0.01$; Figure 3.17 A).

3.3.5.2 Progesterone concentration

Basal plasma progesterone was significantly greater in d5.5 pregnant vs. virgin mice. 24h fast did not alter the concentration of progesterone in virgins vs. control, however, fasting significantly decreased plasma progesterone in d5.5 pregnant mice (2-way ANOVA**,** interaction treatment x group $p<0.001$; Figure 3.17 B).

Figure 3.16

3.3.5.3 IL-6 concentration

IL-6 concentrations of control mice were similar between virgin and pregnant mice. 24h fast treatment had no significant effect on plasma IL-6 concentration in virgins, however, it significantly increased plasma IL-6 in d5.5 mice (2-way ANOVA, $p<0.05$ interaction treatment x group; Figure 3.17 C).

3.3.5.4 Corticosterone concentration

In both virgin and d5.5 pregnant mice the basal corticosterone concentrations were similar. 24h fast treatment significantly elevated corticosterone secretion compared to control mice (2-way ANOVA, $p<0.001$ across treatment; Figure 3.17 D).

3.3.5.5 Activation of TIDA neurones

The basal number of Fos-positive neurones per arcuate profile (Figure 3.18 A-B) was similar in both virgin and pregnant mice and increased following 24 fast similarly in both groups, confirming the expected response to hunger (Johnstone et al., 2006) (2-way ANOVA, $p<0.001$ across treatment; Figure 3.18 C). The basal levels of neurones double labelled for Fos and tyrosine hydroxylase (expressed as % Fos-positive tyrosine hydroxylase cells) in the arcuate nucleus (TIDA neurones) were similar in both virgin and d5.5 pregnant mice. However, % double-labelled cells (activated TIDA neurones) were significantly increased by 24h fast treatment in d5.5 pregnant mice only compared to controls (2-way ANOVA, $p<0.05$ across treatment; Holm-Sidak post hoc test $p<0.05$ across treatment in pregnant group; Figure 3.18 D).

3.3.5.6. Activation of other hypothalamic cell groups

Supraoptic nucleus (SON): In the SON basal expression of Fos was similar in virgin and pregnant mice and 24h fast had no significant effect on either group (2-way ANOVA, ns; Figure 3.19).

Paraventricular nucleus (PVN): Basal Fos expression in the mPVN and pPVN were not altered by either pregnancy or 24h fast treatment (2-way ANOVA, ns; Figure 3.20).

Ventromedial hypothalamus (VMH): Basal Fos expression in the VMH was similar in both virgins and early pregnant mice and 24h fast did not significantly alter expression in either group (2-way ANOVA, ns; Figure 3.21).

Figure 3.17

Figure 3.18

Figure 3.18 cont’d

Figure 3.19

Figure 3.20

Figure 3.20 cont’d

Figure 3.21

Figure 3.21 cont’d

3.3.6 Effect of 24h fast stress in early pregnancy on pregnancy rate

3.3.6.1 Pregnancy rate analysis on d13.5

24 h fast decreased the pregnancy rate from 70% (control) to 36% by day 13.5, this was however not a significant reduction (Chi square, not significant; Figure 3.22A).

3.3.6.2 Prolactin concentration on d13.5

Basal levels of plasma prolactin at d13.5 were similar between pregnant and 'non-pregnant' mice. 24h fast increased plasma prolactin in the 'non-pregnant' group vs. vehicle. The 'non-pregnant' fast group also had higher prolactin levels than the pregnant fasted mice (2-way ANOVA, $p<0.01$ interaction treatment x group; Holm-Sidak $p<0.05$ non-pregnant fast vs. non-pregnant vehicle and vs. pregnant fast; Figure 3.22B). However, statistics should be interpreted with caution as no significant difference was found in the 2-way ANOVA between groups or between treatments alone.

3.3.6.3 Progesterone concentration on d13.5

Pregnant mice had significantly higher plasma progesterone at d13.5 vs. 'non-pregnant' mice, while there was no difference with LPS treatment (2-way ANOVA, $p<0.001$ pregnant vs. non-pregnant; Figure 3.22 C).

Figure 3.22

3.4 Discussion

3.4.1 Control of prolactin secretion in early pregnancy

Post-implantation, dopamine antagonist treatment resulted in a shift to the left of the prolactin dose-response curve. Thus, blocking dopaminergic D2 receptors evoked a higher response in prolactin release in early pregnant compared to virgin mice. This hyper-responsiveness to metoclopramide in early pregnancy may suggest a lower dopamine tone, decreased tyrosine hydroxylase expression, or a reduced DOPAC:dopamine ratio. However, a reduction in basal dopamine activity may be unlikely given the continued stimulation/feedback by prolactin in early pregnancy. It may be that the TIDA neurones themselves are less responsive to prolactin, as they are in late pregnancy, when this allows a nocturnal prolactin surge the day before parturition (Grattan et al., 2008), thus contributing towards the higher basal prolactin observed in early pregnancy compared to virgins (Freeman et al., 2000, Ben-Jonathan et al., 2008). Other potential explanations include altered dopaminergic D2/ prolactin receptor number or sensitivity at the lactotrophs/TIDA neurones. However, this experiment can neither confirm nor reject these possibilities. *In situ* hybridisation could be used to determine whether there is down regulation of D2 or PRL-R gene expression due to stress in early pregnancy, however this may not be likely as this is not what causes the reduced sensitivity to prolactin observed in late pregnancy (Kokay and Grattan, 2005).

It is only at lower doses that we see a difference between early pregnant and virgin mice. This is possibly because at higher doses a much larger proportion of D2 receptors are blocked in both virgin and pregnant groups and therefore, regardless of the number of receptors, sensitivity or basal dopamine present, the maximum prolactin response has been attained.

This experiment did not include a ‘no dopamine antagonist’ control for pregnant mice, which means it is difficult to make a comparison between the basal levels of the experimental groups and, thus, should have been included.

3.4.2 Effects of stress on HPA axis and proinflammatory cytokine responses in early pregnancy

The stressors used were selected as being ethologically relevant to women, mimicking potential situations that could arise in pregnancy, including infection or hunger. Both immune stress and 24h fasting stress activated the HPA axis, increasing corticosterone secretion, as expected, in both virgins and early-mid pregnancy (Neumann et al., 1998, Brunton et al., 2008). Therefore, it is not until later in gestation that HPA axis responses to stressors are attenuated, as previously shown in mice (Douglas et al., 2003), rats (Nakamura et al., 1997, Douglas et al., 2003, Brunton et al., 2005, Brunton et al., 2008) and women (de Weerth and Buitelaar, 2005). In addition, we show that circulating levels of the proinflammatory cytokines IL-6 and TNF-α were significantly increased by treatment with LPS. TNF-α has been implicated to act as a mediator of stress-induced foetal death in early pregnancy. It has been proposed that TNF-α boosts death signalling to kill an embryo if initial stress/events may result in structural anomalies to the foetus, in turn it may stimulate protective mechanisms if the repair of these damages may prevent maldevelopment (Toder et al., 2003, Torchinsky et al., 2005). It has been recently found that LPS treatment in early pregnant mice caused haemorrhages in the placenta and increased the risk of pregnancy loss. In addition, foetuses that did survive show evidence of hypoxia in the brain and impaired foetal neurogenesis, which was found to be dependent upon TNF-α receptor 1 (TNFR1). TNF-α antagonist treatment prevented the placental pathology and foetal loss suggesting that in early gestation the placenta is highly sensitive to proinflammatory signalling. Furthermore, TNF-α inhibition may be an effective prophylactic measure to avoid maternal infection-induced placental defects and associated risks to the developing foetus, including miscarriage and foetal brain development (Carpentier et al., 2011).

IFN-γ, another proinflammatory cytokine, is released in the uterus from natural killer cells in the maternal endometrium, in early pregnancy. In mouse pregnancies it is reported to play an important role in initiation of endometrial vascular remodelling, angiogenesis at implantation sites and maintenance of the placenta. In humans, alterations of these events are believed to contribute to serious complications e.g. foetal loss (Murphy et al., 2009). However, unlike TNF-α, LPS treatment in early

pregnancy did not alter the levels of IFN-γ. TNF-α (and IL-6) are not the only cytokines that have altered secretion following immune stress in early gestation. LPS is known to stimulate production of many cytokines by macrophages (Andersson et al., 1992). For example, LPS administration to rodents has been reported to increase IL-1β, IL-6 and TNF-α, although in late gestation elevated IL-1β and IL-6 are not apparent following LPS treatment (Fofie et al., 2005).

Glucocorticoids are reported to exert adverse effects in late pregnancy on the placenta and foetus, however, their effect in early pregnancy is less well defined. In early gestation, it has been suggested that the actions of glucocorticoids are balanced between positive pregnancy-protective effects (e.g. suppression of natural killer cells (Krukowski et al., 2011)) and adverse pregnancy-threatening effects (e.g. restriction of trophoblast invasion) (Michael and Papageorghiou, 2008). This experiment does not clarify the role of glucocorticoids but implies that high levels are possible at this stage in pregnancy and they may be detrimental to reproductive success.

In women acute psychosocial stress elicits a robust HPA response in early-mid pregnancy (Nierop et al., 2006). Acute physical (water immersion), immune (LPS) or emotional (restraint) stress induces a strong HPA axis secretary response in early-mid pregnant rodents although this response is attenuated in late pregnancy (Nakamura et al., 1997, Neumann et al., 1998). Prolonged or chronic stress is thought to cause long-term detrimental effects on the mother and/or offspring (Douglas, 2010). This has not been directly tested on women due to ethical concerns, although, a correlation between stress perception and cortisol levels show blunted salivary cortisol levels in early pregnant women reporting to have experienced chronic stressful life events (Obel et al., 2005). In contrast, rats exposed to repeated chronic social stress prior to mating, display elevated corticosterone levels during the first half of pregnancy (Stefanski et al., 2005). Unfortunately neither of these papers report pregnancy outcome statistics.

Analysis of the hypothalamic responses of the HPA axis to immune stress in early pregnancy was carried out. Unexpectedly, nur77 and CRH mRNA responses in the pPVN to LPS were significantly attenuated in early-mid gestation compared to virgins, extending previous reports of attenuated responses in late pregnancy (Douglas et al., 2003). Attenuated responses in late gestation in the rat partly result

from increased opioid inhibition and allopregnanolone levels (Brunton and Russell, 2008). However, raised opioid inhibition and allopregnenalone is only apparent later in pregnancy and thus cannot be held accountable for our response in early pregnancy (Douglas et al., 1995, Brunton et al., 2009). Although, allopregnanalone levels in the blood/ brain may increase in early pregnancy as a result of mating behaviours (Frye et al., 2007), treatment with progesterone is unable to reduce stress responses of ovary-intact virgin rats (Douglas et al., 2000). On the other hand, the attenuated response may be related to the post-mating neurogenic reflex experienced in female rodents which induces peaks of gonadotrophin secretion by altering noradrenaline release from the brainstem (Cameron et al., 2004, Szawka et al., 2005). Immune stress is relayed to the HPA axis predominantly via brainstem noradrenaline pathways, therefore, pregnancy-induced changes in pathway signalling may compromise the response to immune stress, including PVN CRH neuronal response.

A dissociation between hypothalamic nur77/CRH mRNA and secretory corticosterone (via pituitary ACTH) responses to immune stress in the peri-implantation period was demonstrated. This confirms and extends previous studies in late pregnant rats showing similar corticosterone secretory responses but reduced CRH release (Nakamura et al., 1998, Neumann et al., 1998). It also supports previous findings that median eminence CRH content remains unchanged following stress in early pregnant rats, implying that stress did not evoke a CRH response/release (Nakamura et al., 1997). As CRH responses were shown to be attenuated very early in pregnancy it is plausible to deduce that the CRH neurone responses are adapted by/under the influence of early pregnancy factors such as the mating-induced neurogenic reflex, pregnancy hormones and cytokines. Conversely, vasopressin responses to the immune stress remained robust, with LPS inducing increased vasopressin mRNA expression in both early pregnancy and virgins, to a similar level. This is intriguing as vasopressin has been found to be synthesised in some of the same pPVN neurones as CRH and appears to be co-released with CRH at the median eminence (Wang and Majzoub, 2011). Vasopressin alone in the absence of CRH appears to drive the high corticosterone responses observed. In addition, it is thought that LPS and cytokines (which can be induced by even non-immune stress) are able

to act directly at the pituitary and adrenals to induce hormone secretion directly, thus bypassing the PVN (John and Buckingham, 2003).

nur77 is a putative regulator of CRH (Douglas et al., 2003), has been shown to bind and regulate the expression of the promoters for both CRH and POMC and is thought to play an important role in the neuroendocrine regulation of HPA axis activity (Murphy and Conneely, 1997). In addition, CRH is also known to regulate and induce nur77 expression (Kovalovsky et al., 2002). Vasopressin's relationship with nur77 is less clear. The vasopressin gene promoter sequence contains a putative nur77 element (also present in the CRH gene), however, *in vitro* studies found that removal of this did not affect regulation of the vasopressin promoter (Iwasaki et al., 1997), implying nur77 is not involved in vasopressin control.

Vasopressin processing/analysis in d10.5 pregnant mice was not carried out and it may have been useful to further support similar gene responses in early and mid-pregnancy. Also the vasopressin analysis was carried out on a different cohort of mice than for CRH/nur77 which is not ideal for direct comparison. However, mice were purchased from the same supplier, housed in a similar environment and age matched. As *in situ* hybridisation analysis may be interpreted differently between experimenters, only one person carried out all analysis for every gene in order to reduce variability and allow more precise group/gene comparisons. *In situ* hybridisation provides information regarding the presence of mRNA markers for a gene of interest. However, this is not a measure of the actual peptide content or of its release and it is possible that it does not directly reflect these. In addition, slightly different LPS doses were given (10 vs. 12.5µg/mouse) in each experiment. However, both are high doses and have previously been shown to induce murine foetal loss (Silver et al., 1994, Parker and Douglas, 2010).

3.4.3 Effect of stress on pregnancy hormones and TIDA neuronal activation

In contrast to HPA axis hormones, key pregnancy hormones were dramatically reduced by both immune and 24h fast stress in early pregnancy. Plasma progesterone was significantly decreased by stress on gestation d5.5, confirming previous reports (Joachim et al., 2003), and for the first time we demonstrated that basal prolactin was strongly and rapidly inhibited by stress in early pregnancy as hypothesised. It is not yet known whether the bi-daily prolactin peaks observed in rodents are also

decreased by stress in early pregnancy and further experiments are required to address this question. Previous reports reveal an important role for the bi-daily prolactin peak secretory pattern in maintaining early pregnancy in rodents and therefore it would be of interest to know the effect of stress on the prolactin system at this time. Basal progesterone and prolactin levels were elevated in early pregnancy as expected, extending previous reports that prolactin increases prior to implantation in pseudopregnancy in mice (Yang et al., 2009, Larsen and Grattan, 2010).

The reduction in prolactin secretion shown following stress may contribute towards the stress-induced decrease in progesterone simultaneously observed, as prolactin is known to enhance progesterone secretion (Freeman et al., 2000). During early pregnancy in rodents, progesterone production in the ovaries is mainly driven by prolactin binding to its receptor expressed in corpus luteal cells and activating the JAK2/STAT5 pathway (Erlebacher et al., 2004). Although we do not provide evidence of prolactin's role in driving progesterone directly in our studies, injecting early pregnant mice with prolactin has been shown to increase progesterone secretion and abrogate stress-induced resorption in mice (Erlebacher et al., 2004). Whether prolactin is able to rapidly effect progesterone secretion via altered gene transcription is unknown.

It should be noted that basal levels of prolactin differ between the two stress experiments. Although the reason behind this is not clear, brief anaesthesia (~3min) before blood sampling after the LPS stress and not after fasting stress may be accountable. The use of pentobarbitone anaesthesia has been reported to increase plasma prolactin in rodents (Turpen and Dunn, 1978) within 15 minutes and thus the brief anaesthetic used may have been sufficient to increase prolactin levels accordingly. We can rule out the possibility that basal levels were different due to the influence of the prolactin surges on the afternoon of proestrus in virgins and the bi-daily peaks in early pregnant mice (Grattan and Kokay, 2008), as in order to avoid these all experiments were conducted in the early-mid light phase of the circadian day (9.00-13.00h).

The stress-induced decrease in plasma prolactin was shown to be associated with a parallel mild, but significant, increase in TIDA neuronal activation following 24h fasting and immune (90 min) stress in early pregnancy, showing these neurones

rapidly respond to stress-induced factors. In virgin mice TIDA neurone activation remain unaltered by either stressor, thus, altered responsiveness in early gestation is acquired post-mating. Interestingly, only the TIDA subpopulation was responsive to stress as no general Fos activation in the arcuate nucleus was apparent following stress in early pregnancy. TIDA neurones have already adapted to mediate the neurogenic reflex in early pregnancy and the bi-daily prolactin peak secretory profile, therefore, these alterations may confer the changes observed with stress. Although expression of Fos in TIDA neurones following an immune challenge has previously been reported to reflect changes in prolactin secretion (Hollis et al., 2005), it should be noted that Fos is not the best indicator of TIDA neurone activation. Fos staining is absent in these neurones under basal conditions despite the fact they are tonically activated and are able to express Fos-related antigens (Hoffman et al., 1994, Hoffman and Lyo, 2002). Therefore, it appears that Fos in TIDA neurones may only serve as a weak marker of TIDA neurone activation following acute stimulation e.g. LPS, which may help explain why we only see a mild increase in Fos-positive TIDA neurones (increase from 2.9% to 12.9% following LPS in early pregnancy; 2-way ANOVA, $p<0.001$ across treatment).

The DOPAC content and DOPAC:dopamine ratio in the median eminence were used as additional measures of dopamine activity (Shieh and Pan, 2001, Andrews and Grattan, 2003). DOPAC levels appeared to be higher in control virgin mice compared to early pregnant mice (not significant), which could partially explain the increased basal prolactin secretion observed in early pregnancy. DOPAC levels also seemed to be higher in control virgin mice compared to LPS-treated virgins. However, the DOPAC levels, and therefore dopamine activity, do not seem to underlie prolactin secretion since this was unchanged by LPS in virgin mice and there is no change in DOPAC to reflect the stress-reduced prolactin levels observed in early pregnancy. DOPAC:dopamine ratios revealed a similar pattern to DOPAC content and it was found that basal levels tended to be higher in virgins vs. pregnant mice. LPS did not significantly alter the DOPAC:dopamine ratio in virgin or pregnant mice. However, LPS-treated virgin mice had a ratio 63% lower than vehicle-treated mice, although this was not significant due to the high variability (which may be due to differences across the oestrous cycle that were not accounted

for). Thus, dopamine activity in the TIDA neurone terminals is not analogous to Fos expression in TIDA neurones in pregnant mice nor does it reflect prolactin levels. Thus, stress does not alter prolactin via dopamine activity and other factors must be considered. One such possible explanation is that LPS, and/or the associated cytokines released, is/are able to directly affect the pituitary lactotrophs to inhibit prolactin secretion (Theas MS, 1998, de Laurentiis A, 2002, O'Connor et al., 2003). Although TIDA neurones are considered to be the main controllers of prolactin, dopamine activity could be altered in other dopaminergic neurones e.g. tuberohypophyseal (THDA) and periventricular hypophyseal dopamine (PHDA) neurones. Dopamine released from all three subpopulations has been demonstrated to topically suppress lactotrophs (DeMaria et al., 1998). THDA neurones release dopamine from their terminals in the anterior pituitary and this has been shown to inhibit oxytocin release (Freeman et al., 2000). This may contribute to reducing prolactin secretion as oxytocin is reported to be a prolactin-releasing factor in pregnancy (McKee et al., 2007). THDA neurones have also been shown to be responsive to LPS and cytokines to control prolactin (de Laurentiis A, 2002).

In contrast to the responses to LPS, fasting generally activated the arcuate neurones in both virgin and early pregnant mice. The fasting-induced Fos observed in virgins verifies previous publications, reflecting activation of other neurones such as neuropeptide Y neurones (NPY) (Becskei et al., 2009). Although food intake is increased during pregnancy (Douglas et al., 2007), with prolactin and placental lactogen contributing to hyperphagia (Ladyman et al., 2010), Fos expression was similar between early pregnant and virgin mice. It is not yet known how these or other appetite-signalling hormone (e.g. leptin, ghrelin), which also rise throughout gestation, may interact with arcuate neurone responses to stress.

These findings support the conclusion that immune and fasting stress, along with other stressors (eg. psychological stress (Joachim et al., 2003)) have detrimental effects on pregnancy maintenance, inducing inhibition of vital pregnancy hormones and thus potentially compromising optimal implantation and pregnancy success.

3.4.4 Effect of stress on neural activation in other hypothalamic nuclei

Other hypothalamic nuclei seem to behave in a different manner to those in the arcuate nucleus. In contrast to the general neuronal population of the arcuate nucleus,

LPS stimulated Fos expression in the SON, PVN and VMH of virgins. This may be a consequence of upstream brainstem activity. The SON is known to be innervated by direct monosynaptic neurones projecting from the brainstem (LC, NTS and VLM) (Michaloudi et al., 1997, Northrop et al., 2006). These hypothalamic neurones normally respond to stress with immediate early gene expression/activity and release neuroendocrine factors involved in producing and controlling responses of the HPA axis (Douglas, 2005b). Basal Fos expression in the PVN was elevated in early pregnancy compared to virgins which is interesting as this has not been previously reported at other gestation periods (Luckman, 1995). The basal activity in the PVN may be changed due to early pregnancy-related alterations in hormones and/or cytokines. The LPS-induced increase in Fos expression in early pregnant mice was attenuated compared to virgins mirroring the blunted response of CRH and nur77 mRNA to immune challenge. The pPVN, which displayed a substantially lower response to LPS in early pregnancy, is known to contain oxytocin neurones, and this may contribute to the decreased prolactin response observed.

