The role of chronic glucocorticoids in the regulation of energy homeostasis

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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<th>Description</th>
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<tbody>
<tr>
<td><strong>11β-HSD1</strong></td>
<td>11 beta-hydroxysteroid dehydrogenase type-1</td>
</tr>
<tr>
<td><strong>11-DHC</strong></td>
<td>11-Dehydrocorticosterone</td>
</tr>
<tr>
<td><strong>3V</strong></td>
<td>Third ventricle</td>
</tr>
<tr>
<td><strong>AAV</strong></td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td><strong>ACTH</strong></td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td><strong>ADX</strong></td>
<td>Adrenalectomy</td>
</tr>
<tr>
<td><strong>AgRP</strong></td>
<td>Agouti related peptide</td>
</tr>
<tr>
<td><strong>αMSH</strong></td>
<td>Alpha-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td><strong>Aif1</strong></td>
<td>Allograft inflammatory factor 1</td>
</tr>
<tr>
<td><strong>αMSH</strong></td>
<td>Alpha-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td><strong>AP</strong></td>
<td>Area postrema</td>
</tr>
<tr>
<td><strong>Arc</strong></td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td><strong>AVP</strong></td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td><strong>BAT</strong></td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td><strong>BBB</strong></td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td><strong>BDNF</strong></td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td><strong>BLQ</strong></td>
<td>Below the limit of quantification</td>
</tr>
<tr>
<td><strong>BMDM</strong></td>
<td>Bone marrow-derived macrophages</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>Caudal</td>
</tr>
<tr>
<td><strong>cAMP</strong></td>
<td>Cyclic adenocine monophosphate</td>
</tr>
<tr>
<td><strong>CE</strong></td>
<td>Collison energy</td>
</tr>
<tr>
<td><strong>CART</strong></td>
<td>Cocaine-amphetamine-related transcript</td>
</tr>
<tr>
<td><strong>Cort</strong></td>
<td>Corticosterone</td>
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<tr>
<td><strong>Cre</strong></td>
<td>Cre recombinase</td>
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<tr>
<td><strong>CRH</strong></td>
<td>Corticotropin-releasing hormone</td>
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<tr>
<td><strong>CXP</strong></td>
<td>Collision exit potential</td>
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<tr>
<td><strong>DAMPs</strong></td>
<td>Damage-associated molecular pattern</td>
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<td>Double distilled water</td>
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<td>DNA-damage inducible transcript 3</td>
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<td>Dorsomedial hypothalamus</td>
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<tr>
<td><strong>DP</strong></td>
<td>Orifice</td>
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<tr>
<td><strong>DREADDs</strong></td>
<td>Designer receptor exclusively activated by designer drugs</td>
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<tr>
<td><strong>E</strong></td>
<td>Energy expenditure</td>
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<tr>
<td><strong>ELISA</strong></td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td><strong>Epi</strong></td>
<td>Epididymal fat</td>
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<tr>
<td><strong>ER</strong></td>
<td>Endoplasmic reticulum</td>
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<td><strong>Fkbp5</strong></td>
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<tr>
<td><strong>GABA</strong></td>
<td>y-aminobutyric acid</td>
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<tr>
<td><strong>GALP</strong></td>
<td>Galanin-like peptide</td>
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<td><strong>Gc</strong></td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td><strong>GFP</strong></td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td><strong>Gilz</strong></td>
<td>Glucocorticoid-induced leucine zipper</td>
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<tr>
<td><strong>GLP-1</strong></td>
<td>Glucagon-like peptide 1</td>
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<tr>
<td><strong>GPCR</strong></td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td><strong>GR</strong></td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td><strong>GRE</strong></td>
<td>Glucocorticoid response element</td>
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<td><strong>HPA</strong></td>
<td>Hypothalamic-pituitary-adrenal axis</td>
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<td><strong>HPLC</strong></td>
<td>High performance liquid chromatography</td>
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<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
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<tr>
<td><strong>Iba1</strong></td>
<td>Ionised calcium-binding adaptor molecule 1</td>
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<td><strong>IgG</strong></td>
<td>Immunoglobulin G</td>
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<td>Nuclear factor of kappa light polypeptide gene enhancer in β cells inhibitor, alpha</td>
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<td>Inhibitor of kappa light polypeptide gene enhancer in β cells</td>
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<td><strong>i.p.</strong></td>
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<td>Inward rectifying potassium channel</td>
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<td><strong>Kir7.1</strong></td>
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</table>
L
LC-MS/MS - Liquid chromatography dual tandem mass spectrometry
LGRKO - Glucocorticoid receptor knockout in the liver
LH - Lateral hypothalamic nucleus
LLE - Liquid/liquid extraction
LLQ - Lower limit of quantification
LPS - Lipopolysaccharide

M
M - Medial
MBH - Mediodasal hypothalamus
MC4R - Melanocortin 4 receptor
Mes - Mesenteric fat
MDR-PGP - Multi-drug resistance p-glycoprotein

N
NAc - Nucleus accumbens
NFkB - Nuclear factor of kappa light polypeptide gene enhancer in β cells
NFkB RE - Nuclear factor of kappa light polypeptide gene enhancer in β cells response element
Nfkbia - Nuclear factor of kappa light polypeptide gene enhancer in β cells inhibitor, alpha
NLR - Nod-like receptor
NLRP3 – NLR family pyrin domain containing 3
NPY - Neuropeptide Y
NTS - Nucleus tractus solitarius

P
PAMPS - Pathogen-associated molecular pattern
PBN - Parabrachial nucleus
PBS - Phosphate buffered saline

P
PCR - Polymerase chain reaction
PFA - Paraformaldehyde
POMC - Pro-opiomelanocortin
PVN - Paraventricular nucleus

Q
qRT-PCR - Quantitative reverse transcription polymerase chain reaction

R
R - Rostral
RER - Respiratory exchange ratio
RPa - Raphe pallidus

S
SCN - Suprachiasmatic nucleus
SF-1 - Steroidogenic factor-1
SNS - Sympathetic nervous system
Socs3 - Suppressor of cytokine signalling-3
SPE - Solid phase extraction
Subcut - Subcutaneous fat

T
TLR - Toll-like receptor
TNFα - Tumour necrosis factor alpha
TRH - Thyrotropin-releasing hormone

U - Z
UCP-1 - Uncoupling protein-1
VMH - Ventromedial hypothalamus
VTA - Ventral tegmentum area
WAT - White adipose tissue
YFP - Yellow fluorescent protein
ZT - Zeitgeber time

Word Count: 51,971
Abstract

The role of chronic glucocorticoids in the regulation of energy homeostasis

Submitted by Charlotte Sefton for the degree of Doctor of Philosophy, The University of Manchester, 2017

The energy-regulatory network of the hypothalamus controls the everyday balance of food intake and energy expenditure. The close proximity of this region to the leaky blood brain barrier (BBB) enables many factors such as hormones to regulate energy homeostasis. This thesis aimed to determine the role of glucocorticoids (Gcs) within the hypothalamus in a model of Gc excess.

Recent research has shown that high-fat diet (HFD) induces hypothalamic inflammation, which is detrimental to the energy-regulatory network. Over a series of time-points, the hypothalamic expression of *Pomc, Npy*, and *Agrp* were quantified and a decrease in orexigenic neuropeptide expression was observed after 4 and 20 weeks. In contrast to predictions, *Pomc* was also decreased after 20 weeks. At all time-points, the mRNA expression of inflammatory markers was too low to detect, however after 20 weeks, microglia morphology indicated activation. Mice gained weight throughout the study, but it is unclear whether the very low hypothalamic inflammation contributed to the development of obesity.

The pleiotropic actions of Gcs enable them to be used as a treatment for a wide number of conditions, including inflammatory diseases, such as rheumatoid arthritis. Long-term Gc treatment is recognised to cause adverse metabolic side-effects including increased body weight gain and hyperphagia. In order to establish the role of hypothalamic Gcs in the development of Gc-induced metabolic syndrome, mice were treated with either corticosterone (Cort) or vehicle (1% EtOH) in the drinking water alongside a HFD or chow for 4 weeks. Exogenous Cort treatment induced a metabolic phenotype as food intake, body weight, and fed glucose increased during the study. Cort treatment also increased hypothalamic corticosterone levels after 24 hours, which remained elevated after the four week treatment period. HFD+Cort treatment exacerbated this effect, despite HFD alone not altering hypothalamic corticosterone levels.

To determine the role of Gcs within the mediobasal hypothalamus (MBH) in the development of Gc-induced obesity and hyperphagia, Cre-lox technology was used to knockdown the glucocorticoid receptor (GR) in the MBH. To ensure that the Cre recombinase enzyme was functional, Cre and YFP expression were co-localized within the MBH of AAV-Cre injected ROSA26-EYFP mice. Although the successful injection of AAV-Cre in GR flox mice should reduce GR expression, the low sensitivity of qRT-PCR in micro-punch and laser capture micro-dissections and dual immunofluorescence prevented confirmation of GR knockdown. After 3 weeks Cort treatment, a number of GR flox mice injected with AAV-Cre had a reduced gain in body weight and food intake. Furthermore, AAV-Cre injected GR flox mice were phenotypically monitored over a 6 month period without Cort treatment. These mice did not gain as much weight over time, suggesting that central Gc actions are important in the control of everyday energy homeostasis.

Collectively, this study has shown that Gcs acting within the hypothalamus have a significant role in the regulation of energy homeostasis. Further investigations will enable the development of co-therapies or ‘drug holidays’ to prevent the negative metabolic sequelae associated with Gc therapies.
Graphical Abstract

↑ Agrp expression  ↑ Body weight
↑ Food intake      ↑ Glucose levels

Bilateral AAV-GFP into the MBH of GR flox mice

Partially protects against Cort-induced body weight gain and food intake

Bilateral AAV-GFP into the MBH of GR flox mice  Bilateral AAV-Cre into the MBH of GR flox mice

Reduced body weight gain over 6 months
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Charlotte Sefton
21/03/17

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Chapter 1

Introduction
1.1 Maintenance of the endogenous glucocorticoid diurnal rhythm

The regulation of many biological mechanisms, including metabolism, inflammation, and the coordination of the stress response is controlled by steroid hormones, known as glucocorticoids (Gcs). The importance of a correctly functioning Gc system is clear, as life is incompatible in its absence (Tronche et al., 1999). The hypothalamic-pituitary-adrenal (HPA) axis regulates the secretion of the primary Gc, cortisol (in humans; corticosterone in rodents) from the zona fasciculata of the adrenal gland in a diurnal manner (figure 1.1, Harno & White, 2010).

With diverse pleiotropic effects, it is unsurprising that the Gc circadian rhythm peaks immediately prior to the start of the subjective day, and levels decrease to a nadir during the subjective night. The suprachiasmatic nucleus (SCN) of the hypothalamus and other higher brain regions maintain the rhythmicity of the Gc diurnal rhythm (Kalafatakis et al., 2016). This daily cycle is composed of 60–90 minute pulses, otherwise known as the Gc ultradian rhythm. The magnitude of these pulses is dependent on the phase of the circadian rhythm (Russell et al., 2014). Furthermore, Gcs feedback at both the pituitary and hypothalamus through self-regulatory negative feedback loops, in order to maintain this stable endogenous daily rhythm (figure 1.1, Harno & White, 2010).
Figure 1.1: The endogenous production of glucocorticoids by the Hypothalamic-Pituitary-Adrenal axis. Activation of the hypothalamic-pituitary-adrenal (HPA) axis stimulates corticotrophin-releasing hormone (CRH) release from the paraventricular nucleus (PVN), which in turn activates the transcription of the pro-opiomelanocortin (Pomc) gene within the anterior pituitary. POMC is post-translationally cleaved to adrenocorticotrophic hormone (ACTH), which stimulates the synthesis of Gcs from the fasciculata of the adrenal cortex.

1.2 Acute vs chronic activation of the hypothalamic-pituitary-adrenal axis

In the presence of a stressor, the HPA axis produces Gcs, which initiate the fight-or-flight response. Together, the acute production of Gcs drives a number of metabolic processes across numerous tissues, including increasing heart rate, slowing digestion, and mobilising glucose stores (Rose et al., 2010). This enables our bodies to respond to stressors, whether it be escaping an approaching lion (acute) or writing your thesis (chronic).
The self-regulatory feedback loops at the level of the hypothalamus and pituitary (figure 1.1) prevent a stressor from chronically activating the stress axis and continually producing Gcs. The importance of this inactivation is identified in patients suffering from disorders of the HPA axis, such as Cushing’s syndrome. Pituitary tumours, adrenal tumours, and ectopic ACTH-secreting tumours lead to the continued secretion of cortisol. The prolonged cortisol exposure that these patients suffer results in the development of a number of metabolic disorders, including abdominal obesity, hyperglycaemia, and insulin resistance (Lacroix et al., 2015).

More recently, it has come to light that it is long-term steroid treatment, which contributes most to the burden of Gc-induced metabolic problems. Used as a direct treatment for conditions such as rheumatoid arthritis, or prophylactically for cancer treatment, Gcs have a wide-medicinal use with >1.2% of the US population prescribed Gcs (Overman et al., 2013). The burden of long-term steroid treatment has recently been quantified in a retrospective study from the University of Manchester. Movahedi and colleagues calculated a 48% increase in the incidence of steroid-induced diabetes in patients suffering from rheumatoid arthritis following 6 months of treatment with Gcs (Movahedi et al., 2016).

Chronically reduced production of cortisol, as observed in Addison’s disease, also causes a number of metabolic sequelae, including loss of body weight, low blood pressure, and hypoglycaemia (Vegiopoulos & Herzig, 2007). Acute increases in Gc levels driven by the activation of the HPA axis are well tolerated, however, in circumstances of chronic excess or chronic insufficiency, the importance of the role of Gcs in metabolic regulation is clear. Therefore, it is important to understand the mechanisms of energy balance and how they are affected by long-term Gc excess in order to develop preventative measures.

1.3 Food intake vs energy expenditure; the everyday fight to maintain body weight

The everyday maintenance of energy homeostasis carefully balances food consumption against energy used to sustain a stable body weight over time. The peripheral metabolic tissues and central networks within the brain are constantly communicating to provide information on whether we need to consume more calories, release energy from stores, or expend more energy (Lenard & Berthoud, 2008).

The central mechanisms controlling food intake are split into two systems; our emotional desire to eat drives the rewarding hedonic pathways (Dallman, 2010), whereas the homeostatic pathways drive food intake when energy stores are low (Morton et al., 2006).
The two complementary systems are modulated by peripherally secreted hunger and satiety signals such as leptin, ghrelin, and insulin.

The neurones within the hypothalamus comprise the homeostatic network of food intake, as these neurones reside next to the leaky blood brain barrier (BBB) they are a primary target for peripheral hunger and satiety signals. These peripheral signals produced by the stomach, pancreas, and adipose tissue shift the balance of neuropeptides, to either drive or prevent the consumption of energy-rich food (Chen et al., 2015).

The hedonic neuronal networks, found primarily outside the hypothalamus, also control food intake. Food can act as a reward signal, activating the mesolimbic dopamine pathways and other limbic regions such as amygdala/hippocampus and cortex (Liu et al., 2014). These pathways are strongly activated in response to palatable food. If not controlled, the continued stimulation of these pathways can lead to food addiction in a similar manner to drugs of abuse (Lenoir et al., 2007).

At the other end of the energy balance spectrum to food intake is energy expenditure. Energy expenditure can be divided into three types of thermogenesis, obligatory, shivering, and non-shivering thermogenesis. Obligatory thermogenesis is more commonly known as basal metabolic rate and comprises the energy required for the everyday maintenance of the body, including ingestion, digestion, and processing of food (Yang & Ruan, 2015). Shivering thermogenesis generates heat from repeated skeletal muscle contractions. In contrast, non-shivering or adaptive thermogenesis produces heat via mechanisms in brown adipose tissue (BAT) in response to changes in physiology and the environment (Morrison, 2016).

The current obesity pandemic clearly indicates that this fine-tuned communication maintaining energy homeostasis can be disrupted easily. In 2014, >350 million women and >250 million men (over half of the adult population) in 114 countries were considered overweight, with a further 422 million adults consequently developing type-2 diabetes. With the trend in obesity continuing to rise (NCD Risk Factor Collaboration, 2016), it has been estimated that by 2030, the UK will spend £2 billion on the treatment of obesity and obesity-related diseases (Wang et al., 2011).

The obesity crisis has primarily been blamed on the unbalanced energy intake vs. energy expenditure. As the use of cars and ‘comfortable’ lifestyle becomes more prevalent, energy expenditure is reduced, and outweighed by the easily accessible western high-fat, high-sugar diet. However, this is increasingly being recognised as an overly simplistic view of obesity, as research has shown how genetics, epigenetics, stress, and disruption
to circadian rhythms (shift work / jetlag) can disrupt the balance of food intake and energy expenditure (Spiegelman & Flier, 2001).

1.3.1 Homeostatic networks of energy balance

The hypothalamus, which is found at the base of the brain next to the third ventricle, consists of a number of nuclei, which interact with each other to form the homeostatic network that controls energy balance. Classical studies have investigated the role of specific hypothalamic nuclei in the regulation of energy balance through the lesioning of nuclei and therefore loss of neurones within specific brain regions (reviewed in Elmquist et al., 1999). The crude electrolytic lesions completed by Hetherington and Ranson which disrupted the neuronal networks within the mediobasal hypothalamus including, the dorsomedial, ventromedial, and arcuate nuclei, the fornix and ventral region to the lateral hypothalamus, caused adiposity and the doubling of body weight in rats (Hetherington & Ranson, 1940). Targeted lesion studies have since been completed in order to decipher the contribution of each individual hypothalamic nucleus. Unlike, the obese phenotype observed with widespread lesioning, lesioning limited to the lateral hypothalamus caused loss of body weight and anorexia (Anand & Brobeck, 1951). Whereas bilateral lesions to the VMH led to hyperphagia and an obesity phenotype as seen in whole MBH lesioning (Stellar, 1954). The VMH and LH were thus named the ‘satiety’ and ‘feeding’ centres respectively. Further, the lesioning of regions outside of the MBH show the importance of a functioning neuronal network within the whole brain. Similar to the VMH, lesions to neurones within the PVN also cause hyperphagia and obesity (Shor-Posner et al., 1985; Sims & Lorden, 1986).

Extrahypothalamic regions, including the nucleus tractus solitarius (NTS), receive inputs from this hypothalamic network in order to regulate food intake. Concomitantly, neurones within the NTS are directly stimulated by peripheral signals and in turn relay information back to the hypothalamic network (figure 1.3). The hypothalamic nuclei are densely populated with neurones, each thought to be primary in controlling different aspects of energy homeostasis (Waterson & Horvath, 2015).

The close proximity of the neurones within the arcuate nucleus (ARC) to the leaky BBB allows easy access to peripheral signals, making these neurones the primary first order neurones to modulate food intake. Within the ARC, the two main sub-populations of neurones antagonistically regulate each other and project to other regions in the brain to form the networks which control food intake (Schwartz et al., 2000).
Unlike the ARC, whose neuronal population and function is well defined, the role of the dorsomedial hypothalamus (DMH) is less clear. Disruption to neuropeptide signalling within the DMH indicates that this region is important in the regulation of glucose homeostasis (Kim & Bi, 2015). Further, the optogenetic stimulation of GABAergic neurones, within the DMH promotes feeding through signalling networks connecting to the paraventricular nucleus (PVN) (Otgon-Uul et al., 2016).

Neurones controlling energy expenditure are also located within hypothalamic nuclei. The steroidogenic factor-1 (SF-1) neurones of the ventromedial nucleus (VMH) project via the sympathetic nervous system (SNS) to regulate BAT thermogenesis (Yang & Ruan, 2015). Similar to the DMH, the feeding and thermogenic effects of VMH neurones are integrated within the PVN prior to signalling to higher centres or the periphery.

The PVN is the principle integrator of signalling from the different nuclei in the hypothalamus. Situated within the PVN, a number of different receptors are present that integrate and process signals from neighbouring hypothalamic nuclei. However, the extensive connections between the primary energy-regulatory melanocortin neurones of the ARC and the PVN ensure that the melanocortin receptors are the most abundant (reviewed in Williams et al., 2001).

The advancement of technology has provided the opportunity to further understand the neuronal networks of the hypothalamus. Designer receptor exclusively activated by designer drugs (DREADDs) and optogenetic studies have shown that these networks are highly complex, with many factors regulating multiple functions. The constant communication between these hypothalamic nuclei, is controlled by two sub-populations of neurones that signal by releasing either appetite-stimulating/orexigenic or appetite-inhibiting/anorexigenic neuropeptides and neurotransmitters (figure 1.2).
**Figure 1.2: Anorexigenic and Orexigenic signals in the hypothalamus**

**Hypothalamic nuclei:** Arcuate nucleus, ARC; dorsomedial nucleus, DMH; lateral hypothalamus, LH; paraventricular nucleus, PVN; ventromedial nucleus, VMH. **Anorexigenic:** α-melanocortin-stimulating hormone, α-MSH; arginine vasopressin, AVP; brain-derived neurotrophic factor, BDNF; cocaine and amphetamine-regulated transcript, CART; corticotropin-releasing factor, CRF; thyrotropin-releasing hormone, TRH; melanin-concentrating hormone, MCH; pro-opiomelanocortin, POMC. **Orexigenic:** glucagon-like peptide 1, GLP-1; neuropeptide y, NPY; agouti-related peptide, AgRP; galanin-like peptide, GALP; γ-aminobutyric acid, GABA. Third ventricle, 3V; blood brain barrier, BBB.

In contrast to neurotransmitters, whose rapid and discrete action at ionotropic receptors are found solely at synaptic sites, neuropeptides are released from many sites along neurones. Neuropeptides can therefore signal across both short and long distances, with the latter aided by G protein-coupled receptor (GPCR) signalling, such as $\text{G}_{\alpha_s}$-coupled...
melanocortin receptors, amplifying small nanomolar neuropeptide concentrations (van den Pol, 2012).

The anorexigenic and orexigenic neuropeptides within the hypothalamus are traditionally viewed as being part of the homeostatic networks of the hypothalamus. However, the recording of these neurones in awake-behaving animals has identified five properties by which these neurones are modulated: 1. the nutritional state of the animal; 2. palatability of the food; 3. food accessibility; 4. the chance of eventual food consumed and 5. an integrative response (Betley et al., 2015; Chen et al., 2015). These studies have shown neuropeptides do not just respond after food consumption, but also in anticipation of food. Furthermore, these responses can be learned, making these neuropeptides important in multiple networks controlling energy balance.

### 1.3.1.1 The anorexigenic neurones which regulate energy balance

The melanocortin system within the central nervous system has been thoroughly characterised in the control of energy balance. Disruption to the pro-opiomelanocortin (Pomc) gene was found in patients suffering from a combination of severe childhood obesity, adrenal insufficiency and pigmentation abnormalities (Krude et al., 1998). This discovery has driven research to further understand the anorexigenic actions of POMC and its derivatives. The homozygous deletion of POMC in the mouse has successfully modelled the phenotypic abnormalities shown in the human (Challis et al., 2004; Smart & Low, 2003; Yaswen et al., 1999).

POMC is cleaved by two pro-hormone convertase enzymes (PCs) and together with other post-translational modifications this results in a number of functionally different peptides (adrenocorticotropic hormone [ACTH] gives α-MSH), β-Lipotropin [β-LPH] produces β-MSH and β-endorphin, and N-POMC is cleaved to γ-MSH), which dependent on the peptide can either bind to melanocortin receptors (MCR) or opioid receptors to elicit there effects (Cone, 2005). The high expression of the MC4R within the PVN indicates that of the melanocortin receptors the MC4R is the most important in the regulation of food intake and energy expenditure (Mountjoy et al., 1994). The global knockout of MC3R induces an acute metabolic phenotype indicating signalling through this receptor also has a minor role in energy regulation (Butler et al., 2001).

Classically the neural connections between the ARC and the PVN indicate α-MSH as the primary neuropeptide to drive the anorexigenic signal. These actions are clear as the central administration of α-MSH into POMC null mice reduces food intake and body weight (Tung et al., 2006). Although β-MSH has a similar affinity for the MC4R as α-MSH,
the intracerebroventricular injection of β-MSH reduces food intake to a lesser extent than α-MSH (Tung et al., 2006). In addition a mutation in the Pomc gene has been described causing the fusion of β-MSH and β-endorphin. The potency of this fusion peptide is dominant as α-MSH cannot overcome its actions and prevent the obesity (Challis et al., 2002). The importance of β-MSH in energy balance was also recently discovered in obese Labradors whose insatiable appetite is driven by the loss of the β-MSH sequence resulting in a truncated POMC (Raffan et al., 2016).

It must be noted that 'normal' POMC processing is not 100% efficient and therefore the precursors of POMC derivatives (including ACTH and POMC) can also bind to MC4R within the PVN to elicit effects (Pritchard et al., 2004; Pritchard et al., 2003). However, not all of POMC derivatives have anorexigenic actions, the cleavage of β-LPH produces β-endorphin which binds to opioid receptors and has an antagonist effect (discussed further in section 1.3.1.2).

With approximately 3000 POMC neurones in the ARC receiving >43,000 inputs from across the brain, it is clear this region of the hypothalamus is the primary site for the melanocortin control of food intake. The majority of these inputs, (approximately 60%), originate from other regions of the hypothalamus, indicating that the ARC is the hub for controlling energy balance (Wang et al., 2015).

With the ability to co-release over 10 peptide hormones, it is not surprising that POMC neurones project broadly throughout the brain. However, the high concentration of melanocortin receptors in the PVN, and the results of anterograde tracing studies from the ARC clearly show the importance the ARC-PVN connection (Millington, 2007). Furthermore, disruption to the melanocortin signalling pathway through knockdown of the MC4R induces hyperphagia and severe obesity (Butler et al., 2001; Coll et al., 2004; Ste Marie et al., 2000), whereas stimulation of POMC neurones suppresses feeding (Aponte et al., 2011; Zhan et al., 2013).

More recent studies have suggested that αMSH signalling via the MC4R is not the only signalling pathway between the ARC and PVN. Classically, ligand binding to the MC4R depolarises the postsynaptic neurone as the Gαs signalling cascade is initiated and cyclic adenosine monophosphate (cAMP) levels rise. New sophisticated technologies have shown that the activity of PVN neurones can be controlled independently of MC4R through the inwardly rectifying potassium channel Kir7.1 (Ghamari-Langroudi et al., 2015).

Investigations into the suppression of food intake have predominantly focussed on the actions of αMSH, however, this is not the only neuropeptide or neurotransmitter in the ARC which has anorexigenic effects. Galanin-like peptide and cocaine-amphetamine-
regulated transcript (CART) are both synthesised by POMC neurones, and have been shown to decrease food intake (Elmquist, 2001; Gundlach, 2002; Vrang et al., 1999). Furthermore, the regulation of POMC neuronal activity is mediated at least in part by γ-aminobutyric acid (GABA), as the genetic disruption to GABA signalling specifically on POMC neurones decreases POMC expression and increases body weight (Ito et al., 2013).

A subset of POMC neurones (<300 neurones), are located in the NTS of the medulla and receive inputs from approximately 22,000 neurones. Located in the hindbrain, the majority of these inputs originate in the pons and medulla (Wang et al., 2015). The NTS alongside the area postrema and the dorsal motor nucleus of the vagus (DMV) neighbour each other and form the dorsal vagal complex (DVC). Retrograde tracing studies mapping the melanocortin axonal projections from both the MBH and NTS indicate how complex the network is (Wang et al., 2015) (figure 1.3). This whole brain mapping study indicated that POMC neurones within the NTS are highly innervated by direct inputs from the brainstem, with strong reciprocal connections between the MBH and NTS. Feeding parameters were investigated following the optogenetic stimulation of POMC neurones which project from the PVN to nuclei within the hindbrain. Only the activation of PVN neurones projecting to the lateral parabrachial nucleus (LPBN) altered food intake parameters (increased latency to feed and decreased total time spent feeding). Whereas the stimulation of neurones from the PVN projecting to the NTS and DMV did not alter food intake (Garfield et al., 2015). However, alongside these neuronal connections to the NTS, peripheral satiety signals directly input to the NTS via the vagus nerve (Williams et al., 2001), which relays back to hypothalamic nuclei (Agostino et al., 2016) (figure 1.3). Although there are significantly fewer POMC neurones in the DVC compared with the hypothalamus, the administration of MC4R agonists and antagonists into the fourth ventricle alters feeding to the same extent as Intracerebroventricular administration (Grill et al., 1998).

Studies have attempted to differentiate the actions of POMC in the ARC and NTS; an immediate decrease in food intake was observed following the acute activation of NTS POMC neurones. Conversely, repeated stimulation of ARC POMC neurones over three days was required before food intake decreased. This difference, identified by a pharmacological approach, was verified through the ablation of POMC neurones in either the ARC or NTS which identified that only the ablation of ARC POMC neurones induced obesity (Zhan et al., 2013).
Figure 1.3: Melanocortin signalling between hypothalamic and extrahypothalamic regions to control energy balance.

Top panel: melanocortin signalling projecting from the hypothalamus, POMC neuronal connections red arrows, AgRP neuronal connections (blue arrows) and secondary connections dotted black line. Bottom panel: melanocortin signalling from the dorsal vagal complex (green arrows).

Bed nucleus of the stria terminalis, BNST; Central amygdala nucleus, CeA; Dorsal vagal complex, DVC; Interomediolateral nucleus, IML; Lateral hypothalamus, LH; Medio basal hypothalamus, MBH; Parabrachial nucleus, PBN; Paraventricular nucleus, PVN

1.3.1.2 The orexigenic neuropeptides of energy balance

Opposing the inhibitory actions of anorexigenic neuropeptides, orexigenic peptides stimulate food intake. As mentioned previously the processing of the anorexigenic peptide POMC produces β-endorphin whose actions at opioid receptors drive orexigenic actions (Mountjoy, 2010). As both derivatives of POMC can stimulate and inhibit food intake it is
unknown whether both subtypes of peptide are produced to regulate each other’s actions. Another possibility is that regulation of the acetylation of αMSH and β-endorphin can modify the function of the peptides and therefore produce very specific effects. Acetylated αMSH is found in the NTS but less in the ARC, while for β-endorphin, acetylated β-endorphin predominates in the NTS and non-acetylated β-endorphin predominates in the ARC (Mountjoy, 2010). However it is difficult to accurately measure the different peptides in the presence of their precursors so the actual ratio of POMC derived peptides present after activation of the POMC neurons may be difficult to assess (Pritchard et al., 2003). The co-administration of both β-endorphin and α-MSH into the third ventricle suggests which peptide is dominant may be dependent on the duration of stimulation. Acute administration of β-endorphin over 2-6 hours increased food intake and reversed the inhibitory actions of NDP-MSH (synthetic analogue of α-MSH). However chronically administrating β-endorphin over 4-7 days fails to antagonize the effects of NDP-MSH (Dutia et al., 2012).

A large number of orexigenic neuropeptides and neurotransmitters have been identified in the hypothalamus (figure 1.2); however, it is the actions of co-localised agouti-related peptide (AgRP), neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) in the ARC, which have been extensively investigated. The rapid starvation, induced by ablation of these neurones within the adult ARC highlights the critical role that these neurones play in food intake (Luquet et al., 2005). Although these neuropeptides and neurotransmitters are co-localised within one neurone subtype, they exert their effects through independent mechanisms.

Unlike both NPY and GABA, AgRP is exclusively expressed within the ARC and has been classically described through its antagonistic actions at the MC4R. Compared with POMC neurones, AgRP neurones receive fewer direct inputs approximately 17,000, with 70% of these originating from nuclei within the hypothalamus (Wang et al., 2015). Food intake can be induced and sustained over time through intracerebroventricular injections of AgRP given directly into the hypothalamus (Small et al., 2001). However, overexpressing Agrp leads to the development of a number of metabolic disorders including obesity and hyperinsulineamia (Graham et al., 1997). Furthermore, through the inclusion of DREADDs specifically on AgRP neurones, feeding can be driven or suppressed, regardless of the animal’s nutritional status (Krashes et al., 2011). Additionally, similar to the mechanistic studies of POMC, AgRP has too been shown to bind to Kir7.1 channels hyperpolarising the MC4R neurones in the PVN to drive food intake (Ghamari-Langroudi et al., 2015).

With multiple orexigenic neuropeptides stimulating food intake, care needs to be taken when trying to distinguish the role of each neuropeptide or neurotransmitter. Congenital
knockouts of NPY are phenotypically normal and have an expected hyperphagic response to fasting, which suggests that other peptides compensate for the lack of NPY (Erickson et al., 1996; Marsh et al., 1999). Electrophysiological studies have shown strengthened GABA signalling from AgRP/NPY neurones in the absence of NPY. Both NPY and GABA signal through their respective receptors NPY1R and GABA<sub>A</sub> to inhibit the oxytocin neurones of the PVN. Consequently, this inhibition stimulates feeding. Blockade of both NPY1R and GABA<sub>A</sub> receptors in the PVN, during AgRP activation, decreases food intake which suggests that both GABA and NPY signalling is critical (Atasoy et al., 2012). These multifunctional neuropeptides are widely spread throughout the hypothalamus. Therefore in order to distinguish the role of neuropeptides within specific nuclei, the knockdown of each neuropeptide within specific nuclei is required. A number of these studies have been conducted, identifying NPY localised in the DMH to be involved in glucose homeostasis, whereas in the ARC it regulates food intake (Kim & Bi, 2015; Hahn et al., 1998). Furthermore, GABA released from AgRP neurones is important for energy expenditure in addition to food intake (Tong et al., 2008).

1.3.2 Glucocorticoids and the neuropeptides of the energy-regulatory network

The close proximity of the orexigenic and anorexigenic neuropeptides within the ARC makes these neurones prime targets of circulating hunger and satiety signals. Although the self-regulatory actions of Gcs at the PVN indicate that circulating Gcs can cross the BBB to act in the hypothalamus, the role of Gcs in regulating the energy-regulatory neuropeptides of the ARC remain unclear.

This uncertainty is in part due to a lack of understanding of the mechanisms within the hypothalamus, which control the tissue levels of Gcs. Within the human brain, the multi-drug p-glycoprotein (MDR-PGP) efflux pump spans the BBB, limiting the concentration of active cortisol which accumulates in the brain (Meijer et al., 2003). However, the role of this efflux pump in the rodent brain is still unknown, as genetic studies investigating the two murine isoforms produce conflicting data (Mason et al., 2012). Further 11β-hydroxysteroid dehydrogenase type-1 (11β-HSD1) is expressed in both human and rodent brains. This enzyme regulates the concentrations of Gcs by converting the inactive form of the steroid (cortisone, humans; 11-dehydrocorticosterone [11-DHC] rodents) to the active form (cortisol, humans; corticosterone, rodents).

The presence of the glucocorticoid receptor (GR) in both AgRP/NPY and POMC/CART neurones indicates that Gcs have a regulatory role within this region (Cintra & Bortolotti, 1992; Unger et al., 2010). Furthermore, glucocorticoid response elements (GREs) are present in the promoter regions of the first order neurones AgRP/NPY and POMC/CART,
providing the capacity for GR to bind (Drouin et al., 1993; Lee et al., 2013; Misaki et al., 1992).

1.3.2.1 Glucocorticoids and the orexigenic neuropeptides

The removal or restriction of food during the active phase can act as a stressor stimulating the HPA axis and increasing the production of Gcs. Gene expression analysis in these studies shows increases in Agrp as the rodents try and increase their food intake (Bi et al., 2003; Makimura et al., 2003; Savontaus et al., 2002). Alternatively, reducing the endogenous production of Gcs by adrenalectomy (ADX) reduces the expression of orexigenic Agrp (Savontaus et al., 2002). Together, these studies show the positive relationship between corticosterone and Agrp. The expression patterns of both AgRP and NPY neuropeptides in the ARC are diurnally rhythmic. The increase in Agrp and Npy at the beginning of the subjective night coincides with the corticosterone rhythm, stimulating feeding at the start of the active period (Akabayashi et al., 1994; Lu et al., 2002).

However, AgRP and NPY’s differing responses to exogenous Gc stimulation leave the relationship between Gcs and orexigenic neuropeptides unclear. Both orexigenic neuropeptides increase when stimulated with the synthetic Gc, dexamethasone (Dex) (Goto et al., 2006; Shimizu et al., 2008), however, only AgRP responds to corticosterone stimulation. The reason behind this differing response between the two neuropeptides is unknown. As both NPY and AgRP are produced by the same neurones, it raises the question of how this differential effect occurs.

