THE ROLE OF HYPOTHALAMIC NEUROPEPTIDES IN THE DEVELOPMENT OF OBESITY-RELATED HYPERTENSION

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Notice 1

Notice 2

Dedicated to my beloved grandmother, Jane Paz, who was robbed of the opportunity to undertake her own tertiary education but who has gained a doctorate in life.

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Abbreviations

3V – Third Cerebral Ventricle

ACE – Angiotensin Converting Enzyme

ACTH – Adrenocorticotropic hormone

AgRP – Agouti-Related Protein

AngII – Angiotensin Two

ARC – Arcuate Nucleus of the Hypothalamus

BAT – Brown Adipose Tissue

BBB – Blood Brain Barrier

BMI – Body Mass Index

BWT – Body Weight

CART – Cocaine and Amphetamine Regulatory Transcript

CE – Cholesterol Esters

Cer – Ceramides

CRH – Corticotropin releasing Hormone

CSF – Cerebrospinal Fluid

CVD – Cardiovascular Disease

CVLM – Caudal Ventrolateral Medulla

DAG - Diaceyglycerides

DIO – Diet Induced Obesity

DMH – Dorsomedial Hypothalamus

EFA – Essential Fatty Acids

GHSR – Growth Hormone Secretatgoge Receptor

HFD – High Fat Diet

HR – Heart Rate

ICV – Intracerebroventricular

IL-6 – Interleukin Six

IL-β – Interleukin Beta

JAK – Janus Tyrosine Kinase

LepRb – Leptin Receptor b

LH – Lateral Hypothalamus

LHPA – Limbic hypothalamic Pituitary Adrenal Axis

MAP – Mean Arterial Pressure

MCH – Melanin Concentrating Hormone

NFD – Normal Fat Diet

NFκ-B – Nuclear Factor Kappa-B

NO – Nitric Oxide

NPY – Neuropeptide Y

NTS – Nucleus Tractus Solitarii

OX1R – Orexin 1 Receptor

OX2R- Orexin 2 Receptor

PI3K – Phosphoinositide 3-Kinase

POMC – Proopiomelanocortin

PVH – Paraventricular Hypothalamus

RSNA – Renal Sympathetic Nerve Activity

RVLM – Rostral ventrolateral medulla

SCN – Suprachiasmatic Nucleus of the Hypothalamus

SNA – Sympathetic Nerve Activity

SNS – Sympathetic Nervous System

SOCS3 – Suppressor of Cytokine Signaling 3

SON – Supraoptic Nucleus of the Hypothalamus

STAT – Signal Transducer and Activator of Transcription

T2D – Type 2 Diabetes

TAG - Triacylglycerides

TLR – Toll Like Receptor

TNF-α – Tumor Necrosis Factor Alpha

UCP1 – Uncoupling Protein 1

VMH – Ventromedial Hypothalamus

WAT- White Adipose Tissue

WHO – World Health Organisation

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 0 original papers published in peer reviewed journals and 4 unpublished publications. The core theme of the thesis is the contribution of hypothalamic neuropeptides to the development of obesity related hypertension. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Neuropharmacology Laboratory at the Baker IDI Heart & Diabetes Institute under the supervision of Professor Geoffrey Head and Dr James Armitage.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of each chapter my contribution to the work involved is detailed on the following page.

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
III	Short Term Fat Feeding Rapidly Increases Plasma Insulin but Does Not Result in Dyslipidaemia	Published in 'Frontiers in Integrative Physiology'.	Conception of experimental design; acquisition, analysis and interpretation of data; the preparation of manuscript for publication.
IV	Specific Role of Dietary Fat in Modifying Cardiovascular and Locomotor Activity Circadian Rhythms	Accepted for publication in 'Chronobiology International'.	Conception of experimental design; acquisition, analysis and interpretation of data; the preparation of manuscript for publication.
V	Hypothalamic Pro-Opiomelanocortin and Neuropeptide Y systems are Altered in the Development of Obesity Related Neurogenic Hypertension	Returned for revision from 'Hypertension' journal.	Conception of experimental design; acquisition, analysis and interpretation of data; the preparation of manuscript for publication.
VI	The Ventromedial Hypothalamus as the origin of aberrant blood pressure and sympathetic regulation in diet induced obesity.	Returned for revision from 'Hypertension' journal.	Conception of experimental design; acquisition, analysis and interpretation of data; the preparation of manuscript for publication.