Oxytocin has been reported to be a crucial prolactin-releasing factor, playing a role in the regulation of prolactin secretion. Anatomically this seems likely as oxytocin (synthesised in the PVN and SON) is transported via axons to the neural lobe where it is released from its terminals and also from the external zone of the median eminence. Oxytocin is then transported to the anterior pituitary via the portal vessels where it has access to the lactotrophs (Freeman et al., 2000). A sub-population of these lactotrophs have been shown to possess oxytocin receptors (Breton et al., 1995). Prolactin release is modulated by oestradiol, which increases between diestrous and proestrous inducing the prolactin surge on the afternoon of proestrous. It has been reported that oxytocin actions at the lactotrophs are elevated at this time with an increase in secretary responses to oxytocin due to an increase in the number of oxytocin-responding lactotrophs and magnitude of their Ca^{2+}_{i} responses. It is, thus, suggested rising oestrogen levels facilitate oxytocin stimulatory actions on lactotrophs and prolactin release (Tabak et al., 2010). The action of oxytocin on controlling the prolactin rhythm has also been suggested to be coordinated by vasoactive intestinal polypeptide (VIP). VIP originates from the suprachiasmatic nucleus (SCN), with its fibres innervating the dopamine neurones in the arcuate

nucleus and oxytocin neurones in the PVN (Egli et al., 2010). Oxytocin neurosecretory cells of the PVN, which have VIP receptors, have been identified using ICC along with retrograde tracing with Fluoro-Gold (method for labelling neurosecretory cells). The injection of a VIP antisense oligonucleotide into the SCN eradicates the afternoon oxytocin and prolactin peaks normally observed in cervically-stimulated ovariectomised rats, suggesting VIP has a role in contributing to oxytocin and prolactin secretion (Egli et al., 2004).

Thyrotropin-releasing hormone (TRH) is also thought to stimulate prolactin release and is known to increase in human pregnancy (Amino et al., 1981), therefore TRH responses may also be altered following stress in early pregnancy, impacting on prolactin secretion. TRH-deficient mice during lactation display significantly reduced serum prolactin compared to the wild type mice. The prolactin mRNA and content were also reduced in the pituitary of these mice, all of which were reversed by TRH treatment, indicating TRH is needed for regulation of pituitary mRNA regulation. Despite the low prolactin levels in the TRH deficient dams they are still fertile, suggesting TRH is not crucial for pregnancy and lactation but is needed for complete function of the lactotrophs, especially during lactation (Yamada et al., 2006). Administration of TRH to rats can induce prolactin secretion, however the temporal release of TRH is not strongly correlated to prolactin secretion (Freeman et al., 2000). The prolactin-stimulating effects of TRH were thought to occur in the pituitary; however, a study in rats has found that TRH works at the hypothalamic level, leading to modulation of the functional output of TIDA neurones (altering firing patterns) and thus reducing its inhibitory effect on prolactin (Lyons et al., 2010, van den Pol, 2010).

Therefore, it would be of interest to determine the effect of stress in early pregnancy on these potential prolactin releasing factors e.g. oxytocin, to see if stress induces changes in their secretion/expression/activity in early pregnancy that could account for the depleted prolactin secretion observed at this time.

Prolactin has also been implicated to be involved in the regulation of oxytocin, supported by the finding that mRNA for the long form of the prolactin receptor (PRL-R_L) is predominantly colocalised with oxytocin mRNA in the SON (over 80% of the total oxytocin neurones). Similarly, in the PVN, PRL-R_L mRNA was also

predominantly located in oxytocin neurones and the proportion of oxytocin neurones exhibiting PRL-R_L mRNA increased with pregnancy (Kokay et al., 2006). Prolactin administration i.c.v increases Fos expression in the SON (Cave et al., 2001) and can increase the electrically stimulated oxytocin release and mRNA content in the hypothalamus (Ghosh and Sladek, 1995). Together these data strongly suggest that prolactin directly regulates the activity of oxytocin neurones. However, no change was observed in the basal Fos response in the SON of early pregnancy mice compared to virgins despite radically different prolactin concentrations. Although, basal Fos expression in the PVN was significantly higher in early pregnant versus virgin mice which may reflect increased activation of oxytocin neurones, and subsequently contribute towards the increased plasma prolactin levels.
In contrast to the general arcuate nucleus response, 24h fasting had no significant effect on SON, PVN or VMH Fos expression, confirming previous reports (Johnstone et al., 2006). Therefore, although both stressors reduce prolactin and progesterone secretion, and increase Fos expression in the arcuate nucleus, they affect other hypothalamic nuclei activity differently. Potential roles for hypothalamic factors, other than dopamine, on prolactin secretion are unclear following fasting. Therefore, the two stressors used display only similar patterns of hypothalamic activation in TIDA neurones, implying that stress responses may generally converge at this neuroendocrine system in early pregnancy.

3.4.5 Effect of stress on tyrosine hydroxylase-positive cell groups in the brainstem

LPS and consequently its cytokine load can access the arcuate nucleus e.g. via the leaky blood brain barrier (BBB) in the median eminence. In addition, cytokines can activate the noradrenergic pathways to the hypothalamus (Brunton and Russell, 2008). The central noradrenergic system originates in the medulla and pons and extensively innervates areas of the brain that are involved in stress response regulation. It is activated by a wide variety of stressful stimuli, both psychological and physical (e.g. cytokine administration) (Pacak et al., 1995a, Pacak et al., 1995b). For example, IL-1 activates neurones in the NTS and VLM when given peripherally to rats. These represent sources of catecholaminergic cells that project to and innervate the PVN (Ericsson A et al., 1994) and result in increased noradrenaline release here (Brunton et al., 2005). Lesions of these projections abolish HPA

responses to IL-1β, reducing PVN CRH and oxytocin neurone activation (Weidenfeld et al., 1989, Melik Parsadaniantz et al., 1995, Buller et al., 2001). The above indicate that noradrenergic projections play a crucial role in mediating HPA responses to IL-1β. IL-1β does not directly act on these neurones: most do not express IL-1β receptors and systemically administered IL-1β is unlikely to cross the BBB (Rivest et al., 2000). IL-1β, like LPS and other cytokines drive prostaglandin synthesise (via cyclo-oxygenase (COX) expression induction) which activates these noradrenergic neurones (Rivest, 2001, Brunton and Russell, 2008). This idea is supported by studies using COX inhibitors, for example, central administration of a COX-1 inhibitor attenuates LPS-induced increase in ACTH and corticosterone secretion (Garcia-Bueno et al., 2009). In late pregnancy, the activity of the noradrenergic system in the PVN is reduced compared to virgin rats, and therefore, may contribute to the attenuated HPA response to stress observed at this time (Douglas, 2005a, Russell et al., 2008).

We show that LPS administration increases neuronal activation in the LC, NTS and RVLM in the brainstem in both virgins and d5.5 pregnant mice. Neurones in the LC show a generally lower response in early pregnancy compared to virgins, potentially leading to the attenuated response of the pPVN. The PVN receives noradrenergic innervations from the A6 cell bodies of the LC, as well as from A1 and A2 cell bodies of the VLM and NTS, respectively (Cunningham and Sawchenko, 1988, Saphier and Feldman, 1989, Hwang et al., 1998).The LC also has a role in activation of the HPA axis (Ziegler and Herman, 2002), supported by the report lesion of the LC suppresses restraint stress-induced ACTH and corticosterone secretion (Ziegler et al., 1999).

Although LC response to stress appeared lower in early pregnancy compared to virgins, the number neurones activated overall in the LC was low. However, neuronal activation in the NTS and RVLM was significantly higher following stress in early pregnancy compared to virgins, suggesting these neurones have adapted with increased sensitivity to stimuli and may contribute towards to the exaggerated response observed by the TIDA neurones. A temporal association was reported between noradrenaline release (in the medial preoptic area) and prolactin secretion on the afternoon of pro-oestrous, suggesting noradrenergic involvement in prolactin

surge regulation (Szawka et al., 2007). Evidence indicates both physiological adrenergic and noradrenergic involvement in the neuronal regulation of the TIDA system. Synaptic interactions reported between the adrenergic and TIDA neuronal systems indicate that adrenergic neurones may also be involved in the regulation of the TIDA system and provide a potential pathway for central adrenergic effects on prolactin secretion (Hrabovszky and Liposits, 1994).

The noradrenergic nucleus LC participates in both proestrous and steroid-induced surges of prolactin. LC lesion decreases noradrenaline release in the preoptic area, PVN and mediobasal hypothalamus, reducing prolactin and luteinising hormone secretion (Szawka et al., 2005). However, LC has not been implicated to be involved in stress-induced prolactin changes (Poletini et al., 2006). The NTS is known to be activated by LPS and cytokines (Brunton et al., 2005, Sparkman et al., 2006) but an association with prolactin secretion has yet to be described. Likewise RVLM neurones are known to be activated by cytokines (Zhang et al., 2003). Although a direct link to prolactin secretion remains unknown at present, arcuate neurones have been observed to be activated by stimulating the RVLM, and the arcuate perikarya showed labelling with a retrograde tracer that was originally microinjected into the RVLM (Li et al., 2009).

We also show an increase in TIDA neurone activation with fasting stress, like LPS, in early pregnancy but we do not know whether this is accompanied by noradrenergic activation in the brainstem as this was not analysed. It has, however, been shown that food ingestion was critical for the expression of Fos in the noradrenergic cells of the LC and NTS in rodents (Johnstone et al., 2006) and therefore we may not expect to observe an increase in Fos activation in the brainstem of fasted mice.

3.4.6 Effect of stress on pregnancy rate and hormones at d13.5

We have shown that LPS stress in early pregnancy dramatically induced complete pregnancy failure, reducing pregnancy rate from 70% (vehicle-treated) to 0% ($p<0.01$). 24h fasting in early pregnancy lead to a milder phenotype, reducing pregnancy rate from 70% to 36%. Although this loss was not significant following 24h fasting this may be due to the way in which the pregnancy rate was calculated. Pregnancy rate was measured as the percentage of mice displaying ≥4 implantation

sites (average of 7 implantation sites). However, a more insightful measure of pregnancy rate/foetal loss can be gained by counting the number of implantation sites and the number of resorption sites in the uterine horns and calculating the percentage of viable implantation sites (Joachim et al., 2003). Never the less, we showed stress (on gestation d5.5) had detrimental effects on pregnancy outcome which supports previous reports of stress-induced abortion in mice (Wiebold et al., 1986, Joachim RA, 2001, Joachim et al., 2003) and increased miscarriage associated with elevated cortisol in early pregnant women (Nepomnaschy et al., 2006). The importance of stress-reduced prolactin on pregnancy outcome is yet to be determined. It would be an interesting experiment to replace the prolactin lost due to stress in early pregnancy (i.e. to keep prolactin levels high) to see if the pregnancy outcome in response to stress can be reversed. This type of hormone replacement study has previously been carried out for progesterone in which a progesterone derivative, dydrogesterone, was found to abrogate murine stress-triggered abortion (Joachim et al., 2003). Low serum progesterone has also been associated with miscarriage in women, as well as increased perceived stress levels (supported by higher circulating CRH concentrations). In very early gestation (women recruited between 4-7 weeks) these risk factors were more pronounced (Arck et al., 2008). LPS has previously been shown to induce occult pregnancy loss in mice (Foerster et al., 2007) , while neutralisation of LPS (using a bacterial permeability increasing protein) or blockage of Toll-like receptor (TLR)-4 for LPS abrogate (sonic) stress-triggered foetal loss in mice (Friebe et al., 2011). Studies into the effects of fever and infection on pregnancy maintenance in animals have reported that hyperthermia in early gestation is associated with resorption, foetal death, and serious malformations e.g. cardiovascular malformations (Edwards, 1969, Poswillo et al., 1974, Smith et al., 1978, Edwards et al., 1995). In humans the data/evidence for whether fever causes foetal loss and complications is not quite so clear. A number of studies show compelling evidence for the increased risk of adverse pregnancy outcomes following fever. For example, Q fever has been shown to increase the incidence of spontaneous abortion, intrauterine growth retardation and preterm labour (Carcopino et al., 2009). In contrast, another group has reported that there is not an association between fever in early pregnancy and risk of foetal death (Andersen et al., 2002). This study

revealed that 18.5% of the recruited women (total 24,000) suffered a fever in the first 16 weeks of pregnancy, indicating that fever is commonly experienced during early pregnancy. However, this study is not able to reflect the group of women who spontaneously aborted in very early pregnancy, before the usual enrolment time. In addition, episodes of fever were only recorded in the first 16 weeks and therefore we do not know about the effects of fever occurring later in pregnancy on foetal loss. Thus, this study cannot absolutely conclude that there is no link between fever and miscarriage.

Mounting evidence indicates that events occurring early in life/*in utero* are critical determinants for problems later in life. Where pregnancy failure does not arise, foetal programming may. Exposure to chronic stress or glucocorticoids during both early and late gestation and the neonatal period induces long-lasting adverse effects in the offspring, for example, cardiovascular disease and depression (Seckl et al., 2000, Seckl and Meaney, 2004). Administration of LPS to mice during mid pregnancy results in a number of physiological and behavioural changes to their male offspring. These males displayed attenuated anxiety behaviour, reduced food/water intake and a lower body weight up until postnatal day 40, indicating prenatal stress can lead to complex and long-term effects on the offspring (Asiaei et al., 2011). It is thought that local glucocorticoid action in the decidua or developing placenta is involved in stress-induced programming during pregnancy and is accompanied by detrimental alterations in 11β-hydroxysteroid dehydrogenases (11β-HSDs) in the decidua/placenta (Seckl and Meaney, 2004, Holmes et al., 2006, Holmes and Seckl, 2006). 11β-HSDs are glucocorticoid metabolising enzymes which modify glucocorticoid actions in a tissue-specific manner. 11β-HSD1 regenerates active glucocorticoids, thus increasing corticosterone/cortisol levels. 11β-HSD2 expression acts as a dehydrogenase, inactivating corticosterone or cortisol through conversion to 11-dehydrocorticosterone and cortisone respectively. Its expression is high in the foetal brain but less so in the adult brain where it is confined to specific areas, indicating its importance in development. Loss of this enzyme from foetal tissues leads to altered cerebellum development and long-term heightened anxiety, consistent with programming (Holmes and Seckl, 2006). Decreased activity of placental 11β-HSD2 increases foetal exposure to maternal cortisol, programming the

foetus for hypertension and metabolic disease later in life. In addition prenatal exposure to cytokines has been shown to result in obesity and gender-specific programming in the offspring (Dahlgren et al., 2001)

Following 24h fasting in early pregnant mice, plasma prolactin levels at d13.5 were significantly elevated in non-pregnant fasted mice compared to both non-pregnant control and pregnant fasted mice. It is unclear by what means levels are significantly higher at this later date following fasting and no such change was found on d13.5 following LPS injection on d5.5 or immediately following 24h fast. Fasted mice that remained pregnant exhibited slightly lower circulating prolactin levels on d13.5 compared to pregnant controls, indicating a pronged effect on prolactin. Conversely, progesterone secretory responses on d13.5 following 24h fast and were similar to control mice (pregnant and non-pregnant) suggesting the fasting treatment did not have a lasting effect on progesterone secretion in pregnancy.

During intrauterine development, one of the principal environmental factors influencing the growth/development of the embryo/foetus, is the maternal diet and subsequent nutrient supply. Epidemiological studies have found that undernutrition during foetal life is associated with increased risk of complex diseases (Chmurzynska, 2010); the incidence of metabolic syndrome, cardiovascular diseases, hypertension, diabetes and obesity have all been found to be higher (McMillen and Robinson, 2005) and appear to be independent of lifestyle risk factors (Drake and Walker, 2004). Maternal protein restriction during foetal development and lactation in rats causes decreased birth weight, delayed sexual maturation and leads to premature aging of reproductive function in female offspring (only data on female progeny reported) (Guzman et al., 2006). The “thrifty phenotype” hypothesis was proposed almost two decades ago (Hales and Barker, 1992) and describes an association between poor nutrition/foetal growth and increased risk of impaired glucose intolerance/diabetes and metabolic syndrome. These finding have been reproduced in many studies since then in different populations and ethnic groups (Hales and Barker, 2001). It suggests that an adaptive response occurs due to poor foetal nutrition, leading to altered metabolism. This could be beneficial if born into a low/poor nutrition environment; however, problems arise when the pre and post-natal conditions do not match.

In summary, we found that circulating prolactin was profoundly inhibited by either LPS immune stress or 24h fasting in early pregnancy. As prolactin is mainly under the inhibitory control of dopamine released from TIDA neurones we hypothesised that stress would increase TIDA neurone activity. In support of this, both stressors significantly increased Fos expression in TIDA neurones in early pregnant mice. However, LPS did not enhance TIDA dopamine activity in the median eminence (as measured by DOPAC content and DOPAC:dopamine ratio). These data imply that increased TIDA neurone activation plays a role in stress-reduced prolactin levels in early pregnant mice but is not souly responsible. Attenuated pPVN neurone recruitment and increased activation of noradrenergic nuclei following stress in early pregnancy indicate that other mechanisms may also contribute to reduced prolactin secretion.

Chapter 4

Mechanisms controlling prolactin secretion/responses following stress in early pregnancy

Chapter 4: Mechanisms controlling prolactin secretion/responses following stress in early pregnancy

Section 4a: Effect of stress on prolactin negative feedback in early pregnancy

4.1 Introduction

4.1.1 Prolactin signal transduction

Different isoforms of the prolactin receptor are expressed in mice, namely the short and long form, with the long form predominantly located in the hypothalamus and more highly expressed in the arcuate nucleus than the short form (Pi and Grattan, 1998, Bakowska and Morrell, 2003).While both forms display identical extracellular portions (and so both bind prolactin), they differ in their ability to activate intracellular pathways (Grattan, 2002). The membrane proximal region of the intracellular domain in the long form of the prolactin receptor is associated with Janus kinase 2 (JAK2). Prolactin binding to its receptor leads to dimerization of the receptor molecules and phosphorylation of JAK2 rapidly (<1min) follows (Freeman et al., 2000). JAK2 recruits and phosphorylates latent cytoplasmic proteins termed signal transducers and activator of transcription (STATs). Phosphorylated (p)STAT molecules then form homo- and hetero-dimers which translocate to the nucleus and bind to specific promoter sequences of target genes (Grattan, 2002). A number of STATs are reported to be involved in prolactin signal transduction in various tissues, including STAT1, STAT3, STAT5a and STAT5b (Bole-Feysot et al., 1998).
The short form of the prolactin receptor does not activate the JAK-STAT pathway but mediates some actions of prolactin through mitogen-activated protein kinase (MAPK) pathways.

4.1.2 Prolactin negative feedback

Pituitary produced prolactin is mainly under the inhibitory control of dopamine released from hypothalamic TIDA neurones in the arcuate nucleus (Grattan, 2002). Prolactin also activates a short-loop feedback system to regulate its own secretion by stimulating TIDA neurones. Prolactin receptors are expressed on these neurones (Kokay and Grattan, 2005) and prolactin has been shown to increase hypothalamic dopamine synthesis and also turnover (Grattan and Kokay, 2008). Activation of the

TIDA neurones via prolactin binding to the long form of its receptor requires activation of the JAK2/ STAT5 signalling transduction pathway (Grattan et al., 2001b, Ma et al., 2005a) and is thought to contribute to the stimulation of tyrosine hydroxylase mRNA expression (Grattan and Kokay, 2008). pSTAT5b has been demonstrated to specifically mediate prolactin negative feedback on the TIDA neurones, with prolactin treatment inducing nuclear translocation of pSTAT5 (Lerant et al., 2001). STAT5b deficient mice express very high serum prolactin levels, although mRNA levels and immunoreactivity of tyrosine hydroxylase are low, indicating signal transduction is impaired and demonstrating that pSTAT5 is essential for mediating prolactin negative feedback in TIDA neurones (Grattan et al., 2001b). In addition, STAT5 deficient mice also display characteristics of prolactin insensitivity, including compromised luteotropic support with attenuated serum progesterone in pregnancy, spontaneous abortion, impairment of mammary gland development and insufficient milk production (Udy et al., 1997).

4.1.2.1 Prolactin negative feedback in early and late pregnancy

During late pregnancy and lactation, TIDA neurones become less able to secrete dopamine in response to prolactin, consequently leading to a state of hyperprolactinemia. There are a number of physiological changes in the regulatory pathways that control prolactin secretion at this time. Firstly, the placenta provides lactogenic hormones, the placental lactogens, in a number of species including rodents (Lee and Voogt, 1999). These are able to bind to and activate prolactin receptors without being subject to the normal inhibitory control by dopamine. Subsequently, the short-feedback loop is bypassed in late pregnancy and lactation allowing continued activation of the prolactin receptors, leading to increased progesterone secretion while simultaneously decreasing prolactin release itself (Grattan, 2002). Secondly, TIDA neurones have a reduced ability to respond to prolactin, with lower TIDA activation compared to early pregnancy (Grattan and Averill, 1995, Andrews et al., 2001), and prolactin secretion resumes despite the continued placental lactogen presence. Thirdly, suckling of the nipple is a potent prolactin stimulus involving a suckling-induced reduction in the activation of TIDA neurones (Selmanoff and Gregerson, 1985).

