The effect of social isolation and leptin on the hypothalamo-pituitary-adrenocortical (HPA) response to acute stress in rats

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Abstract

The hypothalamo-pituitary-adrenal (HPA) axis is the main regulator of stress in mammals, and prolongation of the stress response can lead to manifestation of metabolic and psychiatric disorders. Leptin is an adipokine known to act at the hypothalamus to regulate body fat stores, appetite and energy expenditure, but the role of leptin on the HPA response to stress is still not well understood. Psychological stress, specifically loneliness in humans - referred to as social isolation (SI) in social mammals - can also alter HPA functioning. The work described in this thesis investigates the effects of leptin and SI on the HPA response to acute stress.

My studies suggest that leptin does not influence basal HPA activity, or influence the HPA response to acute endotoxin challenge in rats. They also show that SI does not affect basal HPA activity, but that short term chronic SI does cause hyperactivity of the HPA in response to acute restraint stress, and that the stress axis is hyper-sensitised at the level of the hypothalamus and the adrenal gland after this period of SI. My work suggests that this HPA hyperactivity may be the result of an overactive CRH feed-forward mechanism. I have also shown that short term intermittent SI and long term chronic SI both cause hypoactivity of the HPA axis response to acute restraint stress, with the former regulated by an unknown mechanism and the latter possibly regulated by an overactive glucocorticoid feedback mechanism.

In summary, these studies have highlighted the complex differential activation of the HPA axis in response to different types of stressors; both immunological and psychological. I have also demonstrated the different effects of novel SI paradigms on HPA response to acute stress, and

how such psychological stress can impair the functioning of a key system in the body that may be involved in metabolic and psychiatric disease.

Declaration of Contributors

The majority of the work in this thesis was performed by the author, and all else is appropriately referenced. All assistance with the work described in this thesis is defined below:

Chapter II:

Assistance with leptin and LPS injections and tissue collection was provided by members of the Section of Investigative Medicine.

Chapter III:

Assistance with the restraint stress procedures and tissue collection was provided by members of the Section of Investigative Medicine.

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Finally, I would like to thank my family and my closest friend. You have put up with the late nights, long hours, supported me financially and given me the time and space to pursue what is important to me. You have been the inspiration and the driving force behind my accomplishments.

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Chapter II: Investigating the effects of leptin on the HPA axis response to stress

Chapter IV: General Discussion

Abbreviations

11β-HSD	11 β-hydroxysteroid dehydrogenase
11β-HSD1	11 β-hydroxysteroid dehydrogenase-type 1
11β-HSD2	11 β-hydroxysteroid dehydrogenase-type 2
3β-HSD	*3* β-*hydroxysteroid dehydrogenase*
5-HT	Serotonin
5-HTR	Serotonin receptor
AB-5	Rabbit anti c-fos primary antibody
ABC	Streptavidin-Biotin complex
AChE	Acetyl cholinesterase
ACTH	Adrenocorticotropic hormone
AgRP	Agouti related peptide
AH	Anterior hypothalamic nucleus
AHA	Anterior hypothalamic area
ANOVA	Analysis of variance
AP	Area postrema
AP 1	Activator protein 1
ARC	Arcuate nucleus
AU	Arbitrary units
AUC	Area under the curve
AV3V	Anteroventral area of third ventricle
AVP	Arginine vasopressin
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BCP	1-Bromo-3-chloropropane
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine regulated transcript
CB1	Endocannabinoid receptor 1
CBG	Corticosteroid-binding globulin
CBGR	Corticosteroid-binding globulin receptor
CD14	Cluster of differentiation 14
cDNA	Complementary DNA

c-fos	Cellular fos
CI	Capsula interna
CLP	Cecal ligation and puncture
CNS	Central nervous system
CPE	Carboxypeptidase E
CPM	Count per minute
CREB	cAMP-responsive element-binding protein
$CREB^{-P}$	Phosphorylated cAMP-responsive element-binding protein
CRH	Corticotrophin-releasing hormone
CRH-R	Corticotrophin-releasing hormone-receptor
CRH-R1	Corticotrophin-releasing hormone-receptor type 1
CRH-R2	Corticotrophin-releasing hormone-receptor type 2
CVD	Cardiovascular disease
DA	Dopamine
DAB	3,3' Diaminobenzidine
da-α-MSH	Desacetyl α-MSH
db/db	Leptin receptor deficient mouse
DHEA	Dehydroepiandrosterone
DMN	Dorsomedial nucleus
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DP	Dorsal parvocellular subnucleus of paraventricular nucleus
EBSS	Earle's balanced salt solution
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked Immunosorbent assay
ERK	Extracellular-signal-regulated kinase
EtBr	Ethidium Bromide
FA	Formaldehyde
FS	Folliculostellate
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GABA-R	Gamma-aminobutyric acid-receptor
GDW	Glass-distilled water

GH	Growth hormone
GHRH	Growth-hormone-releasing hormone
GnRH	Gonadotropin-releasing hormone
GpcGR	G-protein-coupled-Glucocorticoid receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive element
HPA	Hypothalamo-pituitary-adrenal
HRP	Horse radish peroxidase
HSP	Heat-shock protein
i.p	Intraperitoneal
ICV	Intracerebroventricular
IFNβ	Interferon β
IgG	Immunoglobulin G
IHC	Immunohistochemistry
I*k*B	Nuclear factor kappa B inhibitor
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-1R	Interleukin-1 receptor
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IR	Insulin receptor
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK4	Interleukin-1 receptor-associated kinase 4
IRF3	Interferon regulatory factor 3
IRMA	Immunoradiometric assay
ISRE	Interferon-sensitive response element
JAK	Janus kinase
JAK2	Janus kinase 2
JNK	C-Jun N-terminal kinase
JP	Joining peptide
LBP	Lipopolysaccharide-binding protein
LC	Locus coeruleus
LepR	Leptin receptor

LepRa-f	Leptin receptor a-f
LH	Luteinizing hormone
LHA	Lateral hypothalamic area
LM	Lateral magnocellular subnucleus of paraventricular nucleus
LPOA	Lateral preoptic area
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAL	MyD88 adaptor-like protein
MAPK	Mitogen-activated protein kinase
MC2R	Melanocortin 2 receptor
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
m-CPP	m-chlorophenylpiperazine
MD-2	Myeloid differentiation factor-2
ME	Median eminence
MnPO	Median preoptic nucleus
MP	Medial parvocellular paraventricular nucleus
MPO	Medial preoptic area
MPON	Medial preoptic nucleus
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
MyD88s	Myeloid differentiation factor 88 splice variant
NAD+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
N-AT	N-acetyltransferase
NF-*k*B	Nuclear factor *kappa* B
NMU	Neuromedin U
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
ob/ob	Leptin deficient mouse
OT	Optic tract
PAM	Peptidyl α-amidating monooxygenase
PBN	Parabrachial nucleus

PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2
PC3	Prohormone convertase 3
pCaMKII	Phosphorylated calcium/calmodulin dependent protein kinase II
pCREB	Phosphorylated cyclic-AMP response element binding protein
PD-1	Programmed death-1
pERK	Phosphorylated extracellular cell-regulated protein kinase
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PNMT	Phenylethanolamine N-methyltransferase
POMC	Pro-opiomelanocortin
PRCP	Prolylcarboxypeptidase
PRL	Prolactin
pSTAT	Phosphorylated signal transduction and transcription
pSTAT3	Phosphorylated signal transduction and transcription 3
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
REM	Rapid eye movement
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	ribosomal RNA
RT	Reverse transcription
SCh	Suprachiasmatic nucleus
SEM	Standard error of the mean
SI	Social isolation
SIGIRR	Single immunoglobulin interleukin-1 receptor-related molecule
SNS	Sympathetic nervous system
SON	Supraoptic nucleus
SPF	Specific pathogen free
SS	Somatostatin
ST	Subthalamic nucleus
StAR	Steroidogenic acute regulatory protein

STAT	Signal transduction and transcription
STAT3	Signal transduction and transcription 3
T2DM	Type 2 diabetes mellitus
TAB1	Transforming growth factor-β-activated kinase 1 binding protein 1
TAB2	Transforming growth factor-β-activated kinase 1 binding protein 2
TAE	Tris-acetate-EDTA
TAK1	Transforming growth factor-β-activated kinase 1
TANK	Tumour necrosis factor receptor-associated factor binding protein
TBK1	Tumour necrosis factor receptor-associated factor binding protein-binding kinase 1
TIR	Toll-interleukin 1 receptor
TLR4	Toll-like receptor 4
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor- α
TRAF6	Tumour necrosis factor receptor-associated factor 6
TRAM	Toll-interleukin 1 receptor-containing adapter molecule-related adapter molecule
TRH	Thyrotropin-releasing hormone
TRIF	Toll-interleukin 1 receptor-containing adapter molecule
Tris	(Hydroxymethyl)aminomethane
TRP	Transient receptor potential
TSH	Thyroid-stimulating hormone
UV	Ultraviolet
v/v	Volume by volume
v-fos	Viral oncogene
VLPO	Ventrolateral preoptic nucleus
VMN	Ventromedial nucleus
VP	Ventral parvocellular subnucleus of paraventricular nucleus
VTA	Ventral tegmental area
w/v	Weight by volume
WAT	White adipose tissue
Y1	Neuropeptide Y receptor type 1
Y5	Neuropeptide Y receptor type 5
α-MSH	α-melanocyte-stimulating hormone

β-LPH β-lipotropic hormone

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Chapter I:

General Introduction

1.1 Stress

Living organisms maintain a dynamic equilibrium with their environment at the behavioural, physiological, cellular and molecular level. Stress is the body's response to an acute disruption of this equilibrium, and survival requires the organism to adapt accordingly, resulting in a controlled response to the perceived stressor (O'Connor et al., 2000). The hypothalamo-pituitary-adrenal (HPA) axis is the main regulator of stress in mammals. While the HPA axis has a well-characterized circadian pattern, physical and emotional stressors can alter this pattern and disrupt normal neuroendocrine function. The adaptive stress response results in a number of behavioural changes that allow the body to focus on the threat. At the physiological level, central and peripheral changes evoke the redirection of energy to sites of stress, muscles and the central nervous system (CNS). Brain function and attention is enhanced, cardiac output and respiration are accelerated, and blood pressure is elevated (Kyrou and Tsigos, 2009). However, prolongation of the stress response results in impairment of key physiological functions, such as metabolism, growth, reproduction, immune-competence and behaviour (McEwen, 1998). The effects of chronic stress on the body's metabolism (Chrousos, 2009) are linked to manifestation of the metabolic syndrome commonly associated with obesity. Metabolic syndrome is a complex disorder comprising abdominal obesity, dyslipidemia, hyperglycaemia, and hypertension that is associated with an increased risk of type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and atherosclerosis (Holvoet, 2008, Laaksonen et al., 2002, Lakka et al., 2002). An exaggerated stress response is also strongly associated with psychiatric disorders, including melancholic depression (O'Connor et al., 2000), anorexia nervosa (Kaye et al., 1987), panic disorder (Gold et al., 1988) and schizophrenia and anxiety disorders (Weiss et al., 2004).

1.1.1 Anatomy & physiology of the hypothalamo-pituitary-adrenocortical (HPA) axis

The HPA axis is comprised of the hypothalamus, and the pituitary and adrenal glands; it is a major neuroendocrine system that regulates the stress response.

1.1.1.2 The hypothalamus

The hypothalamus is a double diencephalic structure in the brain, located just below the thalamus and above the brainstem. The adult human hypothalamus has a volume of approximately 104 cm^3 (Lemaire et al., 2013). It regulates many metabolic processes, including hunger, thirst, body temperature, sleep, circadian cycles and emotional behaviour (Mayer et al., 1952, Rodbard, 1948, Davison and Demuth, 1946). In accord with these many and varied functions, the hypothalamus is connected to a vast number of inputs and outputs, making it responsive to light and olfactory stimuli, steroids, neural transmission from regions such as the heart, stomach and reproductive tract, stressful stimuli and invading microorganisms (Busnardo et al., 2012, Faber and Gebhardt, 1933, Wren et al., 2000, Davison and Demuth, 1946, Osterlund and Spencer, 2011). Peripheral signals can be relayed to the hypothalamus via the brainstem, vagus nerve and directly from the circulation. Hormones and nutrients in the blood can access the hypothalamus via specific transport mechanisms and via the incomplete blood-brain barrier (BBB) at the hypothalamic median eminence (ME) which allows specific circulating factors direct access to the CNS (Gross, 1992). The hypothalamus is comprised of distinct nuclei that release different hormones and respond to different neuropeptides, each with a specific function and target as illustrated in Tables 1.1-1.2 and Figure 1.1. Most hypothalamic-releasing hormones are targeted to the pituitary gland via the hypophyseal portal system (Besser and Mortimer, 1974). The magnocellular cells of two of these nuclei, the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei comprise the posterior pituitary while the

parvocellular neurons of the PVN release corticotrophin-releasing hormone (CRH) into the hypophyseal portal system to act on the anterior pituitary.

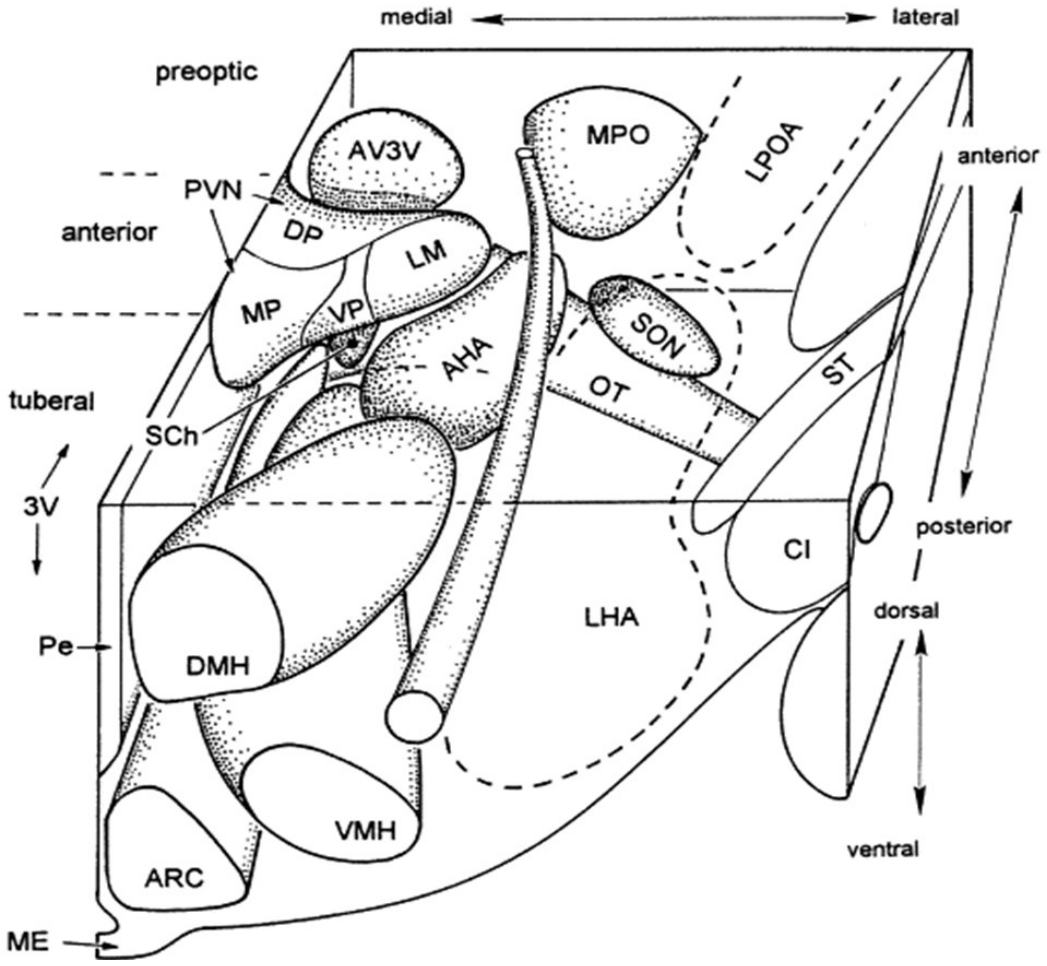

FIGURE 1.1 Schematic three dimensional view of the rat hypothalamus; dorsal and caudal view of the right hemisphere. Abbreviations: AHA, anterior hypothalamic area; ARC, arcuate nucleus; AV3V, anteroventral area of third ventricle; CI, capsula interna; DP, dorsal parvocellular subnucleus of PVN; DMN, dorsomedial nucleus; LHA, lateral hypothalamic area; LM, lateral magnocellular subnucleus of PVN; LPOA, lateral preoptic area; ME, median eminence; MP, medial parvocellular PVN; MPO, medial preoptic area; OT, optic tract; SCh, suprachiasmatic nucleus; SON, supraoptic nucleus; ST, subthalamic nucleus; VMN, ventromedial nucleus; VP, ventral parvocellular subnucleus of PVN. Adapted from (Berthoud, 2002).

TABLE 1.1 Nuclei of the hypothalamus, their secretions and hypothesized roles. Abbreviations: CRH, corticotrophin-releasing hormone; GABA, gamma-aminobutyric acid; GHRH, growth-hormone-releasing hormone; GnRH, gonadotropin-releasing hormone; REM, rapid eye movement; TRH, thyrotropin-releasing hormone.

Hypothalamic Nucleus	Abbreviation	Function
Median preoptic nucleus	MnPO	Secretes noradrenaline, regulates thirst (Miyakubo et al., 2003).
Medial preoptic nucleus	MPON	Secretes GnRH, controls copulation in males (Sinchak and Micevych, 2003).
Ventrolateral preoptic nucleus	VLPO	Secretes inhibitory neurotransmitters such as galanin and GABA, mediates non-REM sleep onset (Luppi, 2010).
Supraoptic nucleus	SO	Secretes oxytocin and vasopressin (Trudel and Bourque, 2012).
Suprachiasmatic nucleus	SCh	Secretes vasopressin, controls circadian rhythms (Giolli et al., 2006).
Paraventricular nucleus	PVN	Secretes CRH, GHRH, GnRH, TRH, oxytocin, vasopressin, somatostatin and dopamine, controls feeding and the stress response (Pego et al., 2010).
Dorsomedial hypothalamic nucleus	DMN	Regulates feeding, drinking, body-weight and circadian activity (Chou et al., 2003).
Median Eminence	ME	Part of the hypothalamus from which regulatory hormones are secreted (Levine et al., 2011).

TABLE 1.1 Continued

Hypothalamic Nucleus	Abbreviation	Function
Ventromedial nucleus	VMN	Neuroendocrine control: regulates satiety, fear, thermoregulation, and sexual activity (Kurrasch et al., 2007).
Arcuate Nucleus	ARC	Secretes GHRH and dopamine, regulates feeding and stress (Arora and Anubhuti, 2006).
Anterior hypothalamic nucleus	AH	Involved in thermoregulation, regulates panting and sweating, and inhibits thyrotropin (Onoe et al., 1992).

TABLE 1.2 Hypothalamic-releasing hormones, their site of production and their effects. Abbreviations: ACTH, adrenocorticotropic hormone; ARC, arcuate nucleus; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; MPON, medial preoptic nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus, TSH, thyroid-stimulating hormone. Adapted from (Schally et al., 1973).

Hormone	Abbreviation	Site of production	Effect
Thyrotropin-releasing hormone	TRH	Parvocellular neurons of the PVN	Stimulates TSH and prolactin release from the anterior pituitary.
Dopamine	DA	ARC	Inhibits prolactin release from the anterior pituitary.
Growth hormone-releasing hormone	GHRH	ARC	Stimulates GH release from the anterior pituitary.
Somatostatin	SS	Periventricular nucleus	Inhibits GH release from the anterior pituitary, inhibits TSH release from the anterior pituitary.
Gonadotropin-releasing hormone	GnRH	MPON	Stimulates FSH release from the anterior pituitary. Stimulates LH release from the anterior pituitary.
Corticotropin-releasing hormone	CRH	Parvocellular neurons of the PVN	Stimulates ACTH release from the anterior pituitary.

TABLE 1.2 Continued

Hormone	Abbreviation	Site of production	Effect
Oxytocin		Magnocellular neurosecretory neurons of the SON and PVN	Controls uterine contraction and lactation.
Vasopressin	AVP	Magnocellular neurosecretory neurons of the SON and PVN	Regulates water retention by increasing absorption in the collecting ducts of the kidney nephron.

The hypothalamus is home to the primary mediators of the stress system; CRH and arginine vasopressin (AVP). Both hypothalamic hormones are secreted in a synchronized fashion, with higher secretory pulses in the early morning that are reduced in magnitude moving into the dark phase in humans (Veldhuis et al., 1990). CRH is a 41 amino acid neuropeptide originally isolated from the ovine hypothalamus (Seasholtz et al., 1991).

CRH was first determined to be involved in the stress response when, in the 1980's, researchers found that intracerebroventricular (ICV) administration of CRH to rats reproduced the stress response, by specifically stimulating adrenocorticotropic hormone (ACTH) and β-endorphin from the pituitary gland (Vale et al., 1981). During an acute stress response, cytokines and inflammatory mediators stimulate CRH and AVP release from the parvocellular neurons of the PVN into the hypophyseal portal system, as illustrated in Figure 1.2. The hypophyseal portal system is a set of blood vessels that link the hypothalamus to the anterior pituitary, allowing cross-talk between the two structures. Once in the portal system, both CRH and AVP regulate ACTH secretion from the anterior pituitary gland (Vale et al., 1981, Lamberts et al., 1984). CRH is a powerful stimulator of ACTH release from the pituitary. Whilst AVP has little effect on its own on basal ACTH release, it acts synergistically with CRH to stimulate ACTH release (Lamberts et al., 1984). During a stress response, additional AVP is thought to be released from the magnocellular cells of the PVN, resulting in a higher CRH-AVP stimulatory effect on pituitary ACTH release (Holmes et al., 1986). CRH and AVP are stimulated by serotonergic and cholinergic signalling systems of the brain, and are inhibited by the gamma-aminobutyric acid (GABA) system, glucocorticoids, ACTH and CRH itself (Chrousos, 1995).

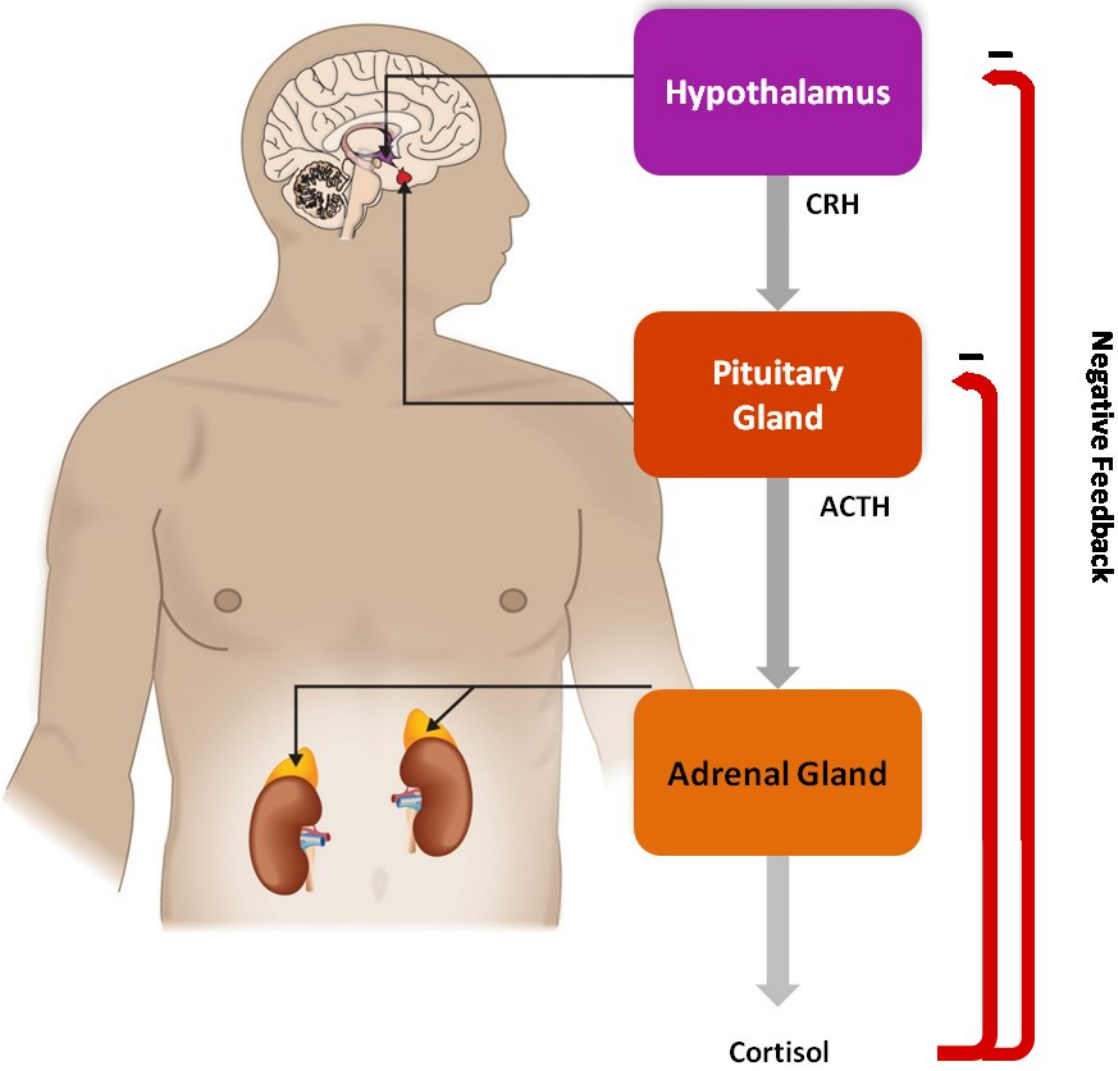

FIGURE 1.2 Schematic diagram of the HPA axis stress cascade and negative feedback mechanism. Abbreviations: CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone. Adapted from (Stangor, 2011).

The hypothalamus also has an important role in appetite regulation. Early experiments involving lesions of the specific regions of the hypothalamus suggested that the lateral hypothalamic area (LHA) controlled hunger and that the ventromedial nucleus (VMN) controlled satiety (Mayer and Thomas, 1967). It is now known that many other hypothalamic nuclei are involved in appetite regulation (Arora and Anubhuti, 2006, Bouret, 2010). The hypothalamic arcuate nucleus (ARC) sits adjacent to the ME and has neuronal projections into the ME. It contains two neuronal populations with opposing effects on food intake. Neurons which express neuropeptide Y (NPY) and agouti related peptide (AgRP) stimulate food intake (Clark et al., 1984, Rossi et al., 1998), whilst neurons expressing pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) inhibit food intake (Kristensen et al., 1998, Aponte et al., 2011). Whilst both neuronal populations project to the PVN, the ARC also projects to the hypothalamic dorsomedial nucleus (DMN), LHA and VMN (Suzuki et al., 2010). POMC neurons release α-melanocyte-stimulating hormone (α-MSH) which binds to melanocortin-4 receptors (MC4R) in regions including the PVN to inhibit feeding (Schwartz et al., 2000). The hyperphagia and obesity observed in MC4R knock-out mice suggests that the melanocortin system plays an important physiological role in the regulation of food intake and body weight (Huszar et al., 1997). The PVN also contains neurons that express thyrotropin-releasing hormone (TRH), the secretagogue of thyroid-stimulating hormone (TSH) from the anterior pituitary. Both hormones regulate growth and thyroid function. TRH and the stress hormone CRH may have an anorexigenic role in appetite regulation (Valassi et al., 2008, Vettor et al., 2002), with central injection of TRH in rats promoting a negative energy balance (al-Arabi and Andrews, 2003) and central administration of CRH reducing body weight in rats (Vettor et al., 2002).

1.1.1.2 Pituitary gland

The pituitary gland is a small endocrine gland of the brain, and in adult humans has a height of approximately 8 mm (Yousem and Grossman, 2010). It sits at the base of the brain and is connected to the hypothalamus by the pituitary stalk and the pars tuberalis. The pituitary gland is split into two main sections; the posterior and anterior lobes. The posterior lobe is directly connected to the hypothalamus. The hypothalamic magnocellular neurosecretory nerve terminals reside in the posterior pituitary, where oxytocin and AVP are secreted. The anterior pituitary is regulated by hypothalamic hormones via the hypothalamo-hypophyseal portal system. The anterior pituitary synthesizes and secretes growth hormone (GH), TSH, prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH), ACTH, β-endorphin and small amounts of α-MSH. The synthesis and secretion of these hormones are regulated by the hypothalamus (Gibo et al., 1993, Knepel et al., 1984). In response to a stressful stimulus, hypothalamic CRH and AVP are not only secreted from parvocellular PVN neurons, but are also released from a ME store and travel in the hypophyseal portal circulation to act directly on corticotroph cells of the anterior pituitary to stimulate the release of ACTH (Lamberts et al., 1984), a 39 amino acid peptide hormone.

ACTH is a product of POMC, which undergoes a series of post-translational modifications involving proteolytic cleavage by endopeptidases (see Figure 1.3). These enzymes break down POMC into various polypeptide fragments including ACTH, α-MSH and β-endorphin (Bertagna, 1994). Release of ACTH is regulated by glucocorticoids which inhibit CRH secretion, and in turn decrease secretion of ACTH. Glucocorticoids can act directly on the pituitary gland to regulate the rate at which the POMC gene is transcribed, as well as the rate at which the POMC peptide is synthesized (de Kloet et al., 2008b). The principal effects of ACTH are to stimulate production and release of corticosteroids from the adrenal gland by binding to the cell surface

receptor, melanocortin-2 receptor (MC2R). Abnormal functioning of the pituitary gland can lead to diseases including hypopituitarism, a form of pituitary hypo-function (Mitchell and Pearce, 2012), or Cushing's disease, a form of adrenal hypercorticism secondary to pituitary hyper-function (Luton et al., 1983).

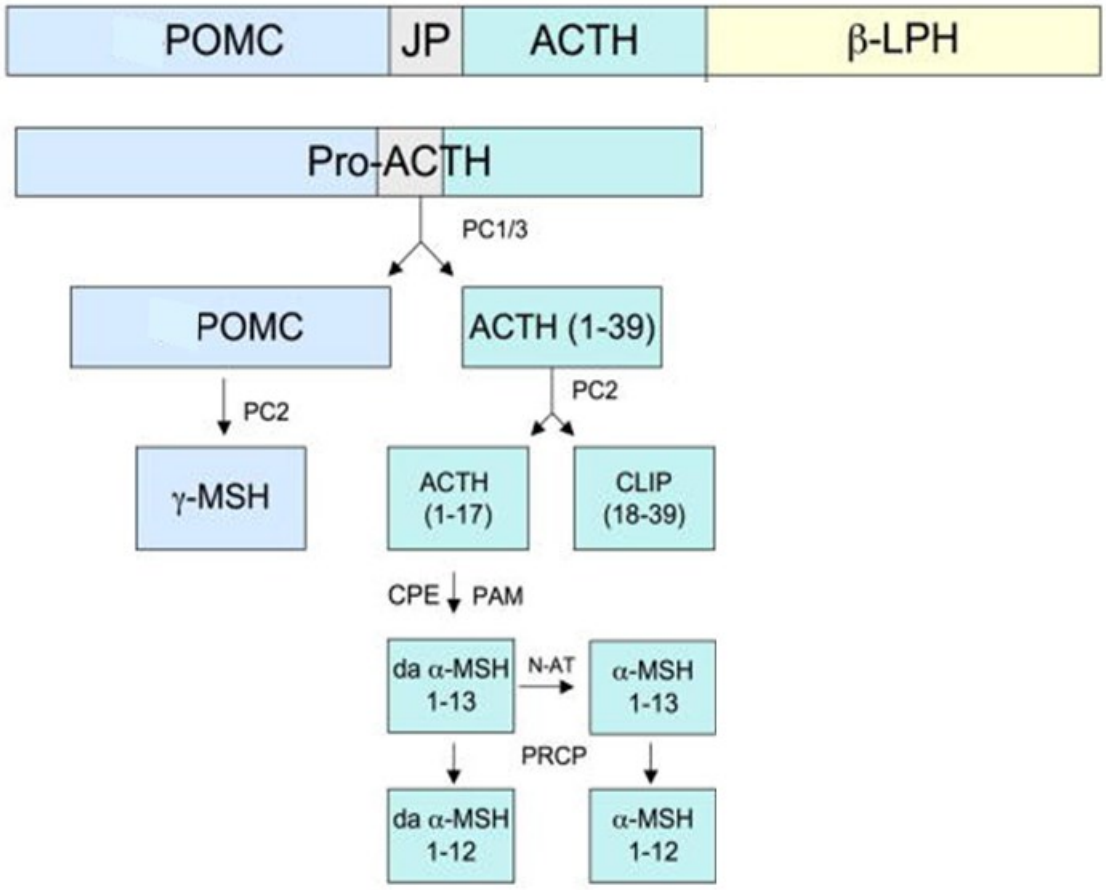

FIGURE 1.3 Schematic diagram of the POMC precursor molecule and its endoproteolytically cleaved peptide products. Abbreviations: POMC, pro-opiomelanocortin; JP, joining peptide; ACTH, adrenocorticotropic hormone; β-LPH, β-lipotropin; CLIP, corticotropin-like-intermediate lobe peptide; da-α-MSH, desacetyl α-MSH; PC1/3 and PC2, prohormone convertases 1/3 and 2; CPE, carboxypeptidase E; N-AT, N-acetyltransferase; PAM, peptidyl α-amidating monooxygenase; PRCP, prolylcarboxypeptidase. Adapted from (Wardlaw, 2011).

1.1.1.3 Adrenal gland

In mammals, the adrenal glands are a pair of endocrine glands located immediately anterior to the kidneys. Each gland measures approximately 13 mm in height and 76 mm in length. In humans, the right adrenal gland is triangular shaped, while the left adrenal gland is semi-lunar shaped. Each adrenal gland has two distinct structures, the outer adrenal cortex and the inner medulla. The adrenal cortex is directly controlled by hormones released from the pituitary gland and primarily produces the glucocorticoids cortisol in humans, or corticosterone in rodents, aldosterone and androgens. The adrenal cortex can be further split into three main zones; 1) the zona glomerulosa which is the outer most layer and is the major site of production of mineralocorticoids, mainly aldosterone, 2) the zona fasciculata which is responsible for producing glucocorticoids such as 11-deoxycorticosterone and cortisol and which secretes low levels of cortisol throughout the day but also produces bursts of cortisol in response to ACTH, and 3) the zona reticularis, the inner most layer of the adrenal cortex which produces androgens, principally dehydroepiandrosterone (DHEA) and androstenedione (Kemppainen and Behrend, 1997).

The second major sub section of the adrenal gland, the medulla, predominantly produces adrenaline and noradrenaline from its chromaffin cells (Kemppainen and Behrend, 1997). These are the main hormones essential for the acute stress response 'fight-or-flight' reaction. The adrenal medulla receives input from the sympathetic nervous system (SNS) which is the major system controlling the medulla, but cortisol from the cortex can also indirectly promote adrenaline synthesis in the medulla (de Kloet et al., 2008b). High levels of cortisol can upregulate phenylethanolamine N-methyltransferase (PNMT), an enzyme that converts noradrenaline to adrenaline, thereby increasing adrenaline synthesis and secretion (Wan and Livett, 1989).

The steroidogenic acute regulatory protein (StAR) plays an extremely important role in steroidogenesis. StAR, primarily produced in steroid-secreting cells, is a transport protein that mediates the first enzymatic reaction in all steroid synthesis; it regulates cholesterol transfer from the outer mitochondrial membrane to the inner mitochondrial membrane, where cholesterol can be cleaved to pregnenolone (Stocco, 2000) (see Figure 1.5).

During a stress response, ACTH from the pituitary gland stimulates secretion of glucocorticoids from the zona fasciculata cells of the adrenal cortex (Chrousos, 2009) (see Figure 1.2). At the intracellular level, ACTH binds to its transmembrane G-protein coupled receptor, MC2R and stimulates production of cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA), leading to the phosphorylation of the cAMP-responsive element-binding protein (CREB). The activation of the transcription factor CREB, results in transcriptional activation of steroidogenic enzymes. MC2R can also interact with the mitogen-activated protein kinases (MAPK), extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, also leading to the activation of CREB and other transcription factors like activator protein 1 (AP 1) (see Figure 1.4).

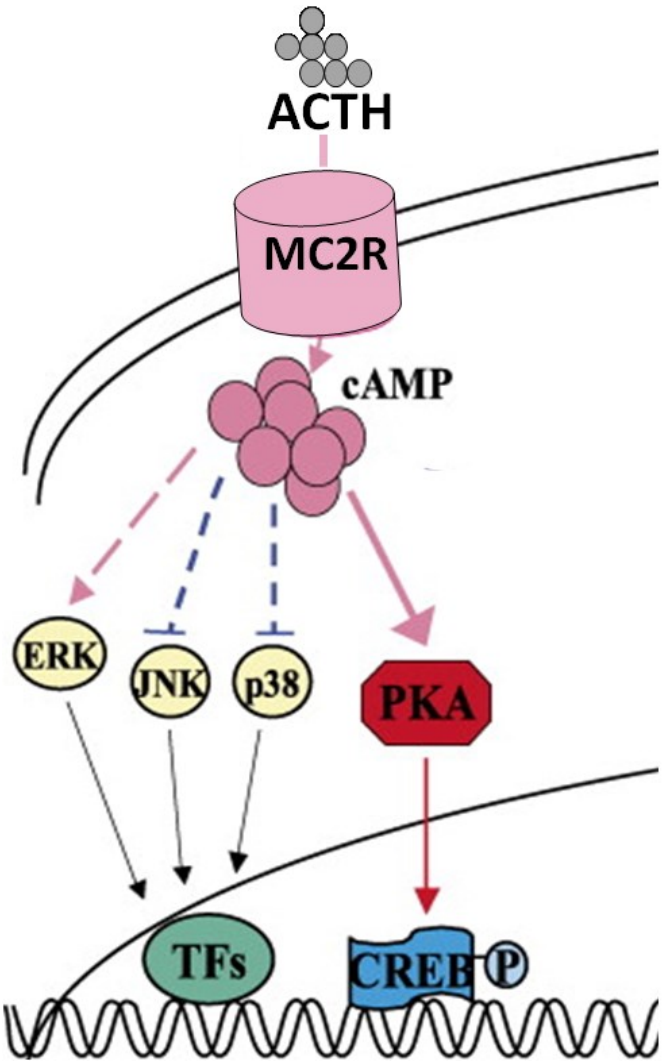

FIGURE 1.4 The ACTH-activated MC2R intracellular signalling pathway. ACTH binds MC2R and stimulates production of cAMP. cAMP activates PKA, leading to the phosphorylation of CREB. MC2R also activates the MAPK's ERK, JKN and p38, also leading to the activation of CREB and other transcription factors, like activator protein 1 (AP 1). This results in transcriptional activation of steroidogenic enzymes. Abbreviations: cAMP, cyclic adenosine monophosphate; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKA, protein kinase A; TFs, transcription factors; $CREB^{-P}$, phosphorylated cAMP-responsive element-binding protein (Lasaga et al., 2008).