I **have** renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: 

Date: …20.03.15……

Acknowledgments

"If I have seen further it was by standing on the shoulders of giants"
- Sir Isaac Newton –

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"We are all travellers in the wilderness of this world, and the best we can find in our travels is an honest friend"

- *Robert Louis Stevenson –*

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"I was taught that the way of progress was neither swift nor easy".

- *Marie Curie –*

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Publications Related to Thesis

Barzel B, Lim K, Burke SL, Armitage J & Head GA. (2015). Specific role of dietary fat in modifying cardiovascular and locomotor activity 24h rhythms. *Chronobiology International***,** in press.

Barzel B, Weir J, Meikle P, Burke SL, Armitage J & Head GA. (2014). Short term fat feeding rapidly increases plasma insulin but does not result in dyslipidaemia. *Front Physiol* **5,** 1-8.

Head GA, Lim K, **Barzel B**, Burke SL & Davern PJ. (2014). Central nervous system dysfunction in obesity induced hypertension. *Curr Hypertens Rep* **16**, 466-474.

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Burke SL, Prior LJ, Lukoshkova EV, Lim K, **Barzel B**, Davern P, Armitage JA & Head GA. (2013). Reduced preprandial dipping accounts for rapid elevation of blood pressure and renal sympathetic nerve activity in rabbits fed a high-fat diet. *Chronobiol Int* **30**, 726-738.

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Scientific Communication

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Barzel B, Burke S, Head G & Armitage J. (2010). Obesity Related Hypertension is associated with altered α-MSH and NPY signalling in the hypothalamus. . In Keystone Symposia. Keystone, Colorado.

Barzel B, Burke SL, Head GA, Weir JM, Meikle PJ & Armitage JA. (2009). Short term fat feeding alters plasma cholesterol ester and ceramide profiles in new zealand white rabbits. In 31th Annual Scientific Meeting of the High Blood Pressure Research Council of Australia, pp. 1495. Hypertension, Sydney, NSW, Australia.

Barzel B, Burke SL, Head GA & Armitage JA. (2008). The role of neuromodulatory peptides in the development of obesity related hypertension. In 30th Annual Scientific Meeting of the High Blood Pressure Research Council of Australia, pp. 1101. Hypertension, Melbourne, Australia.

Abstract

The relationship between body fat and blood pressure is such that relatively minor weight gains are associated with increases in mean arterial pressure (MAP). Whilst the relative risk of cardiovascular disease is greater once systolic and diastolic pressures reach 140/90 mmHg, respectively, it is important to note that there is no specific level of MAP at which disease develops and the correlation between MAP and risk of developing cardiovascular disease is strong, positive and continuous in nature (Carretero & Oparil, 2000). It is in this context that the increase in the prevalence of obesity must be examined. Obesity is now considered a global epidemic placing a considerable economic and health burden on society. Of major concern is the association between obesity and hypertension with some reports suggesting up to 78 % of newly diagnosed hypertension is attributable to obesity.