1.3.2.2 Glucocorticoids and the anorexigenic neuropeptides

The self-regulatory feedback loops of the HPA axis clearly show that Gcs inhibit Pomc expression at the pituitary. Through the analysis of POMC in the pituitary, the binding of GR to the GRE in the promoter region of POMC has been expertly mapped (Drouin et al., 1989), however, studies investigating the role of Gcs on hypothalamic Pomc expression have produced conflicting results. Removal of endogenous Gc production by adrenalectomy has been reported to increase Pomc expression and increase the neuronal resting membrane potential (Gyengesi et al., 2010; Savontaus et al., 2002), while other studies using this technique have shown no change in Pomc (Makimura et al., 2003; Uchoa et al., 2012).
1.3.2.3 Chronically elevated glucocorticoids and the neuropeptides

Although the acute effects of Gcs in the hypothalamus are well established, the long-term effects of chronic corticosterone exposure remain unknown. The metabolic effect of Gcs is evident in the clinic, as patients on long-term treatment for rheumatoid arthritis, or as a prophylactic treatment for cancer, gain weight and have an increased likelihood of developing metabolic disorders such as steroid-induced diabetes (Movahedi et al., 2016). To date, rodent studies have only investigated the phenotypic effects of long-term elevated Gc levels when modelling steroid-induced metabolic syndrome (Cassano et al., 2012; Coll et al., 2005; Karatsoreos et al., 2010; Shpilberg et al., 2012). The development of hyperphagia after two weeks, as seen in these models, indicates a hypothalamic role of increased Gc levels. But it is not known if either the MDR-PGP or 11β-HSD1 can help protect the hypothalamus from these chronically elevated levels.

1.4 The hedonic neuronal networks of food intake

The pleasure and reward of eating nutrient-rich foods drives the intake of food through the central hedonic networks. The prevalence and easily accessible high-fat, high-sugar diets in our modern society are causing this circuitry to be chronically activated, driving our desire to continually eat. The rewarding pleasure of consuming palatable food is so powerful that it can override homeostatic signals of satiety (Kenny, 2011). The power of this circuitry has been shown in studies of addiction; when given the choice between saccharin and cocaine, rats preferred the sweetened water to the highly addictive drug (Lenoir et al., 2007). As palatable foods are highly prevalent in our society, it is important for us to understand the hedonic neuronal networks, and how they interact with the homeostatic mechanisms to control food intake.

The hedonic circuitry traditionally lies outside of the hypothalamus, in what is known as the extrahypothalamic region. Palatable food stimulates the mesolimbic dopamine circuitry, which projects dopamine neurones from the ventral tegmentum area (VTA) to the striatum, higher cortical areas, and nucleus accumbens (NAc), driving the wanting-to-obtain a reward stimulus and the pleasure from the reward respectively (figure 1.5).

To stimulate food intake, the lateral hypothalamic nucleus (LH) integrates signals from the reward pathway, reciprocal connections between itself, and the ARC and orexigenic connections to the NTS. The LH is therefore considered an integration hub combining signals from both hedonic and homeostatic networks driving food intake (Morton et al., 2006).
Figure 1.4: The hedonic networks driving food intake.

The mesolimbic dopamine reward pathway projects dopamine signals from the ventral tegmental area (VTA) project to higher cortical areas, the striatum, and nucleus accumbens (NAc). γ-aminobutyric acid (GABA) projects from the NAc to the lateral hypothalamus (LH). The lateral hypothalamus projects a number of orexigenic signals to the arcuate nucleus (ARC) and dorsal vagal complex (DVC).

1.4.1 Hypothalamic neuropeptides and their hedonic actions

The components of the neuronal networks involved in the control of hedonic and homeostatic mechanisms have traditionally been viewed as independent. More recently, the neuropeptides of the ARC involved in the homeostatic control of food intake have been identified to have hedonic properties. A number of studies have looked at the role of NPY and the melanocortin system in the hedonic regulation of food intake (reviewed in Pandit et al., 2013). Sophisticated studies which target and manipulate specific neuronal populations within the ARC during awake-behavioural studies have determined the role of these neurones in response to hunger and food preference tasks.

The strong orexigenic effects of AgRP have made it a prime target to understand the relationship between the homeostatic and hedonic networks controlling food intake. Immunohistochemistry indicates that AgRP neurones may regulate the hedonic network as they project to the VTA (figure 1.4), part of the dopamine reward signalling pathways (Dietrich et al., 2012). Furthermore, the knockdown of Sirt1 within AgRP neurones increases exploratory behaviour and the preference for cocaine. Electrophysiological
studies have shown that the activation and inhibition of the melanocortin receptors in the VTA do not affect neuronal activity, indicating that it is co-expressed GABA which is released from AgRP neurones mediating the response (Dietrich et al., 2012).

The addition of palatable foods to these studies suggests that AgRP neurones are only important in the food intake of chow food, and the hedonic pathways take over when palatable food is involved (Denis et al., 2015). AgRP neuronal activation stimulates foraging behaviours associated with feeding (Dietrich et al., 2015), however, the sensory detection of food reverses the neuronal activity inhibiting AgRP and increasing POMC activity (Chen et al., 2015). This response is dependent on the palatability of the food, and if the food is not consumed, then the AgRP neurones would be reactivated, driving the foraging behaviour. These studies clearly indicate that AgRP neurones are involved in both hedonic and homeostatic circuits. Investigating both AgRP-driven feeding and foraging behaviours in one study suggests that the neurones of the ARC are heterogeneous, with subsets of neurones involved in different behaviours (Dietrich et al., 2015). To be able to identify the distribution and involvement of these subpopulations requires highly technical in vivo single-cell dynamics.

1.4.2 Glucocorticoid stimulation of the hedonic networks shifts food preference

One of the side-effects of long-term Gc treatment is a shift in food preference. It has long been known that patients treated with Gcs for a short period of time witness the adverse effects of increased food intake and have a shifted food preference towards high-fat, high-sugar foods (Tataranni et al., 1996). However, this effect only worsens with time, as continued elevated Gc levels raise the threshold for which foods are perceived as a reward. Therefore to achieve the same rewarding pleasurable effect, more food needs to be consumed (Moeller et al., 2016).

The actions of Gcs on the hedonic networks are still unclear, as stress-induced increases in Gc levels have variable influences on food intake. Dallman showed that in response to elevated Gcs, approximately 40% of the population increased their food intake, 40% decrease, and the remaining 20% do not change their eating habits (Dallman, 2010). These varying responses are also recorded in rodent models investigating the influences of elevated Gc levels on food intake and preference (Boersma et al., 2016; Liu et al., 2011). The variable results in the literature could be a result of the method used to raise corticosterone levels, the strain or the gender of the animal.

Limited studies have investigated the involvement of Gc signalling in the emotional and top-down processing of food intake. Disruption to the GR in the higher cortical centres has
been used to investigate the Gc role in the hedonic pathways (reviewed in Kolber & Muglia, 2009). Following restraint stress, the amount of sucrose solution consumed is reduced in mice where the GR is knocked down in the forebrain region reducing the amount of sucrose solution consumed compared with control mice, suggesting increased anhedonia (Boyle et al., 2005).

The shift in food preference observed in people with elevated Gc levels clearly indicates a Gc involvement of the hedonic system. Gc signalling has been proven to play a role in the mechanisms controlling reward, hedonic, and homeostatic pathways, however, more studies are required to decipher and distinguish the effect of chronically elevated Gc levels within specific regions of the brain in the control of these systems.

1.5 The central vs peripheral actions of glucocorticoids

Traditionally, it has been thought that pathways, such as glucose homeostasis and lipid metabolism, are controlled by the direct Gc actions on peripheral mechanisms. However, the requirement to mobilise glucose for fuel when an animal is stressed indicates that Gcs play a regulatory role in these processes. Until recently, investigations into the Gc regulation of these metabolic pathways, has been centred on the direct actions of Gcs on peripheral tissues (figure 1.5).

1.5.1 Direct actions of glucocorticoids on adipose tissue

Adipocyte hypertrophy and hyperplasia in patients with chronically elevated corticosterone levels indicates that Gcs have a role in expansion of adipose tissue, albeit directly or indirectly (Lacroix et al., 2015). In addition, chronically elevated Gc levels cause a shift in adiposity specifically increasing central deposition, suggesting adipose bed specific effects. Exogenous administration of corticosterone in the drinking water has successfully modelled this alteration in adiposity distribution (Cassano et al., 2012; Karatsoreos et al., 2010).

GR knockdown within adipocytes (FGRKO) has been used to investigate the Gc actions within this tissue. When challenged with a high-fat diet (HFD), FGRKO mice are protected against the development of diet-induced obesity. Although basal corticosterone levels in FGRKO mice are unaltered, when challenged, both corticosterone and glucose responses to restraint stress are increased (de Kloet et al., 2015). This mouse model has been challenged further by Bose and colleagues, to establish the acute and long-term effects of
Acute injections of dexamethasone increased the expression of a number of genes involved in lipid metabolism. However, long-term exogenous corticosterone treatment has no protective effect on glucose or body composition (Bose et al., 2016). The direct actions of Gcs on adipose tissue are further evident, as prevention of corticosterone regeneration, specifically in adipose tissue (adipocyte 11-βHSD1 KO mice) attenuates the development of hepatic steatosis. Together, these studies indicate inter-tissue mechanisms in the control of glucose and lipid metabolism (Morgan et al., 2014).

In both these KO strains, the inter-tissue signalling and development of compensatory mechanisms makes it difficult to determine the role of adipose tissue in the development of Gc-induced metabolic syndrome. The determination of Gc actions within adipocytes is further complicated, as although functionally different, knockdown of the GR from adipocytes results in the loss of GR from both white and brown adipose tissue.

1.5.2 Glucocorticoid regulation of liver metabolism

Hepatic Gc signalling is highlighted in the modelling of the hepatocyte-specific GR KO mice, as half of these mice die within 48 hours of birth due to hypoglycaemia (Opherk et al., 2004). Disruption to Gc-GR signalling in the liver (LGRKO) protects against the increase in gluconeogenic genes expressed following an acute Dex injection (Bose et al., 2016). Similarly, the use of liver specific GR antagonists reduce hepatic glucose production and improved insulin resistance (Zinker et al., 2007).

However, LGRKO increases the expression of gluconeogenic genes in the kidney when challenged with Dex, potentially masking any effects of whole body changes in glucose and insulin intolerance. This also indicates further inter-tissue communication in the periphery (Bose et al., 2016).

1.5.3 Glucocorticoid regulation of pancreas

The risk of developing steroid-induced diabetes is increased when on long-term Gcs (Movahedi et al., 2016). Pancreatic β-cells control the secretion of insulin and consequently the plasma glucose levels, however, when the β-cells fail to meet increased insulin demand, hyperglycaemia develops (Rafacho et al., 2014).

In the short-term Gcs decrease insulin secretion, increases the availability of glucose for immediate use and reduces long-term glucose storage. The acute actions of Gcs however are dependent on dose, duration, and route of administration. Studies assessing the role of Gcs on pancreatic β-cell function found that oral administration decreases insulin
secretion (Van Raalte et al., 2013), however, a similar study assessing the effect of Gcs by intravenous administration did not see an effect (Vila et al., 2010).

Conversely, the continued administration of Gcs leads to sustained insulin secretion. The prolonged increase in Gc levels leads to up-regulation of β-cell function and continued production of insulin (Beaudry et al., 2013). If glucose levels remain elevated, insulin is continually produced creating a state of hyperinsulineamia and eventually insulin resistance. Consequently, the pancreas tries to counteract the insulin-resistance by producing more insulin, and consequently increases in size. The removal of Gc treatment after 10 days can return glucose and insulin levels to normal in the rat, however, it is still unknown whether these beneficial effects of drug removal are translated into man (Rafacho et al., 2010).
Figure 1.5: The direct and indirect actions of glucocorticoids on peripheral metabolism.

Glucocorticoids produced by the hypothalamic-pituitary-adrenal (HPA) axis negatively feedback at the pituitary, hypothalamus, and hippocampus to self-regulate their production (Red arrows). Glucocorticoids released into circulation by the adrenal directly act on tissues involved in peripheral metabolism; liver, white adipose tissue (WAT), brown adipose tissue (BAT), muscle (Blue arrows). Glucocorticoids can also cross the blood brain barrier targeting energy-regulatory neurones in the mediobasal hypothalamus (MBH) which signal indirectly via either the sympathetic or parasympathetic nervous system to modulate metabolism in peripheral tissues (Green arrows). Paraventricular nucleus, PVN; dorsal vagal complex, DVC; uncoupling protein-1, UCP-1.
1.5.4 The central control of peripheral metabolism

The direct actions of Gcs in the periphery have been thoroughly investigated; however, more recently it has become evident that the brain, at least in part, can regulate these peripheral mechanisms. It has been debated whether these central mechanisms signal to the periphery via the sympathetic or parasympathetic nervous system to modulate these metabolic pathways. Although the mechanisms of this central-peripheral signalling are still unclear, it is evident that the central and peripheral actions of Gcs are not mutually exclusive.

The direct stimulation of the hypothalamic energy-regulatory network with Gcs has been exploited, to investigate the mechanism behind the central-peripheral communication. Intracerebroventricular injections of Dex increase food intake and body weight, and decrease 11β-HSD1 mRNA expression in the liver. This reduced local regeneration of active Gcs suggests a partial compensatory mechanism exists in order to prevent the development of hyperglycaemia. This increase in food intake is likely to be a result of the 2.4 fold increase in hypothalamic Npy levels in Dex treated animals (Cusin et al., 2001; Veyrat-Durebex et al., 2012). The reversal of these effects through vagotomy (Cusin et al., 2001) and the response of NPY to central Gc administration suggests that NPY is acting through the parasympathetic nervous system to induce peripheral metabolic effects (Asensio et al., 2004; Billington et al., 1991; Sainsbury et al., 1997).

Conversely, the selective denervation of sympathetic and parasympathetic inputs to the liver indicates that it is in fact sympathetic signalling which is important for brain – liver communication (Yi et al., 2012). These studies indicate that central-peripheral communication is important in the regulation of metabolism; however, the mechanisms controlling these pathways are still unclear. Furthermore, these studies are limited to investigating the acute actions of central Gcs due to the invasive procedure of intracerebroventricular injections. This technique provides limited information for the role of central-peripheral communication in long-term exogenous Gc treatment. To establish the role of central Gc signalling on peripheral metabolism, further investigations are required.
1.6 High-Fat Diet: the western world challenge against the energy-regulatory network

The increasing accessibility of high-fat and high-sugar food across the globe has turned the western world obesity crisis into a global pandemic (NCD Risk Factor Collaboration, 2016). With some people resistant to the development of diet-induced obesity and others affected by epigenetic modifications, it is important to understand the effect of HFD on the energy-regulatory network (Bergen et al., 1999; Plagemann et al., 2009).

High-fat diet studies have shown that the components of HFD directly affect the neuropeptides of the hypothalamus. The results of pair-feeding studies restricting two cohorts of mice (HF or chow fed), to consume the same amount of calories showed that the composition of the HFD alters the neuronal activity of AgRP neurones and increases body weight (Wei et al., 2015). More recent studies have shown that both short-term and long-term HFD consumption induces inflammation within the hypothalamus. How this hypothalamic inflammation regulates the energy-regulatory network remains unclear.

1.6.1 The direct regulation of neuropeptides by high-fat diet

The effect of short-term high-fat diet

Transcriptomic analysis of the energy regulatory neuropeptides has focussed on the effect of short- and long-term HFD on the primary neuropeptides of the ARC: Pomc, Npy and Agrp. In the short-term the effect of HFD on neuropeptide expression remains unclear as variable effects of HFD have been reported in the literature. Forty-eight hours HFD exposure both increased (Mercer et al., 2014) and had no effect on (Ziotopoulou et al., 2000) anorexigenic Pomc expression, dependent on study design. Continued HFD for one week saw no change in Pomc (Lin et al., 2000; Wang et al., 2002; Ziotopoulou et al., 2000), however after 2 weeks, inconsistent expression changes were observed between studies (Heijboer et al., 2005; Mercer et al., 2014; Ziotopoulou et al., 2000).

This inconsistency between studies is also seen in the expression profiles of orexigenic neuropeptides Npy and Agrp. Decreased expression of these appetite-stimulating neuropeptides were identified after 2 days HFD (Ziotopoulou et al., 2000) which was maintained in some studies after 1 week HFD (Wang et al., 2002); however, other studies recorded no change (Lin et al., 2000; Ziotopoulou et al., 2000). Furthermore, HFD for two weeks did not alter the expression of either Npy or Agrp (Heijboer et al., 2005; Ziotopoulou et al., 2000).
The effect of long-term high-fat diet

In order to combat the extra calories a HFD imposes, neuropeptide analysis after long-term (>19 weeks) HFD has shown a decrease in both orexigenic neuropeptides \textit{Agrp} and \textit{Npy} expression reducing food intake (Huang et al., 2003; Lin et al., 2000). Surprisingly, long-term HFD also decreases the expression of the anorexigenic neuropeptide \textit{Pomc} (Huang et al., 2003; Lin et al., 2000).

It is difficult to interpret the effect of HFD on the energy-regulatory network as the expression data is contradictory between studies. Immunohistochemical analysis of POMC neurones indicated that the number of POMC neurones decreased in the ARC after 8 months HFD, however, a similar study conducted by the same group failed to see this effect after 20 weeks HFD (Berkseth et al., 2014; Thaler et al., 2012). This indicates that neuronal changes resulting from HFD occur at a very late stage and are a consequence, not a cause, of diet-induced obesity (Berkseth et al., 2014). Further to changes in absolute number of neurones, morphological modifications to ARC neurones have been observed following 24 weeks HFD. Increased cell volume and membrane capacitance leave neurones in a state of hyperpolarisation. It is therefore harder for these neurones to reach the threshold potential and fire an action potential (Lemus et al., 2015).

The wide range of time-points investigated to establish the effect of HFD on the hypothalamic energy balance circuitry suggests that the network responds in a temporal manner. However, with the development of diet-induced obesity, it is difficult to distinguish the direct effect of diet itself from secondary side-effects of obesity which may also influence the neuronal network. Such differing responses by hypothalamic neuropeptides to a HFD leave the direct effect of HFD on this network unclear.

1.6.2 Which came first the inflammation or obesity?

The increased prevalence of HFDs has led to research investigating the close link between metabolism and inflammation. Many of the pro-inflammatory cytokines expressed in models of diet-induced obesity have been associated with the development of metabolic sequelae such as insulin resistance (Wellen & Hotamisligil, 2005). Further endogenous Gc production by the HPA axis is thought to be increased by HFD (Sobesky et al., 2016). Yet, the anti-inflammatory role of endogenous Gcs does not prevent the development of chronic HFD-induced hypothalamic inflammation (Thaler et al., 2012). Therefore, it is important to understand the anti-inflammatory role of Gcs, how this is affected by the development of hypothalamic inflammation, and the consequences of diet-induced inflammation on the energy-regulatory network.
Inflammation is an adaptive response triggered by stimuli known as pathogen-associated or damage-associated molecular patterns (PAMPs/DAMPs). Briefly, PAMPs/DAMPs bind to toll-like receptors (TLRs) or nod-like receptors (NLRs) to trigger an immune response. Upon activation, inflammatory mediators such as chemokines and cytokines are produced by macrophages. These inflammatory components work in combination via complex pathways to eliminate the PAMPs/DAMPs and initiate a repair mechanism to resolve the acute inflammatory stimulus (Medzhitov, 2008). However, if inflammation is not fully resolved and levels of DAMPs remain high (for example, the elevated levels of saturated free fatty acids resulting from consumption of HFD), then a state of chronic inflammation may develop (Milanski et al., 2009). This chronic inflammation over time, alongside the continued activation of the immune system, ultimately damages the local tissues and impairs homeostatic mechanisms.

The development of peripheral inflammation, particularly in white adipose tissue (WAT), has been thoroughly investigated following long-term HFD. This research established that chronic peripheral inflammation is associated with the development of insulin and leptin resistance. More recently however, inflammation within the hypothalamus has been recorded following both short-term and long-term HFD (Thaler et al., 2012; Zhang et al., 2008). As the hypothalamus is the primary region which controls food intake, it is vital to understand the mechanisms of diet-induced inflammation in order to establish whether within the central nervous system, inflammation is the cause or consequence of chronic obesity and its associated metabolic sequelae.

1.6.2.1 Peripheral inflammation and obesity

The relationship between inflammation and metabolism was first apparent in obese mice when the overexpression of tumour necrosis factor alpha (TNFα), a pro-inflammatory cytokine, was quantified in WAT (Hotamisligil et al., 1993). Shortly after this overexpression was identified in human WAT of overweight men and women (Hotamisligil et al., 1995; Krogh-Madsen et al., 2006). TNFα expression can be used to show the consequences of chronic elevation of pro-inflammatory cytokines in metabolism. The genetic manipulation of TNFα levels clearly indicates its involvement in both insulin homeostasis, as knockdown improves insulin sensitivity, and overexpression induces insulin resistance (reviewed in Hotamisligil, 2006).

Further adipocyte differentiation increases fat pad mass and weight gain, leading to the infiltration and proliferation of macrophages within the tissue (Amano et al., 2014; Weisberg et al., 2003). The increase in macrophages within the tissue limits the
differentiation of adipocytes in order to prevent weight gain (Lumeng et al., 2007; Weisberg et al., 2003). Consequently, cytokine production is increased as the number of macrophages increases, starting the onset of chronic inflammation (Greenberg & Obin, 2006). Research has indicated that multiple pro-inflammatory chemokines and cytokines are produced within adipose tissue during obesity. Therefore, the disruption to metabolism and development of metabolic sequelae associated with obesity cannot be due to an individual inflammatory mediator, but is a consequence of multiple pathways which interact.

1.6.2.2 High-fat diet heats up the hypothalamus: the development of diet-induced hypothalamic inflammation

Unlike peripheral inflammation, which develops as a consequence of obesity, hypothalamic inflammation has been identified after both short and long-term HFD (Thaler et al., 2012). The HFD-induced temporal inflammatory response within the central nervous system suggests that within the hypothalamus, inflammation may disrupt energy regulation, consequently aiding the development of obesity.

Similar to peripheral inflammation, inflammation within the hypothalamus was first studied in rodent models of chronic HFD. Changes in the expression of pro-inflammatory cytokines indicated that 16 weeks HFD induced inflammation within the hypothalamus of rats (De Souza et al., 2005). Differentiation of components of nutrient excess, has allowed mechanisms of HFD-inflammation to be established. Excess glucose and saturated free fatty acids both act as DAMPs, activating TLR4 and driving the downstream inflammatory signalling cascades (Milanski et al., 2009; Zhang et al., 2008).

Diet-induced inflammation is primarily described by changes in the levels of pro-inflammatory cytokine expression. The association between the IKKβ/NFkB signalling pathway and insulin resistance has made this pathway the most investigated in models of diet-induced low-grade chronic hypothalamic inflammation. The binding of DAMPs to TLR4 activates IKKβ, which phosphorylates IkBα, releasing active NFkB. NFkB translocates and drives the production of pro-inflammatory cytokines (figure 1.6, reviewed in Cai, 2009). Numerous studies have identified that this signalling pathway is activated after long-term HFD (De Souza et al., 2005; Milanski et al., 2009; Posey et al., 2009; Zhang et al., 2008). Further, the activation of IKKβ/NFkB induces endoplasmic reticulum (ER) stress which is associated with the development of diet-induced metabolic sequelae (Milanski et al., 2009; Zhang et al., 2008).
Glucocorticoids anti-inflammatory actions:

Free fatty acids bind to TLR4, activating IKKβ. IKKβ phosphorylates IκBα releasing active NFkB (p50+p65) which drives the production of pro-inflammatory cytokines. Glucocorticoids can exert their anti-inflammatory actions by interacting with p65 and repressing the activity of NFkB.

Glucocorticoids pro-inflammatory actions:

Increased glucocorticoid production by excess free fatty acids activates the GR which upregulates the production of TLR2 and NLRP3. Activation of TLR2 and NLRP3, activates the NFkB pathway which leads to the increased expression of pro-inflammatory cytokines.

Figure 1.6: Diet-induced inflammation and the actions of glucocorticoids.

Glucocorticoid anti-inflammatory actions: Free fatty acids bind to TLR4, activating IκkB. IκkB phosphorylates IκBα releasing active NFkB (p50+p65) which drives the production of pro-inflammatory cytokines. Glucocorticoids can exert their anti-inflammatory actions by interacting with p65 and repressing the activity of NFkB.

Glucocorticoid pro-inflammatory actions: Increased glucocorticoid production by excess free fatty acids activates the GR which upregulates the production of TLR2 and NLRP3. Activation of TLR2 and NLRP3, activates the NFkB pathway which leads to the increased expression of pro-inflammatory cytokines. Abbreviations explained on page 10-11.
More recently, studies have identified that the pro-inflammatory cytokine response to a HFD is induced after only 24 hours (Thaler et al., 2012). However, studies investigating the temporal profile of the pro-inflammatory cytokines have shown that this acute increase in pro-inflammatory cytokines is not sustained throughout a chronic HFD regime. Pro-inflammatory cytokine levels resolve between 1–4 weeks before an inflammatory state is returned with chronic HFD. This temporal expression profile has been described as an ‘on-off-on’ response, however, the mechanisms behind this remain unclear (Fonken et al., 2013; Thaler et al., 2012).

Similar to cytokine expression profiles, the resident macrophages of the brain, are activated before weight gain (figure 1.7, Gao et al., 2013). Microglia were found to increase in number and size after only 3 days HFD (Thaler et al., 2012). However a number of studies establishing the long-term effects of HFD have reported varying microglia responses. No changes in total microglia number were identified after 2 or 6 months HFD (Lemus et al., 2015). However, in similar studies, microglia number and size increased with long-term HFD (Valdearcos et al., 2014). This is further complicated as long-term HFD weakens the BBB, as seen by the increase in immunoglobulin G (IgG) after 16 weeks (Yi et al., 2012), and results in an increase in immune cell migration into the brain (Buckman et al., 2014).
High-fat diet increases the leakiness of the blood brain barrier (BBB) allowing components of high-fat diets, e.g. saturated fatty acids, to get into the arcuate nucleus (ARC). The saturated fatty acids act as damage-associated molecular patterns (DAMPs) to increase cytokine expression and activate microglia and astrocytes. Chronic low-grade inflammation reduces energy-regulatory neuronal expression. Pro-opiomelanocortin, POMC; cocaine-amphetamine-related transcript, CART; agouti-related peptide, AgRP; neuropeptide y, NPY; γ-aminobutyric acid, GABA; third ventricle, 3V.

The multi-functional properties of microglia, including modulation of neuronal activity and anti-inflammatory cytokine release, require studies to investigate the effect of diet on activation state alongside absolute number (Valdearcos et al., 2015). The importance of microglia in diet-induced hypothalamic inflammation is clear as the depletion of microglia from the mediobasal hypothalamus (MBH) prevents the production of pro-inflammatory cytokines when treated with fatty acids in vitro (Valdearcos et al., 2014).

Together, the response of pro-inflammatory cytokines and microglia to over nutrition indicates that inflammation induced by HFD is not restricted to the periphery. The inconsistency between studies has resulted in the mechanisms of diet-induced hypothalamic inflammation development remaining poorly understood (Tran et al., 2016).

1.6.2.3 The effect of hypothalamic inflammation on the energy-regulatory network

The presence of inflammation within the hypothalamus is detrimental to the energy-regulatory network and exacerbates the development of obesity and associated metabolic
disorders. Establishing the indirect effect of HFD through diet-induced inflammation on the energy-regulatory network from the direct effect of HFD is difficult; inconsistencies have been reported between studies investigating the effect of diet-induced inflammation on the energy-regulatory network.

The detrimental effect of diet-induced inflammation on energy-regulatory neuropeptides increases over time. After 8 weeks HFD, apoptosis was found to be increased in AgRP and POMC neurones (Moraes et al., 2009). Expression analysis found decreases in Pomc expression after 10 weeks HFD (Nakata et al., 2016). Conversely, total POMC neuronal number was unaltered after 5 months HFD (Berkseth et al., 2014), however, after 8 months HFD immunohistochemistry identified a decrease in the number of POMC cells with the hypothalamus (Thaler et al., 2012). Not all studies found that inflammation preceded changes in neuropeptides, with 8 weeks HFD decreasing Pomc expression prior to the development of inflammation (Souza et al., 2016).

The negative consequences of inflammation on the energy-regulatory network are demonstrated when you provide an artificial noxious stimulus to induce inflammation. The induction of inflammation using an intraperitoneal injection of lipopolysaccharide (LPS) increases the expression of Agrp within the mouse hypothalamus (Scarlett et al., 2008). Furthermore, prevention of inflammation specifically within neurones of the ARC improves glucose tolerance and protects against HFD-induced weight gain (Benzler et al., 2015; Zhang et al., 2008).

While it is clear that neuropeptides are modulated by the presence of inflammation within the hypothalamus, the mechanisms controlling the development of this low-grade chronic diet-induced inflammation have yet to be elucidated.

1.7 Glucocorticoids and diet-induced hypothalamic inflammation

The development of diet-induced hypothalamic inflammation and the disruption that this evokes on the neuronal network requires the development of therapies to prevent damage to the energy-regulatory network. It has long been known that Gcs are the most potent endogenous anti-inflammatory molecules in the body, and are therefore frequently used as anti-inflammatory agents for many inflammatory diseases. More recently it is has been proposed that the timing and duration of exposure to raised Gc levels influences whether these molecules act in an anti-inflammatory or pro-inflammatory manner (Vasconcelos et al., 2016). With various functions dependent on many factors, it is unknown whether endogenous Gcs act in an anti- or pro-inflammatory state in models of diet-induced hypothalamic inflammation (figure 1.6).
1.7.1 The anti-inflammatory and pro-inflammatory actions of glucocorticoids

Gcs are characteristically anti-inflammatory and therefore numerous Gcs have been synthesised as preventative therapies for a range of inflammatory diseases. Together, glucocorticoids and cytokines regulate each other to maintain and prevent chronic increases in either. In the presence of a noxious stimuli, cytokines are produced which cause the stimulation of Gcs. Through the GR repression of AP-1 and NFkB transcriptional activity, Gcs inhibit the production of pro-inflammatory cytokines (reviewed in Cruz-Topete & Cidlowski, 2015). However, recent studies have shown that dependent on the timing, concentration, and duration of Gc exposure, these classically anti-inflammatory steroid hormones can change their function to a pro-inflammatory state (Duque & Munhoz, 2016).

Typically it has been found that elevated corticosterone levels prior to an LPS challenge exacerbate the increase in pro-inflammatory cytokines (Munhoz et al., 2006; Munhoz et al., 2010). In addition, treating mice with corticosterone prior to challenging harvested microglia ex vivo, increases their pro-inflammatory response. This increases in lba1 and Nlrp3 expression (markers of microglia and inflammasome, respectively) following a range of corticosterone concentrations, indicating that the effect is concentration-dependent (Frank et al., 2014).

Very few studies have investigated the effect of Gc exposure on the inflammatory response in vivo, leading some to question whether under certain circumstances, Gcs switch function from an anti-inflammatory to a pro-inflammatory state (Miller, 2007). Studies investigating the pro-inflammatory role of Gcs suggest that in vivo, Gcs activate microglia, initiating a ‘primed’ state and making them more sensitive to an inflammatory challenge (Busillo et al., 2011; Frank et al., 2014). Conversely, other studies have suggested that it is in fact reduced Gc signalling, or Gc resistance, which consequently leads to the increased inflammation (Raison & Miller, 2003).

In models of diet-induced hypothalamic inflammation, it is unclear why the endogenous anti-inflammatory steroid hormones do not protect and prevent the development of inflammation within the energy-regulatory network. However, a further understanding of the varying functions of Gcs in vivo is required to understand whether a shift from anti-inflammatory to pro-inflammatory actions causes the temporal HFD response.

1.8 Regulation of energy expenditure

The continually changing demands on the energy-regulatory network which drive food intake require energy expenditure to adapt and balance “energy in” vs “energy out” to
prevent weight gain. Although there are three types of thermogenesis, as discussed previously, it is the control of adaptive or non-shivering thermogenesis, which is thought to be most important in maintaining balanced energy homeostasis. Mitochondria-rich brown adipose tissue dissipates energy through increasing the uncoupling-protein 1 (UCP-1) activity, consequently generating heat (figure 1.8). Similar to food intake, this mechanism of energy use is altered by changes in temperature, diet, and stress (reviewed in Zhang & Bi, 2015).

**Figure 1.8: Uncoupling protein-1 production of heat**

The sympathetic nervous system (SNS) releases noradrenaline (NA) to activate β3-Adrenergic Receptor (β3-AR) and driving the cyclic adenosine monophosphate (cAMP) → protein kinase A (PKA) pathway. Production of cAMP, activates PKA which initiates lipolysis of triglycerides. Free fatty acids (FFAs) undergo β-oxidation, which produces and causes protons (H+) to be pumped out through the electron transport chain (ETC) of the mitochondrial matrix. As protons move back into the matrix through uncoupling protein-1 (UCP-1) heat is produced.
BAT is predominantly innervated by the sympathetic nervous system, driving thermogenesis through the β-adrenergic signalling cascade. Retrograde tracing has identified that hypothalamic nuclei, including the PVN and ARC, are important in the central nervous system control of thermoregulation (Bamshad et al., 1999; Zhang & Bi, 2015). Furthermore, neurones of the energy-regulatory network involved in the stimulation or inhibition of food intake have dual functionality, also regulating energy expenditure.

1.8.1 Neuronal control of energy expenditure

As the hypothalamus stimulates BAT adaptive thermogenesis, it is not surprising that mediators of food intake are also pivotal in the regulation on energy expenditure. Peripheral and adiposity signals, known to stimulate and depress orexigenic and anorexigenic neurones to regulate food intake also project to BAT to regulate thermogenesis (figure 1.9).

The high infection of retrograde tracers in the PVN from BAT, indicates that this nuclei is key in the integration and projection of signals from the hypothalamus to BAT (Bamshad et al., 1999). The involvement and importance of SF-1 neurones of the VMH, and NPY neurones of the DMH have been thoroughly investigated. Targeted stimulation of these neurones by a number of peripheral and adiposity signals (including glutamate, insulin, and leptin) indicate that, depending on the stimulant, these neurones activate or depress BAT thermogenesis (Bingham et al., 2008; Kim et al., 2011; reviewed in Zhang & Bi, 2015).

Until recently, investigations into the primary energy-regulatory neuropeptides of the ARC have concentrated on their role in the regulation of food intake. However, disruption to melanocortin signalling (MC4R KO) prevents leptin induced increases in Ucp-1 mRNA expression, indicating their importance in the regulation of energy expenditure (Ste Marie et al., 2000). Overexpression of transcription factors within, and genetic disruption to AgRP/NPY/GABA neurones, indicates that these neurones inhibit BAT function to drive energy availability (Blouet et al., 2012; Tong et al., 2008). Furthermore, a rapid decrease in energy expenditure is induced with the acute activation of AgRP neurones by DREADDs technology (Krashes et al., 2011). On the other hand, leptin stimulation of POMC neurones promotes browning, increasing thermogenic capacity and preventing diet-induced obesity (Dodd et al., 2015).
Figure 1.9: The hypothalamic innervation of brown adipose tissue adaptive thermogenesis.

Neurones within hypothalamic nuclei including the arcuate nucleus (ARC) dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), and ventromedial hypothalamus (VMH) are activated by peripheral signals to regulate brown adipose tissue (BAT) adaptive thermogenesis. The hypothalamic nuclei signal via hindbrain regions including the dorsal vagal complex (DVC) and raphe pallidus (RPa) which project via the sympathetic nervous system to BAT. Agouti-related protein, AgRP; Pro-opiomelanocortin, POMC; glucocorticoids, Gcs.

In humans, the functional significance of BAT remains relatively unknown. Although active in children, BAT transforms into WAT as we age. Thermal imaging has identified that a limited proportion of BAT remains, with important functions in the control of cold-induced adaptive thermogenesis. However, the activation of adaptive thermogenesis in man by dietary factors remains unknown (Sacks & Symonds, 2013). On the other hand, although limited BAT remains in the adult human, WAT can be stimulated to promote ‘browning’ whereby white adipocytes take on characteristics of brown adipocytes. This conversion increases the capacity of adaptive thermogenesis. Recent studies have identified that O-GLcNAc (an enzyme which modifies cytoplasmic and nuclear proteins) within AgRP neurones suppresses the browning of WAT, thus conserving energy (Ruan et al., 2014).
The high prevalence of diet-induced obesity indicates that adaptive thermogenesis is not strong enough to counteract the effects of chronic HFD. Therefore, continued research into the hypothalamic mechanisms controlling energy expenditure is required in order to establish and develop therapies to aid the re-balancing of energy homeostasis.

### 1.8.2 Glucocorticoids and energy expenditure

As we have discussed, Gcs regulate hypothalamic energy-regulatory neuropeptides which in turn regulate energy expenditure. However the effect of chronically elevated hypothalamic Gcs on energy expenditure has not yet been fully characterised. In models of Gc excess, one week Gc treatment decreased *Ucp-1* mRNA expression (Poggioli et al., 2013; Van Den Beukel et al., 2015) and accelerated fatty acid oxidation. Further, in combination with a HFD, Dex decreased energy expenditure which was associated with increased weight gain (Poggioli et al., 2013). However, these studies do not provide the tools to determine whether elevated Gc levels directly stimulate BAT or indirectly regulate energy expenditure through signalling on energy-regulatory neuropeptides.

In order to establish the role of central Gc signalling, Dex was acutely infused into the hypothalamus. This direct infusion of Gcs increased *Npy* in the hypothalamus and decreased *Ucp-1* expression in BAT (Zakrzewska et al., 1999). Furthermore, disruption to Gc signalling on the AgRP/NPY/GABA neurones increased UCP-1 protein expression in female mice on HFD, indicating increased adaptive thermogenesis. Indirect calorimetry in this study identified that knocking out GR on AgRP neurones increased oxygen consumption (VO₂) which suggests an increase in energy expenditure specifically in female mice challenged with HFD (Shibata et al., 2016).