At the end of gestation and during lactation, prolactin loses the ability to induce STAT5b phosphorylation and nuclear translocation, when compared with diestrous rats. This was reported to be associated with an increase in suppressors of cytokine signalling (SOCS) mRNA and may underlie the diminished TIDA neurone dopamine output and hyperprolactinemia observed at this time (Anderson et al., 2006a).
SOCS act as feedback inhibitors of signalling for a number of cytokines that utilize the JAK/STAT pathways. They appear to act as part of an intracellular feedback loop to suppress STAT phosphorylation and consequently to dampen signal transduction (Starr and Hilton, 1999). SOCS proteins contain a central Src homology 2 (SH2) binding domain through which they bind to phosphotyrosine residues located in cytokine receptors (eg. for SOCS2 and 3 and cytokine-inducible SH2-containing protein, CIS) or JAKs (e.g. for SOCS1). They are then able to suppress cytokine signalling in number of ways: by binding to and inhibiting the activity of JAKs; competing for phosphorylated binding sites on receptors with STATs; or by targeting bound signalling proteins for proteasomal degradation (Wormald and Hilton, 2004). Thus, they may play a role in inhibiting prolactin stimulation of STAT5b in late pregnancy and lactation.
On d22 of pregnancy, rats have been found to have increased SOCS1 and SOCS3 in the arcuate nucleus compared to diestrous rats, and on d7 of lactation CIS mRNA levels were significantly elevated, which suppressed prolactin levels to that of basal (Anderson et al., 2006a). These data are consistent with the hypothesis that SOCS/CIS mediate the loss of prolactin-induced activation of STAT5b in TIDA neurones. Administration of prolactin has been shown induce expression of SOCS1, 2 and 3 along with CIS mRNA in the ovary and adrenals (but not mammary glands) of lactating rats deprived for 24h of circulating prolactin. Furthermore, suckling induces SOCS mRNA in the ovary (but not mammary glands) of these rats. After 48h pup-deprivation the mammary gland increases SOCS gene expression in response to prolactin (Tam et al., 2001).
oPRL administration ordinarily increases the STAT5 nuclear:cytoplasmic ratio in lactating mothers whose pups are removed. However, in the presence of the pups prolactin signal transduction through STAT5 in the TIDA neurones is suppressed. It has been reported that CIS mRNA is significantly elevated in suckled lactating rats

along with up-regulation of CIS transcripts, while SOCS1 and 2 mRNA are not. These data imply that loss of sensitivity to prolactin negative feedback is due to heightened CIS expression/action in the TIDA neurones (Anderson et al., 2006b). Prolactin negative feedback is known to be maintained throughout early-pregnancy prolactin surges (Demarest et al., 1983, Grattan et al., 2008). In accordance with prolactin negative feedback during early pregnancy, TIDA neurones display a semicircadian pattern of activity, with reduced dopamine release during the prolactin surges and increased release between them, allowing initiation and termination of the surges (McKay et al., 1982). In early pregnancy, studies have been carried out to see if the nocturnal prolactin surge increases SOCS (SOCS1, SOCS2, SOCS3, CIS) expression in the arcuate nucleus. In contrast to late pregnancy and lactation responses to exogenous prolactin, high (endogenous) prolactin levels in early pregnancy do not induce SOCS expression (Anderson et al., 2006a). It is thought that declining progesterone and high oestrogen during late pregnancy induce SOCS in TIDA neurones. Oestrogen and prolactin were shown to induce SOCS1, SOCS3 and CIS mRNA levels in the arcuate nucleus of ovariectomised late pregnant rats, while progesterone reversed the effect of oestrogen (Steyn et al., 2008). In early pregnancy although negative feedback is apparent during prolactin surges and SOCS is not induced, the effects during basal levels of prolactin are yet to be investigated. In addition, no reports have been published into stress effects on negative feedback in early pregnancy. It is known that stress in early gestation increases circulating cytokine levels, affects TIDA activity and alters prolactin secretion, thus, stress could impact on prolactin negative feedback systems.

Therefore, in order to investigate the effect of stress in early pregnancy on prolactin negative feedback to TIDA neurones, mice were stressed (LPS or 24h fast) and then administered ovine prolactin (oPRL) to drive negative feedback. We aimed to use immunocytochemistry, double labelling for tyrosine hydroxylase and pSTAT5, to determine whether there is an alteration in pSTAT5 signalling in TIDA neurones following stress in early pregnancy, associated with reduced prolactin.

4.1.3 Aim and Hypothesis

Aim:

1. To discover if stress (either alone or in conjunction with oPRL treatment) alters prolactin negative feedback in early pregnancy.

Hypothesis:

1. Prolactin negative feedback is increased in early pregnancy following stress exposure.

4.2 Methods

4.2.1 Effect of LPS stress on prolactin negative feedback

4.2.1.1 Treatment

d5.5 pregnant mice were gently restrained in a holding bag and either injected with lipopolysaccharide (LPS; 12.5µg/100µl saline, i.p;*E.coli* E055:B5, Sigma, Poole, Dorset, UK; n = 14) (Hollis et al., 2005) or vehicle (100µl, i.p; n = 14), at around 10am (3h after lights on). Mice were then returned to their home cages. After 3h mice were injected with ovine prolactin (oPRL; 1µg/g bodyweight i.p; Sigma-Aldrich; vehicle and LPS treated n = 7) (Anderson et al., 2006b) or prolactin buffer (30mM NaHCO3, 150mM NaCl, pH 10.8; n = 7) mixed with saline (1:1) as a control, before being killed 1h later (4h after LPS injection). This time point was selected following a literature search (Anderson et al., 2006b, Anderson et al., 2008).

4.2.1.2 Blood/tissue collection and perfuse fixation

One hour after oPRL injection, mice were quickly moved to an adjacent room and injected with an overdose of sodium pentobarbitone (0.3ml i.p; Centravet, 22106 Didan, France). Once the mouse was deeply anaesthetised a blood sample was taken by cardiac puncture before the mouse was perfused with heparinised saline and fixed with 4% paraformaldehyde (as described in General Methods section 2.9.1). The brain was carefully removed and stored overnight (4ºC) in 4% paraformaldehyde and 15 % sucrose solution. The brain was then stored in a 30% sucrose solution at 4ºC overnight. Following this the brains were frozen before sectioning. Blood was processed for plasma and frozen (-20°C) prior use in assays. Following blood/tissue collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

4.2.1.3 Prolactin ELISA

Plasma samples were diluted 1 in 25 and assayed in duplicate using an adapted protocol (see General Methods section 2.7.2.2) for the mouse prolactin DuoSet ELISA (DY1445, R&D Systems, Inc., Minneapolis, US). To test that there was no cross-reactivity between the murine and ovine prolactin when measuring mouse prolactin using the ELISA a number of controls were carried out. Plasma from

pseudopregnant control mice (saline only, not administered oPRL; n = 4) was pooled and then spiked with (1) 100ng/ml murine prolactin; (2) 20µg/ml ovine prolactin, or (3) 100ng/ml murine prolactin and 20µg/ml ovine prolactin. The plasma samples were then diluted and assayed (n = 4 per treatment) as above. 20µg/ml oPRL was used to spike the plasma as 20µg was the dose given to the mice and it has been estimated that 20g mice contains around 1.2-1.6 ml blood (~1ml plasma). oPRL was dissolved in prolactin buffer (30mM NaHCO3, 150mM NaCl, pH 10.8) before being used to spike plasma samples.

4.2.1.4 Brain sectioning, tyrosine hydroxylase/pSTAT5 ICC and analysis

Brains were sectioned using a freezing microtome (48µm) to provide coronal brain sections containing paraventricular nucleus (PVN), arcuate nucleus, ventromedial hypothalamic nucleus (VMH) and supraoptic nucleus (SON). Sections were stored in cryoprotectant before immunocytochemistry (ICC) was carried out for pSTAT5 and tyrosine hydroxylase (see section 2.10.5). Using a light microscope (20x or 40x objective) on coded slides, pSTAT5-positive cells (those with a dark grey/black nucleus) were counted in roughly 4 sections per mouse for each area of interest (defined using (Franklin K, 1997)). For the PVN, pSTAT5-positive cells were quantified for the magnocellular (m) and parvocellular (p) regions separately. In addition to pSTAT5 only counts, the arcuate nucleus was also analysed for the number of tyrosine hydroxylase-positive cells colocalized with pSTAT5 (expressed as a percentage of the total number of tyrosine hydroxylase cells).

4.2.2 Effect of 24h fast on prolactin negative feedback

4.2.2.1 Treatment

Virgin and d5.5 pregnant were fasted for 24h (n =18, 20 respectively), with food removed from their home cage at 10am (lights on at 7am), controls were allowed to feed normally (n = 16, 21 respectively). After 23h mice were injected with oPRL (1µg/g bodyweight i.p; Sigma-Aldrich; virgin control and fasted n = 8, 10 respectively; d5.5 control and fasted n =11) or prolactin buffer (virgin control and fasted n = 8; d5.5 control and fasted n = 10, 9 respectively) as a control.

4.2.2.2 Blood/tissue collection and perfuse fixation

On hour after oPRL injection blood was collected by cardiac perfusion and mice perfused fixed as described above to provide brains for immunocytochemistry (see section 4.2.1.2). Following blood/tissue collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

4.2.2.3 Prolactin ELISA

Plasma samples were diluted 1 in 25 and assayed in duplicate using an adapted protocol (see General Methods section 2.7.2.2) for the mouse prolactin DuoSet ELISA (DY1445, R&D Systems, Inc., Minneapolis, US).

4.2.2.4 Brain sectioning, tyrosine hydroxylase/pSTAT5 ICC and analysis

Brains were sectioned and processed for pSTAT5 and tyrosine hydroxylase immunocytochemistry as described above (see section 4.2.1.4). The arcuate nucleus was analysed for the number of pSTAT5-positive tyrosine hydroxylase cells (expressed as a percentage of the total number of tyrosine hydroxylase cells).

4.2.3 Statistics

Statistical analysis of the mean prolactin concentrations, pSTAT5 counts and % pSTAT5-positive tyrosine hydroxylase cells, from LPS-treated d5.5 pregnant mice, were determined by 2-way ANOVAs, with Holm-Sidak post-hoc tests. Data from virgin and d5.5 pregnant mice from the fasting stress experiment were analysed using a combination of 2-way and 3-way ANOVAs with a Holm-Sidak post-hoc tests. Data are presented as group means±SEM (excluding outliers: mean±3*SEM).

4.3 Results

4.3.1 Effect of LPS stress on prolactin negative feedback

4.3.1.1 Prolactin concentration

LPS significantly reduced plasma prolactin vs. vehicle in d5.5 pregnant mice treated with prolactin-buffer. Murine plasma prolactin concentrations were significantly lower in oPRL treated mice vs. control (prolactin buffer) (2-way ANOVA, interaction LPS x prolactin treatments $p<0.001$; Figure 4.1). However, LPS treatment in mice given oPRL did not significantly decrease murine plasma prolactin further. Control checks on oPRL cross-reactivity showed that there was a significant difference between prolactin treatments (1 way ANOVA, $p<0.001$ across treatment). Murine prolactin spiking to plasma significantly increased prolactin levels to the same degree as plasma spiked with both ovine and murine prolactin (Holm-Sidak post hoc test, ns). Ovine prolactin alone did not alter the levels of prolactin vs. plasma alone (Holm-Sidak post hoc test, ns; data not shown) measured using the murine prolactin ELISA. Therefore, ovine prolactin levels are not read by the murine prolactin ELISA, nor do they interfere with reading of murine prolactin levels when co-administered with murine prolactin.

4.3.1.2 % pSTAT5-positive TIDA neurones

Very few neurones expressed nuclear pSTAT5 in the arcuate nucleus of mice treated with prolactin buffer (Figure 4.2 A and B) and the number of pSTAT5-positive neurones per arcuate profile was similar in both vehicle and LPS treated mice. This was significantly increased following oPRL treatment in both groups (2-way ANOVA, $p<0.001$ across prolactin treatment; Figure 4.2 C). LPS treatment alone did not alter the percentage of pSTAT5-positive TIDA neurones, however, oPRL treatment increased the proportion of double labelled neurones (presented as % of tyrosine hydroxylase neurones that were pSTAT5-positive) in LPS treated mice (2-way ANOVA, interaction LPS x prolactin treatments $p=0.025$; Figure 4.2 D). When the percentage of double labelled neurones was plotted against mean plasma prolactin data, there appears to be an inverse relationship in all groups except the pregnant stressed mice treated with prolactin buffer, which shows low prolactin and low % pSTAT5-positive tyrosine hydroxylase neurones (Figure 4.3).

Figure 4.1

Figure 4.2

Figure 4.2 cont’d

Figure 4.3

4.3.1.3 pSTAT5 expression on other hypothalamic cell groups

Supraoptic nucleus (SON):

In the SON, expression of pSTAT5 was similar in all groups regardless of LPS or oPRL treatment (2-way ANOVA, ns; Figure 4.4).

Paraventricular nucleus (PVN):

pSTAT5 expression in the mPVN and pPVN was similar in all groups and not altered by LPS or oPRL treatment (2-way ANOVA, ns; Figure 4.5).

Ventromedial hypothalamus (VMH):

LPS treatment alone did not alter the number pSTAT5-positive nuclei in the VMH, however, oPRL treatment increased the pSTAT5-positive cells (2-way ANOVA, $p<0.01$ across oPRL treatment; Figure 4.6).

Figure 4.4

Figure 4.5

Figure 4.5 cont’d

Figure 4.6

Figure 4.6 cont’d

4.3.2 Effect of 24h fast on prolactin negative feedback

4.3.2.1 Prolactin concentration

Neither 24h fast nor oPRL treatment significantly altered plasma prolactin in virgin mice. Murine plasma prolactin concentrations were significantly reduced by fast treatment in d5.5 pregnant mice vs. non-fasted (in both oPRL and prolactin buffer treated groups (3-way ANOVA, interaction fast treatment x group $p<0.001$; 2-way ANOVA for pregnant groups only, $p<0.001$ across treatment; Figure 4.7). oPRL treatment reduced plasma prolactin in non-fasted pregnant mice. However, it did not reduce prolactin further in fasted d5.5 mice.

4.3.2.2 % pSTAT- positive TIDA neurones

The number of pSTAT5-positive neurones per arcuate profile was similar in both virgin and pregnant non-fasted mice treated with prolactin buffer (Figure 4.8 A-D). However, when treated with oPRL the number of pSTAT5 nuclei increased significantly in the non-fasted pregnant mice. Although pSTAT5 expression in non-fasted virgins appears to also mildly increase with oPRL treatment this was not significant (3-way ANOVA, interaction prolactin treatment x group $p<0.01$; Figure 4.8 E). The number of pSTAT5-positive neurones per arcuate profile was similar in both virgin and pregnant fasted mice treated with prolactin buffer and the number of pSTAT5 nuclei increased in both virgin and pregnant mice that were treated with oPRL (3-way ANOVA, interaction fast x prolactin treatments $p=0.001$). Individual 2-way ANOVAs for virgin and pregnant mice alone revealed that 24h fasting increases the pSTAT5 expression in oPRL treated virgin and pregnant mice (2-way ANOVAs, interaction fast x prolactin treatments $p<0.05$; Holm-Sidak post hoc tests, $p<0.05$ across prolactin treatment within fasted mice, $p<0.05$ fast vs. control in prolactin treated mice).

The percentage of pSTAT5-positive tyrosine hydroxylase-positive cells (TIDA neurones) was similar in virgin and pregnant non-fasted mice treated with prolactin buffer. When treated with oPRL the %-pSTAT5 positive TIDA neurones increased slightly in the non-fasted pregnant mice (3-way ANOVA, interaction prolactin treatment x group $p< 0.01$; Figure 4.8 F). The % of pSTAT5-positive tyrosine hydroxylase neurones was low in both virgin and pregnant fasted mice treated with prolactin buffer (3-way ANOVA, interactions fast x group $p<0.05$, fast x prolactin

treatments $p<0.001$, fast x prolactin treatments x group $p=0.001$). Individual 2-way ANOVAs for virgin and pregnant mice alone revealed that 24h fast increased the % pSTAT5-positive TIDA neurones only in oPRL treated pregnant mice (2-way ANOVA, interaction fast x prolactin treatment $p<0.05$; Holm-Sidak post hoc tests, $p<0.05$ fast vs. control in d5.5 prolactin treated mice).

When the % pSTAT5-positive TIDA neurones are plotted against the mean plasma prolactin data there appears to be an inverse relationship in 7 out of the 8 groups, the obvious outlier being the pregnant stressed mice treated with prolactin buffer. They display both low plasma prolactin and low pSTAT5-positive tyrosine hydroxylase neurones (Figure 4.9).

Figure 4.7

Figure 4.8

Figure 4.8 cont’d

Figure 4.9

4.4 Discussion

4.4.1 Effect of LPS stress on prolactin negative feedback

In support of previous data (see section 3.3.2), LPS administration in early pregnancy robustly diminished circulating prolactin in mice treated with prolactin buffer. Additional treatment with oPRL decreased murine plasma prolactin in non-stressed early pregnant mice as expected, demonstrating that prolactin negative feedback had been driven (DeMaria et al., 1999, Anderson et al., 2006a). However, although LPS reduced plasma prolactin, oPRL supplementation did not further decrease it in early pregnancy. This may be because levels have already been substantially reduced and further changes are minimal and thus not significant.

Although stress alone reduces circulating prolactin this is not the case with pSTAT5 signalling. The percentage of pSTAT5-positive TIDA neurones in stressed early pregnant mice was low and comparable to non-stressed mice, showing negative feedback via the pSTAT5 signalling pathway is not altered by stress alone (at basal prolactin levels). However, the addition of oPRL significantly increased the % pSTAT5-positive TIDA of stressed pregnant mice only, demonstrating stress enhances negative feedback in these mice.

Stress and prolactin treatment had no notable effect on pSTAT5 expression in the hypothalamic SON and PVN despite prolactin receptors being expressed in these nuclei (Kokay et al., 2006). In non-pregnant rats prolactin treatment is reported to decrease firing rates of oxytocin neurones, while having no effect on vasopressin neurone activity. Therefore, prolactin seems to directly and specifically regulate oxytocin neurone activity (Kokay et al., 2006). A study in male rats has reported that prolactin has an inhibitory effect on oxytocin release, influencing activation of the JAK-STAT signalling pathway as well as neuronal excitability of oxytocin neurons. Although the functional significance of this is yet to be fully elucidated it has been proposed that it may be important in coordinating secretion of oxytocin with events that alter prolactin secretion, reflecting a feedback pathway to regulate the prolactin-releasing role of oxytocin (Townsend et al., 2005). However, our data show that LPS (with or without prolactin) did not significantly alter pSTAT5 expression in the SON or PVN.

Expression of pSTAT5 nuclei in the VMH was increased with prolactin treatment but was not altered by LPS stress although significance of this unclear. Previous studies have reported limited STAT5 expression in VMH and DMH (dorsomedial hypothalamic nuclei), areas of the hypothalamus known to regulate energy balance and feeding (Lee et al., 2008). This suggests a role for STAT5 and prolactin in energy balance. Appetite and food intake are increased in pregnancy in order to meet the high metabolic demands of the developing offspring and increase fat storage for lactation. For this to occur and be maintained pregnant females become resistant to the anorectic actions of leptin (Ladyman et al., 2010). Prolactin is reported to be orexigenic, acting centrally to increase food intake by inducing leptin resistance and suppressing intracellular signals of leptin receptor activation in the VMH and PVN (Naef and Woodside, 2007). Consistent with these reports, prolactin receptor-deficient mice (male and female) display reduced body weight and fat mass compared to wild-type, supporting the role for prolactin in weight regulation (Freemark et al., 2001).

Therefore, enhanced prolactin negative feedback, via prolactin receptor-induced signalling at the TIDA neurones, does not appear to underlie LPS-induced prolactin suppression in early pregnant mice

4.4.2 Effect of 24h fast stress on prolactin negative feedback

As shown previously (see section 3.3.5), 24h fasting in early pregnancy decreased circulating prolactin whilst having no considerable effect on virgin mice (treated with prolactin buffer). As with LPS stress, additional treatment with oPRL decreased murine plasma prolactin in non-stressed early pregnant mice as expected, demonstrating prolactin negative feedback. In virgin mice there appears to be a general trend of reduction in plasma prolactin with oPRL treatment, however, this is not significant. It may be the case that the dose of oPRL given was too low to see a significant decrease in the already relatively low plasma prolactin levels (compared to pregnant mice). Other studies looking at prolactin negative feedback in mice have observed an increase in negative feedback using much higher doses of oPRL e.g. Brown et al., 2011, used a dose five times higher (5µg/g body weight). The dose chosen for our experiments has been previously shown to increase pSTAT5 nuclear expression compared to vehicle treatment in lactating rats with pups removed for 16h

(Anderson et al., 2006b). However, in our mice the increase in pSTAT5 expression, due to oPRL treatment, may not be high enough to significantly impact on plasma prolactin levels at the time point observed. In addition, the data generated is quite variable and thus may be masking a subtle reduction in plasma prolactin with oPRL treatment. In early pregnancy, although fasting stress reduced plasma prolactin, oPRL supplementation did not further decrease it, suggesting prolactin negative feedback is not enhanced further by stress.

As with immune stress, 24h fast alone did not alter pSTAT5 signalling in either virgin or early pregnant mice. The expression of pSTAT5 in the TIDA neurones was equally low in control and fasted mice administered prolactin buffer only. oPRL increased pSTAT5 expression in the arcuate of virgin and early pregnant mice. The percentage of pSTAT5-positive TIDA neurones in stressed early pregnant mice (treated with prolactin buffer) was low and comparable to non-stressed mice, showing negative feedback was not enhanced by stress alone (at basal prolactin levels). However, the addition of oPRL significantly increased the % pSTAT5-positive TIDA neurones in fasted virgin and pregnant mice, once again demonstrating stress enhances negative feedback in these mice.

The overall expression of pSTAT5-postive neurones in these data is relatively low compared to other studies, this is likely due to using a lower dose of oPRL to drive feedback (Brown et al., 2011, Sjoeholm et al., 2011). However, the dose we chose has been previously used before e.g. in lactating rats whose pups were removed for 16h, oPRL injection significantly increased the STAT5 nuclear: cytoplasmic ratio compared to vehicle (Anderson et al., 2006b).

Prolactin receptor expression is known to increase during pregnancy and lactation in the choroid plexus and a number of hypothalamic nuclei including the arcuate nucleus, PVN and SON (Grattan et al., 2001a). There is mounting evidence that raised prolactin levels increase food intake, with i.c.v. administration of prolactin inducing hyperphagia in virgin female mice in a dose-dependent manner (Sauve´ and Woodside, 1996). In addition, prolactin via i.c.v. increases food intake without influencing oestrous cyclicity, and irrespective of whether administered bi-daily or chronically via infusions (Noel and Woodside, 1993, Sauve´ and Woodside, 1996, Naef and Woodside, 2007). These data suggest that central prolactin receptor

activation leads to increased food intake, independently of steroid hormone levels, and therefore raised prolactin levels during early pregnancy may contribute to hyperphagia (Ladyman et al., 2010).

4.4.3 Relationship between plasma prolactin and STAT5 activity

In general, an inverse relationship seems to exist between prolactin and % pSTAT5 in TIDA neurones in both the LPS and fast studies. However, there is an obvious outlier to this theory in that stress in early pregnancy (no prolactin) suppresses prolactin despite low pSTAT5 expression. This implies that although pSTAT5 expression can be driven following stress in early pregnant mice (with oPRL), it is not exclusively responsible for the stress-reduced prolactin. Data show stress alone causes prolactin suppression but not via increased pSTAT5 expression, thus it is not utilizing the prolactin short-loop feedback system. This cannot explain the mild stress-induced increase in TIDA activation previously observed or the decrease in prolactin secretion in early pregnancy so other stress-related hypothalamic mechanisms must be responsible.