The current thesis examines the effect of consuming a high fat diet (HFD) on metabolic profiles, haemodynamic rhythms and hypothalamic neurons pertinent to energy homeostasis and cardiovascular regulation. Experiments were conducted on New Zealand White rabbits given free access to either a normal fat diet (NFD) or a HFD for three weeks. The main advantage of short-term exposure to an obesogenic stimulus is removal of confounding variables associated with obesity. Therefore, early changes that are key to the development of hypertension can be detected more easily. An additional benefit of the model is that diet-induced obesity in rabbits manifests in a manner analogous to human obesity, namely increased adiposity, hypertension and augmented renal sympathetic nerve activity. Moreover, hypothalamic neuropeptides regulating energy and cardiovascular homeostasis as well as sympathetic tone in humans are well conserved evolutionarily and are thus at play in the rabbit.

Recent evidence suggests specific lipid groups such as ceramides and cholesterol esters play a direct role in obesity-related pathologies. The haemodynamic and sympathetic response to the HFD develops within the first few days of consumption and it is possible that rapid changes in plasma lipid profiles accompany early weight gain and drive, at least initially, the observed hypertension. In addition to dyslipidaemia, hyperinsulinemia and hyperleptinaemia are known to exert a deleterious effect on cardiovascular health. Consequently, the first investigation

described in Chapter 3 examined the effect of three week fat-feeding on metabolic profiles in the rabbit. Circulating insulin and glucose levels were increased within the first three days of the diet in HFD rabbits alone, remaining elevated at week 1 and returning to baseline thereafter. By contrast, plasma leptin levels remained unchanged in both dietary groups, increasing by week 3 in HFD rabbits alone. There was no dyslipidaemia in either NFD or HFD-fed rabbits. The main finding was that plasma insulin and leptin, but not dyslipidaemia, are likely involved in the genesis of hypertension in obesity albeit at different time frames.

Rabbits fed a single meal a day exhibit meal-associated haemodynamic rhythms that are characterised by pre-prandial troughs and post-prandial peaks. Consumption of a HFD *ad libitum* increases pre-prandial values resulting in 'diurnal non-dipping', the failure of blood pressure and heart rate to decrease during the day. This shift in pattern means arterial pressure remains elevated for the duration of the 24-hour cycle subsequently increasing the risk of developing cardiovascular disease. It remains unclear whether the cardiovascular changes are due to the fat content of the diet or increased caloric intake from hyperphagia. Thus the novel aspect introduced to the rabbit model in Chapter 4 was the granting of *ad libitum* access to both NFD and HFD-fed rabbits. Whilst subtle, this change to dietary habits enabled the effect of increased total caloric intake to be distinguished from the effect of increased caloric intake from fat. Rabbits fed a HFD had greater MAP and heart rate (HR) and exhibited marked increases in pre-prandial HR. By contrast, rabbits fed a NFD showed no change in cardiovascular parameters despite consuming more calories. Average daily locomotor activity was decreased in both dietary groups. Thus, increased calories, specifically from fat, affects cardiovascular variables whilst decreased locomotor activity appears to result from greater caloric load irrespective of source.

Obesity is associated with augmented sympathetic nerve activity that is suggested to play a key role in the development of hypertension. The central actions of the hormone leptin, an anorectic adipokine, are thought to link excess adiposity and enhanced sympathetic tone. The actions of leptin are dependent on secondary neurons in the hypothalamus which synthesise and release neuropeptide Y (NPY) and alpha-melanocortin stimulating hormone (α-MSH). Thus, in Chapter 5 the contribution of both neuronal populations to the development of obesity related hypertension was

assessed. HFD-fed rabbits, but not NFD rabbits, exhibited marked renal sympathetic nerve activity (RSNA) and HR responses to intracerebroventricular (ICV) α-MSH which were concomitant with decreased neuronal activity (c-Fos positive cells) in the dorsomedial, ventromedial and paraventricular nuclei of the hypothalamus. ICV NPY reduced MAP in HFD but not control animals. Taken together the results suggest three-week fat-feeding produces marked hypersensitivity to both α-MSH and NPY, enhancing RSNA and resulting in hypertension.