All steroids are biosynthesized from cholesterol, with most of the cholesterol precursor being provided by circulating plasma lipoproteins (Biason-Lauber, 1998). Glucocorticoids are one of three classes of steroid hormones; the other two being mineralocorticoids and sex hormones which are important for salt-water balance and reproduction, respectively. As illustrated in Figure 1.5, cholesterol is converted into 17OH-pregnenolone by the actions of 17-α hydroxylase, which is converted to 17OH-progesterone by the actions of 3β-hydroxysteroid dehydrogenase (3β-HSD), followed by its conversion into 11-deoxycortisol by 21-hydroxylase and then finally into cortisol by the actions of 11β hydroxylase.

These glucocorticoids are released into the systemic circulation by diffusion across a concentration gradient across the plasma membrane of zona fasciculata cells. Whilst glucocorticoids are released into the systemic circulation in a pulsatile manner according to a distinct circadian pattern, glucocorticoids released in response to stress superimpose upon this existing circadian pattern (Buckingham, 2006) and subsequently activate various physiological processes in the body to assist the organism to cope with stress.

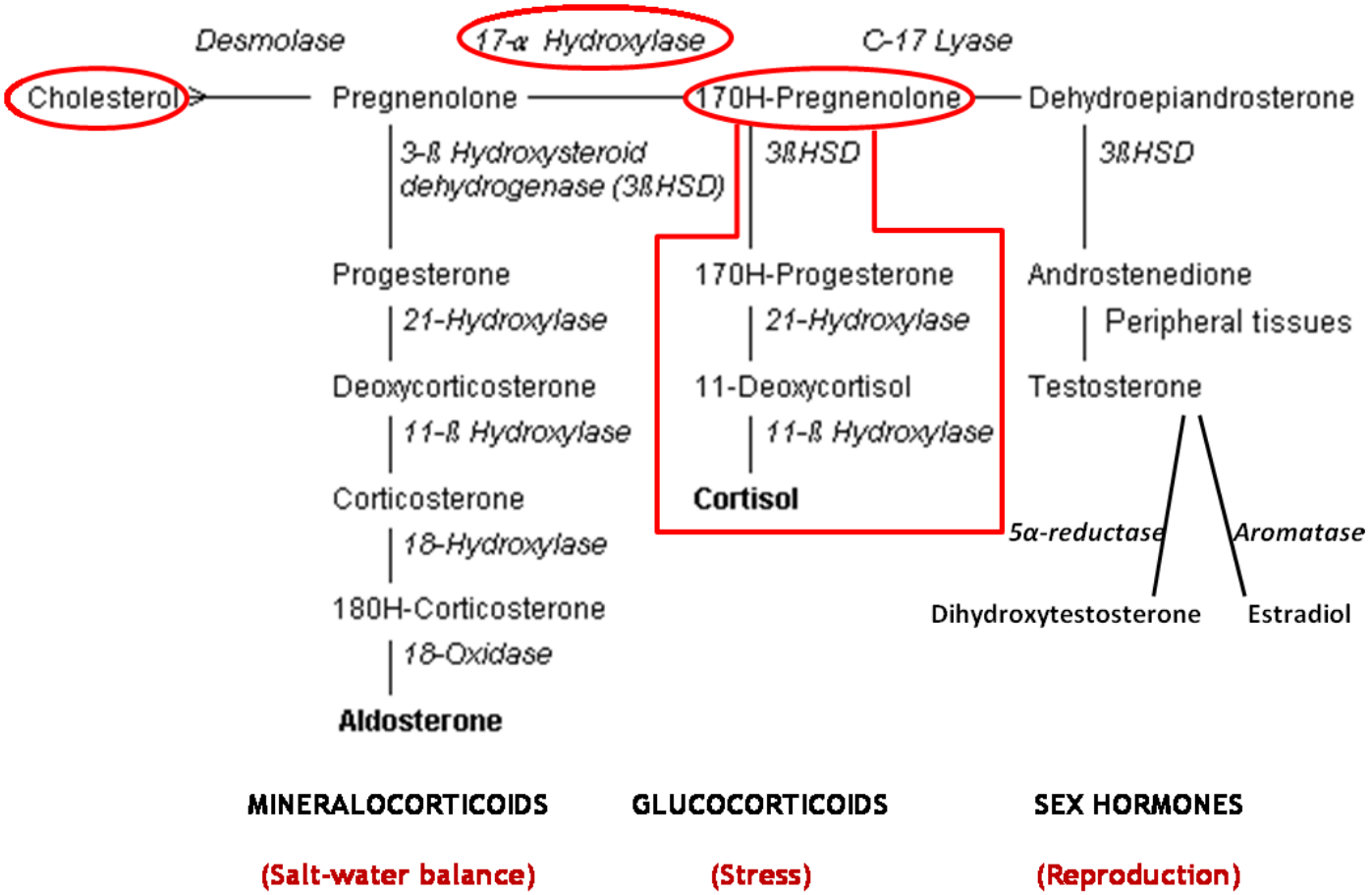

FIGURE 1.5 Enzymatic pathways for conversion of cholesterol to steroids. Red outlined text highlights the pathway for conversion of cholesterol to cortisol. Italics denote enzymes. Adapted from (Deaton et al., 1999, Biason-Lauber, 1998).

1.1.1.4 Mechanism of glucocorticoid action

Glucocorticoid- Glucocorticoid Receptor Binding

Glucocorticoid action is principally mediated via interaction with its intracellular glucocorticoid receptor (GR). The GR is formed of three functionally separate domains; the C-terminal, the central domain and the N-terminal. The C-terminal is the region responsible for binding the glucocorticoid ligand. When unbound to its ligand, the C-terminal of the GR is bound to two heat-shock proteins (HSP) that act as chaperones to prevent the unoccupied GR localizing to the cell nucleus. When the glucocorticoid-GR complex is formed, dissociation of the HSP's from GR occurs, as illustrated in Figure 1.6. The central domain of the GR contains two zinc finger-like projections that interact with DNA; with each finger comprised of a single zinc molecule bound to four cysteine residues. Finally the N-terminal of the GR contains specific amino acid sequences that are involved in activating or inhibiting gene transcription (Schoenmakers et al., 2000, Beato, 1989).

There are three ways through which glucocorticoids exerts their effect. The first is transactivation whereby glucocorticoids bind to GR's to form a glucocorticoid–GR complex. The activated complex, now dissociated from its chaperone HSP's, moves to the cell nucleus where it binds homodimerically to DNA sequences called glucocorticoid-responsive elements (GRE). This allows the recruitment of repressor or activator proteins that inhibit or facilitate the transcription of specific genes (Hebbar and Archer, 2003), for example increasing the transcription of genes encoding anti-inflammatory proteins such as lipocortin-1 and interleukin-10 (IL-10) (Barnes, 1998), while also repressing the pro-inflammatory POMC gene (Drouin et al., 1998). The second way glucocorticoids exert their effects is through interaction of the glucocorticoid–GR complex with transcription factors, such as nuclear factor *k*B (NF-*k*B) (McKay and Cidlowski, 1999). This second mode of glucocorticoid action occurs at lower levels of cortisol than are required for the first mechanism of cortisol action. Finally, the third mechanism involves non-genomic pathways such as glucocorticoid

signaling through membrane-associated receptors. Studies using GR antagonists and GR-knockout mice have demonstrated the involvement of this receptor in non-genomic corticosteroid signaling in neurons of the brain (Karst et al., 2010), and many other studies support their membrane localization (reviewed in (Groeneweg et al., 2012). The fact that intracellularly administered corticosterone does not produce non-genomic effects, and membrane impermeable corticosterone conjugates can induce the same rapid effects as free corticosterone; suggests these receptors are situated outside of the plasma membrane (Groeneweg et al., 2012). Furthermore, electron microscopy studies reveal GR association with neuronal plasma membranes and neuronal postsynaptic membranes (Johnson et al., 2005). In addition, some GR's reside at the plasma membrane in lymphocytes (Bartholome et al., 2004).

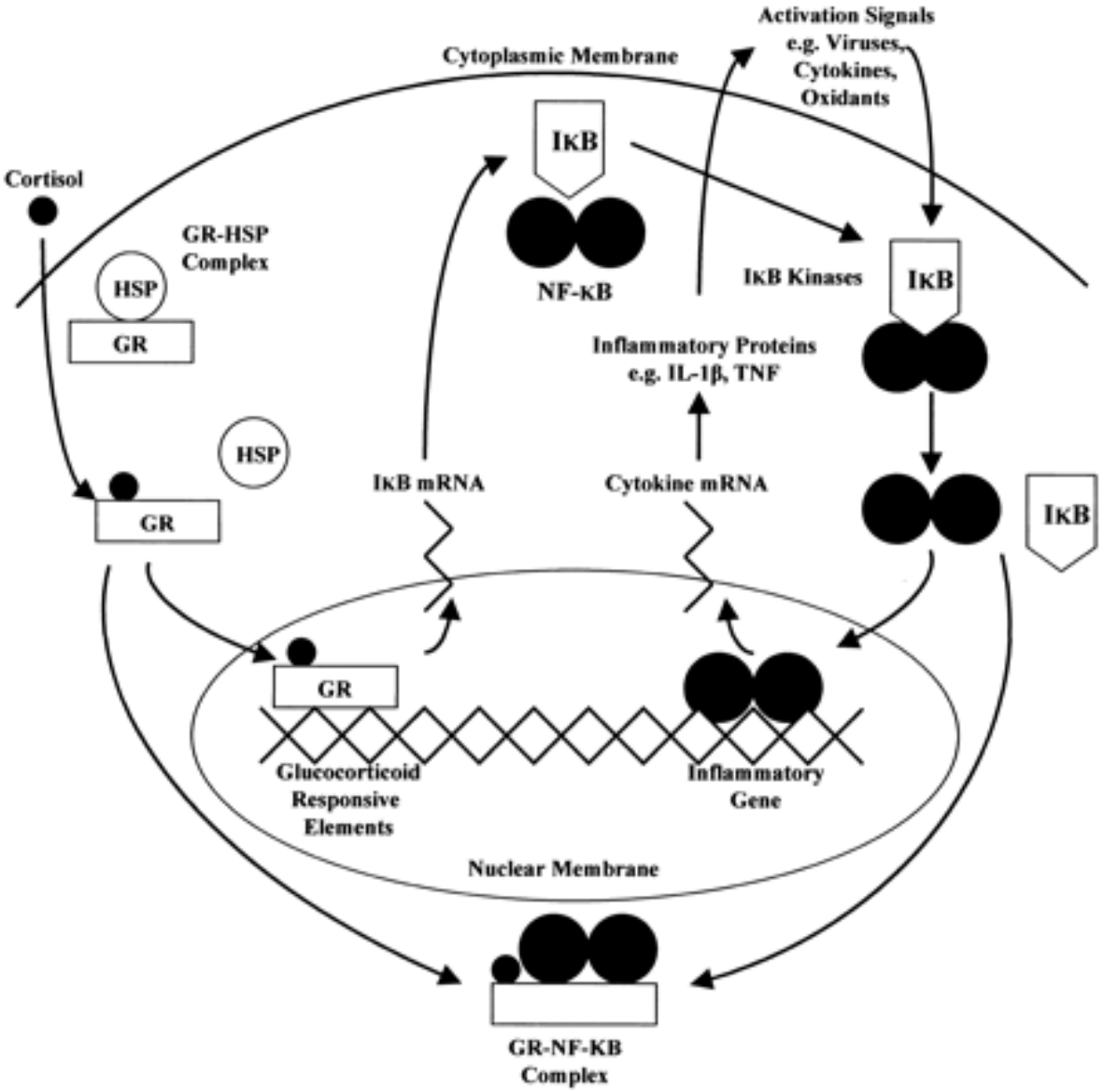

FIGURE 1.6 Diagrammatic representation of glucocorticoid-GR binding and its intracellular effects. Cortisol binds to the GR/HSP complex in the cytoplasm. This leads to HSP dissociation and nuclear translocation of the glucocorticoid-GR complex. Once in the nucleus, the glucocorticoid-GR complex binds to glucocorticoid-responsive elements (GRE) and activates target genes. Abbreviations: GR, glucocorticoid receptor; HSP, heat shock protein; NF-*k*B, nuclear factor kB; I*k*B, NF-*k*B inhibitor; IL-1β, interleukin-1 β; TNF, tumour necrosis factor. Taken from (O'Connor et al., 2000).

Physiological effects of Glucocorticoids

The physiological functions of glucocorticoids are varied, including cardiovascular, metabolic, immunologic, and homeostatic regulation. For example, glucocorticoids have a major effect on glucose homeostasis whereby circulating cortisol levels rise during periods of hunger in an effort to maintain normal blood glucose concentrations via gluconeogenesis and glycogenolysis (Wajchenberg et al., 1984). Their effects on metabolism include promoting the breakdown of carbohydrate and protein and exerting complex effects on lipid deposition and breakdown (McKay and Cidlowski, 2003). They also play a role in the development and homeostasis of T lymphocytes, directly controlling the size of both thymocytes and T-cell numbers (Pazirandeh et al., 2002). Glucocorticoids also have permissive effects on vascular tone, such as raising blood pressure by altering the sensitivity of tissues to catecholamines, and they also exert aldosterone-like actions that regulate electrolyte and water balance (Fan et al., 1975). Glucocorticoids exert complex, and sometimes damaging effects on bone (Kim, 2010) and can have both positive and negative effects on cell growth (Baxter, 1978). Within the CNS, glucocorticoids target neurons and glial cells to support organizational activities in the brain during development, and neuronal plasticity in adulthood (Nichols et al., 2005, de Kloet et al., 2008a). The other central effects of glucocorticoids include changes in mood/behaviour, regulation of food intake, control of body temperature and neuroendocrine function (Pearson Murphy, 2002).

But the most important role of glucocorticoids is most certainly their regulation of the immunologic response to stress. As the end product of the HPA axis, glucocorticoids terminate activation of the stress axis (primarily via a negative feedback mechanism) and regulate the processing, storage and retrieval of stressful experiences (Oitzl et al., 2001, Sapolsky et al., 2000). In rats local infusion of dexamethasone, a synthetic glucocorticoid, into the PVN rapidly inhibits restraint-induced ACTH and corticosterone release (Evanson et al., 2010), while 50 µg/kg intraperitoneal (i.p) injected dexamethasone suppresses the corticosterone response to restraint stress by 80% and completely blocks the otherwise

stimulatory effects of 3 µg/kg i.p injected CRH on corticosterone secretion (Cole et al., 2000).

During a stress response, leukocytes localize at sites of inflammation and activate a cascade of secretory products. To terminate the stress response glucocorticoids inhibit this localisation of leukocytes to inflammatory sites and impede their function, as well as interfering with the function of many other immune cells. The effects of glucocorticoids on endothelial cells are also of profound importance as these cells are the barrier between blood and tissue, and affect hemostasis, vascular permeability and excretion of leukocytes into inflammatory sites (Sternberg and Wilder, 1993). Glucocorticoids inhibit vascular permeability and regulate the expression of adhesion molecules critical to leukocyte localization (Shimizu et al., 1992, Cronstein et al., 1992). In humans, one dose of glucocorticoids can markedly reduce T-lymphocyte levels by redistributing circulating lymphocytes to other areas such as bone marrow (Fauci and Dale, 1974, Fauci et al., 1976); though this effect could be due to changes in the expression of adhesion molecules as described above. Glucocorticoids also antagonize macrophage differentiation (Baybutt and Holsboer, 1990) and therefore indirectly block the release of pro-inflammatory cytokines such as interleukin 1 and 6 (IL-1 and IL-6) and tumour necrosis factor (TNF) (Joyce et al., 1997, Calandra and Bucala, 1997). They inhibit neutrophil adhesion to endothelial cells and at pharmacologic doses impair important neutrophil functions (Sternberg et al., 1992a). Furthermore, glucocorticoids lower circulating eosinophil and basophil levels, and reduce eosinophil and mast cell accumulation at sites of allergic reactions (Sternberg and Wilder, 1993).

1.1.1.4 Other endocrine interactions

Activation of the stress axis also affects growth, reproductive function and the thyroid axis. During an acute stress in humans, glucocorticoids stimulate GH secretion (Casanueva et al., 1990), but during chronic stress GH secretion is attenuated (Raza et al., 1998) and the effects of GH at target tissues are blocked by glucocorticoids (Unterman and Phillips, 1985). This effect of stress on the growth axis may account for the delay in growth seen in children that undergo emotional deprivation, as well as the lack of growth associated with chronic diseases. It is important to note that the effects of acute stress on GH release in rats are oppositional to those seen in humans (Jurcovicova et al., 1984). The stress axis also interacts with the reproductive axis. Many female patients presenting with HPA axis hyperactivity related diseases also experience abnormal menstrual cycles and have impaired reproductive function (Chrousos et al., 1998). This may be because stress mediators such as CRH and glucocorticoids inhibit gonadotropin-releasing hormone (GnRH) release from the hypothalamus, and gonadotropin release from the pituitary gland (Rabin et al., 1988). The thyroid axis is also affected by activation of the stress axis. Somatostatin is an inhibitory neuroendocrine hormone produced in the periventricular nucleus of the hypothalamus. It inhibits the release of GH and TSH from the pituitary gland (Spoudeas et al., 1992) and also inhibits the release of gastrointestinal hormones (Corleto, 2010). CRH-stimulated increases in somatostatin and inflammatory IL-6, a pro-inflammatory cytokine, both inhibit TSH production and secretion (Papanicolaou et al., 1998). In patients with chronic stress system activation, dysregulation of the thyroid axis results in euthyroid 'sick' syndrome whereby thyrotropic feedback control is impaired (Benker et al., 1990).

1.1.2 Regulation of glucocorticoid secretion

Controlled by the hypothalamic suprachiasmatic nucleus (SCh) in mammals (Maywood et al., 2007), circulating glucocorticoids follow a circadian (24 hour) pattern consisting of a succession of pulses of varying amplitude but fixed frequency, of approximately one pulse per hour (ultradian) (Lightman and Conway-Campbell, 2010). Basal levels of glucocorticoids vary throughout the day and peak just before the active phase of the mammal's 24 hour cycle, i.e. in the early morning for humans and in the early evening for nocturnal animals (Cheifetz, 1971) (Van Cauter, 1990). It is thought that this peak in circulating glucocorticoids prepares the organism for the increase in likelihood of experiencing stress throughout their active phase, such as stress from conflict with other organisms or stress from searching for food (Romero, 2002) (Sapolsky et al., 2000). Therefore the strength of the stress response to different types of stress is variable throughout the day. For example, psychological stressors lead to a higher stress response during the resting phase (Kalsbeek et al., 2003), whilst the response to a hypoglycaemic shock leads to a higher stress response at the beginning of the active phase (Gorton et al., 2007).

As mentioned above, the circadian pattern of glucocorticoid release reflects ultradian pulses of hormone release (Conway-Campbell et al., 2012), with these hourly pulses leading to hormone level changes not only in circulation but also in target tissues such as the brain (Droste et al., 2008). The phases of the ultradian pulse are also important in determining the severity of the stress response. Rats are more responsive to noise stress during the rise of the ultradian rhythm whilst their stress response to the same stressor is inhibited during the falling phase of the ultradian rhythm (Windle et al., 1998). It has also been shown that equal spacing of the ultradian pulses is important in maintaining a normal stress response. Chronic stress increases ultradian frequency and decreases responsiveness to an acute stimulus (Windle et al., 2001), with this reduction in responsiveness thought to be a

conditioning of the stress response to the increased probability of the organism experiencing stress due to increases in the number of 'falling phases' within the ultradian rhythm.

Once in circulation, most cortisol is bound to corticosteroid-binding globulins (CBG) in the blood, leaving only approximately 7% cortisol free and therefore biologically active (Westphal, 1971, Kemppainen et al., 1991, Meyer and Rothuizen, 1993). It is thought that this binding of cortisol to CBG's can act as a defence against the possible adverse effects of elevated cortisol levels and can in fact control the accessibility of free cortisol to target tissues and even adjust the rate of cortisol clearance (Breuner and Orchinik, 2002, Siiteri et al., 1982). Only free cortisol is able to enter cells to activate intracellular/membrane receptors and therefore is the only form of cortisol that can be metabolized in the liver (Breuner and Orchinik, 2002). Bound cortisol becomes free by cellular uptake of CBG's. First the cortisol-CBG complex binds to a membrane CBG receptor (CBGR) which transports the complex across the cell membrane. CBG is then cleaved, leaving free unbound cortisol to activate intracellular receptors (Singer et al., 1988, Hryb et al., 1986). Thus CBG-binding can regulate local cortisol levels at specific tissue sites and at the cellular level. There is also evidence of cellular synthesis of CBG which is thought to limit free cortisol availability to intracellular receptors and therefore inhibit cortisol action within specific tissues (Berdusco et al., 1995). Stressful experiences can reduce CBG capacity and therefore increase the availability of local free cortisol to all tissues. For example restraint stress can decrease CBG capacity and cause a prolonged period of elevated free cortisol when total cortisol levels are no longer elevated (Fleshner et al., 1995).

Another major regulator of local glucocorticoid action is 11β-hydroxysteroid dehydrogenase (11β-HSD) (Anagnostis et al., 2009). The 11β-HSD1 isoform is an enzyme that utilizes the reducing agent nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) to hydrogenate inactive cortisol, called cortisone, to its active form. 11β-HSD1 is expressed in

many tissues such as the liver, adipose tissue, fibroblasts, immune cells and the CNS (Cooper and Stewart, 2009). It facilitates glucocorticoid action in target tissue via GR and increased expression of this enzyme has been found in the adipose tissue of obese subjects (Tomlinson et al., 2004). Conversely, the 11β-HSD2 isoform employs the coenzyme nicotinamide adenine dinucleotide (NAD+) to convert active cortisol to cortisone (Yang and Zhang, 2004). 11β-HSD2 is mainly expressed in mineralocorticoid target tissues (Cooper and Stewart, 2009) and rapidly inactivates glucocorticoids to protect the mineralocorticoid receptor (MR) from excess stimulation by cortisol (Cooper and Stewart, 2009).

1.1.2.1 The glucocorticoid feedback mechanism

The HPA axis is regulated in part by a negative feedback effect exerted by glucocorticoids (illustrated in Figure 1.2) at the level of the hypothalamus (Jones et al., 1977), upstream limbic structures such as the hippocampus (Sapolsky et al., 1984), the prefrontal cortex (Hill et al., 2011), and the pituitary gland (Russell et al., 2010). These feedback effects can be fast, mediated by non-genomic mechanisms and occurring as quickly as a few seconds to a few minutes, or slow, involving binding to the GR and changes in gene transcription which can take from 30-60 minutes. To exert a fast feedback effect, glucocorticoids bind to membrane G-protein-coupled-GR's (GpcGR) (Orchinik et al., 1991) on the extracellular surface of CRH-releasing neurons in the PVN. This stimulates endocannabinoid synthesis in post-synaptic neurons, which act on endocannabinoid receptors (CB1) that are localized to glutamatergic terminals impinging on pre-synaptic CRH neurosecretory neurons. Activation of CB1 results in inhibition of pre-synaptic glutamate release, which ultimately reduces the neural activity of parvocellular CRH neurons (Di et al., 2003, Hill and Tasker, 2012). The delayed genomic effects of glucocorticoids are mediated via nuclear GR's (Joels, 1997) and promote recovery and adaptation of the organism to the stress, resulting in normalization of plasma glucocorticoid levels several hours after stress exposure. They include activation of nuclear GR's which raises the threshold of long-term potentiation (LTP)

of signal transmission between neurons, thus reducing the transfer of excitatory information arisen from the stressor (de Kloet et al., 2008c). This results in down regulation of CRH and vasopressin expression in PVN neurons. Glucocorticoids further dampen the stress response by enhancing the inhibitory effects of serotonin (5-HT) and suppressing excitatory β-adrenergic actions (de Kloet et al., 2008b).

1.1.2.2 Diseases linked to HPA dysfunction

HPA dysfunction can lead to, or be the result of, many neuroendocrine diseases. But one common denominator of these diseases is deregulated release of glucocorticoids. Many physical, behavioural and psychiatric disorders are associated with chronic stress i.e. chronic HPA activation, including anxiety, depression, hypertension, obesity, T2DM and insomnia (see Table 1.3). The pathogenesis of these disorders is linked to excessive and constant secretion of stress hormones that influence multiple homeostatic systems (Chrousos and Gold, 1992). Stress mediators, like IL-6, bring about fatigue, nausea and headaches. Chronic hypercortisolism can result in visceral fat accumulation (Chrousos, 2000), insulin hyper-secretion, low GH secretion and hypogonadism-related suppression of libido and fertility (Chrousos et al., 1998, Chrousos and Gold, 1992).

TABLE 1.3 Conditions with altered HPA axis activity. Adapted from (Chrousos, 2009).

Increased activity of the HPA axis	Decreased activity of the HPA axis
Cushing syndrome	Adrenal insufficiency
Chronic stress	Seasonal depression
Melancholic depression	Chronic fatigue syndrome
Anorexia nervosa	Post-traumatic stress disorder in adults
Obsessive-compulsive disorder	Climacteric depression
Panic disorder	Hypothyroidism
Diabetes Mellitus	Rheumatoid arthritis
Post-traumatic stress disorder in children	Asthma/eczema
Hyperthyroidism	Following cessation of glucocorticoid therapy or Cushing syndrome cure
Metabolic syndrome	Following chronic stress

Hyper-activation of the HPA axis, as seen in the genetically CRH-hyper-responsive Fischer rat, which consequently has hypercorticosteronemia, can increase the host's risk of infection and vulnerability to developing tumours, whilst simultaneously reducing susceptibility to inflammatory and autoimmune disease. In contrast, the Lewis rat presents HPA hypo-activation and its resulting hypocortisolism can boost resistance to infection and tumours but increase sensitivity to inflammatory and autoimmune disorders and depression (Sternberg et al., 1992b, Patchev et al., 1992).

HPA-axis abnormalities are also seen in patients with rheumatoid arthritis, who have a weak circadian cortisol rhythm and low ACTH/cortisol levels, despite increased TNF-α, interleukin 1-β (IL-1β) and IL-6 levels (Templ et al., 1996). Rheumatoid arthritis patients often display fatigue and depression, consistent with the behavioural responses seen in the HPA hypoactive Lewis rat. This is also the case with chronic fatigue syndrome patients, who display symptoms of a dysfunctional HPA axis, with low basal cortisol levels in spite of clear ACTH responses to exogenous CRH (Demitrack et al., 1991).

Interestingly, chronic HPA axis hyper-activation can ameliorate the negative effects of excess circulating cortisol. An example of this is the mild immune suppression seen in Cushing's disease, in spite of extremely high hypercortisolism. It is thought that the hypothalamic neurotransmitter, substance P, inhibits CRH neuron activity (Larsen et al., 1993) or that the inflammatory cytokines may be inhibiting the otherwise stimulatory effects of CRH and ACTH on the pituitary and adrenal glands (Jaattela et al., 1991), as seen in septic shock (Rothwell et al., 1991).

1.2 Effects of leptin on HPA physiology

1.2.1 Leptin

In 1949 random single mutations of a gene, named *ob* at the time, were found in a mouse colony at the Jackson Laboratory. These mice, named *ob/ob*, presented with severe obesity, rapidly gaining weight until they were about four times their normal size. They were hyperphagic, had mild diabetes and were sterile (Ingalls et al., 1950). Then in 1965, the db/db was discovered; a mutant obese mouse with severe obesity and hyperphagia, but that, unlike the *ob/ob* mouse, presented with severe life-shortening diabetes (Hummel et al., 1966). Later it was found that the mutated gene in the *ob/ob* mouse codes for a 167 amino acid protein hormone named leptin (Zhang et al., 1994) and that the mutated gene in the *db/db* mouse codes for the leptin receptor (LepR). These findings allowed the first observations of the effects of leptin deficiency/resistance on energy intake and expenditure. Administration of exogenous leptin can reverse the excessive weight gain seen in *ob/ob* mice (Harris et al., 1998). Although the mouse and human leptin genes are located on chromosome 6 and 7 respectively, structurally they closely resemble one another (Sone and Osamura, 2001). Leptin is largely synthesized in the adipocytes of subcutaneous white adipose tissue (WAT) and is secreted in proportion to adipocyte triglyceride content (Scherer and Buettner, 2011). Some leptin is also synthesized in visceral WAT, brown adipose tissue (BAT), the placenta, ovaries, skeletal muscle, the stomach, mammary epithelial cells, bone marrow, the pituitary gland, the liver and the brain (Morash et al., 1999, Hoggard et al., 1997, Bado et al., 1998, Wang et al., 1998, Margetic et al., 2002), though out of these BAT is the only notable contributor to circulating leptin (Margetic et al., 2002).

The LepR is found in numerous peripheral organs, such as the heart, liver, lung, ovaries, pituitary gland, pancreas, skeletal muscle, adrenal gland, testis (De Matteis et al., 1998) and adipose tissue (Siegrist-Kaiser et al., 1997). Within the brain, leptin acts on the choroid plexus, the ventral tegmental area (VTA), and the hypothalamic ARC, PVN, VMN and DMN to signal the extent of the body's fat stores (Collins et al., 1996). Leptin binds to six different isoforms of the LepR, named LepRa-f (Wang et al., 1996).

Leptin has several well characterized actions, including the regulation of body fat by inhibiting food intake, increasing energy expenditure and suppressing lipogenesis, modulating T-cell activity and the mammalian ovulatory cycle, promoting angiogenesis and surfactant expression in type II pneumocytes, regulating bone metabolism and inflammatory responses, and influencing the stress response via the HPA axis (Roubos et al., 2012). Leptin has a major role in energy homeostasis, with leptin-deficient or leptin resistant rodents and humans displaying features of the metabolic syndrome (Ahima et al., 1998, Halaas et al., 1995, Chua et al., 1996).

The satiety inducing effects of leptin are mediated in the brain, at the level of the hypothalamus via activation of the only isoform expressed in this region, LepRb. Leptin activates POMC neurons of the ARC which release α-MSH onto downstream target neurons in regions including the PVN. Here α-MSH acts on MC4R and melanocortin-3 receptors (MC3R) to inhibit food intake and increase energy expenditure (Morton et al., 2006, Elmquist et al., 2005). At the molecular level, the leptin-LepRb complex activates janus kinase 2 (JAK2) (Kloek et al., 2002), of the JAK family of tyrosine kinases, which in turn phosphorylates intracellular signal transduction and transcription (STAT) proteins that stimulate the transcription of target genes to mediate the cellular effects of leptin (Schwartz et al., 2000). Some of leptin's major effects do not require STAT signals and occur through other, STAT-independent, signalling pathways. One of these is the mitogen-activated

protein kinase (MAPK) signal transduction pathway, which can be activated not only by LepRb, but also by the short form of LepR, LepRa (Malendowicz et al., 2006, Bjorbaek et al., 1997).

1.2.2 Leptin Regulation of the Stress axis

Leptin has a putative role in the regulation of the HPA axis (Maffei et al., 1995). Leptin deficient *ob/ob* mice have elevated levels of plasma corticosterone and i.p injection of leptin to *ob/ob* mice reduces food intake and body weight (Halaas et al., 1995), but also significantly reduces plasma corticosterone levels (Ahima et al., 1998), suggesting that leptin exerts an inhibitory influence on HPA function. Leptin administration has also been shown to blunt the stress-induced activation of the HPA axis, with normal fasted mice pre-administered leptin demonstrating a lower rise in plasma corticosterone levels following exposure to restraint stress (Heiman et al., 1997). It has been suggested that the inhibitory effect of leptin on HPA function may be mediated by changes in CRH release from the hypothalamus, since leptin does not directly influence basal or CRH-stimulated ACTH release from rat pituitary cells *in vitro* (Heiman et al., 1997). Leptin has also been shown to act directly on the adrenal gland to inhibit cortisol release (Gaillard et al., 2000).

However, there is also evidence that leptin can act to stimulate the HPA axis under specific conditions. ICV administration of leptin has been shown to increase CRH and CRH-receptor type 2 (CRH-R2) expression in the PVN, and to stimulate ACTH and corticosterone release (Jethwa et al., 2005). Evidence suggests that the stimulatory effects of leptin on HPA axis function may be mediated by the hypothalamic neuropeptide Neuromedin U (NMU). Leptin stimulates the release of NMU from *ex vivo* hypothalamic explants, and hypothalamic NMU mRNA expression is reduced in conditions of low circulating leptin (Jethwa et al., 2005).

Chronic administration of glucocorticoids to rats stimulates leptin gene expression in adipose tissue, an effect which is followed by reduced weight gain and food intake (De Vos et al., 1995). In both humans and rats, corticosteroid treatment increases plasma leptin levels (Slieker et al., 1996, Larsson and Ahren, 1996). However, in some cases, chronic activation of the HPA axis has been shown to reduce basal plasma leptin levels whilst simultaneously elevating plasma corticosterone levels (Lu et al., 2006).

1.3 Effects of social isolation (SI) on HPA physiology

1.3.1 SI and HPA function

Social isolation (SI) is a stressful experience in all species (Weiss et al., 2004, Arango et al., 2001) and has been shown in rodents to increase aggression, reactivity and anxiety in response to human handling, (Hatch et al., 1965). In male rats, SI results in elevated basal plasma stress hormones, corticosterone and ACTH, compared to group-housed rats, as well as a higher stress hormone response to an acoustic stimulation-restraint stress challenge compared to their group-housed counterparts (Weiss et al., 2004, Holson et al., 1991). Thus chronic stress in the form of SI leads not only to increased HPA activity but also increased HPA sensitivity to subsequent stressors. It has been suggested that chronic stress leads to alterations in the glucocorticoid feedback mechanism, resulting in permanently elevated levels of corticosterone under both basal and acute stressful conditions (Ferland and Schrader, 2011).

However, other studies have shown that SI causes HPA hypo-reactivity in response to a novel stressor, suggesting that desensitization to stressful stimuli occurs after a period of SI (Sanchez et al., 1998). In fact, Serra et al found that long term SI lowers basal plasma ACTH levels, increases pituitary sensitivity to CRH and impairs the glucocorticoid negative feedback to the hypothalamus in rats (Serra et al., 2005). In this case, lowered basal ACTH levels after SI could be explained by the constant stimulation of the HPA axis diminishing readily available stores of ACTH.

These neuroendocrine changes are thought to be a result of alterations of neurotransmitter signalling associated with stress. The serotonergic signalling system is the most widely studied transmitter system in the field of social stress. Many different stressors increase 5-HT neurotransmission and its actions are mediated by its receptor, 5-HTR. CRH and AVP are both stimulated by the serotonergic system and in patients with melancholic depression,

an exaggerated and prolonged activation of the stress axis is thought to be partly a result of altered hippocampal serotonin levels (Chrousos, 1995). These SI-induced neurochemical changes can promote anxiolytic/depressive behaviours as seen in male Lewis rat which demonstrates reduced locomotion and increased behavioural reactivity in an elevated plus maze test, compared to group-housed rats (Berton et al., 1997). Stress alters both pre- and postsynaptic transporters for 5HT, increases 5HT binding in the cortex and has been shown to decrease 5HT binding in the hippocampus (Flugge, 1995, McKittrick et al., 1995). Social stress-related HPA dysfunction is also linked with dysregulation of the dopaminergic and noradrenergic signalling systems, as commonly seen in rodent models of schizophrenia (Sontag et al., 2003). Furthermore, a dysfunction of GABA receptor (GABA-R) signal transduction is involved in the pathophysiology of mental disorders such as depression (Romeo et al., 1998). Social stress activates CRH circuits directly connected to the HPA axis and increases pituitary POMC and β-endorphin mRNA levels (Meyerhoff et al., 1988), with an increase in pre-synaptic activity corresponding with a down-regulation of the post-synaptic activity. On the other hand, social stress inhibits AVP circuits involved in aggression and sexual behaviour in rats, but this is thought to be associated with a decrease in testosterone in these animals (Albeck et al., 1997). Behaviours mediated by the amygdaloid vasopressinergic circuit include copulation (Smock et al., 1992) and social recognition (Bluthe et al., 1990), both of which are changed in socially stressed rats and are relevant to altered human behaviours in depressive patients (Yang et al., 2012, Gunderson, 1963).

1.3.2 Diseases linked to SI

1.3.2.1 Depression

Depression is a state of labile mood and comprises a mixture of some of the following symptoms: hypersomnia/insomnia, agitation, anorexia, weight loss/gain, phobic anxiety, somatic complaints, restlessness, fatigue, irritability and feelings of sadness/loss (Bielski and Friedel, 1977). There are many causes of depression including negative life events, side effects of a medical treatment such as beta blockers (Luijendijk et al., 2011), having a genetic predisposition to depression, or having another medical condition such as diabetes (Baskaran et al., 2013).

Abnormalities of the HPA axis are implicated in major depression and many depressed patients have high salivary, plasma and urine cortisol levels, as well as enlarged and hyperactive pituitary and adrenal glands (Nemeroff and Vale, 2005). This is thought to be related to an impaired glucocorticoid feedback mechanism, supported by studies showing that the HPA axis in depressive patients is insensitive to orally administered dexamethasone compared to healthy subjects (Pariante, 2006). Interestingly, successful treatment of depression is related to reinstatement of a fully functioning negative feedback loop (Pariante, 2006). A susceptibility to depression can be programmed through early life events. Separating neonatal rodents from their mothers long term induces permanent HPA axis changes resembling those seen in depressed adult individuals (Sanchez et al., 2001). Serotonergic signalling dysfunction has also been implicated in the pathogenesis of depression (Gardner et al., 2005). Also important are recent findings that show increased plasma IL-6, IL-1β and TNF-α in depressed patients (Raison et al., 2006). These cytokines interact with many of the pathophysiological areas that characterize depression including synaptic plasticity, metabolism of neurotransmitters, neuroendocrine function, and behaviour (Raison et al., 2006). The importance of MR in the regulation of HPA activity is also significant. MR has a higher affinity for glucocorticoids than GR (McEwen et al., 1968), with

MR mainly present in limbic areas such as the hippocampus and GR widely spread across the whole brain (Reul and de Kloet, 1985). The synergy of MR/GR activation is important in mediating the glucocorticoid feedback mechanism and some suggest that major depression is accompanied by a change in MR/GR balance (Young et al., 2003). This has been investigated in humans where Young et al found that MR is more functionally active in depressed patients than GR, and GR function is in fact impaired in these patients compared to controls (Young et al., 2003). Furthermore, MR inhibits hippocampal serotonin release and turnover, while high levels of glucocorticoids can induce GR-stimulated serotonin signalling (Joels and de Kloet, 1994).