4.4.4 Potential pregnancy or stress-related factors targeting TIDA neurones

4.4.4.1 The CRH system

TIDA neurones are thought to be targets for the CRH system and are known to express CRH receptors. CRH R_1 receptor-like immunoreactivity (CRH R_1-ir) is apparent in rat TIDA neurones, however, CRH-ir fibre innervation is very sparse in the arcuate nucleus suggesting it is does not have a major role in TIDA regulation (Campbell et al., 2003). CRH could also have an indirect effect on the TIDA response via activation of the LC which is innervated by CRH-like immunoreactive fibres (Ehlers et al., 1983, Valentino et al., 1992, Jedema and Grace, 2004) and expresses receptors for CRH (Sánchez et al., 1999). The LC is robustly activated following stressful stimuli in experimental animals (e.g. see section 3.3.2.8) and leads to increased noradrenaline (NA) levels in the CNS (Itoi and Sugimoto, 2010). Noradrenaline from the LC plays a role in LH pulse inhibition in response to stress and noradrenaline neurones are known to project to the arcuate nucleus or medial preoptic area (MPOA) (Campbell and Herbison, 2007) directly or indirectly e.g. via the kisspeptin system (Li et al., 2010). Therefore, it is possible the elevated NA,

following stress, may impact directly on the arcuate nucleus, altering TIDA activation.

4.4.4.2 Sex steroids and SOCs

TIDA neurones also have receptors for steroids such as progesterone and oestrogen which change with reproduction state (Steyn et al., 2007). High levels of progesterone are present from the day of mating and, along with the enhanced presence of steroid receptors during pregnancy, supports the idea that steroid hormones may directly alter TIDA neurone activation to regulate prolactin secretion (Steyn et al., 2007, Leite et al., 2008). How this affects TIDA neurone responsiveness to stress stimuli during early pregnancy is unclear from the literature at present. Oestradiol has been shown to greatly augment prolactin-induced STAT5 activation, and increase prolactin receptor, SOCS-3 and CIS mRNA levels in the arcuate nucleus and choroid plexus (Anderson et al., 2008).

As mentioned previously, during late gestation and lactation prolactin loses its ability to drive phosphorylation of STAT5b and this was found to be associated with enhanced SOCS mRNA which may underlie reduced TIDA neurone dopamine output (Anderson et al., 2006a). During stress in early pregnancy, the opposite effect may occur and expression of SOCS may be diminished, thus allowing increased pSTAT5b signalling and suppressed prolactin secretion. However, this seems unlikely as LPS treated mice have been shown to have increased expression of SOCS-3 in the pituitary (Chesnokova et al., 2002) and we found that stress reduces prolactin despite low pSTAT5 levels.

4.4.4.3 Stress-induced cytokines

Another possible stress-related mechanism driving TIDA activation and/or prolactin suppression is activity of cytokines associated with stress/LPS. IL-6 and TNF-α are potential candidates for involvement as they are both released following LPS treatment in early pregnancy (see section 3.3.2.3 and 3.3.3.1, respectively) and are known to be detrimental to pregnancy maintenance (Carpentier et al., 2011). STAT5 A and B are widely expressed and activated by a plethora of cytokines (Hennighausen and Robinson, 2008), including numerous ILs e.g. IL-2 (Laurence et

al., 2007), IL-10 (Wehinger et al., 1996), and IL-4, and growth hormones (Han et al., 1996, Herrington et al., 2000), in addition to prolactin.

In order to investigate the role of proinflammatory cytokines further we selected two cytokines: TNF-α and IL-6, which were administered to early pregnant mice to determine the effect on prolactin, progesterone and TIDA activation (see Section 4B).

Section 4b: Effect of IL-6 and TNF-α on the prolactin system in early pregnancy

4.5 Introduction

4.5.1 The cellular T system

One of the most important components of the cellular immunosystem which adapt in early pregnancy are the helper T (Th) cells (reviewed in (Guerin et al., 2009)). Naive $CD4^+$ T cells differentiate into two polarised forms, Th1 and Th2 cells, which exhibit different effector responses. Th 1 cytokines (e.g.TNF-α) trigger numerous cell-mediated cytotoxic and inflammatory responses, while Th 2 cytokines (e.g. IL-10) are involved with B cell anti-body production and are required for the secretion of hPL and hCG (Druckmann and Druckmann, 2005).

4.5.1.2 The cellular T system in pregnancy

It has been suggested that successful pregnancy is characterised by a Th2 phenomenon, with higher Th2 cytokine production and release involved in foetal allograft survival and pregnancy maintenance (Piccinni et al., 1995, Piccinni, 2006). Recently the Th1:Th2 paradigm has been extended to encompass Th17 cells (which produce the pro-inflammatory cytokine IL-17) and regulatory T (Treg) cells (which regulate Th cells). Th17 cells play a role in induction of inflammation and in the pathogenesis of rejection, while Treg are involved in immunoregulation as well as peripheral tolerance (Saito et al., 2010). In early pregnancy Treg cells are highly expressed in the decidua. Decreased numbers of peripheral and decidual Treg cells have been observed in spontaneous miscarriage cases, suggesting Treg cells contribute to maternal immune tolerance of conceptus antigens and support pregnancy maintenance (Sasaki et al., 2004).

4.5.2 Progesterone as an immunosteroid

We have shown that stress in early pregnancy decreases circulating progesterone and prolactin, both of which may also disrupt the cytokine milieu. Progesterone is known to act as an immunosteroid, aiding maternal tolerance to the foetal 'semi-allograft'. In this capacity, progesterone inhibits very early T-cell lymphopoiesis (generation of lymphocytes) and provides a pregnancy protective immune environment favouring development of Th cells which produce Th2 cytokines (Piccinni et al., 1995).

Progesterone exerts an anti-abortive effect by triggering progesterone induced blocking factor (PIBF) release (Arck et al., 2007). In mice, PIBF reduces NK cell activity (which correlates with resorption rate) (Szekeres-Bartho et al., 1997a) and alters the cytokine profile of activated lymphocytes, favouring production of Th2 cytokines over Th1 cytokines (Szekeres-Bartho and Wegmann, 1996). Stress in early pregnancy decreases circulating progesterone, reduces PIBF and increases resorption rates in mice, all of which have been shown to be abrogated with progesterone treatment (Joachim et al., 2003, Parker and Douglas, 2010). These data highlight the importance of progesterone in establishing and maintaining a suitable cytokine balance in the peri-implantation period.

4.5.3 Prolactin and immune regulation

Prolactin is known to be immunomodulatory, not just through its role in driving progesterone secretion in mice (Freeman et al., 2000). Prolactin has been shown to regulate the cytokine profile and enhance the production of IFN-γ, IL-10 and IL-12 in a stimulus specific manner (Matalka, 2003, Matalka and Ali, 2005). In addition, decidual prolactin silences expression of genes thought to be detrimental to pregnancy, including IL-6 and 20α-HSD in decidua (Bao et al., 2007).

4.5.4 Effects of stress on cytokine balance in pregnancy

Cytokines are reported to be key mediators in the bi-directional interaction between the maternal immune system and the reproductive system throughout pregnancy (Wegmann et al., 1993). Cytokines are not only released following infection or inflammation but have been shown to be secreted following physical and psychological stress (Turnbull and Rivier, 1999). The cytokine profile displayed in early gestation is delicate and can be disturbed by a number of stressors, including immune challenges such as LPS or physical stressors such as hunger (Parker and Douglas, 2010). Psychoemotional stress also disrupts the cytokine balance in mice, resulting in foetal loss (Blois et al., 2004). A study into psychological stress in pregnant women reported a positive correlation between high perceived stress scores and elevated serum proinflammatory cytokines (IL-6 and TNF-α) and lower anti-inflammatory IL-10, compared to women with low stress scores (Coussons-Read et

al., 2005). An increased Th1 profile is also apparent in blood and decidual cells of pregnant mice following stress (Blois et al., 2005b, Blois et al., 2007).

4.5.5 Effects of IL-6 and TNF-α on the HPA axis

Two proteins known to be detrimental for the normal progress of pregnancy are the cytokines IL-6 and TNF-α, both of which are elevated in the serum of women with preeclampsia (Szarka et al., 2010). It has long been known that cytokines are potent activators of the central stress response, constituting part of the feedback loop through which the immune system communicates with the CNS (Chrousos, 1995). LPS administration activates the HPA axis and induces the synthesis and release of cytokines including IL-6 and TNF-α (Turnbull and Rivier, 1999, McCann et al., 2000). TNF-α and IL-6 are able to stimulate activation of the HPA axis either alone, or in synergy with each other (Chrousos, 1995, Tsigos and Chrousos, 2002). The principal action of these cytokines is probably on the release of CRH and vasopressin from the hypothalamus, but they can also have effects at the pituitary level. IL-6 and TNFα have both been shown to stimulate ACTH release from the anterior pituitary *in vitro* and *in vivo* (McCann et al., 1994).

4.5.6 The role of IL-6 in immune regulation

IL-6 is a multifunctional cytokine that is produced from immune cells (e.g. macrophages and dendritic cells) as well as a variety of other cells including endothelial and epithelial cells (Diehl and Rincón, 2002). IL-6 binding to its receptor induces activation of JAK/STAT signalling pathways as well as the CCAAT/enhancer binding protein (C/EBP) pathway (Diehl and Rincón, 2002). Activation of JAK1and JAK 2 by IL-6 causes activation of STAT 3 and mildly activates STAT1.

IL-6 is involved in the control of Th1:Th2 differentiation, promoting Th2 differentiation (dependent upon endogenous IL-4) whilst inhibiting Th1 polarization. IL-4 is the most potent factor that stimulates naive $CD4^+$ T cells to differentiate to the Th2 phenotype and IL-6 has been demonstrated to induce IL-4 production in $CD4^+$ T cells, that then polarizes naive $CD4^+$ T cells to effector Th2 cells (Rincón et al., 1997). IL-6 also upregulates SOCS1 expression in activated $CD4^+$ T cells, resulting in interference of STAT1 phosphorylation by IFN-γ. The inhibition of the

IFN-γ receptor-mediated signals suppresses autoregulation of IFN-γ gene expression during activation of $CD4^+$ T cells. Consequently, Th1 differentiation is prevented (Diehl et al., 2000). Therefore, IL-6 has a dual role in Th1/Th2 differentiation, promoting Th2 and inhibiting Th1 differentiation.

4.5.7 Effects of IL-6 and TNF-α in pregnancy

4.5.7.1 Effects of IL-6 in pregnancy

Mounting evidence indicates that IL-6 can directly modulate gonadal functions. In the ovary IL-6 is produced by rat granulosa cells and can suppress progesterone secretion *in vitro* in rat and porcine granulosa cells (Gorospe et al., 1992, Machelon et al., 1994). IL-6 is not normally expressed in the decidua and both IL-6 and its receptor have been shown to be downregulated in the rat decidua by prolactin and oestradiol (Deb et al., 1999b). Decidual prolactin is a robust repressor of IL-6 expression in the mouse decidua (Bao et al., 2007). Although decidual IL-6 is normally suppressed it is produced in response to inflammation and is thought to have an important role in the pathophysiology of infection-induced preterm labour (Romero et al., 1990, El-Bastawissi et al., 2000, Bao et al., 2007). Production of IL-6 during pregnancy may trigger an inflammatory response and compromise pregnancy success, thus prolactin-induced inhibition of its expression in the decidua could be of major physiological importance.

IL-6 mRNA is also not expressed in the corpus luteum throughout the majority of pregnancy in the rat (only apparent in very early pregnancy and immediately following parturition) but it too has been shown to be induced by LPS. Progesterone inhibits IL-6 expression and therefore it is likely the high levels of this hormone during pregnancy prevent the expression of IL-6 which may have a deleterious effect on corpus luteum function (Telleria et al., 1998).

4.5.7.2 Effects of TNF-α in pregnancy

TNF-α is a pleiotropic cytokine and has been identified throughout the female reproductive tract, placenta and embryonic tissue (Terranova et al., 1995). Elevated levels of TNF-α are thought to be adverse to reproduction and numerous studies have revealed that TNF-α administration to pregnant mice results in foetal loss (Toder et al., 2003). Experiments using CBA/JxDBA/2J mice (model with high levels of

embryonic death) have found that these mice exhibit an increased level of TNF-α in decidual cell cultures (reviewed in (Toder et al., 2003)) and also have elevated expression in the placenta (Tangri and Raghupathy, 1993). In rats, LPS-induced foetal loss can be abrogated by blocking of maternal TNF-α activity (Renaud et al., 2011). In mice, various stressors have been reported to activate TNF-α producing cells at the foetal-maternal interface and increase production of TNF-α. Ultrasonic stress significantly elevates resorption rate with a maximal effect on gestation day 5.5 and increased decidual TNF-α release (Arck et al., 1995). These observations have implicated TNF-α in triggering immunological-induced pregnancy loss (embryo death due to failure to prevent rejection) (Clark et al., 1999, Raghupathy, 2001). TNF-α is thought to act as a mediator in stress-induced embryonic death, possibly through dysfunction of mechanisms required for the uterus to become receptive to blastocysts, thus preventing implantation (Torchinsky et al., 2005). In women the level of TNF-α in the amniotic fluid is significantly higher with uterine infections and is associated with preterm birth incidence (Romero et al., 1990). TNF-α has also been implicated in other pregnancy complications such as preeclampsia in baboons (Sunderland et al., 2011) and humans, where it has been suggested as useful marker for preeclampsia in the first trimester (Serin et al., 2002, Leblanc et al., Epub ahead of print).

TNF-α has been characterised over recent years as a powerful apoptotic activator as well as an activator of anti-apoptotic signalling cascades. It has been described to boost death signalling in order to kill the embryo if an initial adverse situation causes structural abnormalities, while triggering protective mechanisms if damage repair is possible and may avoid maldevelopment (Toder et al., 2003).

4.5.8 IL-6, TNF-α and prolactin

The rat pituitary cell line, MtT/SM, secretes prolactin and has the characteristics of somatomammotrophs. IL-6 has been found to stimulate proliferation of these cells and inhibit the secretion of prolactin (and growth hormone) suggesting IL-6 inhibits the functions of mammotrophs (lactotrophs) and somatotrophs in the pituitary gland (Tomida et al., 2001).

TNF-α, along with other proinflammatory cytokines such as IL-1α and IL-8, have been shown to inhibit decidual prolactin release and decrease prolactin synthesis

from primary human decidual cells from term pregnancies. However, IL-6 treatment was not shown to alter basal decidual prolactin release. This study suggests a paracrine role for cytokines released by decidual macrophages in regulation of decidual prolactin expression (Jikihara and Handwerger, 1994). TNF-α administration to male rats was reported to decrease circulating prolactin but it did not alter the DOPAC:dopamine ratio in the hypothalamus or pituitary. However, both dopamine and DOPAC levels were raised in the anterior pituitary following TNF-α treatment (i.c.v.), indicating an increase in dopamine turnover which could mediate the suppressive effect on prolactin (de Laurentiis A, 2002).
Both IL-6 and TNF-α are released following stress in early pregnancy and are reputed to threaten pregnancy maintenance. Therefore, in order to investigate the effect of TNF-α and IL-6 in early pregnancy on prolactin and its regulation, d5.5 pregnant mice were administered TNF-α or IL-6 (or vehicle). We aimed to use immunocytochemistry, double labelling for tyrosine hydroxylase and Fos, to determine whether there is an alteration in TIDA neurone activation following proinflammatory cytokine treatment in early pregnancy, and whether this is associated with reduced plasma prolactin/progesterone.

4.5.9 Aim and hypotheses

Aim:

1. To determine the effect of TNF- α and IL-6 treatment on prolactin secretion and its regulation in early pregnancy.

Hypotheses:

1. TNF-α and IL-6 treatment decrease circulating prolactin and progesterone in early pregnancy.
2. TNF-α and IL-6 treatment increase TIDA neurone activation in early pregnancy.

4.6 Methods

4.6.1 Effect of IL-6 and TNF-α on hormones and neuronal activation in early pregnancy

4.6.1.1 Treatment

d5.5 pregnant mice were gently restrained in a holding bag and either injected with recombinant mouse IL-6 (200ng in 100µl 0.4% BSA in PBS i.p; R&D Systems, Inc., Minneapolis, US; n = 7 (Zalcman et al., 1998, Dunn, 2006)), recombinant mouse TNF-α (3µg in 100µl 2% BSA in PBS; R&D Systems, Inc., Minneapolis, US; i.p; n = 7 (Sundgren-Andersson et al., 1998)) or vehicle (100µl 0.4% or 2% BSA in PBS i.p; n = 6, 5 respectively) around 10am (3h after lights on). Mice were returned to their home cages. Drugs were reconstituted according to manufacturer's instructions (R&D Systems, Inc., Minneapolis, US) before dilution to the desired dose.

4.6.1.2 Blood/tissue collection and perfuse fixation

After 90 min mice were quickly moved to an adjacent room and injected with an overdose of sodium pentobarbitone (0.3ml i.p; Centravet, 22106 Didan, France). Once the mouse was deeply anaesthetised (no signs of breathing, non-responsive to toe pinch) the heart was exposed. Blood was removed by cardiac puncture before the mouse was perfused with heparinised saline and then fixed with 4% paraformaldehyde (as described in General Methods section 2.9.1). The brains were carefully removed and stored overnight (4ºC) in 4% paraformaldehyde and 15 % sucrose solution. They were then cryoprotected in a 30% sucrose solution at 4ºC overnight. Following this the brains were frozen before sectioning. Blood was processed for plasma and frozen (-20°C) prior to use in assays. Following blood/tissue collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

4.6.1.3 Prolactin ELISA

Plasma samples were diluted 1 in 25 and assayed in duplicate using an adapted protocol (see General Methods section 2.7.2.2) for the mouse prolactin DuoSet ELISA kit (DY1445, R&D Systems, Inc., Minneapolis, US).

4.6.1.4 Progesterone RIA

Plasma samples (undiluted) were assayed in duplicate using a progesterone RIA kit (DSL-3900; kit 1; see section 2.6.1.2).

4.6.1.5 Corticosterone RIA

Plasma samples were diluted 1 in 200 and assayed in duplicate using a cortocosterone RIA kit (Corticosterone ImmunChem Double Antibody RIA kit; MP Biomedicals) (see section 2.6.3.2).

4.6.1.6 Brain sectioning, tyrosine hydroxylase/Fos ICC and analysis

Brains were sectioned using a freezing microtome with slices cut at 48µm. Coronal brain sections (containing PVN, arcuate nucleus, VMH and SON) were stored in cryoprotectant before double immunocytochemistry (ICC) was carried out for Fos and tyrosine hydroxylase (see section 2.10.3). Sections were processed and visualised using the avidin-biotin peroxidase complex (ABC) method with nickel DAB/DAB.

Fos-positive cells (containing a dark grey/black nucleus) were counted in around 4 sections per mouse for each area of interest (defined using (Franklin K, 1997)). This was carried out on coded slides using a light microscope (20x or 40x objective). In addition to Fos only counts, the arcuate nucleus was also analysed the number of tyrosine hydroxylase cells colocalized with Fos (expressed as % of the total number of tyrosine hydroxylase cells). Cells with a dark grey/black nucleus and brown cytoplasmic staining were counted as double labelled (colocalisation).

4.6.2 Statistics

Statistical evaluation of the mean hormone concentrations and neuronal activation were determined by 1-way ANOVAs with a Holm-Sidak post-hoc test. Student t-tests were used to evaluate whether there was a difference between the two vehicle groups (0.4% and 2% BSA in PBS) for each of the above parameters. The data from the two vehicle groups were pooled for the graphs and statistics as they were not significantly different for any of the parameters measured (Students t-test, ns). Data are presented as group means±SEM (excluding outliers: mean±3*SEM).

4.7 Results

4.7.1 Effect of IL-6 and TNF-α on plasma hormones and neuronal activation in early pregnancy.

4.7.1.1 Prolactin concentration

IL-6 treatment had no significant effect on plasma prolactin concentration in d5.5 pregnant mice but TNF-α significantly decreased prolactin compared to vehicle and IL-6 treatment (1-way ANOVA, $p<0.001$ across treatment; Holm-Sidak $p<0.05$ TNF-α vs. vehicle and vs. IL-6; Figure 4.10A).

4.7.1.2 Progesterone concentration

IL-6 treatment had no significant effect on plasma progesterone concentration in early pregnant mice but TNF-α significantly reduced circulating prolactin levels compared to vehicle and IL-6 treatment (1-way ANOVA, $p<0.001$ across treatment; Holm-Sidak $p<0.05$ TNF-α vs. vehicle and vs. IL-6; Figure 4.10B).

4.7.1.3 Corticosterone concentration

IL-6 and TNF-α treatment increased corticosterone levels in early pregnant mice (1-way ANOVA, $p<0.001$ across treatment; Holm-Sidak $p<0.05$ TNF-α vs. vehicle and vs. IL-6; Figure 4.10C). Although a Holm-Sidak test following a 1-way ANOVA did not report a significant increase in corticosterone with IL-6, a Student t-test (IL-6 vs. vehicle) did reveal a significant change ($p<0.05$).

4.7.1.4 Activation of TIDA neurones

IL-6 and TNF-α treatment significantly increased the number of Fos-positive neurones per arcuate profile (Figure 4.11 A and B), in a similar manner, compared to vehicle (1-way ANOVA, $p<0.01$ across treatment; Figure 4.11 C). The proportion of activated TIDA neurones (presented as % Fos-positive tyrosine hydroxylase neurones) mildly increased following both IL-6 and TNF-α injection compared to vehicle (1-way ANOVA, $p<0.01$ across treatment; Figure 4.11 D). TNF-α treatment evoked a higher response than IL-6 (Holm-Sidak, $p<0.05$).

Figure 4.10

Figure 4.11

Figure 4.11 cont’d

4.7.1.5 Activation of other hypothalamic cell groups

Supraoptic nucleus (SON):

In the SON, IL-6 treatment had no significant effect on the number of Fos-positive nuclei in d5.5 pregnant mice compared to vehicle. TNF-α significantly increased Fos expression in the SON compared to both vehicle and IL-6 treatment (1-way ANOVA, $p<0.001$ across treatment; Holm-Sidak $p<0.05$ TNF-α vs. vehicle and vs. IL-6; Figure 4.12).