Of the several hypothalamic nuclei involved in the transduction of leptin signals, the ventromedial hypothalamus (VMH) receives input from both NPY and α-MSH containing neurons in the arcuate (ARC). Moreover, high levels of the leptin receptor are expressed in the VMH whilst projections to hindbrain nuclei regulating sympathetic tone are known to originate in the VMH. In addition, the VMH was the only nucleus in which targeted injections of exogenous leptin increased both cardiovascular parameters and RSNA. Consequently, the study described in Chapter 6 examined the contribution of the VMH to obesity related neurogenic hypertension. Bilateral injections of α-MSH into the VMH of HFD rabbits produced marked renal sympathoexcitation, tachycardia and a small increase in blood pressure. Similarly, microinjections of NPY produce sympathoexcitation but failed to elicit a depressor response observed in NFD-fed animals. Microinjections of the leptin receptor antagonist decreased MAP, HR and RSNA in HFD but not NFD-fed rabbits. Combined, the results obtained in Chapter 6 suggest VMH neurons amplify both leptin and α-MSH signalling, contributing to enhanced sympathetic tone in HFD rabbits. Moreover, the loss of NPY-mediated depressor response concomitant with the fact NPY enhanced RSNA implies subpopulations of NPY neurons are affected in discrete ways by consumption of a HFD.

Collectively, the work presented in this thesis suggests that relatively short-term exposure to a high-fat diet enhances central sensitivity to α-MSH and NPY. These rapid changes occur prior to considerable changes in bodyweight and are preceded by increased circulating insulin and leptin levels. Both hormones affect cardiovascular regulation and are likely to contribute to the functional changes of hypothalamic neurons which lead to obesity related hypertension. The findings presented in this thesis form a new approach to the development of obesity related neurogenic hypertension.

Chapter I – Introduction

1.1 Preface

Obesity presents as a surplus of body adiposity (Caballero, 2007) and is clinically defined as an individual having a body mass index (BMI) greater than or equal to 30 kg/m^2 (Racette *et al.*, 2003). Alone, obesity is neither a medical condition, nor a disease. Nevertheless, obesity is a major risk factor for of non-communicable diseases (Alwan, 2011) and a leading cause of premature death in males and females (Pischon *et al.*, 2008). Obese individuals are more likely to develop cardiovascular diseases, diabetes mellitus, and several types of cancer (Dulloo *et al.*, 2002). In addition, obesity is an independent contributor to the development of several co-morbidities (Figure 1.1) such as sleep apnoea and chronic renal disease (Das, 2010; Foster et al., 2008; Kramer et al., 2005).

The incidence of obesity has been on the rise for the past several decades contributing to an ever increasing socio-economic and health burden (Finucane et al., 2011; Powers et al., 2007; Stewart et al., 2008). The World Health Organisation (WHO) estimated, in 2007, that 100 million new cases of clinical obesity developed over a single decade alone (Powers *et al.*, 2007) whilst it is estimated that in 2008 approximately 600 million individuals were obese worldwide (Finucane *et al.*, 2011). Despite presenting as a global trend, the prevalence of obesity is most prominent in Western countries (Caballero, 2007). In Australia alone, over 1.5 million middle aged individuals are currently obese (Stewart *et al.*, 2008). Similar statistics have been observed in the United States, where roughly two thirds of the adult population is considered overweight, and a third of the adult population considered obese (Powers *et al.*, 2007).

Obesity results from a state of prolonged positive energy balance in which energy intake exceeds energy expenditure. While the causes of such a state are varied, a modern 'Westernised' life style typified by increased food availability and decreased need for energy expenditure is inherently more conducive to the development of obesity (Collins, 2007). Indeed, the growing incidence of obesity has paralleled the increase in the size of meals, their frequency and energy content as well as the increase in an individual's total daily energy intake (Austin et al., 2011; Nielsen & Popkin, 2003; Popkin, 2006; Schroder et al., 2007). In the United States, the shift in dietary habits includes a greater proportion of energy being consumed from fast food