These limited studies suggest the importance of Gc signalling in the hypothalamus, however, to our knowledge, no study has challenged these genetic knock-out models with Gcs. Therefore, to truly establish the effect of chronically elevated hypothalamic Gc levels on energy expenditure these studies are needed.

### 1.9 Summary

Energy homeostasis is controlled in the hypothalamus by a fragile network of neurones, which are highly influenced by a number of external environmental factors. One of these, the easily accessible western world high-fat, high-sugar diet, has driven the world into an obesity pandemic. In trying to understand the consequences of HFDs on the neuronal
network controlling food intake it has become evident that alongside the direct effects of HFDs, diet-induced hypothalamic inflammation develops. This inflammation was hypothesised to indirectly influence the maintenance of energy homeostasis. Models of diet-induced inflammation indicate that within the hypothalamus, inflammation develops with a temporal profile. Furthermore, it is unclear why endogenous anti-inflammatory mechanisms, such as Gcs, do not resolve and prevent the development of chronic low-grade hypothalamic inflammation.

The anti-inflammatory functions of Gcs have driven their use in the clinic, for the treatment of inflammatory diseases such as rheumatoid arthritis. However, these steroid hormones are not solely anti-inflammatory; they also regulate many metabolic pathways. Consequently, long-term exogenous treatment leads to the development of obesity and a number of metabolic sequelae. A recent study has identified that 48% of rheumatoid arthritis patients treated with oral Gcs consequently develop diabetes (Movahedi et al., 2016). With the NHS spending £10 billion a year on the treatment of diabetes (Diabetes, UK), it is important that we understand the mechanisms by which Gcs induce these metabolic disorders.

Patients treated with Gcs are hyperphagic and shift their food preference towards high-fat foods. The energy-regulatory neuropeptides within the hypothalamus control the balance of food intake and energy expenditure. Although the regulatory role of Gcs in carbohydrate, lipid, and protein metabolism highlights Gc metabolic actions within the periphery, the central role of Gcs within the hypothalamus remains unclear. Acute direct Gc stimulation of the hypothalamus modulates hepatic insulin responsiveness and increases body weight gain (Veyrat-Durebex et al., 2012; Yi et al., 2012). However, few studies have used a translational chronic non-invasive Gc administration model to establish the role of hypothalamic Gc signalling in the development of Gc-induced metabolic syndrome. Therefore, the role of hypothalamic Gc-signalling remains unknown in the development of Gc-induced metabolic sequelae. Further understanding of the contribution of central Gc signalling would allow for the development of targeted therapies or ‘drug holidays’ to prevent the development of Gc-induced metabolic sequelae.

1.10 Aims and Objectives

The prevalence of long-term Gc treatments has risen 34% in the past 20 years (Fardet et al., 2011). Consequently, the number of individuals suffering from Gc-induced metabolic sequelae, such as diet-induced obesity, is at an all-time high. As the number of patients on long-term treatments continues to rise, the cost of treating the inevitable co-morbidities
will continue to put a huge strain on the NHS. To prevent the continually increasing costs associated with treatment of the metabolic disorders, an understanding of the mechanisms driving the development of these metabolic sequelae is vital. Therefore the overall aims of this thesis are to establish whether in a model of Gc excess, hypothalamic Gc levels are elevated, and then to understand the contribution of hypothalamic Gcs to the development of Gc-induced metabolic syndrome.

The specific objectives for each study are described in the corresponding chapter. However, the broad objectives for each chapter are described below;

Chapter 3 focusses on the development of a model of diet-induced obesity, to establish the effects of hypothalamic inflammation on the energy-regulatory network of the hypothalamus.

Chapter 4 characterises the phenotypic effect of chronic exogenous corticosterone treatment in a murine model, and investigates the concentration of corticosterone within the hypothalamus following short and long-term corticosterone treatment.

Chapter 5 uses Cre-lox technology to explore the role of hypothalamic Gcs in the development of corticosterone-induced metabolic syndrome. This technology is also used to establish the effect of excess hypothalamic Gcs in age-induced obesity.
Chapter 2

Materials and Methods
Unless otherwise stated materials were supplied by Sigma-Aldrich or Fisher Scientific

Specific methodology for each results chapter is described within that chapter, general methods are described below.

2.1 In Vivo Methods

2.1.1 Animal Husbandry

Mice and rats were housed under a constant 12 hour light – 12 hour dark cycle (lights on 7am, lights off 7pm), with an ambient temperature of 23±1°C and a humidity of approximately 40%. Food and water were available *ad libitum* through all experiments. Animals were allowed to acclimatise to housing conditions 1 week prior to baseline. Baseline measurements were taken 1-2 weeks before treatment dependent on the study.

All experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act, 1986 and approved by The University of Manchester Ethical Review Panel.

2.1.2 Tail-prick micro-sampling

Micro-samples of tail vein blood were taken from the tip of the tail of the mouse using a 23G needle (Terump, Agani needle). Micro-capillary tubes (Drummond) were used to collect blood for corticosterone (5µl in 20µl phosphate buffer saline, PBS) and insulin (20µl). Blood was centrifuged at 16,100 x g for 5 minutes at 4°C and the plasma stored at -80°C.

*Glucose measurements*

Fed blood glucose measurements were measured immediately prior to death (approximately 10am) in fresh tail prick blood samples at the end of the treatment period. The glucose levels (mMol/L) were recorded using a glucometer (Accu-Chek, Roche, West Sussex, UK).

2.2 Molecular Methods

2.2.1 RNA extraction

*Mouse and rat hypothalami / Cell pellets*

Hypothalami were homogenised in fast prep tubes (Lysing Matrix D, MP Biomedicals) and bone marrow derived macrophages (BMDM) cells were homogenised using a
Qiashredder, both in RLT buffer supplemented with β-Mercaptoethanol (1:10) (Qiagen). RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. All samples underwent an RNase-free DNase step to remove any genomic DNA contamination (Qiagen). RNA was eluted in 30μl RNA-free water and the elute was re-eluted through the RNeasy spin column to increase RNA yield.

The total RNA concentration was measured and integrity was verified by the 260/280 ratio (Nanodrop, ND100; Nano-Drop Technologies). RNA was diluted with RNA-free water to 5ng/μl for mRNA analysis.

**Micro-punches and laser capture micro-dissections**

Micro-dissected samples from MBH micro-punches were needle homogenised in RLT-plus buffer supplemented in β-Mercaptoethanol (1:10) (Qiagen). Laser capture micro-dissections were homogenised in RLT-plus buffer supplemented with β-Mercaptoethanol by vortexing for 30 seconds. RNA was extracted from both micro-punches and laser-dissected using the Qiagen Micro plus kit according to the manufacturer’s instructions. RNA was eluted in 20μl RNA-free water and the elute was re-eluted through the RNeasy spin column to increase RNA yield.

Quantification of RNA was performed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) as described within individual chapters.

**2.2.3 Agarose gel electrophoresis**

Polymerase chain reaction (PCR) products were run on a 2% agarose gel and visualised using a Gel doc (Biorad). The agarose gel was made in 1xTAE buffer and 10μl safe stain (Safeview) was added to visualise the samples. Samples were run alongside a 1kb ladder (Hyperladder IV, Bioline). The gel was run in 1xTAE buffer for 45 minutes at 90V unless otherwise stated.

**2.2.4 Microscopy**

**Brightfield and fluorescence images taken on slidescanner**

Images were acquired using a 20x/0.80 Plan Apo objective using the 3D Histech Pannoramic 250 Flash II slide scanner. Pannaromic Viewer software (3D Histech) was
used to analyse and take images offline. Images were then processed and analysed using Fiji ImageJ (FIJI).

2.2.5 Buffers

**Citrate buffer pH6.0**
9ml stock solution A (0.1M Citric acid) is added to 41ml stock solution B (0.1M Sodium citrate) and made up to 500ml with distilled water. A pH of 6 must be ensured.

**Phosphate-Buffered Saline (PBS)**
100 PBS tablets (Dulbecco A) diluted in 1l distilled water to give a 10x solution. This was diluted 1:10 in distilled water to give a 1x solution.

**50x TAE Buffer**
Tris base 242g, Glacial acetic acid 57.1ml, 0.5M Na$_2$EDTA, pH8.0 (186.12g in 850ml, pH to 8.0 with 10M NaOH, and made up to final volume of 1000ml).

Made up to 1000ml final volume with double distilled water and diluted to 1x with double distilled water for running agarose gels.

2.3 Statistical analyses

All data are presented as mean+/sem. Statistical analysis was performed using Graphpad Prism software (Prism 7, Graphpad Software Inc. La Jolla CA). Housekeeper optimisation was conducted using EST Database software.
Chapter 3

The effects of high-fat diet on the energy-regulatory network of the hypothalamus
3.1 Introduction

The evolution of the western diet culture is one factor that has driven the development of the current obesity pandemic. It has been proposed that the consumption of a high-fat diet (HFD) increases both the circulating and hypothalamic free fatty-acid levels which directly regulate the energy-regulatory network of the hypothalamus (Tran et al., 2016). The hypothalamus is formed of a carefully balanced network of energy-regulating neurones and supporting glial cells whose stimulation either increases or decreases food intake. A number of studies have investigated the effect of HFD on this network, however, the impact of a HFD on the primary energy-regulatory neurones is highly dependent on the paradigm investigated (Bergen et al., 1999; Gout et al., 2008; Lin et al., 2000).

Emerging evidence has suggested that alongside the direct effects of HFD on the energy-regulatory neuropeptides, consumption of a HFD can induce inflammation within the hypothalamus (reviewed in Valdearcos et al., 2015). This hypothalamic inflammation increases the complexity of how energy balance is regulated. In a number of rodent models, both short and long-term HFD induces hypothalamic inflammation (Fonken et al., 2013; Milanski et al., 2009; Thaler et al., 2012; Zhang et al., 2008). Interestingly, hypothalamic inflammation monitored over a series of time-points in a single model of HFD has indicated a temporal response (Thaler et al., 2012). However, the mechanisms controlling this on-off-on response within the hypothalamus are unknown.

Administration of a HFD has been associated with altering the endogenous production of glucocorticoids (Gc) by the hypothalamic-pituitary-adrenal (HPA) axis (Auvinen et al., 2012; McNeilly et al., 2015). Gcs classically suppress the innate and adaptive immune responses through multiple mechanisms, repressing a plethora of pro-inflammatory genes encoding a wide number of cytokines and chemokines (Cruz-Topete & Cidlowski, 2015). Chronic exposure to the anti-inflammatory Gc hormones can alter their actions and shift the response to a pro-inflammatory state (Frank et al., 2014). It is not known whether this shift in Gcs inflammatory actions contributes to the temporal on-off-on hypothalamic inflammatory response to a HFD.

3.1.1 Aims and Objectives

This chapter aimed to establish a murine model of HFD-induced hypothalamic inflammation to determine the inflammatory effects of Gcs within the hypothalamus. Long-term HFD has been shown to chronically activate the HPA axis, elevating the concentrations of circulating anti-inflammatory Gcs (Kohsaka et al., 2007; Tannenbaum et
al., 1997). However, the timing, duration, and concentration of Gc exposure is thought to alter these classically anti-inflammatory steroid hormones to a pro-inflammatory state, consequently, exacerbating inflammation. The interaction between HFD-induced hypothalamic inflammation and chronic exposure to elevated Gcs is unknown.

Therefore, this chapter initially aimed to establish a model of high-fat feeding (HFF) to determine the effect of HFD on neuropeptides within the hypothalamus and elucidate the temporal development of diet-induced hypothalamic inflammation.

1. To investigate the effect of high-fat diet on energy-regulatory neuropeptides

Models of HFD administration have produced variable effects on hypothalamic energy-regulatory neuropeptide expression. Unsurprisingly, transcriptomic analysis of the orexigenic neuropeptides *Agrp* and *Npy* across a number of studies display similar expression profiles. Expression of both *Agrp* and *Npy* decrease after 2 days HFD (Ziotopoulou et al., 2000), however, variable results have been recorded with long-term HFD (*Agrp* no change Gout et al., 2008; *Agrp* decrease Huang et al., 2003; Wang et al., 2002) (*Npy*, no change Bergen et al., 1999; *Npy* decrease, Lin et al., 2000; Wang et al., 2002). This variability in mRNA expression has also been recorded for the anorexigenic neuropeptide *Pomc* after 2 days HFD (increase Mercer et al., 2014; decrease Ziotopoulou et al., 2000). Similarly, in studying the long-term effect of HFD on *Pomc*, both decreases (Huang et al., 2003; Lin et al., 2000) and no change (Bergen et al., 1999; Gout et al., 2008) in expression have been recorded.

Only a small number of studies have established the effect of HFD on both anorexigenic and orexigenic neuropeptides across time in one study. Therefore, this chapter aims to examine the effect of HFD over a series of time points on both anorexigenic and orexigenic hypothalamic neuropeptides.

2. To develop a model of high-fat diet induced hypothalamic inflammation

High-fat feeding increases circulating and hypothalamic free fatty acids, which initiate the innate immune response (Milanski et al., 2009). Cytokine expression profiles over a series of time points have shown that hypothalamic inflammation induced by a HFD has a temporal response (Thaler et al., 2012). Furthermore, the supporting glial cells, astrocytes
and microglia, are activated with chronic HFF. How this diet-induced activation of the immune response alters the energy-regulatory network is unknown.

This study aims to develop and characterise a mouse model of HFD-induced hypothalamic inflammation.

### 3.2 Methods

#### 3.2.1 High-fat diet studies

**Short-term high-fat diet study in mice**

C57BL/6J male mice (10 weeks of age, Harlan laboratories) were group housed in cohorts of 5 or 6. Baseline measurements were taken for 1 week prior to start of the study. Mice were assigned either a high-fat diet (60% energy from fat diet, D12492, Research Diets) or a control diet (10% energy from fat – sucrose matched diet, D12450J, Research diets) based on their baseline body weights. Mice were then placed on the test diet and body weights were measured after 24 hours or 1 week treatment at 10am. At the end of the study mice were culled by rising CO₂ at approximately 10am and tissues were dissected, snap frozen, and stored at -80°C for future analysis. The whole brain was removed and the whole hypothalamus was immediately removed from the ventral side of the brain (~20mg, section 3.2.4).

**Long-term high-fat diet study in mice**

Male C57BL/6J mice (10 weeks of age, Harlan laboratories) were group housed in cohorts of 2-6. Baseline body weight was measured for 1 week prior to the start of the study. Mice were assigned either a high-fat diet (60% energy from fat diet, D12492, Research Diets) or a control diet (10% energy from fat – sucrose matched diet, D12450J, Research diets) for 1 week, 2 weeks, 3 weeks, 4 weeks or 20 weeks (n=10 / group for each time point) based on baseline measurements. Body weights were measured weekly at 10am. At the end of the study mice were culled by rising CO₂ at approximately 10am, and tissues were dissected, snap frozen and stored at -80°C for future analysis. The whole brain was removed and either snap frozen for immunohistochemistry (section 3.2.6) or the whole hypothalamus was immediately removed from the ventral side of the brain (~20mg, section 3.2.4).
**Short-term high-fat diet study in rats.**

Sprague Dawley male rats (515-610g, Charles River) were singly housed. Baseline measurements were taken 1 week prior to start of the study. Rats were assigned either a high-fat diet (60% energy from fat diet, D12492, Research Diets) or a control diet (10% energy from fat – sucrose matched diet, D12450J, Research diets) for 3 days (n=9/group) based on baseline measurements. Body weights were measured at the start and end of the study at 10am. At the end of the study rats were culled by rising CO\textsubscript{2} at approximately 10am and the hypothalamus was dissected from the brain (section 3.2.4) and frozen for mRNA expression analysis.

**3.2.2 Cardiac perfusion**

Whole brains were taken from mice following cardiac perfusion. Mice were overdosed with an intraperitoneal (i.p.) injection of urethane (w/v 30%). A cardiac perfusion was performed with 0.9% NaCl followed by 4% paraformaldehyde (PFA in 0.1M phosphate buffer). Whole brains were removed and post-fixed in 4% (w/v) PFA for 24 hours before paraffin embedding for Iba1 immunohistochemistry.

**3.2.3 Intraperitoneal lipopolysaccharide injection**

Male C57BL/6J mice (10 weeks of age, Harlan laboratories) received either an i.p. injection of lipopolysaccharide (LPS) at 10µg/mouse (E.coli, serotype 0127:B8) or vehicle (sterile saline) at 5ml/kg (n=6/group) at 10am. After 2 hours the mice were culled by rising CO\textsubscript{2} and tissues were snap frozen for mRNA expression analysis. The whole brain was removed and the whole hypothalamus was immediately removed from the ventral side of the brain (∼20mg, section 3.2.4).

**3.2.4 Dissection of the hypothalamus**

To isolate the hypothalamus for mRNA expression analysis from mouse and rat brains, micro-dissection scissors were used to cut immediately caudal to the optic chiasm and dorsally by the mammillothalamic tract. The dissection was limited laterally by the hypothalamic sulci. The entire hypothalamus was removed including the arcuate, ventromedial, dorsomedial and paraventricular nuclei. Whole hypothalami removed from the rat brain were dissected in two down the sagittal plane. All tissues were stored in ‘RNA-later’ (Ambion) at -80°C.
To ensure the entire medial region of the brain was hydrated during processing for immunohistochemistry, rostral and caudal regions of the whole brain were dissected away using a flat edged dissection blade.

3.2.5 Lipopolysaccharide stimulation of bone marrow derived macrophages

A bone marrow derived macrophage (BMDM) cell line (donated by Dr. Brough, University of Manchester) was cultured in Dulbecco modified medium supplemented with 10% Foetal Bovine Serum (FBS, SeraLab, West Sussex, UK), 1% Penicillin and 1% L-glutamine (Gibco). The adherent cell line was cultured in T75 filter capped vented at 37°C in 5% CO₂. Cells were split, 1:10, every 5 days or when confluent. Confluent BMDM cells were plated at 500,000 cells/ml in a 6 well plate and incubated at 37°C. After 24 hours BMDM cells were stimulated with 1 µg/ml LPS (E.coli 026:B6) for 4 hours. Following stimulation, cells were removed and centrifuged at 380 xg for 5 minutes, a cell pellet was collected and RNA extracted (section 2.2.1) to provide a positive control to make the standard curves for qRT-PCR.

3.2.6 One step Quantitative Reverse Transcription Polymerase Chain Reaction

RNA was extracted from whole mouse hypothalami, half rat hypothalami or BMDM cells according to general methods (section 2.2.1). Gene expression analysis was performed by one step real-time (reverse transcription) quantitative-PCR (qRT-PCR) using ABI 7900 Sequence Detection System instrument and software (Applied Biosystems). Specific forward and reverse primers for each gene (3pmol/µl of each) were used to amplify RNA (10ng) in duplicate in 10µl volumes on a 384 well plate (table 3.1). Standard cycling conditions were used with Power SYBR Green RNA-to-CT™ 1 step kit (Applied Biosystems) and AgRP expression was performed using TaqMan RNA-to-CT™ 1 step kit (Applied Biosystems) to quantify relative gene expression (table 3.2, 3.3). Gene expression levels were normalised to a housekeeping gene using standard curve analysis. Standards were prepared from pooled RNA which was serially diluted 1:2 in DEPC-treated water, to create a 7 point standard curve ranging from 40ng/µl to 0.625ng/µl. RNA samples were diluted 1:40 in DEPC-treated water. RT negative samples were run alongside samples in singlicate as a control.

Three housekeeping genes were tested, and based on analysis gene expression levels were normalised to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (Hprt) using standard curve analysis.
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<tr>
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<tr>
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<td><strong>Glucocorticoid marker genes</strong></td>
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<tr>
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<td>Nr3c1</td>
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<tr>
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<tr>
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<td>TACTGGCCACATCAACAGGA</td>
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<td>Pomp</td>
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<td>CCTCACTGGGCCCCCTTGTG</td>
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<tr>
<td><strong>Glucocorticoid marker genes</strong></td>
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<td></td>
<td></td>
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<tr>
<td>GR</td>
<td>Nr3c1</td>
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<td><strong>Inflammatory genes</strong></td>
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<td>Il-6</td>
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<td>CAGTGCACTACGCTGCTTTCCATA</td>
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</tbody>
</table>

**Table 3.1:** Mouse and rat primer sequences for genes quantified by qRT-PCR. For definitions see abbreviations on page 9-10.
3.2.7 Primer design and validation

Primer3 software was used to design specific primer pairs, approximately 20 base pairs in length, for each gene across exon-exon boundaries to prevent the amplification of genomic DNA (table 3.1). Primers were designed to amplify a single product between 80–
base pairs in length with a 40-60% GC content and a Tm of approximately 60°C. Specificity of primers was ensured through the visualisation of PCR products on a 2% agarose gel (section 2.2.3) and if a single product was present the product was purified using a QiaQuick PCR purification kit (Qiagen), sequenced and compared to the original gene sequence.

### 3.2.8 Processing brain tissue for paraffin sectioning

Rostral and caudal brain regions were dissected away to ensure the whole hypothalamic region was hydrated during processing (section 3.2.4). Tissue was processed using a Leica TP1050 processor. Processing involved incubation in increasing concentrations of ethanol from 70-100%, incubation in xylene and finally in hot wax. Once processed, the hypothalamic brain region was embedded in wax, rostral side down, to ensure microtome sectioning in the same plane.

### 3.2.9 Iba1 immunohistochemistry

Brains were processed as described in section 3.2.7. Sections (10µm) were cut on a microtome (Leica RM2255) and alternate sections were mounted on superfrost plus slides (Thermo scientific) throughout the hypothalamic region and incubated at 30°C overnight before being stored at room temperature.

All slides were ‘spotted’ (slides were placed on a hot surface so that the wax surrounding the section melted, 60°C, and then transferred to a cool surface) before xylene dewaxing. All remaining paraffin wax residues were removed from slides in xylene and sections were hydrated through a series of decreasing ethanol concentrations (100%-70%) washes, for 2 minutes each, finishing in water. An antigen retrieval step was carried out by incubating slides in citrate buffer at 97.5°C for 30 minutes. They were then allowed to cool in the citrate buffer for a further 20 minutes. Sections were individually isolated with a Barrier pen (Ambion), and peroxidase blocked for 20 minutes (Dako). After 20 minutes the peroxidase block was washed with 1xPBS (3 x 5 minutes washes) and incubated for 60 minutes in 10% Seablock (Calbiochem) (diluted in 1xPBS). Primary antibody Iba1 (019-19741, Wako) was diluted in 10% Seablock (1:1000) and incubated for 1 hour at room temperature. Primary antibody was washed off in 1xPBS, 3 x 5 minute washes, after which sections were incubated in horseradish peroxidase (HRP) labelled secondary anti-rabbit antibody (1:100, diluted in 1 x PBS) (Dako) for 30 minutes at room temperature. Slides were washed, 3 x 5 minutes in 1xPBS, and incubated in chromagen substrate (1ml...
Diaminobenzidine, DAB, substrate in 20µl chromagen, Dako) for 10 minutes. Sections were washed in 1xPBS before rehydrated in increasing concentrations of ethanol (70-100%). Slides were washed in xylene before mounted with DPX (Sigma). Slides were left in the hotbox at 37°C overnight. Images were acquired using a 20x/0.80 Plan Apo objective using the 3D Histech Pannoramic 250 Flash II slide scanner. Pannoramic Viewer software used to analyse and take images offline.

3.2.10 Blind semi-quantitative lba1 analysis

Activated microglia were quantified in the rostral, central and caudal regions of the ARC (table 3.4) following 1 week, 4 weeks and 20 weeks HFD in mice. Image files were renamed to allow double-blind analysis.

<table>
<thead>
<tr>
<th>Region</th>
<th>Bregma Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rostral</td>
<td>-1.22/-1.58</td>
</tr>
<tr>
<td>Central</td>
<td>-1.58/-1.94</td>
</tr>
<tr>
<td>Caudal</td>
<td>-1.94/-2.18</td>
</tr>
</tbody>
</table>

Table 3.4: Regions for quantification of microglia.

Image analysis of microglia morphology was conducted across three sections per region (n=5 / group). Each section was given a score (0-3) on the level of microglia activation within the arcuate nucleus (table 3.5). Images were assessed by two individuals on two separate occasions.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
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<td>++</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
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</tr>
<tr>
<td>+/-</td>
<td>N/A</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.5: Microglia activation score.
3.3 Results

3.3.1 The effect of short- and long-term high-fat diet on body weight

To investigate the effect of HFD-induced hypothalamic inflammation, we set up a model of diet-induced obesity. Mice were fed either 60% energy from fat diet (HFD) or 10% energy from fat diet (control) for 24 hours, 1 week (figure 3.1) or 20 weeks (figure 3.2). In both cohorts of mice body weight was increased after 1 week (figure 3.1B, figure 3.2A). Increased body weight remained elevated throughout the 20 week treatment period (figure 3.2A). After twenty weeks HFD, epididymal fat mass was unaltered (figure 3.2B).

Figure 3.1: Short-term high-fat diet increases body weight in mice.
Body weights were monitored over (A) 24 hours (B) 1 week high-fat diet (HFD). High-fat diet, HFD, 60% energy from fat diet; Control, 10% energy from fat diet. (A, B) Two way ANOVA, Sidak’s multiple comparisons test ** p<0.01 vs 0 days HFD, n=6/group. (See table 8.1 of appendix for F values).
Figure 3.2: Long-term high-fat diet increases body weight in mice.

Body weights were monitored over (A) 20 weeks high-fat diet (HFD) (B) epididymal fat mass was measured after 20 weeks. High-fat diet, HFD, 60% energy from fat diet; Control, 10% energy from fat diet.

(A) Two way ANOVA, Sidak’s multiple comparisons test *** p<0.001 vs control, n=10/group (B) Unpaired t-test, p>0.05, n=10/group. (See table 8.2 of appendix for F values).

3.3.2 The effect of high-fat diet on energy-regulatory neuropeptides of the hypothalamus.

The expression of hypothalamic orexigenic and anorexigenic neuropeptides was quantified in the hypothalamus over a series of time points, to determine the effect of HFD on the energy-regulatory network in mice. Expression of orexigenic neuropeptides, Agrp and Npy, was unaltered after 24 hours HFD (figure 3.3A). A trend towards an increase in expression of the anorexigenic neuropeptide Pomc was quantified after 24 hours HFD (figure 3.3A), with expression increasing after 1 week HFD. After 1 week HFD, mRNA expression of Npy and Agrp indicated a trend towards an increase (figure 3.3B).
**Figure 3.3: Short-term high-fat diet increases hypothalamic pro-opiomelanocortin expression in mice.**

Energy-regulatory neuropeptide, neuropeptide y (Npy), agouti-related peptide (Agrp) and pro-opiomelanocortin (Pomc) mRNA expression was quantified in whole hypothalami of mice fed either 60% energy from fat diet (HFD) or 10% energy from fat diet (Control) for (A) 24 hours or (B) 1 week. Mann Whitney t-test, ** p<0.01 vs. control, n=6/group.

Orexigenic neuropeptides Agrp and Npy mRNA expression decreased after 2 weeks HFD. A trend towards a decrease in Npy expression compared with control fed mice continued during the treatment period, with Agrp mRNA expression maintaining decreased expression after 4 and 20 weeks HFD. Unlike the first cohort of mice, anorexigenic Pomc expression was not decreased after 1 week HFD (figure 3.4C), but similar to orexigenic neuropeptide expression, Pomc expression was also decreased after 20 weeks HFD (figure 3.4C). Differences in gene expression were analysed over time within the separate
experimental groups (control fed, 60% energy from fat diet). In the group fed control diet, an increase in hypothalamic \textit{Agrp} was quantified after 2 weeks (figure 3.4A) and \textit{Pomc} expression increased after 20 weeks compared with 1 week feeding (figure 3.4C).

\begin{figure}[h]
\centering
\begin{subfigure}{0.45\textwidth}
\centering
\includegraphics[width=\textwidth]{Agrp.png}
\caption{\textit{Agrp} expression}\label{fig:Agrp}
\end{subfigure}
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\begin{subfigure}{0.45\textwidth}
\centering
\includegraphics[width=\textwidth]{Npy.png}
\caption{\textit{Npy} expression}\label{fig:Npy}
\end{subfigure}
\hspace{1cm}
\begin{subfigure}{0.45\textwidth}
\centering
\includegraphics[width=\textwidth]{Pomc.png}
\caption{\textit{Pomc} expression}\label{fig:Pomc}
\end{subfigure}
\caption{Hypothalamic neuropeptide expression decreases with a high-fat diet.}
\end{figure}

Mice were fed either 60\% energy from fat diet (HFD) or 10\% energy from fat diet (Control) for 1 week, 2 weeks, 3 weeks, 4 weeks or 20 weeks. Energy regulatory neuropeptide mRNA expression; (A) agouti-related peptide (Agrp), (B) neuropeptide y (Npy) and (C) pro-opiomelanocortin (Pomo) and was quantified in the whole hypothalamus at each time point.

Two way ANOVA, Tukey’s multiple comparisons test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control, # $p<0.05$, ## $p<0.01$, ### $p<0.001$ vs week 1, n=5-10. (See table 8.3 of appendix for $F$ values).
3.3.3 Lipopolysaccharide induced hypothalamic inflammation

To establish a method to assess hypothalamic inflammation, inflammatory marker mRNA expression was quantified in the hypothalamus of lipopolysaccharide (LPS) injected and saline injected mice. Two hours after LPS stimulation there was increased mRNA expression of the pro-inflammatory cytokines *Il-1β*, *Il-6* and *Nfkbia*, however *Ikbkb* mRNA expression did not alter. *Il-1β*, *Il-6* and *Nfkbia* were not detectable in the hypothalamus of saline-injected mice (figure 3.5A). Expression of *Ail1* (markers of microglia, figure 3.5B) *Ddit3* and *Dnajb9* (markers of ER stress, figure 3.5C) did not alter in the hypothalamus following LPS stimulation.

![Graphs showing mRNA expression](image)

**Figure 3.5: Lipopolysaccharide induces markers of inflammation within the hypothalamus.**

Mice were injected intraperitoneally with either lipopolysaccharide (LPS, 10µg/mouse) or saline (5ml/kg). After 2 hours stimulation mRNA expression of (A) pro-inflammatory markers, interleukin-1 beta (*Il-1β*), interleukin-6 (*Il-6*), nuclear factor of kappa light chain gene enhancer in B cells inhibitor alpha (*NfkBia*), inhibitor of nuclear factor kappa B kinase subunit beta (*Ikbkb*), (B) microglia marker, allograft inflammatory factor 1 (*Ail1*), and (C) ER stress markers, DNA-damage inducible transcript 3 (*Ddit3*) and DnaJ heat shock protein family member B9 (*Dnajb9*) were quantified in the hypothalamus. Not detectable; ND.

Mann Whitney t-test, *p<0.05 **p<0.01 vs. saline, n=6/group.
3.3.4 High-fat diet induced hypothalamic inflammation

Transcriptomic analysis was performed using whole hypothalamic RNA following 24 hours, 1-4 weeks and 20 weeks of HFD. Inflammatory marker expression (Il-1β, Il-6, Tnfa, Nfκbia) was not detectable in the hypothalamus at any time point (data not shown). Expression of the ER stress markers, endoplasmic reticulum BiP co-factor, Dnajb9, and C/EBP homologous protein, Ddit3, was unaltered after 24 hours and 1 week HFD (figure 3.6A, B). After 20 weeks HFD, the expression of Dnajb9 increased, whereas Ddit3 expression did not alter (figure 3.6C, D). Expression of Ilkbkb and Aif1 (marker of microglia) did not alter after HFD at any time-point (figure 3.6E, F).
Figure 3.6: Long-term high-fat diet does not induce hypothalamic inflammation.

(A) The hypothalamic mRNA expression of DNA-damage inducible transcript 3 (Ddit3) and DnaJ heat shock protein family member B9 (Dnajb9) was quantified after 24 hours or 1 week high-fat diet (HFD). (B) Ddit3 and (C) Dnajb9 mRNA expression was quantified in the whole hypothalamus after 2, 3, 4 and 20 weeks HFD. (E) Inhibitor of kappa light polypeptide gene enhancer in β cells (Ikbkb) and (F) Allograft inflammatory factor 1 (Aif1) expression was quantified after 1, 2, 3, 4 and 20 weeks HFD. HFD; 60% energy from fat diet, Control; 10% energy from fat diet.

(A, B) Mann Whitney t-test, p>0.05, n=3/group (C) Two way ANOVA, Tukey’s multiple comparisons test, * p<0.05, ** p<0.01 vs. control, n=6-10/group (E, F) p>0.05, n=6-10/group. (See table 8.4 of appendix for F values).
To get an assessment of the glial response to a HFD, the morphology of activated microglia was quantified in the ARC after 1, 4 and 20 weeks HFD (figure 3.7). Iba1 immunohistochemistry indicates that the number of activated microglia within the medial region of the ARC (Bregma coordinates, rostral -1.22/-1.58mm, medial -1.58/-1.94mm, caudal -1.94/2.18mm) increases after 20 weeks of HFD (figure 3.7D).
Figure 3.7: Twenty weeks high-fat diet increases the number of activated microglia. Activated microglia were quantified in the rostral (R), medial (M) and caudal (C) sections of the arcuate nucleus following 1 week, 4 weeks and 20 weeks high-fat diet (HFD) (A) Activated microglia classification key (B) Representative images of medial (M) hypothalamic Iba1 immunohistochemistry and quantification of microglia activation after 1 week, (C) 4 weeks and (D) 20 weeks HFD. 60% energy from fat diet (HFD) or 10% energy from fat diet (Control), Unpaired t-test, * p<0.05 vs. control, n=5.
3.3.5 The effect of high-fat diet on hypothalamic inflammation in rats.

To determine if the development of hypothalamic inflammation was dependent on the animal species used, rats were fed HFD for three days. This increased body weight and food intake (figure 3.8A and B). Expression of the anorexigenic neuropeptide Pomc was decreased (figure 3.8C). Glucocorticoid receptor (Nr3c1) expression did not alter after three days HFD (figure 3.8C). Expression of suppressor of cytokine signalling 3 (Socs3) mRNA was the only inflammatory marker decreased after three days HFD, Ikkb and Il-6 expression remained unaltered (figure 3.9).

![Figure 3.8: Three days high-fat diet increased body weight and food intake in rats.](image)

Rats were fed either 60% energy from fat diet (HFD) or 10% energy from fat diet (Control) for three days. (A) Body weight and (B) food intake were monitored over the three day treatment. (C) Pro-opiomelanocortin (Pomc) and (D) glucocorticoid receptor (Nr3c1) mRNA expression was quantified in the hypothalamus after three days HFD.

(A) Two way ANOVA, Sidak's multiple comparisons test, *** p<0.001 vs day 0, HFD (B) Unpaired t-test, ** p<0.01 vs control (C) Mann Whitney t-test, * p<0.01 vs. control (D) Mann Whitney t-test, p>0.05, n=9. (See table 8.5 of appendix for F values).
Figure 3.9: Short-term high-fat diet does not induce hypothalamic inflammation in rats.
Rats were fed 60% energy from fat diet (HFD) or 10% energy from fat diet (Control) for three days. Pro-inflammatory inhibitor of kappa light polypeptide gene enhancer in β cells (Ikkb), interleukin-6 (Il-6), and suppressor of cytokine signalling 3 (Socs3) mRNA expression was quantified in the hypothalamus after three days HFD. Mann Whitney t-test, * p<0.05 vs. control, n=9.

3.3.6 The effect of high-fat diet on glucocorticoid target genes in mice.
As markers of glucocorticoid activity, glucocorticoid-induced leucine zipper (Tsc22d3), and glucocorticoid receptor (Nr3c1) expression was measured in the hypothalamus of mice fed a HFD. Nr3c1 expression was unaltered after 24 hours and 1 week of HFD (figure 3.10). High-fat diet did not alter the expression of Nr3c1 compared to mice fed control diet, and this was consistent for all time-points across the 20 week treatment period. However, Nr3c1 expression increased in both treatment groups over time. A transient activation of the GR after two weeks HFD was indicated, as hypothalamic Tsc22d3 expression was increased only after two weeks HFD. Tsc22d3 expression decreased after 2 weeks feeding of both control and HFD (figure 3.10C).
Figure 3.10: The effect of high-fat diet on glucocorticoid receptor expression and activity.

Mice were randomly assigned either 60% energy from fat diet (HFD) or 10% energy from fat diet (Control) for 24 hours, 1 week, 2 weeks, 3 weeks, 4 weeks or 20 weeks. Hypothalamic mRNA expression of glucocorticoid target genes; glucocorticoid induced leucine zipper (Tsc22d3) and glucocorticoid receptor (Nr3c1) were quantified at each time point.

Two way ANOVA, Tukey’s multiple comparisons test, **p<0.01 vs control, # p<0.05, ### p<0.001 vs time, n=5-10. (See table 8.6 of appendix for F values).
3.4 Discussion

Although studies have investigated the effects of HFD on the regulation of neuropeptides at different time points, inconsistent results between the studies have prevented an understanding of the evolution of neuropeptide changes associated with HFD. Furthermore, acute HFD has been found to induce hypothalamic inflammation thought to be involved in the development of obesity; however, the mechanisms behind this development are still unknown.