1.3.2.2 Anxiety

Anxiety, the feeling of fear and worry, is characterized by somatic, emotional, cognitive, and behavioural modifications (Ghadirian, 1981), and is usually accompanied by restlessness, fatigue and muscle tension. Although a normal reaction to a stressor, anxiety can become overwhelming and manifest as an anxiety disorder. As in depression, HPA axis hyperactivity is found in patients with anxiety disorders such as panic disorder (Abelson et al., 2007) and social anxiety disorder (Condren et al., 2002). It is thought that both glucocorticoid insensitivity and CRH over-expression contribute to overall HPA axis hyperactivity (Kunugi et al., 2006) in these patients. Over-activity of the amygdala (Pfleiderer et al., 2007) is also associated with the pathogenesis of anxiety disorders. Many anxiety patients have over-activation of the amygdala. The known role of the amygdala in fear and aggression, and as one of the main extra-hypothalamic sources of CRH, gives rise to the idea that amygdala-secreted CRH may drive this over-activation in these patients.

1.3.2.3 Schizophrenia

Schizophrenia is a mental disorder defined by a breakdown of thought processes and poor emotional responsiveness (Krystal et al., 2001). Symptoms include hallucinations, paranoia, disorganized speech and thinking, and social dysfunction (Braff and Geyer, 1990). Whilst genetics play an important part in the incidence of schizophrenia, the environment, psychological and social processes are also contributory factors (Corcoran et al., 2003) (Krystal et al., 2001). Schizophrenia affects cognition, behaviour and emotion, and patients thus commonly present additional conditions such as depression and anxiety (Krystal et al., 2001).

Stressful life events increase the risk of schizophrenia (Ellenbroek et al., 1998). Schizophrenic patients have higher-than-normal plasma noradrenaline and cortisol levels (Meltzer, 1987), and generally have a higher stress hormone response to a novel stressor (Lammers et al., 1995). This has been shown in studies in which treatment with 2-deoxy-D-glucose and dexamethasone induced a higher level of metabolic stress in schizophrenics than in controls, as determined by elevated plasma ACTH levels (Elman et al., 1998, Muck-Seler et al., 1999). Although 2-deoxy-D-glucose is not a classic stressor, pharmacological doses of this glucose analogue block glycolysis and thus prevent neuronal glucose uptake. This results in hypoglycemia (Horton et al., 1973) which, although different from environmental stress that may be more relevant to the schizophrenic state, still activates appropriate stress-related neuroendocrine systems (Breier, 1989).

Others found that the benzodiazepine antagonist iomazenil and the serotonin agonist, m-chlorophenylpiperazine (m-CPP) act synergistically to increase cortisol levels and produce mild psychosis in normal people (D'Souza et al., 2006), but induce a more robust cortisol response accompanied by more severe psychotic symptoms when administered to schizophrenics, (Lindenmayer et al., 1997). Reduced numbers of hippocampal GR's is one

potential source of elevated glucocorticoid levels in schizophrenic patients (Corcoran et al., 2003). Reduced GR mRNA expression and reduced prefrontal cortex MR mRNA expression has been found in the amygdala of schizophrenics (Perlman et al., 2004) (Xing et al., 2004). Some schizophrenics have also been found to have decreased GR mRNA levels in the frontal cortex, dentate gyrus, and hippocampus (Webster et al., 2002).

1.4 Hypotheses and aims

Leptin has several well characterized functions, including regulation of body fat stores, food intake, and energy expenditure (Collins et al., 1996, Jethwa et al., 2005, Hamann and Matthaei, 1996). There is evidence to suggest that leptin can also have both stimulatory and inhibitory effects on the HPA response to stress (Ahima et al., 1998, Heiman et al., 1997, Jethwa et al., 2006). The alterations in the HPA axis observed in obesity may be mediated by leptin as most obese patients have elevated circulating levels of leptin (McGregor et al., 1996). The role of leptin in the regulation of HPA activity therefore remains unclear. This project will further investigate the effect of leptin on the HPA axis

SI can alter HPA function in rats and humans (Thornton et al., 2010, Weiss et al., 2004). In rats, SI is sufficiently stressful to lead to aggression and anxiety in response to human handling (Hatch et al., 1965, Weiss et al., 2004). In humans, loneliness can lead to the pathogenesis of psychiatric disorders such as schizophrenia and depression (Pariante and Lightman, 2008, Kaye et al., 1987, Corcoran et al., 2003). While some evidence suggests that SI causes hyper-activation of the HPA axis in response to a novel stress (Weiss et al., 2004), possibly caused by the impairment of the glucocorticoid negative feedback loop (Serra et al., 2005, Raone et al., 2007), others show that SI causes hypo-activation of the HPA axis in response to an acute stress, indicating desensitization to stressful stimuli (Sanchez et al., 1998). This poses the question of whether the three components of the HPA axis are more sensitive or less sensitive to stressful stimuli after a period of SI and how these changes within the stress axis actually occur. We therefore aimed to investigate an animal model of SI using the Sprague Dawley rat; specifically looking at the effects of SI on HPA function within multiple HPA tissues.

1.4.1 Hypotheses

1.4.1.1 Hypothesis 1

I hypothesize that leptin will modulate the acute HPA response to LPS treatment in rats.

To elucidate the effects of leptin on the HPA axis I will:

- Investigate the effects of acute leptin administration on basal/acute LPS-stimulated HPA axis activity and neuronal activation in hypothalamic, and other stress-related brain centres in male rats.

1.4.1.2 Hypothesis 2

SI can alter the HPA axis response to a novel acute stress in the adult rat, by sensitizing and/or desensitizing specific components of the axis.

To test this hypothesis I aim to:

- Investigate the effects of chronic and intermittent SI on HPA function within multiple HPA tissues in adult rats; specifically looking at basal HPA activity, differences in HPA axis responsiveness to acute stress and neuronal activation in stress related hypothalamic centres.
- Examine the stress hormone profiles of isolated compared to group-housed rats.
- Investigate possible changes in the HPA-glucocorticoid feedback mechanism.
- Investigate possible changes in the expression of stress-related genes in specific components of the HPA axis

Chapter II:

Investigating the effects of leptin on the HPA axis response to stress

2.1 Introduction

2.1.1 The HPA axis as a target of leptin

As briefly described in Chapter 1, leptin has been reported to exert contradictory effects on the HPA axis, both basally and in response to stress (Halaas et al., 1995, Ahima et al., 1998, Jethwa et al., 2005). It also plays an important role in the regulation of appetite by inhibiting both secretion of gastric ghrelin and the stimulation of feeding by ghrelin (Kalra et al., 2005), and simultaneously reducing orexigenic hypothalamic NPY and increasing anorexigenic hypothalamic α-MSH expression (De Vos et al., 1995) (Dhillon et al., 2000). Below I outline the known effects of leptin on individual components of the HPA axis.

2.1.1.1 Leptin and the hypothalamus

Circulating leptin is thought to cross the BBB to access the brain by a mechanism involving its binding to the short form of the leptin receptor, LepRa (Kastin et al., 1999), though other LepR isoforms may also be involved (Kastin et al., 1999). Although the mechanisms are not well understood, it is thought that the LepR's involved are located in non-neuronal cells of the choroid plexus, meninges and blood vessels (Price et al., 2010).

The hypothalamus is a major central target of leptin. LepRb mRNA is widely distributed in the hypothalamus, being expressed in the ARC, DMN, VMN, periventricular hypothalamic nucleus, LHA, medial mammillary nucleus and posterior hypothalamic nucleus (Elmquist et al., 1998b). It is thought that upon binding to leptin, the hypothalamic LepRb is internalized via clathrin-coated vesicles into early endosomes (Sweeney, 2002). Subsequent oligomerization of the leptin-LepRb complex allows segregation of JAKs which then phosphorylate and activate each other (Sweeney, 2002), LepRb itself (Bjorbaek et al., 1997) and STAT proteins (Ghilardi et al., 1996). Phosphorylated STAT (pSTAT) then dimerizes and translocates to the cell nucleus to bind to DNA and activate gene transcription (Roubos

et al., 2012) (see Figure 2.1). Peripheral and intra-hypothalamic injection of leptin induce pSTAT3 in the hypothalami of *ob*/*ob* and wild type mice (Tartaglia et al., 1995, El-Haschimi et al., 2000), suggesting that the hypothalamus is a direct target of leptin (Vaisse et al., 1996). In accord with this, ob/ob mice and leptin-deficient humans present with hypothalamic neuroendocrine disorders such as hypothalamic hypothyroidism (van der Kroon et al., 1981, Ozata et al., 1999) and hypothalamic amenorrhea in humans (von Schnurbein et al., 2012). These abnormalities are reversible via leptin replacement therapy (Chan et al., 2006).

The effects of leptin on the stress response are largely thought to be mediated in the hypothalamic PVN. But the actions of leptin on the hypothalamus are a source of controversial debate as leptin can both inhibit and stimulate the release of the same neuro-hormones from the hypothalamus, by acting under different circumstances or differentially on specific neuronal circuits. Leptin can down-regulate CRH expression and CRH release from the PVN of adrenalectomized *ob/ob* mice (Arvaniti et al., 2001), suggesting an inhibitory role of leptin on the hypothalamic component of the stress axis. In obese Zucker rats, a fasting-induced reduction circulating leptin levels is coupled with increased cellular-fos (c-fos) mRNA expression, an early marker of neuronal activation, in the ARC and PVN. This c-fos activation can be by a CRH antagonist (Doyon et al., 2007), suggesting a stimulatory role of leptin on hypothalamic CRH expression.

But there is also evidence that leptin can have a stimulatory effect on the HPA axis, with ICV or systemic leptin administration previously shown to increase hypothalamic CRH 2 hrs after administration (Uehara et al., 1998) and incubation of rat and mouse hypothalamic explants with leptin resulting in enhanced CRH release (Costa et al., 1997, Raber et al., 1997). Leptin injection into 40hr-fasted rats increases CRH mRNA levels in the PVN (Schwartz et al., 1996).

Whilst the PVN is LepRb rich, there is evidence that much of leptin's control over the PVN actually occurs via the ARC (Elmquist et al., 1998a, Elmquist et al., 1998c, Patterson et al., 2011, Schwartz et al., 1996). Lesioning ARC-to-PVN projecting fibres in rats increases plasma leptin concentrations and reduces HPA-axis activity, as demonstrated by reduced plasma ACTH concentrations in response to restraint stress (Bell et al., 2000). These lesioned rats also show increased food intake, weight gain, and loss of α-MSH in the ARC. This strongly suggests that the ARC plays a role in leptin regulation of the PVN (Bell et al., 2000).

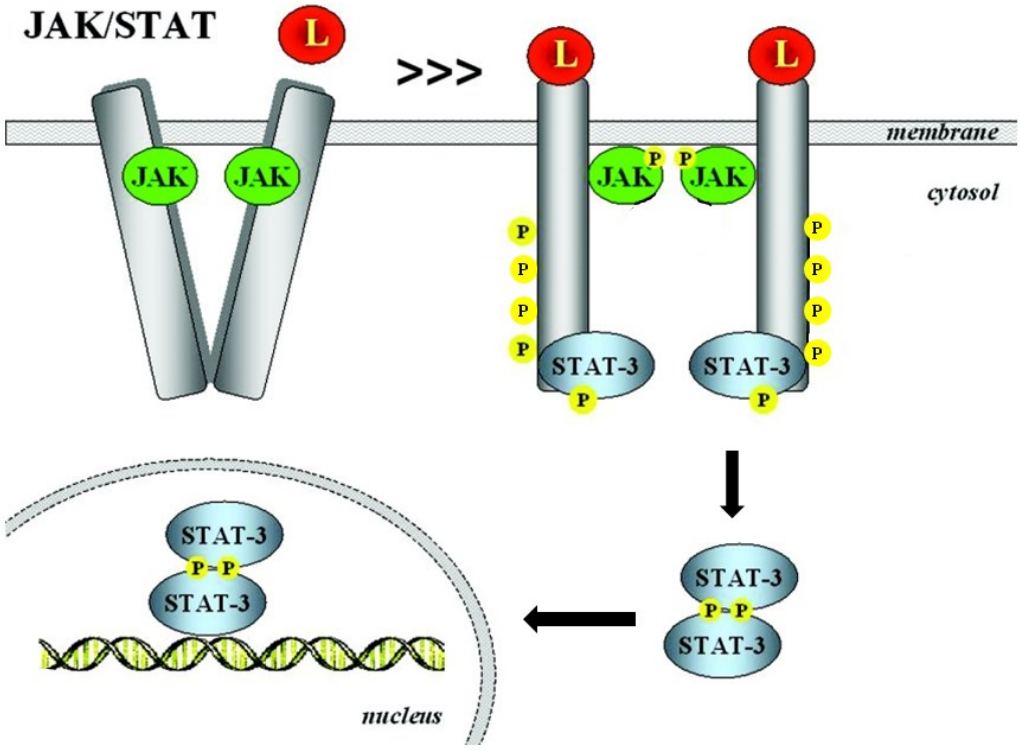

FIGURE 2.1 Leptin and LepRb activation of the JAK/STAT intracellular signalling pathway. Leptin binds to LepRb and causes a conformational change that allows segregation of JAKs, which then become activated and tyrosine-phosphorylate other JAKs, LepRb's and STAT's. This phosphorylation of STATs leads to their dissociation from LepRb, dimerization and then translocation to the cell nucleus to regulate gene expression by binding to promoter regions of target genes. Abbreviations: LepRb, leptin receptor B; JAK, janus kinase; STAT3, signal transduction and transcription 3; P; phosphate, L; leptin. Adapted from (Fruhbeck, 2006).

2.1.1.2 Leptin actions on the pituitary gland

Leptin regulation of hypothalamic CRH, whether inhibitory or stimulatory, affects ACTH secretion from corticotroph cells of the anterior pituitary and thus glucocorticoid production and secretion from the adrenal glands (Heiman et al., 1997, Jethwa et al., 2006). However, it is also possible that leptin acts directly on the pituitary gland. Zamorano *et al* showed that LepR mRNA is expressed in the rat anterior pituitary, albeit at low levels (Zamorano et al., 1997), and others have shown LepR immunoreactivity in corticotroph cells of the ovine anterior pituitary (Iqbal et al., 2000). Furthermore, obese humans that lack leptin signalling have pituitary dysfunction (Clement et al., 1998), though this may reflect a lack of leptin signalling in the hypothalamus rather than defective signalling in the pituitary itself. Other studies show that incubation of rat primary pituitary cells with leptin *in vitro* does not affect ACTH release (Heiman et al., 1997). Leptin is also found in corticotroph cells and in the posterior pituitary (Louis et al., 2011, Ogasawara et al., 2008, Jin et al., 1999, Morash et al., 1999, Siawrys et al., 2007), suggesting that cells of the pituitary gland may self-regulate by autocrine or paracrine leptin-based mechanisms.

2.1.1.3 Leptin and the adrenal gland

As mentioned above, evidence suggests that leptin can indirectly suppress glucocorticoid secretion from the adrenal gland by inhibiting hypothalamic CRH and pituitary ACTH release in rats (Malendowicz et al., 2007). But the adrenal gland may also be a direct target of leptin as LepRa-f mRNA and protein have been found in human, rat and bovine adrenocortical cells(Glasow and Bornstein, 2000, Pralong et al., 1998, Hoggard et al., 1997, Malendowicz et al., 1997, Bornstein et al., 1997). Pre-incubation of human primary adrenocortical cells with leptin inhibits ACTH-stimulated cortisol release, while leptin inhibits ACTH-stimulated corticosterone secretion from primary rat adrenocortical cells (Pralong et al., 1998). The authors suggest these effects are likely mediated by LepRb as its transcript was found to be expressed in the adrenal tissue and leptin had no inhibitory effect in adrenal glands obtained from db/db mice (Pralong et al., 1998). A similar effect is seen in primary bovine adrenocortical cells which, after incubation with leptin at concentrations comparable to those observed in obese individuals, have reduced basal cortisol secretion and attenuated ACTH-stimulated cortisol release (Bornstein et al., 1997).

2.1.2 Interactions of leptin with appetite regulating neuropeptides and hormones

Leptin interacts with many appetite-regulating neuropeptides and hormones (see Figure 2.2). While leptin inhibits the activity of orexigenic NPY /AgRP-releasing neurons (Hahn et al., 1998), it stimulates the activity of anorexigenic CART/POMC-derived α-MSH releasing neurons of the ARC, thus promoting a state of negative energy balance (Elias et al., 1998, Elias et al., 1999). The melanocortin system is a neural system that regulates energy homeostasis, and within the hypothalamus, controls body weight (Cone, 1999, Hagan et al., 1999). While the melanocortin system is involved in leptin-appetite regulation, it is also thought to interact with the stress axis as both α-MSH and AgRP stimulate the HPA axis (Dhillo et al., 2002).

Leptin also influences peripheral appetite-regulating hormones such as ghrelin, the orexigenic gut hormone (Inui et al., 2004). Circulating leptin and ghrelin demonstrate a reciprocal relationship with ghrelin levels rising and leptin levels falling before a meal and vice versa postprandially (Crowley et al., 2005, Xu et al., 1999). There is little evidence to suggest leptin directly inhibits ghrelin, with the majority of evidence suggesting that leptin restrains the ghrelin induced activation of hypothalamic appetite-stimulating circuits. Leptin can directly inhibit ghrelin transcription *in vitro* (Zhao et al., 2008) and decrease ghrelin release from isolated rat stomach (Kamegai et al., 2004). In the hypothalamic ARC, ghrelin binds to ghrelin receptors to stimulate NPY/AgRP-releasing neurons (Kalra et al., 2003) to release NPY, AgrP, and GABA in the PVN and ultimately induce feeding (Kalra et al., 2003, Kalra et al., 1999, Kalra and Kalra, 2004, Bagnasco et al., 2003, Yokosuka et al., 1998). Leptin counteracts this by suppressing NPY synthesis, release, and action in the ARC and PVN (Kalra et al., 1999), as demonstrated in Figure 2.2.

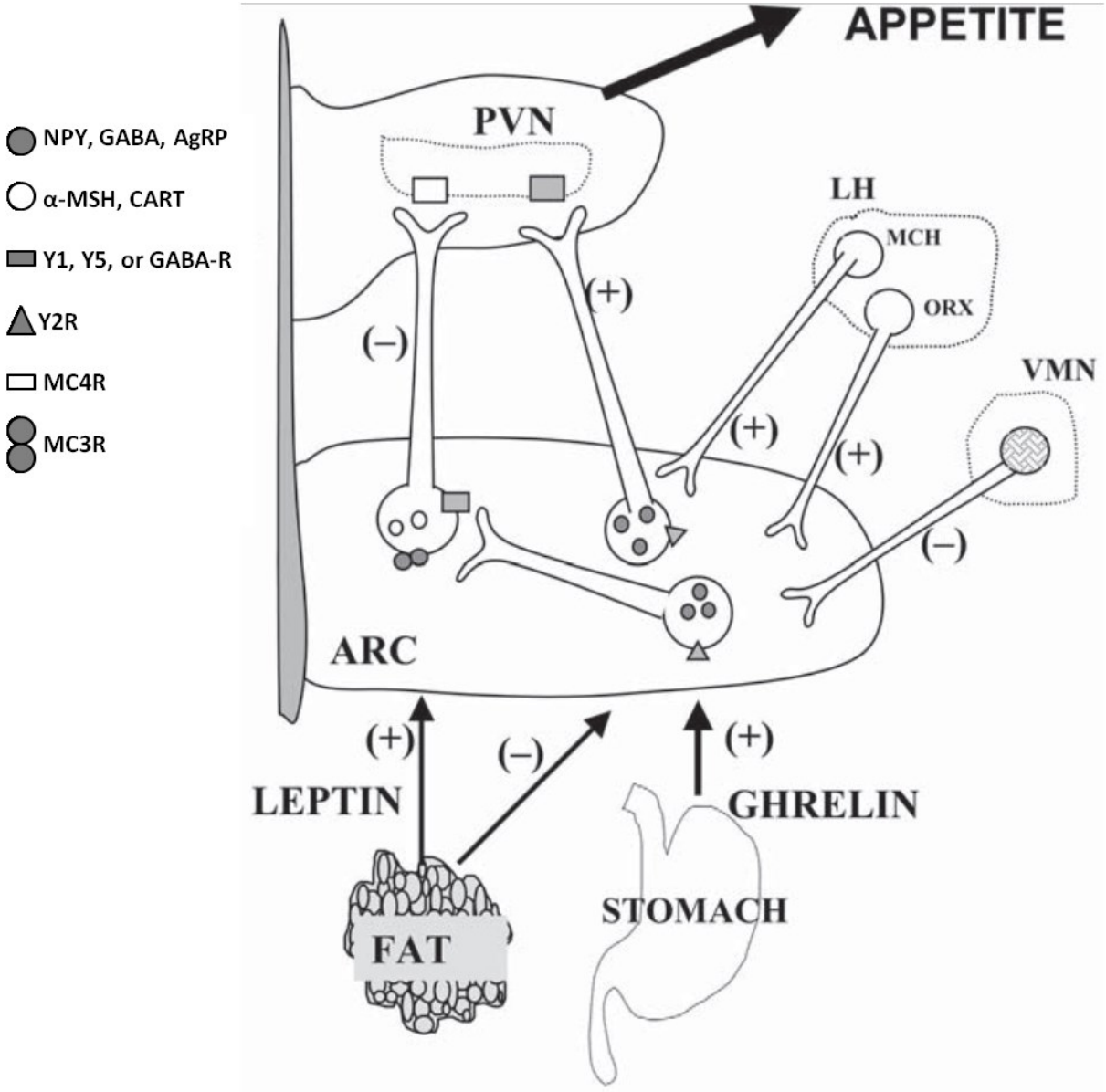

FIGURE 2.2 Diagrammatic representation of leptin and ghrelin modulation of the hypothalamic appetite-regulating circuitry. NPY/AgRP and GABA-expressing neurons in the ARC project into the PVN where NPY, AgrP, and GABA stimulate appetite by activating Y1/Y5, MC4R, and GABA-R respectively. Simultaneously, NPY, AgRP, and GABA released in the ARC act on Y1/Y5, MC3R, and GABA-R to suppress POMC/α-MSH/CART-expressing neurons' restraint on appetite. Leptin inhibits appetite by restraining NPYergic signalling and stimulating melanocortin signalling, while ghrelin stimulates appetite enhancing NPYergic signalling. Abbreviations: PVN, paraventricular nucleus; ARC, arcuate nucleus; NPY,

neuropeptide Y; GABA, gamma-aminobutyric acid; AgRP, agouti related peptide; α-MSH, α-melanocyte-stimulating hormone; CART, cocaine and amphetamine regulated transcript; Y1/Y5, NPY receptor type 1 and type 5 respectively; GABA-R, GABA receptor; Y2R, NPY receptor type 2; MC4R, melanocortin 4 receptor; MC3R, melanocortin 3 receptor. Adapted from (Kalra et al., 2003).

2.1.3 Bacterial Lipopolysaccharide

Lipopolysaccharide (LPS) is an endotoxin that elicits a strong immune response in mammals and which at very high doses can cause septic shock (Priest et al., 1989). It is a major component of the outer surface membrane of gram-negative bacteria (Webster et al., 1955) and plays a role in maintaining the structural integrity of the bacteria as well as protecting their cell membrane from attack. LPS consists of a polysaccharide component, covalently bonded to a lipid moiety called lipid A which anchors the polysaccharide into the outer bacterial membrane (Seydel et al., 2003).

2.1.4.1 Lipopolysaccharide signalling

The LPS signalling pathway, illustrated in Figure 2.3, begins with circulating LPS being recognized by LPS-binding protein (LBP) (Schumann et al., 1990), which chaperones LPS to soluble cluster of differentiation 14 (CD14), forming a ternary complex (Hailman et al., 1994, Tobias et al., 1995). LBP and CD14 then aid the loading and docking of LPS onto the LPS receptor complex, which is composed of dimerized toll-like receptor 4 (TLR4) receptors and two molecules of the extracellular adapter myeloid differentiation factor-2 (MD-2), all situated at the cell membrane (Tobias et al., 1995, Hailman et al., 1994). The LPS-LPS receptor complex utilizes adapter proteins in order to operate, including myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (MAL), toll-IL-1 receptor (TIR)-containing adapter molecule (TRIF) and TRIF-related adapter molecule (TRAM) (Wesche et al., 1997, Jiang et al., 2003, Fitzgerald et al., 2001, Yamamoto et al., 2003a, Yamamoto et al., 2003b).

Once activated, TLR4 signalling follows an early a) MyD88-dependent pathway and a delayed b) MyD88-independent pathway, with both resulting in the activation of NF-*k*B and interferon regulatory factor 3 (IRF3) pathways (Palsson-McDermott and O'Neill, 2004, Jiang et al., 2003, Yamamoto et al., 2003a, Fitzgerald et al., 2003b). For the early phase MyD88-dependent pathway, TLR4 recruits MyD88 and MAL to the receptor complex, an action

negatively regulated by single immunoglobulin IL-1 receptor-related molecule (SIGIRR), ST2 and MyD88-splice variant (MyD88s) (Jiang et al., 2003, Oshiumi et al., 2003a, Horng et al., 2002, Horng et al., 2001, Wald et al., 2003, Thomassen et al., 1999, Brint et al., 2004). MyD88 then facilitates an association with interleukin-1 receptor-associated kinase 4 (IRAK4), leading to the phosphorylation of interleukin-1 receptor-associated kinase 1 (IRAK1) and the binding of tumour necrosis factor receptor-associated factor 6 (TRAF6) to this complex (Suzuki et al., 2002) (Qin et al., 2004, Cao et al., 1996). The IRAK1-TRAF6 complex breaks away from the receptor and interacts with a transforming growth factor-β-activated kinase 1 (TAK1)-TAK-1 binding protein 1 (TAB1)-TAB2 complex at the plasma membrane (Yamaguchi et al., 1995, Shirakabe et al., 1997, Ninomiya-Tsuji et al., 1999).

The adaptors TRIF and TRAM are responsible for facilitating the late phase LPS response. TLR4 recruits TRIF and TRAM to the receptor complex, an action negatively regulated by TRIAD3A (Yamamoto et al., 2003a, Oshiumi et al., 2003b, Chuang and Ulevitch, 2004). TRIF then binds to either TRAF6 or TRAF-binding protein (TANK)-binding kinase 1 (TBK1), with the TRIF-TRAF6 complex resulting in NF-*k*B release (Sato et al., 2003, Fitzgerald et al., 2003a) and the TRIF-TBK1 complex resulting in the activation of IRF3 by acting as an IRF3 kinase (Fitzgerald et al., 2003a). Once phosphorylated, IRF3 can then bind to the interferon-sensitive response element (ISRE)(Wietek et al., 2003) and the transcription factor, p65, to induce interferon β (IFNβ) release (Schafer et al., 1998, Kim et al., 2000, Wathelet et al., 1998).

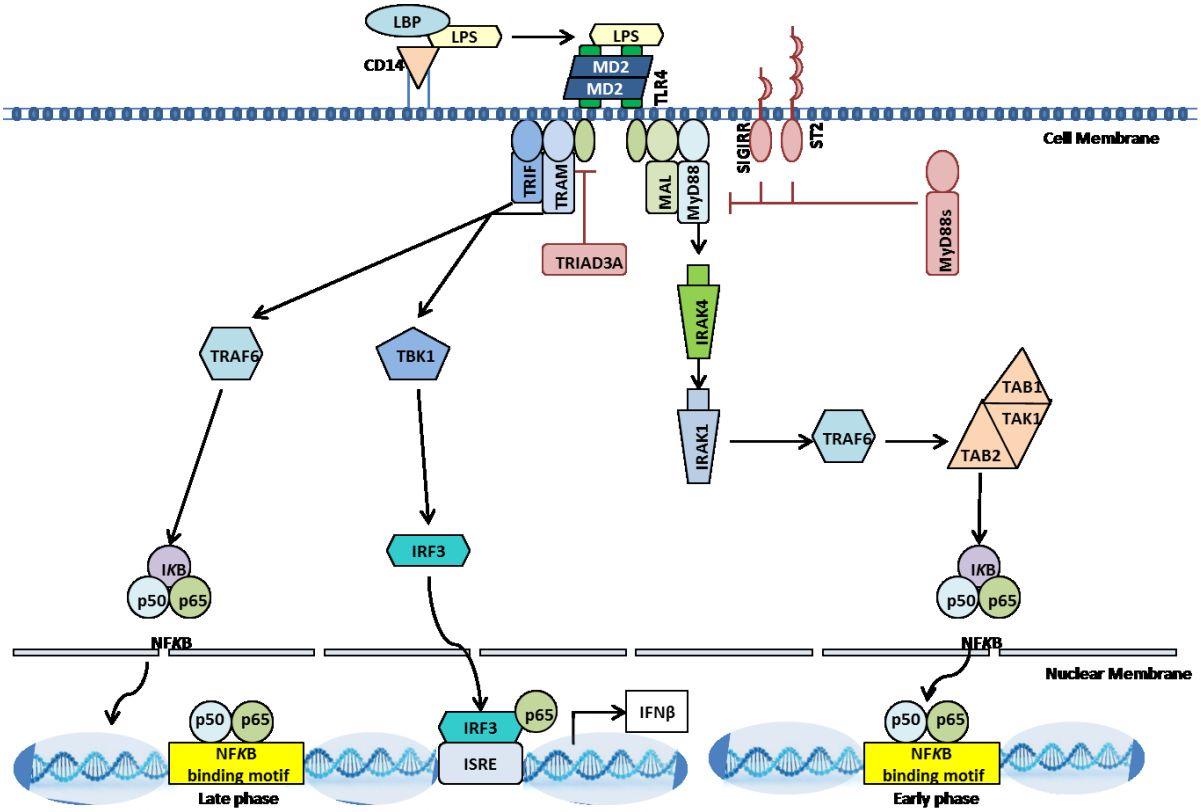

FIGURE 2.3 The LPS intracellular signalling cascade. Soluble LBP chaperones LPS to CD14. CD14 loads the LPS-LBP complex onto the LPS receptor, made of dimerized TLR4's and two molecules of MD-2. MyD88 and MAL-dependent TLR4 signalling results in the NF-*k*B release while TRIF and TRAM-dependent signalling results in NF-*k*B and IFNβ release. Abbreviations: LBP, LPS-binding protein; CD14, cluster of differentiation 14; LPS, lipopolysaccharide; MD-2, myeloid differentiation factor-2; TLR4, toll-like receptor 4; TRIF, toll-IL-1 receptor-containing adapter molecule; TRAM, Toll-IL-1 receptor-containing adapter molecule-related adapter molecule; MAL, MyD88 adaptor-like protein; MyD88, myeloid differentiation factor 88; SIGIRR, single immunoglobulin interleukin-1 receptor-related molecule; IRAK4, IL-1 receptor-associated kinase 4; TBK1, tumour necrosis factor receptor-associated factor binding protein-binding kinase 1; TRAF6, tumour necrosis factor receptor-associated factor 6; TAB1, transforming growth factor-β-activated kinase 1 binding protein 1; TAK1, transforming growth factor-β-activated kinase 1; NF-*k*B, nuclear factor kappa B; I*k*B,

NF-*k*B inhibitor; IRF3, interferon regulatory factor 3; ISRE, interferon-sensitive response element; IFNβ, interferon β. Adapted from (Palsson-McDermott and O'Neill, 2004).

2.1.4.2 Effects of lipopolysaccharide on the HPA axis

As mentioned above, LPS induces strong immune responses in mammals, and acute administration of LPS stimulates the HPA axis (Leshin and Malven, 1984, Rivier et al., 1989). The stimulatory effects of LPS on the HPA axis are mediated partly by pro-inflammatory mediators such as IL-1, Il-6 and TNF-α, which stimulate the release of CRH and AVP that instigate the neuroendocrine stress cascade (John and Buckingham, 2003, Turnbull and Rivier, 1999). Both systemically and centrally administered LPS increase CRH mRNA levels in the PVN (Kakucska et al., 1993), as well as CRH release (Givalois et al., 1995), and both lesions of the hypothalamus (Yasuda and Greer, 1978) and pharmacological blockade of CRH-R1 (Moberg, 1971) inhibit the effects of LPS on the pituitary-adrenocortical response. Centrally administered LPS can stimulate AVP heteronuclear RNA levels in the magnocellular and parvocellular areas of the PVN (Xia and Krukoff, 2003), and can also stimulate AVP release from hypothalamic SCh neurons *ex vivo* (Nava et al., 2000). Furthermore, LPS can directly stimulate ACTH release from primary pituitary cells (Elenkov et al., 1992). LPS-stimulated IL-6 release from folliculostellate (FS) cells in the anterior pituitary acts with hypothalamic CRH to stimulate ACTH release from pituitary corticotroph cells (Gloddek et al., 2001). The effects of LPS on all cells are mainly exerted via NF-*k*B (Libermann and Baltimore, 1990). Evidence for this includes LPS stimulation of IL-6 release from FS cells via a glucocorticoid-sensitive, NF-*k*B -dependent mechanism, and LPS-stimulated activation of NF-*k*B in corticotroph cells (Mehet et al., 2012). *In vivo*, LPS increases intrapituitary NF-*k*B, whereas an NF-*k*B inhibitor attenuates the LPS-induced release of ACTH (Mehet et al., 2012) in rats.

Repeated LPS challenge dampens the HPA response, as shown by reduced ACTH and corticosterone release in mice injected with LPS for 5 days compared to mice that received one individual LPS injection on day 1 only (Hadid et al., 1995). Rats repeatedly injected with LPS demonstrate reduced c-fos mRNA, PVN CRH mRNA and pituitary POMC mRNA levels

compared to rats receiving a single LPS challenge (Takemura et al., 1997). It is thought that this decreased pituitary-adrenal responsiveness is partly due to a reduced hypothalamic drive (Takemura et al., 1997).

As described above, leptin's influences on basal and activated HPA activity (Ahima et al., 1998, Heiman et al., 1997, Jethwa et al., 2006) are controversial. As a potent stimulator of the HPA axis, LPS may be a reliable stressor with which to examine the role of leptin in the HPA axis response to stress.

2.1.4 Hypothesis

I hypothesize that leptin will modulate the acute HPA response to LPS treatment in rats.

2.1.5 Aims

To elucidate the effects of leptin on the HPA axis I will determine:

- The effects of a low dose of acute leptin administration on basal and acute LPS-stimulated HPA axis activity in male rats, using an established dose of LPS
- The effects of different doses of acute LPS stimulation on neuronal activation, as measured by c-fos protein expression, in regions of the hypothalamus and brain stem implicated in the stress response and/or energy homeostasis
- The effects of a higher dose of acute leptin administration on basal and acute LPS-stimulated HPA axis activity in male rats.

2.2 Methods

2.2.1 Animals and housing

All animal procedures undertaken were approved by the British Home Office Animals Scientific Procedures Act 1986 (Project License Number: 70/6623). On arrival, specific pathogen free (SPF) male Wistar rats (Charles River, Kent, UK) were group housed in fours and maintained at 21-23˚C on a 12 hr light: 12 hr dark cycle (light period 07:00-19:00 hrs) with *ad-libitum* access to RM1 standard chow (RM1 diet, Special Diet Services Ltd., Witham, Essex) and water. After the 72 hour acclimatization period, all rats were handled daily for 3 minutes each for two weeks to minimize the risk of neuronal activation due to environmental stressors, as acute restraint stress has been shown to induce high levels of c-fos in rats (Trneckova et al., 2006). At two weeks, the experiments were carried out in the early light phase.

2.2.2 Materials

2.2.2.1 Injectables

Recombinant human leptin (NIDDK, Bethesda, USA)

Recombinant rat leptin (R&D Systems, Abingdon, UK)

Lipopolysaccharide from Escherichia coli Serotype 0111:B4 (Sigma-Aldrich, Dorset, UK)

Saline (B. Braun Medical Ltd., Sheffield, UK)

2.2.2.2 Plasma hormone immunoassay kits

Rat leptin enzyme-linked immunosorbent assay (ELISA) (Crystal Chem Inc., Illinois, USA)

ACTH immunoradiometric assay (IRMA) (DiaSorin, Dublin, Ireland)

Corticosterone enzyme immunoassay (EIA) (Cayman Chemical Company, Michigan, USA)

Rat IL-1β Quantikine ELISA (R&D Systems)

2.2.2.3 Materials for immunohistochemistry

0.01M Phosphate buffered saline (PBS) (see Appendix I)

0.2M PBS (see Appendix I)

PBS Formaldehyde (PBS FA) solution (see Appendix I)

4% (*v*/*v*) Formaldehyde (VWR International Ltd., Dorset, UK)

40% (*w*/*v*) Sucrose solution (see Appendix I)

Antifreeze (see Appendix I)

Net-well meshed-insert wells and Net-well reagent trays (VWR International Ltd)

PBS Tween (PBST) solution (see Appendix I)

Methanol-Hydrogen peroxide (H_2O_2) solution (see Appendix I)

Blocking solution (see Appendix I)

Primary antibody diluent (same as blocking solution)

Rabbit anti c-fos (AB-5) primary antibody (Merck Chemicals Ltd., Nottinghamshire, UK)

Secondary antibody diluent (see Appendix I)

Biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Peterborough, UK)

0.05M (hydroxymethyl)aminomethane Hydrogen Chloride (TrisHCl) solution (pH 7.6) (see Appendix I)

Streptavidin-Biotin complex (ABC)-HRP conjugated kit (Dako UK Ltd., Cambridgeshire, UK)

3,3' Diaminobenzidine (DAB) solution (see Appendix I)

Poly-L-lysine-coated slides (VWR International Ltd)

50% (*v*/*v*), 70% (*v*/*v*) and 100% Ethanol solutions (see Appendix I)

Xylene (BDH, Dorset, UK)

Di-N-Butyl Phthalate in Xylene (DPX) mounting medium (BDH)

2.2.3 Study designs

2.2.3.1 The effect of low dose leptin on the HPA axis response to high dose lipopolysaccharide

Method

Forty eight male Wistar rats, weighing 300g after two weeks of housing, were randomized into 6 body weight matched groups of 8. Rats were first i.p. injected with either 100µl saline, 100µg/kg recombinant human leptin, or were not injected at all, and were then returned to their cages. One hour and 45 minutes later rats were i.p. injected with 250µg/kg LPS or were not injected at all, and were again returned to their cages. This resulted in the following treatment groups (n = 8): saline, leptin, saline + LPS, leptin + LPS, LPS and no treatment. Leptin and LPS were reconstituted in water and aliquots were stored at -20˚C prior to the study. On the day of the study, leptin and LPS were further diluted in saline, to be administered at a final volume of 100µl.