outlets and restaurants, as opposed to home cooked meals (Nielsen *et al.*, 2002). This nutritional shift is significant and due to greater fat and energy content as well as energy density of fast foods (Bowman & Vinyard, 2004). Accompanying this trend was a near tripling in the total amount of energy obtained from sweetened beverages (Nielsen & Popkin, 2004). Thus it is difficult to demarcate the contribution of calories obtained from fat or sugar as opposed to those obtained by hyperphagia alone. These changes are not isolated to the United States. Poor dietary habits have accompanied increased obesity rates in European, Middle-Eastern and Asian countries (Parizkova, 2000; Popkin, 2001) which suggests that the increase in global prevalence of obesity is likely to be an ongoing trend. It is evident that changes in diet and exercise patterns play a significant role in the development of obesity, although the precise contribution of either factor remains contentious (Bassett et al., 2008; Prentice & Jebb, 1995). Lifestyle changes such as prolonged shift work and excessive travel (jet lag) may also result in obesity as they often disturb food intake and metabolic rhythms further contributing to a positive energy balance (Gonnissen *et al.*, 2013). Obesity is exceptionally difficult to treat, with approximately 90% of obese patients reported to re-gain weight following successful weight loss (Rosenbaum *et al.*, 1997; Sumithran *et al.*, 2011).

A slow but inexorable rise in blood pressure (the regulation of which in healthy subjects will be reviewed in section 1.4) is found in a large portion of obese individuals (Kannel *et al.*, 1967) but in the main, hypertension does not become symptomatic. Nonetheless, there are considerable ramifications of untreated hypertension (defined as diastolic blood pressure ≥ 90 and or a systolic blood pressure ≥ 140 (Stergiou *et al.*, 2004). It is estimated that approximately 70% of newly diagnosed hypertension patients in the Framingham cohort were obese (Garrison *et al.*, 1987). Despite the delays in diagnosis, treatment of obesity related hypertension consumes a vast amount of resources including hospitalisation and medical attention (Powers *et al.*, 2007) which creates a considerable global economic burden (Stewart *et al.*, 2008).

To help alleviate the health and economic burdens of obesity related hypertension, a greater understanding of the mechanisms that contribute to its development is required. Key to this appears to be the activation of the sympathetic nervous system (reviewed in section 1.6.2 Head *et al.*, 2014) which occurs as a result of peripheral obesity related metabolic signals acting at the hypothalamus. The current thesis focuses on the central pathways and seeks to delineate the contribution of two key hypothalamic neuromodulatory systems; neuropeptide Y (NPY) and alpha melanocortin-stimulating hormone (α-MSH) in the development of obesity related hypertension. Importantly, perturbations to circadian processes as well as exposure to stress also contribute to the development of hypertension (Korner, 2007). These will be discussed at greater length in section 1.5 of this literature review. The following sections will discuss the pathogenesis of obesity in greater detail, as well as the likely mechanisms that contribute to an obese state. These include peripheral signalling molecules and the central nervous system (CNS) responses to these factors.