Paraventricular nucleus (PVN):

In both the mPVN and the pPVN, IL-6 treatment had no significant effect on the number of Fos-positive nuclei in early pregnant mice compared to vehicle. TNF-α significantly increased Fos expression in both PVN regions compared to vehicle and IL-6 treatment (1-way ANOVAs, $p<0.001$ across treatment; Holm-Sidak $p<0.05$ TNF-α vs. vehicle and vs. IL-6; Figure 4.13).

Ventromedial hypothalamus (VMH):

In the VMH, IL-6 treatment mildly increased the number of Fos-positive nuclei in d5.5 pregnant mice compared to vehicle. TNF-α significantly increased Fos expression in the VMH compared to both vehicle and IL-6 treatment (1-way ANOVA, $p<0.001$ across treatment; Holm-Sidak $p<0.05$ IL-6 vs. vehicle, $p<0.05$ TNF-α vs. vehicle and vs. IL-6; Figure 4.14).

Figure 4.12

Figure 4.13

Figure 4.13 cont’d

Figure 4.14

Figure 4.14 cont’d

4.8 Discussion

4.8.1 Effect of IL-6 and TNF-α on plasma hormones and TIDA neuronal activation in early pregnancy.

The activity of cytokines associated with stress/LPS in early pregnancy may influence prolactin regulation, lead to suppression of prolactin secretion and threaten foetal survival. IL-6 and TNF-α are known to be detrimental to pregnancy maintenance (Carpentier et al., 2011) and were candidates for our study into the effect of stress-induced pro-inflammatory cytokines on prolactin and its regulation. Serum TNF-α levels in early pregnant women have been reported to be elevated before miscarriage and may play a significant role in recurrent pregnancy loss (Çolakoglu et al., 2004). However, the role of IL-6 in pregnancy maintenance/loss is less clear. There are reports that IL-6 serum levels are higher in healthy pregnancies compared to women undergoing recurrent spontaneous abortion (Makhseed et al., 2000) implying that IL-6 is pregnancy protective. However, decidual IL-6 expression and content in the amniotic fluid is associated with inflammation and preterm labour (El-Bastawissi et al., 2000, Bao et al., 2007).

In support of a possible role for IL-6 and TNF-α in stress-induced adverse effect on pregnancy we have shown that circulating levels of these cytokines are increased following LPS treatment in early pregnancy (see section 3.3.2.3 and 3.3.3.1, respectively). IL-6 administration to d5.5 pregnant mice was found to mildly increase corticosterone levels, indicating activation the HPA stress axis, however, it had no significant effect on circulating prolactin or progesterone compared to vehicle. Thus stress-induced IL-6 release does not seem to underlie prolactin suppression following stress in early pregnancy. Conversely, TNF-α treatment significantly reduced both plasma prolactin and progesterone levels in early pregnant mice, in addition to robustly elevating corticosterone levels. Double ICC for Fos and tyrosine hydroxylase expression in the arcuate nucleus revealed that both IL-6 and TNF-α mildly increased activation of TIDA neurones. Therefore, data demonstrate that TNF-α may be partly responsible for stress-reduced prolactin secretion through action directly at the pituitary lactotrophs since it does not strongly alter TIDA activation. In support of this theory, *in vitro* studies have revealed that LPS treatment

increases TNF-α release from lactotrophs while also decreasing prolactin release (Theas MS, 1998).

4.8.2 Effect of IL-6 and TNF-α on neuronal activation in other hypothalamic nuclei

Other hypothalamic nuclei seem to behave in a slightly different manner to those in the arcuate nucleus following IL-6 treatment. In contrast to the general increase in the activation of the neuronal population of the arcuate nucleus, IL-6 did not stimulate Fos expression in the SON or PVN and only mildly increased Fos expression in the VMH. TNF-α treatment also increased Fos expression throughout the arcuate nucleus to a similar level as IL-6. However, TNF-α (like LPS) robustly increased activation in the SON, pPVN and VMH. Whether, as with LPS treatment, the pPVN response to TNF-α is attenuated in early pregnancy compared to virgins (possible reflecting oxytocin neurone response) is unknown at present as this study did not include the virgin groups required for comparison. Oxytocin is reported to be a key prolactin-releasing factor and is involved in the regulation of prolactin secretion (Freeman et al., 2000). For example, oxytocin is essential for the bi-daily prolactin surges observed in rats following cervical stimulation (McKee et al., 2007). Therefore, it is possible that TNF-α induces changes in oxytocin neurone activity and secretion of oxytocin in early pregnancy that could contribute to the reduced prolactin levels observed at this time.

LPS treatment to d5.5 pregnant mice profoundly affected pregnancy rate, resulting in occult foetal loss. However, it is not known what effect the pro-inflammatory cytokines IL-6 and TNF-α have on resorption rate as all mice were sacrificed after 90 min on d5.5 of pregnancy. In order to determine the effect on foetal survival the experiment would need to be repeated and mice sacrificed on d13.5 to allow resorption site analysis.

IL-6 treatment had a minimal effect on neurone activation in a number of hypothalamic nuclei, compared to TNF-α. It is possible that if IL-6 was administered at a higher dose we may see a more profound effect on the pregnancy hormones/neuronal activation, as although the HPA axis was activated this was only observed as a relatively mild increase in corticosterone vs. vehicle when compared to TNF-α. However, previous studies using the same dose have found that although plasma corticosterone levels varied only slightly or not significantly at all following

IL-6 treatment, this dose was high enough to induce behavioural effects, mesocortical dopamine activity in the hippocampus and prefrontal cortex, and increase serotonin levels from the hypothalamus (Zalcman et al., 1998, Dunn, 2006). The TNF-α dose used has previously been used in a study into the role of TNF-α in fever and was demonstrated to elicit hypothermal reactions (dependent on IL-6) but we did not measure body temperature (Sundgren-Andersson et al., 1998).

Although both stressors activated the HPA axis and mildly increased TIDA activation (as measured by Fos expression in the TIDA neurones) only TNF-α reduced prolactin and progesterone secretion, and activated a number of other hypothalamic nuclei. As both IL-6 and TNF-α mildly increased TIDA activation, but IL-6 had no effect on prolactin secretion, it does not appear that stress-reduced prolactin is completely due to the effects of IL-6 or TNF-α on TIDA neurone dopamine activity. Rather, stress-reduced prolactin in early pregnancy may be as a result of proinflammatory cytokine effects on the TIDA neurones in combination with other mechanisms. For example, TNF-α in early pregnancy may act directly at the pituitary lactotrophs to suppress prolactin release possibly through triggering apoptosis of lactotrophs and consequently decreasing in prolactin secretion.

The neuroendocrine mechanisms controlling prolactin are highly sensitive to cytokines and in order to test these mechanisms further experiments need to be carried out. A number of cytokines, including TNF-α, are known to activate the JAK2/STAT3 signalling pathway (Miscia et al., 2002, Romanatto et al., 2007). For example, Romanatto et al. have demonstrated that TNF-α activates JAK2/STAT3 and modulates insulin and leptin signalling and action in the hypothalamus inhibiting food intake. To investigate the effect of stress on pro-inflammatory cytokine signalling in hypothalamus, ICC could be carried out for pSTAT3 nuclear expression in stressed early pregnant mice. In addition, to investigate the role of cytokines in prolactin inhibition a range of cytokine receptor antagonists or blockers could be injected centrally prior to stress exposure and then TIDA/dopamine activity and prolactin secretion measured. This would allow us to test for cross-talk between cytokines/receptors and help ascertain the mechanism behind cytokine/stress-reduced prolactin in early pregnancy.

Chapter 5

Prolactin and anxiety in early pregnancy

Chapter 5: Prolactin and anxiety in early pregnancy

5.1 Introduction

5.1.1 Stress and anxiety during pregnancy

Anxiety is thought to be a psychophysiological sign that the stress response has been initiated. The locus coeruleus (LC) is robustly activated following stressful stimuli and thus is regarded as an important part of the central 'stress circuitry'. Mounting evidence also suggests a relationship between the central noradrenergic system and fear/anxiety states and depression (Itoi and Sugimoto, 2010). Around 30% of women experience some type of anxiety disorder during their lifetime and symptoms may change in these women during pregnancy and the postpartum period (Levine et al., 2003).

Maternal anxiety has adverse effects on pregnancy (Dayan et al., 2002, Orr et al., 2007) and women who experience more stress and anxiety during pregnancy have significantly higher rates of adverse birth outcomes e.g. low birth weight. Multiple studies report a relationship between increased risk of preterm birth and 'pregnancy-related' or 'state' (response to environmental stressors) anxiety (Lobel et al., 1992, Rini et al., 1999, Dole et al., 2003). Pregnancy-specific anxiety refers to maternal worries or concerns related to pregnancy such as labour, delivery and health of the offspring (Rini et al., 1999). An early study found that anxiety in the first trimester (as well as the second and third) is associated with abnormalities during pregnancy and parturition e.g. threatened abortion, preterm labour (Gorsuch and Key, 1974). Anxiety and depression in the first trimester are also linked with the subsequent risk for preeclampsia (Kurki et al., 2000). In addition, maternal anxiety or depression are related to functioning and development of the offspring. For example, women who were anxious during pregnancy have infants with an elevated risk of having non-optimal neuromotor development (van Batenburg-Eddes et al., 2009).

5.1.2 Reproductive hormones and anxiety

Both pregnancy and lactation are accompanied by behavioural changes, including an altered responsiveness to stress. For example, during late pregnancy (d21) rats display increased anxiety-like behaviour on the elevated plus maze (EPM), compared

to virgin females (Neumann et al., 1998). These alterations have been associated with the changes in reproductive hormones at different reproductive states (Young and Cook, 2004). Systemic administration of progesterone to ovariectomised rats leads to reduced anxiety-like responses, with an increase in open-arm exploration on the EPM. The addition of oestradiol attenuates this effect. It has therefore been suggested that anxiety behaviour varies across the oestrous cycle due to the modulatory effects of ovarian hormones (Mora et al., 1996). In males rats, progesterone administration i.p rapidly produces significant anxiolytic behavioural effects 30 min after administration (Gomez et al., 2002).

The effect of oestradiol on anxiety is unclear; some studies report an anxiolytic effect of oestradiol administration in rats (Marcondes et al., 2001, Bowman et al., 2002), but others report an anxiogenic effect of oestradiol treatment in rats and mice (Mora et al., 1996, Morgan and Pfaff, 2001).Marcondes et al. (2001) report that anxiety levels of female rats were lower in proestrous than during di-estrous, and conclude that the levels of oestradiol modulate this response. Another study also demonstrated changes in anxiety across the reproductive cycle in rats, reporting low levels of anxiety during late proestrus when progesterone levels are elevated and high levels of anxiety at stages when progesterone levels are low (oestrous, metoestrous, dioestrous and d21 of gestation) (Zuluaga et al., 2005). Although these studies conclude that oestradiol or progesterone, respectively, lead to reduced anxiety-like behaviour during proestrous, prolactin levels are also greater on the afternoon of proestrous (Freeman et al., 2000) and may contribute to reduced anxiety. In support of this, i.c.v administration of prolactin to rats decreases anxiety-like behaviours on the EPM in a dose dependent manner. In contrast, down-regulation of brain prolactin receptors (long-form), using an antisense oligodeoxynucleotide infusion, increases anxiety-like behaviour (Torner et al., 2001). This demonstrates an anxiolytic effect of prolactin acting at the brain level. Furthermore, infusion of ovine prolactin to female rats attenuated the stress-induced increase in CRH. Accordingly, the CRH response was further elevated by antisense targeting of the prolactin receptors. This suggests that prolactin reduces responsiveness of the HPA axis via prolactin receptors in the brain, in addition to acting as an endogenous anxiolytic (Torner et al., 2001, Torner,

2002). In humans, a correlation between anxiety levels and serum prolactin has also been reported (Jeffcoate et al., 1986).

5.1.3 Behavioural assays for anxiety

Measuring anxiety-like behaviour in rodents has mostly been undertaken using animal models of anxiety, such as the EPM and the open field test (OFT). These tests are both based on exposure to aversive unfamiliar places and exploit the natural avoidance behaviours of rodents to exposed and unprotected areas (Belzung and Griebel, 2001). Thus, these tests are based on an anxiogenic agent e.g. unprotected arm on the EPM or open area in OFT, and the anxiety level is determined from the number of entries to unprotected areas and the time spent in these regions (Carola et al., 2002). However, the conclusions drawn from these two tests are often contradictory. For example, one study comparing C57BL/6 and BALB/6 strains report that C57BL/6 mice are not very anxious and highly active, while BALB/6 mice are highly anxious and not very active in the OFT (Crawley et al., 1997). In contrast, another report states that these two strains have similar anxiety and locomotor activity profiles when using a number of behaviour tests such as the EPM and OFT (Rogers et al., 1999). Discrepancies between studies from different groups may reflect differences in behavioural analysis criteria. In addition, Rogers et al. (1999) found that data obtained from different anxiety tests were discordant. Strain differences on the EPM did not correlate with the OFT, implying that they do not measure the same behavioural traits.

Behavioural responses are typically recorded for 5 min as early studies revealed that rats demonstrated the most robust avoidance behaviours in this period (Montgomery, 1955). Therefore, to test for anxiety behaviour following stress in early pregnancy, two commonly used behavioural tests were carried out for 5 min: the EPM and the OFT. As prolactin and progesterone are both reported to be anxiolytic stressed-reduced levels of these hormones in early pregnant mice may impact on anxiety-like behaviour.

5.1.4 Aim and hypotheses

Aim:

1. To determine the effect of stress (LPS or 24h fasting) on anxiety behaviours in early pregnant mice.

Hypotheses:

1. LPS stress will increase anxiety-like behaviour in early pregnancy.
2. 24h fast will increase anxiety-like behaviour in early pregnancy.

5.2 Methods

5.2.1 Effect of LPS stress on anxiety behaviour and plasma hormones

5.2.1.1 Treatment

Virgin and d5.5 pregnant mice were gently restrained in a holding bag and injected with lipopolysaccharide (LPS; 12.5µg/100µl saline, i.p; *E.coli* E055:B5, Sigma, Poole, Dorset, UK; n = 6) (Hollis et al., 2005) or vehicle (saline; 100µl, i.p; n = 8) around 10am (3h after lights on). Mice were then returned to their home cages before carrying out the EPM. A second cohort of virgin and d5.5 pregnant mice were treated in the same way (LPS, n = 8, 11 respectively; vehicle, n = 7, 11 respectively) to provide another set of mice to carry out the OFT.

5.2.1.2 Behavioural tests and blood collection

A. Elevated plus maze

Following 24h mice were removed from their home cage and immediately moved to an adjacent room where the EPM was set up (see General Methods section 2.13.1 and Figure 2.21). The EPM is comprised of four arms (2 open, 2 closed) coming out from a central area (5x5cm square) on a 1m high cross-stand. The closed arms were enclosed by walls (15cm high x 30cm long x 5cm wide), while the open arms were completely open (30 cm long x 5cm wide (Carola et al., 2002)). The mice were then placed into the centre area (neutral area) of EPM and allowed to roam freely for 5 min whilst being video recorded (experimenter left the behavioural room for 5 min). Immediately following the EPM test mice were moved to an adjacent room and killed by conscious decapitation (<1 min after removal from maze). Trunk blood was collected, processed for plasma and frozen (-20 ^{o}C) prior to use in assays. Following blood collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

The behavioural video was analysed manually for typical anxiety behaviour measures (Carola et al., 2002): the latency to first open arm entry, number of open arm entries, total number of entries (crude measure of overall locomotor activity) and percentage time spent open arms. In addition, investigation of the open arms was measured by counting the number of stretch attends performed (stretching out from enclosed arms into open arms) (Kimura et al., 2009).

Ashley Boyle (3rd year Physiology undergraduate summer student, The University of Edinburgh) assisted in carrying out this experiment and in video analysis.

B. Open field test

Mice were removed from their home cage, 24h after treatment, to an adjacent room containing the OFT (see General Methods section 2.13.2 and Figure 2.22). The OFT was carried out on an arena: board divided into 25 squares (5 x 5 squares; 10cm^2) with a 15cm high wall (Kimura et al., 2009). The central 9 squares are considered to be the inner zone (more anxiogenic) and the outer 16 squares the outer zones (less anxiogenic). The mouse was placed onto the central square (in the inner zone) and allowed to move freely over the arena whilst being video recorded for 5 min (experimenter left the behavioural room for 5 min). Immediately following the test mice were moved to an adjacent room and killed by conscious decapitation (<1 min after removal from maze). Trunk blood was collected, processed for plasma and frozen (-20 ^{o}C) prior to use in assays. Following blood collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

The behavioural video was analysed manually for percentage of time spent in the inner zone, number of inner zone entries, the number of crossings over the grid lines (to give indication of locomoter activity) and the number of times the mouse reared up/explored the outer wall (indication of activity and exploratory behaviour). Ciorstaidh McGlone (Neuroscience Masters student, The University of Edinburgh) assisted in carrying out this experiment and in video analysis.

5.2.1.3 Prolactin ELISA

Plasma samples were diluted 1 in 25 and assayed in duplicate using an adapted protocol (see General Methods section 2.7.2.2) for the mouse prolactin DuoSet ELISA kit (DY1445, R&D Systems, Inc., Minneapolis, US).

5.2.1.4 Corticosterone RIA

Plasma samples were diluted 1 in 200 and assayed in duplicate using a cortocosterone RIA kit (Corticosterone ImmunChem Double Antibody RIA kit; MP Biomedicals) (see section 2.6.3.2).

5.2.2 Effect of 24h fast on anxiety behaviour and plasma hormones

5.2.2.1 Treatment

Virgin and d5.5 pregnant mice were fasted for 24h (n = 6, 9 respectively), with food removed from their home cage at 10am (lights on at 7am), controls were allowed to feed normally (n = 7, 8 respectively) to provide mice for the EPM test. A second cohort of virgin and d5.5 pregnant mice were treated in the same way (24h fast, n =6, 9 respectively; vehicle, n = 6, 10 respectively) to provide another set of mice to carry out the OFT.

Kevin Hung (Physiology Honours student, The University of Edinburgh) assisted in carrying out these experiments and in video analysis.

5.2.2.2 Behavioural tests and blood collection

A. Elevated plus maze

Following 24h mice were removed from their home cage and immediately moved to an adjacent room where the EPM was set up. As with the LPS treatment experiment mice were recorded on the maze for 5 min and were killed by conscious decapitation immediately following the test. Trunk blood was collected, processed for plasma and frozen (-20 ^{o}C) prior to use in assays. Following blood collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites. The behavioural video was analysed manually for the same anxiety behaviour measures as before (see section 5.2.1.2 A).

B. Open field test

Mice were removed from home cage following 24h to an adjacent room containing the OFT and were recorded on the maze for 5 min and were killed by conscious decapitation immediately following the test. Trunk blood was collected, processed for plasma and frozen (-20 ^{o}C) prior to use in assays. Following blood collection pregnancy status was checked by laparotomy and inspection of the uterine horns for evidence of implantation sites.

The behavioural video was analysed manually for the same anxiety behaviour measures as before (see section 5.2.1.2 B).

5.2.2.3 Plasma hormone levels

Plasma samples were measured for prolactin and corticosterone using the same dilutions as described previously (section 5.2.1).

5.2.3 Statistics

Statistical evaluation of the mean hormone concentrations (prolactin and corticosterone) and behavioural parameters for each group were determined by 2-way ANOVAs with a Holm-Sidak post-hoc test. Individual Student t-tests were carried out to compare treatments in virgin groups only for latency to open arm entry, and also to compare plasma prolactin levels following the behavioural tests with previously measured control prolactin levels where no behavioural tests were performed. $p<0.05$ was considered significant. Data are presented as group means±SEM (excluding outliers: mean±3*SEM).

5.3 Results

5.3.1 Effect of LPS stress on EPM performance and plasma hormones

5.3.1.1 Effect of LPS stress on EPM performance

Latency to first open arm entry:

Latency to first open arm entry showed no significant difference with treatment or group using a 2-way ANOVA (ns; for virgins: vehicle 7.8±7.1 sec, LPS 148.5±40.6 sec; for pregnant: 109.8±73.3 sec, LPS 119.6±62.9). The latency was increased after LPS treatment in virgin mice only (Students t-test, $p<0.001$).

Number of entries into open arms and total entries:

In vehicle treated mice, early pregnant mice showed fewer entries into the open arms (calculated as a % of the total entries to open and closed arms) than virgin mice (2-way ANOVA, interaction group x treatment $p=0.001$; Holm-Sidak post hoc, $p<0.05$ vs. virgin vehicle; Figure 5.1 A). LPS treatment decreased the number of open arm entries in virgin mice (Holm-Sidak post hoc, $p<0.05$ vs. vehicle) but had had no significant effect in pregnant mice.

The total number of entries (combine open and closed arm entries) was also lower in early pregnant mice than in virgins in the vehicle groups (2-way ANOVA, $p<0.01$ across group, $p<0.05$ across treatment; Holm-Sidak post hoc, $p<0.05$ vs. virgin vehicle; Figure 5.1 B). LPS treatment reduced the total number of entries in the virgin group only (Holm-Sidak post hoc, $p<0.05$ vs. vehicle).

Percentage time in open/closed arms:

The percentage time spent in the open arms was lower in early pregnant mice than in virgin mice in the vehicle group (2-way ANOVA, interaction group x treatment $p=0.001$; Holm Sidak post hoc, $p<0.05$ vs. virgin vehicle; Figure 5.1 C). LPS treatment decreased the % time in the open arms in virgin mice (Holm-Sidak post hoc, $p<0.05$ vs. vehicle) but had no significant effect in pregnant mice.

Early pregnant mice spent more time in the closed arms than virgin mice in the vehicle groups (2-way ANOVA, interaction group x treatment $p<0.05$; Holm Sidak post hoc, $p<0.05$ vs. virgin vehicle; Figure 5.1 D) and LPS treatment increased the time spent in the closed arms in virgin mice only (Holm-Sidak post hoc, $p<0.05$).

Figure 5.1

Number of stretch attends:

Early pregnant mice displayed fewer stretch attends than virgin mice in both the vehicle and LPS groups (2-way ANOVA, $p<0.001$ across group, $p=0.001$ across treatment; Holm Sidak post hoc, $p<0.05$ vs. virgin vehicle, $p<0.05$ vs. virgin LPS; Figure 5.2). After LPS treatment, there were fewer stretch attends in both groups, but this was only significant in the virgin group (Holm-Sidak post hoc, $p<0.05$).