This chapter aimed to establish a model of HFD to investigate the development and evolution of diet-induced hypothalamic inflammation, in order to determine the direct and indirect effects of HFD on the energy-regulatory network. There was an expected increase in body weight after 1 week HFD, which persisted leading to obesity after 20 weeks. However, surprisingly, both orexigenic and anorexigenic neuropeptide mRNA expression decreased with long-term HFD in mice. Unlike LPS stimulation, which increased the expression of pro-inflammatory cytokines within the hypothalamus, HFD did not increase their expression at any time point in either mice or rats. Further, HFD did not alter the expression of ER stress markers which have been associated with HFD and obesity. Conversely, after 20 weeks HFD microglia were activated in the ARC.

For Gcs to have an anti-inflammatory effect there would need to be clear evidence of HFF influencing Gc effects. Gc actions are highly dependent on the expression of the GR. Throughout this study, HFF did not alter Nr3c1 (GR) expression in the whole hypothalamus; however there was an increase in the expression of Tsc22d3, a marker of GR activity (GILZ) which suggests that GR is activated at this time point.

3.4.1 The effect of high-fat diet on hypothalamic neuropeptide expression

The quantification of neuropeptide mRNA expression within the whole hypothalamus over a series of time points can be used to understand how chronic HFD alters the energy-regulatory network. In this study we identified decreases in both Npy and Agrp expression after 20 weeks. Others have found similar effects, in that given a high energy from fat diet, mice decrease food intake in order to maintain energy homeostasis, and therefore orexigenic neuropeptide expression decreases (Heijboer et al., 2005; Huang et al., 2003; Lin et al., 2000; H. Wang et al., 2002).

Opposite to orexigenic neuropeptides, an increase in the expression of anorexigenic neuropeptides (such as Pomc) would be expected in order to counteract a gain in body weight on a HFD. Acute (1 week) HFD in this study increased Pomc expression as
expected, although, it should be noted that at the same time-point in a separate cohort of mice (long-term HFD) this increase in Pomc was not observed. Further, Pomc expression did not remain elevated as predicted, but long-term HFD decreased Pomc expression. This decrease in Pomc mRNA expression has been quantified in other chronic HFD studies (Huang et al., 2003; Lin et al., 2000).

This surprising response of Pomc to chronic HFD might be a consequence of elevated leptin levels, no longer stimulating Pomc to decrease food intake as leptin resistance develops (Münzberg et al, 2004). Contrary to the dogma that leptin levels control Pomc expression, a recent study dissociated the effects of leptin on POMC. It was suggested that in response to a chronic HFD, mice decrease their food intake each day in order to maintain body weight (Mercer et al., 2014).

It should be noted that alongside the mRNA expression changes seen in this model, long-term HFD is associated with the loss of energy-regulatory neurones within the hypothalamus. A 25% decrease in POMC neurones was quantified by immunohistochemistry after 8 months HFD (Thaler et al., 2012). It is unknown whether this detrimental effect of chronic HFD on neuronal populations, is due to the HFD or a consequence of HFD-induced hypothalamic inflammation.

3.4.2 High-fat diet-induced hypothalamic inflammation

Unlike peripheral inflammation, diet-induced hypothalamic inflammation, is thought to develop after both acute and chronic HFD (De Souza et al., 2005; Thaler et al., 2012). This suggests that hypothalamic inflammation may play a role in the development of diet-induced obesity.

In this study, we did not see any changes in the expression of inflammatory or ER stress markers at any time point in either the short-term or long-term mouse models of HFD. The quantification of hypothalamic inflammatory markers post-LPS injection, indicates that our method of cytokine quantification is an effective technique for the measurement of large increases in inflammatory marker expression. However, studies reporting diet-induced hypothalamic inflammation have quantified <1.5 fold increases in inflammatory markers (Thaler et al., 2012). Therefore, if our samples only contain a small proportion of cytokine producing cells the threshold for detection may not have been reached (Amsen et al., 2009). Further, inflammatory markers were quantified in the hypothalamus of rats, to establish whether the short-term development of HFD-induced inflammation differed between rodent species. Although studies have quantified inflammation in the
hypothalamus of rats after HFD (De Souza et al., 2005; Milanski et al., 2009; Thaler et al., 2012), similar to the mouse model hypothalamic inflammation was not quantified in this study.

With many studies examining the effect of HFD on relatively small increases in a select number of inflammatory markers, it should be considered whether these changes in inflammatory markers are pathophysiologically relevant (Ren et al., 2014). Furthermore, the forced activation of the inflammatory Ikkβ / NFκB pathway within hypothalamic neurones, does not lead to an increase in hypothalamic pro-inflammatory cytokine production (X. Zhang et al., 2008). Therefore, these results may suggest that activation of the Ikkβ / NFκB pathway for the production of inflammatory cytokines is more important in the non-neuronal cells of the hypothalamus.

In this study long-term HFF increased the number of activated microglia within the ARC after 20 weeks. Although we did not see an activation of microglia until 20 weeks, a recent study has identified that microglia activation is dependent on diet and hormones but not increased body weight (Gao et al., 2013). Further, the depletion and inhibition of microglia activation prevents the increase in pro-inflammatory cytokines and neuronal stress markers induced by a HFD (Andre et al., 2016; Valdearcos et al., 2014). Activated microglia, both phagocytose debris and secrete inflammatory cytokines; in response to a HFD challenge the timings of these actions is disputed. Thaler and colleagues identified a neuroprotective response of microglia as activation of these cells was induced following the immediate increase in inflammatory markers aiding the decrease in these cytokines expression. Conversely, microglia were activated immediately, contributing to the increase in pro-inflammatory cytokine production in a different model of HFD-induced inflammation (Valdearcos et al., 2014). Although these studies show differing responses to acute HFD, both studies identified increases in microglia activation with chronic HFD. The chronic activation of microglia is thought to be detrimental to the synaptic plasticity of the neuronal network impairing the function of energy-regulatory neurones (Tran et al., 2016). The increased activation of microglia in this study after 20 weeks HFD could be associated with the decrease in expression of Agrp, Npy, and Pomc as a consequence of the damaging microglia effects on these neurones. Together, these studies show the detrimental effects of chronic microglia activation; however, their role in the regulation of short-term HFD remains unclear.

This study has highlighted the variability observed in the modelling of diet-induced inflammation. It may be that the differing diets used within this field have prevented a clear understanding of the mechanisms driving hypothalamic inflammation. The separation of high-fat from high-sugar indicates that a HFD primes the neuronal cells but does not
induce inflammation (Sobesky et al., 2016). However, other studies have shown that excess saturated fatty acids induce the production of inflammatory cytokines through the activation of TLR4 signalling (Milanski et al., 2009). Therefore, with such variability in the development of hypothalamic inflammation and its regulatory role on the energy-regulatory network, the pathophysiological relevance of diet-induced hypothalamic inflammation is still unclear. This makes it impossible to investigate the role of Gcs in modulating hypothalamic inflammation if a reproducible model is not available.
Chapter 4

Hypothalamic glucocorticoid concentrations in an *in vivo* model of glucocorticoid-induced metabolic syndrome
4.1 Introduction

The metabolic consequences of elevated circulating glucocorticoids (Gcs) have been recognised because of hypothalamic-pituitary-adrenal (HPA) axis disorders and exogenous Gc therapy. Long-term Gc treatment is used for a wide number of inflammatory and malignant conditions; consequently, these individuals develop adverse metabolic sequelae including obesity, hyperphagia, and hyperglycaemia. The control of food intake and energy expenditure by neurones within the hypothalamus, suggest these metabolic side-effects could be mediated through Gcs acting within the hypothalamus (Sohn et al., 2013). There is evidence that the glucocorticoid receptor (GR) is co-localised with the primary neurones of the hypothalamus, i.e. AgRP/NPY/GABA (Unger et al., 2010) and POMC/CART (Cintra & Bortolotti, 1992) and there are glucocorticoid-response-elements (GREs) in the promotor region of these energy-regulatory neuropeptides (Drouin et al., 1993; Lee et al., 2013; Misaki et al., 1992).

To mimic the consequences of Gc-induced metabolic syndrome, a method of non-invasive Gc administration to adrenally intact mice has been developed (Karatsoreos et al., 2010). This method of exogenous Gc administration is successful in inducing obesity, hyperphagia, and impairing glucose homeostasis, all of which are hallmarks of Gc-induced metabolic syndrome. However, this model has yet to be used to establish the mechanisms behind the development of these metabolic disorders.

4.1.1 Aims and Objectives

This chapter investigates the effect of exogenous Gc treatment on corticosterone levels within the hypothalamus and establishes whether increases in hypothalamic corticosterone levels are associated with the development of metabolic sequelae.

1. To analyse hypothalamic corticosterone levels in a non-invasive murine model of peripheral corticosterone treatment

The self-regulatory Gc feedback inhibition at the paraventricular nucleus (PVN) regulates the magnitude and duration of adrenal Gc production (Kalafatakis et al., 2016). This clearly indicates that endogenous Gcs can translocate to and act within the hypothalamus to regulate the HPA axis. To assess the effect of excess Gcs seen in patients on long-term Gc treatment, recent studies have developed a translational murine model whereby corticosterone is administered in the drinking water (Karatsoreos et al., 2010; Shpilberg et
al., 2012). However, these studies have not assessed whether central Gc signalling might contribute to the observed body weight gain and hyperphagia. In this chapter, a model whereby corticosterone administered in the drinking water (75µg/ml) was used to mimic the metabolic consequences of Gcs at pharmacological doses.

To determine whether corticosterone given in the drinking water translocates into the hypothalamus after corticosterone treatment, a method of liquid/liquid extraction followed by solid phase extraction was developed. This allowed quantification of corticosterone in a hypothalamic-enriched region by liquid chromatography dual tandem mass spectrometry (LC-MS/MS). Hypothalamic corticosterone concentrations were quantified after acute (24 and 48 hours) and chronic (4 weeks) corticosterone treatment, to establish whether hypothalamic corticosterone levels remain elevated over time. To elucidate whether increased hypothalamic Cort levels are associated with the development of metabolic syndrome, body weight, food intake, and glucose were recorded during the study.

2. To assess the effect of corticosterone and high-fat diet co-treatment on hypothalamic corticosterone levels and metabolic phenotype.

Elevated corticosterone levels resulting from Cushing’s syndrome or long-term Gc therapy increases an individual’s drive to eat (Geer et al., 2016; Moeller et al., 2016). The increased levels of Gcs that these patients’ experience, can cause a change in food preference to high-fat calorie dense food (Auvinen et al., 2013; Dallman, 2010), however, the mechanisms driving this food preference shift have not been investigated. Exogenous Gc treatment also alters the expression of energy-regulatory neuropeptides resulting in an increase in food intake (White Lab). This suggests that the mechanisms behind the Gc-induced hyperphagia are due to Gcs acting in the hypothalamus; however, the effect of corticosterone and HFD co-treatment on hypothalamic corticosterone levels has not been investigated.

To examine the effect of corticosterone and HFD on hypothalamic corticosterone concentrations, corticosterone in the drinking water (75µg/ml) was co-administered alongside 60% energy from fat diet. Hypothalamic corticosterone concentrations were quantified after four weeks treatment to determine whether co-administration of HFD+Cort increases the levels of hypothalamic corticosterone. Finally, body weight and food intake were monitored throughout the study, to establish whether corticosterone and HFD co-treatment exacerbates Gc-induced metabolic phenotype.
4.2 Methods

4.2.1 Administration of corticosterone to mice

Ten week old male C57Bl/6J mice (Charles River) were singly housed, food intake and body weight were monitored twice weekly over three weeks to record baseline measurements. Mice were pseudo-randomised by baseline body weights and administered either corticosterone (Cort; 75µg/ml) dissolved in 100% ethanol (final concentration, 1% ethanol) or vehicle (final concentration 1% ethanol) in their drinking water for 24 hours, 48 hours, or 4 weeks (n=12/group). Mice treated with corticosterone for four weeks received either a high-fat diet (60% energy from fat diet, D12492, Research Diets, HFD-Cort) or standard chow (RM1). Body weights, food, and water intake were measured twice weekly at 10am.

4.2.2 Tail-prick micro-sampling

Blood samples were taken as described in section 2.1.2. At the end of the 4 weeks Cort treatment tail-blood micro-samples were taken at 7am (ZT = 1) and 7pm (ZT = 13) for circulating corticosterone measurements.

At the end of each study (24 hours, 48 hours and 4 weeks Cort treatment), blood was taken by tail-prick micro-sampling for immediate glucose levels. Samples for analysis of circulating corticosterone levels were taken and stored as described in section 2.1.2. Immediately following sampling, mice were culled by cervical dislocation and tissues were dissected and snap-frozen on dry ice for future analysis.

4.2.3 Dissection of hypothalamic-enriched and extra-hypothalamic regions

Micro-dissection scissors were used to dissect a hypothalamic-enriched region from the ventral side of the frozen brain (~50mg). Two coronal micro-dissection cuts were made at the levels of the optic chiasm and mammillary bodies. The brain was rotated and cuts were made either side to dissect out the hypothalamus as a rectangle. The rest of the brain was homogenised as one and described as the extrahypothalamic region (~375mg).
4.2.4 Plasma Corticosterone assay

Plasma corticosterone levels were quantified using an ELISA kit according to the manufacturer’s instructions (4 week Cort treatment: Cayman, Cambridge Bioscience, UK; 24 and 48 hours Cort treatment: Abcam, UK). Corticosterone ELISA was switched between studies due to the Cayman kit being discontinued. Samples were read at 455nm absorbance with an absorbance of 655nm subtracted.

4.2.5 Tissue steroid extraction and LC-MS/MS quantification

Tissue corticosterone levels were measured in hypothalamic-enriched and extrahypothalamic regions (rest of the brain) by liquid chromatography dual tandem mass spectrometry (LC-MS/MS). Both regions were dissected and homogenised in sterile water using a rotor homogeniser (200mg/ml). A 100µl sample aliquot was enriched with corticosterone-D8 major (US Biological) as internal standard to a concentration of 10µg/ml.

A liquid/liquid extraction (LLE) was performed followed by a solid phase extraction (SPE). Briefly, 500µl ethyl acetate was added to each sample, aspirated and then centrifuged (45xg, 10mins) to separate the layers. The aqueous layer was discarded while the solvent layer was dried under nitrogen (~50L/minute, Porvair science, Minivap) and then reconstituted in 30% methanol. An SPE extraction was performed using a C-18 plate (Strata C18-E, Phenomenex, UK) which was conditioned with methanol and water. Samples were loaded in 30% methanol, washed with double distilled H₂O and 30% methanol before elution in 100% methanol (2 x 250µl, CVC3000 vacuubrand). The eluents were dried under nitrogen (~50L/minute, Porvair science, Minivap) and reconstituted in 50% mobile phase A/50% mobile phase B. Mobile phase A was 2mM ammonium acetate in methanol/water (10/90 v/v) containing 0.027% formic acid. Mobile phase B was 2mM ammonium acetate in methanol/water (90/10 v/v) containing 0.027% formic acid.

Chromatographic separation of corticosterone and 11-DHC was performed by Dr Helen Small (Cancer Research UK Manchester Institute) using reverse phase chromatography (Kinetex® 5µm XB-C18 100 Å, 50 x 2.1 mm, Phenomenex, UK) on an Agilent 1200 binary pump HPLC system running a gradient from 100% A to 100% B over 1.5 minutes, hold for 1.5 minutes then immediately back to 100%A and hold for 3.5 minutes. Total run time was 6.5 minutes with flow rate of 500µl/min and an injection volume of 10µl. The system was run at room temperature.
The chromatography system was coupled to a Sciex API4000 Qtrap mass spectrometer. The instrument was operated in multiple reaction monitoring (MRM). Corticosterone was monitored in positive ion mode; the following parameters were implemented in the ion source: spray voltage 4.5 kV, temperature 550°C, CUR at 20, GS1 at 50 and GS2 at 60. The transitions used were: Cort; 347.1/329.3 with CE = 22, DP = 37 and CXP 15. D8-Cort (internal standard); 355.3/73 with CE = 50, DP = 117 and CXP = 15. 11-DHC was monitored in negative ion mode; the following parameters were implemented in the ion source: spray voltage -4.5 kV, temperature 550°C, CUR at 20, GS1 at 50 and GS2 at 60. The transitions used were:11-DHC; 343.1/328.0 with CE = -28, DP = -100 and CXP -15. D8-Cort (internal standard); 353.1/246.1 with CE = -25, DP = -100 and CXP = -15.

Data was processed using Analyst Software (Applied Biosystems, UK) and signal intensities obtained by standard peak integration methods. Quantitation was performed by comparison against a standard curve generated from multiple dilutions of brain tissue spiked with analyte of interest in methanol (10 ng/μl – 0.01 ng/μl). Final analyte quantification was derived from a mean of two biological replicates. The lower limit of accurate quantification (LLQ) was set at a level twice the peak area measured in blank brain tissue, to ensure robust and confident discrimination of analytes from low-level background peaks which may co-elute. The mean LLQ was 0.3 ng/ml Cort, 0.5 ng/ml 11-DHC. When samples were measured as BLQ statistical analysis was performed using a value of zero.
4.3 Results

4.3.1 Hypothalamic corticosterone increases after short-term Cort treatment

Corticosterone levels within the hypothalamus were increased after 24 (figure 4.1A) and 48 hours treatment (figure 4.2A). The correlation between hypothalamic and circulating corticosterone levels strengthened after 48 hours (figure 4.1B, 4.2B). Extrahypothalamic (rest of the brain) levels were increased alongside hypothalamic corticosterone levels at both time points (figure 4.1C, 4.2C).
Figure 4.1: Hypothalamic corticosterone levels increase after 24 hours Cort treatment.

Corticosterone levels were quantified by liquid chromatography dual tandem mass spectrometry (LC-MS/MS) in hypothalamic and extrahypothalamic brain regions of corticosterone (75µg/ml, Cort) or 1% ethanol (vehicle, V) treated mice. (A) Hypothalamic corticosterone levels (B) hypothalamic corticosterone vs circulating corticosterone levels and (C) hypothalamic vs extrahypothalamic corticosterone levels after 24h Cort treatment. Below the levels of quantification, BLQ.

(A) Unpaired t-test * p<0.05, (B) D’Agostino normality test, Pearson’s correlation p>0.05 (C) D’Agostino normality test, Pearson’s correlation *** p<0.001, n=7-12.
Figure 4.2: Hypothalamic corticosterone levels increase after 48 hours Cort treatment.

Corticosterone levels were quantified by liquid chromatography dual tandem mass spectrometry (LC-MS/MS) in hypothalamic and extrahypothalamic brain regions of corticosterone (75µg/ml, Cort) or 1% ethanol (vehicle, V) treated mice. (A) Hypothalamic corticosterone (B) hypothalamic vs circulating corticosterone and (C) hypothalamic vs extrahypothalamic corticosterone levels after 48 hours Cort treatment. Below the levels of quantification, BLQ.

(A) Unpaired t-test * p<0.05, (B) D’Agostino normality test, Spearman’s correlation *** p<0.001 (C) D’Agostino normality test, Pearson’s correlation *** p<0.001, n=7-12.
4.3.2 Exogenous Cort treatment does not affect adrenal weight

Adrenal weight does not alter after short-term Cort treatment (figure 4.3A). A decrease in spleen weight after 24 and 48 hours Cort treatment indicates that administration of corticosterone in the drinking water has immediate effects on immune suppression (figure 4.3B).

**Figure 4.3: Short-term corticosterone treatment does not affect the hypothalamic-pituitary-adrenal axis.**

*Mice were treated with either corticosterone (75μg/ml, Cort) or 1% ethanol (Vehicle) in their drinking water 24 hours or 48 hours. Adrenal and spleen mass were measured at the end of the study.*  
*Unpaired t-test, *** p<0.001, n=12/group.*

4.3.3 The effect of short-term Cort treatment on body weight, food intake, and glucose homeostasis

Exogenous Cort administered in the drinking water increased food intake after 24 hours which remained elevated at 48 hours (figure 4.4A). Cort treatment did not affect body weight after 24 or 48 hours (figure 4.4B). Fat pad mass remained unaltered across the three fat pad beds measured (epididymal, mesenteric, and subcutaneous) after short-term Cort treatment (figure 4.4C, D). Twenty-four and forty-eight hours Cort treatment did not alter glucose levels (figure 4.4E).
Figure 4.4: Food intake increases after 24 hours Cort treatment.

Mice were treated with either corticosterone (75µg/ml, Cort) or 1% ethanol (Vehicle) in drinking water for 24 or 48 hours. (A) Food intake (B) body weight (C, D) epididymal (Epi), mesenteric (Mes) and subcutaneous (Sub) fat pad mass and (E) fed glucose were monitored after 24 and 48 hours Cort treatment.

Unpaired t-test, *** p<0.001, n=12/group.
4.3.4 Four weeks Cort treatment increases hypothalamic corticosterone levels

Hypothalamic corticosterone levels were increased 4-fold after four weeks Cort treatment. This increase was exacerbated by co-treatment with HFD (9-fold); however, corticosterone levels in the hypothalamus of mice treated with HFD alone were below the limit of quantification (figure 4.5). Corticosterone levels in the extrahypothalamic region were also increased after four weeks Cort treatment. Similar to the hypothalamic region, extrahypothalamic corticosterone levels were increased after Cort treatment alongside 60% energy from fat diet (H+C), however, HFD treatment alone did not alter corticosterone levels (figure 4.5).

In a parallel study after four weeks Cort treatment +/- HFD, Nr3c1 (GR) mRNA expression decreased in the hypothalamus. However, the residual GR still mediated affects as mRNA expression of Gc activity markers; Gc-induced leucine zipper (Tsc22d3), and FK506 binding protein (Fkbp5), increased with Cort treatment (parallel study completed by Dr. Erika Harno and Mrs Alison Davies; Sefton et al., 2015).
Figure 4.5: Hypothalamic and extrahypothalamic corticosterone levels following four weeks corticosterone treatment.

Corticosterone levels were quantified by liquid chromatography dual tandem mass spectrometry (LC-MS/MS) in hypothalamic and extrahypothalamic brain regions of mice treated with either corticosterone (75µg/ml, C) or 1% ethanol (V) alongside 60% energy from fat diet (H+C, H) or standard chow diet. BLQ = below the limit of quantification.

One way ANOVA * p<0.05, ** p<0.01, *** p<0.001 n=12 (See table 8.7 of appendix for F values).

Both hypothalamic and extrahypothalamic corticosterone levels increased alongside rising circulating corticosterone levels (figure 4.6A, B). Further, hypothalamic corticosterone levels increased alongside extrahypothalamic levels (figure 4.6C).

In a parallel study completed by Dr. Erika Harno and Mrs Alison Davies, Cort treatment decreased expression of \( HSD11b1 \) within the hypothalamus, indicating a reduced regeneration of endogenous corticosterone. Furthermore, mRNA expression of the steroid efflux pump \( Abcb1 \) remains unaltered with Cort treatment (Sefton et al., 2015).
Figure 4.6: Hypothalamic corticosterone levels are positively correlated with circulating corticosterone after four weeks treatment.

Mice treated with corticosterone (75µg/ml, Cort) or 1% ethanol (Vehicle) in drinking water alongside 60% energy from fat diet (HFD+Cort, HFD) or standard chow for four weeks. (A) Circulating vs hypothalamic corticosterone levels (B) circulating vs extrahypothalamic corticosterone levels (C) hypothalamic vs extrahypothalamic corticosterone levels. (A, B, C) D’Agostino normality test, Spearman’s correlation *** p<0.001, n=12.
4.3.5 Four weeks exogenous Cort treatment alters the circulating corticosterone diurnal rhythm

Despite exogenous Cort treatment suppressing the HPA axis, continued administration of corticosterone in the drinking water over a four week period increased plasma corticosterone levels 9-fold at zeitgeber time = 1 (ZT=0, lights on 7am) and 2-fold higher at ZT=13 compared with vehicle treated (figure 4.7). Exogenous Cort treatment alongside HFD also increased plasma corticosterone levels at both time points, abolishing the corticosterone diurnal rhythm (figure 4.7). Treatment with 1% ethanol +/- HFD did not affect the plasma corticosterone levels or corticosterone diurnal rhythm (figure 4.7).

![Figure 4.7: Corticosterone treatment increases circulating corticosterone levels at both the nadir and peak.](image)

Plasma circulating corticosterone levels were quantified after 4 weeks treatment with either corticosterone (75µg/ml, Cort) or 1% ethanol (Vehicle) alongside 60% energy from fat diet (HFD+Cort, HFD). Zeitgeber time, ZT; ZT=1, 8am; ZT=13, 8pm. Paired t-test ** p<0.01, n=5-6.
4.3.6 Exogenous Cort suppresses the hypothalamic-pituitary-adrenal axis after 4 weeks

Unlike short-term Cort treatment, at the end of the four week treatment period, a reduced adrenal weight was measured in both Cort and HFD+Cort groups, suggesting that the increase in Gcs had suppressed the HPA axis. HFD alone did not affect the adrenal weight (figure 4.8A). As expected, Cort +/- HFD decreased the spleen weight after four weeks treatment (figure 4.8B).

Figure 4.8: Corticosterone treatment dampens the hypothalamic-pituitary-adrenal axis.
Mice were treated with either corticosterone (75µg/ml, C) or 1% ethanol (V) alongside 60% energy from fat (H+C & H) or standard chow diet. (A) Adrenal gland and spleen weight measured after four weeks treatment. One way ANOVA *** p<0.001 vs Vehicle, n=12/group (See table 8.8 of appendix for F values).
4.3.7 Four weeks Cort treatment induces obesity

Body weight increased after 14 days Cort treatment alone (figure 4.9A). HFD with or without Cort increased body weight after 7 days and remained elevated throughout the 4 week treatment period. Adiposity increased with Cort treatment across all three fat pads measured, epididymal, subcutaneous, and mesenteric (figure 4.9B). HFD increased fat pad mass, but combination of HFD+Cort did not exaggerate the effect.

**Figure 4.9: Four weeks corticosterone induces obesity.**
Mice were treated with either corticosterone (75µg/ml, Cort, C) or 1% ethanol (Vehicle, V) alongside 60% energy from fat (HFD+Cort, H+C & HFD, H) or standard chow diet. (A) Body weight measured twice weekly across the four week treatment period. (B) Epididymal, subcutaneous, and mesenteric fat pad mass measured after four weeks treatment.

(A) Two way ANOVA, Sidak's multiple comparisons test *** vs vehicle p<0.001 n=18-20

(B) One way ANOVA *** vs vehicle, p<0.001, n=12. (See table 8.9 of appendix for F values).
4.3.8 Four weeks Cort increases food intake expression.

Food intake increased after 10 days and remained elevated compared with vehicle throughout the treatment period (figure 4.10A). HFD treated mice, with/without Cort, decreased their food intake to control for the increased calories (figure 4.10B). Cort treatment +/- HFD increased water intake (figure 4.10C). Glucose levels were increased after four weeks HFD+Cort treatment, but HFD and Cort treatment alone did not alter glucose levels (figure 4.10D).

Hypothalamic neuropeptide mRNA expression was quantified in a separate cohort of Cort treated mice after 4 weeks. Expression of the orexigenic neuropeptide Agrp increased three-fold, with no changes in Npy, Cart or Pomc after Cort treatment (parallel study completed by Dr. Erika Harno and Mrs Alison Davies: Sefton et al., 2015).
Figure 4.10: Cort treatment induces hyperphagia.
Mice were treated with either corticosterone (75µg/ml, Cort, C) or 1% ethanol (Vehicle, V) alongside 60% energy from fat (HFD+Cort, H+C & HFD, H) or standard chow diet. (A, B) Food intake and (C) water intake measured twice weekly and (D) fed glucose measured at the end of four weeks treatment.

(A, B, C) Two way ANOVA Sidak’s multiple comparisons test, * p<0.05, **p<0.01, *** p<0.001 vs vehicle n=12/group, (D) One way ANOVA *p<0.05, ** p<0.01 n=6/group. (See table 8.10 of appendix for F values).
At the end of the study, circulating but not hypothalamic corticosterone levels increased in relation to body weight but only in the HFD group, and not in either Cort or HFD+Cort treatment groups (figure 4.11A, C). Unsurprisingly, at the end of the study cumulative food intake increased with circulating corticosterone levels in Cort and HFD+Cort treated groups (figure 4.11B). This positive correlation was not seen with hypothalamic corticosterone levels and cumulative food intake in any treatment group (figure 4.11D).

**Figure 4.11: Body weight and food intake correlation analysis against corticosterone levels.** Mice were treated with either corticosterone (75µg/ml, Cort) or 1% ethanol (Vehicle) alongside 60% energy from fat (HFD+Cort & HFD) or standard chow diet. Correlation analysis of circulating vs final body weight (A) and food intake (B) and hypothalamic corticosterone levels vs final body weight (C) and food intake (D).

(A) D'Agostino normality test, Pearson’s correlation; HFD * p<0.05, Vehicle, Cort, HFD+Cort p>0.05; n=12 / group.

(B) D’Agostino normality test, Pearson’s correlation; Cort & HFD+Cort * p<0.05, HFD & Vehicle p>0.05; n=12 / group.

(C) D’Agostino normality test, Pearson’s correlation, p>0.05, n=12 / group.

(D) D’Agostino normality test, Pearson’s correlation, p>0.05, n=12 / group.
4.4 Discussion

Obesity and hyperphagia are two of the metabolic consequences which patients treated with long-term Gcs develop. Although some co-therapies are available to treat specific adverse effects, for example bisphosphonate treatment for steroid-induced osteoporosis, other side-effects such as steroid-induced diabetes and obesity are more complex to treat.

The development of a mouse model which mimics Gc-induced obesity, hyperphagia, and hyperglycaemia enables us to investigate the mechanisms driving these side-effects. In this chapter, hypothalamic corticosterone levels increased immediately and remained elevated throughout chronic Gc treatment. In combination with HFD, Cort (HFD+Cort) exacerbates the increase in hypothalamic corticosterone levels despite HFD alone not altering hypothalamic corticosterone concentrations. These increases in hypothalamic corticosterone levels can be associated with decreases in hypothalamic Nr3c1 (GR) expression and decreased hypothalamic Gc regeneration (Harno and Davies) in Cort treated animals. Further, phenotypic monitoring identified the increase in hypothalamic corticosterone levels to be associated with increased food intake after 24 hours, with body weight increasing after 14 days. Additionally, the increase in hypothalamic corticosterone levels and food intake is associated with increased orexigenic Agrp expression in the hypothalamus (Harno and Davies).

4.4.1 Hypothalamic corticosterone concentrations increase rapidly and are sustained by chronic exogenous Cort treatment

The energy-regulatory network of the hypothalamus contains the primary neurones which control food intake and therefore it is important to establish the role of Gcs within this region. Although it is dogma that Gcs act at the PVN as part of the self-regulatory feedback loops of the HPA axis (Russell et al., 2014), it is unknown whether the hypothalamus can protect itself from chronically elevated Gc concentrations. Therefore, it was necessary to determine if chronic increases in Gcs lead to long-term elevation of Gc levels in the hypothalamus. To achieve this, a method of steroid extraction was optimised from a hypothalamic-enriched region and then LC-MS/MS quantification of corticosterone was performed.

It was clear from the data that hypothalamic corticosterone levels increase after 24 hours and remain elevated throughout Cort treatment. After 48 hours, increased hypothalamic Cort concentrations were directly correlated with increased circulating corticosterone levels. This correlation was sustained after the 4 week Cort treatment. In addition, decreased adrenal weight after 4 weeks indicated that there was reduced endogenous Gc
production, demonstrating that it is the increased circulating corticosterone which is translocating into the hypothalamus.

Furthermore, in a parallel study (conducted by Harno and Davies), markers of Gc regeneration and transportation out of the brain were investigated. Similar models of chronic elevated corticosterone have found increases in \textit{Hsd11B1}, the enzyme which converts the inactive 11-DHC to Cort, in the liver (Harno et al., 2013) and adipose tissue (Morgan et al., 2014). However, in our model we identify decreases in \textit{Hsd11b1} mRNA expression within the hypothalamus after 4 weeks. This decrease protects the hypothalamus from further increases in Cort, as the down-regulation prevents regeneration of Cort from inactive 11-DHC. Additionally, the efflux transporter \textit{Abcb1} is able to transport Gc from the brain. As hypothalamic mRNA expression of this transporter is unchanged in our model, it is unlikely that the transportation of Gcs out of the brain is altered.

The expression of the glucocorticoid receptor (\textit{Nr3c1}) was also decreased in the hypothalamus. It is considered dogma that Gcs negatively feedback at the PVN, decreasing CRH expression, to counteract the feedback inhibition by excess Gcs. However, the effect of chronic exogenous Cort treatment on \textit{Nr3c1} expression has not been previously quantified. Although the expression of \textit{Nr3c1} was decreased in the whole hypothalamus, the expression was not completely down-regulated allowing continued Gc action. The activity of Gcs in the hypothalamus is in fact elevated in our model, despite the decrease in \textit{Nr3c1}, as expression of Gc activity markers increases after 4 weeks treatment (\textit{Tsc22d3} and \textit{Fkbp5}).

Further, the levels of corticosterone quantified in the rest of the brain (extrahypothalamic region) were 9-fold higher than in the hypothalamic region. This suggests that the mechanisms controlling Cort regeneration and translocation may differ between brain regions, as indicated by the lower Cort concentration in the hypothalamic region compared with the extrahypothalamic region.

4.4.2 Combined corticosterone and high-fat diet exacerbates hypothalamic corticosterone levels

Exogenous Cort treatment has been associated with a shift in food preference towards high-fat, high-sugar foods (Tataranni et al., 1996). It is not known how this shift in food preference affects the development of Gc-induced metabolic syndrome. Therefore, through the combined treatment of Cort and HFD for 4 weeks, this model established the
effect of HFD+Cort on hypothalamic corticosterone concentrations and the activity of these Gcs within the hypothalamus.

Hypothalamic corticosterone concentrations increased even further, when mice were treated with both Cort and HFD for 4 weeks compared with Cort alone. No difference in water intake was observed between the two Cort treatment groups throughout the study, indicating that as Cort was administered in the drinking water, both Cort treated groups received the same dose of Cort across the treatment period.

Alterations in the HPA axis have been identified in different models of elevated corticosterone (Cassano et al., 2012; Karatsoreos et al., 2010). In our model, HFD alone did not alter either circulating or hypothalamic corticosterone levels. Furthermore, no change in adrenal weight was measured at the end of the study, indicating that the endogenous production of Gcs was not altered by treatment. This indicates that the exacerbated response to combined HFD+Cort treatment is not due to the HFD activating the HPA axis. Similarly, the addition of HFD alongside Cort treatment decreased Hsd11β-1 and Nr3c1 expression to the same extent as Cort treatment alone. This indicates that the increased hypothalamic Cort treatment is not a consequence of alterations to the mechanisms which control Gc levels.

Consequently, the exacerbated increase in hypothalamic corticosterone levels in the HFD+Cort group is probably due to increased transportation of circulating corticosterone into the hypothalamus. Long-term HFD studies investigating the effect of HFD on hypothalamic inflammation, have identified that chronic HFD increases the BBB permeability (Yi et al., 2012). Therefore, the addition of HFD alongside Cort treatment could be increasing the BBB permeability enabling the movement of Gcs across it. To confirm this, further investigations into the effect of combined HFD+Cort treatment on the BBB permeability are needed.

4.4.3 Elevated hypothalamic corticosterone concentrations are associated with obesity and hyperphagia

The increased hypothalamic corticosterone levels observed in our model throughout the 4 week treatment period are associated with the development of Gc-induced metabolic syndrome. Although the effects of peripheral Gcs are well established, it is likely that the increase in Gcs within the hypothalamus play an important role in the development of the metabolic side-effects associated with Gc treatment.
The hypothalamus contains both orexigenic and anorexigenic neurones and is situated alongside the leaky BBB making this region a prime site in regulating food intake. In our model, hypothalamic Gc levels increase after 24 hours, which is associated with an immediate increase in food intake. Further, elevated hypothalamic Gc concentrations are associated with increased expression of the orexigenic neuropeptide Agrp (Sefton et al., 2016). The actions of Agrp on food intake are well known, however, more recent studies have indicated that AgRP neurones can signal to peripheral tissues to regulate glucose homeostasis and energy expenditure (Steculorum et al., 2016; Yi et al., 2012). Further investigations are required to establish the role of the primary energy-regulatory neurones of the MBH in the development of Gc-induced metabolic side-effects.

Unlike food intake, body weight does not increase till day 14 of Cort treatment. The combination of HFD+Cort advances this increase in body weight to day 7, however, those animals on combined HFD+Cort are similar in body weight to those on HFD alone. The administration of Cort in the drinking water is a successful model for investigating the mechanisms of Gc-induced side-effects, as alongside the obesity and hyperphagia, Cort treatment trends towards increasing glucose levels and HFD+Cort treatment induces hyperglycaemia.