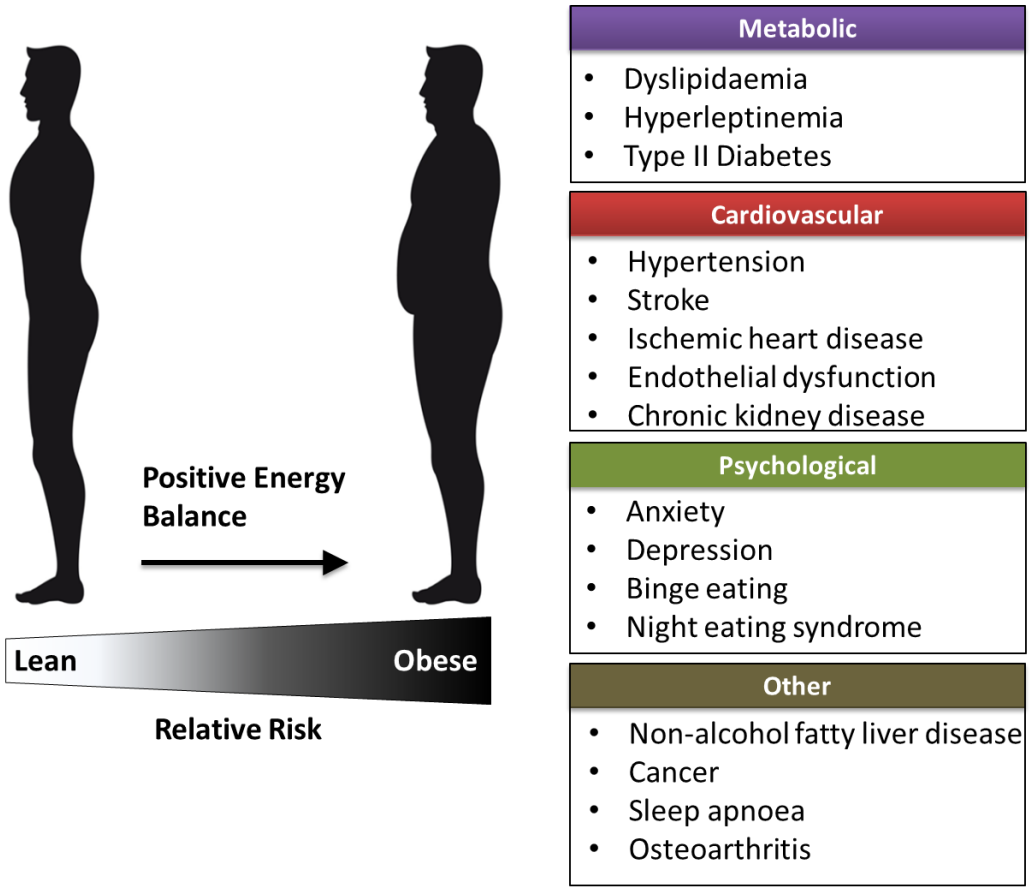

Figure 1.1: Deleterious health conditions associated with obesity.

The impact of a positive energy balance transforms individuals from lean to overweight and obese resulting in a marked increase in the relative risk of ill health. These include metabolic disorders of dyslipidaemia and hyperleptinaemia and an increased risk of type II diabetes. Cardiovascular consequences include hypertension, ischaemic and haemorrhagic stroke, ischaemic heart disease, endothelial function and chronic kidney disease. In addition there are considerable psychological dysfunctions including anxiety and depression as well as other disease consequences including cancer, sleep apnoea and osteoarthritis (Haslam & James, 2005).

1.2 Consequences of Increased White Adipose Tissue

White adipose tissue (WAT) was initially thought to be passively accumulated fat thus allowing the body to utilise stored energy during periods of low food availability (Trayhurn & Beattie, 2001). However, this view was abandoned after the discovery in 1994 that WAT released leptin and had endocrine like functions (Hassan et al., 2012; Zhang et al., 1994). It is now well recognised that WAT secretes a multitude of chemical factors (adipokines) that are central to energy and glucose homeostasis, inflammation, sympathetic output and blood pressure (Hassan *et al.*, 2012). By volume, the largest secretions from WAT are fatty acids. While primarily used as energy substrates to fuel various functions around the body, excess free fatty acids are capable of stimulating numerous inflammatory pathways (For review see Calder, 2011). For instance, free fatty acids engage toll-like receptors (TLR), a pathogen-sensing family of proteins belonging to the innate immune system (Shi *et al.*, 2006). Diet-induced obese (DIO) mice exhibit augmented TLR4 mRNA in adipose tissue and increased inflammatory markers. Importantly, these changes are not observed in $TLR4^{-/-}$ mice despite being fed the same diet (Shi *et al.*, 2006). Similarly, increased free fatty acid plasma concentrations in lean humans are associated with the activation of nuclear factor κ-B (NFκ-B), a pro-inflammatory agent (Tripathy *et al.*, 2003). Interestingly, the likelihood of developing Type 2 Diabetes (T2D) is greater with increased inflammatory markers (Schmidt *et al.*, 1999). Thus, increased circulating free fatty acids may unfavourably affect glucose homeostasis in obese individuals. Indeed, inflammation, particularly if localised to WAT, has been implicated in the development of insulin resistance (Hotamisligil, 2006; Xu et al., 2003) whilst treatment of obese patients with anti-inflammatory agents improves insulin sensitisation despite no change in body weight (Hundal *et al.*, 2002).