5.3.1.2 Effect of LPS stress on plasma prolactin and corticosterone (following EPM)

Plasma prolactin levels were lower in early pregnant mice than in virgin mice (vehicle-treated) following the EPM (2-way ANOVA, $p< 0.01$ across group, $p<0.001$ across treatment; Holm-Sidak $p<0.05$ vs. virgin vehicle; Figure 5.3 A). The prolactin levels of vehicle-treated virgin mice (24h) following the EPM were elevated compared to vehicle-treated virgin mice (90 min) that were not tested on the EPM (see chapter 3, Figure 3.3; Students t-test, $p<0.01$), suggesting the test itself is stimulating prolactin secretion in these mice. In contrast, the prolactin levels of vehicle-treated early pregnant mice (24h) following the EPM were reduced compared to vehicle-treated early pregnant mice (90 min) that were not tested on the EPM (Students t-test, $p<0.01$), suggesting the test itself is inhibiting prolactin secretion. LPS treatment reduced prolactin levels in virgin mice only (Holm-Sidak $p<0.05$ vs. vehicle) to a similar level as LPS-treated early pregnant mice. LPS treatment did not appear to significantly affect plasma prolactin levels in early pregnant mice but this may be a ceiling effect due to the already suppressed prolactin levels following the EPM.

Plasma corticosterone levels were lower in early pregnant mice than in virgin mice (vehicle-treated) following the EPM (2-way ANOVA, $p<0.05$ across group; Holm-Sidak $p<0.05$ vs. virgin vehicle; Figure 5.3 B). LPS treatment did not significantly increase corticosterone levels in either group.

Figure 5.2

Figure 5.3

5.3.2 Effect of LPS stress on OFT performance and plasma hormones

5.3.2.1 Effect of LPS stress on OFT performance

The percentage time spent in the inner zone was similar in both virgin and early pregnant mice and was not significantly altered by LPS treatment (2-way ANOVA, ns; Figure 5.4 A). The number of entries to the inner zone was similar in virgin and early pregnant mice and was not significantly altered by LPS treatment (2-way ANOVA, ns; Figure 5.4 B).

The number of inner zone crossings over the gridlines from one inner zone 'box' to another inner zone 'box' (expressed as a percentage of the total crossings) was not significantly different between virgin and early pregnant mice. LPS treatment reduced the number of inner zone crossings in both virgin and pregnant mice (2-way ANOVA, $p<0.01$ across treatment; Holm-Sidak post hoc, $p<0.05$ vs. vehicle in both virgin and pregnant mice; Figure 5.4 C). The total number of crossings over the arena was similar in both virgin and early pregnant mice and was not significantly altered by LPS treatment (2-way ANOVA, ns; Figure 5.4 D).

Wall rears:

The total number of wall rears was lower in early pregnant mice than in virgin mice and was not significantly altered by LPS treatment (2-way ANOVA, $p<0.001$ across group; Holm-Sidak post hoc, $p<0.05$ vs. virgin; Figure 5.5).

5.3.2.2 Effect of LPS stress on plasma prolactin and corticosterone (following OFT)

Plasma prolactin levels were lower in early pregnant mice than in virgin mice following the OFT and was not significantly altered by treatment (2-way ANOVA, $p<0.001$ across group; Figure 5.6 A). As with the EPM prolactin levels of vehicle-treated early pregnant mice (24h) following the OFT were reduced compared to vehicle-treated early pregnant mice (90 min) that were not tested on the OFT (Students t-test, $p<0.01$), suggesting the OFT is altering prolactin secretion, although in virgins the corresponding vehicle-treated mice (90 min) are not significantly different. Plasma corticosterone levels were similar in both virgin and pregnant vehicle-treated mice. LPS treatment significantly increased plasma corticosterone levels in both groups but more so in virgin mice (2-way ANOVA, $p<0.05$ across group, $p<0.001$ across treatment, interaction treatmentxgroup $p=0.05$; Figure 5.6 B).

Figure 5.4

Figure 5.5

Figure 5.6

5.3.3 Effect of 24h fast on EPM performance and plasma hormones

5.3.3.1 Effect of 24h fast on EPM performance

Latency to first open arm entry:

The latency to first open arm entry showed no significant differences between virgin and pregnant groups or between fasting and control groups (2-way ANOVA, ns; for virgins: control 17.0±10.0 sec, fast 3.5±1.9 sec; for pregnant: control 24.8±12.3 sec, fast 10.4±9.1 sec).

Number of entries into open arms and total entries:

The number of entries into the open arms (calculated as a % of the total entries to open and closed arms) was similar in virgin and early pregnant mice in the vehicle group. 24h fast increased the number of open arm entries in both virgin and pregnant but more so in virgin mice (2-way ANOVA, $p<0.01$ across group, $p<0.001$ across treatment, interaction treatment x group $p=0.053$; Holm-Sidak post hoc, $p<0.05$ vs. virgin 24h fast; Figure 5.7 A).

The total number of entries was similar in virgin and pregnant control groups but was significantly higher in virgin vs. pregnant fasted groups (2-way ANOVA, $p<0.05$ across group; Holm-Sidak post hoc, $p<0.05$ vs. virgin fast; Figure 5.7 B). 24h fast stress did not significantly affect the number of entries in either group.

Percentage time in open/closed arms:

The percentage time spent in the open arms was similar in virgin and early pregnant mice in the vehicle group. 24h fasting increased the time spent in the open arms (2-way ANOVA, $p<0.05$ across treatment; Figure 5.7 C).

The percentage time spent in the closed arms was similar in virgin and early pregnant mice in the vehicle group. 24h fasting decreased the time spent in the closed arms in both virgin and early pregnant mice (2-way ANOVA, $p<0.001$ across treatment; Holm-Sidak post hoc, $p<0.05$ vs. vehicle; Figure 5.7 D).

Number of stretch attends:

Early pregnant mice showed more stretch attends than virgins in control groups (2-way ANOVA, interaction group x treatment $p<0.001$; Holm Sidak post hoc, $p<0.05$

Figure 5.7

vs. virgin vehicle; Figure 5.8). 24h fasting increased the number of stretch attends in virgins, but decreased it in pregnant mice (Holm-Sidak post hoc, $p<0.05$ vs. control).

5.3.3.2 Effect of 24h fast on plasma prolactin and corticosterone (following EPM)

Plasma prolactin levels were similar in virgin and early pregnant mice (vehicle-treated) following the EPM. The prolactin levels of control virgin mice following the EPM were elevated compared to control virgin mice that were not tested on the EPM (see chapter 3, Figure 3.17; Students t-test, $p<0.01$), again suggesting the EPM test itself is stimulating prolactin secretion in these mice. 24h fasting decreased prolactin in early pregnancy only (2-way ANOVA, interaction group x treatment; Holm-Sidak $p<0.05$ vs. pregnant control, $p<0.05$ vs. virgin fast; Figure 5.9 A).

Plasma corticosterone levels were similar in virgin and d5.5 pregnant control mice following the EPM. 24h fasting significantly increased corticosterone levels in both groups equally (2-way ANOVA, $p<0.001$ across treatment; Figure 5.9 B).

5.3.4 Effect of 24h fast on OFT performance and plasma hormones

5.3.4.1 Effect of 24h fast on OFT performance

The percentage time spent in the inner zone was similar in both virgin and early pregnant mice and was not significantly altered by fasting (2-way ANOVA, ns; Figure 5.10 A). The number of entries to the inner zone was similar in both control virgin and early pregnant mice. 24h fasting decreased the number of inner zone entries in early pregnant mice (2-way ANOVA, $p<0.05$ across treatment; Figure 5.10 B).

The number of inner zone crossings (expressed as a percentage of the total crossings) was not significantly different between virgin and early pregnant control mice. However, 24h fasting increased the number of crossings in the inner zone in early pregnant mice (2-way ANOVA, $p<0.01$ across group; Holm-Sidak post hoc, $p<0.05$ vs. virgin fast group; Figure 5.10 C). The total number of crossings over the arena was similar in both virgin and early pregnant mice and was not significantly altered by 24h fasting (2-way ANOVA, ns; Figure 5.10 D).

Figure 5.8

Figure 5.9

Figure 5.10

Number of wall rears:

The total number of wall rears was significantly higher in early pregnant vs. virgin control mice (2-way ANOVA, interaction group x treatment $p<0.05$; Holm-sidak post hoc, $p<0.05$ vs. virgin control; Figure 5.11). 24h fasting significantly reduced the number of rears against the wall in early pregnant mice to a similar to virgin mice (Holm-Sidak post hoc, $p<0.05$ vs. virgin fast).

5.3.4.2 Effect of 24h fast on plasma prolactin and corticosterone (following OFT)

Plasma prolactin levels were similar in both virgin and d5.5 pregnant mice in control groups following the OFT. Fasting for 24h caused a significant reduction in plasma prolactin in the early pregnant group following the OFT (2-way ANOVA, $p< 0.01$ across treatment; Holm-Sidak post hoc, $p<0.05$ vs. control in pregnant group only; Figure 5.12 A).

Plasma corticosterone levels were significantly higher in virgin than early pregnant control mice. 24h fasting significantly increased corticosterone levels following the OFT in both groups to a similar level (2-way ANOVA, $p<0.05$ across group, $p<0.001$ across treatment; Holm-Sidak, $p<0.05$ vs. virgin control; Figure 5.12 B).

Figure 5.11

Figure 5.12

5.4 Discussion

5.4.1 Effect of LPS on EPM and hormone response

A number of immune stressors, including LPS, have previously been shown to activate the HPA axis and induce anxiety-like behaviours in rodents (Lacosta et al., 1999). IL-1β and LPS injection (i.p) to male mice has been demonstrated to decrease open-arm entries and time spent in the open arms of an EPM, in a dose-dependent manner. In addition, the total number of entries to all arms were decreased indicating a reduction in locomotion and general activity (Swiergiel and Dunn, 2007). Central administration of IL-1β and TNF-α to male rats also revealed anxiogenic effects on the EPM (Connor et al., 1998). Thus, we hypothesised that LPS administration would increase anxiety in virgin and early pregnant mice. Furthermore, we postulated that the anxiogenic response would be heightened in early pregnancy following LPS stress due to a reduction of anxiolytic prolactin.

LPS administration had profound anxiogenic effects on the virgin mice, with the latency to enter the open arms increasing with LPS, while the time spent in the open arms and number of entries to the open arms significantly decreased. In addition, the total number of arm entries (open and closed) and stretch attends performed were reduced by LPS, indicating a reduction in locomotion and risk assessment behaviour respectively.

Control early pregnant mice displayed higher anxiety-like behaviours compared to virgin mice, as they entered the open arm less often and spent less time in open arms than virgin control mice. LPS treatment in early pregnant mice appeared to have no effect on the anxiety-behavioural parameters measured compared to controls. However, as anxiety levels were already high in early pregnancy (compared to virgins) this may be a ‘ceiling effect’ which masks any LPS-induced changes. Early pregnant mice behaved similarly to stressed virgin mice regardless of LPS treatment, indicating that early pregnant mice are more anxious in general than virgin mice. This is different to what is reported in mid pregnant (d11) mice which showed reduced anxiety on the light/dark choice test (Maestripieri and D'Amato, 1991) and in late pregnant (d18) rats (Neumann et al., 1998, Neumann, 2003) where they display reduced fear and anxiety on the EPM (and HPA responses) compared to non-pregnant animals (Neumann et al., 1998, Slattery and Neumann, 2008).

Plasma prolactin levels were elevated in vehicle-treated (24 h) virgin mice following the EPM compared to previously analysed vehicle-treated (90 min) virgin mice that were not tested on the EPM prior to blood sampling. This indicates that the EPM itself may be stimulating prolactin secretion, but LPS-treatment/stress prevents this. In contrast, prolactin levels of vehicle-treated early pregnant mice were suppressed following the EPM when compared to previously analysed vehicle-treated (90 min) early pregnant mice that were not tested on the EPM. This might indicate that the early pregnant mice were more stressed by the EPM and hence the prolactin levels decreased in vehicle-treated mice as well as LPS-treated mice. However, corticosterone responses do not appear to support this. Another possibility is that there could be some kind of delayed hyporesponsiveness in early pregnant mice to the injection 24h before.

Prolactin levels in early pregnant mice did not differ with treatment which may contribute towards the lack of change observed in anxiety-like behaviours that were expected. However, in LPS-treated virgins there were increased anxiety behaviours following stress accompanied by decreased prolactin levels.

Plasma corticosterone levels following the EPM were not significantly affected by LPS treatment administered 24h prior to the test. Therefore, LPS-induced corticosterone (as observed 90 min after injection; see Figure 3.3) is not sustained and after 24 h is only moderately affected. Circulating prolactin concentrations were not altered by LPS in early pregnant mice, but they too were lower than in virgin mice.

It is difficult to conclude the effect of the LPS vs. the EPM itself on corticosterone and prolactin within this experiment as there were not any ‘no EPM’ controls included. A comparison using data for mice sacrificed 90 min after treatment provided an insight into the effect of the EPM itself on prolactin. However, it would have been useful to include virgin and pregnant mice that were treated with saline or LPS and were then killed 24h later to provide plasma from this time point for assays without completing the EPM.

The measurement of latency to first open arm entry does not appear to be a good indicator of anxiety as in this experiment the data was very variable, mainly due to the problem of mice entering whichever arm they happened to be looking at when

placed in the centre square. The aim had been to place the animal at an angle/position that they were neither looking directly at the open nor closed arm (diagonally across the centre square) but this proved problematic due to the mice moving around, coupled with the handler wanting to restrain them as little as possible. Thus, it would have been better to place all animals pointing specifically at the closed arm as described in other studies (Connor et al., 1998, Swiergiel and Dunn, 2007).
High levels of oestrogens (as associated with oestrous) are known to have anxiolytic effects and therefore virgin mice in oestrous should have been discarded. However, virgin mice were not smear tested and therefore the day of the cycle was unknown for each mouse.

5.4.2 Effect of LPS on OFT and hormone response

It has been demonstrated that IL-1β or LPS injections (i.p) to male mice decrease the number of crossing in the centre of the arena (inner zone) of an OFT (Swiergiel and Dunn, 2007). However, Swiergiel and Dunn (2007) also reported a decrease in the number of crossings and rears in the outer zone, indicating a reduction in general activity and thus do not provide unequivocal support for IL-1β or LPS-induced anxiety (Swiergiel and Dunn, 2007). In our study we found that there was no significant difference in the percentage time spent in the inner zone or number of entries to the inner zone with either treatment or group (virgin vs. pregnant mice). The mice spent around 80% of their time in the outer zone, demonstrating thigmotaxis (tendency to remain close to the outer walls). There was, however, a significant decrease in the number of crossing made in the inner zone with LPS treatment in both virgins and pregnant mice. As there was no significant change in the total number of crossings (indicating there was no alteration in the general activity of any of the mice), the reduction in inner zone exploration (crossings) indicates an increase in anxiety-like behaviour with LPS treatment. The number of rears against the wall ('vertical exploration') was not affected by LPS treatment although pregnant mice reared less than virgin mice. Therefore, it appears that LPS treatment mildly induces anxiety-like behaviour, decreasing exploration of the inner zone but this effect was not more pronounced in early pregnant mice as was hypothesised.

As described earlier with the EPM, prolactin levels following the OFT in early pregnant mice were suppressed (compared to 90 min data) and did not differ with treatment. This may again be a 'ceiling effect' of the already robustly suppressed prolactin levels. Therefore, we cannot conclude that LPS is definitely not having an effect on prolactin secretion. As a difference in plasma prolactin levels with treatment was not apparent following the OFT this may partly explain why only very mild changes in anxiety-like behaviours were observed. In virgin mice there was also no change in prolactin levels with LPS treatment but levels were higher in virgins than early pregnant mice. There does not appear to be an association with decreased prolactin levels and increased anxiety behaviours following stress as early pregnant mice (who have lower prolactin than virgins) have very similar anxiety behaviour profiles to virgin mice with the exception of performing less outer zone rears. This is in contrast to the EPM where it was found that LPS had a more profound effect on virgin mice, increasing anxiety behaviours. This is curious as it has been shown in a relations study of EPM and OFT in C57BL/6 mice that movements and rears in the OFT were linearly correlated with enclosed arm visits on the EPM (Lalonde and Strazielle, 2008).

The plasma corticosterone levels following the OFT were increased by LPS treatment in both virgin and early pregnant mice. This differs to the corticosterone levels following the EPM where a difference with treatment was not observed. However, the levels are all in a similar range to the previous experiment, although differences with LPS were mild and not significant following the EPM. It may be that the anxiety tests themselves are eliciting varying degrees of stress responses.

5.4.3 Effect of 24h fast on EPM and hormone response

It was hypothesised that 24h fasting would lead to increased anxiety-like behaviour in early pregnant mice, possibly via predicated increased corticosterone (and CRH) and decreased prolactin secretion. In addition, the levels of circulating ghrelin are reported to increase with fasting in humans and rodents (Cummings et al., 2001, Briatore et al., 2006) and ghrelin is considered to be anxiogenic (Carlini et al., 2002). Ghrelin is a peptide found in the hypothalamus and the stomach and is known to stimulate release of growth hormone from the pituitary (Kojima et al., 1999). Plasma ghrelin levels are acutely regulated by food intake, increasing with fasting and

decreasing following food consumption (Cummings et al., 2001). In rodents it has been shown that ghrelin plays a role in hypothalamic regulation of homeostasis. The administration of ghrelin (i.c.v) to rats robustly stimulates feeding, increasing food intake and body weight (Asakawa et al., 2001a, Nakazato et al., 2001). Studies looking into the behavioural effects of ghrelin have reported that central administration of ghrelin to male rats increased anxiety-like behaviours on the EPM (Carlini et al., 2002). Similarly, effects on anxiety-like behaviour in mice have been described after both i.p or i.c.v ghrelin administration (Asakawa et al., 2001b). It has been reported that the underlying mechanism behind the anxiogenic effects of ghrelin involves CRH. Administration of a CRH receptor antagonist significantly reduced the ghrelin-induced anxiogenic effects on an EPM. In addition, administration of ghrelin to male mice increased CRH mRNA expression in the hypothalamus (Asakawa et al., 2001b). CRH receptor type 1 is reported to mediate emotional stress-induced disruption of food intake and behavioural changes in rats (Hotta et al., 1999). Tail pinch stress increases expression of ghrelin in the stomach (Asakawa et al., 2001b) as does starvation stress (48h fasting) (Asakawa et al., 2001a).
Despite previous reports indicating that fasting may lead to an increase in anxiety-like behaviour (possibly via increased CRH or ghrelin) we did not demonstrate this. Both virgin and early pregnant fasted mice spent significantly more time on the open arms of the EPM and entered them more often than control animals, implying that they are less anxious. However, as these animals were fasted it is more likely that this demonstrates increased exploration activity in the search to find food and is motivated by hunger. Fasted early pregnant mice entered the open arms significantly less often than fasted virgin mice and also displayed a reduction in total entries (locomotor activity) indicating fasted pregnant mice are less active than fasted virgin mice, which could account for the reduced number of entries to the open arm. The latency to enter the open arms tended to be shorter in fasted mice although this was not significant and data was highly variable. Control pregnant mice displayed heightened risk assessment behaviour with more stretch attends performed compared to virgin mice, perhaps indicating they are more cautious. However, fasted pregnant mice performed less stress attends compared to both control pregnant mice and fasted virgin mice. The reasons behind these changes is not clear and it could be the

case that they are ‘false-positives’ as they do not seem to correlate with the other parameters measured.

Overall fasting appears to reduce anxiety-like/increase exploratory behaviour in spite of the fact 24h fasting robustly increases corticosterone levels in both virgin and pregnant mice. As with the LPS study, the EPM itself appears to stimulate prolactin release in virgin mice, although this is independent of fasting treatment. Control early pregnant prolactin levels were similar to both the virgin groups and as expected 24h fasting suppressed plasma prolactin levels in early pregnant mice. However, plasma prolactin concentrations do not seem to have an association with anxiety behaviours as predicted. Although prolactin levels decrease with fasting in early pregnant mice this was not reflected in EPM performance.

It would be interesting to measure the circulating ghrelin levels of our 24h fasted mice to establish if these levels have risen as is assumed. If not this could be part of the reason why we do not see increased anxiety with 24h fasting. However, our study was not the only one to find this as another fasting study (15h fast) on mice reported no significant effect of fasting on anxiety measures (Hata et al., 2001).

5.4.4 Effect of 24h fast on OFT and hormone response

As with the EPM, it was hypothesised that 24h fasting would lead to increased anxiety-like behaviours in early pregnant mice on the OFT. As already mentioned it has been reported that fasting increases ghrelin expression (Asakawa et al., 2001a) and central administration of ghrelin increases anxiety as measured by an EPM (Carlini et al., 2002). However, Carlini et al. also reported that central administration of ghrelin to male rats had no effect on any OFT parameter measured (e.g. time in the inner zone, locomotion, rearing) except for causing an increase in freezing behaviour (total absence of body or head movement) at high doses (3nmol/μl) (Carlini et al., 2002).

We have found that 24h fasting had no significant effect on the time spent in the inner zone in either virgin or early pregnant mice. The number of inner zone entries and crossings were also not altered by fasting in virgins. Although the number of entries into the inner zone significantly decreased in fasted pregnant mice, the number of inner crossings increased and overall there was not a change in locomotion/exploring activity (total crossings) in either virgin or early pregnant

mice. Consistent with the idea that rears against the wall represent 'vertical exploration' the number of rears carried out by virgin mice was not altered with fasting either. Although, rearing behaviour was higher in the pregnant vs. virgin control group and was decreased by fasting in early pregnant mice, behaviour on the OFT overall did not indicate an increase in anxiety behaviour in either virgins or pregnant mice. In addition, OFT behaviour following 24h fasting did not indicate an increase in exploratory behaviour (as it did in the EPM). A study looking into the effect of chronic food deprivation (maintenance at 80% original body weight) also did not observe an increase in anxiety-like behaviour but they did report increased activity in an OFT in food deprived rats (after 8 days on food restricted diet) along with increased circulating corticosterone (Heiderstadt et al., 2000).