Cort-induced hyperphagia and increased Agrp expression are associated with the elevated hypothalamic corticosterone concentrations observed in our model. However, further studies to manipulate the hypothalamic Gc signalling pathways are required to establish whether these alterations in Agrp drive other Gc-induced metabolic side-effects observed in our model.
Chapter 5

Does GR knockdown in the MBH protect against glucocorticoid-induced metabolic syndrome?
5.1 Introduction

The pleiotropic functions of Gcs mean that they are used in many clinical situations e.g. as treatments for inflammatory diseases and cancer. As a consequence of long-term high dose treatment, patients treated with chronic Gcs suffer from adverse metabolic side-effects including obesity and Gc-induced diabetes. In our lab, we have successfully set up a non-invasive mouse model of exogenous corticosterone therapy, which mimics the development of these metabolic side-effects, including increased body weight and hyperphagia.

Genetic disruption to GR signalling within the PVN, has defined the role of Gc negative feedback as part of the HPA axis. Initially, using two separate strains of GR flox mice, this study determined that phenotypic changes are dependent on which exon is targeted to knockdown the GR. GR immunofluorescence identified a 43% reduction in PVN GR expression when exon 2 was deleted, compared with an 87% reduction when exon 3 was removed (Laryea et al., 2013). Unlike the knockdown of exon 2, which is thought to still produce truncated function-retaining GR products, the removal of exon 3 completely inactivates the gene, as out-of-frame splicing terminates translation early (Kellendonk et al., 2002; Tronche et al., 1999). This indicates that the removal of exon 3, which contains the zinc-finger of the DNA binding domain, is the ‘cleaner’ knockout to investigate Gc actions.

The mediobasal hypothalamus (MBH) consists of a number of hypothalamic nuclei including the arcuate nucleus (ARC) and ventromedial hypothalamus (VMH). The neuronal networks within these nuclei form the part of the energy-regulatory network which controls food intake. Both orexigenic and anorexigenic neurones within the MBH express the GR, yet the function of these neurones in the development of Gc-induced metabolic syndrome is unknown. In chapter 4, we identified that exogenous Cort treatment increases Cort concentrations within the hypothalamus. However, the role of these increased Cort levels and the mechanisms of Gc-induced obesity and hyperphagia are still unclear.

Through the genetic alteration or disruption to Gc actions within targeted brain regions we can determine the mechanisms driving these diseases. The knockdown of Agrp, induces a late onset lean phenotype (Wortley et al., 2005), and the knockdown of Pomc specifically within the hypothalamus increases body weight and food intake (Smart et al., 2006). Together, this indicates the importance of these two neurone populations in the control of body weight and food intake. However, the energy-regulatory network is highly plastic and influenced by many peripheral hormones. Both the anorexigenic POMC and orexigenic AgRP neurones originate from the same Pomc-expressing progenitor cells
(Padilla et al., 2010). Consequently, it is unknown whether compensatory mechanisms develop in congenital neuropeptide knockout animal models. Furthermore with age, AgRP innervation of POMC neurones increases and therefore the communication between the neurones is important in the regulation of energy balance (Newton et al., 2012).

In order to establish the effect of Gcs within the ARC, GR has been knocked down in AgRP neurones. This knockdown protects against body weight gain induced by a HFD. However, this study found that GR knockdown in AgRP neurones decreased 

Ag

expression but did not affect the co-localised 

Np

expression (Shibata et al., 2016), indicating these neuropeptides have differing sensitivity to Gcs. In order to establish the role of these neuropeptides in the development of Gc-induced metabolic diseases these knockout models need to be challenged with Gcs.

Developments in technology have provided the opportunity to target specific signalling pathways within small brain regions of adult mice. Cre-lox technology is one such example which has been widely used to better understand different neuronal networks within the central nervous system. Cre recombinase packaged into adeno-associated viral vectors (AAV) can be intracranially injected to knockdown or overexpress the gene of interest in specific distinct brain regions. This method of genetic modification can be completed in adult mice, which prevents the development of compensatory mechanisms often seen in congenital models.

5.1.1 Aims and Objectives

By using virus mediated Cre-lox technology, this chapter aims to understand the mechanisms driving Gc-induced obesity and hyperphagia by knocking-down the GR in the MBH and challenging with exogenous Gcs.

1. To optimise bilateral injections of adeno-associated viral vectors into the MBH.

To establish the effect of hypothalamic Gcs in the development of Gc-induced metabolic syndrome, stereotaxic recovery surgery was used to optimise bilateral AAV-Cre or AAV-GFP injections into the MBH of mice. As other hypothalamic regions, such as the PVN, are involved in the regulation of food intake and energy expenditure, the intracranial injections were optimised to ensure that the virus did not translocate and infect these regions.
2. To validate GR knockdown by AAV-Cre injection into the MBH.

In order to establish the role of MBH Gcs in the development of Gc-induced metabolic syndrome, GR was knocked down in the MBH of GR flox mice. A number of techniques were optimised to verify the knockdown of GR in the Cre-infected MBH. In the GR floxed mouse line used, the loxP sites are orientated in the same direction either side of exon 3, therefore in the presence of Cre recombinase, exon 3 is deleted, inactivating the GR gene (figure 5.1).

Stereotaxic recovery surgery was used to deliver the Cre recombinase enzyme directly into the MBH of GR flox mice. To establish that exon 3 was excised and that GR was no longer functional in the presence of Cre recombinase, micro-punch dissection, laser capture micro-dissection, mRNA expression quantification and immunofluorescence were used.

**Figure 5.1: Cre-lox technology used to knockdown glucocorticoid receptor in the MBH.**

Cre recombinase proteins bind to the loxP sites either side of Nr3c1 exon 3 (glucocorticoid receptor) forming a dimer. The two dimers bind to form a tetramer. The DNA is then cleaved at both loxP sites by the Cre recombinase protein excising exon 3 from the gene.
Furthermore, to establish whether the Cre recombinase enzyme injected into the MBH of GR flox mice was functional, bilateral AAV-Cre injections into the MBH of ROSA26-EYFP mice were completed. Cre and YFP antibody detection within the same region indicates that the Cre recombinase enzyme is functional.

3. **To determine whether GR knockdown in the MBH protects against Gc-induced metabolic syndrome.**

To establish the role of hypothalamic Gcs in the development of Gc-induced metabolic syndrome, GR was knocked down within the MBH of GR flox mice. In order to specifically knock down the GR within the MBH, stereotaxic recovery surgery was used to deliver the Cre recombinase enzyme directly into the MBH. To determine whether GR knockdown within the MBH is beneficial against the development of Gc-induced metabolic syndrome AAV-Cre injected mice were challenged with corticosterone treatment and phenotypically characterised during the treatment period.

4. **To investigate if knockdown of the GR in the MBH protects against weight gain over time.**

Body weight and food intake alter as mice age, leading to a gain in body weight. Although the mechanisms behind this are unknown, the plasticity of the energy-regulatory neuronal network may drive the development of obesity with time. Furthermore, the effects of Gcs on the energy-regulatory network over time are unknown. Therefore, to establish the effect of Gcs within the MBH over a longer time period, GR was knocked down within the MBH using AAV-Cre injections. Over a 6 month period, the objective was to phenotypically characterise the AAV-Cre injected GR flox mice through monitoring of body weight, food intake, and energy expenditure.
5.2 Methods

5.2.1 Transgenic mice

**GR flox**
GR flox reporter mice (B6.129P2-Nr3c1^tmGsc/leg, EMMA strain #02124, C57BL/6J background, Tronche et al., 1999) express loxP sites either side of exon 3, the DNA-binding domain, of the glucocorticoid receptor (\textit{Nr3c1}). In the presence of Cre recombinase exon 3 is deleted preventing the translation of functional GR. Mice were true bred by crossing male and female GR flox mice.

**ROSA26-EYFP**
ROSA26-EYFP reporter mice (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J (JAX strain #006148; C57BL/6J background; Srinivas et al., 2001)) express an enhanced yellow fluorescent protein (EYFP) gene, inserted into the Gt(ROSA)26Sor locus (a kind gift from Prof. Simon Luckman, University of Manchester). The expression of EYFP is prevented by an upstream STOP sequence which is flanked by two \textit{loxP} sites. In the presence of Cre recombinase the STOP sequence is deleted and EYFP is expressed.

5.2.2 Genotyping of GR flox mice
All DNA extraction and genotyping was performed by Miss TJ Allen. DNA was extracted for genotyping from ear snips or liver of all GR flox mice using KAPA2G mouse genotyping kit according to manufacturer's instructions (Kapa Biosystems). DNA was extracted in 1x KAPA express extract buffer, supplemented with KAPA express extract enzyme (2µl/reaction). Samples were lysed for 10 minutes at 75°C followed by enzyme inactivation 95°C for 5 minutes. Samples were diluted 1:10 with 10mM Tris-HCl for PCR.

Genotyping PCR was performed using GoTaq Hot Start Polymerase kit (Promega). GR PCR master mix was prepared according to the manufacturer's instructions (23µl per reaction) and combined with 2µl gDNA in PCR tubes. Primer sequences and thermocycler conditions are described in table 5.1 and 5.2 respectively.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward (5’-3’)</td>
<td>5’- GGCATGCACATTACTGGGCTTCT -3’</td>
</tr>
<tr>
<td>Reverse 1 (5’-3’)</td>
<td>5’- GTGTAGCAGCCAGCTTACAGGA -3’</td>
</tr>
<tr>
<td>Reverse 2 (5’-3’)</td>
<td>5’- CCTTCTCATTCCATGTCAGCATG -3’</td>
</tr>
</tbody>
</table>

\textit{Table 5.1: Primer sequences for genotyping of GR flox mice.}
Table 5.2: GR flox genotyping polymerase chain reaction conditions.

<table>
<thead>
<tr>
<th>Cycling step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation/Melting</td>
<td>95</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Repeat 35 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>63</td>
<td>1 minute</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1 minute</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>12</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

PCR products were run on a 2% agarose gel (section 2.2.3) for 1 hour at 100V. Agarose gels were visualised using a Gel doc (Biorad) and homozygous GR flox mice were identified by a single band at 275bp, wildtype mice identified by a single band at 225bp and heterozygote GR flox mice were identified by two bands at 225bp and 275bp. Only GR flox mice were taken forward for analysis.

5.2.3 Stereotaxic Injections

GR flox and ROSA26-EYFP mice were singly housed for 1 week before surgery and throughout the studies. Mice (22-34g, approximately 10 weeks of age) were initially anesthetised under 3% isoflurane in medical oxygen in an anaesthesia box. The animal's head was shaved and securely placed into a stereotaxic frame. Depth of anaesthesia was maintained at 1.5–2% isoflurane in medical oxygen, dependent of loss of foot pinch response and breathing rhythmicity. Throughout the procedure gaseous anaesthesia was maintained through the attachment of a nose cone to the stereotaxic frame (Kopf Instruments, Bilaney Consultants).

Iodine (10% w/w cutaneous solution, Ecolab) was used to clean the shaved head and an incision was made to expose the underlying skull and bregma. The skull was levelled so that bregma and lambda were less than ±0.1mm recorded on the digital display unit connected to the stereotaxic frame. Bilateral burr holes were drilled ~0.5mm in diameter (Fine Science Tools) at -1.5mm, ±0.3mm from Bregma according to Allen Brain atlas.

A 10μl Hamilton syringe and needle (33 gauge, Thermo Fisher) was attached to the stereotaxic frame filled with either AAV7-CAG-oCRE (AAV-Cre) vectors (titer 1.7e13 vg/ml, donated kindly by Fatima Bosch, Universitat Autònoma de Barcelona, Spain) or AAV7-CAG-GFP (AAV-GFP) vectors (titer 9.3e12 vg/ml, donated kindly by Fatima Bosch,
The Hamilton syringe was positioned according to the bregma co-ordinates and lowered slowly to a depth of -6.2mm before raising to -6.0mm. At depth of -6.0mm, 100nl of virus was slowly injected following which the needle was allowed to rest for 10 minutes before being raised in a stepwise manner. To prevent sharp changes in pressure the needle was raised slowly with 2 minute pauses at -4.0mm and -2.0mm.

Sterile saline (0.9% NaCl) was applied to the skull to thoroughly clean the incision site before the incision was closed using Vetbond glue (3M Science). The mouse was given a subcutaneous injection of Buprenorphine (2mg/kg) and topical lidocaine (5% EMLA) was applied to the wound before the removal of isoflurane. The mice were allowed to recover in a 37°C incubator and once fully recovered from anaesthesia and self-sufficient at obtaining food and water the mouse was returned individually housed to the BSF unit, University of Manchester. To ensure the mice were consuming food and water post-surgery, body weight was monitored daily for 1 week post-surgery. Table 5.3 describes the different cohorts of AAV-injected mice and their treatment during the study.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Injection</th>
<th>Treatment</th>
<th>Duration</th>
<th>Group size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR flox</td>
<td>AAV-Cre or AAV-GFP</td>
<td>Corticosterone (75µg/ml) or Vehicle (1% EtOH) in the drinking water</td>
<td>3 weeks</td>
<td>n=10-11 per group</td>
</tr>
<tr>
<td>GR flox</td>
<td>AAV-Cre or AAV-GFP</td>
<td>Phenotypically monitored without Cort treatment</td>
<td>6 months</td>
<td>n=8 per group</td>
</tr>
<tr>
<td>GR flox</td>
<td>AAV-Cre or AAV-GFP</td>
<td>Monitoring of energy expenditure without Cort treatment</td>
<td>15 weeks</td>
<td>n=7-8 per group</td>
</tr>
<tr>
<td>GR flox</td>
<td>AAV-Cre or AAV-GFP</td>
<td>Confirmation of GR knockdown</td>
<td>2 weeks</td>
<td>n=3-5 per group</td>
</tr>
<tr>
<td>ROSA26-EYFP</td>
<td>AAV-Cre</td>
<td>Phenotypically monitored without Cort treatment</td>
<td>3 weeks</td>
<td>n=4</td>
</tr>
</tbody>
</table>

*Table 5.3: Summary of AAV injections and treatment studies.*

### 5.2.4 Three week corticosterone treatment

Two weeks post-surgery, body weight, food intake, and water intake were monitored twice weekly for baseline measurements. AAV-injected mice were then randomly assigned either corticosterone (75µg/ml, Cort n=11, Cre-Cort n=11) dissolved in ethanol or 1% ethanol (Vehicle n=10, Cre n=11) in the drinking water for three weeks (table 5.4).
### Table 5.4: Three week Cort treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Injection</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>AAV-GFP</td>
<td>1% Ethanol</td>
</tr>
<tr>
<td>Cort</td>
<td>AAV-GFP</td>
<td>75μg/ml Cort</td>
</tr>
<tr>
<td>Cre</td>
<td>AAV-Cre</td>
<td>1% Ethanol</td>
</tr>
<tr>
<td>Cre-Cort</td>
<td>AAV-Cre</td>
<td>75μg/ml Cort</td>
</tr>
</tbody>
</table>

Throughout the three week treatment, body weight, food intake, and water intake were measured twice weekly. At the end of the study mice were culled by rising CO2 at approximately 10am and tissues were harvested, weighed, and snap frozen on dry ice for future analysis.

#### 5.2.5 Phenotypic monitoring of mice without Cort treatment

AAV-Cre and AAV-GFP mice (n=8 per group) were allowed to recover for two weeks post-surgery before phenotypic monitoring over a 6 month period. Body weight, food intake, and water intake were monitored twice weekly at 10am. Mice were fed standard chow and water *ad libitum* throughout the experiment.

Glucose measurements were taken from tail-prick micro-sampling every 4 weeks as described in section 2.1.2. At the end of the study mice were culled by rising CO2 and tissues were harvested, weighed, and snap frozen.

#### 5.2.6 Indirect Calorimetry

AAV-Cre and AAV-GFP mice (n=7-8 per group) were allowed to recover for two weeks post-surgery. Body weight, food intake, and water intake were monitored twice weekly at 10am for 15 weeks. Mice were fed standard chow and water *ad libitum* throughout the experiment. Indirect calorimetry cages (Columbus Instruments, Columbus) were used to measure metabolic gases (oxygen consumption, VO2 and carbon dioxide production, CO2) at three time points across the 15 week study. During indirect calorimetry recording mice were moved into calorimetric cages and allowed to acclimatise to these cages for 48 hours before recording for 72 hours. Measurements were taken during weeks 2, 10 and 15 of the study. Oxygen consumption (VO2) carbon dioxide production (CO2) and
respiratory quotient (RQ) were measured every 10 minutes. At the end of the study mice were culled by rising CO₂ and tissues were harvested, weighed, and snap frozen.

5.2.7 Micro-punch mediobasal hypothalamus dissection

The lateral side of the left hemisphere was cut to allow visualisation of the brain sections. The cerebellum was cut to provide a flat service to mount whole snap-frozen brains on a cryostat using OCT. Sections (14µm) were taken from the beginning of the hypothalamus until the ARC, cresyl violet stain was used to visualise mouse brain architecture (described in section 5.2.8). On positive confirmation of the beginning of the ARC, three 14µm sections were placed onto superfrost plus slides and stored at -80°C for future immunofluorescence. The ARC was punched using a sample corer 0.5mm diameter (Interfocus, Fine Science Tools). The tissue was immediately placed in RLT buffer and frozen on dry ice until RNA-extraction at the end of the day (Qiagen, section 2.2.1).

5.2.8 Cresyl violet staining

Sections (14µm) taken during micro-punching (section 5.2.7) were hydrated for 30 seconds in decreasing concentrations of ethanol (90–70%). Sections were immersed in 20% cresyl violet (Histology department, University of Manchester) for 30 seconds. Cresyl violet solution was washed off in water before dehydrating in increasing concentrations of ethanol for 30 seconds each (70–100%). Slides were washed in xylene, 1 minute, before mounted with DPX (CellPath).

5.2.9 Immunofluorescence

Dual GR-Cre immunofluorescence

Fourteen micrometre thick sections taken immediately before the micro-punch were stained for dual GR-Cre immunofluorescence. Before GR-Cre immunofluorescence, sections were allowed to defrost. Each section was isolated using a Barrier pen (Ambion) and sections were fixed in 4% paraformaldehyde for 20 minutes. Post-fixation sections were washed 3 x 5 minutes in 1xPBS and permeabilised in 0.25% Triton-X (diluted in 1xPBS, peroxidase block) for 10 minutes. The peroxidase block was washed off in 3 x 5 minutes 1xPBS and incubated for 1 hour at room temperature in 10% Seablock (Calbiochem) (diluted in 1xPBS). Seablock was removed and each section was incubated in 100µl primary antibody; GR anti-mouse monoclonal, 1:200, 3 hours at RT (GR32L, Calbiochem), Cre anti-rabbit polyclonal, 1:1000, overnight at 4°C (69050-3, Novagen).
Primary antibodies were added consecutively. Primary antibody was washed off in 3 x 5 minutes 1xPBS, and secondary antibodies were added consecutively. Secondary antibodies (Life technologies) were diluted in 10% Seablock (Calbiochem); anti-mouse Alexa Fluor 594, 1:100, room temperature for 1 hour, anti-rabbit Alexa Fluor 488, 1:100, for 1 hour at room temperature. Secondary antibodies were washed off 3 x 5 minutes 1xPBS and endogenous brain fluorescence was blocked by 10 minutes incubation in sudan black (0.1%). Sudan black was washed off in 3 x 5 minutes 1xPBS washes before mounted with Prolong Gold antifade mountant with DAPI (Thermo Scientific). Sections were allowed to dry at room temperature in the dark before imaging.

Consecutive GR-Cre immunofluorescence
Whole brains taken from AAV-Cre injected GR flox mice were mounted on the cryostat as previously described section 5.2.7. Sections (14µm) were taken throughout the whole hypothalamus and placed on alternate superfrost plus slides. Consecutive sections were stained for either Cre or GR as described above in Dual GR-Cre immunofluorescence. Primary antibodies were incubated overnight at 4°C; GR anti-rabbit polyclonal, 1:200 (M-20, Santa Cruz), Cre anti-rabbit polyclonal 1:2000 (69050-3, Novagen). Secondary antibodies (Life Technologies) were incubated for 1 hour at room temperature; GR anti-rabbit Alexa Fluor 488, 1:100, Cre anti-rabbit Alexa Fluor 594, 1:100.

YFP immunofluorescence
Whole brains taken from AAV-Cre injected ROSA26-EYFP mice were mounted on the cryostat as previously described section 5.2.7. Sections (14µm) were taken throughout the whole hypothalamus and placed on alternate superfrost plus slides. Consecutive sections were stained for either Cre or YFP (described in Dual GR-Cre immunofluorescence). Primary antibodies were incubated overnight at 4°C; GFP anti-chicken polyclonal, 1:1000 (ab13970, abcam); Cre anti-rabbit polyclonal 1:2000 (69050-3, Novagen). Secondary antibodies were incubated at room temperature (Life Technologies); anti-chicken Alexa Fluor 594 1:300, 3 hours; anti-rabbit Alexa Fluor 594, 1:100 1 hour.

5.2.10 Laser-Capture Micro-dissection
AAV-Cre injected GR flox mice were mounted on the cryostat as previously described in section 5.2.7. Sections (30µm) were taken throughout the hypothalamus and consecutive sections were placed on superfrost plus slides and MMI Membrane slide (Molecular Machines and Industries). Sections were allowed to air dry before storage at -80°C. Samples were kept on dry ice before RNase free cresyl violet staining was completed to
aid laser micro-dissection (section 5.2.10.1). Samples were laser micro-dissected within 2-3 hours of cresyl violet staining. Cre immunofluorescence and cresyl violet staining (section 5.2.9, 5.2.10.1) were used to guide which sections should be dissected. Adhesive MMI Isolation caps were used to collect PVN and ARC samples. Samples were collected in 100µl RLT-Plus buffer (Qiagen) and stored on dry ice. RNA extraction was completed at the end of the day on all samples (section 2.2.1).

5.2.10.1 RNase free cresyl violet

Sections (30µm) mounted on MMI Membrane slides for laser capture micro-dissection were stored on dry ice before cresyl violet staining (Ambion). All plastic coplin jars were cleaned with RNase Zap (Thermo Scientific) before use. Two slides at a time were rehydrated in decreasing concentrations of RNA-free ethanol for 30 seconds (100–50%, diluted in DEPC-treated water). Cresyl violet stain (100µl of 20%) was pipetted onto slides and incubated for 30 seconds (Ambion LCM staining kit). Slides were then dehydrated in increasing concentrations of ethanol for 30 seconds (50–100%, diluted in DEPC-treated water). Slides were left to air dry for 5 minutes and taken immediately to the laser capture microscope for micro-dissection. A maximum of 6 slides were completed per batch to allow micro-dissection within 2 hours.

5.2.11 Two-step Quantitative Reverse Transcription Polymerase Chain Reaction

5.2.11.1 RNA conversion to cDNA

RNA from micro-dissected samples (micro-punches and laser capture micro-dissections) underwent reverse transcription (RT) to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems). Equal quantities of RNA and mastermix (7µl each for RT positive reaction, 5µl each for RT negative reaction) were added to individual PCR tubes for conversion to cDNA (table 5.5). Reverse transcription was run on a DNA engine Tetrad PTC-225 thermal cycler. Samples were incubated at 25°C for 10 minutes, 37°C for 120 minutes and the reaction was ended by incubating at 85°C for 5 minutes.
<table>
<thead>
<tr>
<th>Mastermix component</th>
<th>RT +ve (per reaction)</th>
<th>RT –ve (per reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT buffer</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>25x NTP (100nM)</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>10x Primers</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Multiscribe RT</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease free-water</td>
<td>3.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Table 5.5: RNA to cDNA reverse transcription mastermix.**

### 5.2.11.2 Pre-amplification of cDNA

cDNA was amplified using TaqMan PreAmp Master Mix Kit (Applied Biosystems) for a pre-set list of genes before quantitative analysis (table 5.6). Taqman probes and SYBR primers (table 5.6) were pre-amplified at a final concentration (0.2X, each assay) under standard conditions (table 5.7).

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre</td>
<td>Cre</td>
<td>GAGAACCGACTTCGACCAGG</td>
<td>ATCTCGGCGATTCTCAGCAG</td>
</tr>
<tr>
<td>TBP</td>
<td>Tbp</td>
<td>GGGAGAATCATGGACCAGAA</td>
<td>GATGGGAATTCCAGGAGTCA</td>
</tr>
<tr>
<td><strong>Taqman</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBP</td>
<td>Tbp</td>
<td>Mm00446973_m1</td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>Hprt</td>
<td>Mm03024075_m1</td>
<td></td>
</tr>
<tr>
<td>GR exon 3</td>
<td>Nr3c1 exon 3</td>
<td>Mm01260496_m1</td>
<td></td>
</tr>
<tr>
<td>GR exon 4</td>
<td>Nr3c1 exon 4</td>
<td>Mm01260496_m1</td>
<td></td>
</tr>
<tr>
<td>GR exon 8/9</td>
<td>Nr3c1 exon 8</td>
<td>Mm01260500_m1</td>
<td></td>
</tr>
<tr>
<td>GR exon 1-2</td>
<td>Nr3c1 exon 1-2</td>
<td>Mm00433832_m1</td>
<td></td>
</tr>
<tr>
<td>Gilz</td>
<td>Tsc22d3</td>
<td>Mm01304886_g1</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.6: Mouse primer sequences and Taqman probes for genes quantified by qRT-PCR.**
<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>10 minutes</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>15 seconds</td>
<td>14</td>
</tr>
<tr>
<td>60</td>
<td>4 minutes</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.7: cDNA pre-amplification conditions.

5.2.11.3 Quantitative Reverse Transcription Polymerase Chain Reaction

Amplified cDNA was quantified for each gene using two-step qRT-PCR. All samples were run in triplicate on a 384 well plate ABI 7900 Sequence Detection system instrument and software (Applied Biosystems). Standard cycling conditions with GoTaq qPCR Mastermix (Promega) and Taqman Universal mastermix II (Life Technologies) was performed to quantify relative gene expression (table 5.8, 5.9). Gene expression levels were normalised to a housekeeping gene using standard curve analysis. Standards were prepared from pooled pre-amplified cDNA. Pre-amplified cDNA was pooled and diluted 1:5 in DEPC-treated water to create a stock top standard. The diluted pooled cDNA was then serially diluted 1:2 in DEPC-treated water, to create a standard curve ranging from 1:5 - 1:10240.

All pre-amplified cDNA samples were diluted 1:40 (Hprt, Tbp), 1:320 (Tsc22d3, Nr3c1 exon 3, Nr3c1 exon 4, Nr3c1 exon 8/9, Nr3c1 exon 1-2) or 1:1280 (Cre) in DEPC-treated water and run against standard curve. RT negative samples were run alongside samples in singlicate as a control.

<table>
<thead>
<tr>
<th>Two Step qRT-PCR</th>
<th>Reaction Component</th>
<th>Volume (µl/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR, Promega</td>
<td>cDNA template</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2x Go Taq Mix</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>CxR dye</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>DEPC water</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Primers (3pmol/µl)</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Two Step qRT-PCR</th>
<th>Reaction Component</th>
<th>Volume (µl/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taqman, Life technologies</td>
<td>cDNA template</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Assay on Demand (20X)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Mastermix</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.8: Preparation of mastermix for qRT-PCR.
### Conditions for Taqman Two step qRT-PCR

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Activation</td>
<td>95</td>
<td>10 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denature</td>
<td>95</td>
<td>15 seconds</td>
<td>40</td>
</tr>
<tr>
<td>Anneal/Extend</td>
<td>60</td>
<td>1 minute</td>
<td></td>
</tr>
</tbody>
</table>

### Conditions for SYBR Two step qRT-PCR

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
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<td>Melt curve</td>
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*Table 5.9: Quantitative reverse transcription polymerase chain reaction conditions.*
5.3 Results

5.3.1 Intracranial injection of AAV7-CAG-Cre into the MBH

The intracranial injection of virus-mediated Cre recombinase (AAV-Cre) into GR flox mice knocks down the GR within the targeted region. loxP sites situated either side of exon 3 are excised in the presence of Cre recombinase, preventing the production of a functional GR. Therefore, it is necessary to optimise the bilateral intracranial injections into the MBH of GR flox mice, in order to determine the role of this region in the development of Gc-induced metabolic syndrome.

AAV7-CAG-Cre (AAV-Cre) or AAV7-CAG-GFP (AAV-GFP) virus (as a control) were injected into the MBH and after two weeks recovery AAV-Cre virus expression was identified by Cre immunofluorescence (figure 5.2A). AAV-GFP injections into the MBH were optimised as a control (figure 5.2B). The unilateral injection of AAV-GFP indicates that the intracranial injection of our AAV virus does not translocate across into the contralateral hemisphere (figure 5.2B). Further, the optimisation of bilateral intracranial injections indicated that 100nl injection each side and removal of the needle in a stepwise manner ensures the virus remains within the MBH. This targeted approach confirms that GR knockdown can be limited to the MBH without effecting GR expression in other hypothalamic regions including the PVN, which are also involved in energy regulation. Additionally, DAPI staining indicates that neither the injection of AAV-Cre or AAV-GFP virus into the MBH induced cell death.
Figure 5.2: AAV-GFP and AAV-Cre injections into the mediobasal hypothalamus of GR flox mice.

Representative immunofluorescence images of (A) bilateral AAV-Cre injection into mediobasal hypothalamus (MBH) of GR flox mice, (red = Cre, blue = DAPI). (B) Unilateral AAV-GFP injection into the MBH of GR flox mouse, (green = GFP, blue = DAPI) Scale bars; top panels 500µm, bottom panels 20µm. Third ventricle, 3V; glucocorticoid receptor, GR; green fluorescent protein, GFP.

5.3.2 Maintenance and genotyping of GR flox mice

GR flox mice were pure bred in the BSF unit at the University of Manchester. To ensure pure breeding, litters were genotyped every 10 generations. During the duration of this study, it was discovered that a ‘leaky’ gene infiltrated the pure breeding pairs, producing a range of genotypes (wildtype, heterozygote, flox homozygote). On this discovery, all of
the mice injected with either AAV-Cre or AAV-GFP were genotyped to verify their genotype. Mice that were not GR flox and therefore did not contain the loxP sites either side of exon 3 were excluded from all analysis. 7/10, AAV-GFP vehicle treated; 8/11, AAV-GFP Cort treated; 6/11, AAV-Cre vehicle treated; 10/11 AAV-Cre Cort treated were GR flox and included in future analysis.

5.3.3 Quantification of Cre mRNA expression in MBH micro-punches

In order to determine the effect of GR knockdown in our model of Cort treatment, it is necessary to establish a method to validate the successful injection of AAV-Cre into the MBH and consequently the knockdown of GR within this region. To ensure that the AAV-Cre virus ‘hit’ the MBH, micro-punch MBH dissections were taken (figure 5.3A) and Cre mRNA expression was quantified (figure 5.3B). Cre mRNA expression was present in 7/10 AAV-Cre injected mice treated with Cort (Cre-Cort) and 3/6 AAV-Cre injected mice treated with vehicle (Cre) (figure 5.3B). A CT value >30 was classified as undetectable and therefore any AAV-Cre injected mice with undetectable, Cre expression were excluded from all further gene expression and phenotypic analysis. Cre expression was not detectable in the micro-punch MBH dissections of AAV-GFP mice treated with either Cort or Vehicle.
Figure 5.3 Quantification of Cre mRNA expression in MBH micro-punch dissections.

(A) Architecture of the mouse mediobasal hypothalamus (MBH) and representative image of micro-punch region taken for mRNA expression analysis.  
(B) Cre mRNA expression was quantified in the MBH of AAV-Cre and AAV-GFP mice treated with or without Cort for 3 weeks. Samples whose CT value >30 were classified as undetectable and excluded from future analysis. (GFP, AAV-GFP & Vehicle 1%EtOH; Cort, AAV-GFP & Cort 75μg/ml; Cre, AAV-Cre & Vehicle 1%EtOH; Cre-Cort, AAV-Cre & Cort 75μg/ml). Green fluorescent protein, GFP.  
Kruskal-Wallis test, Dunn’s multiple comparisons test, ** p<0.01 vs GFP and Cort treatment groups. (See table 8.11 of appendix for F values).
5.3.4 Quantification of \textit{Nr3c1} mRNA expression in MBH micro-punches

To establish that the intracranial bilateral injection of AAV-Cre virus successfully knocked down GR in the MBH, mRNA expression of \textit{Nr3c1} (GR) was quantified in the micro-punch MBH dissections. In our GR flox mouse line, the \textit{loxP} sites are situated either side of exon 3 (figure 5.4A). Orientated in the same direction the two \textit{loxP} sites are cleaved by the \textit{Cre} recombinase enzyme deleting exon 3 and preventing the translation of \textit{Nr3c1} mRNA into a functional GR.

Taqman probes targeting exon 3 of the \textit{Nr3c1} indicate no change in \textit{Nr3c1} expression (figure 5.4B). \textit{Tsc22d3} (GILZ), a marker of GR activity, was increased in Cort-treated mice; in addition this increase was not reduced in Cre-Cort mice. This is unexpected, as \textit{Nr3c1} should be knocked down in the MBH of AAV-Cre injected GR flox mice, therefore reducing the stimulation of \textit{Tsc22d3} (figure 5.4C). Taqman probes targeting other regions of the \textit{Nr3c1} gene, identified no changes in \textit{Nr3c1} mRNA expression in AAV-Cre injected GR flox mice (figure 5.4D). No changes in \textit{Nr3c1} expression were identified when normalised against a second housekeeping gene \textit{Tbp} (data not shown).
Figure 5.4: Quantification of Nr3c1 and Tsc22d3 mRNA expression in the MBH.

(A) Schematic diagram of the glucocorticoid receptor gene (Nr3c1) indicating loxP sites situated either side of exon 3 in GR flox mice. (B) Taqman primers targeting Nr3c1 Exon 3 (C) Tsc22d3 (GILZ) and (D) Nr3c1 Exon 1-2, Nr3c1 Exon 4 and Nr3c1 Exon 8 used to quantify mRNA expression in the mediobasal hypothalamus of AAV-Cre and AAV-GFP treated with or without Cort for 3 weeks. Cre mRNA expression where CT>30 were excluded. (GFP, AAV-GFP & Vehicle 1%EtOH; Cort, AAV-GFP & Cort 75μg/ml; Cre, AAV-Cre & Vehicle 1%EtOH; Cre-Cort, AAV-Cre Cort 75μg/ml). Green fluorescent protein, GFP.

Two way ANOVA, Tukey’s multiple comparisons test * p<0.05, ** p<0.01. (See table 8.12 of appendix for F values).
5.3.5 GR and Cre expression visualised by dual immunofluorescence

In order to identify whether the GR was co-localised in the same region as Cre expression, dual GR-Cre immunofluorescence was completed on frozen sections either side of the MBH micro-punch. GR expression is not altered throughout the brain by the addition of loxP sites either side of exon 3 in GR flox mice (Tronche et al., 1999; Tronche et al., 1999). In the presence of functional Cre recombinase, the GR should be knocked down, and therefore GR protein should not be expressed in Cre-infected MBH (figure 5.5A).

In a proportion of GR flox mice injected with AAV-Cre and treated with either Cort or Vehicle for three weeks, Cre expression was visualised immediately prior to the MBH micro-punch (5/10; representative images in appendix 8.1). In a Cre-Cort mouse, Cre expression was only visualised in one hemisphere despite a bilateral injection (figure 5.5A). This unilateral expression allows the direct comparison of GR expression between Cre-infected and non-infected regions in the MBH (figure 5.5B). Higher magnification images identify that GR expression was unaltered between Cre-infected and non-infected MBH (figure 5.5C). Similarly, bilateral injection of GFP did not alter GR expression between the GFP infected MBH and the non-infected cortex (appendix 8.2; representative images of AAV-GFP injected GR flox mice).
Figure 5.5: Dual GR-Cre immunofluorescence in AAV-Cre injected mice.

(A) Schematic representation of glucocorticoid receptor (GR) expression within the brain (left panel, red = GR), in the presence of Cre recombinase GR should not be expressed (right panel, green = cre recombinase, red = GR). (B) Representative images of Cre and GR expression in the mediobasal hypothalamus (MBH), scale bars = 200µm (C) Higher magnification images of GR and Cre expression in the left hemisphere and right hemisphere of the MBH (scale bar = 100µm) Green = Cre, Red = GR, Blue = DAPI. Images are representative of 2-4 sections/animal. Third ventricle, 3V.
Detection of GR by the mouse monoclonal antibody (Merck Millipore, figure 5.5B) was weak throughout the brain (figure 5.5). The poor detection of GR prevents clear visualisation of GR expression in both non Cre-infected regions and the Cre-infected MBH. Therefore, the antibody detection of GR was optimised using a different antibody (Santa Cruz), which has previously been used to quantify brain GR expression in similar models (Laryea et al., 2013; Shibata et al., 2016). As both anti-GR (Santa Cruz) and anti-Cre (Novagen) antibodies were raised against the same species, the immunofluorescence was performed on consecutive sections (figure 5.6A). A separate cohort of GR flox mice was injected with AAV-Cre to determine that GR expression was reduced in the presence of Cre (n=2, appendix 8.3). GR expression was detected throughout the brain, and was not altered in the same region as Cre recombinase expression compared with non-Cre infected regions (figure 5.6B, C). DAPI staining was unchanged in all sections.
**Figure 5.6: Cre and GR immunofluorescence in consecutive brain sections.**

(A) Schematic representation of glucocorticoid receptor (GR) expression within the brain (left panel, green = GR), in the presence of Cre recombinase GR should not be expressed (red = cre recombinase, green = GR). (B) Representative images of Cre and GR in the mediobasal hypothalamus (MBH), scale bar = 100µm (C) Higher magnification images of GR and Cre expression in the left hemisphere and right hemisphere of the MBH (scale bar = 100µm) Red = Cre, Green = GR, Blue = DAPI. Images represent three sections/animal. Third ventricle, 3V.
5.3.6 Quantification of \textit{Nr3c1} mRNA expression in Cre-infected MBH

In order to understand why the \textit{Nr3c1} expression was not decreased in the MBH micro-punches or immunofluorescence sections, a separate cohort of GR flox mice were injected with either AAV-Cre (n=3) or AAV-GFP (n=3). As GR is ubiquitously expressed, any extra tissue containing GR collected from the surrounding AAV target site (MBH) may conceal the Cre recombinase knockdown of GR. Therefore, Cre-infected regions were dissected by laser capture micro-dissection, in order to reduce the dissection of surrounding non-infected regions.