Dyslipidaemia, aberrant plasma lipid profiles, is an additional consequence of obesity that has been linked with metabolic syndrome, pulmonary embolisms, deep venous thrombosis and atherosclerotic arterial disease (Lawrence & Kopelman, 2004). In humans, obesity related dyslipidemia manifests as high plasma concentrations of low density lipoproteins, triacylglycerides and ceramides as well as reduced plasma high density lipoprotein levels (Haus et al., 2009; Howard et al.,

2003; Williams & Krauss, 1997). Crucially, all of these trends are associated with insulin resistance and inflammation (Fernandez-Real & Ricart, 2003).

1.3 Quantifying Adiposity

1.3.1 Abdominal Adiposity in the Development of Comorbidities in Obesity

Guidelines published by the WHO highlight a recent shift in how cardiovascular risk is assessed in obese individuals. In its report (2000) the WHO recommended anthropometric measures ancillary to BMI to enable a more accurate assessment of cardiovascular risk among the obese. The move followed recognition that whilst total adiposity strongly correlates with metabolic syndrome and cardiovascular risk, specific WAT deposits are associated with different risk profiles and contribute differently to the total risk of cardiovascular disease (Hassan *et al.*, 2012; Muller *et al.*, 2012). Body composition may be a better predictor of disease than body mass. Stefan and colleagues (2008) show that visceral adiposity was a strong predictor of insulin resistance among individuals of normal-weight. Moreover, ectopic fat depots in skeletal muscle and the liver were greater indicators of insulin resistance among obese individuals. By contrast, benign obese individuals (showing no deleterious metabolic profile) had 54 % less ectopic fat than obese individuals who were insulin resistant (Stefan *et al.*, 2008). Similarly, pericardial fat is a strong predictor of vascular calcification but abdominal adiposity, particularly in the viscera, correlates more strongly with cardiovascular disease (Rosito *et al.*, 2008). Furthermore, participants in the Quebec Heart Institute's lifestyle modification program lost only 0.3 Kg of weight following 1 year on the program. This minor loss of weight translated into a 6 cm reduction in waist circumference and a 17.5 % loss of visceral adiposity concurrent with a pronounced improvement in metabolic profile (Despres *et al.*, 2008).

The relevance of body composition to predicting cardiovascular risk is further evident in several ethnic populations, notably, Asian Indians (defined as individuals originating from India, Pakistan, Sri Lanka, Bangladesh or Nepal). Members of this population have a surprisingly high rate of insulin resistance despite being non-obese