Prolactin levels were unchanged by fasting in virgin mice following the OFT, but were decreased by fasting in early pregnant mice as expected. Once again circulating prolactin levels did not reflect anxiety-related behaviour levels. Our data suggest that fasting does not alter anxiety-like behaviour as measured by the OFT despite a strong increase in corticosterone levels following 24h fasting in both virgins and early pregnant mice.

Overall, 24h fasting lead to enhanced exploratory behaviour (reduced anxiety) in early pregnant mice. In hindsight 24h fasting may not have been a suitable treatment to use in order to determine the effects of stress in early pregnancy on anxiety behaviour. The drive to explore (presumably motivated by hunger in order to find food) following fasting appears to be robust and to take over/mask stress-induced anxiety behaviours.

Chapter 6

General Discussion

Chapter 6: General Discussion

6.1 Stress effects on pregnancy hormones in early pregnancy

Stress exposure in early pregnancy in mice significantly reduces levels of the crucial pregnancy hormones which are ordinarily elevated at this time e.g. progesterone (Joachim et al., 2003). We found that stress exposure (immune and 24h fast) on pregnancy day d5.5 diminished circulating progesterone levels and we also demonstrated for the first time that basal plasma prolactin levels were robustly reduced by stress in early pregnancy as hypothesised. The effect of stress on the bi-daily prolactin peaks observed in rodents in early pregnancy is not known at present but we expect that these peaks would also be reduced by stress. An additional experiment involving stress exposure during these peaks would be needed to confirm this. As prolactin is known to facilitate progesterone secretion from the corpus luteum in early pregnancy in rodents (Erlebacher et al., 2004), the attenuated prolactin response to stress may contribute to the stress-reduced progesterone levels simultaneously observed. In addition, stress profoundly affected pregnancy maintenance, with LPS treatment leading to occult foetal loss, while 24h fast reduced pregnancy rate from 70% to 36%.

6.2 Potential mechanisms leading to stress-reduced prolactin in early pregnancy

6.2.1 TIDA activation and dopamine activity

We found that stress significantly decreases circulating prolactin in mice during early pregnancy and thus we sought to establish the mechanism(s) behind this. As dopamine derived from the TIDA neurones is the main inhibitor of prolactin release from the lactotrophs we hypothesised that a reduction in prolactin secretion would be associated with a corresponding increase in TIDA neurone activity. Indeed the stress-induced attenuation in circulating prolactin levels was accompanied by a parallel increase in TIDA neurone activation following either LPS immune stress or 24h fast in early pregnancy, as indicated by increased Fos expression in tyrosine-positive neurones in the arcuate nucleus. The rise in TIDA activation with stress in early pregnancy was significant but mild (<15% of TIDA neurones activated with either stressor). In virgin mice this stress-induced elevation of TIDA activity was not observed, thus the altered responsiveness of TIDA neurones seems to be acquired

post-copulation. It is known that these neurones have already adapted by d5.5 to mediate the neurogenic reflex and initiate the bi-daily prolactin surge profile observed in rodents, therefore, these alterations may confer the slight changes observed with stress.

Although it has been previously reported that Fos expression in TIDA neurones following an immune challenge reflects alterations in prolactin secretion (Hollis et al., 2005), Fos is not considered the most accurate indicator of TIDA neurone activation (Hoffman et al., 1994, Hoffman and Lyo, 2002). Thus, Fos expression in TIDA neurones may only serve as a weak marker of TIDA neurone activation following acute stimulation, e.g. LPS, which may explain why we see only a relatively mild increase in TIDA activation. Therefore, due to this fact, we also measured the DOPAC content and the DOPAC:dopamine ratio in the median eminence, both of which have been utilized previously to measure dopamine activity (Andrews and Grattan, 2003). The DOPAC content levels were found to be higher in virgin than early pregnant vehicle-treated mice (though not significant, likely due to the high variability), which may account for the increase in basal prolactin secretion in early pregnancy. DOPAC levels were appeared lower in LPS-treated virgins compared to vehicle-treated virgins. However, surprisingly, no change in DOPAC levels were observed with LPS treatment in early pregnant mice, despite a robust reduction in plasma prolactin. The ratio of DOPAC:dopamine revealed a similar pattern to DOPAC content alone in the median eminence. Although the basal DOPAC:dopamine ratios tended to be higher in virgins compared to pregnant mice, we found that again LPS immune stress did not significantly alter the DOPAC:dopamine ratio in early pregnant mice. Thus, DOPAC content and the DOPAC:dopamine ratios did not reflect the stress-altered prolactin levels observed in early pregnant mice, or indeed the unaltered prolactin levels displayed in virgin mice. Overall, alterations in dopamine activity in the median eminence do not appear to underlie the stress-reduced prolactin secretion observed in early pregnant mice. In addition, dopamine activity in the TIDA neurone terminals is not analogous to Fos expression in TIDA neurones in early pregnant mice. As these data imply that stressors do not alter prolactin levels via TIDA dopamine secretion, it is not likely that a reduction in the prolactin-releasing factors which act to inhibit TIDA activity

are responsible e.g. kisspeptin (Szawka et al., 2010) and the opioids (Andrews and Grattan, 2002). However, it is possible that stress-induced reduction of other prolactin-releasing factors which are reported to act directly at the lactotrophs contribute towards prolactin suppression e.g. oxytocin, TRH (Ben-Jonathan et al., 2008). Although, regarding TRH, it has recently been shown that direct application of TRH to mouse pituitary slices did not stimulate prolactin secretion from lactotrophs (unlike oxytocin) as observed in rats (He et al., 2011).

6.2.2 Prolactin negative feedback

In support of the conclusion that stress does not lead to reduced prolactin release by increased TIDA activity we also found that stress does not alter prolactin negative feedback to the TIDA neurones in early pregnancy.

As previously described, LPS administration and 24h fast in early pregnancy both robustly diminish circulating prolactin in mice. Additional treatment with oPRL was found to decrease murine plasma prolactin in non-stressed early pregnant mice as expected, demonstrating that prolactin negative feedback had been driven (DeMaria et al., 1999, Anderson et al., 2006a). However, although stress reduced plasma prolactin, oPRL supplementation did not further decrease it in early pregnancy, perhaps because levels have already been substantially reduced and further changes are minimal. Although stress alone decreased plasma prolactin levels in early pregnancy this was not the case with STAT5 signalling. In stressed early pregnant mice the percentage of pSTAT5-positive TIDA neurones was found to be low and comparable to non-stressed mice, indicating that negative feedback via the pSTAT5 signalling pathway was not altered by stress alone (at basal prolactin levels).

In contrast, it is possible that at the time of the bi-daily prolactin surges in early pregnancy enhanced prolactin negative feedback does contribute to the assumed reduction in these prolactin peaks. We demonstrated a robust increase in the expression of pSTAT5 in TIDA neurones following stress (indicating elevated prolactin signal transduction) in early pregnant mice that were co-administered oPRL. This suggests that when prolactin levels are high (e.g. during prolactin surges in early pregnancy) stress exposure enhances prolactin negative feedback to the TIDA neurones, suppressing further prolactin release and consequently reducing progesterone secretion, threatening pregnancy maintenance.

To conclude, enhanced prolactin negative feedback, via prolactin receptor-induced signalling, does not underlie stress-induced suppression of basal prolactin in early pregnancy. This further reinforces the idea that stress-reduced prolactin is not due to enhanced TIDA activity, responsiveness or sensitivity. Thus, other stress-related factors must be responsible, perhaps directly affecting prolactin secretion at the lactotrophs.

6.2.3 Stress-induced cytokines

One possible stress-related mechanism driving prolactin suppression is increased activity of cytokines associated with stress/LPS. We have found that both LPS and 24h fasting lead to an increase in circulating IL-6 and LPS treatment also robustly increases plasma TNF-α in early pregnancy. IL-6 and TNF-α are both known to be detrimental to pregnancy maintenance and also can activate the HPA axis (Chrousos, 1995, Tsigos and Chrousos, 2002). The principal action of these cytokines has been suggested to be through stimulating the release of CRH and vasopressin from the hypothalamus, however, we do not show this in early pregnancy where the CRH response to stress was attenuated. Proinflammatory cytokines can also cause direct effects at the pituitary level inducing ACTH release from the anterior pituitary *in vitro* and *in vivo* (McCann et al., 1994).

IL-6 administration to early pregnant mice was found to mildly increase corticosterone levels, indicating activation of the HPA stress axis as expected, however, it had no significant effect on either circulating prolactin or progesterone levels compared to vehicle. Therefore, it does not seem that stress-induced IL-6 release is responsible for prolactin suppression following stress in early pregnancy. Conversely, TNF-α treatment in early pregnancy significantly decreased both plasma prolactin and progesterone levels, in addition to robustly elevating corticosterone levels. While we found that both pro-inflammatory cytokines led to a mild increase in TIDA activation (increased Fos expression), only TNF-α reduced plasma progesterone and prolactin in early pregnancy, thus again supporting the notion that TIDA activity is not accountable for stress-reduced prolactin. So, as it does not strongly induce TIDA activation data demonstrate that TNF-α may be partly responsible for stress-reduced prolactin secretion through other mechanisms such as direct action at the pituitary lactotrophs.

6.2.4 Sex steroids and TNF-α

6.2.4.1 Effects of progesterone on prolactin regulation

It has been reported in rodents that TIDA neurones express steroid receptors (progesterone and oestrogen) during pregnancy. Thus, these hormones may contribute towards regulation of maternal prolactin secretion by direct actions on the TIDA neurones (Steyn et al., 2007). Progesterone is able to suppress TIDA neurone activity on the afternoon of proestrous and enhances the pre-ovulatory prolactin surge in rats. Phosphorylated tyrosine hydroxylase levels and tyrosine hydroxylase activity in the median eminence are reported to decrease concomitantly with the pre-ovulatory rise in progesterone (Liu and Arbogast, 2008). In addition, progesterone administration prior to the preovulatory progesterone surge in rats also decreases tyrosine hydroxylase phosphorylation state, while increasing activity of phosphatase 2A (potential mediator of phosphorylation of serine sites on tyrosine hydroxylase) in the median eminence. These changes were also accompanied by a premature rise in serum prolactin, indicating a role for progesterone in stimulating prolactin release on the afternoon of proestrous (Liu and Arbogast, 2008, Liu and Arbogast, 2010).

Less is reported about the effects of progesterone on TIDA neurones and prolactin secretion in early pregnancy. Whether stress rapidly affects the level of expression/action of progesterone receptors on TIDA neurones in early pregnancy is unknown at present. However, this seems unlikely as we have found that altered TIDA responsiveness is not responsible for stress-reduced prolactin in early pregnancy.

A human study looking into the regulatory role of progesterone on decidual prolactin production in early pregnancy reported that progesterone plays only a facilitatory role in prolactin regulation. Patients were treated with the anti-progestin mifepristone for 24-36h before elective pregnancy termination and decidual tissue was collected from these patients as well as from women who had not been pre-treated. Production of prolactin by decidual parietalis (part of the decidua lining the uterus) decreased over time and production from patients pre-treated with mifepristone was found to be significantly lower than controls. However, decidual prolactin production by decidua capsularis (to which trophoblast was attached) was maintained over the 5 culture days was unaffected by mifepristone pre-treatment. These findings suggest that

progesterone may facilitate in prolactin production by maintaining decidual differentiation and other trophoblast derived factors are able to maintain decidual prolactin production (Wu et al., 1990). Subsequent studies also suggest that the induction of prolactin synthesis is dependent upon progesterone-induced decidualisation (Brosens et al., 1999).

6.2.4.2 Progesterone and pro-inflammatory cytokines

Progesterone is thought to play a facilitatory role in providing a pregnancy protective immune milieu e.g. by reducing Th1 cytokine production and increasing Th2 cytokine production (Arck et al., 2007). For example, it has been shown that progesterone reduces levels of TNF-α mRNA and protein production from LPS-treated mouse macrophages *in vitro* (Miller and Hunt, 1998). However, we have shown that shown that TNF-α administration to early pregnant mice robustly reduces circulating progesterone levels and therefore a stress-induced increase in TNF-α may prevent or mask the inhibitory actions of progesterone.

6.2.4.3 Oestrogen and TNF-α action at the lactotrophs

Although receptors for progesterone and oestrogen are reported on the lactotrophs little is known about a role for progesterone directly at the lactotrophs on prolactin regulation, however more is reported on effects of oestrogen at lactotrophs (Stefaneanu, 1997). Typically oestrogen is considered to be a prolactin releasing factor. However, stress(restraint)-induced oestrogen secretion in early pregnant rats (MacNiven et al., 1992) and chronic oestrogenisation of ovariectomised rats is reported to raise TNF-α levels. In support of a proposed role for TNF-α in stress-reduced prolactin secretion, *in vitro* studies have revealed that LPS treatment increases TNF-α release from lactotrophs while also decreasing prolactin release (Theas MS, 1998). TNF-α abrogates oestrogen-induced prolactin release in both lactotroph cultures and in the pituitary cells from ovariectomised rats (Theas MS, 1998, Theas et al., 2000). Therefore, it seems that stress-induced elevated oestrogen levels counteract their stimulatory actions on prolactin release by enhancing TNF-α levels. It has been confirmed that TNF-α reduces anterior pituitary cell proliferation and prolactin release in an oestrogen-dependent manner (Candolfi, Zaldivar et al.

2002). Thus, stress-induced changes in oestrogen may impact on TNF-α activity at the anterior pituitary and subsequently affect prolactin production and release.

6.2.4.4 Oestrogen-dependent TNF-α action on lactotrophs

Both mitosis and apoptosis occur during the oestrous cycle, pregnancy and lactation in the pituitary cells (Yin and Arita, 2000, Candolfi et al., 2006). Oestrogens are widely accepted to be modulators of pituitary cell renewal and sensitise cells to mitogenic and apoptotic signals (Seilicovich, 2010, Zaldivar et al., 2011). It has been recently suggested that oestrogen sensitises anterior pituitary cells to TNF-α and LPS-induced apoptosis through suppression of pro-inflammatory NF-κB (regulator of cell survival) (Eijo et al., 2011) whose signalling is activated by LPS and TNF-α (Li and Stark, 2002). Oestrogen administration to anterior pituitary cells from female rats increases expression of both TNF-α and TNF-α receptors (TNFR1), particularly in the lactotrophs (Zaldivar et al., 2011). Furthermore, TNF-α release and TNFR1 expression is higher in the anterior pituitary glands of proestrous rats (when oestradiol levels are elevated) compared to diestrous rats (Theas, Pisera et al. 2000; Zaldivar, Magri et al. 2011). Thus, stress-induced increase in TNF-α and oestrogen in early pregnancy may directly impact on the pituitary lactotrophs to enhance apoptosis of lactotrophs and consequently decrease prolactin secretion. However, whether stress can rapidly induce lactotroph apoptosis via TNF-α in only 90min remains to be determined as previously mentioned studies measured prolactin release and active cell number from cell cultures after an 8h-2d incubation with TNF-α.

6.2.4.5 TNF-α induced activation of hypothalamic nuclei

We also found that TNF-α robustly increased activation in the SON, pPVN and VMH. Whether the pPVN response to TNF-α is similar to LPS and is attenuated in early pregnancy compared to virgins (possibly reflecting oxytocin neurone response) is currently unknown. Increased activation in the SON (as indicated by Fos expression) may imply an increase in oxytocin activity/secretion, which would not lead to suppression of prolactin release. However, there are studies which indicate that Fos expression does not always correlate with the spike activity of magnocellular neurones in the SON (Luckman et al., 1994) and enhanced Fos expression in the SON does not necessarily reflect increased peripheral oxytocin

secretion. For example, central administration of the peptide α-melanocyte-stimulating hormone or an agonist for its receptor MC4 induces Fos expression in the SON, however, they both reduce electrical activity of oxytocin neurones in the SON (*in vivo*). Accordingly, central administration of α-melanocyte-stimulating hormone reduces oxytocin secretion into the bloodstream in rats (Sabatier et al., 2003). In contrast, central administration of MC4 receptor agonists increase oxytocin release from dendrites in isolated SONs. Thus, it seems that α-melanocyte-stimulating hormone can induce different effects on systemic and dendritic oxytocin release which is dissociated from Fos expression and electrical activity (Sabatier et al., 2003). Therefore, although following stress in early pregnant mice we observed increased Fos expression in the SON, oxytocin secretion may not have risen and thus does not stimulate prolactin secretion.

6.2.4.6 Future ideas to investigate stress-induced proinflammatory cytokines and prolactin secretion in early pregnancy

In order to investigate the role of proinflammatory cytokines, including TNF-α, on the mechanisms controlling prolactin secretion further experiments need to be conducted. A number of cytokines, including TNF-α, activate the JAK2/STAT3 signalling pathway (Miscia et al., 2002, Romanatto et al., 2007). The effect of stress on pro-inflammatory cytokine signalling in the hypothalamus and pituitary could be carried out by immunocytochemistry for pSTAT3 nuclear expression in stressed and control early pregnant mice. Furthermore, by blocking a range of pro-inflammatory cytokines (e.g. through direct administration of cytokine specific antagonists to the brain) prior to stress exposure in early pregnancy, the role of certain cytokines in prolactin inhibition could be determined. This would help to ascertain the mechanism(s) responsible for cytokine/stress-reduced prolactin in early pregnancy. A new powerful system to monitor hormone secretion from rodent pituitary slices has recently been described. This is achieved by using an adapted fluorescence microscope and transgenic rodents with fluorescent proteins targeted to specific secretory vesicles e.g. prolactin granules of lactotrophs, and thus, these are co-secreted with the hormone. Using this system in transgenic mice dopamine has been shown to decrease fluorescence protein output and oxytocin to increase it in a dose dependent manner, which correlated to hormone (prolactin) output (as measured by

RIA) (He et al., 2011). It may therefore be possible to introduce a range of (stress-induced) pro-inflammatory cytokines (including TNF-α) to pituitary slices of virgin and early pregnant mice and monitor their effect on prolactin secretion from the lactotrophs. As this technique allows long perfusion times (up to 8h) the same slice may be stimulated multiple times with various factors/cytokines (with sufficient washing) which would reduce the number of animals required and enhance the reliability when comparing different experimental variables. This system further enhances our understanding of secretion from a large population of cells in their normal topographical organisation (which is important for secretory responses in the pituitary) and importantly fills the void between single cell monitoring (exocytosis; milliseconds) and release into the bloodstream (minutes) (He et al., 2011).

6.3 The effect of stress in early pregnancy on the HPA axis

Our data demonstrate that stress, both immune and fasting, activates the HPA axis, leading to the expected hormonal secretory responses in early and mid pregnant mice, as observed in virgins (Brunton et al., 2008). This emphasises the reports that the hyporesponsiveness of the HPA axis to stress in late pregnancy does not emerge until after d10.5 (Douglas et al., 2003). Consequently high glucocorticoid levels induced by stress exposure may impact on a variety of mechanisms required for pregnancy establishment and maintenance.

In early gestation, it has been suggested that the actions of glucocorticoids are balanced between positive pregnancy-protective effects (e.g. suppression of natural killer cells (Krukowski et al., 2011), promotion of trophoblast growth and invasion, stimulation of hCG) and adverse pregnancy-threatening effects (e.g. inhibition of placental growth, induction of placental/decidual apoptosis) (Michael and Papageorghiou, 2008). Although we do not address the specific roles of glucocorticoids, our data implies that high levels of glucocorticoids are possible at this stage in pregnancy and they may be detrimental to reproductive success.

Analysis of the hypothalamic responses to LPS stress in early pregnancy unexpectedly revealed that nur77 and CRH mRNA responses in the pPVN were attenuated in early-mid gestation compared to virgins, extending previous reports of attenuated responses in late pregnancy (Douglas et al., 2003). This is in contrast to the corticosterone response, thus, the CRH PVN responses seem to be dissociated

from the secretory HPA axis responses to LPS (at least at this time-point after stress exposure). As CRH responses were attenuated very early in pregnancy it may be that CRH neurone responses are adapted by early pregnancy factors such as the mating-induced neurogenic reflex, pregnancy hormones and cytokines. It is not clear how neuronal activity can be attenuated while secretory responses remain robust, although we found that pPVN vasopressin mRNA responses to LPS in early pregnancy were maintained and similar to virgins. This is intriguing as vasopressin is reported to be synthesised in some of the same pPVN neurones as CRH and appears to be co-released with CRH at the median eminence (Wang and Majzoub, 2011). The robust vasopressin response to LPS suggests that vasopressin (synergistic secretagogue), in the absence of CRH, may alone drive the high corticosterone responses observed. In addition, LPS and other stress-induced cytokines may directly act at the pituitary and adrenals to induce hormone secretion directly, thus bypassing the PVN (John and Buckingham, 2003).

The reduction of CRH responses in the pPVN mirrors the lack of strong TIDA neurone activation following stress in early pregnancy. A study looking into the distribution of CRH-receptor-like immunoreactivity in the rat hypothalamus found it to be expressed in arcuate nucleus populations, including TIDA neurones. However, CRH-immunoreactive fiber innervation of the arcuate nucleus was very sparse and therefore it seems unlikely that they play a major role in TIDA neurone regulation (Campbell et al., 2003).

Unlike pPVN CRH neurones not all neuronal responses are suppressed by stress, for example the responses from brainstem nuclei (NTS, RVLM) and the vasopressin responses from the pPVN are robust following stress in early pregnancy. The discovery that stress-induced vasopressin and CRH responses differ in early pregnancy reveals a curious dissociation between different subsets of pPVN neuronal populations and how this occurs remains to be determined. Differential activation of various neuronal populations in the hypothalamus and brainstem following stress exposure indicate that stress leads to selective activation of neurones through complex and thus far unknown/untested pregnancy-related mechanisms. For example, the distribution of sex steroid receptors on neurones and whether they respond to the neurogenic reflex may be involved in selectivity of activation.