Cre immunofluorescence (figure 5.7A) was used as a guide to dissect Cre-infected regions. Of the three AAV-Cre injected animals, one did not express Cre recombinase within the MBH, and therefore was included in the study as a control (AAV-Cre = 2, AAV-GFP = 4). Cresyl violet staining was used to show the architecture of the brain, which aided the dissection of the PVN and MBH (figure 5.7A). The PVN was dissected from approximately six 30µm sections, and the Cre-infected MBH was dissected from approximately twelve 30µm sections. \textit{Cre} mRNA expression indicated that Cre recombinase was present only in the MBH of AAV-Cre injected animals (figure 5.7B). \textit{Nr3c1} Exon 3 and \textit{Tsc22d3} (GILZ, a marker of GR activity) expression did not alter between AAV-Cre and AAV-GFP injected GR flox mice (figure 5.7C and D).
Figure 5.7: Validation of GR knockdown in AAV-Cre and AAV-GFP injected mice by laser capture micro-dissection

(A) Architecture of the mouse hypothalamus (left panel), Cre immunofluorescence (middle panel, red = Cre, blue = DAPI, scale bar 100 µm) and cresyl violet staining (right panel, scale bar 200µm) used to guide dissection of the paraventricular nucleus (PVN) and Cre-infected regions of the mediobasal hypothalamus (MBH) (B) Cre (C) Nr3c1 (GR) and (D) Tsc22d3 (GLILZ) mRNA expression was quantified in the PVN and MBH in AAV-Cre and AAV-GFP mice two weeks post-surgery. Green fluorescent protein, GFP.

AAV-GFP; n=4, AAV-Cre; n=2
5.3.7 AAV-Cre injections into the MBH of ROSA26-EYFP mice

To confirm that the AAV-Cre virus used was functionally active, bilateral intracranial injections were performed on four ROSA26-EYFP mice. If functional, the Cre recombinase enzyme injected into the MBH would cleave the \textit{loxP} sites situated either side of the stop codon driving the YFP expression (figure 5.8A). Therefore, YFP fluorescence should be present in the same region as Cre expression.

The immunofluorescence detecting Cre expression indicated that the AAV-Cre injection successfully targeted the MBH (figure 5.8B). YFP expression was visualised by YFP immunofluorescence in consecutive sections to those containing Cre expression (figure 5.8B). YFP expression was located in the same region as Cre expression, indicating that when the Cre virus was present it was functionally active (figure 5.8B). Cre and YFP immunofluorescence was visualised across the MBH in three of the four AAV-Cre injected ROSA26-EYFP mice. Expression of both Cre and YFP was visualised in twelve – sixteen 30\textmu m sections per animal (appendix 8.4; representative images of each AAV-Cre injected ROSA26-EYFP mouse). Images were taken in the cortex, using the same exposure times, as an internal negative control neither Cre nor YFP expression was visualised within this region (figure 5.8C).
Figure 5.8: Cre and YFP immunofluorescence in AAV-Cre injected ROSA26-EYFP mice.

(A) Schematic diagram of Cre recombinase action to drive YFP expression. (B) Representative images of Cre recombinase and YFP expression across consecutive sections in the mediobasal hypothalamus (MBH) and (C) cortex from AAV-Cre injected ROSA26-EYFP mice. Red = Cre, Green = YFP, Blue = DAPI. Scale bars = 100μm. Images are representative of 12-16 sections per mouse. Third ventricle, 3V; yellow fluorescent protein, YFP.
5.3.8 The effect of AAV-Cre injection in GR flox mice on body weight and food intake after three weeks Cort treatment

In parallel with the verification of GR knockdown in AAV-Cre injected GR flox mice, phenotypic analysis was conducted of body weight and food intake throughout the three week Cort treatment. Intracranial injections of AAV-Cre into the MBH of ROSA26-EYFP mice identified that the AAV-Cre virus used to determine the role of MBH Gcs in the development of Gc-induced metabolic syndrome is functionally active. Furthermore, the GR flox mouse line used in this study, has been used in genetic Cre-lox crosses to successfully knockdown GR (Laryea et al., 2013; Tronche et al., 1998). Therefore, the successful injection of Cre recombinase into the MBH, should cleave the loxP sites either side of Nr3c1 Exon 3 rendering the GR protein non-functional within the Cre-infected MBH.

Two weeks post-surgery AAV-injected mice were deemed fully recovered. Body weight and food intake were measured across two weeks to ensure a stable baseline before Cort treatment (data not shown). AAV-Cre and AAV-GFP injected mice were then randomly assigned Cort (75μg/ml) or vehicle (1%EtOH) administered in the drinking water for 3 weeks (table 5.4). Body weight and food intake were monitored throughout the Cort treatment periods. AAV-Cre injected mice whose Cre mRNA expression was undetectable in MBH micro-punch dissections (figure 5.3B) were excluded from phenotypic analysis.

Mice injected with AAV-GFP and treated with Cort (Cort) increased body weight (figure 5.9A, B) and food intake (figure 5.9F, G) over the three week period compared with control groups (GFP and Cre, AAV-Cre injected treated with vehicle). Mice injected with AAV-Cre and treated with Cort (Cre-Cort) had a trend towards increase in body weight but this increase was not statistically different from either Cort or Cre treatment groups (figure 5.9B, D, E). Cre-Cort mice increased food intake compared with GFP treated mice, but did not alter compared with Cre or Cort treated mice (figure 5.9G, I, J). Mice injected with AAV-Cre and treated with vehicle (Cre) did not alter body weight or food intake over the three week period (figure 5.9A, B, F, G).
Figure 5.9: Phenotypic analysis of AAV-GFP and AAV-Cre injected mice on Cort treatment.

Mice injected with AAV-Cre or AAV-GFP were treated with or without Cort treatment for 3 weeks. (A) Body weight, (B-E) percentage body weight change, (F) food intake and (G-J) percentage food intake change were monitored throughout the treatment period. GFP, AAV-GFP & Vehicle 1%EtOH; Cort, AAV-GFP & Cort 75μg/ml; Cre, AAV-Cre & Vehicle 1%EtOH; Cre-Cort, AAV-Cre& Cort 75μg/ml. Green fluorescent protein, GFP.

Two way ANOVA, Sidak's multiple comparisons test * p<0.05, **<0.01 ***<0.001 vs GFP and Cre (See table 8.13 of appendix for F values).
Both Cort and Cre-Cort groups had trends towards increases in the three fat pads and liver weights at the end of the study compared to GFP and Cre treatment groups (figure 5.10A, B and C). A trend towards an increase in fed glucose was measured immediately prior to death in Cort and Cre-Cort treatment groups (figure 5.10D). The overall effect of Cort at the end of the 3 week treatment period increased body fat pad mass (subcutaneous and mesenteric), final body weight, liver weight and glucose compared with vehicle treated mice (two-way ANOVA, * p<0.05).

**Figure 5.10: The effect of AAV-Cre and AAV-GFP on fat pad, liver mass and glucose**

Mice injected with AAV-Cre and AAV-GFP were treated with or without Cort for 3 weeks. (A) Epididymal, subcutaneous and mesenteric fat pad mass was weighed at the end of the study. (B) Body weight (BW) was recorded at the end of the study. (C) Whole liver was weighed at the end of the study. (D) Fed glucose measurements were taken immediately prior to death at day 21 Cort treatment. GFP, AAV-GFP & Vehicle 1%EtOH; Cort, AAV-GFP & Cort 75μg/ml; Cre, AAV-Cre & Vehicle 1%EtOH; Cre-Cort, AAV-Cre & Cort 75μg/ml. Green fluorescent protein, GFP. (See table 8.14 of appendix for F values). Two Way ANOVA, Tukey’s multiple comparisons p>0.05
5.3.9 The effect of AAV-Cre injections on Cort intake

As Cort treatment was administered in the drinking water (75μg/ml), water intake was monitored throughout the treatment period to ensure that AAV-Cre bilateral injections into the MBH did not alter intake of the Cort treatment. Cort increased water intake throughout the study. Cre-Cort treatment had a trend towards an increase in water intake at day 21 (figure 5.11A). Adrenal weight measured at the end of the 21 day treatment resulted in a trend towards a decrease in both Cort treatment groups (figure 5.11B, Cort and Cre-Cort). Spleen weight was decreased in both Cort treatment groups, (figure 5.11C, Cort and Cre-Cort) after three weeks treatment. AAV-Cre injection alone (Cre) did not affect water intake, adrenal, or spleen weight.

![Graph A](image.png)

**Figure 5.11: The effect of AAV-Cre injection on Cort intake**

GR flox mice injected with AAV-Cre or AAV-GFP were treated with vehicle (1%EtOH) or Cort (75μg/ml) for three weeks. (A) Water intake was monitored throughout the three week treatment period. (B) Adrenal and (C) spleen weight was measured at the end of the three week treatment period. GFP, AAV-GFP & Vehicle 1%EtOH; Cort, AAV-GFP & Cort 75μg/ml; Cre, AAV-Cre & Vehicle 1%EtOH; Cre-Cort, AAV-Cre & Cort 75μg/ml. Green fluorescent protein, GFP. (See table 8.15 of appendix for F values).

Water intake, Two way ANOVA Sidak’s multiple comparisons, * p<0.05 vs GFP, Cre, ** p<0.01 vs GFP. Adrenal and spleen, Two way ANOVA Tukey’s multiple comparisons *** p<0.001.
5.3.10 The effect of AAV-Cre injection on body weight and food intake over time

To establish the effect of hypothalamic Gcs over time, a cohort of GR flox mice were bilaterally injected with either AAV-Cre (n=10) or AAV-GFP (n=9). AAV injected animals were phenotypically monitored over a 6 month period without exogenous Cort treatment. AAV-Cre injected mice did not gain as much weight over the 6 month period as AAV-GFP injected mice (figure 5.12A, two way ANOVA, * p<0.05). Fat pad mass was unaltered at the end of the study between treatment groups (figure 5.12B). Food intake was unaltered between treatment groups throughout the 6 month period (figure 5.12C).
Figure 5.12: Phenotypic analysis of AAV-Cre injected animals over 6 months.
GR flox mice received bilateral injections of either AAV-Cre (n=9) or AAV-GFP (n=8) into the mediobasal hypothalamus (MBH). Body weight and food intake were measured twice weekly throughout the 6 month period. (A) Percentage body weight change (B) epididymal (Epi), mesenteric (Mes) and subcutaneous (Subcut) fat pad mass was measured at the end of the study. (C) Percentage food intake change. Green fluorescent protein, GFP.

%Body weight change and %food intake change, Two Way ANOVA, Sidak’s multiple comparisons test, * p<0.05 vs AAV-GFP, Fat pad mass, Unpaired t-test, p>0.05 (See table 8.16 of appendix for F values).
5.3.11 The effect of AAV-Cre and AAV-GFP MBH injection on tissue weight over time.

To ensure that the AAV-Cre virus injected into the MBH had not translocated to the PVN, adrenal mass was measured at the end of the sixth month monitoring period. Adrenal mass was unaltered at the end of the 6 months (figure 5.13A). Whereas, spleen weight was decreased after 6 months (figure 5.13B). BAT and skeletal muscle weight were unaltered, while liver weight was decreased at the end of the study (figure 5.13C).

**Figure 5.13: The effect of AAV-Cre on peripheral tissue weight after 6 months phenotypic monitoring**

GR flox mice were injected with either AAV-Cre (n=9) or AAV-GFP (n=8) into the mediobasal hypothalamus and monitored over 6 months. At the end of the study (A) adrenal weight (B) spleen (C) brown adipose tissue (BAT), skeletal muscle and liver weight were measured. Green fluorescent protein, GFP.

Unpaired t-test *p<0.05, **p<0.01.
Fed glucose was monitored every four weeks over the sixth month period, AAV-Cre mice had a trend towards a decrease in fed glucose during the monitoring period (figure 5.14).

*Figure 5.14: The effect of AAV-Cre injection on fed glucose monitored monthly over the sixth month period.*

Every four weeks fed glucose was measured in tail-prick micro samples from AAV-GFP (n=8) and AAV-Cre (n=9) injected mice. Green fluorescent protein, GFP. Unpaired t-test **p<0.01
5.3.12 The effect of AAV-Cre and AAV-GFP injections on energy expenditure over time.

To determine whether the reduced body weight gain after AAV-Cre injection in the MBH over the 6 month monitoring period (figure 5.15A) was due to an increase in energy expenditure, this was measured using indirect calorimetry at three time points across 15 weeks. A parallel cohort of GR flox mice injected with either AAV-Cre (n=8) or AAV-GFP (n=7) were used for this study.

AAV-injected mice were allowed to recover for two weeks post surgery prior to the 15 weeks monitoring. Energy expenditure (EE) and respiratory exchange ratio (RER) were measured at three time points during the 15 week study; run 1, week 2 (days 10-13), run 2, week 10 (days 70-73); run 3, week 15 (days 101-104). Mice were placed into the indirect calorimetry cages two days prior to recordings for acclimatisation. Due to a limited number of indirect calorimetry cages, this study was run in two cohorts. Unfortunately, technical difficulties prevented cohort 1, run 1 and cohort 2, run 3 from being completed. Energy expenditure and RER were unaltered throughout the study; days 10-13 (ANCOVA p=0.921), days 70-73 (ANCOVA p=0.184), and days 101-104 (ANCOVA p=0.813).
Figure 5.15: The effect of AAV-Cre injections into the MBH on energy expenditure and respiratory exchange ratio.

Indirect calorimetry cages were used to measure energy expenditure (EE) and respiratory exchange ratio (RER) in GR flox mice were injected with either AAV-Cre or AAV-GFP virus into the mediobasal hypothalamus (MBH). (A) Days 10-13, AAV-GFP n=4, AAV-Cre n=4, (B) Day 70-73, AAV-GFP n=7, AAV-Cre n=8, (C) Days 101-104, AAV-GFP n=4, AAV-Cre n=4. Green fluorescent protein, GFP. ANCOVA analysis.
Body weight and food intake were monitored throughout the 15 week period. AAV-Cre injected mice had a trend towards decreased body weight over the 15 weeks (figure 5.16A). Fat pad mass at the end of the study was unaltered between AAV-GFP and AAV-Cre (figure 5.16B). Food intake was unaltered between treatment groups throughout the study (figure 5.16C).

At the end of the 15 week monitoring period adrenal weight was unaltered, indicating that AAV-Cre virus did not translocate to the PVN (figure 5.16D). Spleen, muscle, BAT and liver weight were not altered after the 15 week period (data not shown). Fed glucose measured at the end of the study did not alter between AAV-injected animals (data not shown).
Figure 5.16: The effect of AAV-Cre and AAV-GFP on body weight and food intake over time.

GR flox mice were injected with either AAV-Cre or AAV-GFP (n=7) AAV-Cre (n=8). Body weight and food intake were measured twice weekly throughout the 15 week study. (A) Percentage body weight change (B) epididymal (Epi), mesenteric (Mes) and subcutaneous (Subcut) measured at the end of the study (C) food intake and percentage food intake change. (D) Adrenal weight was measured at the end of the study. Green fluorescent protein, GFP.

%Body weight change, food intake and %food intake change, Two way ANOVA, Sidak’s multiple comparisons test, p>0.05. Fat pad mass, adrenal weight unpaired t-test p>0.05. (See table 8.17 of appendix for F values).
5.4 Discussion

The close proximity of the hypothalamic energy-regulatory network to the BBB makes the neurones within this region susceptible to elevated peripheral corticosterone levels. Targeting GR knockdown within the MBH, provides the opportunity to determine the role of Gc actions in the development of Gc-induced hyperphagia.

AAV-Cre injected GR flox mice were challenged with chronic Cort, to determine the involvement of hypothalamic Gcs in the development of Cort-induced obesity and hyperphagia. Through the bilateral injection of AAV-Cre into the MBH of ROSA26-EYFP mice the Cre recombinase was confirmed functional. A number of techniques, including qRT-PCR of micro-punch and laser micro-dissected regions and immunofluorescence, were employed to establish knockdown of GR in Cre-infected MBH. However, adequate detection of GR knockdown in the Cre-infected MBH was not achieved. The low sensitivity of these assays, alongside the ubiquitous expression of GR within the brain, may have prevented the quantification of GR knockdown in Cre-infected MBH.

Phenotypic monitoring throughout the Cort treatment period indicated that AAV-Cre injected Cort treated GR flox mice (Cre-Cort) were partially protected from body weight gain and hyperphagia at the end of the 3 weeks. Furthermore, GR flox mice injected with AAV-Cre and monitored over 6 months (without exogenous Cort challenge) did not gain as much weight over time as controls. Therefore, together these results suggest that Gc actions in the MBH are important in the regulation of energy homeostasis both with and without chronic exogenous Cort treatment.

5.4.1 Hypothalamic glucocorticoid actions in the development of Gc-induced metabolic syndrome

Through intracranial injections of viral vectors, Cre recombinase can be targeted to specific brain regions to induce knockdown of a gene. The GR within the MBH was targeted in this study as chronic Cort treatment induces obesity and hyperphagia, the latter of which is at least partially controlled by the energy-regulatory network within the MBH.

The knockdown of GR was not confirmed using qRT-PCR or immunofluorescence (discussed in section 5.4.3) in AAV-Cre injected mice. The successful injection of AAV-Cre into ROSA26-EYFP mice identified the Cre recombinase to be functional, as YFP was expressed in the same region as Cre recombinase. Therefore, it is likely that when Cre recombinase is present in the MBH of GR flox mice, the GR is successfully knocked down
(Kaspar et al., 2002). Cre expression was quantified in the MBH micro-punches of Cre and Cre-Cort mice. However, the expression of Cre within the micro-punches was highly variable between animals. This variability could be due to a number of factors including differing transduction efficiency between animals, differing success of viral injection, or differences in micro-punch sampling. Further, in validation of GR knockdown, the micro-punch sampling of both Cre-infected and non-Cre infected areas cannot be controlled. Therefore, reduction in Nr3c1 expression is probably masked in Cre-injected mice. In addition, the differing expression of Cre within this cohort of mice would produce differing percentage knockdown of GR. Consequently, the differing percentage knockdown of GR means that it is difficult to determine the contribution of hypothalamic Gcs in the development of Cort-induced obesity and hyperphagia. Phenotypic data of Cre-Cort mice suggests that the treatment may be protective in the long-term against the increase in food intake and body weight caused by exogenous Gcs.

The AAV7-CAG-oCre virus used in this study deletes exon 3 from Nr3c1 gene, consequently, preventing the translation of a functional GR in all transduced cell types including both neurones and glial cells. In order for the hypothalamic energy-regulatory network to function successfully, constant communication between different hypothalamic nuclei and neurones-glial cells is required. Cre immunofluorescence indicated that the AAV-Cre virus translocates throughout the MBH. Importantly, Cre expression was not found in the PVN, and therefore the HPA axis remained intact.

The increase in Agrp expression by chronic Cort treatment discovered in our lab (Sefton et al., 2016), identifies the importance of this orexigenic neuropeptide. However, the complex nature of the energy-regulatory network requires an approach to investigate the Gc actions in the whole MBH, in order to establish the contribution of hypothalamic Gcs in the development of Cort-induced hyperphagia and obesity.

**The role of the glucocorticoid receptor in the arcuate nucleus**

To date, studies have investigated the acute effects of Gc stimulation, or have used highly invasive techniques, to establish the actions of Gcs within the hypothalamus. Increases in both Npy and Agrp expression have been quantified after acute stimulation with Dex (Goto et al., 2006; Shimizu et al., 2008). Removal of endogenous Gc production, by ADX, either increases or does not alter Pomp expression (Makimura et al., 2003; Savontaus et al., 2002; Uchoa et al., 2012). Further, ADX has shown that Gcs regulate the membrane potential of both orexigenic and anorexigenic neurones (Gyengesi et al., 2010). To our knowledge, our study is the first to investigate the effect of chronic non-invasive administration of corticosterone on ARC neuropeptide expression. After 3 weeks, AgRP is
the only neuropeptide with increased mRNA expression, signifying its importance in the
development of Gc-induced hyperphagia. Although the knockdown of GR on
AgRP/NPY/GABA neurones does not specifically target Gc actions on AgRP, it does
prevent compensation for the lack of AgRP signalling by co-expressing orexigenic
neuropeptides. In addition, GR KO on AgRP neurones has been linked to increases in
energy expenditure in a HFD model (Shibata et al., 2016), however, this mouse line has
not been challenged with chronic Gcs.

**The role of the glucocorticoid receptor in the ventromedial hypothalamus**
Although the GR is highly expressed in the VMH, very few studies have investigated the
involvement of the ‘satiety centre’ when challenged with Gcs. Removal of the endogenous
Gc production through ADX in rats, decreases NPY receptors expression specifically in
the VMH (Wisialowski et al., 2010). This suggests that there is a role for Gcs within the
VMH in the regulation of orexigenic neuropeptide signalling.

### 5.4.2 Gc actions in the MBH delay the increase in body weight over time

The monitoring of body weight and food intake over a 6-month period for mice injected
with either AAV-Cre or AAV-GFP indicated that AAV-Cre injected GR flox mice did not
gain as much weight over time. Although neither Cre expression nor GR expression has
been quantified in this study, lack of changes in adrenal weight between AAV-Cre and
AAV-GFP mice indicates that Cre recombinase did not hit the PVN. The reduced body
weight gain of AAV-Cre mice was not associated with a decrease in food intake. Similarly,
alterations in food intake have not been shown in chow fed mice over 15 weeks when GR
was knocked out specifically on AgRP neurones (Shibata et al., 2016).

The direct actions of Gcs on adipocytes, has recently been identified to prevent body
weight gain over time and protect against altered glucose homeostasis and hepatic
steatosis (Mueller et al., 2016). However, although the hypothalamic neuropeptides of the
energy-regulatory network project to peripheral WAT and BAT to regulate thermogenesis
the actions of hypothalamic Gcs in the regulation of energy expenditure are still unknown.
Both populations of neurones within the ARC have been associated with the regulation of
energy expenditure (reviewed in Zhang & Bi, 2015). Peripheral hormones, leptin and
insulin acting through POMC neurones, are key in the stimulation of WAT browning
(Dodd et al., 2015). Therefore, in a separate cohort of GR flox mice, Gc actions were
disrupted through the knockdown of GR within the MBH, and energy expenditure was
recorded across 15 weeks. No changes in energy expenditure or RER were recorded,
however, technical difficulties have resulted in highly variable data (indirect calorimetry cages did not record 2/6 runs).

The plasticity of the neuronal network of the MBH alters with time. As we age, AgRP innervation on to POMC neurones increases, thereby increasing inhibition of POMC (Newton et al., 2013). The age-induced alterations to synaptic inputs on the two neuronal sub-populations have been linked to corticosterone. In a model of ADX, POMC neurones are depolarised and AgRP neurones are hyperpolarised (Gyengesi et al., 2010). Furthermore, corticosterone levels increase as we age (Dalm et al., 2005). Therefore in our model, if GR is successfully knocked down in the MBH, depolarised POMC neurones would provide the opportunity for other stimuli to increase POMC activity, ultimately preventing the gain in body weight.

5.4.3 Difficulties in the quantification of GR knockdown within the MBH

The virus mediated delivery of Cre recombinase enables the role of discrete regions, such as the MBH, to be investigated in the development of disease. The specific targeting through bilateral AAV-Cre injections requires confirmation of Cre recombinase expression and knockdown of the targeted gene in the correct region of each individual animal. In this study qRT-PCR and immunofluorescence were used to confirm the successful injection of AAV-Cre into the MBH (10/16 animals). However, these techniques did not provide the sensitivity required to confirm GR knockdown. The ubiquitous expression of GR and translocation of viral vectors within brain tissue means that wherever Cre recombinase is expressed the GR should be knocked down. Confirmation of GR knockdown needs to therefore be quantified in each individual animal. This study quantified GR expression within Cre-infected tissue at two time points; two weeks after AAV-Cre surgery and 5 weeks after surgery, when Cort treatment has been completed. However, the ubiquitous expression of GR means that if non Cre-infected regions were sampled in the tissue taken for analysis, then the knockdown effect of recombinase may be masked. As Cre recombinase expression is abundant in the MBH and it cuts the \( \text{loxp} \) sites situated either side of GR exon 3 in GR flox mice, then the GR should be knocked down in this study.

Quantification of \( \text{Nr3c1} \) mRNA expression displayed a trend towards a decrease in both Cort and Cre-Cort groups compared with vehicle mice, therefore indicating that Cort treatment itself has an effect on MBH GR expression. Previous studies in our lab have identified that 4 weeks Cort treatment reduces expression of GR in the whole hypothalamus (PVN and the MBH) up to 50% (Sefton et al., 2016). It is dogma that Gcs
negatively feedback at the PVN to regulate the HPA axis. As a consequence, the continued activation of the HPA axis during chronic stress decreases GR expression specifically within the PVN (Russell et al., 2014). Therefore, the decrease in whole hypothalamic GR (as seen in chapter 4) is greater than Cort-induced reduction specifically within the MBH.

Immunofluorescence was used to visualise the successful injection of Cre recombinase into the MBH. The deletion of exon 3 from the \(\text{Nr}3c1\) (GR) gene by Cre recombinase induces out-of-frame splicing and premature translation termination, preventing the translation of a functional protein. Therefore, in the imaging of dual immunofluorescence, the GR should not be expressed in the same region as Cre. As GR expression is not affected by the addition of \(\text{loxP}\) sites (Tronche et al., 1999; Tronche et al., 1999), the reduction of GR should be clear between the Cre-infected MBH and non Cre-infected regions. Within this study two different GR antibodies were used, however, due to the overall poor detection of the anti-GR (GR32L, Calbiochem), and both anti-GR (M-20, Santa Cruz) and anti-Cre (69050-3, Novagen) antibodies being raised against the same species preventing dual immunofluorescence, a reduction in the GR was not detected in the Cre-infected MBH.

Congenital knockout studies investigating the action of Gcs in the PVN (Laryea et al., 2013) and forebrain regions (Boyle et al., 2005), have deciphered that GR has a critical threshold for successful Gc actions. Laryea and colleagues compared the effect of GR within the PVN on adiposity using two GR flox mouse lines. Deletion of exon 2 decreased GR expression 43%, whereas deletion of exon 3 decreased GR expression 87% in the PVN. Only the severe (87%) decrease in GR expression induced a metabolic syndrome-like phenotype. This breach in normal functions was also found when GR was knocked out of forebrain regions (Boyle et al., 2005). Therefore, a severe knockdown of GR is required to determine the effects of Gcs within the MBH in the development of Gc-induced metabolic syndrome.
Chapter 6

General Discussion
6.1 Overview

The mechanisms underlying the development of obesity, hyperphagia, and hyperglycaemia in patients treated with long-term Cort are complex and there is a tantalising suggestion that Cort acts within the hypothalamus as well as the periphery. This thesis initially investigated if Gcs were involved in acting on inflammatory effects in the hypothalamus caused by HFD, but unfortunately, it was not possible to establish a reproducible model of HFD-induced hypothalamic inflammation. Secondly, this thesis has examined the role of hypothalamic Gc actions in the development of Gc-induced metabolic sequelae, through the development of an in vivo model of chronic Cort administration in the drinking water. Further, by the targeted knockdown of the GR within the MBH, using Cre-lox technology, it has been possible to identify hypothalamic Gc actions involved in the regulation of energy balance as it effects steroid-induced metabolic syndrome.

The administration of Cort in the drinking water generates a reliable and reproducible in vivo model, to establish the mechanisms behind the development of the associated metabolic sequelae. This study found that hypothalamic Cort levels rapidly increase with exogenous Cort treatment and these concentrations remain elevated after chronic Cort treatment. The elevated hypothalamic Cort levels are associated with increased Agrp expression after 4 weeks (Sefton et al., 2016). These associations suggest that Cort induced increases in Agrp drive the Gc-induced hyperphagia and hyperglycaemia.

Furthermore, the phenotypic monitoring of Cre-Cort GR flox mice provides preliminary evidence, that the development of Gc-induced metabolic sequelae is partially driven by Gc actions within the MBH. In addition, the reduced body weight gain of AAV-Cre injected GR flox mice without Cort treatment identifies the importance of Gc actions within the hypothalamus over time. In order to calculate the contribution of hypothalamic Gcs in the development of Gc-induced metabolic syndrome, future studies will need to quantify the percentage of GR knockdown within the MBH.

6.1.1 Hypothalamic inflammation is not easily reproducible in mouse models of high-fat diet

The anti-inflammatory vs pro-inflammatory actions of Gcs within the central nervous system is currently under much debate (Miller, 2007). Hypothalamic inflammation caused by HFD administration in rodents has been well characterised (Thaler et al., 2012; Valdearcos et al., 2014; Wisse & Schwartz, 2009) and mechanistic pathways have been identified (Dalvi et al., 2016; Milanski et al., 2012; Zhang et al., 2008). However, HFD
models have established that the development of the associated hypothalamic inflammation is highly dependent on the duration, diet, and species studied (De Souza et al., 2005; Fonken et al., 2013; Thaler et al., 2012; Wang et al., 2012). The difficulties found in this thesis to develop a reproducible model of HFD-induced inflammation, supports the unwieldy time-frames of hypothalamic inflammation reported in the literature. In addition, the pathophysiological relevance of the HFD-induced inflammation recorded remains unknown, as inflammation within the hypothalamus was only reported by the activation of microglia after 20 weeks.

Although hypothalamic inflammation induced by HFF has been reported in the literature, these studies do not agree on the development and timing of when specific gene expression, protein expression, or immune cell responses occur. Thaler and colleagues reported variability in the small changes in pro-inflammatory cytokine expression within a single study across a number of time points (Thaler et al., 2012). However, even within this single study, expression changes were not consistent at various time points. These small changes in gene expression makes it difficult to use this model to understand mechanisms.

Alternatively a number of studies have described diet-induced inflammation in terms of the response of immune cells, microglia and astrocytes (Gao et al., 2013; Lemus et al., 2015; Thaler et al., 2012). However in our model of HFF we found that this low-grade activation develops at a very late stage (20 weeks). Additionally we found that establishing the levels of activated microglia is a highly subjective technique. It is therefore difficult to understand the importance of this type of hypothalamic inflammation when its characterisation is unpredictable and temperamental and varies from lab to lab.

In models where hypothalamic inflammation has been measured it is tantalising to suggest that the temporal on-off-on response is driven by a switch in endogenous Gc actions, from anti-inflammatory to pro-inflammatory with continued HFD. The resolution of short-term HFD-induced hypothalamic inflammation after 1-2 weeks (Fonken et al., 2013; Thaler et al., 2012) may be controlled by endogenous Gcs. Recent research suggests that the timing, duration, and concentration of Gc exposure switches the anti-inflammatory actions of Gcs to a pro-inflammatory mode (Cruz-Topete & Cidlowski, 2015). Therefore, continued chronic HFF may induce this shift in Gc actions, allowing hypothalamic inflammation to resume (Meijer et al., 2003).

When establishing models of hypothalamic inflammation induced by HFF, multiple inflammatory markers have been measured. Yet it still remains unclear which is the most important inflammatory pathway. A number of papers have suggested the importance of the IKKβ/NFκB pathway in the development of hypothalamic inflammation, although in our
model we could not detect changes in components of the IKKβ/NFκB pathway. However the disruption to this signalling pathway specifically within AgRP neurones (Zhang et al., 2008) or within astrocytes which protects against diet-induced obesity, signifies its importance (Douglass et al., 2017). In addition, as the IKKβ/NFκB pathway remains inactive in a ‘healthy’ hypothalamus, pharmaceutical inhibition of this pathway may provide a therapy against this type of obesity.

The temporal profile of diet-induced hypothalamic inflammation recorded in the literature (Fonken et al., 2013; Thaler et al., 2012) suggests there are endogenous mechanisms which can resolve acute HFF induced hypothalamic inflammation. The anti-inflammatory actions of endogenous Gcs make them a prime candidate to control the temporal hypothalamic response reported in the literature. There has been much debate over whether the long-term feeding of a HFD activates the HPA axis to drive the production of Gcs, enabling the resolution of hypothalamic inflammation (Auvinen et al., 2012; Namvar et al., 2016). Alternatively, pro-inflammatory cytokines can feedback onto the HPA axis as a self-regulatory feedback mechanism to increase the production of anti-inflammatory Gcs. A number of cytokines including IL-6 and TNFα (both reported to be increased by HFF (Thaler et al., 2012)) drive the CRF secretion from the PVN and ACTH secretion from the pituitary driving the production of Gcs from the adrenal (reviewed in Turnbull & Rivier, 1999). Therefore either the stress activation by HFF or the feedback of cytokines onto the HPA axis could increase Gc levels within models of HFF.

In this thesis, elevated circulating Gc levels induced by exogenous Gc therapy have shown circulating Gcs to be able to translocate into and remain elevated within the hypothalamus over time. This could provide the opportunity for activated GR to block the actions of NFκB, preventing the continued production of pro-inflammatory cytokines (Cruz-Topete & Cidlowski, 2015). Therefore the ability for hypothalamic Gc concentrations to remain elevated over time would enable Gcs to reduce and protect against a HFF-induced inflammatory response.

However it is unknown why endogenous Gcs cannot resolve hypothalamic inflammation induced by long-term HFF. The continued administration of a HFD could be driving the HPA axis to sustain Gc concentrations at a chronically elevated level over time. This continued production of Gcs may induce Gc resistance or switch the classically Gc anti-inflammatory actions to a pro-inflammatory state.

What causes the Gc actions to switch from anti-inflammatory to pro-inflammatory is not known. Chronic exposure to Gcs is one mechanism thought to cause the Gc response to switch to produce pro-inflammatory actions (Frank et al., 2014). Gcs have been shown to increase Nlrp3 (a component of the inflammasome) which drives the production of pro-
inflammatory cytokines, additionally Gcs can also increase expression of TLR2 which can then be activated by PAMPs/DAMPs (including free fatty acids) to produce inflammatory cytokines.

With many different potential functions, anti-inflammatory or pro-inflammatory, the role of Gcs in the control of HFD-induced inflammation needs to be further investigated. However at present the temporal and unpredictability of current HFF models could itself cause instability in the mapping of Gc actions within these models. If a stable and reproducible model of hypothalamic inflammation were to be developed and Gcs were proved to be causative agents, the levels of Gcs within the hypothalamus could be manipulated over time as a means to treating this form of obesity.

6.1.2 Glucocorticoid-induced metabolic syndrome: Is the brain to blame?

In excess, Gcs lead to the development of metabolic disorders including; obesity, hyperphagia, and hyperglycaemia. Chronic corticosterone administration in the drinking water provides a successful, reproducible, non-invasive in vivo model by which the mechanisms driving the development of these metabolic sequelae can be determined. The administration of Gcs in this manner enables Gcs to act directly on peripheral metabolic tissues, thereby increasing hepatic gluconeogenesis and decreasing BAT thermogenesis.

However, the rapid and sustained increase in food intake identified by this model, which is known to be regulated by the hypothalamus, identifies the importance of the hypothalamus in the development of Gc-induced hyperphagia. Furthermore, the recent advances mapping sympathetic connections between the hypothalamus and peripheral metabolic tissues (Steculorum et al., 2015; Yi et al., 2012), suggests that hypothalamic Gc actions may also indirectly regulate the development of other metabolic sequelae including Gc-induced hyperglycaemia.

6.1.2.1 Glucocorticoid actions within the hypothalamus

The close proximity of energy-regulatory neurones to the leaky BBB enables circulating Gcs to cross into the brain and stimulate these hypothalamic appetite-regulatory neurones. This study identified that in models of exogenous corticosterone treatment, hypothalamic corticosterone levels increase alongside elevated circulating corticosterone. In addition, Cort decreases hypothalamic expression of the enzyme which regenerates inactive Gcs (11-DHC) to active corticosterone (11β-HDSD1). Therefore, the increased levels of Cort within the hypothalamus are a direct result of the Cort treatment.
These elevated Cort levels were found to increase rapidly (within 24 hours), and remain elevated at the same level throughout chronic Cort treatment. As Cort levels increased with exogenous Cort treatment, it is suggestive that the hypothalamus is unable to effectively prevent or remove Cort which accumulates within the hypothalamus. Present in the capillary endothelial cells of the BBB, the multi-drug resistant efflux pump transports Gcs from the intracellular compartment to the extracellular space (Meijer et al., 2003). Although RNA levels of the efflux pump did not change with Cort treatment in this study, it is not known whether the activity of the efflux pump is increased as a consequence of elevated hypothalamic Cort levels.