Maximum c-fos protein expression occurs 90 minutes after stimulation by a stressor, and 15 minutes was considered a reasonable time for the LPS to generate a stress response (Gillen and Briski, 1997). All rats were therefore decapitated by guillotine another 1 hr and 45 minutes later. Trunk blood was collected into ethylenediaminetetraacetic acid (EDTA) lined tubes and spun at room temperature at 6000rpm (Boeco Centrifuge S-8, Boeco, Hamburg, Germany) for 10 minutes to separate the plasma. Samples were stored at -20˚C until measurement of plasma leptin and IL-1β concentrations by ELISA, and plasma corticosterone by EIA and plasma ACTH concentrations by IRMA, as described in 2.2.4.

2.2.3.2 The dose response effect of lipopolysaccharide on the HPA axis stress response

Method

This study was carried out to determine the lowest dose of LPS capable of inducing a robust stress response, as the dose of 250µg/kg induced an extremely high stress response in rats from study 2.2.3.1.

Forty male Wistar rats, weighing 300-390g after two weeks of housing, were randomized into 5 body weight matched groups of 8. Rats were i.p. injected with either 100µl saline or LPS at concentrations of 10, 25, 100 or 250µg/kg, before being returned to their cages. Aliquots of reconstituted LPS had been stored at -20˚C prior to injection. LPS was administered at a final volume of 100µl. Two hours later all rats were decapitated by guillotine. This time point allowed for maximum c-fos protein expression, which as described earlier occurs 90 minutes after stimulation by a stressor. This 2 hr time point is also in line with an earlier study investigating the effect of leptin on the HPA axis response to acute restraint stress (Heiman et al., 1997). So for this study (2.2.3.2) as well as study 2.2.3.3 it was decided to use the same 2 hr time point between leptin administration, the acute stress procedure and decapitation, making it possible to compare results from multiple studies. The study was completed within the early light phase to avoid ultradian rhythmic changes in circulating plasma stress hormones. Trunk blood was collected and plasma was separated and stored as described in 2.2.3.1, for measurement of plasma corticosterone and ACTH concentrations by EIA and IRMA respectively, as described in 2.2.4.2 - 2.2.4.3. Whole rat brains were removed and separated from their brainstems. Brains and brainstems were then submerged in 4% formaldehyde (FA) solution overnight at 4˚C to allow fixation of the tissue. Brains were then transferred in to a 40% sucrose solution for 4 days at 4˚C to dehydrate them for cryopreservation. Finally they were frozen in isopentane chilled to -45˚C,

before being stored at -80˚C until sectioning and immunohistochemical staining for c-fos at a later stage, as described in 2.2.5.

2.2.3.3 The effect of a high dose of leptin on the HPA axis response to a low dose of lipopolysaccharide

Forty male Wistar rats, weighing 300-390g after two weeks of housing, were randomized into 4 body weight matched groups of 10. Rats were first i.p. injected with either saline or 2mg/kg recombinant rat leptin and returned to their cages. Two hours later rats were i.p. injected with either saline or 25µg/kg LPS and again returned to their cages. This resulted in the following treatment groups (n = 10): saline + saline (saline), leptin + saline (leptin), saline + LPS (LPS) and leptin + LPS. Leptin and LPS had been reconstituted with water and aliquots stored at -20˚C prior to the study. All LPS injections were administered at a final volume of 100µl, while leptin was administered in a final volume of 700µl.

Two hours after being returned to their cages, all rats were decapitated by guillotine. Trunk blood was collected and plasma was separated and stored as described in 2.2.3.1, for measurement of plasma leptin, corticosterone and IL-1β concentrations by ELISA, and plasma ACTH concentrations by IRMA, as described in 2.2.4.

2.2.4 Leptin, ACTH, corticosterone and interleukin-1β assays

2.2.4.1 Leptin enzyme-linked immunosorbent assay

A commercial rat leptin ELISA kit was used for the quantitative determination of plasma leptin levels, following the manufacturer's instructions. Standards, buffers and reference preparations were supplied. Each kit provided a 96-well rabbit anti-leptin antibody pre-coated plate. Lyophilised leptin standard was reconstituted with 100µl of sample diluent to make up the leptin stock solution. Leptin standards of a known concentration were made up by serial dilution of the stock solution using the sample diluent. Each well was washed twice with wash buffer, after which 45µl of sample diluent and 50µl of guinea pig anti-leptin serum was added to each well. To this, 5µl of sample or standard was added to the plate in duplicates (samples were diluted to concentrations of 1:5 for samples from study 2.2.3.1, and 1:50 and 1:1000 for samples from study 2.2.3.3) and left to incubate at 4ºC for 16-20 hours. During this incubation period the leptin in the standards and plasma samples bound to the rabbit anti-leptin antibody pre-coated on the plate, as well as simultaneously binding to the anti-leptin Immunoglobulin G (IgG) of the guinea pig anti-serum. After the incubation period any unbound material was removed by washing five times, before adding 100µl of a horse radish peroxidase (HRP)-conjugated anti-guinea pig IgG antibody, which bound to the guinea pig anti-leptin IgG of the complex already immobilized to the wells. This was left to incubate at room temperature for three hours, after which further washing the plate seven times removed excess HRP-conjugated antibody. This was followed by an enzyme reaction in which the bound HRP-conjugated antibody in the wells was detected by adding a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution. This reaction was incubated for 30 minutes at room temperature in the dark and was stopped by addition of 100µl 1M sulphuric acid. The absorbance was measured using a Labsystems Multiscan RC plate reader (Thermo Scientific, Massachusetts, USA) at a wavelength of 450nm while the background was read at 630nm. Leptin concentrations were determined via interpolation of the unknown

concentrations against the standard curve generated from the known standard concentrations.

2.2.4.2 ACTH immunoradiometric assay

A commercial human ACTH IRMA kit, that cross-reacts with rat ACTH and is highly specific for ACTH 1-39, was used for the quantitative determination of plasma ACTH levels following the manufacturer's instructions. Calibrators, controls, buffers and reference preparations were supplied. Lyophilised ACTH calibrators and standards of a known concentration were reconstituted with 2ml GDW. Two hundred microlitres of calibrators, controls and samples were added to 12 x 17 mm polypropylene tubes, along with 50µl of iodinated tracer and one mouse anti-goat coated polystyrene bead in each tube. In this assay two ACTH antibodies are used; each one specific for different regions of the ACTH molecule. The iodinated tracer contains a purified polyclonal goat antibody specific for ACTH 26-39 and an iodine-125 labelled monoclonal antibody specific for ACTH 1-17. When calibrators, controls and samples are incubated with the tracer and mouse anti-goat coated polystyrene beads, the ACTH-specific antibodies bind to the ACTH present in the calibrators, controls and samples. Only ACTH 1-39 present in the calibrators, controls and samples binds to both antibodies to form an antibody complex. To allow sufficient time for this reaction to occur, the tubes were covered with para-film and left at room temperature for 20 hours. Following the 20 hour incubation period, tubes were washed three times to remove any unbound radioactivity, before all radioactivity bound to each bead was measured using a gamma counter for 60 seconds. The counts per minute (CPM) from the calibrators were then used to construct a standard curve in order to interpolate the unknown ACTH concentrations of the plasma samples. All calibrators, controls and samples were measured in duplicate and the average reading used in subsequent analysis.

2.2.4.3 Corticosterone enzyme immunoassay

A commercial corticosterone EIA kit was used for the quantitative determination of plasma corticosterone levels, following the manufacturer's instructions. Standards, buffers and reference preparations were supplied. Each kit provided a 96-well rabbit anti-sheep IgG pre-coated plate. A corticosterone stock standard solution was serially diluted in assay buffer to make up further corticosterone standards of known concentrations. Eight wells of each plate were prepared as controls, with three containing nothing (2 x 'Blank' wells + 1 x 'Total Activity' well), two 'Non-specific Binding' wells that contained 100µl of assay buffer + 50µl of corticosterone acetyl cholinesterase (AChE) tracer and three 'maximum Binding' wells called B_0 that contained 50µl of assay buffer + 50µl of corticosterone AChE tracer + 50µl of sheep antiserum. To the remainder of the plate, 50µl of standards or samples (samples diluted to concentrations of 1:10 for samples from study 2.2.3.1, 1:750 for samples from study 2.2.3.2 and 1:500 for samples from study 2.2.3.3) were added in duplicate, followed by 50µl of corticosterone AChE tracer and 50µl of sheep antiserum. The plates were then covered in plastic film and incubated at room temperature on a flat orbital shaker at 400rpm for 2 hours. This assay is based on the competition between corticosterone and a corticosterone AChE conjugate for a limited number of corticosterone-specific sheep antiserum binding sites. Because the concentration of the AChE tracer is constant while the concentration of corticosterone varies, the amount of AChE tracer that is able to bind to the sheep antiserum is inversely proportional to the concentration of corticosterone in the well. This sheep antiserum-corticosterone complex binds to the rabbit polyclonal anti-sheep IgG previously attached to the well. After the 2 hour incubation step, the plates were washed five times to remove any unbound reagents and then 200µl of an AChE substrate was added to each well. At the same time 5µl of AChE tracer was added to the 'Total Activity' well of each plate, after which the plates were covered with plastic film and incubated at room temperature, on a flat orbital shaker at 400rpm and in the dark, for 60 minutes. The product of this enzymatic reaction has a distinct yellow colour and its absorbance was measured

spectrophotometrically using a Labsystems Multiscan RC plate reader at a wavelength of 420nm. The intensity of the colour is proportional to the amount of AChE tracer bound to the well, which is inversely proportional to the amount of free corticosterone present in the well during the incubation. Corticosterone concentrations were determined via interpolation of the unknown concentrations against the standard curve generated from the known standard concentrations.

2.2.4.4 Interleukin-1β enzyme immunoassay

A commercial rat IL-1β quantikine ELISA kit was used for the quantitative determination of plasma IL-1β levels, following the manufacturer's instructions. Controls, standards, buffers and reference preparations were supplied. Each kit provided 2 x 96-well rat IL-1β-specific polyclonal antibody pre-coated plates. A lyophilised rat IL-1β control was reconstituted with 1ml of GDW and a lyophilised rat IL-1β standard was reconstituted with 2ml of calibrator diluent to create a standard stock solution. This solution was further serially diluted with calibrator diluent to produce 6 more standards of known concentrations of IL-1β. Fifty microlitres of assay diluent was then added to each well, followed by 50µl of standard, control or sample per well (samples were diluted with calibrator diluent to a concentration of 1:3 as per kit instructions). The plates were then covered with plastic film and incubated at room temperature for 2 hours. This allowed any IL-1β in the samples, controls or standards to bind to the immobilized antibody already bound to the wells. After the 2 hour incubation step, all the wells were washed five times to remove any unbound reagents before adding 100µl of a rat IL-1β conjugate, an enzyme-linked polyclonal antibody that is specific for rat IL-1β, and incubating again at room temperature for 2 hours. After this second incubation step, all the wells were washed again five times to remove any unbound IL-1β conjugate and then 100µl of a light-sensitive substrate solution was added to each well. This was incubated for 30 minutes at room temperature, away from light. Then 100µl of a stop solution was added to each well and the optical density of each well was measured using a

Labsystems Multiscan RC plate reader at a wavelength of 450nm. Samples were measured in duplicate and the average reading used in subsequent analysis. IL-1β concentrations were determined via interpolation of the unknown concentrations against the standard curve generated from the known standard concentrations.

2.2.5 Immunohistochemistry

2.2.5.1 Cellular fos-like immunoreactivity

C-fos was named after the viral oncogene (v-fos) encoded by the Finkel-Biskis-Jinkins murine osteosarcoma virus, and is itself encoded by the fos gene (Morgan and Curran, 1991). Fos is a proto-oncogene of the immediate early gene family of transcription factors which, in response to stimulation, is rapidly and temporarily expressed in specific neurons. Hence it is used as an early marker of neuronal activity in the CNS (Morgan and Curran, 1991). The fos gene is transcribed within minutes of neuronal stimulation, leading to peak c-fos mRNA accumulation at approximately 30 minutes after stimulation, and peak c-fos protein levels accumulating at 90 minutes after stimulation (Gillen and Briski, 1997). C-fos protein is routinely detected using immunohistochemical methods. Several different types of stimulus, including many pharmacological agents, have been shown to induce c-fos expression in neurons of the CNS (Herrera and Robertson, 1996). The c-fos like immunoreactivity observed following peripheral administration of LPS would indicate the neurons this agent activates either directly or indirectly. Furthermore, leptin treatment may affect such neuronal activation of LPS-injected rats and indicate which stress or non stress-related neurons are sensitive to leptin action.

2.2.5.2 Immunohistochemical staining for cellular-fos

Thirty micrometers coronal sections of the whole brain and brainstem were cut using a freezing sled microtome (Shandon Southern Products Ltd., Cheshire, UK) and collected into 24-well plates containing antifreeze. Sections were stored in antifreeze at -20°C until IHC analysis. For IHC, selected sections were transferred into net-well meshed-insert wells held in net-well reagent trays and first washed in PBS three times for five minutes each time. They were then incubated in a H_2O_2-methanol solution for 15 minutes to quench endogenous peroxidase enzyme activity. Sections were then washed in PBS a further three times before being incubated in a blocking solution for two hours, in an effort to reduce background staining due to non-specific binding. A 1:20,000 dilution of a rabbit anti c-fos primary antibody (AB-5) was prepared in primary antibody diluent. The sections were then incubated overnight in the primary antibody solution, after which they were washed with PBST three times for five minutes each. A 1:400 dilution of a biotinylated goat anti-rabbit IgG secondary antibody was prepared in secondary antibody diluent. The sections were then incubated in the secondary antibody solution for two hours and then washed in PBST three times for five minutes each time. A working solution of ABC-HRP was prepared by diluting (1:200) the separate streptavidin and biotin reagents from the ABC-HRP kit in a TrisHCl solution. The biotin on the secondary antibody binds to sites available on the avidin, which is already conjugated with an enzyme. Sections were incubated in the ABC-HRP solution for one hour, before being washed in PBST three times again. Horseradish peroxidase activity was then visualized by staining the sections in a DAB solution for approximately ten minutes or until darkened sufficiently, before being washed with PBS to stop excessive darkening of the sections. The DAB chromogen reacts with the enzyme on the streptavidin-biotin complex to reveal a dark brown stain representing c-fos. The sections were then floated onto poly-L-lysine-coated slides and allowed to dry for a few hours before being dehydrated in 50, 70 and then 100% ethanol, delipidated in xylene and mounted and cover-slipped using DPX mounting medium. The slides were then allowed to dry overnight.

For quantification of c-fos-like positive nuclei, slides were observed under a light microscope and manual blinded cell counting was carried out. The numbers were counted within defined hypothalamic nuclei using the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007).

2.2.6 Statistics

Plasma hormone data and immunohistochemical c-fos analyses were analyzed using one way analysis of variance (ANOVA) with a post-hoc Tukey's test to identify statistical significance between all relevant pairs of treatment groups. The Pearson's correlation coefficient was used to determine whether a correlation existed between LPS dose and c-fos counts, where 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation. All data is expressed as mean ± SEM and significance was taken at $P \leq 0.05$ throughout. All data were analyzed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA).

2.3 Results

2.3.1 The effect of a low dose of leptin on the HPA axis response to a high dose of lipopolysaccharide: Plasma leptin, ACTH, corticosterone and interleukin-1β concentrations

There was no significant difference in body weight between any of the experimental groups prior to experimentation (Control: 294±5.41g, Saline: 294±4.89g, Leptin: 300±5.23g, Saline + LPS: 304±5.98g, LPS: 303±5.42g, Leptin + LPS: 294±4.30, $P = 0.51$, $n = 8$). Rats that received only leptin demonstrated a significant increase in plasma leptin levels compared to controls, 3.5 hrs after leptin administration. Rats that received leptin + LPS demonstrated significantly higher plasma leptin concentrations compared to all other experimental groups that did not receive leptin. LPS alone did not influence plasma leptin concentrations (Figure 2.4A).

Saline + LPS and leptin + LPS significantly increased plasma ACTH levels 1 hr and 45 minutes after injection. LPS alone caused a non-significant rise in plasma ACTH levels. Leptin alone had no effect on plasma ACTH concentrations and did not significantly influence LPS-induced ACTH secretion. Plasma ACTH concentrations were significantly greater in rats that received saline + LPS compared to rats receiving leptin + LPS ($P \leq 0.01$) (Figure 2.4B).

LPS significantly increased plasma corticosterone levels 105 minutes after injection. Leptin alone had no effect on basal plasma corticosterone levels. Leptin in combination with LPS did result in a reduced plasma corticosterone increase compared to LPS-injected rats, but this result was not statistically significant (Figure 2.4C).

Plasma IL-1β levels were significantly increased by injection of LPS, but were not influenced by injection of leptin alone. Co-injection of leptin + LPS did not influence LPS-induced increases in IL-1β levels (Figure 2.4D). No other significant differences were observed between any of the experimental groups.

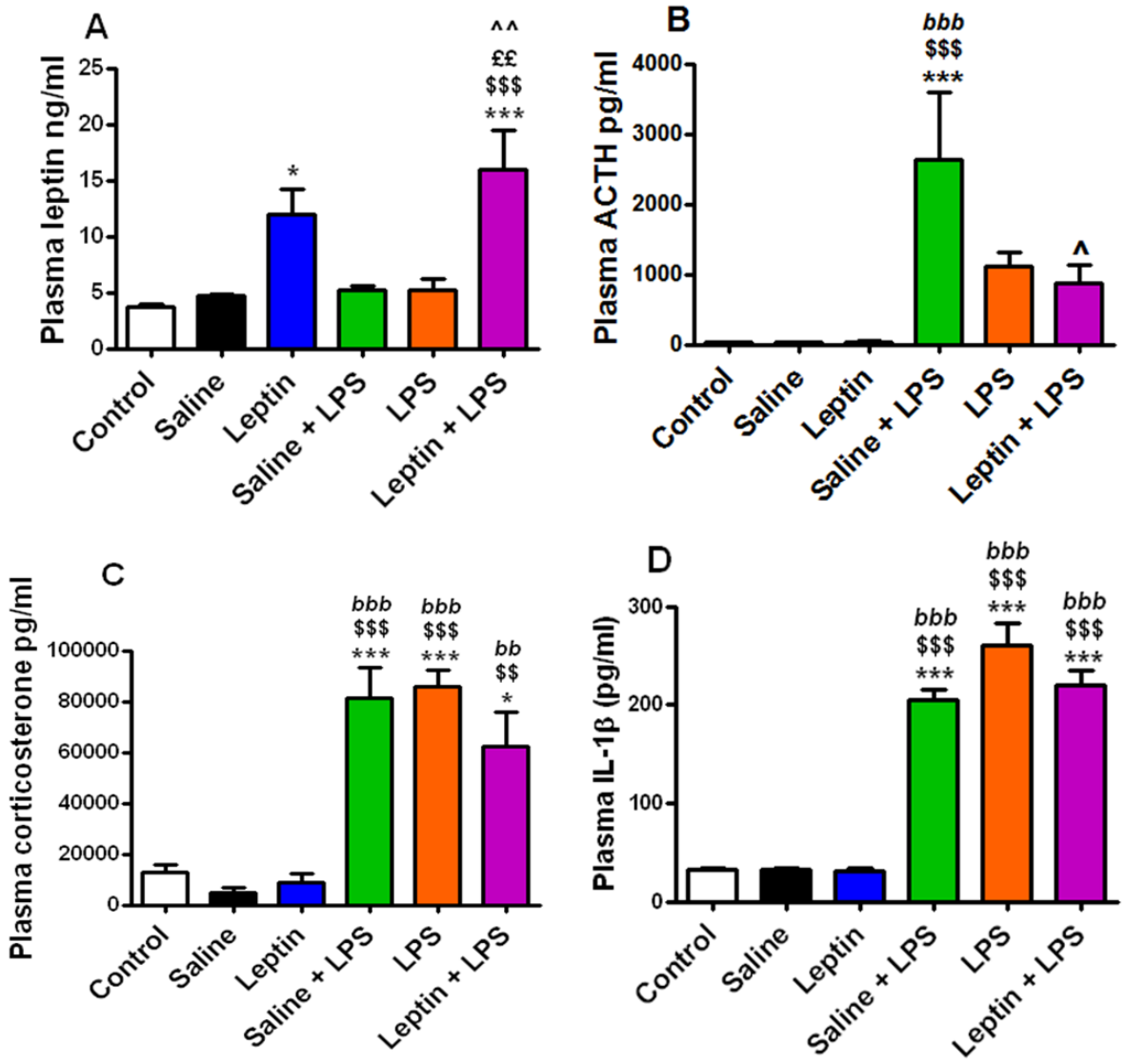

FIGURE 2.4 The effect of i.p. administration of leptin on LPS-stimulated increases in plasma (A) leptin, (B) ACTH, (C) corticosterone and (D) IL-1β concentrations. Male Wistar rats received one of the following i.p. treatments; saline at 0 minutes, 100µg/kg leptin at 0 minutes, saline at 0 minutes and 250µg/kg LPS at 1 hr and 45 minutes, 250µg/kg LPS at 1 hr and 45 minutes, or 100µg/kg leptin at 0 minutes and 250µg/kg LPS at 1 hr and 45 minutes. Rats were decapitated 3 hrs and 30 minutes after the first injection at 0 minutes. Data are presented as mean ± SEM; one way ANOVA with post-hoc Tukey's tests. * = P ≤ 0.05 vs. control, *** = P ≤ 0.001 vs. control, $$ = P ≤ 0.01 vs. saline, $$$ = P ≤ 0.001 vs. saline, *bb* = P ≤ 0.01 vs. leptin, *bbb* = P ≤ 0.001 vs. leptin, ££ = P ≤ 0.01 vs. LPS, ^ = P ≤ 0.05 vs. saline + LPS, ^^ = P ≤ 0.01 vs. saline + LPS; n = 3-8.

2.3.2 The dose response effect of lipopolysaccharide on the HPA axis stress response

2.3.2.1 Plasma ACTH and corticosterone concentrations

There was no difference in body weight between any of the experimental groups prior to experimentation (Saline: 346±6.25g, 10μg/kg LPS: 350±7.79g, 25μg/kg LPS: 356±6.62g, 100μg/kg LPS: 353±9.30g, 250μg/kg LPS: 345±7.65, P = 0.83, n = 10). Doses of LPS ranging from 25μg/kg to 250μg/kg induced a neuroendocrine stress response. One hundred micrograms per kilogram and 250μg/kg LPS induced the strongest responses in plasma ACTH levels compared to the saline-treated group ($P \leq 0.05$ and $P \leq 0.01$ respectively), 120 minutes after injection (Figure 2.5A). Twenty five micrograms per kilogram and 250μg/kg LPS induced greatest plasma corticosterone response compared to the saline-treated group ($P \leq 0.05$ and $P \leq 0.01$ respectively) (Figure 2.5B). The lowest dose of LPS tested, 10μg/kg, induced small rises in plasma ACTH and corticosterone but these changes were not statistically significant. No other significant differences were observed between any of the experimental groups.

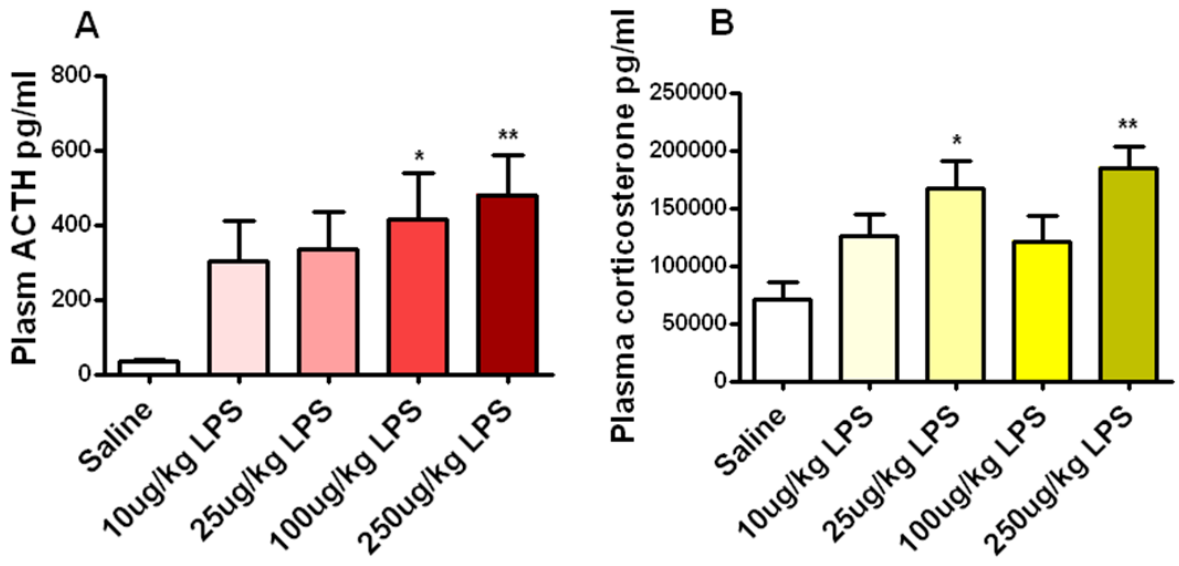

FIGURE 2.5 The dose response effect of i.p. LPS on plasma (A) ACTH and (B) corticosterone concentrations. Rats received either i.p. saline or i.p. LPS at 10, 25, 100 or 250µg/kg LPS. Data are presented as mean ± SEM; one way ANOVA with post-hoc Tukey's tests. * = $P \leq 0.05$ vs. saline, ** = $P \leq 0.01$ vs. saline; n = 10.

2.3.2.2 C-fos-like immunoreactivity in the hypothalamus and brainstem

Intraperitoneal injection of different doses of LPS increased hypothalamic c-fos expression in the ARC and VMN compared to saline-treated rats; with level of expression increasing alongside dose, albeit insignificantly (Table 2.1 and Figure 2.6A and D). LPS also induced c-fos expression in the PVN (Table 2.1 and Figure 2B) but not in a dose-dependent manner. Twenty five and 250µg/kg LPS induced an increase in c-fos expression in the hypothalamic SON but this was also not statistically significant (Table 2.1 and Figure 2.6C). Sections from rat hypothalami from the 100µg/kg LPS treated group were not available for inclusion in the analysis due to the lack of matched sections from this treatment group.

In the rat brainstem, LPS appeared to induce c-fos expression in the caudal and rostral nucleus of the solitary tract **(**NTS), parabrachial nucleus (PBN), area postrema (AP) and locus coeruleus (LC), but these effects were not statistically significant (Table 2.2 and Figure 2.7A-E). Note that the absence of bars above named treatment groups on the graphs reflects c-fos-like counts of 0, and the absence of an error bar on the 250µg/kg LPS-injected group within the PVN reflects that only a single section from this group was analyzed.

TABLE 2.1 The dose-response effect of i.p. LPS on c-fos protein expression in rat hypothalamic areas. Table summarizing c-fos-like positive cell counts observed in hypothalamic areas following i.p. injection of saline or LPS at 10, 25, 100 or 250µg/kg. Data are presented as mean ± SEM; one way ANOVA with post-hoc Tukey's test, non significant, n = 2-4.

Hypothalamic nucleus	c-fos-like positive counts				
	Saline	10µg/kg LPS	25µg/kg LPS	100µg/kg LPS	250µg/kg LPS
ARC	69±10.6	142±38	215±87.5	258±43	281±57.6
PVN	20.5±11.9	215±48.5	176±149	116±76.5	177±13.5
SON	5.33±5.33	5.50±5.50	14±14	no sections available	54.3±32.8
VMN	108±48.5	192±10.5	242±37.5	255±56	294±39.5

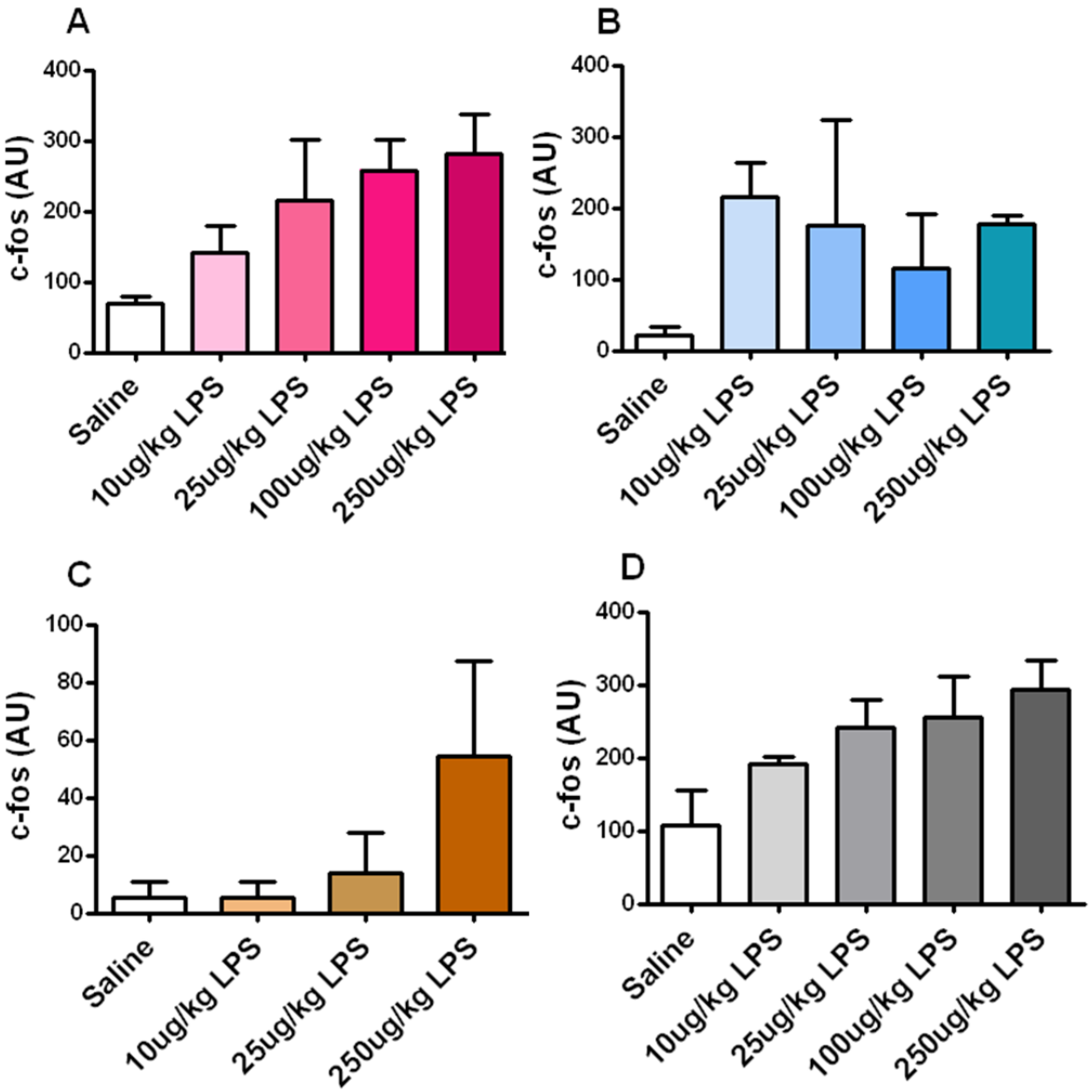

FIGURE 2.6 The dose-response effect of i.p. LPS on c-fos protein expression in the rat hypothalamic (A) ARC, (B) PVN, (C) SON and (D) VMN. Rats received either i.p. saline or i.p. LPS at 10, 25, 100 or 250µg/kg. Values are measured in arbitrary units (AU) and presented as mean ± SEM; one way ANOVA with by post-hoc Tukey's test, ns; n=2-4. Pearson's correlation coefficient between LPS dose and c-fos expression = 0.

TABLE 2.2 The dose-response effect of i.p. LPS on c-fos protein expression in rat brainstem areas. Table summarising c-fos-like positive cell counts observed in brainstem areas following i.p. injection of saline or LPS at 10μg/kg, 25μg/kg, 100μg/kg or 250μg/kg. Data are presented as mean ± SEM; one way ANOVA with post-hoc Tukey's test, ns; n = 1-4.

Brainstem nucleus	c-fos-like positive counts				
	Saline	10μg/kg LPS	25μg/kg LPS	100μg/kg LPS	250μg/kg LPS
Caudal NTS	0±0	15.3±7.86	no sections available	51±16	96 (1 section)
Rostral NTS	6.75±2.66	45.7±13.9	120±120	177±42	78.5±78.5
PBN	0±0	0±0	0±0	56.3±29.9	83±21
AP	1±1	14.5±5.50	no sections available	10.5±2.50	no sections available
LC	8.5±4.5	0±0	0±0	13.3±9.61	12.3±7.88

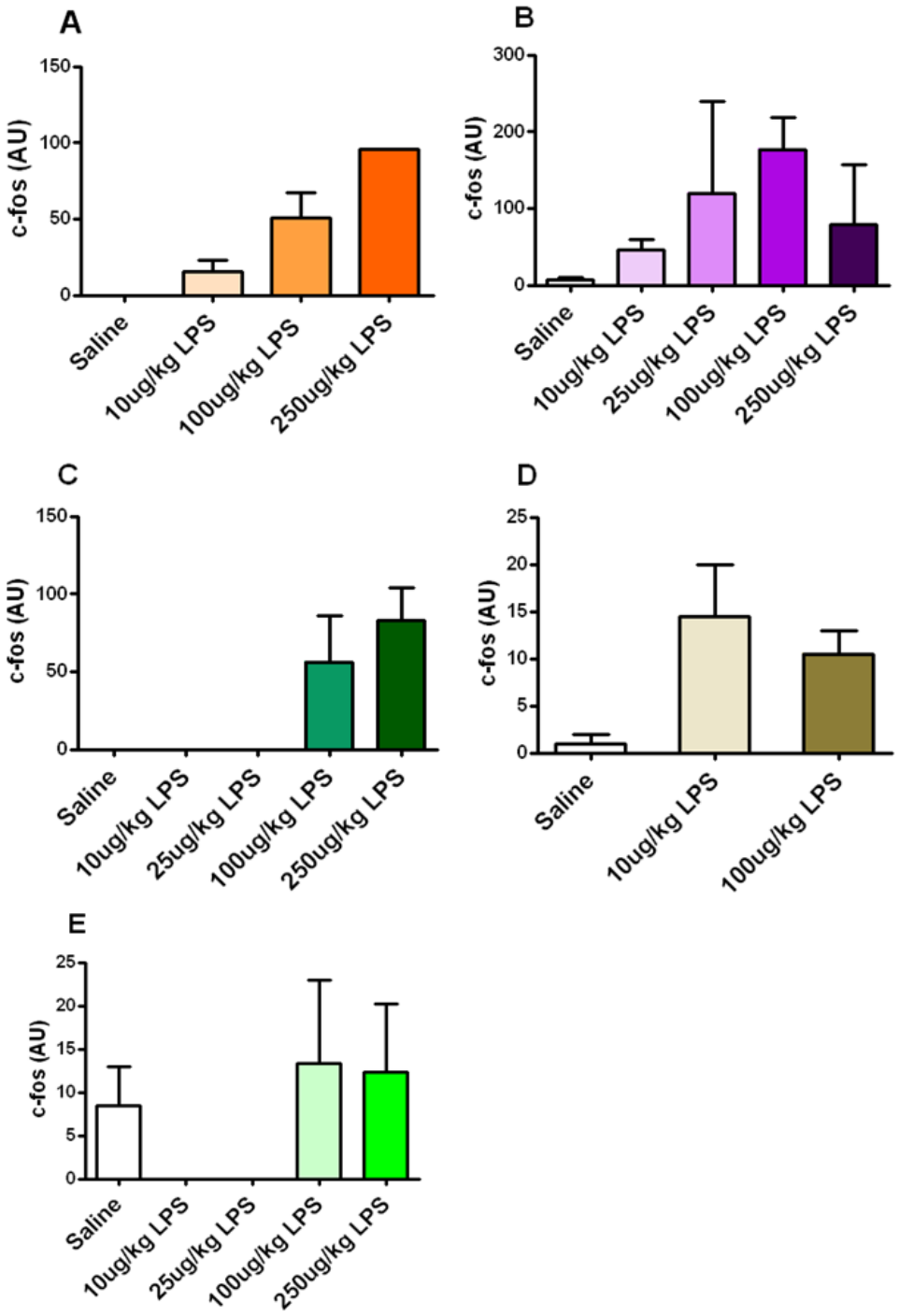

FIGURE 2.7 The dose-response effect of i.p. LPS on c-fos protein expression in the (A) caudal NTS, (B) rostral NTS, (C) PBN, (D) AP and (E) LC of the rat brainstem. Rats received either i.p. saline or i.p. LPS at 10, 25, 100 or 250µg/kg. Values are measured in arbitrary units (AU) and presented as mean ± SEM; one way ANOVA with by post-hoc Tukey's test, ns; n=1-4.

2.3.3 The effect of a high dose of leptin on the HPA axis response to a low dose of lipopolysaccharide: Plasma leptin, ACTH, corticosterone and interleukin-1β concentrations

There was no difference in body weight between any of the experimental groups prior to experimentation (Saline + saline: 346±6.97g, Leptin + saline: 345±4.79g, Saline + LPS: 346±5.54g, Leptin + LPS: 3464±6.72g, $P = 0.99$, $n = 9$-10). Rats that received only leptin demonstrated a significant increase in plasma leptin levels 4 hrs after leptin administration compared to saline-injected rats ($P \leq 0.001$), whilst LPS alone had no effect on plasma leptin levels (Figure 2.8A). Rats that received LPS demonstrated significantly higher plasma ACTH and corticosterone levels compared rats that did not receive LPS ($P \leq 0.05$ and $P \leq 0.001$ respectively) (Figure 2.8B and C respectively). Leptin, whether injected alone or whether co-administered with LPS, did not affect plasma ACTH or corticosterone levels (Figure 2.8B and C respectively). Plasma IL-1β levels were significantly increased in rats by injection of LPS ($P \leq 0.01$), but were not influenced by injection of leptin alone (Figure 2.8D), and co-administration of leptin + LPS did not influence LPS-induced increases in IL-1β levels (Figure 2.8D). No other significant differences were observed between any of the experimental groups.