6.4 The effect of stress in early pregnancy on the PVN and brainstem noradrenergic pathways

LPS, via cytokines, is able to activate the brainstem noradrenergic pathways to the hypothalamus (Brunton et al., 2008). We found that LPS stress increases the activation of LC neurones in early pregnant and virgin mice. However, neurones in the LC appear to display a generally lower response in early pregnancy compared to virgins, which may potentially contribute to the attenuated response of the pPVN (e.g. lower CRH and nur77 mRNA and Fos nuclear response compared to virgins following LPS). In addition, although the LC response to stress appeared lower in early pregnancy compared to virgins, the overall number of neurones activated in the LC was low. However, the response to LPS was greater in the noradrenergic neurones of the NTS and RVLM, which respond more to LPS administration in early pregnancy compared to virgin mice. This suggests that an adaptation has occurred in these neurones to increase their sensitivity to certain stimuli which may contribute towards to the mildly exaggerated response to stress observed by the TIDA neurones. The release of noradrenaline in the hypothalamus has been temporally linked with plasma secretory surges of prolactin in the rat oestrous cycle (Szawka et al., 2007), with the LC implicated to be involved in regulating prolactin surges during oestrous (Szawka et al., 2005). However, evidence indicates that neurones of the LC are not involved stress-induced changes in prolactin secretion (Poletini et al., 2006). Likewise, although the NTS and RVLM are activated by cytokines, no association with prolactin secretion has been reported at present.

As with LPS, we also show a mild increase in TIDA neurone activation with 24h fasting stress in early pregnancy but whether this is accompanied by noradrenergic activation in the NTS, LC or RVLM is not known as this was not analysed. However, it has been reported that food ingestion is critical for the expression of Fos in the noradrenergic cells of the LC and NTS in rodents (Johnstone et al., 2006) and therefore in fasted mice we may not expect to observe an increase in Fos activation in the brainstem.

6.5 The effect of immune stress in early pregnancy on anxiety-related behaviour

A number of immune stressors, including LPS, have been reported to activate the HPA axis and induce anxiety-like behaviours in rodents (Lacosta et al., 1999).

Therefore, we hypothesised that LPS administration would increase anxiety-like behaviours in virgin and early pregnant mice and further postulated that in early pregnancy the anxiogenic response following LPS stress would be enhanced because of the reduction in anxiolytic prolactin (and progesterone). Increased prolactin levels during pregnancy elevate neurogenesis in the subventricular zone of the lateral ventricle of the maternal brain and is essential for normal postpartum behaviour (Larsen and Grattan, 2010). It has been reported that in early pregnancy low prolactin leads to a reduction in neurogenesis and is associated with later heightened postpartum anxiety. In addition, the female offspring of these mothers also display increased anxiety (Larsen and Grattan, in press). However, it has not been reported what the acute effects of stress or low prolactin are on maternal anxiety during early pregnancy itself.

We found that LPS administration (24h before behavioural testing) had profound anxiogenic effects on the virgin mice and also decreased their locomotion activity compared to vehicle-treated mice when tested on the EPM. However, in early pregnant mice LPS treatment appeared to have no effect on the anxiety-behavioural parameters measured compared to controls. This may be partly explained by the fact that vehicle-treated pregnant mice displayed higher anxiety-like behaviours compared to virgins and as anxiety levels were already high in early pregnancy this may be a 'ceiling effect' which masks any LPS-induced changes. Data indicate that mice are generally more anxious in early pregnancy compared to virgins which is in contrast to reports in mid (d11) (Maestripieri and D'Amato, 1991) and in late pregnancy (d18) (Neumann et al., 1998, Neumann, 2003).

The lack of effect of stress on anxiety in early pregnancy may be associated with the relatively unaltered levels of circulating prolactin following the EPM. It seems that the EPM itself has a profound effect on prolactin levels in early pregnant mice reducing them (compared to mice not tested on the EPM) regardless of treatment. However, stressed virgin mice displayed heightened anxiety responses to stress compared to both controls and early pregnant mice, which was accompanied by a reduction in the anxiolytic prolactin (similar to levels in the pregnant mice).

Further analysis of anxiety-like behaviour using an OFT revealed a mild increase in anxiety behaviour following LPS treatment but this effect was not more pronounced

in early pregnant mice as was hypothesised. Once again, in early pregnancy a difference in plasma prolactin levels with treatment was not apparent following the OFT which may partly explain why only very mild of changes in anxiety-like behaviours were observed.

In addition, the lack of predicted increase in anxiety-responsiveness in early pregnancy may be related to the attenuated PVN responses to stress that we have previously reported. It has been shown in many studies that CRH administration or overproduction of CRH increases anxiety-like behaviours in rodents (Van Gaalen et al., 2002). In addition, vasopressin is also reported to modulate anxiety-related behaviours. Studies using rats that are bred for either a high or low anxiety-related trait reveal that the increased anxiety-levels in the former are due to a polymorphism in the promoter region of the vasopressin gene, resulting in increased hypothalamic vasopressin activity. High anxiety bred rats display increased expression of vasopressin mRNA in the PVN and release. In support of an anxiogenic role for vasopressin, studies using these high anxiety rats have revealed that intra-PVN treatment of a vasopressin receptor antagonist reduces anxiety behaviours (Wigger et al., 2004). In mice, preliminary data also indicates that there is a correlation between anxiety behaviour and the expression of vasopressin in the PVN. In mice bred for high anxiety the intra-PVN expression of vasopressin is significantly higher than in mice bred for low anxiety (Landgraf et al., 2007).

Therefore, a combination of high CRH and vasopressin PVN responses in virgin mice may contribute towards the heightened anxiety-behaviours observed on the EPM following LPS. In contrast, in early pregnancy the attenuated CRH response and lower PVN activation (Fos expression) following stress compared to virgins may partly explain why the anxiety responses to LPS were lower in early pregnancy than virgins.

6.6 The effect of 24h fast in early pregnancy on anxiety-related behaviour

It was also hypothesised that 24h fasting would lead to increased anxiety-like behaviour in early pregnant mice, possibly via predicated increased corticosterone, decreased prolactin secretion and elevated levels of circulating ghrelin which is reported to increase with fasting in rodents (Cummings et al., 2001, Briatore et al., 2006) and is considered to be anxiogenic (Carlini et al., 2002). We did not

demonstrate this though and found that both virgin and early pregnant fasted mice displayed reduced anxiety-like and increased exploratory behaviours (likely motivated by hunger) despite increased corticosterone responses.

Although 24h fasting suppressed plasma prolactin levels in early pregnant mice as expected, plasma prolactin concentrations do not seem to have an association with anxiety behaviours. It would be interesting to know what the circulating ghrelin levels of our 24h fasted mice are to determine if they have raised as is assumed. If not this could also contribute to explaining why we do not see heightened anxiety-like behaviour with 24h fasting as predicted. In addition, as previously indicated Fos expression in the SON, VMH and PVN was very low and was not altered by 24h fasting. Therefore, a change in the CRH and vasopressin responses to fasting (as seen following LPS) are unlikely to be accountable for the mild changes observed in anxiety/exploratory behaviours. We did, however, observe an increase in activation of neurones in the arcuate nucleus following fasting which may indicate an impact on appetite-regulating factors such as neuropeptide Y (NPY). Mild fasting (24h) in male rats has been reported to increase NPY mRNA expression in the hypothalamic arcuate and dorsomedial nuclei (Marks et al., 1992) and NPY administration dose-dependently suppresses prolactin secretion from lactotrophs *in vitro* (Wang et al., 1996). Therefore, it may be that fasting in early pregnant mice heightens the NPY response compared to virgins due to an unknown pregnancy-related factor and thus contributes to reducing plasma prolactin levels. In addition, NPY is considered a potent anxiolytic agent and this role is supported by reports of the expression of receptors for NPY in brain regions associated with anxiety such as the amygdala (Sajdyk et al., 2004). Multiple studies have demonstrated that central administration of NPY produces anxiolytic responses under stress or anxiety-inducing situations, for example NPY treatment (i.c.v) in male mice reduced anxiety-related behaviours on the EPM, light/dark test and also increased locomotor activity in the OFT (Karlsson et al., 2005). Therefore, presumed increased NPY due to fasting in both virgin and early pregnant mice may be partly responsible for the reduced anxiety-like behaviours and increased exploratory behaviours observed following 24h fasting.

In contrast to the EPM, the OFT did not indicate an increase in anxiety behaviour or exploratory behaviour in either virgins or pregnant mice with 24 fasting and although

prolactin levels decreased by fasting in early pregnant mice as expected, circulating prolactin levels once again did not reflect anxiety-related behaviour levels.
Overall, data from both behavioural experiments do not support our hypothesis that fasting would lead to enhanced anxiety-like behaviour in early pregnant mice and due to the drive to explore (likely motivated by hunger) 24h fasting may not have been a suitable treatment to use to measure the effects of stress in early pregnancy on anxiety behaviour.

6.6 Summary

To summarise, we have found in mice that the secretory HPA response to stress in early pregnancy is robust and comparable to the virgins, although early pregnant mice seem to display enhanced anxiety levels compared to virgins. Stress in early pregnancy profoundly reduces plasma progesterone and prolactin levels, increases circulating levels of pregnancy-threatening proinflammatory cytokines (e.g. TNF-α) and consequently threatens pregnancy maintenance. Stress-induced suppression of prolactin is not entirely due to enhanced TIDA neurone activation, dopamine activity or prolactin negative feedback as expected. Therefore, other stress-related factors must also be responsible, for example, stress may lead to a decrease in prolactin-releasing factors that act at the pituitary level (this remains to be tested). Stress-induced cytokines e.g. TNF-α are likely contenders and may act directly at lactotrophs to impede prolactin secretion in early pregnancy (see Figure 6.1). In support of this theory lactotrophs are known to express receptors for TNF-α and TNF-α treatment reduces anterior pituitary cell proliferation and prolactin release in an oestrogen-dependent manner. We have shown that TNF-α treatment in early pregnancy decreases both progesterone and prolactin section, while only mildly increasing TIDA activation, suggesting that TNF-α acts directly at the pituitary to suppress prolactin. Further work is required to fully investigate the mechanism behind this.
Furthermore, we propose that there is an overlapping role for stress-increased glucocorticoid action on reduced pregnancy hormone secretion by enhancing pro-inflammatory cytokine release following stress in early pregnancy (see Figure 6.2). Increased glucocorticoid levels following stress in early pregnancy may drive the

production and release of proinflammatory cytokines (e.g. TNF-α) and result in a decrease of pregnancy hormone levels. Low circulating progesterone and prolactin would allow proinflammatory levels to further rise as the inhibitory effect of these hormones on pro-inflammatory cytokines would be suppressed. A rise in glucocorticoids/proinflammatory cytokine levels together with depleted plasma progesterone/prolactin in early gestation may increase the risk of pregnancy complications such as pregnancy failure and adverse foetal programming.

Figure 6.1

Figure 6.2

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Appendix A: Suppliers/Chemicals index

Reagent	Company	Code
Acetic anhydride	Sigma-Aldrich	A6404
Anti-fos Ab	Santa Cruz	SC-52
Anti-TH Ab	Millipore	AB152
Ammonium chloride	BDH	271495R
'AnalR' formamide	BDH	103264R
Aprotinin	Sigma-Aldrich	A1153 - 10MG
Biotinylated anti rabbit Ig	Vector	BA-1000
Bovine serum albumin, fraction V	Millipore	82-045
Chromium potassium sulphate 12 hydrate	BDH	2775845
Corticosterone RIA	MP Biomedicals	7120102
Denhardt's solution	Sigma-Aldrich	D2532
Deionised Formamide	Sigma-Aldrich	F9037
Developer Kodak D19	Ilford/Lizars	
Dextran Sulphate	Sigma-Aldrich	D8906
Diaminobenzidine tetrachloride (DAB)	Sigma-Aldrich	D5637
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich	D5758
DPX mountant	VWR	360294
Disodium hydrogen orthophosphate	BDH	103834G
Dithiothretol (DTT)	Sigma-Aldrich	D9779
Emulsion (Ilford)	Calumet	P9281
Ethylene diamine tetracetic acid (EDTA)	Sigma-Aldrich	E5134
Ethylene glycol	Sigma-Aldrich	102466
FBS (heat-inactivated)	Invitrogen	10108157 (100ml)
Gelatine	BDH	440454B
Glycerol	Fisher Scientific	G/0600/17
Heparin	Sigma-Aldrich	H0777-500KU
Hydrogen peroxide (30%)	Sigma-Aldrich	H1009
Interleukin-6 (recombinant mouse)	R&D System	406-ML-005
Lipopolysaccharide (LPS) E055:B5 (E.coli)	Sigma-Aldrich	L2880 - 10mg
Metoclopramide hydrochloride (10g)	Sigma-Aldrich	M0763
Mouse IL-6 DuoSet ELISA	R&D Systems	DY406
Mouse prolactin DuoSet ELISA	R&D Stems	DY1445
Nickel II sulphate	BDH	101674A

Normal sheep serum	Sigma-Aldrich	S2263
Ovine prolactin	Sigma-Aldrich	L6520
Paraformaldehyde	Sigma-Aldrich	P6148
Phenol:Chloroform isoamyl alcohol	Sigma-Aldrich	P3803
Phospho-Stat5 (Tyr694) Antibody	Cell Signalling Tech (supplied by New England Biolabs)	9351-S
Potassium chloride	Sigma-Aldrich	P3911 or 9541
Progesterone radioimmunoassay (kit 1)	Oxford Bioinnovations	DSL 3900
Progesterone radioimmunoassay (kit 2)	MP Biomedicals	07814701
Reagent Diluent (DuoSet ELISA)	R&D Systems	DY995
RNase A	Sigma-Aldrich	
QIAGEN quick nucleotide removal kit	QIAGEN	28304
S35 dATP 9.25MB	Perkin Elmer	NEG034H
Salmon sperm DNA	Sigma-Aldrich	D9156
Sodium acetate	Sigma-Aldrich	S9513
Sodium bicarbonate	Sigma-Aldrich	S8875
Sodium chloride	Sigma-Aldrich	S7663
Sodium dihydrogen orthophosphate	BDH	1024555
Substrate solution (DuoSet ELISA)	R&D Systems	DY999
Sucrose	Sigma-Aldrich	S7903 or 179949
Toludine blue	Sigma-Aldrich	
Triethanolamine (TEA)	Sigma-Aldrich	T1377
Tris	Sigma-Aldrich	T8524
Tris-HCL	VWR	441514A
Tri-sodium citrate	Sigma-Aldrich	S4641
Triton X-100	Sigma-Aldrich	T9284
Tumour necrosis factor-α (recombinant mouse)	R&D Systems	410-MT-050
Vectastain ABC elite kit	Vector Labs	PK6101
Wash buffer (DuoSet ELISA)	R&D Systems	WA126
Xylene	BDH	1330-20-7

Appendix B: General solutions

Solutions for perfuse fixation

- Heparinised saline (0.9%): 1L dH_2O + 9g NaCl + 129mg Heparin
- Phosphate buffer (PB) (1L of 1M PB; pH 7.3-7.4): 1L dH_2O (heated) + 115g disodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$) + 27.2g Sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$).
- 4% paraformaldehyde in phosphate buffer (PB) (pH7.3-7.4): 500ml dH_2O (heated) + 40g PAF + few drops of 10M NaOH solution (to dissolve the powder), + 500ml 0.2M PB.
- 2% PFA + 15% sucrose in 0.1M PB: (for 500ml) 250mls 30% sucrose + 250mls 4% PFA.
- 30% sucrose: 30g sucrose / 100ml in 0.1M PB.
- Cryoprotectant: (for 1L) 200ml glycerol + 300ml ethylene glycol + 500ml 0.1M PBS.

Solutions for immunocytochemistry

- Phosphate Buffer (see section above)
- 0.1M PB + 0.3% Triton X-100 (PBT): 3ml Triton X-100 + 1000ml 0.1M PB.
- 0.1M phosphate buffered saline (PBS; 0.9%): 9g NaCl + 1000ml 0.1M PB.
- 3% normal sheep serum (NSS) in 0.1M PBT: 3ml NSS + 97ml 0.1M PBT.
- 0.3% hydrogen peroxide in 0.1M PB: 1ml 30% H_2O_2 + 99ml 0.1M PB.
- 1M sodium acetate buffer: 82g sodium acetate anhydrous + 1000ml dH_2O, pH 6 with glacial acetic acid.
- DAB concentrate (25mg/ml):1g Diaminobenzidine tetrachloride + 40ml dH_2O (warm).
- Nickel-DAB visualisation solution: (for 100ml) 50ml 0.2M sodium acetate buffer + 2.5g Nickel II sulphate + 0.08g ammonium chloride + 25mg in 1ml DAB + 49ml dH_2O +100µl H_2O_2.
- DAB visualisation solution: (for 100ml) 50ml 0.2M sodium acetate buffer + 25mg in 1ml DAB + 49ml dH_2O +100µl H_2O_2.
- Tris buffered saline (TBS; 0.05M Trizma, 0.15M NaCl; pH 7.6): 6.05g Trizma + 8.85g NaCl + 1000ml dH_2O.
- 'Incubation solution' for pSTAT5 protocol (0.05M TBS,0.3% triton X-100, 0.25% BSA): 600ml TBS + 1.8ml triton-x +1.5g BSA.
- 0.01M Tris-HCl (pH 10): 0.157g Tris-HCL + 100ml dH_2O.

Solutions for mounting and staining brain sections

- Gelatine for subbing slides: 2.5g gelatine to 500ml dH_2O, add 0.25g chromium potassium sulphate.
- Acetic Alcohol Fixative (AAF): 10ml 40% formaldehyde + 5ml glacial acetic acid (100%) + 85ml absolute alcohol.
- Toludine Blue: 1% toluidine blue in dH_2O.

Solutions for in situ hybridisation

- 1M Phosphate buffered saline (PBS; pH7.3-7.4): 1L dH_2O (heated) + 115g Disodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$) + 29.64g Sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) + 85g NaCL
- 4% Paraformaldehyde (PAF) in 0.1M PBS (pH 7.2): (250ml) 10g PAF + 250ml 0.1M PBS + few drops of 10M NaCl.
- Triethanolamine (TEA)/actetic anhydride solution: 3.725ml TEA + 0.625ml acetic anhydride + 1050µl concentrated HCl, made up to 250ml with ddH_2O.
- RNase Buffer : 1.12g Tris 8.0 (10mM) + 23.2g NaCl (0.5M) + 0.32g EDTA (1mM), made up tp 800ml in ddH_2O.
- Diethylpyrocarbonate (DEPC) water (0.01%): 1ml DEPC + 999ml ddH_2O.
- Hybridisation Buffer: To make 50ml combine the following: 12ml 5M NaCl (292mg/ml) + 1ml Tris pH 7.6 (149mg/ml) + 1ml Denhardts solution (1.6% BSA 200mg/ml, 6% Ficoll 100mg/ml, 6% PVP 100mg/ml) + 400µl EDTA (250mM) + 5ml dextran sulphate 25% + 500µl NaPPI 5% (50mg/ml) + 200µl yeast tRNA (35mg/ml) + 250µl yeast total RNA (20mg/ml) + 1ml salmon testes DNA (10mg/ml) + 330µl Poly(A) (15mg/ml) + 25ml formamide, made up to 50ml with ddH_2O.
- 20 X Standard sodium citrate (SSC) stock: 175.4g NaCl + 88.2g Tri-sodium citrate, made up to 1L with sterile ddH_2O.
- Developer (Kodak D19): 157g + 500ml ddH_2O.
- Fixer (Ilford Hypam): 100ml fixer stock + 400ml ddH_2O.

Solutions for haemotoxylin and eosin counter staining

- Haemotoxylin: bought in ready made (0.4% haemotoxylin)
- 70% Acid alcohol: 700ml absolute alcohol (100%) + 300ml dH_2O + 10ml HCl (conc)
- Scott's Tap Water Substitute (STWS): 20g magnesium sulphate + 3.5g sodium bicarbonate + 1000ml dH_2O
- 1% Eosin: 10g + 1000mls dH_2O
- 5% Potassium Alum: 5g aluminium potassium sulphate + 100mls dH_2O

Appendix C: Publications and presented abstracts

Publications:

Parker VJ, Menzies JR, and Douglas AJ (2011) Differential changes in the hypothalamic-pituitary-adrenal axis and prolactin responses to stress in early pregnant mice. *Journal of Neuroendocrinology*, **23**: 1066-1078 (Original article).

Parker VJ, Arck PC, and Douglas AJ (2011) Reciprocal brain-body neuro-endocrine-immune interactions: role in maintaining pregnancy. *Advances in Neuroimmune Biology* (in press) (Review).

Parker VJ and Douglas AJ (2010) Stress in early pregnancy: maternal neuro-endocrine-immune responses and effects. *Journal of Reproductive Immunology*, **85**: 86-92 (Review).

Conference Abstracts:

Parker VJ, Menzies JR and Douglas AJ (2011) Hypothalamic mechanisms mediating inhibition of prolactin secretion following stress in early pregnancy. Physiology 2011, The Physiological Society (Oral).

Parker VJ, Menzies JR and Douglas AJ (2011) Hypothalamic mechanisms mediating inhibition of prolactin secretion following stress in early pregnancy. British Society for Neuroendocrinology Annual Meeting (poster).

Parker VJ, and Douglas AJ (2010) Hypothalamic mechanisms mediating inhibition of prolactin secretion following stress in early pregnancy. The Parental Brain international conference (Oral).

Parker VJ, and Douglas AJ (2010) Hypothalamic mechanisms mediating inhibition of prolactin secretion following stress in early pregnancy. The 7th International Congress of Neuroendocrinology (Poster).

Parker VJ, and Douglas AJ (2010) Mechanisms of stress-induced pregnancy failure in mice: role of maternal neuro-endocrine-immune responses. ENDO 2010 The 92nd Annual Meeting & Expo (Poster).

Parker VJ, and Douglas AJ (2009) Pregnancy alters prolactin and cytokine responses to stress in mice. The 7th European Congress on Reproductive Immunology (Oral).

Parker VJ, and Douglas AJ (2009) Control of basal and stress-induced prolactin secretion in early pregnancy. British Society for Neuroendocrinology Annual Meeting (Poster).

Parker VJ, Arck PC, and Douglas AJ (2008) Effect of an immune stress on neuroendocrine responses in early pregnancy. British Society for Neuroendocrinology Annual Meeting (Poster).

Parker VJ, and Douglas AJ (2008) Effect of an immune stress on prolactin secretion in early pregnancy. The 4th EMBIC Summer School. *American Journal of Reproductive Immunology* **60**, 88-89 (2008) (Poster).

Parker VJ, Arck PC, and Douglas AJ (2007) Neuroendocrine responses to immune stress in early pregnancy. The3rd EMBIC Summer School. *American Journal of Reproductive Immunology* **58**, 177-180 (2007) (Oral).