As part of the HPA axis it is well known that Gcs can feedback at the GR within the PVN of the hypothalamus in order to self-regulate and dampen the production of endogenous Gcs from the adrenal (Kalafatakis et al., 2016). However the regulation of GR on the energy-regulatory network has not been as well documented. This study therefore identified that although GR expression decreases within the PVN (as part of the HPA axis) and may alter within the MBH (the energy regulatory network) Gcs do translocate into the brain and remain elevated over time.

Despite these mechanisms to limit Cort levels within the hypothalamus, the elevated hypothalamic Cort levels increase Gc activity as indicated by increased Tsc22d3 expression. This increased Gc activity driven by a rapid increase in hypothalamic Gc levels is associated with an increase in food intake which is regulated by mechanisms within the hypothalamus. Parallel studies within the lab, have identified that orexigenic Agrp expression increases after 24 hours and is sustained throughout Cort treatment (Harno et al., 2016). Although we did not see changes in Pomc expression within our studies, other labs have shown that in the absence of Gcs, Pomc expression can both increase (Gyengesi et al., 2010) and decrease (Savontaus et al., 2002). Together these studies provide a strong indication of the importance which Gc signalling within the hypothalamus provides in the development of Gc-induced metabolic syndrome. Further the consistent, reproducible increase in Agrp, identified within our lab across a number of in vivo studies, puts Agrp at the forefront of candidates to be driving the metabolic side-effects caused by elevated Gc levels.

This direct relationship between hypothalamic Cort levels and Agrp expression is further shown in challenging Cort alongside a HFD. HFD+Cort exacerbated the increases in hypothalamic Cort levels after 4 weeks, which in turn was paralleled by further increases in Agrp expression (unpublished). As patients treated with Gcs report a shift in food preference to highly palatable foods (Dallman, 2010), the addition of HFD to this study was completed to determine whether the shift in food intake exacerbates the metabolic
phenotype induced by Gc. The increased hypothalamic Cort levels and consequences this has on neuropeptide expression described above may be due to HFD disrupting the BBB and increasing the ability for Cort to translocate into the hypothalamus (Yi et al., 2012). Although the increases in hypothalamic Cort and Agrp levels are exacerbated in the HFD+Cort group, compared with Cort alone, HFD+Cort does not lead to even greater increases in body weight during the treatment period.

Unlike humans, when on a HFD, mice do not over eat as they reduce the number of total grams of food consumed to counteract the increased fat content of food. Therefore despite the increased Agrp expression driving food intake, the Cort-induced hyperphagia recorded in the Cort group alone cannot overcome the mice’s ability to control food intake when on a HFD.

Though we did not see an exacerbation of metabolic phenotype with this study, similar studies co-treating rodents with HFD and Cort have found that the combined treatment of HFD+Cort worsens the ability to control glucose homeostasis (Beaudry et al., 2013; Shpilberg et al., 2012). Further this aggravated insulin response to HFD+Cort treatment persists after the cessation of treatment (Auvinen et al., 2013). The differing response between humans and rodents in the overconsumption of HFD or palatable foods limits this model in determining the additive effect of Cort and HFD. Therefore it is difficult to give advice on healthcare policy when elevated Cort levels are combined with HFD are involved.

The importance of this model in mapping the development of Gc-induced metabolic consequences should not be forgotten. The strong and reproducible increase in hypothalamic Cort levels associated with alterations in orexigenic neuropeptide expression provides an excellent model for determining the hypothalamic role of Gcs in driving Gc-induced hyperphagia and obesity.

6.1.2.2 Disrupting glucocorticoid actions within the hypothalamus

Through disrupting the Gc actions specifically within the MBH and challenging with Cort, this study aimed to establish the role of central Gcs in the development of Gc-induced metabolic syndrome. Although alterations in Agrp expression with acute and chronic Cort treatment found AgRP to be a prime target of Cort-induced hyperphagia, the contribution of other neuropeptides in the development of other metabolic sequelae is unknown. The use of congenital knockouts in understanding the role of neuropeptides has often resulted in the development of compensatory mechanisms due to co-localisation of either
orexigenic or anorexigenic neuropeptides within one neurone (Luquet et al., 2005; Shibata et al., 2016).

This study therefore provides initial evidence on the importance of the whole MBH by targeting GR knockdown within all neuronal subtypes. Unfortunately, due to low assay sensitivity (discussed in section 5.4.3) it was not possible to confirm GR knockdown within the MBH of AAV-Cre injected GR flox mice. Despite this, the injection of AAV-Cre into the MBH of ROSA26-EYFP mice confirmed that the Cre recombinase enzyme was functionally active. Therefore, it is likely that GR is knocked down in the presence of Cre recombinase (Kaspar et al., 2002).

Throughout Cort treatment of AAV-Cre injected GR flox mice (Cre-Cort), phenotypic monitoring identified that the body weight of Cre-Cort mice did not increase to the same extent as AAV-GFP injected Cort-treated mice. Therefore, this suggests that GR knockdown within the MBH may partially protect mice from Gc-induced obesity. Similarly, this separation between Cort and Cre-Cort mice towards the end of the three week Cort treatment was paralleled in the monitoring of food intake. As Cort treatment increases Agrp and food intake within 24 hours (Harno et al., 2016), the partial protective effect on body weight and food intake gain in Cre-Cort mice, suggests an association between hypothalamic Gc actions and metabolic disorders which develop with chronic Cort treatment.

It is also possible however, that the development of Cort-induced metabolic sequelae in this model of exogenous Cort treatment is driven by Gc actions within peripheral metabolic tissues. If the bilateral injection of AAV-Cre into the MBH of GR flox mice has severely knocked down the GR within the targeted region, yet only partially protected against the development of the metabolic sequelae, then consequently Cort actions within the periphery or other brain regions are important.

It must be acknowledged that the knockdown of GR by virus mediated Cre recombinase does not induce a complete knockout within the MBH. The actions of residual GR are identified both in our exogenous Cort treatment model and in investigating the effects of GR within different brain regions. Cort treatment alone reduces Nr3c1 expression by up to 50% within the whole hypothalamus. Yet this decrease in expression does not prevent Gc actions, as expression of Tsc22d3 and Agrp still increase (Sefton et al., 2016). In addition, residual GR in knockouts of different potencies can still induce phenotypic effects (Boyle et al., 2005; Laryea et al., 2013). Despite the Cort-induced reduction in GR, the increase in Agrp expression in our model is robust. Conversely, a recent paper identified that 25% of POMC neurones express high levels of Agrp (Lam et al., 2017), and equally a proportion of AgRP neurones express low levels of Pomp (Henry et al., 2015). Although the
percentage of POMC and AgRP neurones expressing GR was not stated, the neuronal heterogeneity within the ARC suggests that it may not be possible to distinguish between AgRP or POMC effects even when GR has specifically been knocked out on one neuronal subtype. Therefore, the confirmation and percentage of GR knockdown is required in order to establish the contribution of Gc actions within the hypothalamus in our model of chronic elevated Cort levels.

6.1.3 Central vs peripheral mechanisms driving the development of Gc-induced metabolic syndrome.

Gcs are well known to have direct effects on peripheral tissues and although this model shows a strong response within the hypothalamus these direct peripheral effects cannot be ignored. In phenotyping our model of Gc-induced metabolic syndrome we have successfully mimicked the development of a number of metabolic consequences seen in patients treated with exogenous Gcs (see figure 6.1). Increased adipocyte differentiation, hepatic steatosis, and BAT thermogenesis could be driven by Gcs acting directly within the tissues (de Kloet et al., 2015; Zinker et al., 2007). On the other hand recent studies suggest that central mechanisms can control what has previously been thought of as Gcs acting directly in the periphery, through a brain – periphery axis (Steculorum et al., 2016; Yi et al., 2012). This study therefore provides a basis for future experiments to determine the contribution of hypothalamic corticosterone mechanisms.

6.1.3.1 Gcs actions on glucose homeostasis and insulin signalling

The increased circulating corticosterone concentrations in our model allow Gcs to act directly within the periphery to regulate glucose and insulin signalling. In our lab we have identified that insulin levels increase rapidly whereas hyperglycemia does not develop until the end of a 3 week Cort treatment period (unpublished data). The rapid increase in insulin released from the pancreas is unsurprising due to insulin’s antagonistic effects on Cort actions (Rafacho et al., 2014). Furthermore Cort is known to act directly on the liver to increase gluconeogenesis (Opherk et al., 2004). Surprisingly, in our model of Cort treatment, hyperglycaemia does not develop until late on in the study (Harno et al., 2016). This could be as chronically elevated insulin levels can no longer function or the circulating insulin cannot control the increased production of glucose and therefore insulin resistance develops. However more recently the retrodialysis of dexamethasone into the
ARC inhibits hepatic insulin sensitivity indicating that central Gcs can regulate peripheral regulation of glucose (Yi et al., 2012).

6.1.3.2 Gcs actions on white adipose tissue

We identified in our model that chronic Cort treatment is required for adiposity to increase across the three adipose tissue beds measured (epididymal, mesenteric, and subcutaneous). The known direct actions of Gcs on adipose tissue could be attributable to this effect. It is well known from the studies of patients with chronically elevated endogenous Gcs that the increased Gc levels cause adipocyte differentiation and lipolysis resulting in increased circulating fatty acids (Geer et al., 2014; Lacroix et al., 2015). However, in response to the increased adipose tissue deposition, leptin is released. These elevated leptin levels are known to have secondary anorexigenic effects within in the hypothalamus and are discussed in section 6.1.3.4. In addition, more recent studies have shown that AgRP neuronal signalling suppresses browning of WAT (Ruan et al., 2014) which may indicate a mechanism by which central Gc actions could modulate WAT functions.
Figure 6.1: Proposed mechanisms of Gc-induced metabolic syndrome

Chronic corticosterone (Cort) treatment increases hypothalamic corticosterone levels which are associated with increased Agrp expression leading to hyperphagia. Thermogenesis, adipocyte differentiation and glucose homeostasis could be regulated by either direct stimulation of peripheral tissues (peripheral mechanism) or AgRP signalling via the sympathetic nervous system to peripheral tissues (central mechanism).

6.1.3.3 Gcs actions on brown adipose tissue

The importance of the hypothalamic control of BAT in energy expenditure however has been clarified by recent studies. These have identified that alongside the direct stimulation of BAT to alter function, increased signalling from hypothalamic neuronal networks can alter BAT thermogenesis. With the increase in hypothalamic corticosterone identified in our model it is tantalising to speculate that the hypothalamic neuronal networks may play a role in the control of BAT thermogenesis. The rapid activation of AgRP neurones within the ARC using DREADD technology results in reduced energy expenditure (Krashes et al., 2011). Contrary to this, the disruption to different mechanisms of action within AgRP neurones, including the deletion of vesicular GABA transporter or thioredoxin interacting protein increases energy expenditure (Blouet et al., 2012; Tong et al., 2008). These studies show the complexity of identifying hypothalamic mechanisms which drive energy expenditure and food intake. A more recent study has prevented the actions of Gcs directly on AgRP neurones through the knock-down of GR. In the presence of a HFD,
mice lacking GR on AgRP neurones were mildly protected from the development of metabolic effects and displayed increased energy expenditure (Shibata et al., 2016). This study taunts us with further evidence that the elevated hypothalamic corticosterone levels within our model can regulate BAT thermogenesis through a central mechanism.

The rapid, sustained, and reproducible increases in hypothalamic corticosterone levels induced by chronic exogenous Cort treatment, provide the opportunity to determine how Gc actions within the hypothalamus affect food intake and energy expenditure.

6.1.3.4 Early vs late Gc mechanisms driving time-dependent metabolic consequences

Surprisingly, although food intake increases within 24 hours, Gc-induced body weight gain is not observed until day 10. This is similar to previous studies investigating the metabolic consequences of chronic corticosterone exposure (Harno et al., 2016; Karatsoreos et al., 2010). The mechanisms behind this delayed increase in body weight, despite the rapid increase in food intake are still unknown.

Muscle atrophy

The delayed gain in body weight could be accounted for by the direct actions of corticosterone on skeletal muscle. Gc-induced muscle atrophy is a well-known side effect of Gcs (Schakman et al., 2003). Therefore acute muscle atrophy induced by excess circulating corticosterone could decrease body weight and counteract any increases in fat pad mass. The use of dual-energy X-ray absorptiometry (DEXA) or Echo MRI scans in rodent models can be used measure fat redistribution, from which lean mass can be calculated. Multiple scans throughout our chronic Cort treatment model could help establish whether acute muscle atrophy contributes to the delayed body weight gain. However, as the animals would be restrained or anaesthetised this would cause stress and therefore unpredictable changes in endogenous Gcs it might give spurious results.

Energy Expenditure

The time-dependent metabolic effects of Cort treatment could be driven by alterations in energy expenditure. Multiple studies have identified that Gcs can directly regulate BAT, the body’s main site of non-shivering thermogenesis (Soumano et al., 2000; Strack et al., 1995; Van Den Beukel et al., 2015). The thermogenic capacity of BAT is dependent on UCP-1 expression within the tissue’s mitochondria. Increasing the levels of circulating
corticosterone through the implantation of a subcutaneous Cort pellet decreases UCP-1 expression in mice, suppressing BAT activity and thermogenesis (Van Den Beukel et al., 2015). Conversely, the stimulation of human BAT with Gcs indicates that acute stimulation may increase BAT thermogenic activity (Ramage et al., 2016).

Using our model of chronic Gc treatment, BAT weight was increased after 24 hours, probably caused by the increased lipid deposition identified by H&E staining (unpublished data). The increased lipid within this highly thermogenic tissue could provide the fuel to increase thermogenesis, counteracting the increase in food intake, and preventing the increase in body weight. Conversely to the thermogenic response to acute Cort stimulation, after 4 weeks Cort treatment UCP-1 expression decreases within BAT indicating decreased thermogenesis (Harno et al., 2016). However why the increased thermogenic activity of BAT with short term Cort stimulation becomes dysfunctional with chronic Cort treatment leading to decreased thermogenesis is unknown.

The measurement of thermogenic genes in BAT or WAT should only be used as a marker of thermogenesis as it may not represent true changes in the effect of Gcs on energy expenditure. A number of studies use changes in thermogenic genes alongside other techniques such as indirect calorimetry in order to understand the effect on energy expenditure. However changes in markers of thermogenesis (including UCP-1) do not always translate across to changes in whole body energy expenditure (Ruan et al., 2014). This may be due to poor sensitivity of the methodology used to be able to detect changes in energy expenditure. The investigation of how Gc-induced metabolic sequelae develop over time could help the development of effective treatment regimes (see section 6.1.5).

6.1.3.5 Secondary effects of Gc-induced metabolic syndrome

This model of exogenous Gc treatment used within our lab and by other groups has shown how rapid the effects of elevated circulating corticosterone levels are on other peripheral hormones including leptin and insulin (Harno et al., 2016; Karatsoreos et al., 2010). The alterations in these peripheral hormones (insulin, leptin) as well as alterations in other peripheral factors including glucose and free fatty acids should be considered as they too have roles in energy homeostasis.

Elevated insulin levels are known to have antagonistic actions compared with Gcs (Warne et al., 2009). It is well known that Gcs can act directly on your pancreas to produce insulin (Rafacho et al., 2010). However more recently Gc signalling within the ARC has also been shown to modulate hepatic insulin responsiveness via NPY and the sympathetic nervous system signalling (Yi et al., 2012). Furthermore elevated insulin levels can act on AgRP neurones to suppress the production of hepatic glucose (Könner et al., 2007). Together
these studies show the complex relationship between Gcs and insulin signalling and suggest that in our model direct actions of Gcs within the periphery could initiate secondary effects controlled by the hypothalamus to regulate metabolism.

Similar to insulin, leptin concentrations are increased rapidly with exogenous Cort treatment and remain elevated leading to leptin resistance. Alongside the known actions of leptin to stimulate Pomc to reduce food intake (Mizuno et al., 1998), leptin more recently has been shown to act on POMC neurones to regulate glucose homeostasis and hepatic insulin sensitivity (Berglund et al., 2012). Together these show the complexity between Gcs and other peripheral hormones in the development of metabolic sequelae.

Over time continued Gc treatment in our model causes insulin and leptin resistance (unpublished) therefore the antagonistic actions of these hormones can no longer limit the metabolic effects of Gcs. Maintaining the sensitivity of insulin and leptin signalling could prevent some of the latter metabolic consequence developing. The importance of these secondary effects could be investigated through knockdown or disruption to hypothalamic insulin or leptin signalling within our model of exogenous Gc treatment.

6.1.4 Age-related changes to the neuronal hypothalamic network

The neuronal network is a highly plastic system which alters over time and it is unknown how these changes in neuronal plasticity impact the action of Gc within the hypothalamus. Phenotypic analysis resulting from chronic Gc treatment has recently been completed in adolescent and adult mice in order to model how disruption to endogenous Gc levels affects patients suffering from Cushing’s syndrome (Kinlein et al., 2017). Administrating Cort through drinking water Kinlein and colleagues found that unlike adults, adolescents reduced body weight gain and lean mass while increasing adiposity during the 4 week treatment period. This study further investigated how both adolescents and adults respond when the Cort is removed from the drinking water. During the 4 week ‘recovery’ period adult mice normalized their body weight however adolescents increased adiposity persisted. This study shows that as the hypothalamic neuronal network develops the effects of excess Gcs vary resulting in different metabolic phenotype.

Although the effects of exogenous Cort have not been investigated in aged mice this thesis investigated how knockdown of the GR within the mediobasal hypothalamus affects body weight over time without Cort treatment. While confirmation of GR knockdown is still required in this cohort of AAV-Cre injected GR flox mice the reduced gain in body weight over time observed in these animals suggests the importance of endogenous Gc actions
within the hypothalamus. As Gcs concentration is thought to increase as we age, this protective effect of GR knockdown within the MBH may be a consequence of chronically elevated Gcs no longer being able to act on energy-regulatory neurones. Furthermore removal of endogenous Cort by ADX results in the membrane potential of POMC neurones to depolarise while NPY/AgRP neurones hyperpolarise. These changes alongside the altered synaptic arrangement of these neurones indicates the importance of Gcs in the regulation of the energy-regulatory network in adult mice (Gyengesi et al., 2010).

6.1.5 Impact of Gc-induced metabolic syndrome

The wide number of functions and low cost of Gcs ensures a very effective therapy which can be prescribed for many patients suffering from a variety of diseases. The wide use of Gcs in the clinic means that the side-effects of their long-term use are well known by doctors. Although some of these side effects can be controlled and treated, such a bisphosphonate treatment for steroid-induced osteoporosis, many of the side-effects including increased body weight, food intake, and altered glucose homeostasis are harder to treat.

This thesis therefore was aimed at studying whether we could identify the mechanisms leading to the development of Gc-induced metabolic syndrome in order to suggest ways in which patients could be treated and yet prevent the adverse side-effects. Avoiding the development of Gc-induced sequelae in this way is highly important as it is a goal which would have an immediate impact. This could be achieved through the re-classification of current co-treatments already available to patients or the targeting of different mechanisms by using different Gcs.

My project has shown that Gcs are acting via hypothalamic mechanisms to drive food intake inducing hyperphagia. The increased Agrp expression recorded may also be regulating metabolic mechanisms within peripheral tissues delaying body weight gain which has been shown to develop after 10 days. Developing synthetic Gcs which do not target the brain could prevent these centrally driven mechanisms.

Changes in insulin and leptin signalling within this model (Harno et al., 2016) suggest that Gcs are acting directly on peripheral tissues. As these peripheral hormones can themselves influence energy metabolism an understanding is required on how the direct peripheral actions of Gcs may impact the hypothalamus. Further knowledge of these
peripheral mechanisms could provide targets for co-treatments which would lessen the impact of the Gc-induced metabolic side-effects.

The future studies described below firstly address some of the issues found in determining the contribution of hypothalamic Gc signalling and secondly suggests in vivo experiments which could inform clinical studies on how to address (co-therapies vs treatment regimes) the development of Gc-induced obesity and diabetes.

6.1.6 Future Studies

Currently, the National Institute for Health and Care Excellence (NICE) guidelines only advise on precautions that patients can take to minimize the risk of developing these side-effects. Consequently, as metabolic sequelae such as Gc-induced diabetes inevitably develop, anti-diabetic drugs can be prescribed. With a 48% incidence of Gc-induced diabetes in rheumatoid arthritis patients treated with Gcs for >6 months (Movahedi et al., 2016), there is a need to develop preventative medicines.

This study has begun the process of understanding how Gc actions in the hypothalamus might contribute to the metabolic side-effects of Gc therapy. By developing a robust model of chronic Gc treatment it has been possible to characterise the increase in hypothalamic corticosterone levels within 24 hours. These increased hypothalamic corticosterone levels are associated with changes in orexigenic Agrp and Gc-induced hyperphagia. Therefore these studies highlight the need for future work to focus on limiting translocation of exogenous Gcs into the brain or preventing hypothalamic Gc actions, in order to avoid the development of Gc-induced metabolic sequelae. Together, these future studies will allow the development of preventative treatments for Gc-induced obesity, hyperphagia, and diabetes to relieve the socio-economic burden that chronic steroid therapy currently places on the NHS.

6.1.6.1 Validation of GR knockdown within the MBH of AAV-Cre injected GR flox mice

The investigation of Gc actions within the MBH in this thesis, provides preliminary evidence that Gc actions within the hypothalamus are involved in the development of Gc-induced metabolic disorders. Although the Cre recombinase injected into the MBH was confirmed as a functional enzyme, multiple techniques including qRT-PCR and dual immunofluorescence have failed to provide the sensitivity required to quantify GR knockdown within Cre-infected regions.
As residual GR within the MBH remains functionally active (Boyle et al., 2005; Laryea et al., 2013; Sefton et al., 2016), future injections into the MBH of GR flox mice may require a higher titred AAV-Cre virus to increase the percentage GR knockdown in order to inhibit Gc actions. The insertion of loxP sites either side of exon 3, containing the DNA-binding domain of GR, should in the presence of Cre recombinase induce premature translation termination of the GR. Therefore, use of an antibody which recognises the C-terminus of GR there should not be a signal in Cre-infected regions (Laryea et al., 2013). Further, decreased Nr3c1 expression within Cre-infected regions may be quantified by qRT-PCR by increasing the titre of the AAV-Cre virus and therefore consequently increasing GR knockdown.

6.1.6.2 Could brain impenetrable Gcs prevent the development of a Gc-induced metabolic syndrome?

The rapid and sustained increase in hypothalamic corticosterone levels quantified by LC-MS/MS, associated with the rapid and sustained increases in Agrp, indicates the importance of elevated hypothalamic corticosterone in the development of metabolic sequelae. Therefore, preventing the accumulation of Gcs within the hypothalamus could protect against Gc-induced obesity, hyperphagia, and hyperglycaemia.

This study modelled the Gc-induced metabolic syndrome by allowing the ad libitum access to a pharmacological dose of corticosterone in the drinking water. The reduced hypothalamic Gc levels compared with the extra-hypothalamic region, suggests that the hypothalamus may be able to protect itself in part by from Gc accumulation. The peripheral administration delivery of different synthetic Gcs, indicates they can each get across the BBB (Karssen et al., 2002; Mason et al., 2012). However, disruption to the efflux multi-drug resistant pump in the BBB causes an accumulation of Gcs in the brain (Meijer et al., 2003). Therefore, either the development of brain impenetrable Gcs or increasing the efflux to prevent Gc accumulation within the MBH, may protect against the hypothalamic driven metabolic disorders.

6.1.6.3 Can targeted therapies protect against the development of Gc-induced metabolic syndrome?

These studies have shown that different aspects of Gc-induced metabolic syndrome develop at different times, yet hypothalamic Gc levels are elevated rapidly and are sustained after chronic treatment. Therefore, targeting the timing of Gc treatment or developing co-therapies, could prevent or delay Gc-induced metabolic sequelae.
‘Drug holidays’

Chronic exogenous Cort treatment increases hypothalamic Cort levels and food intake after 24 hours; however, increases in body weight are delayed until day 10. Therefore, the removal of Cort treatment for an acute period of time (24 – 48 hours) may be beneficial in further delaying the onset of Gc-induced obesity or diabetes.

The effect of treatment cessation has been investigated in mice through the monitoring of body weight and food intake for 8 weeks, after 4 weeks HFD+Cort combined therapy was stopped. On removal of treatment, body weight, circulating Cort levels, and food intake all return to normal after 1-2 weeks. However, insulin levels remained elevated in Cort treated mice with and without HFD (Auvinen et al., 2013). This positive reversal of Cort induced phenotypic alterations, suggests that the acute removal of Cort may be beneficial. However, as this study used a lower dose of Cort, which alone did not increase body weight, it is unknown whether this metabolic reversal will occur in our model.

It is highly important in studies investigating the beneficial effects of removing exogenous Cort on metabolism, which the required anti-inflammatory actions of the Gc-therapy remain. Further, when the treatment period ends for patients treated with exogenous Gcs chronically (>3 weeks), Gcs are required to be withdrawn gradually as long-term Gcs have dampened their HPA axis. Therefore, the complete cessation of Gc treatment may not be plausible, rather a lower dose known to not induce metabolic effects may be required for a short period of time (Karatsoreos et al., 2010).

The rapid Cort-induced increase in food intake identified in this thesis, suggests, that the implementation of ‘drug holidays’ may only be beneficial in delaying the development of chronic metabolic disorders. A recent study has identified that a fasting diet regime can restore insulin generation in patients with type-1 diabetes and reverse the phenotype of type-1 and type-2 diabetes (Cheng et al., 2017). Consequently, Gc-induced diabetes may be protected by the implementation of a fasting regime such as the 5:2 diet.

Selective glucocorticoid receptor modulators

The successful use of GR antagonists, such as mifepristone (RU-486), to improve the metabolic profile of patients with elevated endogenous circulating cortisol, in the treatment of Cushing’s syndrome, indicates the benefit of preventing Gc metabolic actions (Johanssen & Allolio, 2007). However, global GR antagonism would not be possible alongside the treatment of exogenous Gc therapy, as this would prevent both the ‘wanted' anti-inflammatory and ‘unwanted' metabolic effects.
The development of dual-functional drugs has proved that the beneficial anti-inflammatory effects remain without the development of side-effects. To date these drugs have not been used in the treatment of inflammatory diseases. Mice fed HFD for 4 weeks while being treated with C108297 (Corcept), had reduced diet-induced obesity and WAT inflammation by GR antagonism and GR agonism respectively (van den Heuvel et al., 2016). Similarly, body weight gain and fat deposition were protected against in a HFD model, when co-treated with a selective GR/MR antagonist (CORT118335, Corcept) (Mammi et al., 2016). It is unknown whether these drugs would have both anti-inflammatory and metabolic beneficial effects in a model of chronic inflammation such as rheumatoid arthritis.

Conversely, the targeting of GR antagonism to specific tissues can prevent the development of metabolic side-effects. The development of a GLP-1-estrogen conjugate (Finan et al., 2012), which limits the activation of estrogen within GLP-1 targeted tissues can reverse diet-induced metabolic syndrome. However, in order to prevent the dampening of the HPA axis, the targeting of the GR antagonism to the hypothalamus would need to specifically antagonise energy-regulatory neuropeptides within the MBH.

**Directing combination therapies to the hypothalamus**

Exogenous Cort treatment in this study indicates the importance of hypothalamic Gc actions, as increased Gc levels within the hypothalamus are associated with increased Agrp expression. Increased Agrp expression and signalling both lead to increased food intake, and more recently, has been associated with reducing energy expenditure (Blouet et al., 2012; Krashes et al., 2011; Small et al., 2001).

Therefore, preventing AgRP signalling through co-treating with an MC4R agonist alongside exogenous Gcs, may prevent or delay the development of Gc-induced hyperphagia or obesity. Initial studies using the MC4R agonist Setmelanotide (RM-493, Rhythm Pharmaceuticals), have investigated the beneficial metabolic effects of activating MC4R signalling. Deficiency in functional POMC signalling leads to severe hyperphagia and extreme obesity, both of which can be reversed after treatment with daily RM-493 (Kuhnhen et al., 2016). Further, the 72 hour infusion of RM-493 into obese, but otherwise healthy individuals, increased energy expenditure indicating that prevention of AgRP action is both beneficial at rebalancing energy intake and energy expenditure (K. Y. Chen et al., 2015). In addition, the combined therapy of this MC4R agonist alongside a GLP-1R receptor agonist potentiates the beneficial effects, increasing loss of body weight and improving glycaemic control, compared with each treatment individually in diet induced obese mice (Clemmensen et al., 2015). These studies deliver initial evidence of the
benefits that MC4R agonism provides. However, it is unknown how long-term MC4R agonist co-therapy would interact with chronic Gc therapy.

6.1.7 Final conclusions

The delivery of Cort in the drinking water provides an excellent reproducible, non-invasive *in vivo* mouse model to investigate the mechanisms driving the development of the Gc-induced metabolic syndrome. The analyses of Cort levels within the hypothalamus following acute and chronic Cort treatment indicate the speed and persistent actions of elevated hypothalamic Gcs. To investigate the role of these elevated hypothalamic Cort levels, the actions of Gcs were disrupted in the MBH by AAV-Cre injection. Confirming the percentage GR knockdown within this model proved technically difficult, however, phenotypic monitoring throughout the study hinted at the importance of elevated Cort levels within this region. Future studies, can further dissect the mechanisms of elevated hypothalamic Gcs, and co-therapies to prevent the development of Gc-induced metabolic sequelae can be developed through utilising this model of Gc excess.
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Nakata, M., Yamamoto, S., Okada, T., & Yada, T. (2016). AAV-mediated IL-10 gene transfer counteracts inflammation in the hypothalamic arcuate nucleus and obesity
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study of the central effects of specific proopiomelanocortin (POMC)-derived melanocortin peptides on food intake and body weight in Pomc null mice. 


Yi, C.-X., Foppen, E., Abplanalp, W., Gao, Y., Alkemade, A., la Fleur, S. E., … Kalsbeek,


Appendix, Chapter 3

Table 8.1: Figure 3.1 ANOVA F values

<table>
<thead>
<tr>
<th>Two Way repeated measures ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 10) = 0.06</td>
<td>P=0.81</td>
</tr>
<tr>
<td>Time</td>
<td>F (1, 10) = 0.80</td>
<td>P=0.39</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (1, 10) = 0.62</td>
<td>P=0.45</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (10, 10) = 1.0</td>
<td>P=0.49</td>
</tr>
</tbody>
</table>

Panel B: 1 week HFD, Body weight

<table>
<thead>
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<th>Two Way repeated measures ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 10) = 11.83</td>
<td>P=0.006</td>
</tr>
<tr>
<td>Time</td>
<td>F (1, 10) = 15.18</td>
<td>P=0.003</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (1, 10) = 0.20</td>
<td>P=0.65</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (10, 10) = 4.04</td>
<td>P=0.018</td>
</tr>
</tbody>
</table>

Table 8.2: Figure 3.2 ANOVA F values

Figures 3.1 and 3.2 illustrate the effects of a high-fat diet on body weight in mice over different time periods. Table 8.1 and 8.2 present the ANOVA F values for these comparisons.

Table 8.3: Figure 3.4 ANOVA F values

<table>
<thead>
<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (4, 77) = 2.87</td>
<td>P=0.028</td>
</tr>
<tr>
<td>Time</td>
<td>F (4, 77) = 16.8</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Diet</td>
<td>F (1, 77) = 12.98</td>
<td>P=0.0006</td>
</tr>
</tbody>
</table>

Panel B: Npy expression

<table>
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<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Interaction</td>
<td>F (4, 77) = 1.87</td>
<td>P=0.12</td>
</tr>
<tr>
<td>Time</td>
<td>F (4, 77) = 4.31</td>
<td>P=0.003</td>
</tr>
<tr>
<td>Diet</td>
<td>F (1, 77) = 6.13</td>
<td>P=0.015</td>
</tr>
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</table>

Panel C: Pomc expression

<table>
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<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (4, 76) = 3.40</td>
<td>P=0.012</td>
</tr>
<tr>
<td>Time</td>
<td>F (4, 76) = 5.27</td>
<td>P=0.0008</td>
</tr>
<tr>
<td>Diet</td>
<td>F (1, 76) = 0.31</td>
<td>P=0.57</td>
</tr>
<tr>
<td>Table 8.4: Figure 3.6 ANOVA F values</td>
<td></td>
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<td>-------------------------------------</td>
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<tr>
<td><strong>Panel C: Dnajb9 expression</strong></td>
<td></td>
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</tr>
<tr>
<td>Two Way ANOVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (3, 52) = 5.23 P=0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (3, 52) = 1.00 P=0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (1, 52) = 5.56 P=0.02</td>
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</tr>
<tr>
<td><strong>Panel D: Ddit3 expression</strong></td>
<td></td>
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<tr>
<td>Two Way ANOVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (3, 53) = 0.37 P=0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (3, 53) = 0.62 P=0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (1, 53) = 0.88 P=0.35</td>
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<td></td>
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<tr>
<td><strong>Panel E: Ikbkb expression</strong></td>
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<tr>
<td>Two Way ANOVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (4, 82) = 0.96 P=0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (4, 83) = 1.18 P=0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (1, 83) = 1.06 P=0.3</td>
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<tr>
<td><strong>Panel F: Aif1 expression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two Way ANOVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (4, 82) = 0.10 P=0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
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<tr>
<td>F (4, 82) = 0.06 P=0.99</td>
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<tr>
<td>Diet</td>
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<tr>
<td>F (1, 82) = 0.59 P=0.44</td>
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**Table 8.4: Figure 3.6 ANOVA F values**

<table>
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<tr>
<th>Table 8.5: Figure 3.8 ANOVA F values</th>
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<tbody>
<tr>
<td><strong>Panel A: 3 days HFD, Body weight Rats</strong></td>
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<tr>
<td>Two Way repeated measures ANOVA</td>
</tr>
<tr>
<td>Interaction</td>
</tr>
<tr>
<td>F (1, 16) = 24.09 P=0.0002</td>
</tr>
<tr>
<td>Time</td>
</tr>
<tr>
<td>F (1, 16) = 15.48 P=0.0012</td>
</tr>
<tr>
<td>Diet</td>
</tr>
<tr>
<td>F (1, 16) = 0.30 P=0.58</td>
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<td>Subjects (matching)</td>
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<td>F (16, 16) = 114.1 P&lt;0.0001</td>
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**Table 8.5: Figure 3.8 ANOVA F values**
<table>
<thead>
<tr>
<th>Figure 3.10</th>
<th>The effect of high-fat diet on glucocorticoid receptor expression and activity.</th>
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</table>

<table>
<thead>
<tr>
<th>Panel B: <em>Nr3c1</em> expression</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Interaction</td>
<td>F (4, 77) = 0.55</td>
<td>P=0.69</td>
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<tr>
<td>Time</td>
<td>F (4, 77) = 12.8</td>
<td>P&lt;0.0001</td>
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<tr>
<td>Diet</td>
<td>F (1, 77) = 0.08</td>
<td>P=0.77</td>
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<table>
<thead>
<tr>
<th>Panel C: <em>Tsc22d3</em> expression</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
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<tr>
<td>Interaction</td>
<td>F (4, 78) = 2.80</td>
<td>P=0.03</td>
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<tr>
<td>Time</td>
<td>F (4, 78) = 40.52</td>
<td>P&lt;0.0001</td>
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<tr>
<td>Diet</td>
<td>F (1, 78) = 9.53</td>
<td>P=0.002</td>
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</table>

*Table 8.6: Figure 3.10 ANOVA F values*
Appendix, Chapter 4

Table 8.7: Figure 4.5 ANOVA F values

<table>
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<tr>
<th>Figure 4.5</th>
<th>Hypothalamic and extrahypothalamic corticosterone levels following four weeks corticosterone treatment.</th>
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</thead>
<tbody>
<tr>
<td><strong>Panel: Hypothalamic Corticosterone</strong></td>
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</tr>
<tr>
<td>One-Way ANOVA</td>
<td>F (3, 43) = 16.28</td>
</tr>
<tr>
<td><strong>Panel: Extrahypothalamic Corticosterone</strong></td>
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</tr>
<tr>
<td>One-Way ANOVA</td>
<td>F (3, 43) = 11.79</td>
</tr>
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</table>

Table 8.8: Figure 4.8 ANOVA F values

<table>
<thead>
<tr>
<th>Figure 4.8</th>
<th>Corticosterone treatment dampens the hypothalamic-pituitary-adrenal axis.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Panel A: Adrenal</strong></td>
<td></td>
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<tr>
<td>One-Way ANOVA</td>
<td>F (3, 42) = 21.77</td>
</tr>
<tr>
<td><strong>Panel B: Spleen</strong></td>
<td></td>
</tr>
<tr>
<td>One-Way ANOVA</td>
<td>F (3, 42) = 49.09</td>
</tr>
</tbody>
</table>

Figure 4.9: Four weeks corticosterone induces obesity.