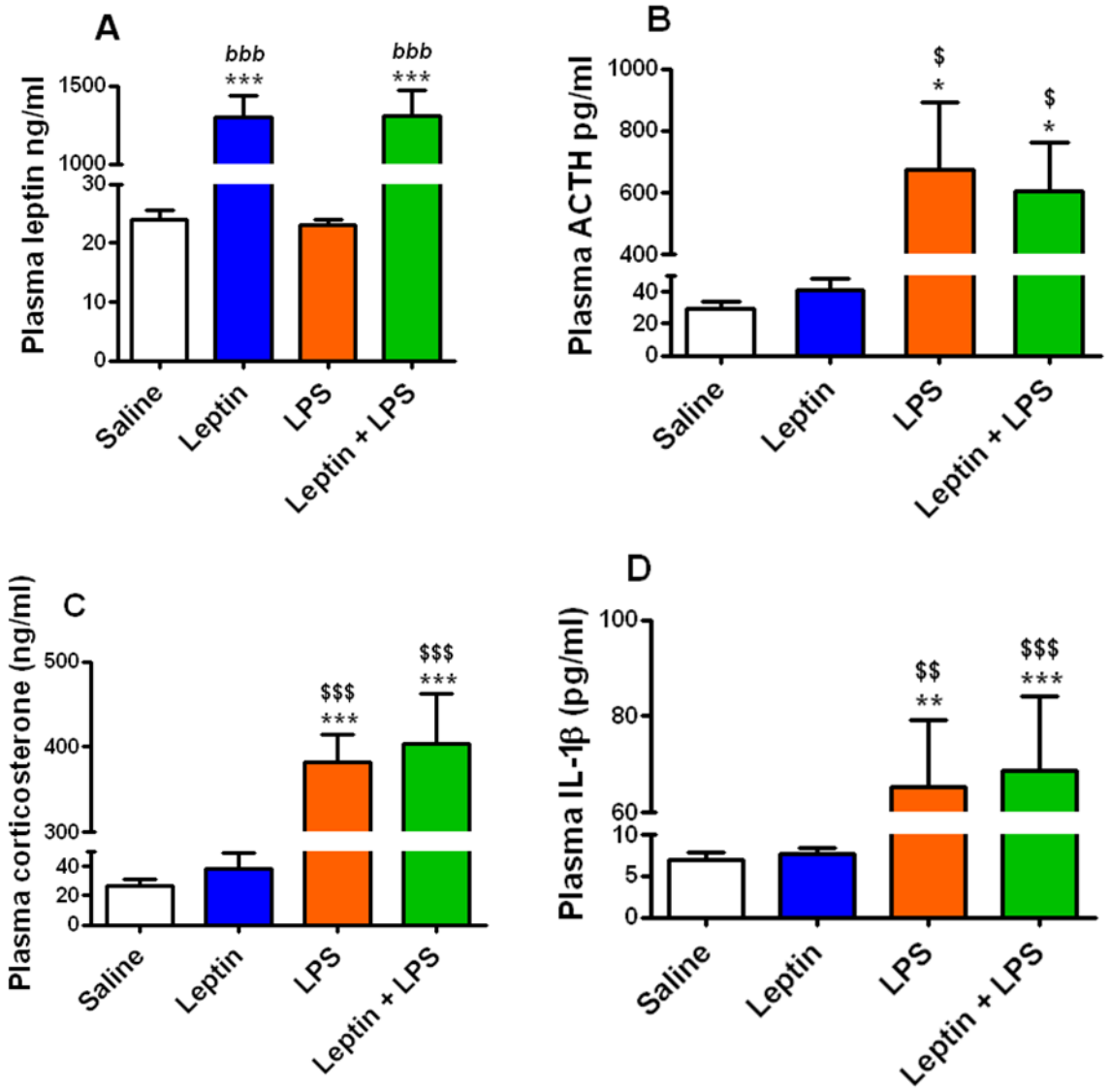

FIGURE 2.8 The effect of i.p. administration of leptin on LPS-stimulated increases in plasma (A) leptin, (B) ACTH, (C) corticosterone and (D) IL-1β concentrations. Male Wistar rats received one of the following i.p. treatments; saline at 0 and 120 minutes, 2mg/kg leptin at 0 minutes + saline at 120 minutes, saline at 0 minutes + 25µg/kg LPS at 120 minutes or 2mg/kg leptin at 0 minutes + 25µg/kg LPS at 120 minutes. Data are presented as mean ± SEM; one way ANOVA with post-hoc Tukey's test. * = P ≤ 0.05 vs. saline, ** = P ≤ 0.01 vs. saline, *** = P ≤ 0.001 vs. saline, $ = P ≤ 0.05 vs. leptin, $$ = P ≤ 0.01 vs. leptin, $$$ = P ≤ 0.001 vs. leptin, *bbb* = P ≤ 0.001 vs. LPS; n = 8-10.

2.4 Discussion

The studies described in this chapter aimed to elucidate the effects of leptin on the HPA response to stress. They suggest that peripheral leptin administration does not influence basal HPA activity or influence the HPA response to acute endotoxin challenge.

Two mg/kg leptin (Figure 2.8A), but not 100µg/kg leptin (Figure 2.4A), significantly raised circulating plasma leptin levels in adult male Wistar rats. LPS, at doses above 25µg/kg, induced a strong stress response as demonstrated by significantly elevated plasma stress hormones, ACTH and corticosterone, as well as significantly elevated plasma levels of the cytokine IL-1β. At doses above 25µg/kg, LPS appeared to drive neuronal activation in the hypothalamic ARC, PVN, SON and VMN, and at doses above 100µg/kg, neuronal activation in the NTS, PBN, AP and LE of the brainstem. However, these effects on neuronal activation were not statistically significant in the current studies. Neither 2mg/kg nor 100µg/kg leptin influenced the HPA response to acute LPS challenge.

The effects of LPS on HPA activity are mediated via a number of cytokines (Uehara et al., 1987, Sharp et al., 1989, Sapolsky et al., 1987). Leptin is a cytokine itself, and some have reported a stimulatory effect of LPS on leptin secretion (Landman et al., 2003, Mastronardi et al., 2001). For example, LPS has been reported to restore the leptin in fasted rats to levels observed in the fed state (Sarraf et al., 1997). However, it is perhaps unsurprising that LPS alone had no effect on plasma leptin levels in LPS-injected rats in study 2.2.3.1 (Figure 2.4A). This is because most studies that report a stimulatory effect of LPS on leptin are only able to demonstrate a statistically significant rise in plasma or serum leptin levels in LPS-injected mice and rats, compared to controls, at time points of 4 hrs or longer after LPS injection (Sarraf et al., 1997, Wang et al., 2004, Kim et al., 2007, Finck et al., 1998).

In addition to measurement of plasma stress hormones, IL-1β was measured as an early marker of endotoxemia in studies 2.2.3.1 and 2.2.3.2, as it is one of the cytokines most strongly associated with endotoxemia, and has been shown to act as an 'initiator' cytokine that stimulates a cytokine cascade, ultimately driving many of the physiological changes seen in endotoxemia, in both human and experimental endotoxemic models (Blackwell and Christman, 1996). Plasma ACTH, corticosterone and IL-1β reached extremely high levels in response to this endotoxin challenge and it is therefore possible that these stress hormones may have reached a maximum level of release due to optimal steroidogenesis. This may have masked any inhibitory, or possibly any additive effect, that leptin may have had on the HPA response to this acute challenge. Therefore the next experiment (2.2.3.2) was designed to determine the lowest dose of LPS capable of inducing a robust stress response. Such a response might be more amenable to modulation by leptin than the effect observed in the earlier high dose LPS study.

The results from this study show that while LPS doses from 25µg/kg upwards did stimulate the HPA axis (Figure 2.5), they did not do so in a dose dependent manner, and some effects did not achieve statistical significance. It is well known that after a stress response plasma ACTH levels start to normalise from 30 minutes, whilst corticosterone levels return to resting levels much later. Therefore it is possible that this study missed any significant effect of the 25µg/kg LPS dose on circulating ACTH at the 2 hr time point for plasma collection. C-fos protein levels in the hypothalamic ARC, PVN, SON and VMN and the caudal and rostral NTS, PBN, AP and LC were measured (Tables 2.3 and 2.4 and Figures 2.6 and 2.7). Although LPS induced c-fos protein in the hypothalamus and brainstem, there was no statistical significance between treatment groups. The lack of statistical significance likely stems from the use of low ‘n’ numbers of sliced brain and brainstem sections in the statistical analyses; in some cases resulting in the loss of specific treatment groups usable in the analyses. The main reason for the use of low ‘n’ numbers was the lack of matched sections across treatment groups. This was largely due to damage caused to brain tissues during the

processes of cryoprotection and slicing. In conclusion, the measurement of c-fos protein levels in the hypothalamus indicated that 25ug/kg LPS was the lowest dose to i) stimulate all the hypothalamic nuclei we investigated and ii) induce a statistically significant hormonal response compared to saline-injected rats.

The variability of the stress response between individual animals and between cohorts is a problem in stress studies. For example, corticosterone levels were higher for all doses of LPS in the dose response study (Figure 2.5B) than in study 2.2.3.1 (Figure 2.4C). This was not the case with ACTH levels. The difference between corticosterone responses between these two studies may have arisen from batch variability between the LPS used, as similar doses of LPS have been reported in the literature to induced variable stress responses (Huang et al., 1998, Castanon et al., 2003). There are also known age-related changes in adrenal sensitivity to stress (Heidinger et al., 2008), though the age difference of only 4 weeks in our study is unlikely to be sufficient to explain the differences observed. Based on the results of the LPS dose-response study, an LPS dose of 25ug/kg was chosen to investigate the effects of a higher dose of leptin on the HPA response to acute LPS challenge. In support of this, other studies have shown that 25μg/kg LPS induces a strong but reversible stress response, as demonstrated by the inhibition of LPS-induced fever by systemic administration of α-MSH in rats (Huang et al., 1998). Study 2.2.3.3 was therefore carried out wherein rats were administered leptin at a dose of 2mg/kg which has previously been shown to i) result in circulating plasma leptin levels well above those seen in obese rats (Beck and Richy, 2009), ii) reduce basal corticosterone levels in rats (Clark et al., 2008), and iii) inhibit the HPA response to restraint stress in rats (Heiman et al., 1997). Rats were then injected with 25μg/kg LPS. The results show that plasma ACTH and corticosterone concentrations in LPS-treated rats (Figure 2.8B-C) reached similar levels to those observed in the study by Huang and colleagues (Huang et al., 1998). Plasma IL-1β levels achieved in LPS-treated rats (Figure 2.8D) were also similar to levels observed in septic humans (Kurt et al., 2007, Sankar and Webster, 2012) and fell into the range of IL-1β levels observed in

animal models of endotoxemia (Rodrı et al., 2002). But again, this higher dose of leptin failed to influence the LPS-induced increase in LPS-induced ACTH, corticosterone and IL-1β levels (Figure 2.8B-D).

To date, all other studies have investigated the effects of leptin on the HPA response to milder stressors such as fasting and restraint stress, whereas the studies described in this chapter have focused on the effects of leptin on the HPA response to a powerful immunological stressor. It is interesting to note that whilst different stressor types e.g. head trauma (Ott et al., 1987), stroke (Kang et al., 2008), autoimmune diseases (Hu et al., 1993) and psychological stress (Connor and Leonard, 1998) ultimately activate the HPA axis via a convergence at the hypothalamus to precipitate the CRH-ACTH-glucocorticoid cascade, there is surprisingly little overlap in the sets of genes within the brain that are induced or repressed by severe immunological stress compared to mild psychological stressors such as restraint stress (Reyes et al., 2003). In addition to the convergent effect at the hypothalamus, LPS can also act directly at the pituitary gland to stimulate the inter-pituitary release of IL-6, which subsequently upregulates ACTH secretion (Gloddek et al., 2001, Watanobe and Yoneda, 2003). LPS can also act directly on the adrenal gland to stimulate cortisol release which is mediated via cyclooxygenase-dependent mechanisms, and LPS can also increase IL-1 receptor (IL-1R) and IL-1α and β mRNA expression in the mouse adrenal gland (Mohn et al., 2011, Pournajafi Nazarloo et al., 2003, Andreis et al., 1991). Thus the fact that LPS induces a different activational response within the brain compared to mild stressors, and activates the HPA axis at multiple levels may provide an explanation as to why leptin failed to attenuate the LPS induced HPA response in my studies.

In conclusion, these findings suggest that the stimulation of the HPA axis by a commonly used endotoxin is not affected by peripheral leptin administration. Due to the observed lack of effect of leptin on the HPA axis response to stress in the current studies, I decided to

concentrate on the effects of a long-term stressor, namely SI, on the HPA axis response to acute stress in rats.

Chapter III:

Investigating the effects of SI on the HPA axis response to stress

3.1 Introduction

SI, known more commonly as loneliness, is a chronic form of distress (Weiss, 1974). Longitudinal studies in humans have identified behavioural, neural, hormonal, cellular and genetic changes as a result of loneliness in humans (Cacioppo et al., 2006, Cacioppo and Hawkley, 2009). SI in humans has been linked to stress-associated increases in vascular resistance (Cacioppo et al., 2002b), blood pressure (Hawkley et al., 2006, Hawkley et al., 2010) and morning cortisol (Adam 2006), as well as reduced quality of sleep (Hawkley et al., 2010, Cacioppo et al., 2002a), and sedentary lifestyles (Hawkley et al., 2009). SI has also been linked to increased blood pressure in rats (Andrews et al., 2003). The effect of SI in nonhuman social mammals is evident from studies that show isolation can cause stress-associated changes such as increased sympathetic tone and HPA activation, and decreased immunity and expression of glucocorticoid-regulating genes (Adam et al., 2006, Cole et al., 2007, Cole, 2008). SI is indeed a stressful experience in rodents as it can result in increased aggression and anxiety in response to human handling (Hatch et al., 1965).

3.1.1 Effects of SI on HPA function

3.1.1.1 Changes in circadian patterns of stress hormone release

SI of 5-week old male rats for four weeks increases corticosterone secretion and alters its 24-hour pattern of release (Perello et al., 2006). Isolated rats demonstrate a slight drop in plasma corticosterone at 2100 h and then a sharp increase in corticosterone levels at 0100 h, suggesting they are more stressed then control rats during their active phase (Perello et al., 2006). This is not accompanied by changes in the plasma ACTH profile, suggesting that isolation stress may affect adrenal cell function directly, either through autonomic innervations of the adrenal gland or through circulating catecholamines (Kalsbeek et al., 2003, Perello et al., 2006). The circadian patterns of circulating levels of the pituitary-released stress hormones, PRL and GH, are also altered as a result of SI, and their total release is decreased (Perello et al., 2006).

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3.1.1.2 Behavioural changes

Many changes in behaviour have been observed in animal models of SI, notably including several that are comparable to the negative symptoms seen in humans with psychiatric disorders. One of the most profound changes that occur in isolated rats and mice is the increase in aggression, after even just brief periods of individual housing (Brain, 1975, Baumel et al., 1970). Another study found that group size has an impact on multiple aspects of behaviour, particularly during the first two months of housing after rats are first introduced to each other. Here it was found that isolated rats spend more time sniffing, chewing cage bars and dashing around their cages- behaviours attributed to escape attempts- whilst group housed rats spend more time sleeping and feeding (Hurst et al., 1999). In post-weaning male Wistar rats, 13 weeks of SI causes an anxiogenic behavioural profile and behavioural hyperactivity under basal and stressful conditions. Specifically, isolated rats demonstrate a 60% reduction in entries into the open arms of a plus maze during a plus maze test, as well

as reduced locomotion on the entire maze, compared to group housed rats (Weiss et al., 2004). Furthermore, during a startle session comprised of a restraint stress session followed by high intensity acoustic stimulation, isolated rats showed a trend towards higher startle reactivity compared to group housed rats (Weiss et al., 2004).

3.1.2 Effects of SI on activated HPA function: Hypo-reactivity or hyper-reactivity?

3.1.2.1 HPA Hypo-reactivity

A few studies have demonstrated HPA hypoactivity in response to a novel stressor after periods of SI (Brain and Nowell, 1971, Viveros et al., 1988). Sanchez et al. showed that long term SI reduces the corticosterone response to restraint stress in rats, resulting in fewer ACTH-immunoreactive pituitary corticotrophs in response to a restraint stress procedure in 11-week old isolated rats, compared to group housed rats. Corticotrophs were significantly smaller in isolated rats and exhibited less ACTH immunoreactivity than control rats (Sanchez et al., 1998). It is suggested that SI, in this case, either caused desensitization to stressful stimuli, or that the reduced activation of corticotrophs in response to acute restraint stress was due to changes in the normal maturation of these cells, as rats in this study were prematurely weaned at the age of 16 days (Sanchez et al., 1998).

3.1.2.2 HPA Hyper-reactivity

Most of the literature points to HPA hyperactivity in response to a novel stressor following periods of SI. In male rats, SI results in a higher stress hormone response to an acoustic stimulation-restraint stress challenge compared to their group-housed counterparts (Weiss et al., 2004, Holson et al., 1991). Chronic SI also enhances HPA responsiveness to a range of new stressors such as immobilization, cold stress and foot shock. One study shows that SI exaggerates the proinflammatory cytokine response to cardiac arrest and exacerbates

stroke-induced depressive-like behaviour in mice (Akana et al., 1992, Norman et al., 2010). This is supported by studies showing that long-term SI increases the corticosterone response to acute stress in mice, rats and hamsters compared to their group housed counterparts (Hermes et al., 2006, Dronjak et al., 2004, Ferland and Schrader, 2011, Toth et al., 2011, Weiss et al., 2004). SI can also increase the cortisol response to stress in larger mammals such as pigs and cattle (Creel and Albright, 1988, Kanitz et al., 2009).

Evidence suggests that HPA hyperactivity has profound negative effects on glucocorticoid-regulated processes, such as neuronal activity in the brain. In group housed rats, running induces hippocampal neurogenesis, but has the opposite effect in isolated rats (Stranahan et al., 2006). Immobilization stress results in increased cortisol and impaired wound healing in isolated hamsters but not pair-housed hamsters. Removal of endogenous cortisol via adrenalectomy eliminates the effects of immobilization stress on wound healing in isolated hamsters, whilst blocking of the cortisol stress response by oxytocin injection facilitates wound healing (Detillion et al., 2004). This shows that removal of the primary source of glucocorticoids or blocking the effects of glucocorticoids downstream can abolish the effect of SI on wound healing, suggesting the response to immobilization stress observed in isolated hamsters is due to increased cortisol release. These wound healing effects are consistent with the known immunosuppressive effects of cortisol. This work is relevant to impaired immunity in lonely humans; elevated urinary cortisol levels were accompanied by lower natural killer cell activity and poorer T-lymphocyte responses to mitogen stimulation in lonely psychiatric patients compared to non-lonely psychiatric patients (Kiecolt-Glaser et al., 1984a, Kiecolt-Glaser et al., 1984b). In non-human animals, increased prenatal exposure to glucocorticoids reduces birth weight and causes hypertension, hyperglycaemia and increased HPA activity in the offspring in later life, whilst in humans it causes low birth weight and increased HPA activity, and high blood pressure, insulin resistance, glucose intolerance and hyperlipidemia in subsequent adulthood (Seckl and Meaney, 2004). All these conditions have an inflammatory component and accord with findings that link perceived isolation in

humans with a pro-inflammatory gene expression profile (Cole et al., 2007). This supports the notion that chronically lonely individuals are at increased risk of inflammatory disease (Cole et al., 2007, Cole et al., 2012, Fredrickson et al., 2013).

As described in chapter 1, loneliness can promote the pathogenesis of psychiatric disorders such as schizophrenia and depression (Pariante and Lightman, 2008, Kaye et al., 1987, Corcoran et al., 2003), and an overactive HPA axis is implicated in major depression (Nemeroff and Vale, 2005, Sanchez et al., 2001). Many depressed patients have increased plasma cortisol levels, as well as enlarged and hyperactive pituitary and adrenal glands (Nemeroff and Vale, 2005). This is thought to be due to an impaired glucocorticoid feedback mechanism, as studies have shown that the HPA axis is insensitive to orally administered dexamethasone in depressive patients compared to healthy subjects (Pariante, 2006). HPA axis hyperactivity is also found in patients with anxiety disorders (Abelson et al., 2007, Condren et al., 2002). It is thought that both glucocorticoid insensitivity and CRH over-expression may contribute to overall HPA axis hyperactivity in these patients (Kunugi et al., 2006). It has also been found that stressful life events increase the risk of schizophrenia (Ellenbroek et al., 1998), and that schizophrenic patients have elevated plasma cortisol levels (Meltzer, 1987) and a higher stress hormone response to a novel stressor (Lammers et al., 1995).

3.1.2.3 Age-related differences in HPA dysregulation

The developmental timing of SI is key, and pre-weaning effects of isolation may differ from post-weaning effects (Weiss et al., 2001), as may the effects measured sometime after the SI procedure compared with immediately after SI. In evolutionary terms, this may reflect that SI presents different survival challenges to an infant animal compared to an adult animal. For infants, protection against the negative effects of excess glucocorticoid exposure on the developing CNS (Sapolsky and Meaney, 1986) may be the reason hypo-responsiveness in response to an acute stress is seen in pre-weaning animals (Sapolsky and Meaney, 1986). On the other hand, sexually mature animals or post-weaning animals are no longer protected from the stress of these survival challenges, which may explain why these animals exhibit HPA hyper-reactivity in response to novel stressors.

There is much inconsistency between, and within, animal species in studies of SI and HPA stress responsiveness. Hawkley et al. have reviewed and summarized the major studies done to date in this area (Table 3.1) (Hawkley et al., 2012). Inconsistencies arise because of experimental differences such as the timing of glucocorticoid measurements or the source of cortisol samples e.g. plasma or saliva. These inconsistencies make it difficult to draw conclusions regarding the effects of SI on HPA-regulated physiological processes in social mammals, and thus to translate them into human studies. More definitive studies investigating the effects of SI on the HPA response to stress are needed in a commonly used social mammal. This would allow such invasive experimental studies that are required, to provide more detailed information into the mechanisms involved in SI induced HPA dysfunction. One such mammal is the rat.

TABLE 3.1 Effects of SI on HPA functioning in social mammals. n.s.; non significant. Adapted from (Hawkley et al., 2012).

Species	Reference	Age at isolation	Isolation duration	Age at testing	Basal Glucocorticoid + ACTH	Glucocorticoid reactivity to stress
Mice	(Chida et al., 2005)	Post-weaning (5 weeks)	9 weeks	Adult	n.s. glucocorticoid different at baseline. Greater glucocorticoid increase from baseline in isolated than group housed animals at 9 weeks; group housed animals continued to show glucocorticoid increase; at 19 weeks, control glucocorticoid > isolated glucocorticoid	Not investigated
Mice	(Williams et al., 2009)	Post-weaning	Chronic	Adult		Increased glucocorticoid response to stress
Voles	(Grippo et al., 2007a)	Adult	60 days	Adult	n.s. difference	Increased glucocorticoid response to resident intruder
Voles	(Grippo et al., 2007b)	Adult	4 weeks	Adult	n.s. difference	Increased glucocorticoid response to resident intruder
Voles	(Pournajafi-Nazarloo et al., 2011)	Adult	1 h, or 4 weeks of daily 1h (separated from same-sex sibling partner)	Adult	Increased glucocorticoid	Not investigated
Voles	(Pournajafi-Nazarloo et al., 2011)	Adult	4 continuous weeks	Adult	n.s. difference	Not investigated

TABLE 3.1 Continued

Species	Reference	Age at isolation	Isolation duration	Age at testing	Basal Glucocorticoid + ACTH	Glucocorticoid reactivity to stress
Voles	(Ruscio et al., 2007)	Post-weaning	4 or 21 days	Juvenile	Increased glucocorticoid (and increased CRH in hypothalamus)	Not investigated
Voles	(Stowe et al., 2005)	adult, 3-4 months of age	24 h or 2 weeks	Adult, 3-4 months of age	n.s. difference	Not investigated
Rats	(Djordjevic et al., 2010)	Adult	21 days individual housing, followed, or not, by 30' immobilization	Adult	Decreased glucocorticoid	n.s. different in cortisol response to immobilization
Rats	(Dronjak et al., 2004)	Adult	long-term	Adult	Increased glucocorticoid relative to long-□term crowded animals	Increased glucocorticoid response to acute immobilization and cold stressors relative to long-□term crowding
Rats	(Ferland and Schrader, 2011)	Adult (56 days)	Overnight for 14 consecutive nights, with or without chronic variable stress (warm/cold swim, cold room, rotation)	Adult		Increased glucocorticoid in response to separation with or without chronic variable stress
Rats	(Hermes et al., 2006)	Post-weaning (~ 28 days)	3 months	Adult, 4 months	Decreased glucocorticoid	Increased glucocorticoid response to restraint stress in females
Rats	(Lukkes et al., 2009)	Juvenile, day 21	3 weeks, followed by 2 weeks group housing	Adult (8 weeks)		n.s. difference in cortisol response to restraint stress
Rats	(Perello et al., 2006)	Post-puberty (35 days)	4 weeks	Adult, 9 weeks	Glucocorticoid increased in plasma, decreased in adrenal gland	Not investigated

TABLE 3.1 Continued

Species	Reference	Age at isolation	Isolation duration	Age at testing	Basal Glucocorticoid + ACTH	Glucocorticoid reactivity to stress
Rats	(Sanchez et al., 1998)	Post-weaning (early at 16 days)	2 months	Adult	Decreased glucocorticoid	Decreased glucocorticoid response to immobilization stress
Rats	(Scaccianoce et al., 2006)	Adult (2 months)	8 weeks		Glucocorticoid n.s. different from pair housed rats	Not investigated
Rats	(Serra et al., 2005)	Early life	chronic, 4 weeks	Adult	Decreased basal ACTH	Not investigated
Rats	(Toth et al., 2011)	Post-weaning (21days)	7-8 weeks	Adult	Glucocorticoid n.s. differently than socially housed animals	Increase response to aggressive encounter
Rats	(Weintraub et al., 2010)	Adolescent (30 days)	3 weeks (days 30-50); then housed in same-sex groups	Adolescent (50 days) and adult (70 days)		Adolescence: greater glucocorticoid recovery in males and less glucocorticoid recovery in females following acute restraint stress. Adult: decreased glucocorticoid response in males and increased glucocorticoid response in females following restraint stress.
Rats	(Weiss et al., 2004)	Post-weaning	Chronic	Adult (4 months)	ACTH increased in males, but not females	Increased glucocorticoid and ACTH response to (open field and elevated plus maze) stress in males, but not females
Hamsters, Siberian dwarf	(Castro and Matt, 1997)	Adult	Males separated from mate for 4 weeks	Adult	Increased glucocorticoid in isolated relative to mate housed animals?	Not investigated

TABLE 3.1 Continued

Species	Reference	Age at isolation	Isolation duration	Age at testing	Basal Glucocorticoid + ACTH	Glucocorticoid reactivity to stress
Hamsters, Siberian dwarf	(Detillion et al., 2004)	Post-weaning (2-3 months)		Adult	glucocorticoid n.s. different than pair housed animals	Increased response to immobilization
Cattle	(Creel and Albright, 1988)	Neonatal	10 weeks	Post-puberty, 1 year of age	n.s. glucocorticoid increase relative to control calves at 10 weeks of age	Increased response to acute stress (approach and flight distances from humans) at 10 weeks of age
Pigs	(Kanitz et al., 2009, Tuchscherer et al., 2010)	Pre-weaning	Single 4 h bout	Infant: 7, 21, and 35 days of age		Increased glucocorticoid response to immobilization
Pigs	(Kanitz et al., 2004)	Pre-weaning	2 h daily, Days 3-11	Pre-weaning	Increased glucocorticoid	Not investigated
Pigs	(Tuchscherer et al., 2004)	Pre-weaning	2 h daily, Days 3-11	Pre-weaning	glucocorticoid and ACTH increased at Day 12 and Day 56 vs. control piglets n.s. different cortisol response to peripheral LPS in isolated vs. control animals	Not investigated
Marmosets	(Cross et al., 2004)	Adult	15 min	Adult	Increased glucocorticoid	Not investigated
Marmosets	(Smith et al., 2011)	Adult	6 to 20 weeks	Adult	Increased glucocorticoid	Not investigated

TABLE 3.1 Continued

Species	Reference	Age at isolation	Isolation duration	Age at testing	Basal Glucocorticoid + ACTH	Glucocorticoid reactivity to stress
Marmosets	(Smith and French, 1997)	Adult	11 h; 11 h plus 5 min handling stress after net capture	Adult	Increased glucocorticoid	Not investigated
Monkeys (rhesus)	(Feng et al., 2011)	Pre-weaning (at birth)	1 month, then peer or mother-reared	Puberty: 2 and 3.5 years	Basal hair cortisol lower at both time points in peer than mother-reared animals	Delayed plasma cortisol recovery from capture and restraint stress in peer reared relative to mother-reared animals.
Monkeys (rhesus)	(Higley et al., 1992)	Juvenile: 6 and 18 months	4 sequential 4-day separation from mothers or peer group	Juvenile: 6 and 18 months (prior to and on last day of separation)	See glucocorticoid reactivity to isolation stress	Increased plasma cortisol response to social separation; persistently elevated cortisol throughout separation
Monkeys (squirrel)	(Levine and Mody, 2003)	Pre-weaning	4-6 h bouts for 2 months	Adult (2-3 years of age)	Decreased glucocorticoid	Not investigated
Monkeys (squirrel)	(Lyons et al., 1995)	Juvenile	7 days individual housing	Juvenile	glucocorticoid increased; ACTH decreased	Not investigated
Monkeys (squirrel)	(Lyons et al., 1999)	Adolescent, late (31-33 months)	6 days housed alone (vs. in pairs and in 4-monkey groups)	Adolescent, late	glucocorticoid increased 1 day after separation vs. paired condition; ACTH increased up to 1 h post-separation, then decreased 1 day post separation vs. paired condition (cortisol inversely related to ACTH)	Not investigated
Monkeys (squirrel)	(Mendoza and Mason, 1986)	Adult	1h in home cage separated from pair-mate	Adult	See glucocorticoid reactivity to isolation stress	No effect on plasma corticosteroid 1 h post separation
Monkeys	(Mendoza and Mason, 1986)	Adult	1h in home cage separated from pair-mate	Adult	See glucocorticoid reactivity to isolation stress	Increased plasma cortisol 1 h post-separation

TABLE 3.1 Continued

Species	Reference	Age at isolation	Isolation duration	Age at testing	Basal Glucocorticoid + ACTH	Glucocorticoid reactivity to stress
Baboons	(Sapolsky et al., 1997)	Adult and juvenile	SI relative to median values	Adult and juvenile	Increased glucocorticoid	Not investigated
Humans	(Arnetz et al., 1983)	Adult, older	Senior citizen housing (social activity program vs. control)	Adult, older	Increased glucocorticoid	Not investigated
	(Ebrecht et al., 2004)	Adult	Loneliness	Adult	n.s. association with morning rise in cortisol	Not investigated
Humans	(Grant et al., 2009)	Adult, middle-aged	Smaller social network	Adult, middle-aged	Increased glucocorticoid (daily integrated "area-under-the curve"	Not investigated
Humans	(Kiecolt-Glaser et al., 1984a, Kiecolt-Glaser et al., 1984b)	Adult	Loneliness in psychiatric patients	Adult	Increased glucocorticoid (urinary)	Not investigated
Humans	(Pressman et al., 2005)	Adult, young	Loneliness	Adult, young	increased glucocorticoid	Not investigated
Humans	(Adam et al., 2006)	Adult, middle-aged	Loneliness adult, middle-aged	Increased morning rise in cortisol as a function of trait loneliness and as a function of daily fluctuations in loneliness		Not investigated
Humans	(Steptoe et al., 2004)	Adult, middle-aged	Loneliness	Adult, middle-aged	Increased morning rise in cortisol as a function of trait loneliness; n.s. association with total cortisol or cortisol slope over working day	n.s. association with cortisol response to Stroop mirror-tracing tasks
Humans	(Doane and Adam, 2010)	Adult, middle-aged	Loneliness	Adult, young	Increased morning rise in cortisol as a function of daily fluctuations in loneliness, and flatter diurnal slope as a function of trait loneliness	Not investigated

3.1.3 Hypothesis

SI can alter the HPA axis response to a novel acute stress in the adult rat, by sensitizing and/or desensitizing specific components of the axis.

3.1.4 Aims

To test this hypothesis I aim to:

- Investigate the effects of 7-day and 3-week SI on HPA function within multiple HPA tissues in adult rats, examining basal HPA activity, HPA axis responsiveness to acute stress and neuronal activation in stress related hypothalamic centres.
- Examine the stress hormone profiles of isolated rats compared to group-housed rats.
- Investigate possible SI-induced changes in the HPA-glucocorticoid feedback mechanism.
- Investigate possible SI-induced changes in the expression of stress-related genes in specific components of the HPA axis.

3.2 Methods

3.2.1 Animals and housing

All animal procedures were carried out under the British Home Office Animals Scientific Procedures Act 1986 (Project License Number: 70/6623). On arrival, specific pathogen free (SPF) male Sprague Dawley rats (Charles River) were either group housed in fours or individually housed, and maintained at 21-23˚C on a 12 hr light: 12 hr dark cycle (light period 07:00-19:00 hrs) with *ad-libitum* access to RM1 standard chow (Special Diet Services Ltd) and water. After the 72 hour acclimatization period, all rats were handled daily for 3 minutes each for 7 days or 3 weeks depending on the study designs outlined in 3.2.2. This was to minimize the risk of non-specific stress-induced increases in HPA activation, as this could confound experimental stress-induced activation of the HPA axis. At 7 days or 3 weeks, the experiments were carried out in the early light phase.

3.2.2 Materials

3.2.2.1 Restraint apparatus

Tailveiner Restrainer (Braintree Scientific Inc., Massachusetts, USA).

3.2.2.2 Hormone immunoassay kits

ACTH IRMA and corticosterone EIA kits were obtained, as listed in chapter 2 (2.2.2.2).

3.2.2.3 Materials for immunohistochemistry

As listed in chapter 2 (2.2.2.3).

3.2.2.4 Materials for primary adrenal culture

Earle's Balanced Salt Solution – phenol red free (EBSS) (+2.2g/l sodium bicarbonate and pregassed with O_2) (Sigma-Aldrich)

Collagenase from Clostridium Histolyticum (Sigma-Aldrich)

Trypan Blue (Sigma-Aldrich)

3.2.2.5 Materials for hypothalamic qPCR

RNA extraction:

Tri-reagent (Sigma-Aldrich)

1-Bromo-3-chloropropane (BCP) (Sigma-Aldrich)

70% (*v*/*v*) ethanol (see Appendix I)

PureLink RNA Mini Kit (Invitrogen, Paisley, UK)

- Wash Buffer 1
- Wash buffer 2
- RNase free water

27.3 U/ul PureLink DNase (Invitrogen)

- 10% DNase I reaction buffer
- 12.5% DNase
- 77.5% RNase free water

Agarose gel electrophoresis:

1% Agarose Gel (see Appendix I)

50x TAE Buffer (see Appendix I)

Ethidium Bromide (EtBr) (Thermo Scientific)

Running Buffer (see Appendix I)

6 x Orange DNA loading dye (Thermo Scientific)

Reverse transcription:

Rat hypothalamic RNA (at a concentration of 10µg/µl)

High Capacity complementary DNA (cDNA) Reverse Transcription (RT) Kit (Invitrogen)

2 x RT master mix (20µl reaction per sample):

2ul 10x RT buffer

0.8ul 25x deoxyribonucleotide triphosphate (dNTP) Mix (100mM)

2ul 10x RT Random primers

1ul Multiscribe Reverse Transcriptase

10.2ul Nuclease-free H_2O

Quantitative polymerase chain reaction (qPCR):

Rat hypothalamic cDNA

Taqman gene expression Master mix (Applied Biosystems, Texas, USA)

β-actin primer (ID: Rn00667869_m1) (Invitrogen)

Glucocorticoid receptor (GR) primer (ID: Rn00561369_m1) (Invitrogen)

CRH primer (ID: Rn01462137_m1) (Invitrogen)

RNase-free H_2O

3.2.2 Study design

3.2.2.1 The effect of 7-day SI on the HPA axis response to acute restraint stress

Twenty male Sprague Dawley rats weighing approximately 200g were split into two groups of ten, with one group housed in fives and the others housed individually to isolate them, for 7 days. Rats were weighed everyday to monitor body weight gain. After 7 days, rats underwent experimentation in which 5 rats from each group (group housed and isolated) were restrained in a Tailveiner Restrainer (Braintree Scientific Inc) for 15 minutes before being returned to their cages, whilst the other 5 rats from each group were left in their cages. Two hours after being returned to their cages, all restrained rats, as well as all non-restrained rats, were decapitated by guillotine. This time point not only took into account the 90 minute time point at which maximum c-fos protein expression is reached after stimulation by a stressor, as described in chapter 2, but is also in line with time points investigated after stress exposure in isolated rats in other studies (Dronjak et al., 2004, Lukkes et al., 2009, Weiss et al., 2004, Ferland and Schrader, 2011). This made it possible to draw direct comparisons with other studies, as discussed later in this chapter. All tissues and blood were collected within the early light phase to minimize ultradian rhythmic changes in circulating plasma stress hormones.

Trunk blood was collected and plasma was separated and stored for measurement of plasma corticosterone and ACTH concentrations by EIA and IRMA respectively, as described in chapter 2 (2.2.3.1, 2.2.4.2 - 2.2.4.3). Whole rat brains were removed and separated from their brainstems. Brains were then fixed, dehydrated and stored as described in chapter 2 (2.2.3.2) until sectioning and immunohistochemical staining for c-fos at a later stage, also described in chapter 2 (2.2.5). Adrenal glands were dissected and placed into 5ml of pre-prepared EBSS. Adrenals were kept at room temperature in this solution until dispersion and culture for measurement of basal corticosterone release from

adrenal cells. This was conducted immediately after tissues and blood had been taken from all the rats.

3.2.2.2 The effect of 7-day SI on total plasma stress hormone release in response to acute restraint stress

This study aimed to investigate the temporal profile of HPA hormone release in response to stress in group housed and isolated rats. It sought to determine whether there is an increase in total hormone release in response to restraint stress, or whether the response has been temporally shifted and is in fact of a similar magnitude between the two housing groups.

Seventy-two male Sprague Dawley rats, weighing approximately 170g were split into two groups of 36, with one group housed in fours and the others housed individually to isolate them, for 7 days. Rats were weighed every day to monitor body weight gain. After 7 days, rats underwent experimentation in which 18 rats from each group (group housed and isolated) were restrained in a Tailveiner Restrainer for 15 minutes before being returned to their cages, whilst the other 18 rats from each group were left in their cages. Restrained rats were decapitated by guillotine 0, 15, 30, 60 or 180 minutes after cessation of the restraint stress procedure; rats from the 0 time point group were not returned to their cages and were decapitated immediately after cessation of restraint stress. All rats, including those that had not undergone restraint stress, were decapitated during the early light phase to minimize ultradian rhythmic changes in circulating plasma stress hormones.

The chosen time points allowed collection of plasma from the point at which stress hormone levels were expected to increase, all the way through to the point at which stress hormone levels were expected to return to resting levels (Vahl et al., 2005, Krause et al., 2011). This

allowed a complete time profile of the stress response, as well as calculation of the total amount of stress hormone release from each group.

Trunk blood was collected and plasma was separated and stored for measurement of plasma corticosterone and ACTH concentrations by EIA and IRMA respectively, as described in chapter 2 (2.2.3.1, 2.2.4.2 - 2.2.4.3).

3.2.2.3 The effect of 7-day, 7-day overnight or 3-week SI on the HPA response to acute restraint stress

The lack of difference in total stress hormone release in group housed rats compared to isolated rats in study 3.2.2.2 suggested that short term SI does not affect the stress response to restraint, but this finding was not in line with the results from study 3.2.2.1. As discussed, in section 3.4.2, group housed rats may have undergone additional stress due to factors such as cage-mate separation during the restraint stress procedure. The amount of time this additional stress response may have lasted and the extent to which it may have activated the HPA axis is unknown and may have influenced the time profile response observed after a restraint stress procedure. Therefore this particular study design may not be suitable for such investigations.

Others have shown that 3 weeks of SI (Dronjak et al., 2004, Ruscio et al., 2007, Smith et al., 2011) and intermittent overnight SI (Ferland and Schrader, 2011) cause dysregulation of the basal and acutely activated HPA axis, yet the mechanism by which these changes occur have not yet fully been investigated. Thus the study described in this section (3.2.2.3) was designed to investigate the mechanism by which the changes in the HPA response to restraint stress in isolated rats, in study 3.2.2.1, occurred. This study also investigated the effects of short term overnight SI and 3 weeks of SI on HPA responses to restraint stress in rats, as well as the mechanism by which these changes occur.

Sixty male Sprague Dawley rats, weighing approximately 145g were split into two groups of 36 and 24; with the first group being group housed in fours and the second group being housed individually to isolate each rat. Twelve group housed rats were maintained for 7 days and 12 were maintained for 3 weeks, and 12 isolated rats were maintained for 7 days and 12 were maintained for 3 weeks. An additional group of 12 group housed rats were isolated only in the active dark phase (19:00 hrs to 07:00 hrs) but returned to their original groups during the inactive light phase (07:00 hrs to 19:00 hrs). This resulted in 5 groups; 7-day group housed, 3-week group housed, 7-day isolated, 3-week isolated and 7-day overnight isolated rats. Rats were weighed every day to monitor body weight gain, and behavioural observations were carried out on all rats at 07:30 hrs and 18:30 hrs every other day. The behaviours monitored are listed in Table 3.2 and were scored according to this classification to determine whether there was a difference in behaviours exhibited between i) group housed and isolated rats and ii) group housed and overnight isolated rats, when overnight isolated rats were returned to their original groups.

After 7 days or 3 weeks, rats underwent experimentation in which 6 rats from each group (group housed, isolated and overnight isolated) were restrained in a Tailveiner Restrainer for 15 minutes before being returned to their cages, whilst the other 6 rats from each group were left in their cages. One hour and 45 minutes later, restrained rats, as well as all non-restrained rats were decapitated by guillotine. These studies were carried out during the early light phase to minimize ultradian rhythmic changes in circulating plasma stress hormones. Rats that had not undergone restraint stress were also decapitated during the early light phase. Trunk blood was collected and plasma was separated and stored for measurement of plasma corticosterone and ACTH concentrations by EIA and IRMA respectively, as described in chapter 2 (2.2.3.1, 2.2.4.2 - 2.2.4.3). Whole brains were removed from brainstems and trimmed to reveal the hypothalamus. Hypothalami were snap

frozen in liquid nitrogen and stored at -80˚C prior to RNA extraction and qPCR, to determine hypothalamic GR and CRH mRNA levels (described in section 3.2.8).

3.2.3 Behavioural Observations

Behavioural observations were carried out every other day. Once overnight isolated rats were returned to their groups, behaviours were observed at 07:30 hrs and 18:30 hrs. Behaviours were classified into one of 12 categories: feeding, drinking, rearing, locomotion, grooming, stationary, pica, head down, sleeping, burrowing, climbing or bed-making (Table 3.2), adapted from (Abbott et al., 2001). Within 30 minutes of the time points stated above (07:30 hrs and 18.30 hrs), each rat was observed for 5 seconds, 6 times each, and the behaviour displayed most within those 5 seconds was recorded.

TABLE 3.2 Classification of behaviours monitored for scoring of rat behaviours. Adapted from (Abbott et al., 2001).

Behaviour	Description of Behaviour
Feeding	Rat eating chow
Drinking	Rat drinking from water bottle
Rearing	Rat with front paws off floor
Locomotion	Rat with all four legs moving
Grooming	Rat grooming
Stationary	Rat standing or sitting still
Pica	Rat eating something other than chow
Head Down	Rat standing with nose lowered
Sleeping	Rat sleeping
Burrowing	Rat burrowing in cage floor covering
Climbing	Rat climbing cage
Bed Making	Rat bed making with paper strips

3.2.4 ACTH immunoradiometric assay

Plasma ACTH levels were measured using a commercial human ACTH IRMA kit as described in chapter 2 (2.2.4.2).

3.2.5 Corticosterone enzyme immunoassay

Plasma and cell culture media corticosterone levels were measured using a commercial corticosterone EIA kit as described in chapter 2 (2.2.4.3).

3.2.6 Immunohistochemical staining for cellular-fos

Brain sections were stained for c-fos using IHC methods described in chapter 2 (2.2.5.2).

3.2.7 Primary Adrenal Culture

The method described by Cover et al., (Cover et al., 2001) was modified for the culture of primary adrenal cells. Adrenals were grouped together dependent on experimental groups and chopped into small fragments using a razor blade on a glass Petri dish. The adrenal fragments were transferred to 2ml EBSS (+2.2g/l sodium bicarbonate) with collagenase from *Clostridium histolyticum* (2mg/ml) and incubated at 37ºC with a minimal flow of 95%O_2 / 5%CO_2 for 60 minutes. During this incubation, cells were mechanically dispersed every 10 minutes using a P1000 Gilson pipette with a trimmed 1000µl tip. The cells were filtered through a 100µm gauze and subsequently centrifuged at 1000g for 15min. The supernatant was removed and the cell suspension was resuspended in 2ml EBSS and centrifuged again as above. The cell suspension was then resuspended in 3ml EBSS and a 10µl sample was taken for staining with Trypan blue for cell counting. A cell count was performed with a haemocytometer and automated counter. Trypan blue stained any dead cells a medium-dark blue colour and cell viability was calculated as follows:

$$\frac{\text{Live cell count}}{\text{Total cell count}} \times 100 = \text{percentage viability}$$

The target cell count was 10^6 cells/ml and the volume of the cell suspension was adjusted accordingly with EBSS. Two hundred microlitres of the cell suspension was transferred to a 96 well plate which was pre-incubated in a humidifying chamber with a flow of 95%O_2 / 5%CO_2 for 60 minutes. Plates were removed, covered and centrifuged at 2000g for 10 seconds in a Hettich Rotina 420R centrifuge (Hettich Lab Technology, Tuttlingen, Germany), and the supernatant discarded by upturning the plates and blotting on tissue paper. Two hundred microliters of fresh EBSS was then added to each well and the plates were then incubated as above for 2 hrs, followed by centrifugation at 2000g for 10 seconds

and finally the supernatant was collected and stored at -20ºC until analysis of corticosterone levels by EIA.

3.2.8 Hypothalamic qPCR

3.2.8.1 RNA extraction

Frozen tissue was homogenized in 1ml/100mg Tri-reagent (Sigma-Aldrich) using a Tissuelyser II (Qiagen, Venlo, Netherlands) and RNA was separated by adding 100µl BCP (Sigma-Aldrich) to each sample and spinning at 12000rpm for 10 minutes. Total RNA was then extracted using a PureLink RNA Mini Kit, according to the manufacturer's protocol. Briefly, 350µl of 70% ethanol was added to each RNA sample and the solutions were transferred to filter cartridges. The cartridges were spun at 10700rpm for 15 seconds in a Microcentrifuge 5417R (Eppendorf, Hamburg, Germany). The flow-through was disposed of and 350µl wash buffer 1 was then added to each RNA sample. This wash solution was passed through the filter cartridges by spinning and the flow-through discarded again. Eighty microlitres PureLink DNase was then added to each sample and incubated at room temperature for 15 minutes. Three hundred and fifty microlitres of wash buffer 1 was again added to each cartridge and spun again for 15 seconds, the flow-through discarded and 500µl wash buffer 2 was added to each cartridge and also spun for 15 seconds. The flow-through was discarded and another wash step using wash buffer 2 was carried out. Cartridges were then spun for 1 minute to remove excess flow-through, before being inserted into recovery tubes. Thirty microlitres of RNase free water was added to the centre of each spin cartridge, incubated at room temp for 1minute and then spun a final time for 1 minute. For each sample, purified RNA in 30µl RNase free water was stored at -20˚C for determination of quantity and quality of RNA, and RT at a later date.

3.2.8.2 Agarose gel electrophoresis

The RNA concentration of samples was determined using a NanoDrop Lite Spectrophotometer (Thermo Scientific). One microlitre of RNA from each sample was pipetted onto the measuring platform and RNA concentration was measured twice for each sample, for the calculation of an average reading. The integrity of the RNA was confirmed by running 10µg of each RNA sample on a standard 1% agarose gel (Appendix I). Briefly for 2 large gels, 3.3g agarose and 330ml GDW were heated in a glass beaker until bubbling, allowed to cool and then mixed with 7ml 50 x TAE buffer (Appendix I) and 16.5µl EtBr. This mixture was poured into two gel frames and allowed to set for 30 minutes. Ten micrograms of RNA from each sample was heated to 70˚C for 1 minute, mixed with 5µl loading dye and kept on ice until loaded on to the gel. Once all samples were loaded, the gel frames were filled with running buffer (Appendix I) and run at 110 volts for 1 hour. The gels were then viewed on an ultraviolet (UV) light box to confirm the presence of two sharp bands for each intact total RNA sample; representing the 28s and 18s bands which are subunits of ribosomal RNA (rRNA) in eukaryotic RNA. The 28s rRNA band should be approximately twice the intensity of the 18s rRNA band, indicating fully intact RNA; whereas partially degraded RNA will either be smeared, lack sharp rRNA bands, or will not exhibit the correct difference in intensity between the 28s and 18s bands. Completely degraded RNA will appear as a very low molecular weight smear (Imbeaud et al., 2005).

3.2.8.3 Reverse transcription

Complementary DNA (cDNA) was synthesized from rat hypothalamic RNA using a High Capacity cDNA RT Kit. Briefly, 16µl 2x RT master mix (Appendix I) and 500ng RNA sample were added to PCR tubes in triplicates. Each tube was made up to 20µl with RNase free water and loaded into a PTC-100 Programmable Thermal Controller (MJ Research Inc., Quebec, Canada) under the following conditions: 25˚C for 10 minutes, 37˚C for 120 minutes, 85˚C for 5 seconds, and 4˚C until transferred to -20˚C for storage for qPCR at a later stage.

3.2.8.4 qPCR

Ten microlitres of Taqman gene expression Master mix and 8µl RNase-free water was added to each well of a 384-well reaction plate. One microlitre of cDNA sample (approximately 31ng) was then added to each well, in triplicate. One microlitre of β-actin primer, GR primer or CRH primer was then added to each corresponding well depending on the gene of interest, with β-actin used as the endogenous reference gene. The 384-well reaction plate was covered with optical adhesive film and centrifuged briefly to minimize air bubbles. The reaction well plate was then loaded into a 7900HT Fast Real-Time PCR machine (Applied Biosystems) and PCR performed using the following conditions: i) 50ºC for 2 minutes, ii) 95ºC for 10 minutes, iii) 95ºC for 15 seconds and iv) 60ºC for 1 minute (steps iii and iv were repeated for 40 cycles). Data was analyzed using RQ Manager Software (Applied Biosystems) and gene expression levels were quantified relative to β-actin RNA and using the $2^{-\Delta\Delta C}$ T method. The $2^{-\Delta\Delta C}$ T method calculates the fold change in gene expression (in this case GR or CRH) normalized to an endogenous reference gene (β-actin) and relative to the control (group-housed rats that did not undergo restraint stress).

3.2.9 Statistics

Body weight data was analyzed using the unpaired t-test. Behavioural data are represented as median frequency and interquartile range, with Kruskal-wallis and post-hoc Dunn's test for 7-day housed rats, and Mann-Whitney U test for 3-week housed rats, as data is non-parametric.

Plasma and primary adrenal culture stress hormone data, immunohistochemical c-fos counts and mRNA expression data were analyzed using two way analysis of variance (ANOVA) with a post-hoc Bonferroni test. Incremental area under the curve (AUC) analysis was used for measurement of total stress hormone release, followed by an unpaired t-test. Data is expressed as mean ± SEM.

Significance was taken at $P \leq 0.05$ throughout. All data were analyzed using GraphPad Prism version 5 for Windows (GraphPad Software).

3.3 Results

3.3.1 The effect of 7-day SI on the HPA axis response to acute restraint stress

3.3.1.1 Body weight

There was no difference in average body weight gain between group housed and isolated rats after 7 days of housing (Figure 3.1).

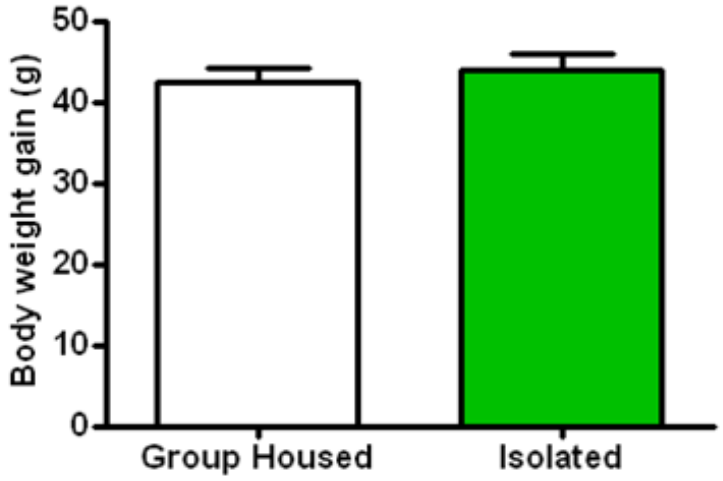


FIGURE 3.1 Average body weight gain in group housed and isolated rats. Male Sprague Dawley rats were group housed in fives or individually housed, for 7 days. Data are presented as mean ± SEM. unpaired t-test, ns; n = 10.

3.3.1.2 Plasma ACTH and corticosterone concentrations

Fifteen minutes of restraint induced a neuroendocrine stress response in rats. Isolated-restraint rats had significantly higher plasma ACTH levels than group housed-restraint and isolated-no restraint rats ($P \leq 0.05$), 2 hrs after cessation of restraint stress (Figure 3.2A). Group housed-restraint rats had significantly higher plasma corticosterone levels than their no-restraint controls ($P \leq 0.05$), while isolated-restraint rats had significantly higher plasma corticosterone levels than their no-restraint controls ($P \leq 0.01$) and group housed-restraint rats ($P \leq 0.01$) (Figure 3.2B). Two-way ANOVA revealed a significant interaction effect of housing on the response to restraint stress for both ACTH and corticosterone responses ($P \leq 0.05$). However there was no difference in basal ACTH or corticosterone levels between group housed and isolated rats (Figure 3.2A and B respectively). No other significant differences were observed between any of the experimental groups.

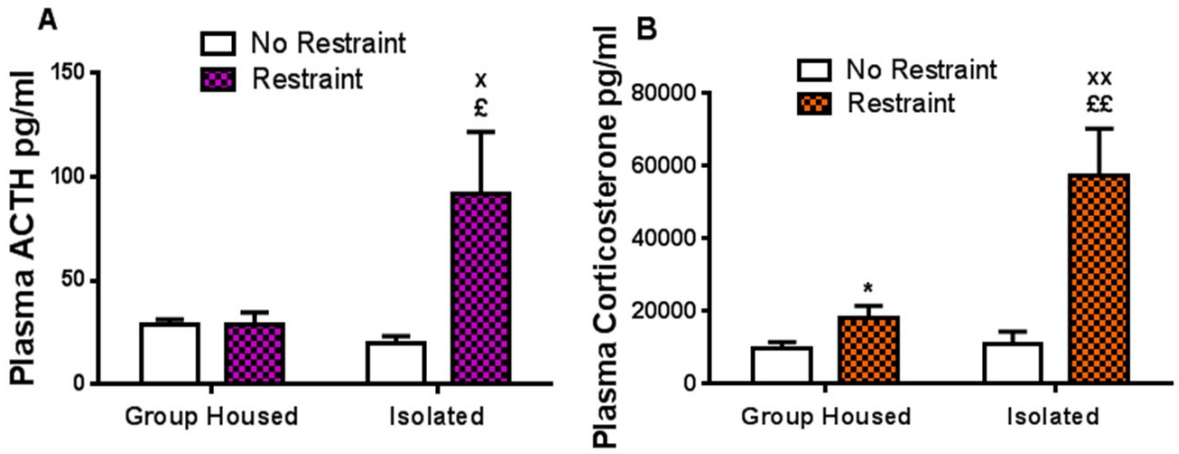


FIGURE 3.2 The effect of 15 minutes of restraint stress on plasma (A) ACTH and (B) corticosterone concentrations in group housed and isolated male Sprague Dawley rats. Rats were decapitated 2 hrs after cessation of restraint stress. Data are presented as mean ± SEM; two-way ANOVA with post-hoc Bonferroni correction. * = P ≤ 0.05 vs. group housed-no restraint, x = P ≤ 0.05 vs. group housed-restraint, xx = P ≤ 0.01 vs. group housed-restraint, £ = P ≤ 0.05 vs. isolated-no restraint, ££ = P ≤ 0.01 vs. isolated-no restraint; Bonferroni interaction value = P ≤ 0.05 for both **A-B**; n = 4-5.

3.3.1.3 Basal corticosterone release from rat primary adrenal cells

Primary adrenal cells from group housed-restraint rats demonstrated a significantly higher basal corticosterone release than those from group housed-no restraint rats (P ≤ 0.001), as did adrenal cells from isolated-restraint rats compared to those from isolated-no restraint rats (P ≤ 0.001) and group housed-restraint rats (P ≤ 0.001) (Figure 3.3). Two-way ANOVA showed there was no interaction effect of housing on corticosterone release from adrenal cells of all experimental groups. There was no significant difference in corticosterone release between adrenal cells from group housed-no restraint and isolated-no restraint rats (Figure 3.3).

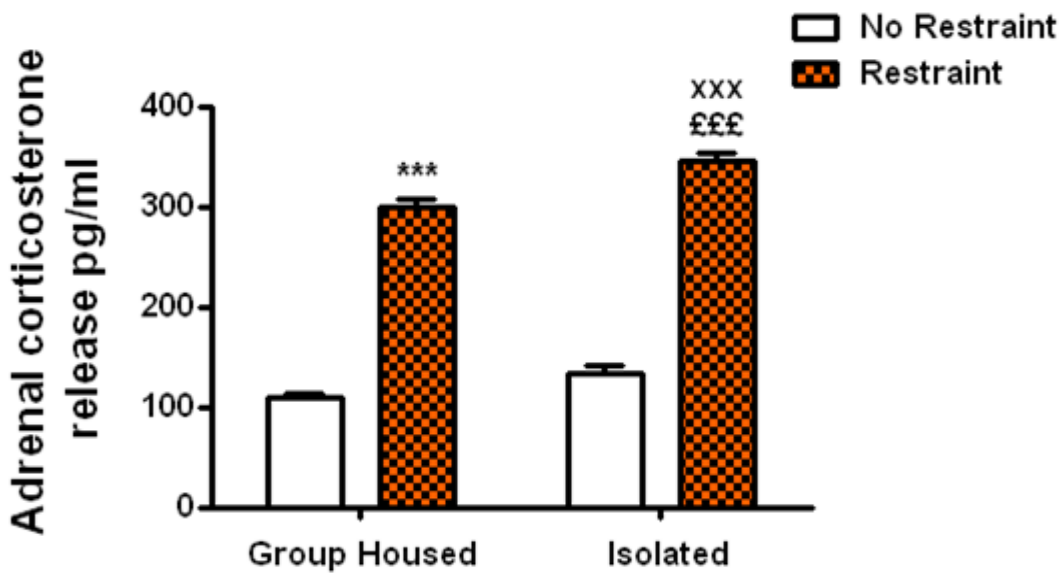


FIGURE 3.3 The effect of SI on basal corticosterone release from primary adrenal cells in male Sprague Dawley rats. Adrenals were dissected and grouped together dependant on treatment group. Adrenals were dispersed and cultured before being incubated for 2 hrs. Data are presented as mean ± SEM; two-way ANOVA with post-hoc Bonferroni correction. *** = P ≤ 0.001 vs. group housed-no restraint, xxx = P ≤ 0.001 vs. group housed-restraint, £££ = P ≤ 0.001 vs. isolated-no restraint; Bonferroni interaction value = ns and P ≤ 0.01; n = 9-11.

3.3.1.4 C-fos-like immunoreactivity in the hypothalamus

Fifteen minutes of restraint significantly increased c-fos expression in the hypothalamic ARC of group housed-restraint and isolated-restraint rats ($P \leq 0.05$ compared to their respective no-restraint controls) (Table 3.3, Figure 3.4A and Figure 3.5a-d), 2 hours after cessation of the restraint stress procedure. Restraint also significantly increased c-fos expression in the hypothalamic PVN of group housed-restraint and isolated-restraint rats ($P \leq 0.001$ and $P \leq 0.01$ respectively, compared to their no-restraint controls) (Table 3.3, Figure 3.4B and Figure 3.5e-h). Isolated rats demonstrated a significantly higher c-fos response in the PVN than group housed rats after restraint stress ($P \leq 0.05$) (Table 3.3, Figure 3.4B and Figure 3.5f and h respectively). There was no difference in c-fos protein expression in the ARC between group housed-no restraint and isolated-no restraint rats (Table 3.3, Figure 3.4A and Figure 3.5a and c respectively). There was also no difference in c-fos protein expression in the PVN between group housed-no restraint and isolated-no restraint rats (Table 3.3, Figure 3.4B and Figure e and g respectively). Two-way ANOVA revealed a significant interaction effect of housing on restraint stress induced c-fos protein expression in the PVN ($P \leq 0.05$) but not the ARC.

TABLE 3.3 The effect of 15 minutes of restraint on c-fos protein expression in rat hypothalamic areas of the group housed and isolated rats. Table summarizing c-fos-like positive cell counts observed in hypothalamic areas following 15 minutes of restraint stress in group housed and isolated male Sprague Dawley rats. Data are presented as mean ± SEM; two-way ANOVA with post-hoc Bonferroni correction; * = P ≤ 0.05 vs. group housed-no restraint, *** = P ≤ 0.001 vs. group housed-no restraint, x = P ≤ 0.05 vs. group housed-restraint, £ = P ≤ 0.05 vs. isolated-no restraint, ££ = P ≤ 0.01 vs. isolated-no restraint; Bonferroni interaction value = ns and P ≤ 0.05 for **ARC** and **PVN** respectively, n = 2-4.

Hypothalamic nucleus	**c-fos-like positive counts**			
	Group housed		**Isolated**	
	No restraint	**Restraint**	**No restraint**	**Restraint**
ARC	34 ± 19	125.7 ± 35.86*	52 ± 16.11	187.50 ± 51.50£
PVN	16.50 ± 1.50	198 ± 11.80***	34 ± 7.02	467.33 ± 82.93x, ££

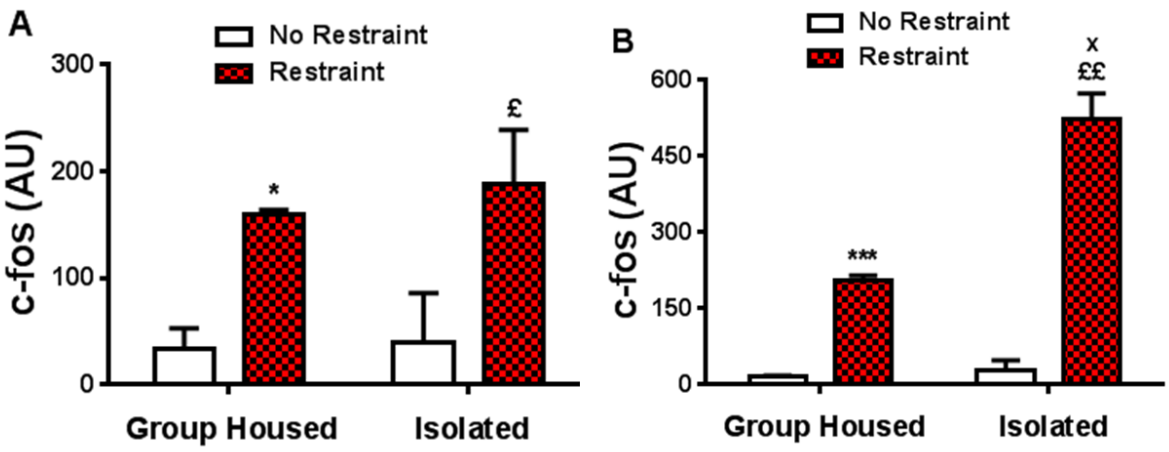


FIGURE 3.4 The effect of 15 minutes of restraint on c-fos protein expression in the hypothalamic (A) ARC and (B) PVN in group housed vs. isolated male Sprague Dawley rats. Rats were decapitated 2 hrs after cessation of restraint stress and hypothalami were immunostained for c-fos protein. Counts are represented as arbitrary units and data are presented mean ± SEM two-way ANOVA with post-hoc Bonferroni correction. * = P ≤ 0.05 vs. group housed-no restraint, *** = P ≤ 0.001 vs. group housed-no restraint, x = P ≤ 0.05 vs. group housed-restraint, £ = P ≤ 0.05 vs. isolated-no restraint, ££ = P ≤ 0.01 vs. isolated-no restraint; Bonferroni interaction value = ns and P ≤ 0.05 for A and B respectively; n = 2-4.

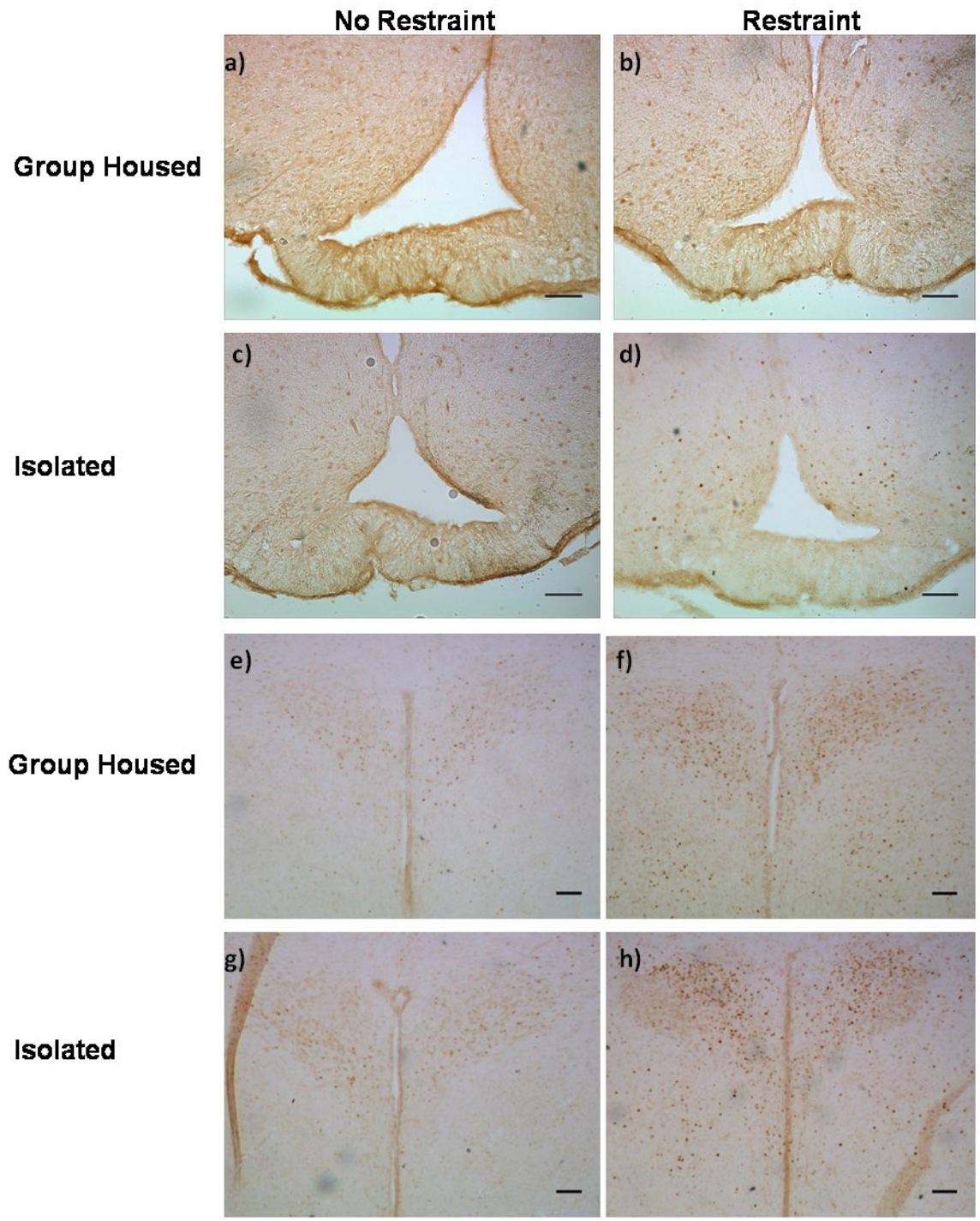


FIGURE 3.5 Representative photomicrographs of c-fos protein in coronal sections of the hypothalamic (a-d) ARC and (e-h) PVN. Images derived from **(a and e)** group housed-no restraint, **(b and f)** group housed-restraint, **(c and g)** isolated-no restraint and **(d and h)** isolated-restraint rats. The level in relation to the Bregma of the illustrated sections is Arc; -2.52mm and PVN; -1.72mm, according to the rat brain atlas (Paxinos and Watson 2007). Scale bar = 100μm.

3.3.2 The effect of 7-day SI on total plasma stress hormone release in response to acute restraint stress

3.3.2.1 Body weight

Isolated rats gained significantly more body weight compared to group housed rats after 7 days of housing ($P \leq 0.001$) (Figure 3.6).

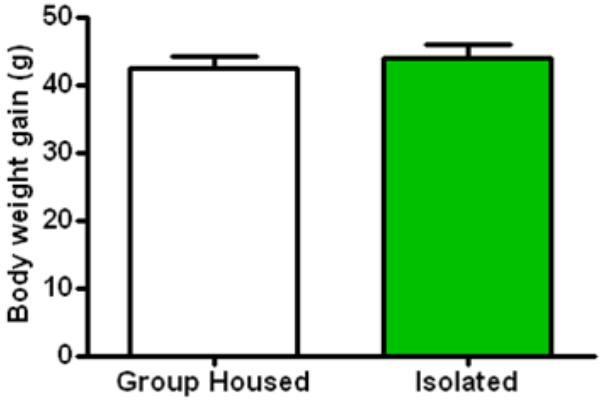


FIGURE 3.6 Average body weight gain (g) in group housed and isolated rats. Male Sprague Dawley rats were group housed in fours or individually housed, for 7 days. Data are presented as mean ± SEM. unpaired t-test, $P \leq 0.001$; n = 36.

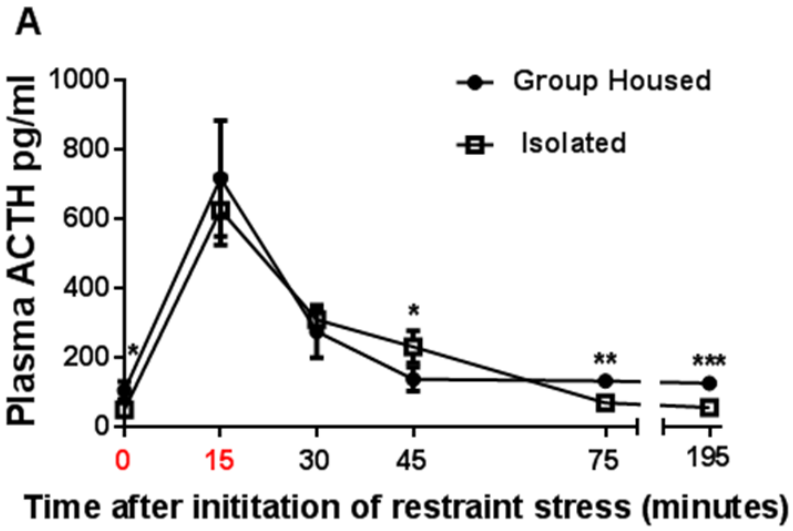
3.3.2.2 Plasma ACTH and corticosterone concentrations

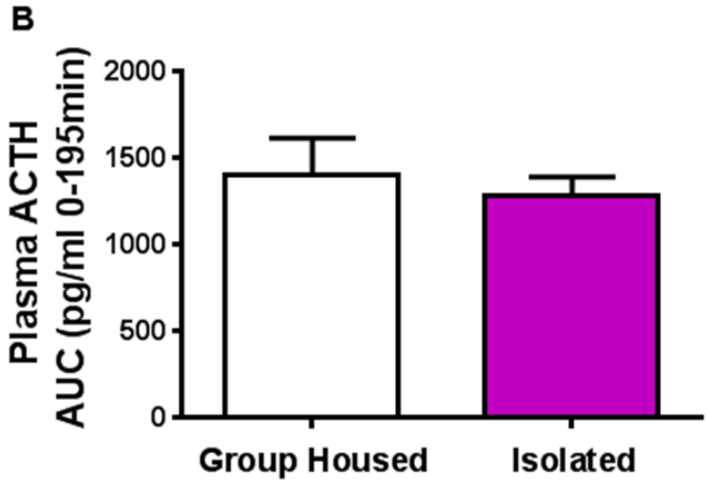
Basal plasma ACTH concentrations of group housed rats were significantly higher than those of isolated rats ($P \leq 0.05$) (Figure 3.7A), but there was no difference in basal plasma corticosterone concentrations between group housed and isolated rats. Fifteen minutes of restraint induced a strong stress response in both group housed and isolated rats, with plasma ACTH and corticosterone peaking at 15 and 30 minutes respectively, and returning close to resting levels at 75 minutes after the start of the restraint stress procedure (Figure 3.7A and C).

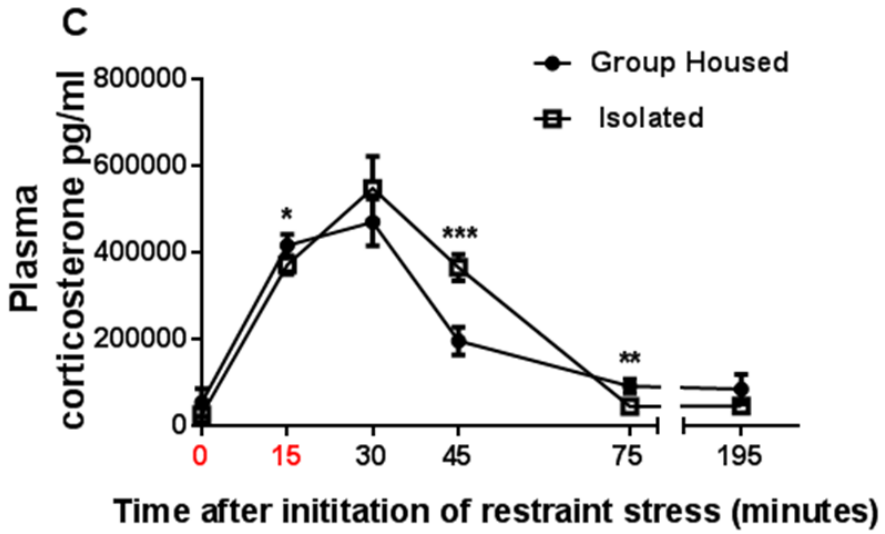
Restraint significantly increased plasma ACTH concentrations in isolated rats compared to group housed rats, 45 minutes after the start of the restraint procedure ($P \leq 0.05$) (Figure 3.7A). At 75 and 195 minutes, plasma ACTH levels of group housed rats were significantly higher than those of isolated rats ($P \leq 0.01$ and $P \leq 0.001$ respectively) (Figure 3.7A). A two-way ANOVA revealed no significant interaction effect of housing on ACTH response to restraint stress. Incremental AUC analysis revealed no significant difference in total ACTH release from 0-195 minutes between group housed and isolated rats (Figure 3.7B).

Plasma corticosterone concentrations were significantly higher in group housed rats than isolated rats, 15 minutes after the start of the restraint procedure ($P \leq 0.05$) (Figure 3.7C). At 45 minutes, plasma corticosterone levels of isolated rats were significantly higher than those of group housed rats ($P \leq 0.001$), but at 75 minutes group housed rats had significantly higher corticosterone levels than isolated rats ($P \leq 0.01$) (Figure 3.7C). Two-way ANOVA revealed a significant interaction effect of housing on corticosterone response to restraint stress ($P \leq 0.05$). But again, incremental AUC analysis revealed no significant difference in total corticosterone release from 0-195 minutes between group housed and isolated rats (Figure 3.7D). As both plasma ACTH and corticosterone concentrations return to resting levels at 75 minutes after the start of the restraint stress procedure, for both group

housed and isolated rats (Figure 3.7A and C), analysis of total plasma ACTH and corticosterone release from 0-75 minutes was also considered. But this also revealed no difference in total plasma ACTH and corticosterone release from 0-75 minutes between group housed and isolated rats.


A
Plasma ACTH pg/ml
1000
800
600
400
200
0
Group Housed
Isolated
*
*
**
***
0
15
30
45
75
195
Time after inititation of restraint stress (minutes)


B
Plasma ACTH
AUC (pg/ml 0-195min)
2000
1500
1000
500
0
Group Housed
Isolated


C
Plasma
corticosterone pg/ml
800000
600000
400000
200000
0
Group Housed
Isolated
*
***
**
0
15
30
45
75
195
Time after inititation of restraint stress (minutes)

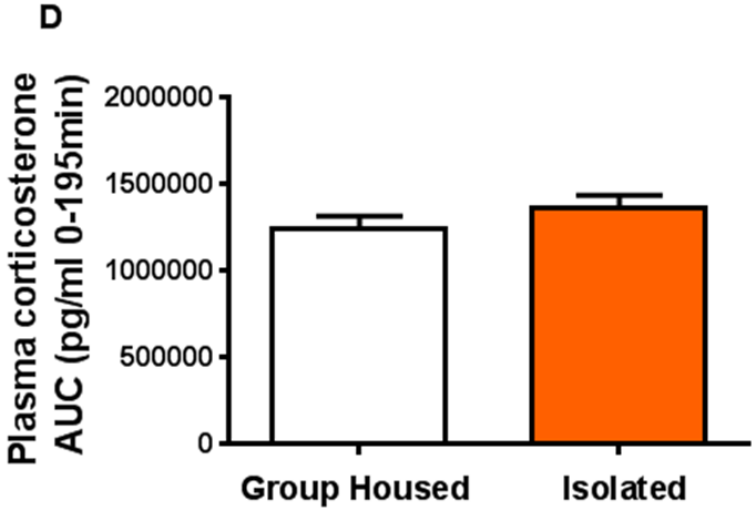

D
Plasma corticosterone
AUC (pg/ml 0-195min)
2000000
1500000
1000000
500000
0
Group Housed
Isolated

FIGURE 3.7 Time profile (A and C) and AUC (B and D) of plasma (A-B) ACTH and (C-D) corticosterone release, after 15 minutes of restraint stress in group housed and isolated male Sprague Dawley rats. Rats were decapitated immediately after cessation of restraint stress (15 minutes) or 30, 45, 75 and 195 minutes after the start of restraint stress. Red time-points refer to the start (0 minutes) and cessation (15 minutes) of the restraint stress period. A and C: data are presented as mean ± SEM; two-way ANOVA with post-hoc Bonferroni correction; * = $P \leq 0.001$, ** = $P \leq 0.01$, *** = $P \leq 0.05$; Bonferroni interaction value = ns and $P \leq 0.05$ for A and C respectively. B and D: unpaired t-test; ns. n = 6.