<table>
<thead>
<tr>
<th>Figure 4.9</th>
<th>Four weeks corticosterone induces obesity.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Panel A: % Body weight change</strong></td>
<td></td>
</tr>
<tr>
<td>Two-Way repeated measures ANOVA</td>
<td>F (DFn, DFd)</td>
</tr>
<tr>
<td>Interaction</td>
<td>F (24, 344) = 12.28</td>
</tr>
<tr>
<td>Time</td>
<td>F (8, 344) = 199.3</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (3, 43) = 15.78</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (43, 344) = 17.99</td>
</tr>
<tr>
<td><strong>Panel B: Epididymal Fat</strong></td>
<td></td>
</tr>
<tr>
<td>One-Way ANOVA</td>
<td>F (3, 43) = 18.62</td>
</tr>
<tr>
<td><strong>Panel B: Subcutaneous Fat</strong></td>
<td></td>
</tr>
<tr>
<td>One-Way ANOVA</td>
<td>F (3, 43) = 39.44</td>
</tr>
<tr>
<td><strong>Panel B: Mesenteric Fat</strong></td>
<td></td>
</tr>
<tr>
<td>One-Way ANOVA</td>
<td>F (3, 43) = 16.12</td>
</tr>
</tbody>
</table>
**Figure 4.10** Cort treatment induces hyperphagia.

<table>
<thead>
<tr>
<th>Panel A: % Percentage food intake</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (24, 344) = 3.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>F (8, 344) = 3.37</td>
<td>0.0010</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (3, 43) = 11.81</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (43, 344) = 2.76</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel B: Calorie (kcal/day)</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (24, 344) = 4.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>F (8, 344) = 6.32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Column Factor</td>
<td>F (3, 43) = 9.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (43, 344) = 3.68</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel C: Water Intake</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (24, 336) = 2.34</td>
<td>0.0005</td>
</tr>
<tr>
<td>Time</td>
<td>F (8, 336) = 1.81</td>
<td>0.074</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (3, 42) = 8.92</td>
<td>0.0001</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (42, 336) = 7.87</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel D: Fed Glucose</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-Way ANOVA</td>
<td>F (3, 20) = 5.80</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Table 8.10: Figure 4.10 ANOVA F values**
## Appendix, Chapter 5

### Figure 5.3

**Quantification of Cre mRNA expression in MBH micro-punch dissections.**

<table>
<thead>
<tr>
<th>Panel B: Cre expression</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
<td>F (3, 25) = 3.39</td>
</tr>
</tbody>
</table>

### Table 8.11: Figure 5.3 ANOVA F values

### Figure 5.4

**Quantification of Nr3c1 and Tsc22d3 mRNA expression in the MBH.**

**Panel B: Nr3c1 exon 3 / Hprt expression**

<table>
<thead>
<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 17) = 0.25</td>
<td>P = 0.62</td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 17) = 3.19</td>
<td>P = 0.09</td>
</tr>
<tr>
<td>AAV injection</td>
<td>F (1, 17) = 0.29</td>
<td>P = 0.59</td>
</tr>
</tbody>
</table>

**Panel C: Tsc22d3 expression**

<table>
<thead>
<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.43</td>
<td>P = 0.51</td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 21.5</td>
<td>P = 0.0002</td>
</tr>
<tr>
<td>AAV injection</td>
<td>F (1, 18) = 0.47</td>
<td>P = 0.50</td>
</tr>
</tbody>
</table>

**Panel D: Nr3c1 exon1-2 / Hprt expression**

<table>
<thead>
<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.26</td>
<td>P = 0.61</td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 0.39</td>
<td>P = 0.53</td>
</tr>
<tr>
<td>AAV injection</td>
<td>F (1, 18) = 0.46</td>
<td>P = 0.50</td>
</tr>
</tbody>
</table>

**Panel E: Nr3c1 exon 4 / Hprt expression**

<table>
<thead>
<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.11</td>
<td>P = 0.73</td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 1.3</td>
<td>P = 0.26</td>
</tr>
<tr>
<td>AAV injection</td>
<td>F (1, 18) = 0.003</td>
<td>P = 0.95</td>
</tr>
</tbody>
</table>

**Panel F: Nr3c1 exon 8 / Hprt expression**

<table>
<thead>
<tr>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.089</td>
<td>P = 0.76</td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 2.41</td>
<td>P = 0.13</td>
</tr>
<tr>
<td>AAV injection</td>
<td>F (1, 18) = 0.052</td>
<td>P = 0.82</td>
</tr>
</tbody>
</table>

### Table 8.12: Figure 5.4 ANOVA F values
Figure 8.1: Dual GR-Cre immunofluorescence in AAV-Cre injected GR flox mice. Representative images of Cre and glucocorticoid receptor (GR) in the mediobasal hypothalamus (MBH) and cortex of AAV-Cre injected, Cort treated GR flox mice. Cre = green, GR = Red, DAPI = blue, Scale bars = 200µm. Third ventricle, 3V.
Figure 8.2: Dual GR-Cre immunofluorescence in AAV-GFP injected GR flox mice. Representative images of green fluorescent protein (GFP) and glucocorticoid receptor (GR) expression in the mediobasal hypothalamus (MBH) and cortex of (A) AAV-GFP injected, vehicle treated GR flox mice (B) AAV-GFP injected, Cort treated GR flox mice. Green = GFP, Red = GR, Blue = DAPI. Images are representative of 2-4 sections/animal. Scale bars = 200µm. Third ventricle, 3V.
**Figure 8.3:** Cre and GR immunofluorescence in consecutive brain sections of AAV-Cre injected GR flox mice.

Representative images of (A) Cre and (B) glucocorticoid receptor (GR) in the mediobasal hypothalamus (MBH) of AAV-Cre injected GR flox mice, scale bar = 100µm. Third ventricle, 3V. Red = Cre, Green = GR, Blue = DAPI.
Figure 8.4: Cre and YFP expression in AAV-Cre injected ROSA26-EYFP mice. Representative images of Cre recombinase and yellow fluorescent protein (YFP) expression across consecutive sections in the (A,B) mediobasal hypothalamus (MBH) and (C,D) cortex from AAV-Cre injected ROSA26-EYFP mice. Red = Cre, Green = YFP, Blue = DAPI. Scale bars = 200µm. Images are representative of 12-16 sections per mouse. Third ventricle, 3V.
<table>
<thead>
<tr>
<th>Figure 5.9 Phenotypic analysis of AAV-GFP and AAV-Cre injected mice on Cort treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Panel A: Body weight</strong></td>
</tr>
<tr>
<td>Two Way repeated measures ANOVA</td>
</tr>
<tr>
<td>Interaction</td>
</tr>
<tr>
<td>Time</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Subjects (matching)</td>
</tr>
<tr>
<td><strong>Panel B: % Body weight change</strong></td>
</tr>
<tr>
<td>Two Way repeated measures ANOVA</td>
</tr>
<tr>
<td>Interaction</td>
</tr>
<tr>
<td>Time</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Subjects (matching)</td>
</tr>
<tr>
<td><strong>Panel F: Food intake</strong></td>
</tr>
<tr>
<td>Two Way repeated measures ANOVA</td>
</tr>
<tr>
<td>Interaction</td>
</tr>
<tr>
<td>Time</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Subjects (matching)</td>
</tr>
<tr>
<td><strong>Panel G: % Food intake change</strong></td>
</tr>
<tr>
<td>Two Way Repeated measures ANOVA</td>
</tr>
<tr>
<td>Interaction</td>
</tr>
<tr>
<td>Time</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Subjects (matching)</td>
</tr>
</tbody>
</table>

*Table 8.13: Figure 5.9 ANOVA F values*
Table 8.14: Figure 5.10 ANOVA F values

<table>
<thead>
<tr>
<th>Panel A: Epididymal fat</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 1.46</td>
<td>P=0.24</td>
<td></td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 3.59</td>
<td>P=0.07</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>F (1, 18) = 0.087</td>
<td>P=0.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel A: Subcutaneous fat</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.093</td>
<td>P=0.76</td>
<td></td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 5.85</td>
<td>P=0.02</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>F (1, 18) = 0.47</td>
<td>P=0.49</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel A: Mesenteric fat</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.49</td>
<td>P=0.49</td>
<td></td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 6.73</td>
<td>P=0.01</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>F (1, 18) = 0.097</td>
<td>P=0.75</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel B: Final body weight</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.12</td>
<td>P=0.72</td>
<td></td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 5.44</td>
<td>P=0.03</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>F (1, 18) = 0.010</td>
<td>P=0.91</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel C: Whole liver mass</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.12</td>
<td>P=0.7</td>
<td></td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 6.47</td>
<td>P=0.02</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>F (1, 18) = 0.0004</td>
<td>P=0.98</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel D: Fed glucose</th>
<th>Two Way ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.12</td>
<td>P=0.73</td>
<td></td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 7.2</td>
<td>P=0.01</td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>F (1, 18) = 0.0017</td>
<td>P=0.96</td>
<td></td>
</tr>
</tbody>
</table>
### Figure 5.11 The effect of AAV-Cre injection on Cort intake.

#### Panel A: Water intake

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (18, 108) = 1.36</td>
<td>P=0.16</td>
</tr>
<tr>
<td>Time</td>
<td>F (6, 108) = 2.16</td>
<td>P=0.05</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (3, 18) = 2.47</td>
<td>P=0.09</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (18, 108) = 5.5</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

#### Panel B: Adrenal mass

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 15) = 0.029</td>
<td>P=0.86</td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 15) = 2.43</td>
<td>P=0.13</td>
</tr>
<tr>
<td>AAV Injection</td>
<td>F (1, 15) = 1.37</td>
<td>P=0.25</td>
</tr>
</tbody>
</table>

#### Panel C: Spleen weight

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (1, 18) = 0.19</td>
<td>P=0.66</td>
</tr>
<tr>
<td>Cort treatment</td>
<td>F (1, 18) = 51.4</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>AAV Injection</td>
<td>F (1, 18) = 0.18</td>
<td>P=0.67</td>
</tr>
</tbody>
</table>

**Table 8.15: Figure 5.11 ANOVA F values**

### Figure 5.12 Phenotypic analysis of AAV-Cre injected animals over 6 months.

#### Panel A: % Body weight change

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (50, 750) = 1.6</td>
<td>P=0.002</td>
</tr>
<tr>
<td>Time</td>
<td>F (50, 750) = 232.8</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (1, 15) = 6.2</td>
<td>P=0.02</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (15, 750) = 100.4</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

#### Panel C: % Food intake change

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (49, 735) = 0.62</td>
<td>P=0.97</td>
</tr>
<tr>
<td>Time</td>
<td>F (49, 735) = 3.4</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>F (1, 15) = 0.047</td>
<td>P=0.83</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (15, 735) = 63.89</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

**Table 8.16: Figure 5.12 ANOVA F values**
Table 8.16: Figure 5.16 ANOVA F values

<table>
<thead>
<tr>
<th>Panel A: % Body weight change</th>
<th>Two way repeated measures ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (31, 403) = 0.27</td>
<td>P &gt; 0.99</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>F (31, 403) = 92.11</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>F (1, 13) = 0.20</td>
<td>P = 0.65</td>
<td></td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (13, 403) = 96.62</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel C: Food intake</th>
<th>Two way repeated measures ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (32, 416) = 0.52</td>
<td>P = 0.98</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>F (32, 416) = 2.57</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>F (1, 13) = 0.31</td>
<td>P = 0.58</td>
<td></td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (13, 416) = 34.51</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel D % Food intake change</th>
<th>Two way repeated measures ANOVA</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>F (33, 429) = 2.26</td>
<td>P = 0.98</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>F (33, 429) = 2.26</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>F (1, 13) = 0.32</td>
<td>P = 0.5</td>
<td></td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>F (13, 429) = 66.41</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>
Elevated hypothalamic glucocorticoid levels are associated with obesity and hyperphagia in male mice

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Glucocorticoid (Gc) excess, from endogenous overproduction in disorders of the hypothalamic-pituitary-adrenal axis or exogenous medical therapy, is recognised to cause adverse metabolic side effects. The Gc receptor (GR) is widely expressed throughout the body, including brain regions such as the hypothalamus. However the extent to which chronic Gcs affect Gc concentrations in the hypothalamus and impact on GR and target genes is unknown. To investigate this, we used a murine model of corticosterone-induced obesity, and analysed corticosterone levels in the hypothalamus and expression of genes relevant to Gc action. Mice were administered corticosterone (75 µg/ml, Cort) or ethanol (1%, vehicle) in drinking water for 4 weeks. Cort-treated mice had increased body weight, food intake and adiposity. As expected, Cort increased plasma corticosterone levels at both ZT1 and ZT13, ablating the diurnal rhythm. LC-MS/MS revealed a 4-fold increase in hypothalamic corticosterone, which correlated with circulating levels and concentrations of corticosterone in other brain regions. This occurred despite decreased 11β-hydroxysteroid dehydrogenase (Hsd11b1) expression, the gene encoding the enzyme which regenerates active Gcs, while efflux transporter Abcb1 mRNA was unaltered. In addition, while Cort decreased hypothalamic GR (Nr3c1) expression 2-fold, the glucocorticoid-induced leucine zipper (Tsc22d3) mRNA increased which indicated elevated GR activation. In keeping with the development of hyperphagia and obesity, Cort increased Agrp, but there were no changes in Pomc, Npy or Cart mRNA in the hypothalamus. In summary, chronic Cort treatment causes chronic increases in hypothalamic corticosterone levels, and a persistent elevation in Agrp, a mediator in the development of metabolic disturbances.
ameliorate the unwanted actions of this successful class of therapeutic agents.

The peripheral mechanisms involved in the development of Gc-induced metabolic disorders have been widely documented. Gc actions in liver, adipose tissue and skeletal muscle, highlight their regulatory role in carbohydrate, lipid and protein metabolism (reviewed in (2)). In addition, the tissue-specific regeneration of Gcs within liver and adipose tissue provides an important mechanism for their actions within the periphery (3). Glucocorticoid receptors (GRs) are also widely distributed throughout the brain and yet the contribution of Gc actions in brain regions known to regulate energy balance is often ignored when considering the effects of excess Gcs on metabolic regulation.

Pharmacological concentrations of Gcs acting in the hypothalamus are ideal candidates for generating abnormal networking, which could lead to metabolic side effects. Gcs are known to regulate a range of orexigenic and anorexigenic neuropeptides in centers of the brain with a role in the control of food intake and body weight although present data is somewhat contradictory. Dependent on the method and duration of corticosterone (Cort) administration, Pro-opiomelanocortin (Pomc) expression has both increased (4, 5) and decreased (6) following treatment. Similarly Agouti-related peptide (Agrp) expression follows this inconsistency in response to Cort treatment (7, 8). Although the glucocorticoid response elements (GREs) are present within the promotor regions of Agrp (9), Pomc (10), and Neuropeptide Y (Npy) (11) the effect of chronic Gc treatment is still unclear.

It is dogma that Gcs act on CRH neurones as part of the self-regulatory feedback of the HPA axis, consequently downregulating GR expression in the paraventricular nucleus (PVN). To prevent accumulation of Gcs in the brain the efflux transporter (MDR-PGP) removes Gcs from the brain (12), conversely the enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) converts inactive 11-dehydrocorticosterone (11-DHC) to corticosterone contributing to the levels of corticosterone in the hypothalamus. However the effects of chronic Cort treatment on these mechanisms which control the absolute concentrations of corticosterone in the hypothalamus are unknown.

To investigate the effects of chronic glucocorticoid treatment on hypothalamic regulation of energy balance requires a well-characterized model. Unfortunately there is a history of conflicting data describing the effects of Cort on body weight in rodent models. The effects of exogenous Cort treatment can increase body weight (13, 14) or decrease it (15, 16).

An elegant study in 2010, used a translational model of Cort administration in the drinking water to mimic the excess Gcs seen in patients on long-term Gc treatment (17). However, the levels of Gcs that are able to translocate to and remain in the brain to regulate central metabolic effects are unclear. Therefore, this study aimed to analyze Gc levels within the hypothalamus following Cort treatment at doses that cause metabolic side effects. We also evaluated the effects of Gcs on genes regulating hypothalamic Gc concentrations and on Gc target genes including those known to contribute to an adverse metabolic phenotype.

Materials and Methods

Animal Husbandry and administration of corticosterone treatment

Ten-week-old male C57Bl/6J mice (Charles River, UK) were singly housed under a constant 12hr light-dark cycle (lights on 0700h, ZT = 0, lights off 1900h, ZT = 12), with an ambient temperature of 23 ± 1°C and a humidity of approximately 40%. Food and water were available ad libitum through all experiments. All experiments were performed in accordance with the UK Animals (Scientific Procedures Act, 1986) using procedures approved by The University of Manchester Ethical Review Panel.

Food intake and body weight measurements were measured twice weekly throughout the experiment. After three weeks baseline; mice were randomly assigned a treatment group by body weight and administered either corticosterone (75 μg/ml, ‘Cort’, Sigma-Aldrich, UK) dissolved in 1% ethanol or vehicle (1% ethanol) for 24 hours, 48 hours or 4 weeks. Separate cohorts were used to allow the analysis of both hypothalamic corticosterone levels (LC-MS/MS, n = 12 per group) and gene expression analysis (qRT-PCR, n = 8 per group, in situ n = 3 per group). At the end of each study, blood was taken by tail-prick sampling for analysis of circulating corticosterone; plasma was removed and stored at −80°C for future analysis. Immediately following sampling, mice were culled by cervical dislocation. Tissues were dissected and snap-frozen on dry ice for future analysis. For mRNA analysis, the whole hypothalamus (~20 mg) was removed immediately from the ventral side of the brain. microdissection scissors cut immediately caudal to the optic chiasm. The dissection was limited laterally by the hypothalamic sulci and dorsally by the mammillothalamic tract. The entire hypothalamus was removed including the arcuate, ventromedial, dorsomedial and paraventricular nuclei and stored in “RNA-later” for future analysis.

Plasma corticosterone quantification

Plasma corticosterone levels were quantified using an ELISA according to the manufacturer’s instructions (4 week Cort treatment, Cayman, Cambridge Bioscience, UK; for 24 and 48 hour Cort treatment, Abcam, UK, as the Cayman kit was discontinued).

Tissue corticosterone and 11-dehydrocorticosterone quantification

Tissue corticosterone levels were measured in hypothalamic-enriched and extrahypothalamic regions (rest of the brain) by
liquid chromatography dual tandem mass spectrometry (LC-MS/MS). A hypothalamic-enriched region was dissected from the ventral side of the frozen brain (~50 mg). Two coronal microdissection cuts were made at the level of the optic chiasm and the mammillary bodies, the brain was then rotated and two more cuts were made to dissect out the hypothalamus as a rectangle. The rest of the brain was homogenized as one and is described as the extrahypothalamic region (~375 mg). Both hypothalamic-enriched and extrahypothalamic regions were homogenized in sterile water (200 mg/ml) using a rotator homogenizer. A 100μl sample aliquot was enriched with corticosterone-D8 major as internal standard to a concentration of 10 μg/ml. A liquid/liquid extraction (LLE) was performed followed by a solid phase extraction (SPE). Briefly, 500μl ethyl acetate was added to each sample, aspirated and then centrifuged (500 rpm, 10mins) to separate the layers. The aqueous layer was discarded while the solvent layer was blown to dryness under nitrogen and then reconstituted in 30% methanol. An SPE extraction was performed using a C-18 plate (Strata C18-E, Phenomenex, UK) reconstituted in 30% methanol. An SPE extraction was performed using a C-18 plate (Strata C18-E, Phenomenex, UK) which was conditioned with methanol and water. Samples were loaded in 30% methanol, washed with double distilled H2O and which was conditioned with methanol and water. An SPE extraction was performed. Briefly, 500μl ethyl acetate was added to each sample, aspirated and then centrifuged (500 rpm, 10mins) to separate the layers. The aqueous layer was discarded while the solvent layer was blown to dryness under nitrogen and then reconstituted in 30% methanol before elution in 100% methanol (2 × 250μl). The eluents were dried under nitrogen and reconstituted in 50% mobile phase A/50% mobile phase B. Mobile phase A was 2 mM ammonium acetate in methanol/water (10/90 v/v) containing 0.027% formic acid. Mobile phase B was 2 mM ammonium acetate in methanol/water (90/10 v/v) containing 0.027% formic acid.

Chromatographic separation of corticosterone and 11-dehydrocorticosterone (11-DHC) was performed using reverse phase chromatography (Kinnetx® 5 μm XB-C18 100 Å, 50 × 2.1 mm, Phenomenex, UK) on an Agilent 1200 binary pump HPLC system running a gradient from 100% A to 100% B over 1.5mins, hold for 1.5mins then immediately back to 100% A and hold for 3.5mins. Total run time was 6.5mins with flow rate of 500μl/min and an injection volume of 10μl. The system was run at room temperature.

The chromatography system was coupled to a Sciex API4000 Qtrap mass spectrometer. The instrument was operated in multiple reaction monitoring (MRM). Corticosterone was monitored in positive ion mode; the following parameters were implemented in the ion source: spray voltage 4.5 kV, temperature 550 °C, CUR at 20, GS1 at 50 and GS2 at 60. The transitions used were: CORT; 347.1/329.3 with CE –4.5, DP –100 and CXP –15. D8-CORT (internal standard); 355.3/73 with CE = 50, DP = 117 and CXP = 15.

11-DHC was monitored in negative ion mode; the following parameters were implemented in the ion source: spray voltage –4.5 kV, temperature 550 °C, CUR at 20, GS1 at 50 and GS2 at 60. The transitions used were:11-DHC; 343.1/328.0 with CE = –28, DP = –100 and CXP = –15. D8-CORT (internal standard); 353.1/246.1 with CE = –25, DP = –100 and CXP = –15.

Data was processed using Analyst Software (Applied Biosystems, UK) and signal intensities obtained by standard peak integration methods. Quantitations were performed by comparison against a standard curve generated from multiple dilutions of brain tissue spiked with analyte of interest in methanol (10ng/μl = 0.01ng/μl). Final analyte quantification was derived from a mean of two biological replicates. The lower limit of accurate quantification (LLQ) was set at a level twice the peak area measured in blank brain tissue, to ensure robust and confident differentiation of analytes from low-level background peaks which may coelute. The mean LLQ was 0.3ng/ml CORT, 0.5ng/ml 11-DHC. When samples were measured as BLQ statistical analysis was performed using a value of zero.

Quantification of mRNA by real-time quantitative PCR

RNA was extracted using an RNeasy mini kit (Qiagen, UK) with on column genomic DNA digestion according to the manufacturer’s protocol. RNA integrity and quantification was confirmed using a NanoDrop ND2000 (Thermo Scientific, UK). Transcript levels were determined by reverse transcription and qRT-PCR on a Prism 7900HT Sequence Detection System (Applied Biosystems, UK) with either TaqMan Gene Expression Assays and Taqman RNA-to-Ct 1 step kit (Applied Biosystems, UK) or SYBR assays designed with primer-BLAST software (NCBI) and Power SYBR Green RNA-to-Ct 1 step kit (Applied Biosystems, UK). Relative quantification was achieved using a standard curve approach with normalization to the reference genes, HPRT or TBP for Taqman or SYBR assays respectively, and nomination of the vehicle group as calibrator. The primer sequences used in this study were: Aebb1 forward 5’-TTTG-GCAAAGCCGGAGATG-3’ and reverse 5’-CCAGCT-TATATCTGTGTCAGCA-3’; Agrp Mm00475829_g1; Cartpt Mm04210469_m1; Fkbp5 forward 5’-AGCAACGGTAA- AAAHTCCACCT-3’ and reverse 5’-TTCCTCAACAAACGAA- CACCA-3’; Hprt Mm03024075_m1; HSD11b1 Mm00476182_m1; Npy forward 5’-ATGCAGATGAA- CAAGGAATGGG-3’ and reverse 5’-GTGAGAGATGAGTT-3’. The transitions used were:11-DHC; 343.1/328.0 with CE –4.5, DP –100 and CXP –15. D8-CORT (internal standard); 353.1/246.1 with CE = –25, DP = –100 and CXP = –15.

In situ hybridization

Frozen whole brains were sectioned (12 μm) using a cryostat freezing microtome. Coronal sections were taken of the entire hypothalamus from each animal starting from immediately prior to the paraventricular nucleus to the end of the third ventricle. Representative sections through the hypothalamus from each animal were then analyzed to ensure inclusion of all anatomical levels.

In situ riboprobes were generated by synthesis and sub cloning of the sequence (GeneArt, UK) into pGM-5ZF(+) vector (Promega, UK). The resultant plasmid constructs were linearized using appropriate restriction enzymes, to generate templates for sense and antisense transcripts. Antisense and sense riboprobes were synthesized with an SP6/T7 in vitro transcription system (Promega, UK) in the presence of 35S-uridine triphosphate (PerkinElmer, UK). Probes were hybridized overnight at 60°C, and hybridization visualized by film autoradiography (Kodak Bio-Max MR film, Kodak, USA). Films were scanned using a CoolSNAP-Pro camera (Photometrics, UK) while on a light box. Signal intensity was quantified by densitometry analysis or autoradiographs. Optical density (relative units, RU) within the
target region was calculated on each section with a minimum of 7 sections per animal, 3 animals per group.

**Statistical analysis**

All data are represented as mean ± SEM and results considered statistically significant $P < .05$. Hypothalamic, extrahypothalamic corticosterone levels and absolute tissue weights (unpaired $t$ test), Correlation analyses (Pearson’s and Spearman’s rank correlation), qRT-PCR (Mann Whitney unpaired $t$ test), circulating corticosterone, food intake and body weight (two-way ANOVA). All statistical analyses were performed with GraphPad Prism (La Jolla, CA, USA) version 6.00.

**Results**

**Exogenous Cort treatment alters circulating corticosterone**

After 4 weeks of exogenous Cort treatment, the plasma corticosterone was 9-fold higher in the Cort-treated mice at zeitgeber time $= 1$, (ZT), and 2-fold higher at ZT = 13 compared with vehicle (figure 1A, $P < .01$). A reduction in adrenal weight was observed after 4 weeks Cort treatment (figure 1B, $P < .001$), suggesting that the chronic increase in Gcs had suppressed the HPA axis. As expected, four week Cort treatment reduced spleen weight (figure 1B, $P < .001$), with this effect seen as early as 24 hours (figure 1C, $P < .01$). Whole pituitary Nr3c1 (GR) mRNA expression was not altered after 4 weeks Cort treatment (data not shown).

**Exogenous Cort treatment increases hypothalamic corticosterone levels after 24 and 48 hours**

To determine if Cort treatment resulted in elevation of corticosterone in the hypothalamus within 24 hours or whether it gradually accumulated over time, levels were quantified after 24 and 48 hours Cort treatment. Corticosterone levels in the hypothalamus were increased compared to vehicle treated animals and this was similar after 24 and 48 hours Cort treatment (figure 2A and B, $P < .05$). Corticosterone levels were correlated between hypothalamic and circulating corticosterone levels in the Cort treated group (figure 2C, $P = .5$ and D, $P < .01$). Similarly, within the rest of the brain (described as the extrahypothalamic region) corticosterone levels increased alongside hypothalamic corticosterone levels after 24 and 48 hours Cort treatment (figure 2E and F, $P < .001$). Vehicle treated mice were not included in correlation analysis. 11-DHC can be converted to corticosterone by 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) in the hypothalamus and therefore 11-DHC levels were also assessed. However, hypothalamic and extrahypothalamic levels of 11-DHC were below the limit of quantification (data not shown).

**Four weeks exogenous Cort treatment increases hypothalamic corticosterone levels**

Hypothalamic corticosterone levels were increased 4-fold with four weeks Cort treatment (figure 3A, $P < .01$). This increase is comparable to the increase seen after 24 and 48 hours. Similarly, extrahypothalamic corticosterone levels increased in mice treated with Cort (figure 3B, $P < .01$). Both hypothalamic and extrahypothalamic corticosterone levels were positively correlated with cir-
culating corticosterone (figure 3C, \( P = .07 \)) and with each other (figure 3D, \( P = .43 \)). Only Cort treated mice were included in correlation analysis. Hypothalamic and extra-hypothalamic levels of 11-DHC were below the limit of quantification (data not shown).

There was a marked decrease in \textit{Hsd11b1} (11\( \beta \)-HSD1) mRNA expression after Cort treatment indicating reduced endogenous corticosterone regeneration from the inactive 11-Dehydrocorticosterone (11-DHC) (figure 3E, \( P < .01 \)). In comparison, \textit{Abcb1} (gene for MDR1a, an efflux transporter known to remove Gcs across the blood brain barrier) did not change between treatment groups (figure 3F).

**Four weeks Cort increases GR activity in the hypothalamus**

After four weeks Cort, the increased hypothalamic corticosterone levels decreased the hypothalamic mRNA expression of \textit{Nr3c1} (GR) (figure 4A, \( P < .01 \)). However, there was increased expression of \textit{Tsc22d3}, (glucocorticoid-induced leucine zipper, GILZ), a transcription factor known to be regulated by Gcs (figure 4B, \( P < .01 \)) and a trend towards an increase in FK506 binding protein 5 (\textit{Fkbp5}) within the hypothalamus (figure 4C) indicating increased Gc activity. Mineralocorticoid receptor (\textit{Nr3c2}) mRNA expression did not change with Cort treatment (data not shown). Hypothalamic \textit{Crh} expression was at the limit of detection and therefore this data set has not been included.

**Exogenous Cort alters hypothalamic AgRP mRNA expression**

Exogenous Cort treatment significantly increased \textit{Agrp} mRNA expression in the hypothalamus (figure 5A, \( P < .01 \)). However, hypothalamic mRNA expression of energy regulatory neuropeptides; \textit{Npy}, \textit{Pomc} and \textit{Cart}, were not altered after 4 weeks treatment with Cort (figure 5A). In situ hybridization confirmed increased \textit{Agrp} expression throughout the arcuate nucleus, while \textit{Pomc} expression was not altered following Cort treatment (Figure 5B, \( P < .05 \) for \textit{Agrp}).

Associated with the rise in hypothalamic \textit{Agrp} expression, food intake was increased after three days and remained elevated throughout the four weeks treatment (figure 6A, \( P < .01 \)). Body weight significantly increased after 2 weeks Cort compared to vehicle-treated animals (figure 6B, \( P < .001 \)). Fat pad mass was also increased after 4 weeks Cort treatment, with epididymal fat increased 2-fold, subcutaneous fat 2.6-fold and mesenteric fat 1.5-fold (figure 6C, \( P < .001 \)).

**Discussion**

In this model of pharmacological treatment with Gcs to induce obesity, it is evident that corticosterone accumulates in the brain and this study provides the first evidence that it is chronically elevated in the hypothalamus. This increase was seen after 24 hours and hypothalamic corticosterone levels remained elevated after 4 weeks of treatment, even though there was a reduction in Gc regeneration, suggesting a continuing direct influx of the administered Cort from the circulation. The increases in corticosterone were associated with elevated Gc activity in the hypothalamus, despite a decrease in hypothalamic GR. Treatment with Gcs increased the expression of the orexigenic neuropeptide, \textit{Agrp}, in the hypothalamus and as might be expected there was increased food intake. At the end of the 4 week treatment period, mice had developed...
marked hyperphagia which could, in part, be driving the obesity.

We have used delivery of Cort in the drinking water, which represents a relatively stress-free approach to mimicking pharmacological administration of Gcs because of the implications of uncontrolled stress in the chronic paradigm. As mice eat and drink steadily throughout their active phase in this model of Cort administration mice continue to take in a steady dose of Cort throughout the dark phase. A similar model was used by Karatsoreos and colleagues, and although a higher dose of Cort (100 μg/ml) was given over four weeks, a comparable increase in circulating corticosterone was achieved (17). In the current study, the administration of Cort in the drinking water gave a greater increase in plasma corticosterone levels at ZT = 1 compared to ZT = 13. We believe this is due to mice starting their active phase at ZT = 112 and therefore eating and drinking at this time. As in this study, Karatsoreos et al (17) found that increased corticosterone caused adrenal atrophy during the treatment period, which is a marker of inhibition of HPA activity. However they did not assess whether central effects of the elevated corticosterone might contribute to the body weight gain.

In the current study, treatment with Gcs resulted in markedly increased corticosterone in the hypothalamus, which remained chronically elevated throughout treatment. We cannot exclude the possibility that corticosterone from blood was present and quantified alongside tissue-specific corticosterone in both the hypothalamic and extrahypothalamic brain regions. However studies have shown that blood contamination of whole brain samples has little or no effect on brain steroid levels and saline-perfusions can be detrimental because they can alter ste-
roid concentrations in a region-specific manner (18). While it is accepted that Gcs act in the hypothalamus to decrease corticotrophin releasing hormone (CRH) as part of the HPA axis, the concentrations of Gcs which exist after chronic Cort treatment have not previously been identified, in a model such as this. There are several mechanisms which exist to modify the levels and activity of Gcs. The efflux transporter (Abcb1) is able to remove Gcs from the brain (19) although it is unlikely to be effective in our model as its expression is not altered. There is also the 11β-HSD1 conversion of 11-DHC to corticosterone, which could be the main contributor to the levels of corticosterone in the hypothalamus and could be up-regulated as found in liver (20) and adipose tissue (3) or down-regulated to prevent further accumulation of corticosterone. In our study there is decreased expression of hypothalamic 11β-HSD1, implying a protective mechanism whereby with excess Gcs there is a downregulation of the enzyme to prevent regeneration of additional corticosterone from inactive 11-DHC. This indicates that increased hypothalamic Gc levels are more likely a result of the increased circulating corticosterone translocating into the brain and not a consequence of increased corticosterone regeneration. Further the mechanisms controlling corticosterone entry and clearance may differ between brain regions as indicated by the lower corticosterone concentration in the hypothalamic region compared to extra-hypothalamic region.

Perhaps more importantly, it is dogma from the HPA axis that chronic Gcs down-regulate the GR in the pituitary and hypothalamus. In the HPA axis, the reduction in GR is considered a compensatory mechanism to counteract the feedback inhibition by Gcs (21). We have found it hard to find any literature pertaining to this in a similar model to ours. Indeed our novel findings show that Gcs do down-regulate the GR in our model, but most importantly

Figure 5. Exogenous Cort alters Agrp mRNA expression (A) Agrp (agouti-related protein), Npy (neuropeptide Y), Cart (cocaine and amphetamine regulated transcript) Pomc (pro-opiomelanocortin) mRNA expression analysis following 4 weeks Cort treatment in the whole hypothalamus (Mann Whitney t test; ** P < .01, n = 5–8 / treatment group). B, Representative images and densitometry of in situ hybridization AgRP and Pomc expression in the arcuate nucleus (Unpaired t test ** P < .01, n = 3 / treatment group).
the receptor is not completely down-regulated. Gcs can still act in the hypothalamus to increase known target genes such as GILZ, a transcription factor, known to be sensitive to Gc activation (22, 23), and also maintain the decreases in the 11β-HSD1 and the GR. Although there is a 50% decrease in GR expression at 4 weeks, GILZ, is increased at 24h (unpublished data) and remained elevated after 4 weeks treatment. Therefore the chronic exogenous treatment of Gcs down-regulates GR in our model, but most importantly this partial down-regulation allows the continued action of Gcs in the hypothalamus to increase known target genes such as GILZ.

The increased hypothalamic Gcs, led us to investigate their effect on energy-regulating neuropeptides within the hypothalamus. Surprisingly we did not see any change in Pomc mRNA in the hypothalamus by qRT-PCR or in situ hybridization. There are well-known mechanisms whereby Gcs inhibit pituitary-derived POMC. The binding of the GR to GREs in the promoter region of the POMC gene has been expertly mapped (24). However the impact of Gc regulation on the neuron specific promoter regions of POMC (25) which regulate hypothalamic POMC expression is less clear (4, 5, 26).

In a previous study, we observed an increase in Agrp expression in the hypothalamus with 25 μg/ml CORT in drinking water but the mice did not develop hyperphagia or obesity (7). In the current study with 75 μg/ml CORT, in drinking water, there was a clear increase in Agrp expression and this was associated with the development of hyperphagia and obesity. It has previously been shown that fasting induces increases in Gcs which activate transcription of the Agrp gene via GR binding directly to the AgRP-GRE (9). The elevated Agrp expression in our model, without alterations in other hypothalamic neuropeptide expression, suggests the importance of AgRP in central Gc signaling. While AgRP neurons also release orexigenic GABA and NPY (27, 28), our studies suggest Gcs do not increase NPY in this model. Given that NPY is predominantly expressed in the arcuate nucleus, it is unlikely that Gcs are modifying the NPY neurons that act on energy balance, but we cannot discount the fact that they may be causing changes in other regulatory functions in other hypothalamic regions.

In the current model, the effect of chronic treatment with Gcs is to increase hypothalamic Gcs, and compensatory mechanisms in Gc regulation do not lead to down-regulation of the system, but rather allow a sustained enhanced effect of Gcs in the hypothalamus. This leads us to speculate that Gc-induced increases in Agrp may be mediating peripheral mechanisms alongside the development of hyperphagia leading to the development of obesity. Therefore it is highly likely that Gc actions in the brain and periphery are not mutually exclusive. Indeed, while Gcs have well-recognized peripheral effects in regulating metabolism, this study suggests that chronically elevated Gcs acting in the hypothalamus may be the cause of abnormalities in neuronal regulation of energy balance which contribute to the plethora of metabolic side-effects.

Figure 6. Exogenous Cort treatment induces hyperphagia and obesity (A) Food intake, (B) body weight and (C) adiposity following 4 weeks exogenous Cort treatment ((A, B) Two-way ANOVA and (C) unpaired t test, *** P < .001, n = 19/ treatment group) Closed square represents Cort treated, open circle represent vehicle.

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