3.3.3 The effect of 7-day, 7-day overnight or 3-week SI on the HPA response to acute restraint stress

3.3.3.1 Body weight

There was no significant difference in body weight gain between group housed, isolated and overnight isolated rats after 7 days of housing (Figure 3.8A), or between group housed and isolated rats after 3 weeks of housing (Figure 3.8B).

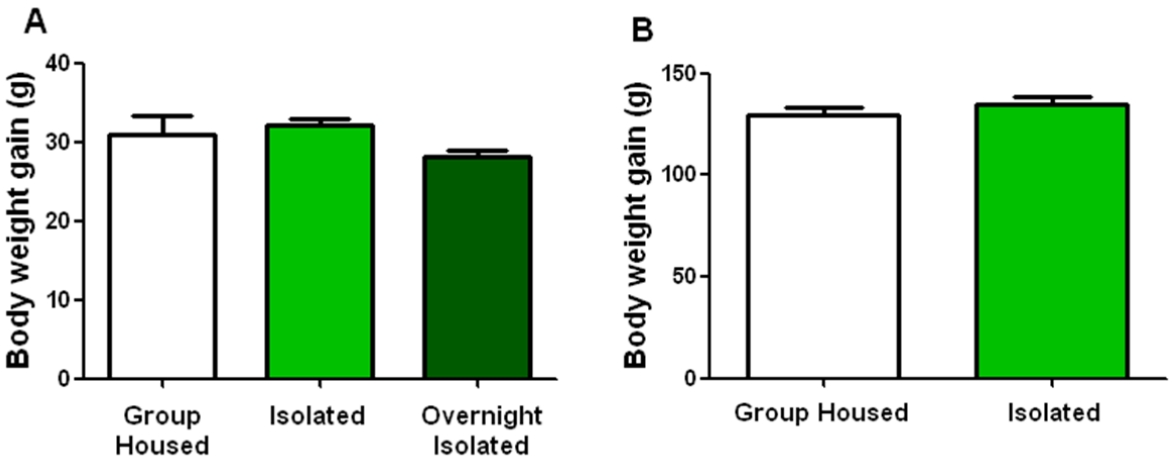


FIGURE 3.8 Average body weight gain (g) in (A) 7-day group housed, isolated and overnig

ht isolated and (B) 3-week group housed and isolated male Sprague Dawley rats. Rats were grouped in fours or housed individually for 7 days or 3 weeks. Overnight isolated rats were grouped in fours during the light phase and housed individually during the dark phase, for 7 days. Data are presented as mean ± SEM. unpaired t-test, ns. n = 12.

3.3.3.2 Behavioural Observations

Morning behavioural observations revealed that from day 2 to day 4 of housing, 7-day isolated rats had significantly reduced time spent sleeping ($P \leq 0.001$) compared to group housed rats (Figure 3.9). Overnight isolated rats had significantly increased locomotive behaviour ($P \leq 0.05$) and significantly reduced time spent sleeping ($P \leq 0.001$) compared to group housed rats (Figure 3.9). Although overnight isolated rats increased time spent climbing, this was not significant in comparison to group housed or isolated rats (Figure 3.9). All three housing groups reduced time spent remaining stationary from day 2 to day 4, with isolated rats reducing this behaviour more than group housed rats, and overnight isolated rats reducing this behaviour more than both isolated and group housed rats (Figure 3.9). However these differences were not statistically significant.

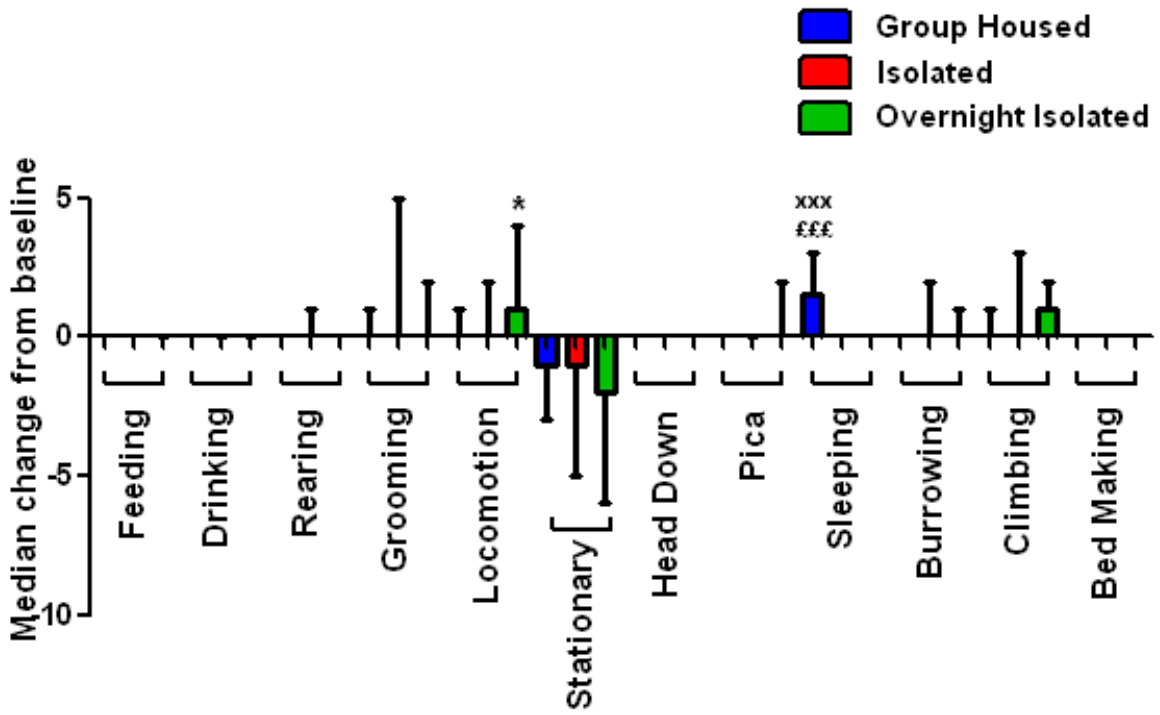


FIGURE 3.9 Light phase behaviour in 7-day group housed, isolated and overnight isolated male Sprague Dawley rats. Rats were housed in fours (group housed), individually (isolated), or in fours during the light phase and individually during the dark phase (overnight isolated). Rats were observed at the start of the light phase (07:30 hrs), after overnight isolated rats had been returned to their original groups. Within 18 minutes of the time point stated above (07:30 hrs), each rat was observed for 5 seconds, 6 times. Data are presented as median change from day 2 (interquartile range); Kruskal-Wallis one-way ANOVA on ranks with post-hoc Dunn's test. * = $P \leq 0.05$ vs. group housed, xxx = $P \leq 0.001$ vs. isolated, £££ = $P \leq 0.001$ vs. overnight isolated; n = 12.

Evening behavioural observations revealed that from day 2 to day 6 of housing, 7-day isolated rats had significantly increased time spent remaining stationary ($P \leq 0.05$) compared to group housed rats (Figure 3.10). Both group housed and overnight isolated rats spent reduced time remaining stationary, but this was not significant between the two groups, or between overnight isolated and isolated rats (Figure 3.10). Overnight isolated rats spent increased time feeding and drinking, while group housed and isolated rats spent increased time grooming, but these differences were not statistically significant (Figure 3.10).

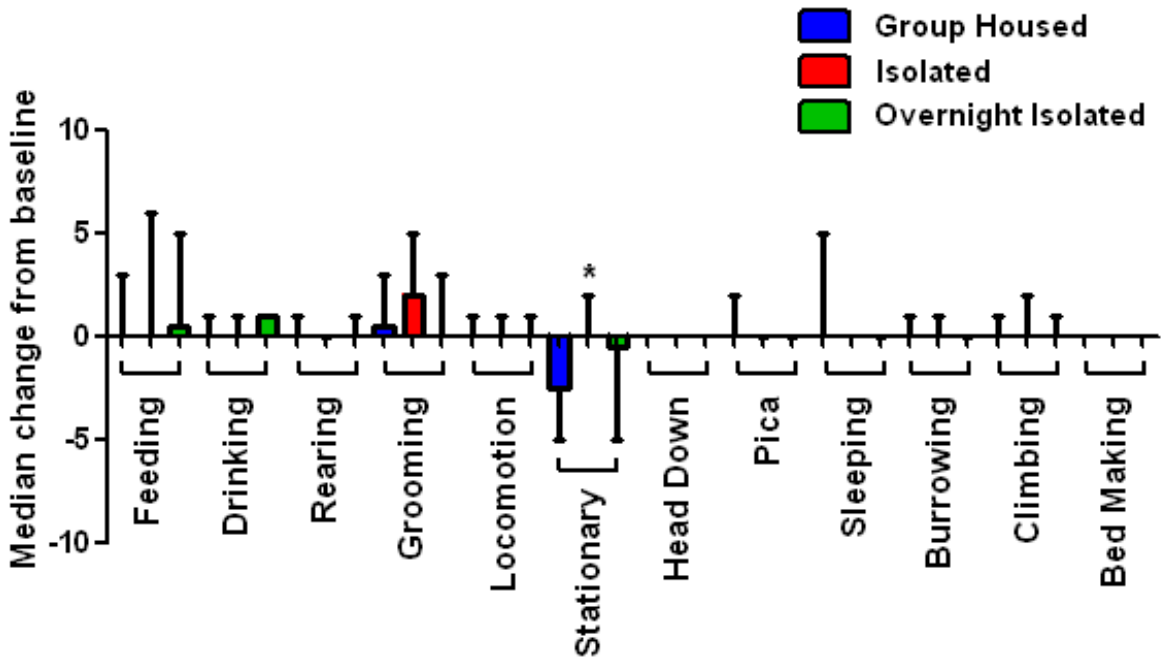


FIGURE 3.10 Dark phase behaviour in 7-day group housed, isolated and overnight isolated male Sprague Dawley rats. Rats were housed in fours (group housed), individually (isolated), or in fours during the light phase and individually during the dark phase (overnight isolated). Rats were observed at the end of the light phase (18:30 hrs), before overnight isolated rats were housed individually for the duration of the dark phase. Within 18 minutes of the time point stated above (18.30 hrs), each rat was observed for 5 seconds, 6 times. Data are presented as median change from day 2 (interquartile range); Kruskal-Wallis one-way ANOVA on ranks with post-hoc Dunn's test. * = $P \leq 0.05$ vs. group housed; n = 12.

Morning behavioural observations revealed that from day 2 to day 18 of housing, 3-week isolated rats had spent significantly reduced time grooming ($P \leq 0.001$), remaining stationary ($P \leq 0.01$), pica ($P \leq 0.05$) and climbing ($P \leq 0.001$) compared to group housed rats (Figure 3.11). Although group housed rats demonstrated an increase in feeding, locomotive and burrowing behaviours, these differences were not statistically significant (Figure 3.11).

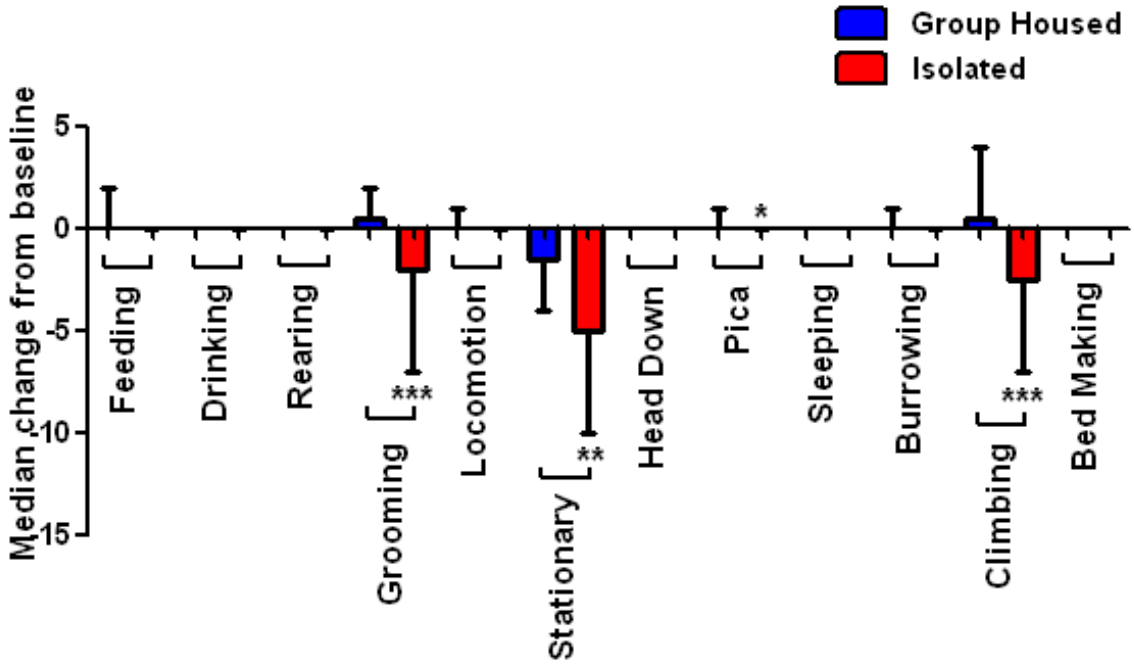


FIGURE 3.11 Light phase behaviour in 3-week group housed and isolated male Sprague Dawley rats. Rats were housed in fours (group housed) or individually (isolated). Rats were observed at the start of the light phase (07:30 hrs). Within 12 minutes of the time point stated above (07:30 hrs), each rat was observed for 5 seconds, 6 times. Data are presented as median change from day 2 (interquartile range); Mann-Whitney U test. * = $P \leq 0.05$ vs. group housed, ** = $P \leq 0.01$ vs. group housed, *** = $P \leq 0.001$ vs. group housed; n = 12.

Evening behavioural observations revealed that from day 2 to day 18 of housing, 3-week isolated rats had spent significantly increased time feeding ($P \leq 0.05$) compared to group housed rats, with group housed rats in fact reducing the amount of time spent feeding (Figure 3.12). Isolated rats spent significantly increased time grooming ($P \leq 0.05$) compared to group housed rats, and significantly reduced time climbing ($P \leq 0.01$) compared to group housed rats, with group housed rats in fact increasing their time spent climbing over the housing period (Figure 3.12). Group housed rats demonstrated a greater increase in rearing, locomotive and burrowing behaviour, while isolated rats exhibited a larger reduction in time spent remaining stationary and a greater increase in pica behaviour. However these differences in behaviour were not statistically significant (Figure 3.12).

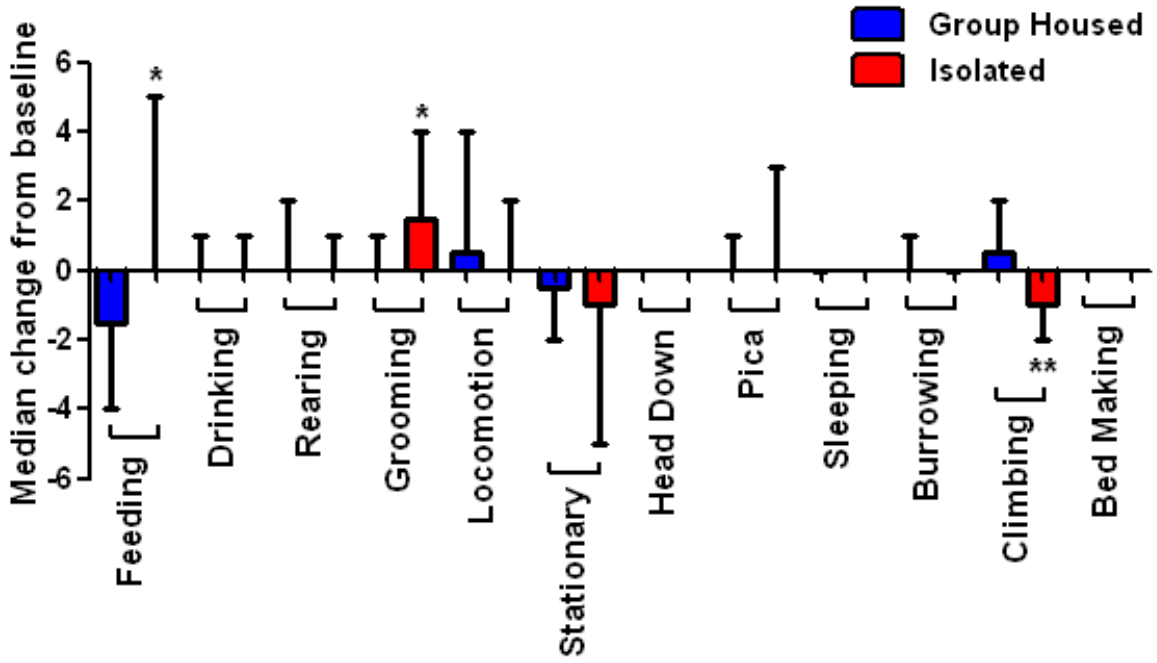


FIGURE 3.12 Dark phase behaviour in 3-week group housed and isolated male Sprague Dawley rats. Rats were housed in fours (group housed) or individually (isolated). Rats were observed at the end of the light phase (18:30 hrs). Within 12 minutes of the time point stated above (18:30 hrs), each rat was observed for 5 seconds, 6 times. Data are presented as median change from day 2(interquartile range); Mann-Whitney U test. * = P ≤ 0.05 vs. group housed, ** = P ≤ 0.01 vs. group housed; n = 12.

3.3.3.3 Plasma ACTH and corticosterone concentrations

In 7-day housed rats, basal plasma ACTH concentrations were surprisingly significantly lower in isolated rats compared to group-housed rats ($P \leq 0.05$), but there was no difference between isolated and overnight isolated rats, or group housed and overnight isolated rats (Figure 3.13A). Restraint did not cause a significant increase in plasma ACTH concentrations in any of the housing groups. Isolated-restraint rats demonstrated significantly lower plasma ACTH levels compared to group housed-restraint rats ($P \leq 0.001$), 2 hours after cessation of a 15 minute restraint procedure (Figure 3.13A). There was no difference between isolated-restraint rats and overnight isolated-restraint rats. Two-way ANOVA revealed no significant interaction effect of housing on the ACTH response to restraint stress.

In 3-week housed rats, restraint induced a significant rise in plasma ACTH levels in group housed rats ($P \leq 0.01$ compared to group housed-no restraint rats) but not in isolated rats compared to their no-restraint controls (Figure 3.13B). ACTH concentrations were significantly higher in group housed-restraint rats compared to isolated-restraint rats ($P \leq 0.01$) (Figure 3.13B). There was no difference in basal ACTH levels between group housed and isolated rats. Two-way ANOVA revealed a significant interaction effect of housing on the ACTH response to restraint stress ($P \leq 0.01$).

In 7-day housed rats, restraint did not induce an increase in plasma corticosterone in group housed or overnight isolated rats compared to their no-restraint controls, but did induce a significant rise in corticosterone levels in isolated rats ($P \leq 0.01$) (Figure 3.13C). Basal plasma corticosterone concentrations were significantly lower in isolated rats compared to group housed rats ($P \leq 0.05$), but there was no difference between isolated and overnight isolated rats, or group housed and overnight isolated rats (Figure 3.13C). Plasma corticosterone concentrations were significantly lower in overnight isolated-restraint rats

compared to isolated-restraint rats ($P \leq 0.01$) but there was no difference between overnight isolated-restraint rats and group housed-restraint rats, or isolated-restraint rats and group housed-restraint rats (Figure 3.13C). Two-way ANOVA revealed no significant interaction effect of housing on the corticosterone response to restraint stress.

In 3-week housed rats, restraint induced a rise in plasma corticosterone levels in group housed rats but this was not significantly different to group housed-no restraint rats. Restraint induced a significant rise in plasma corticosterone levels in isolated rats compared to their no-restraint controls ($P \leq 0.05$) (Figure 3.13D). There was no significant difference between corticosterone concentrations in group housed-restraint rats and isolated-restraint rats, and there was also no difference between basal corticosterone levels in group housed and isolated rats (Figure 3.13D). Two-way ANOVA revealed no significant interaction effect of housing on the corticosterone response to restraint stress.

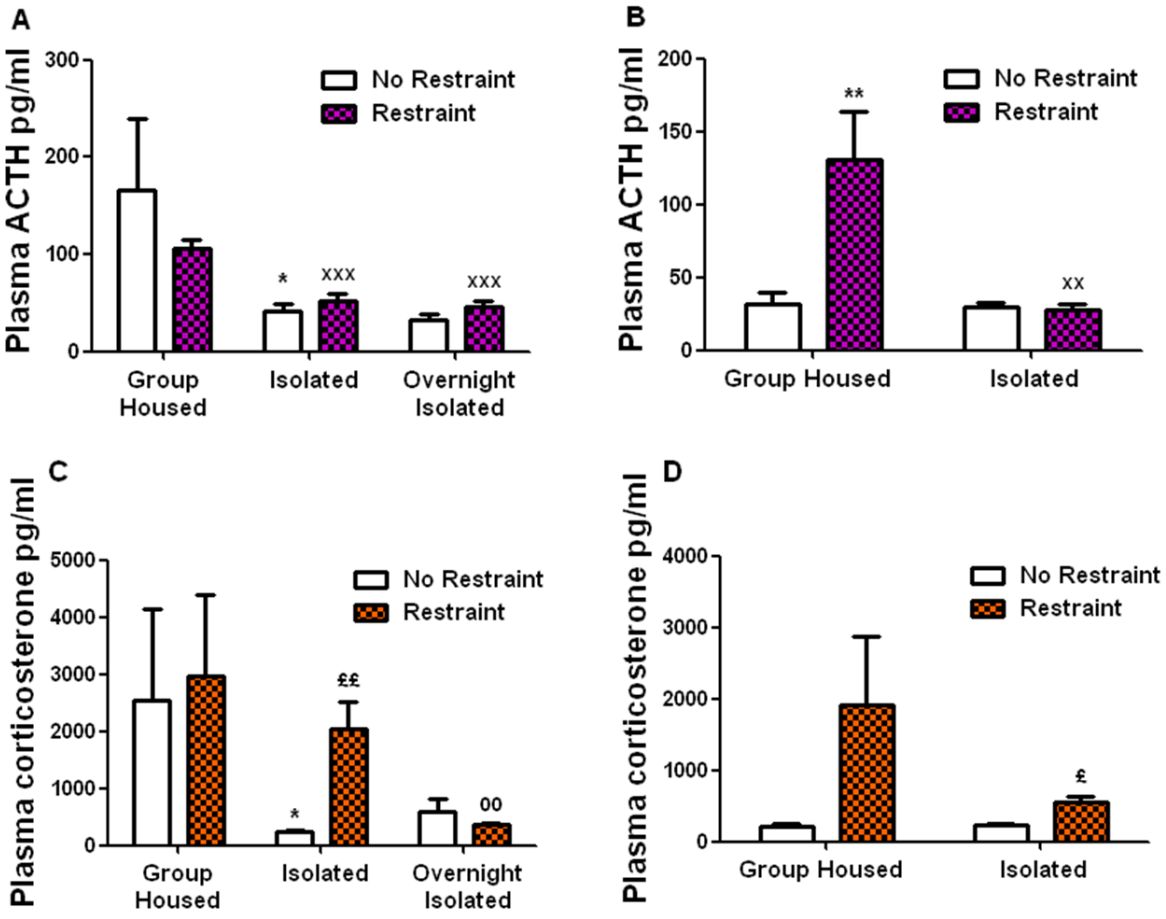


FIGURE 3.13 The effect of 15 minutes of restraint stress on plasma (A-B) ACTH and (B-D) corticosterone concentrations in (A and C) 7-day group housed, isolated and overnight isolated and (B and D) 3-week group housed or isolated male Sprague Dawley rats. Rats were decapitated 2 hrs after cessation of restraint stress. Data are presented as ± SEM; two-way ANOVA with post-hoc Bonferroni correction. * = P ≤ 0.05 vs. group housed-no restraint, ** = P ≤ 0.01 vs. group housed-no restraint, xx = P ≤ 0.01 vs. group housed-restraint, xxx = P ≤ 0.001 vs. group housed-restraint, £ = P ≤ 0.05 vs. isolated-no restraint, ££ = P ≤ 0.01 vs. isolated-no restraint; 0 = P ≤ 0.05 vs. isolated-restraint. Bonferroni interaction value = **(A, C-D)** ns, **(B)** P ≤ 0.01; n = 5-6.

3.3.3.4 Glucocorticoid receptor and corticotrophin-releasing hormone mRNA expression in the hypothalamus

The quality of RNA extracted from each sample was determined by visualizing samples on a 1% agarose gel. All samples, except 12-14 and E3, presented two bands representing the 28s and 18s rRNA subunits of total RNA, suggesting good quality RNA had been extracted (Figure 3.14). Samples 12-14 were smudged suggesting possible degradation of RNA or, as the three smudged samples are directly next to each other, there may have been problems with loading samples on that particular section of the gel. No band was visible for sample E3 suggesting there may have been an error when loading the gel with this sample. Therefore all 60 samples were included in the RT and qPCR stages to follow.

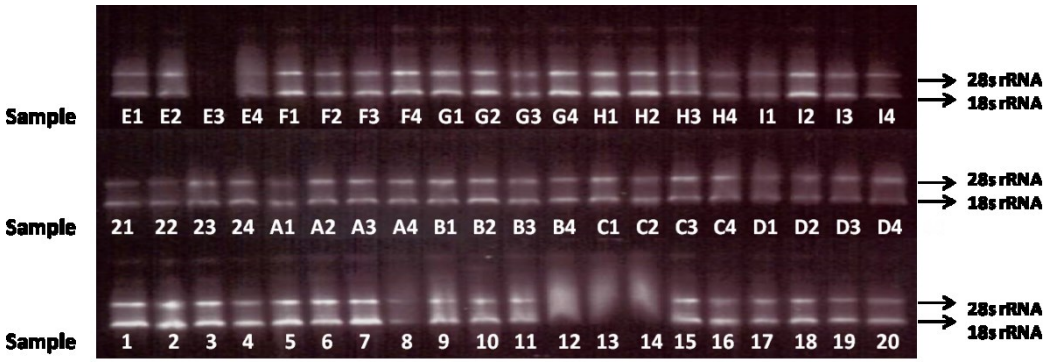


FIGURE 3.14 Agarose gel electrophoresis of total RNA isolated from hypothalami of male Sprague Dawley rats. Following RNA isolation, the quality of RNA was determined by running RNA samples on a 1% agarose gel with 50 x TAE buffer and EtBr. Each intact total RNA sample should present two bands, representing the 28s and 18s rRNA subunits. Sample numbers are denoted under each set of bands.

In 7-day housed rats, basal hypothalamic GR mRNA expression levels were not significantly different between group housed and isolated rats. Basal GR mRNA expression was lower in overnight isolated rats compared to group housed and isolated rats, but this effect was not statistically significant (Figure 3.15A). Restraint increased GR mRNA expression in all three housing groups, but this effect did not achieve statistical significance when compared to their respective no-restraint controls (Figure 3.15A). GR mRNA levels were significantly higher in overnight isolated-restraint rats compared to group housed-restraint and isolated-restraint rats ($P \leq 0.01$ and $P \leq 0.05$ respectively), but there was no difference in GR mRNA expressions levels between group housed-restraint and isolated-restraint rats (Figure 3.15A).

In 3-week housed rats, there was no difference between basal hypothalamic GR mRNA expression levels in group housed and isolated rats. Restraint increased GR expression in both group housed and isolated rats, but this difference was not statistically significant when compared to their respective no-restraint controls (Figure 3.15B). GR mRNA expression was higher in isolated-restraint rats compared to group housed-restraint rats, but again this difference was not statistically significant (Figure 3.15B).

In 7-day housed rats, there was no difference between basal hypothalamic CRH mRNA expression levels in group housed and isolated rats, but basal CRH mRNA expression was significantly higher in overnight isolated rats compared to isolated rats ($P \leq 0.01$) (Figure 3.15C). Restraint increased CRH mRNA expression levels in group housed and isolated rats ($P \leq 0.05$ compared to their respective no-restraint controls) but did not affect CRH mRNA expression levels in overnight isolated rats (Figure 3.15C). There was no difference between CRH mRNA expression levels in group housed-restraint, isolated-restraint and overnight isolated-restraint rats (Figure 3.15C).

In 3-week housed rats, there was no difference between basal hypothalamic CRH mRNA expression levels in group housed and isolated rats. Restraint only increased CRH expression in isolated rats, but this difference was not statistically significant when compared to isolated-no restraint rats (Figure 3.15D). CRH mRNA expression was significantly higher in isolated-restraint rats compared to group housed-restraint rats ($P \leq 0.05$) (Figure 3.15D).

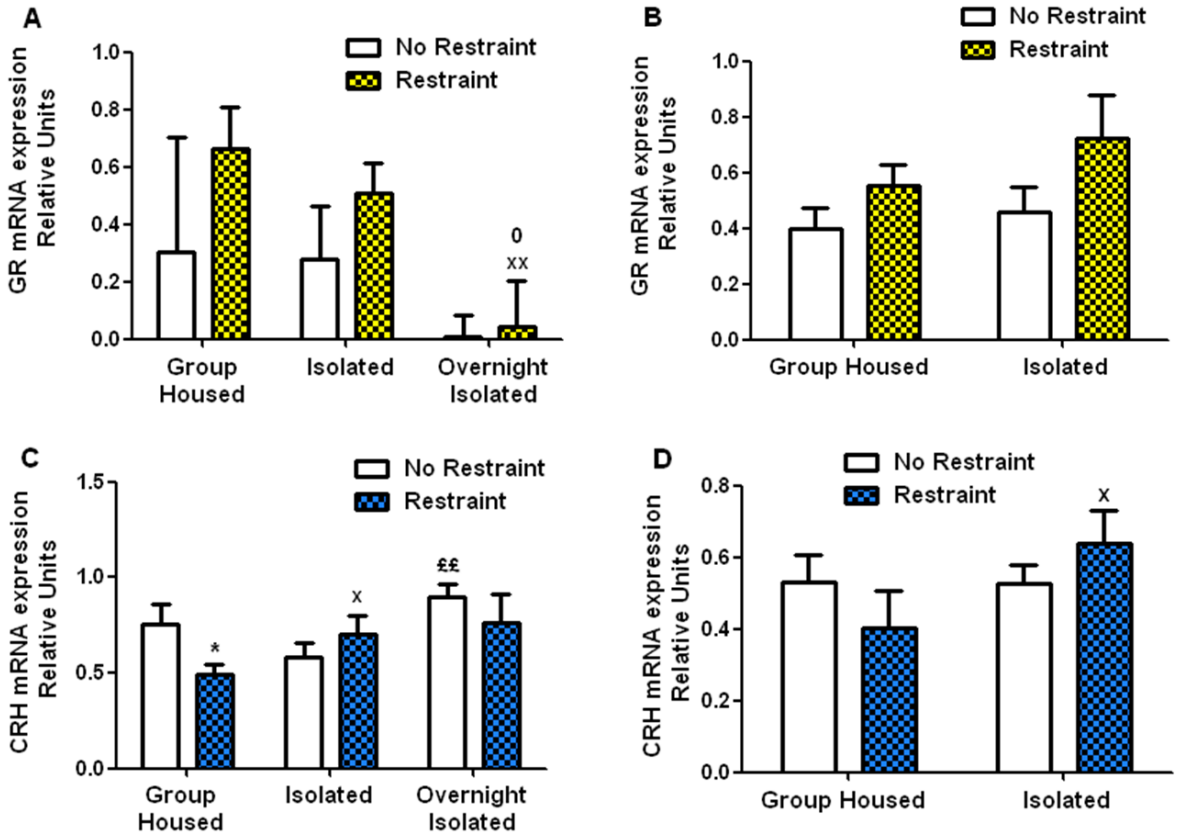


FIGURE 3.15 The effect of 15 minutes of restraint stress on hypothalamic (A-B) GR and (C-D) CRH mRNA expression in (A and C) 7-day group housed, isolated and overnight isolated and (B and D) 3-week group housed or isolated male Sprague Dawley rats. Rats were decapitated 2 hrs after cessation of restraint stress. Data are presented as mean ± SEM; two-way ANOVA with post-hoc Bonferroni correction. * = P ≤ 0.05 vs. group housed-no restraint, x = P ≤ 0.05 vs. group housed-restraint, xx = P ≤ 0.01 vs. group housed-restraint, ££ = P ≤ 0.01 vs. isolated-no restraint; 0 = P ≤ 0.05 vs. isolated-restraint. Bonferroni interaction value = ns for **A-D**; n = 5-6.

3.4 Discussion

3.4.1 Summary of findings

The stress response to 15 minutes of restraint in male Sprague Dawley rats was altered in 7-day isolated rats when compared to group housed rats. Seven days of SI resulted in:

- significantly increased plasma ACTH and corticosterone concentrations,
- significantly increased corticosterone release from primary adrenal cells,
- increased c-fos protein expression in the hypothalamic ARC and PVN, in response to acute restraint stress.

However 7-day isolation did not affect basal plasma stress hormone concentrations, basal adrenal corticosterone release or basal hypothalamic c-fos protein expression levels.

Time profiles of plasma stress hormone release in response to a 15 minute restraint procedure showed that plasma ACTH and corticosterone were significantly higher at 45-195 minutes and 45-75 minutes after the start of the restraint procedure, respectively, in 7-day isolated rats compared to group housed rats. However incremental AUC analyses showed that 7-day isolation does not affect total ACTH or corticosterone release in response to 15 minutes of restraint stress in male Sprague Dawley rats.

Both 7-day and 3-week isolation paradigms caused increases in depressive-like behaviours in male Sprague Dawley rats, by Days 2-6 and Day 18 of housing respectively. Feeding, grooming, locomotion, remaining stationary, sleeping, climbing and pica were the behaviours affected by SI, but differently so between 7-day and 3-week housed rats. Seven-day isolation and overnight isolation-induced stress caused a general increase in activity, and reduced sleeping in response to morning awakening. On the other hand, 3-week-isolation induced stress caused a general reduction in activity when startled with morning awakening, and excessive stress-associated grooming in the evenings.

Seven-day isolation and overnight isolation caused behavioural changes in male Sprague Dawley rats by day 4-6, in both the light and dark phase. This was demonstrated by reduced time sleeping and increased locomotive behaviour during the light phase, and increased time spent remaining stationary in overnight isolated rats during the dark phase. Seven days of overnight isolation reduced the ACTH and corticosterone response to 15 minutes restraint stress in male Sprague Dawley rats, compared to 7-day group housed rats, and in the case of corticosterone 7-day isolated rats. Overnight isolation reduced basal, and significantly reduced restraint-induced, GR mRNA expression compared to group housed and isolated rats. Seven days of isolation increased restraint-induced hypothalamic CRH mRNA expression compared to group housed rats, while overnight isolation increased basal CRH mRNA expression but had no effect on restraint-induced CRH mRNA expression.

Three-week isolation also caused behavioural changes in rats, in both the light and dark phase. This was demonstrated by reduced time spent remaining stationary, reduced grooming and climbing, and less pica during the light phase, and increased feeding and grooming and reduced time spent climbing during the dark phase. In response to restraint stress, 3-week isolation reduced the ACTH and corticosterone response and increased hypothalamic GR mRNA expression 2 hours after cessation of restraint stress, compared to group housed rats. Three-week isolation also significantly increased hypothalamic CRH mRNA expression in response to restraint stress.

3.4.2 Detailed discussion

The studies described in this chapter investigated the effects of SI on the HPA axis response to acute stress, in rats. They suggest that SI can cause hyper-activation or hypo-activation of the HPA axis response to acute restraint stress, depending on the length of the SI period. This hyper-activation is evident at the hypothalamic and adrenal level, and is demonstrated peripherally by changes in circulating plasma stress hormone concentrations.

Acute restraint stress produces a robust HPA response in rats, making it a reliable acute stressor (Hermes et al., 2006, Lukkes et al., 2009, Weintraub et al., 2010). Individual restraint stress periods lasting 30, 60, and 120 minutes have been found to trigger the induction and phosphorylation of several signalling molecules in the hypothalamic PVN and ARC, as measured by increased c-fos, phosphorylated extracellular cell-regulated protein kinase (pERK), phosphorylated calcium/calmodulin dependent protein kinase II (pCaMKII) and phosphorylated cyclic-AMP response element binding protein (pCREB) immunoreactivity (Kwon et al., 2006). Restraint stress also increases the number of CRH immunoreactive fibres in the hypothalamic ME, stimulates ACTH production in the anterior pituitary as measured by increased ACTH immunoreactive cells, and increases plasma ACTH and corticosterone concentrations, compared with control animals (Sanchez et al., 1998, Gadek-Michalska and Bugajski, 2003).

To date, all major SI studies in rats have housed rats for three weeks or more (reviewed in (Hawkley et al., 2012), but studies in other species such as voles (Pournajafi-Nazarloo et al., 2011, Ruscio et al., 2007, Stowe et al., 2005), pigs (Kanitz et al., 2009, Tuchscherer et al., 2010, Kanitz et al., 2004, Tuchscherer et al., 2004) and monkeys (Higley et al., 1992, Lyons et al., 1995, Lyons et al., 1999, Mendoza and Mason, 1986) show significant effects of short-term SI on the HPA axis. Study 3.2.2.1 was carried out to determine the effects of short-

term SI on the HPA axis response to stress after just seven days of SI in rats. In all three studies outlined in this chapter, rats from all housing groups demonstrated excessive defecation and urination during the restraint procedure, suggesting acute restraint was certainly a stressful experience for them. Figure 3.2 shows that group housed rats responded to 15 minutes of restraint with significant increases in plasma corticosterone concentrations. Although plasma ACTH concentrations were not higher in group housed-restraint rats than group housed-no restraint rats (Figure 3.2A), their significantly higher corticosterone concentrations (Figure 3.2B) suggest there was a significantly different plasma stress hormone response between the two groups and thus that restraint stress is indeed stressful for group housed rats. Seven days of SI did not affect basal activity of the HPA axis, but it did cause hyper-activation of the HPA axis response to restraint as seen by significantly increased plasma ACTH and corticosterone concentrations, compared to group housed rats. As mentioned in chapter 2, it is well known that after a stress response, plasma ACTH levels start to normalise from 30 minutes, whilst corticosterone levels return to resting levels much later. Therefore it is possible that a significant effect of restraint stress on circulating ACTH levels was missed at the 2 hr time point. Nevertheless, these results are in line with other studies that support the notion that SI can cause hyper-activation of the HPA axis. For example, Ferland and Schrader show that three weeks of SI in rats increases the corticosterone response to cage-mate separation stress (Ferland and Schrader, 2011), while Weiss et al., show that 13-week isolated rats have increased ACTH and corticosterone responses to open field and elevated plus maze stress tests (Weiss et al., 2004).

Increased corticosterone release from cultured primary adrenal cells of isolated rats (Figure 3.3) suggests that the adrenal gland is more sensitive to acute restraint stress after a period of SI. This correlates with available evidence suggesting that a dysfunctional adrenal gland may be involved in the pathogenesis of disease associated with HPA dysregulation. Fourteen day chronic variable stress - hypoxia, warm swim, cold swim, cold room, shaker platform, restraint and overnight isolation - has been found to cause hyperplasia and

hypertrophy of the adrenal gland, leading to increased corticosterone responses to ACTH (Ulrich-Lai et al., 2006). Interestingly, depressed patients have been found to have enlarged and hyperactive adrenal glands (Nemeroff and Vale, 2005), and the adrenal glands of isolated rats have been shown to be larger than those of group housed rats. In addition, the adrenal glands of isolated rats are more responsive to electric shock stress compared to those of group housed rats (Viveros et al., 1988).

To determine whether the hypothalamic component of the HPA axis is directly affected by SI, hypothalamic c-fos protein expression in response to 15 minutes of restraint stress was also measured in isolated and group housed rats. The ARC and PVN are hypothalamic nuclei known to be activated in response to restraint stress (Kwon et al., 2006), and by others stressors such as LPS (Dallaporta et al., 2007) and air jet stress (Porter and Hayward, 2011). Restraint significantly increased c-fos protein expression in the ARC (Figure 3.4A) and PVN (Figure 3.4B) of both SI and group housed rats, supporting the above finding that restraint is a stressful experience for rats. But increased ARC, and significantly higher PVN c-fos protein expression, is evident in isolated-restraint rats when compared to group housed-restraint rats, suggesting that the hypothalamus is hyper-responsive to acute stress following a period of SI. This is in accord with other studies reporting 6 weeks of SI in prairie voles increases CRH mRNA expression in the PVN in response to an elevated plus maze test, suggesting enhanced hypothalamic activity in response to acute stress (Pan et al., 2009).

Collectively, the results (3.3.1) from study 3.2.2.1 suggested that 7-day SI caused hyper-activation of the HPA axis response to acute restraint stress, and that this hyper-activation was occurring at the hypothalamic and adrenal level. However, it was unknown whether isolated rats had a higher amount of total peripheral ACTH and corticosterone release, in response to 15 minutes of restraint stress, compared to group housed rats, or whether SI caused a time-shift in HPA responsiveness to acute stress, and thus the effects we observed

in 3.3.1 were the result of an earlier peak in HPA responsivity in isolated rats. Therefore study 3.2.2.2 was designed to investigate this by measuring plasma ACTH and corticosterone levels in male Sprague Dawley rats at 0 15, 30, 45, 75 and 195 minutes after the start of a 15 minutes restraint stress period, in group housed and isolated rats. Unlike the previous study, SI influenced body weight in these rats; the body weight of isolated rats was significantly higher than that of group housed rats, after just seven days of SI. This could be because isolated rats had much more time available to feed as they were not interacting with other rats, because they were not being disturbed when feeding by other rats, or because they may have been less physically active, possibly because of the lack of interaction or 'playtime' with other rats or their housing in a single level cage, rather than the double-levelled cage that the group housed rats were housed in. The ACTH time profile (Figure 3.7A) revealed that although plasma ACTH levels were significantly higher in isolated rats compared to group housed rats at 45 minutes after the start of the restraint stress procedure, total ACTH release was not different from that of group housed rats (Figure 3.7B). A similar pattern in the corticosterone time profile and AUC (Figure 3.7C-D) suggested that SI did not have a large effect on this particular cohort's HPA response to acute restraint stress. ACTH and corticosterone levels peaked at the same time for both group housed and isolated rats, and followed a very similar profile returning to resting levels (Figure 3.7), though there were differences at specific time points. At time point 0 minutes, group housed rats had significantly higher plasma ACTH levels than isolated rats, yet isolated rats displayed a significantly higher ACTH response to restraint up to 75 minutes after the start of the restraint stress. But at 75 and195 minutes group housed ACTH levels were significantly higher than those of isolated rats. This suggests that any significant difference in total ACTH release may have been masked by these significant differences in basal (Figure 3.7A) and resting concentrations. Based on the results of study 3.3.1, in which levels of both stress hormones were still significantly higher in isolated rats than in group housed rats at 2 hrs after cessation of restraint stress, it is surprising that in study 3.3.2 both plasma ACTH and corticosterone were significantly higher in group housed rats than in

isolated rats at a similar time point on the profile (Figure 3.7). A possible reason for this could be a differing environmental experience that both housing groups may have encountered to the previous experiments, particularly on the day of the study. As mentioned in chapter 2, it is very difficult to control for external events that can affect social stress in any animal. It is possible that loud noises, abnormal changes in lighting or rough handling by animal facility staff may have contributed to stress in these rats, and therefore could have acted as long-term stressors themselves. Another reason could be the stress of removing rats from grouped cages for the restraint procedure on the day of experimentation. This is particularly important in the case of group housed rats because they temporarily lose cage mates whilst some are being restrained and others returned to their cages. Cage mate separation has been found to induce an acute stress response in rats (Ferland and Schrader, 2011) and in the rats in study 3.3.2 this may have exaggerated the stress responses observed. Of course this may also be true for rats in study 3.3.1, but it is difficult to determine the extent to which such additional stressors may have affected these two cohorts. The amount of time this additional stress response may have lasted and the extent to which it may have activated the HPA axis is unknown and could have influenced the time profile response observed after a restraint stress procedure. Any possible effects of cage mate separation were not evident in study 3.3.1 as stress levels were not measured before or throughout the study period on the day of experimentation. In conclusion, the results of study 3.3.2 were difficult to interpret, and it was therefore decided that investigation of different and potentially more stressful SI paradigms was required to support the findings of study 3.3.1, as well as to investigate the mechanisms by which SI might influence HPA responsiveness and which might mediate the putative changes observed in study 3.3.1.

Building upon the 7-day isolation paradigm in study 3.2.2.1, study 3.2.2.3 investigated the effects of 7 days of overnight isolation on the HPA axis response to acute restraint stress, and compared this to the previously investigated paradigm of 7-day isolated rats. Another arm of this study investigated the effects of 3-week chronic SI on the HPA response to acute

restraint stress in rats. Similar experimental paradigms have previously triggered different HPA responses to acute stress. Fourteen days of intermittent SI has been shown to increase plasma corticosterone levels (Ferland and Schrader, 2011, Pournajafi-Nazarloo et al., 2011), increase hypothalamic CRH mRNA expression, and increase CRH-R1 mRNA but decrease CRH-R2 mRNA expression in the pituitary gland after each isolation session in rats (Pournajafi-Nazarloo et al., 2011). On the other hand, three weeks of chronic SI in the vole increases basal plasma corticosterone levels and CRH immunoreactivity in the PVN, and reduces basal AVP immunoreactivity in the PVN compared to pair-housed control animals (Ruscio et al., 2007). The HPA response to acute stress was not investigated in these chronically stressed animals. In rats, 3 weeks of SI also reduces basal corticosterone levels, but investigation of the HPA response to acute stress in these rats shows that chronic 3-week SI does not affect the corticosterone response to acute immobilization stress, compared to group housed rats (Djordjevic et al., 2010). The mechanisms by which these changes occur were not investigated in this study. Therefore study 3.2.2.3 compared the effects of intermittent SI, but for 7 days instead of 14 days, on the HPA axis response to acute restraint stress. This 7-day paradigm allows direct comparison with the results of study 3.2.2.1. The 3-week chronic SI paradigm was chosen as a longer chronic stress period that had previously been shown to induce changes in the HPA response to acute stress in rats, and which may be more stressful than just 7 days of chronic SI as investigated in study 3.2.2.1.

Firstly, morning and evening behaviour was observed in these rats. Behavioural changes in rats have been attributed to SI, such as increased aggression in response to handling as a consequence of isolation housing. Such behaviours can be categorized into stressful or not-stressful behaviours, and further characterised as depressive-like or anxiolytic behaviours that may be linked to psychiatric disorders such as schizophrenia, depression and anxiety (Weiss et al., 2004, Lukkes et al., 2009, Landgraf and Wigger, 2002, Djordjevic et al., 2012, Qi et al., 2006, Burman et al., 2008, Kalueff and Tuohimaa, 2005). In study 3.3.3,

behavioural observations were carried out with the aim of establishing whether SI caused behavioural changes which were attributable to stress. Other studies observe behaviour during specific stress-tests, such as the open field test, elevated plus maze test and forced-swim test (Einon and Morgan, 1977, Djordjevic et al., 2012, Qi et al., 2006). But the observations made during these tests cannot solely be attributed to SI, as these tests themselves may affect behaviour (Weiss et al., 2004). This is because additional stress may be caused by the new test-environment and equipment, as well as momentary isolation of animals that are used to being group housed at all times. The behavioural observation technique described in this chapter is an adaptation of a technique used in two previous studies (Fray et al., 1980, Molloy and Waddington, 1987), and is a novel approach that observes grouped rats whilst still in their respective cages. This method allows observations to be made in a non-invasive manner and attributes any stressful behaviour observed to the housing paradigm alone.

Although morning and evening behaviours were both observed throughout study 3.2.2.3, the evening behaviours are of particular interest as rats are nocturnal animals and are therefore active during the dark phase. It is notable that methodological differences between morning and evening observations also contribute to the possibility that the morning observations may be unreliable. As mentioned above, before morning observations were carried out, overnight isolated rats were returned to their original grouped cages. This was an interruption to their natural routine as rats are usually sleeping at this point in the light phase, therefore adding to the possibility that any behaviour recorded at this time point may have been unnatural. To control for this additional disturbance in overnight isolated rats, group housed rats and chronically isolated rats were also briefly picked up and returned to their cages immediately at this time point. A further limitation to the analysis of morning behavioural data was that Day 6 behavioural data for 7-day housed rats was, although initially recorded, not available due to loss of data. Thus morning behavioural observations presented in Figure 3.9 included the change in behaviour between days 2 and 4 only,

whereas evening behavioural observations presented in Figure 3.10 included the change in behaviour between days 2 and days 6. Collectively, these confounding factors suggest evening behavioural observations may be more reliable than morning observations.

The increased activity and reduced sleeping time of overnight isolated rats and 7-day isolated rats in the morning (Figure 3.9) could perhaps be attributed to increased startle-response to the awakening procedure and increased time taken to overcome this startle-effect when compared to other housing groups. This suggests that both isolation and overnight isolation are stressful, with overnight isolation more so, as increased activity and restlessness has been linked to an anxiolytic behavioural phenotype in rats (Hilakivi et al., 1989, Varty et al., 2000). Although not significantly different, the increased evening grooming behaviour observed in 7-day isolated rats (Figure 3.10) indicates a possible pro-depressive phenotype of these rats, as increased grooming is linked to a depressive behavioural profile in rats (Landgraf and Wigger, 2002), and is thought to be a coping mechanism for rats exposed to stress (Smolinsky et al., 2009).

Morning behavioural observations revealed that 3-week isolated rats significantly reduced time spent grooming and climbing compared to group housed rats (Figure 3.11). Isolated rats also demonstrated a greater reduction in time spent remaining stationary compared to group housed rats (Figure 3.11). This is surprising because of the links between increased grooming and stress described above. However this could be explained by the fact that waking the rats immediately before morning observations startles them, as they are usually sleeping at that point. Isolated rats may remain startled for longer while group housed rats may overcome the startle-effect more quickly, allowing them to engage in normal behaviours immediately after being startled.

Significantly increased evening feeding behaviour in 3-week isolated rats compared to 3-week group housed rats (Figure 3.12) may suggest isolated rats were more stressed as

excessive feeding can be associated with stress (Badiani et al., 1996). Hyperphagia has been associated with depression and isolation so increased feeding could be indicative of this (Hall et al., 1998, Nakhate et al., 2011). Isolated rats groomed more than group housed rats in the evening (Figure 3.12), again suggesting they are more stressed than their grouped counterparts and possibly pro-depressive (Landgraf and Wigger, 2002, Smolinsky et al., 2009). Reduced climbing in isolated rats compared to group housed rats in the evening, coupled with the lack of a difference in other activity behaviours such as rearing and burrowing between the two housing groups (Figure 3.12), suggests isolated rats were overall less active than group housed rats. This is in line with more classic associations of depression and stress with reduced locomotion (Strekalova et al., 2005) and increased immobility (Griebel et al., 2005, Elizalde et al., 2008, Griebel et al., 2002).

In conclusion, both 7-day and 3-week isolation paradigms caused increases in depressive-like behaviours in male Sprague Dawley rats. Feeding, sleeping, grooming, locomotion, remaining stationary and climbing were the behaviours most affected, but differently so between 7-day and 3-week housed rats. Seven-day isolation and overnight isolation-induced stress caused an exaggerated and elongated startle response to morning awakening, and a general increase in activity in the morning. Three weeks of SI induced stress caused a general reduction in activity when startled with morning awakening, and excessive stress-associated grooming in the evenings.

The reduced basal and restraint-induced ACTH and corticosterone response in overnight isolated rats (Figure 3.13A and C) suggests hypo-activation of the HPA axis and contrasts the HPA hyperactivity in response to restraint stress seen in 7-day chronically isolated rats, in study 3.3.1 (Figure 3.2) and study 3.3.3 (Figure 3.13 A and C). It is notable that the hormonal responses of 7-day group housed rats in study 3.3.3 (Figure 3.13A and C) were extremely high compared to those seen in all previous experiments, suggesting that this group of rats was particularly stressed before or during experimentation. This is likely due to

the putative environmental reasons discussed above, in which case it can be assumed that these results for group housed rats are distorted. This makes it difficult to compare the hormonal responses of 7-day isolated rats and overnight isolated rats to group housed controls. Basal and restraint stress-induced hypothalamic GR mRNA expression was reduced in overnight isolated rats compared to group housed and 7-day isolated rats (Figure 3.15A). The HPA axis is partly regulated by a glucocorticoid negative feedback mechanism, with some of this feedback occurring at the level of the hypothalamus via the GR (Jones et al., 1977). Glucocorticoids bind to the GR to promote normalization of plasma glucocorticoid levels after stress exposure. This results in down regulation of CRH and vasopressin expression in PVN neurons, and therefore a dampening of the downstream mechanisms that are initially activated after stress exposure (de Kloet et al., 2008c). Reduced GR expression in the hypothalami of overnight isolated rats would therefore suggest a down-regulated glucocorticoid feedback mechanism. Basal hypothalamic CRH mRNA expression was increased in overnight isolated rats compared to 7-day isolated rats (Figure 3.15C).

During an acute stress response, CRH is released from the parvocellular neurons of the PVN and stimulates ACTH secretion from pituitary corticotrophs (Vale et al., 1981, Lamberts et al., 1984), and thus activation of the HPA axis. Increased CRH expression in the hypothalami of overnight isolated rats would therefore suggest an overactive CRH feed-forward output. Collectively, these GR and CRH mRNA expression levels would suggest an overactive HPA response with a less effective glucocorticoid mechanism in overnight isolated rats, yet the reduced plasma stress hormone levels of these rats suggests another regulatory mechanism is responsible for this hypo-activation observed in overnight isolated rats. This is at odds with the findings of previous studies by other groups showing that fourteen days of intermittent SI causes hyper-activation of the HPA axis in rats (Ferland and Schrader, 2011, Pournajafi-Nazarloo et al., 2011) and increases hypothalamic CRH mRNA and pituitary CRH-R1 mRNA expression (Pournajafi-Nazarloo et al., 2011). However GR expression was not measured in these studies. One explanation for the HPA hypo-

activation in overnight isolated rats could be that the increased CRH output may be ineffective due to a possible reduction in pituitary CRH-R1 expression, which would accord with the reduced GR expression in the hypothalamus, as a hypo-active HPA axis would not require high levels of GR for normalization of a stress response. In 7-day isolated rats, GR mRNA expression was comparable to that seen in group housed rats, while CRH mRNA expression was significantly higher in response to restraint, when compared to group housed rats (Figure 3.15A and C). This would suggest that an overactive CRH feed-forward mechanism is responsible for the HPA hyper-activation seen in 7-day isolated rats, and that the glucocorticoid feedback mechanism is not altered in these rats. This is the first study to investigate the effects of just 7 days of SI in rat HPA activity and is therefore not directly comparable to other SI paradigms.

The decreased plasma ACTH and corticosterone response to restraint in 3-week isolated rats, compared to 3-week group housed rats, suggests hypo-activation of the HPA axis in response to acute novel stress (Figure 3.13A and D). The increased GR and CRH mRNA expression levels in response to restraint in 3-week isolated rats suggest an increased stress response which is down-regulated by an exaggerated glucocorticoid feedback mechanism. This feedback could be occurring at multiple levels of the HPA axis, as it is well known that glucocorticoids can act directly on GR at the hypothalamus (Jones et al., 1977) and pituitary (Russell et al., 2010), as well as upstream limbic structures such as the hippocampus (Sapolsky et al., 1984) and the prefrontal cortex (Hill et al., 2011). This may explain the lack of effect of such an increased CRH drive seen in 3-week isolated rats. Others have also shown HPA hypo-activation of the HPA axis in rats after 3 weeks of SI (Djordjevic et al., 2010), but the mechanism behind these changes has not previously been investigated.

Given the potential confounding factors within individual studies described in this chapter, such as the possible effects of cage mate separation in study 3.3.2, and the surprisingly

increased basal and restraint stress induced plasma ACTH and corticosterone levels in 7-day group housed rats in study 3.3.3, as well as the limitations described above for the analysis of morning behavioural data from study 3.3.3, it is not possible to draw definitive conclusions from a) the results of study 3.3.3 or b) direct comparisons of study 3.3.2 to study 3.3.3. This reflects the difficulty in controlling for a wide range of environmental stressors in stress-related studies, as well as the natural variability of the stress response between individual rats of the same species and sex. Nonetheless, these data are the first to suggest that 7-day SI causes hyper-activation of the HPA axis during an acute stress response, and that the hypothalamus and the adrenal gland, are both hyper-sensitive after a 7-day period of SI. These data also demonstrate for the first time that 7-day overnight isolation and 3-week SI cause hypo-activation of the HPA axis response to acute stress, with the former regulated by an unknown mechanism and the latter possibly regulated by an overactive feedback mechanism.

Chapter IV:

General Discussion

4.1 Introduction

The HPA axis plays a crucial role in regulating the response to emotional and physiological stressors. In response to a stressor, hypothalamic hormones, particularly CRH, target the anterior pituitary gland to regulate ACTH secretion, which itself targets the adrenal gland to stimulate the release of glucocorticoids (Chrousos, 2009, Vale et al., 1981, Lamberts et al., 1984). Evidence suggests that a chronically activated HPA axis can lead to the pathogenesis of obesity and diabetes (Chrousos, 2009) by altering key physiological processes involved in appetite regulation and energy homeostasis (McEwen, 2004), such as glucocorticoid regulation of the hypothalamic peptides CRH and AVP (Cavagnini et al., 2000, Savontaus et al., 2002) and other metabolically active hormones such as insulin, leptin and ghrelin (Brindley and Rolland, 1989, Cavagnini et al., 2000, la Fleur, 2006, Jahng et al., 2008). Leptin has a putative role in the regulation of the HPA axis and can signal to the hypothalamus, where appropriate downstream circuits are activated to modulate the stress response (Maffei et al., 1995). Some evidence suggests that the obesity-associated alterations in the HPA axis may be mediated by leptin (McGregor et al., 1996).

Chronic HPA activation is also implicated in the pathogenesis of psychiatric disorders (O'Connor et al., 2000, Gold et al., 1988, Weiss et al., 2004), by disruption of key brain functions such as altered hypothalamic and pituitary CRH-R1 expression (Kehne and Cain, 2010) and disrupted endocannabinoid signalling in upstream brain limbic structures and within the hypothalamus (Gorzalka et al., 2008, Finn, 2010). Loneliness is a specific form of chronic stress in humans, and is referred to as SI in animals, whereby social mammals are deprived of natural social interactions with animals of the same species. This form of chronic stress can cause changes in HPA function and is implicated in the pathogenesis of psychiatric disorders such as schizophrenia and depression (Thornton et al., 2010, Weiss et

al., 2004, Hatch et al., 1965, Pariante and Lightman, 2008, Kaye et al., 1987, Corcoran et al., 2003).

This thesis has described work investigating the effect of leptin and SI on the HPA axis response to acute stress.

4.2 Investigating the effects of leptin on the HPA axis response to stress

Discovered in 1994, leptin is a 167 amino acid (Zhang et al., 1994) adipokine, and leptin deficiency causes severe obesity, rapid weight gain, hyperphagia, mild diabetes and infertility (Ingalls et al., 1950). Leptin is largely synthesized in WAT (Scherer and Buettner, 2011), and the LepR is found in numerous peripheral organs (De Matteis et al., 1998, Siegrist-Kaiser et al., 1997) and within the brain, where leptin acts on the choroid plexus, VTA and the hypothalamic ARC, PVN, VMN and DMN to signal the extent of the body's fat stores (Collins et al., 1996). Leptin has a well characterised role in the regulation of body fat which it influences by inhibiting food intake and increasing energy expenditure (Ahima et al., 1998, Halaas et al., 1995, Chua et al., 1996). My studies focused on the effect of leptin on the HPA axis response to acute stress, in the form of acute LPS challenge in male Wistar rats.

My work suggests that peripheral leptin administration does not influence basal HPA activity or influence the HPA response to acute endotoxin challenge. LPS induced a strong stress response, as demonstrated by increased levels of the plasma stress hormones ACTH and corticosterone and the cytokine IL-1β, increased neuronal activation in the hypothalamic ARC, PVN, SON and VMN, as well as the NTS, PBN, AP and LC of the brainstem. However, pre-treatment with leptin did not influence the HPA response to acute LPS challenge.

The studies described in chapter 2 are the first to investigate the effects of leptin on the HPA response to a powerful immunological stressor. Different types of stressors (Ott et al., 1987, Kang et al., 2008, Hu et al., 1993, Connor and Leonard, 1998) ultimately activate the HPA axis via the hypothalamus, stimulating the CRH-ACTH-glucocorticoid cascade. However different sets of genes within the brain are induced or repressed by severe immunological

stress, such as LPS challenge, compared to those induced or repressed by mild psychological stress such as restraint stress (Reyes et al., 2003). Furthermore, LPS has been shown to stimulate ACTH and glucocorticoid release from the pituitary and adrenal glands respectively (Gloddek et al., 2001, Watanobe and Yoneda, 2003, Mohn et al., 2011, Pournajafi Nazarloo et al., 2003, Andreis et al., 1991). The fact that LPS induces a different activational response within the brain compared to mild stressors, and activates the HPA axis at multiple levels, may explain why leptin failed to attenuate the LPS induced HPA response in my studies, yet was effective in blunting the restraint stress-induced HPA response in other studies (Heiman et al., 1997)

To further investigate the role leptin plays in the HPA axis response to stress, it would be interesting to determine which stress pathways are influenced by leptin in response to mild stressors. In mice, acute restraint stress induced activation of the HPA axis can be influenced by pre-treatment with leptin (Heiman et al., 1997), but the mechanisms behind these changes have not yet been investigated. It would be interesting to test the effects of leptin on the stress response of individual components of the HPA axis, *ex vivo*. If an inhibitory effect of leptin is observed, the exact mechanisms by which these changes occur could be examined by investigating expression levels of genes known to be activated/repressed by stress in the HPA axis of these mice, and how the expression of these genes is affected by leptin.

Extending this to chronically stressed rodent models - specifically in rats because of the various stress models available in this species - could shed light on whether acute leptin administration influences a chronically activated HPA axis, in response to a new stressor. It is well known that chronic stress-induced HPA axis activation can lead to chronically elevated circulating leptin levels (McGregor et al., 1996, Beck and Richy, 2009). Obese rodents also have chronically elevated leptin levels, but suffer from leptin resistance. This suggests that acute leptin pre-treatment may not influence the HPA axis response to a novel

stressor in chronically stressed rats as they may also be leptin insensitive. If this is indeed the case, then it would be interesting to investigate which of the stress related genes and signalling pathways, looked at in the acute stress studies described above, are changed to allow this leptin insensitivity to occur.

It would also be interesting to carry out these studies in obese rats and directly compare the effects of acute leptin treatment on the HPA response to a novel stressor between chronically stressed rats and obese rats. As obese rats present with a constantly activated HPA axis - as evidenced by their chronically elevated circulating glucocorticoid levels (Mattsson et al., 2003, Buchenauer et al., 2009) - as well as with elevated basal circulating leptin levels (Maffei et al., 1995), it would be interesting to see whether the same signalling pathways contribute to chronic HPA activation in both models, and may further our understanding of why leptin insensitivity occurs in obese rats. This could be important in understanding the association between chronic psychological stress and the manifestation of the metabolic syndrome (Chrousos, 2009).

4.3 Investigating the effects of SI on the HPA axis response to stress

SI is a chronic form of stress in mammals (Weiss, 1974) and causes behavioural, neural, hormonal, cellular and genetic changes in humans, rodents and other social mammals (Cacioppo et al., 2006, Cacioppo and Hawkley, 2009, Hatch et al., 1965, Weiss et al., 2004, Holson et al., 1991). In rats, SI has generally been shown to result in increased HPA activity and increased HPA sensitivity to subsequent stressors (Weiss et al., 2004, Holson et al., 1991), but some studies suggest that HPA hypo-reactivity in response to a novel stressor can also occur (Sanchez et al., 1998) (Serra et al., 2005). These neuroendocrine changes are thought to result from the alterations of neurotransmitter signalling associated with stress, such as changes in the serotonergic (Chrousos, 1995, Berton et al., 1997, Flugge, 1995, McKittrick et al., 1995) dopaminergic, noradrenergic (Sontag et al., 2003) and GABA-ergic (Romeo et al., 1998) signalling systems.

The results from the studies described in chapter 2 prompted the exploration of a milder stressor in rats, and the effects of this stressor on HPA function. This work is also an extension of studies which have investigated SI in rats. To date all SI studies in rats have housed the animals for three weeks or more (reviewed in (Hawkley et al., 2012). The studies described in chapter 3 investigated the effects of intermittent, short term and long term chronic SI on the HPA axis response to acute restraint stress in rats. I am the first to show that 7-day chronic SI causes hyperactivity of the HPA in response to acute restraint stress, and that the hypothalamus and the adrenal gland are both hyper-sensitive after this period of SI. These data also demonstrate, for the first time that 7-day overnight SI and 3-week chronic SI cause hypo-activation of the HPA axis response to acute restraint stress, with the former regulated by an unknown mechanism and the latter possibly regulated by an overactive feedback mechanism.

The exploration of acute restraint stress as a mild psychological stressor revealed that it indeed produced a robust stress response, and that it resulted in a more reproducible stress response compared to LPS challenge. The same dose of LPS induced hugely different levels of stress in rats, as seen by widely variable fold increases in plasma ACTH and corticosterone between studies. On the other hand, restraint stress induced a similar stress response in rats across studies that used the same time points, as seen by similar fold increases in plasma ACTH and corticosterone between these studies. Thus restraint stress appears a useful model acute stressor for the future leptin experiments described earlier in this chapter.

The importance of HPA hyperactivity in disease is still not well understood. Some evidence suggests that HPA hyperactivity has negative effects on glucocorticoid-regulated processes. In rodents, SI-induced HPA hyperactivity results in increased circulating basal corticosterone levels, which directly reduces hippocampal neurogenesis and impairs wound healing (Stranahan et al., 2006, Detillion et al., 2004). Compared to non-lonely psychiatric patients, lonely psychiatric patients have higher urinary cortisol levels and lower natural killer cell and T-lymphocyte levels, in response to mitogen stimulation (Kiecolt-Glaser et al., 1984a, Kiecolt-Glaser et al., 1984b). These results suggest an immunosuppressive effect of excess cortisol and that HPA hyperactivity can result in impaired immunity. Prenatal over-exposure to glucocorticoids in non-human animals can cause hypertension, hyperglycaemia and increased HPA activity in the offspring in later life, whilst in humans it causes increased HPA activity all the way from early life, as well as high blood pressure, insulin resistance, glucose intolerance and hyperlipidemia in subsequent adulthood (Seckl and Meaney, 2004). All these conditions are pro-inflammatory, which suggests that chronically lonely individuals are at increased risk of inflammatory disease (Cole et al., 2007, Cole et al., 2012, Fredrickson et al., 2013).

An overactive HPA axis is also implicated in major depression (Nemeroff and Vale, 2005, Sanchez et al., 2001). Many depressed patients have increased plasma cortisol levels, as well as enlarged and hyperactive pituitary and adrenal glands (Nemeroff and Vale, 2005). This is thought to be due to an impaired glucocorticoid feedback mechanism (Pariante, 2006). Stressful life events also increase the risk of schizophrenia (Ellenbroek et al., 1998), and many schizophrenic patients have elevated plasma cortisol levels (Meltzer, 1987) and a higher cortisol response to a novel stressor (Lammers et al., 1995). Patients with anxiety disorders present with HPA hyperactivity (Abelson et al., 2007, Condren et al., 2002) and this is thought to be due to glucocorticoid insensitivity and CRH over-expression (Kunugi et al., 2006).

The HPA hyperactivity seen in my study of 7-day chronically isolated rats may also be caused by an overactive CRH feed-forward mechanism, as hypothalamic GR gene expression was not changed and hypothalamic CRH gene expression was significantly higher in response to restraint stress in these rats when compared to group housed rats. These findings demonstrate a physiological change in gene expression of stress related genes by SI, after just 7 days of chronic SI. Although this duration of SI may not be directly applicable to humans, these data do suggest that loneliness in humans could begin contributing to the pathogenesis of metabolic and psychiatric diseases after relatively brief periods of loneliness.

Some studies have shown that SI causes HPA hypoactivity in response to a novel stressor, and that this may be due to desensitization to stressful stimuli (Sanchez et al., 1998) and impairment of the mechanism by which glucocorticoids negatively feedback to the hypothalamus (Serra et al., 2005). However, there is little evidence in the current literature to suggest that HPA hypoactivity is important in the pathogenesis of metabolic and psychiatric disorders. HPA hypoactivity is seen in patients with rheumatoid arthritis, who also often display fatigue and depression (Templ et al., 1996). Patients with chronic fatigue

syndrome also display symptoms of HPA hypoactivity (Demitrack et al., 1991). The mechanisms behind the HPA hypoactivity observed in my study of 7-day overnight isolated rats are unknown. While there was a reduced plasma ACTH and corticosterone response to restraint stress, hypothalamic GR gene expression was reduced and hypothalamic CRH gene expression was increased. These gene expression data would suggest a down-regulated glucocorticoid feedback mechanism and overactive CRH feed-forward mechanism, yet this does not accord with the plasma stress hormone responses to restraint stress in these rats. It is therefore important to investigate this further by, as discussed in chapter 3, looking at CRH-R1 expression in the pituitary gland. Reduced CRH-R1 gene expression in the pituitary would counteract an increased hypothalamic CRH output, and may explain the reduced GR expression in the hypothalami of these rats. This is because a hypo-active HPA axis would not require high levels of GR for normalization of a stress response.

Rats chronically isolated for three weeks demonstrated HPA hypoactivity, with increased hypothalamic GR and CRH gene expression. This suggests that although there is an increased stress response in these rats, an exaggerated glucocorticoid feedback mechanism may have dampened this response. As this feedback mechanism could be occurring at multiple levels of the HPA axis, (Jones et al., 1977, Russell et al., 2010), as well as at upstream limbic structures (Sapolsky et al., 1984, Hill et al., 2011), the increased CRH drive may have had little effect on the stress response in these rats. It would therefore be interesting to extend investigations of the mechanisms behind HPA hypoactivity in 3-week chronically isolated rats by determining gene expression levels of GR in the pituitary and adrenal glands, as well as in the hippocampus and prefrontal cortex.

Throughout all the experiments described in chapter 3, basal corticosterone concentrations were not affected by SI, and neither were circulating leptin levels measured in animals from the first SI study (data not shown). As a way of understanding HPA dysfunction in obese

rats, it may be interesting to alter the SI paradigm from my studies and create a chronically stressed rat model that displays elevated basal circulating corticosterone and leptin concentrations, as did Perello et al., (Perello et al., 2006), in an effort to mimic the stress hormone profile of obese rats, and then determine the acute stress-induced HPA response in both models. These experiments could be very important in understanding the link between chronic stress-associated HPA dysregulation in obesity.

4.4 Final Summary

In the work described in this thesis I have shown that the adipokine leptin likely does not acutely influence the HPA response to a powerful immunological stressor. This may reflect that leptin may only be involved in the regulation of stress signalling pathways activated by non-endotoxin stressors. I have also demonstrated that SI can influence the HPA response to acute stress, and that varying lengths of SI can induce different changes in HPA function, resulting in HPA hyper/hypoactivity in response to acute stress. More work is required to further understand the long term effects of HPA dysregulation and how this may be important in metabolic and psychiatric disease.

Appendix I - Solutions

0.01M Phosphate Buffered Saline (PBS)

0.1M PBS was diluted 1:10 in Glass Distilled Water (GDW)

0.05M (hydroxymethyl)aminomethane Hydrogen Chloride (TrisHCl) solution (pH 7.6)

6.05g Trizma base was dissolved in 1 litre GDW and the pH was adjusted to 7.6 with HCl

0.1M PBS

28.2g $Na_2HPO_4.2H_20$, 5.44g KH_2PO_4 and 174g NaCl (VWR International Ltd., Dorset, UK) were made up to 2 litres in GDW, the pH was measured and confirmed to be 7.4 and this solution was stored at 4°C

0.2M PBS

56.4g $Na_2HPO_4.2H_20$, 10.88g KH_2PO_4 and 348g NaCl (VWR International Ltd., Dorset, UK) were made up to 2 litres in GDW, the pH was measured and confirmed to be 7.4 and this solution was stored at 4°C

1% Agarose Gel (for two large gels)

3.3g Agarose (VWR International Ltd., Dorset, UK)

330ml GDW

16.5ul Ethylenediaminetetraacetic acid (EDTA) (VWR International Ltd., Dorset, UK)

7ml 50 x Tris-acetate - EDTA (TAE) buffer

3,3' Diaminobenzidine (DAB) solution

0.01M PBS

50mg/ml DAB (Sigma-Aldrich, Dorset, UK)

30% (*v/v*) Hydrogen Peroxide (H_2O_2) (VWR International Ltd., Dorset, UK)

40% Sucrose solution

400g of sucrose (Tate & Lyle, London, UK)

Dissolved in GDW up to a volume of 1 litre

50% (*v*/*v*), 70% (*v*/*v*) and 100% Ethanol solutions

Absolute ethanol was diluted in GDW to obtain various concentrations of ethanol

50x TAE Buffer

242g Trizma Base (Sigma-Aldrich, Dorset, UK)

57.1ml Acetic acid (Sigma-Aldrich, Dorset, UK)

18.6g EDTA (Sigma-Aldrich, Dorset, UK)

Made up to 1L with GDW

Antifreeze

0.2M PBS

30% (*v*/*v*) Ethylene glycol (VWR International Ltd., Dorset, UK)

20% (*v*/*v*) Glycerol (VWR International Ltd., Dorset, UK)

GDW

Blocking solution

0.01M PBS

3% (*v/v*) Goat serum (Generon, Berkshire, UK)

0.25% (*v/v*) Triton X-100 (VWR International Ltd., Dorset, UK)

H_2O_2-Methanol solution

Methanol (VWR International Ltd., Dorset, UK)

0.6% (*v/v*) H_2O_2 (VWR International Ltd., Dorset, UK)

PBS Formaldehyde (FA) solution

0.01M PBS

4% (*v*/*v*) FA (VWR International Ltd., Dorset, UK)

PBS Tween solution (PBST)

0.01M PBS

0.05% (*v/v*) Tween-20 (VWR International Ltd., Dorset, UK)

Primary antibody diluent (same as blocking solution)

Running Buffer

20ml 50 x TAE

75ul Ethidium Bromide (EtBr) (Fisher Scientific, Loughborough, UK)

1L GDW

Secondary antibody diluent

0.01M PBS

0.3% (*w/v*) Bovine serum albumin (BSA) (Sigma-Aldrich, Dorset, UK)

0.25% (*v/v*) Triton X-100 (VWR International Ltd., Dorset, UK)

Appendix II - List of Suppliers

Applied Biosystems, Texas, USA

B. Braun Medical Ltd., Sheffield, UK

BDH, Dorset, UK

Boeco, Hamburg, Germany

Braintree Scientific Inc., Massachusetts, USA

Cayman Chemical Company, Michigan, USA

Charles River, Kent, UK

Crystal Chem Inc., Illinois, USA

Dako UK Ltd., Cambridgeshire, UK

DiaSorin, Dublin, Ireland

Eppendorf, Hamburg, Germany

Generon, Berkshire, UK

GraphPad Software Inc., California, USA

Hettich Lab Technology, Tuttlingen, Germany

Invitrogen, Paisley, UK

Merck Chemicals Ltd., Nottinghamshire, UK

MJ Research Inc., Quebec, Canada

NIDDK, Bethesda, USA

Qiagen, Venlo, Netherlands

R&D Systems, Abingdon, UK

Shandon Southern Products Ltd., Cheshire, UK

Sigma-Aldrich, Dorset, UK

Special Diet Services Ltd., Witham, Essex

Tate & Lyle, London, UK

Thermo Scientific, Massachusetts, USA

Vector Laboratories, Peterborough, UK

VWR International Ltd., Dorset, UK

Publications Arising

Basharat S, McGavigan A, Parker JA, Murphy KG, Bloom SR, Buckingham JC, John CD (2013) Leptin fails to blunt the lipopolysaccharide-induced activation of the hypothalamo-pituitary-adrenal (HPA) axis in rats. *In:* Journal of Endocrinology.

Poster Presentations

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Basharat S, Parker JA, Murphy KG, Bloom SR, Buckingham JC, John CD (2012) The physiological role of leptin in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis response to stress.
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