as defined by BMI values of around 24 kg/m^2 (Banerji *et al.*, 1999). Furthermore, the high cardiovascular mortality rate found in this population cannot be explained by traditional risk factors such as smoking and the prevalence of hypertension (Beckles *et al.*, 1986). A distinguishing feature of this population is the propensity to greater abdominal adiposity and waist circumference compared with BMI and age-matched Caucasians (Banerji *et al.*, 1999; Raji *et al.*, 2001). Chandalia and colleages (2007) matched non-diabetic Asian Indian men with non-diabetic Caucasian men according to age, weight, waist circumference and BMI. They reported that Asian Indians had a greater total body fat content and that this tended to localise in truncal subcutaneous pads (Chandalia *et al.*, 2007). Intriguingly, visceral adiposity was similar between the two groups, suggesting the observed insulin resistance in Asian Indians was due to accumulation of subcutaneous abdominal fat (Chandalia *et al.*, 2007). Comparable differences exist between American white and black females. Non-diabetic subjects of similar age, BMI, total fat mass and waist to hip ratio had their plasma lipid profiles and insulin sensitivity levels assessed relative to body composition (Albu *et al.*, 1997). While greater abdominal adiposity was associated with poorer health outcomes in both ethnic groups, black women had less visceral fat than white women suggesting the pathological effect of body composition may vary between races (Albu *et al.*, 1997). As fat pads' contribution to total adiposity differs between ethnic groups so do the associated consequences. Guidelines for quantifying obesity and predicting associated risk must therefore be gender and ethnic specific.

In addition to visceral adiposity, epicardial pad thickness strongly correlates with BMI and waist circumference in both males and females (Kim *et al.*, 2012). Situated in the visceral pericardium cavity, this fat pad lies in close proximity to the myocardium and shares the same microvasculature as the heart (For a more detailed review see Sacks & Fain, 2007). In twins discordant for obesity, cardiac dimensions were similar within pairs yet augmented epicardial adiposity and low-grade inflammation were isolated to the obese twin (Graner *et al.*, 2012). These data suggest epicardial adiposity is intimately linked with general obesity and is independent of genetic influences. Importantly, epicardial fat may indirectly impede cardiac function due to its ability to induce inflammation (Graner *et al.*, 2012). Indeed, epicardial adipose tissue in humans is known to secrete a range of pro-inflammatory adipokines such as tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Local

expression of these adipokines, as well as localised cardiac inflammation, may be poorly represented in plasma adipokine concentrations (Mazurek *et al.*, 2003). Not surprisingly, epicardial fat correlates with coronary artery calcification and one or both of ischemia or stenosis (Nakazato *et al.*, 2012).

The past two decades have seen our understanding of WAT, specifically in the context of obesity and metabolic syndrome, advance considerably. Numerous adipokines released from WAT pads (and their target sites) have been identified and these have been implicated in the development of diabetes, hypertension and cardiovascular disease. It is conceivable that increased total adiposity upsets the hormonal milieu of the body thus perturbing central homeostatic mechanisms responsible for the regulation of energy, glucose and blood pressure homeostasis. The hypothalamus is a key link between excess adiposity and hypertension. Lacking an effective blood brain barrier (BBB), hypothalamic nuclei receive multiple signals from the periphery pertaining to the state of energy homeostasis (Johnson & Gross, 1993). As discussed above, metabolic substrates such as FFA and glucose, hormones such as insulin and adipokines such as leptin are all capable of stimulating arcuate (ARC) neurons, directly modulating central homeostatic pathways that are central to blood pressure maintenance and energy usage. This is likely to be the mechanism through which obesity related hypertension develops and will now be discussed in greater detail.

1.3.2 Adipokines

Adipokines, the collective term applied to proteins secreted by adipocytes, constitute a large portion of WAT secretions and are widely associated with regulating a variety of physiological processes, in particular energy and glucose homeostasis (Ahima & Lazar, 2008; Trayhurn *et al.*, 2011). Crucially, adipokines have been implicated in the pathogenesis of obesity due to their role as functional links between WAT and the CNS (Ahima & Lazar, 2008). It is currently estimated over 100 different adipokines are secreted from WAT (Trayhurn, 2013). Whilst the role and functions of all adipokines cannot be presented in full, the following section presents key adipokines pertinent to the development of obesity, metabolic syndrome and hypertension (Table 1.1 and Figure 1.2). The adipokine leptin will be discussed at greater length given it is the primary focus of this thesis.

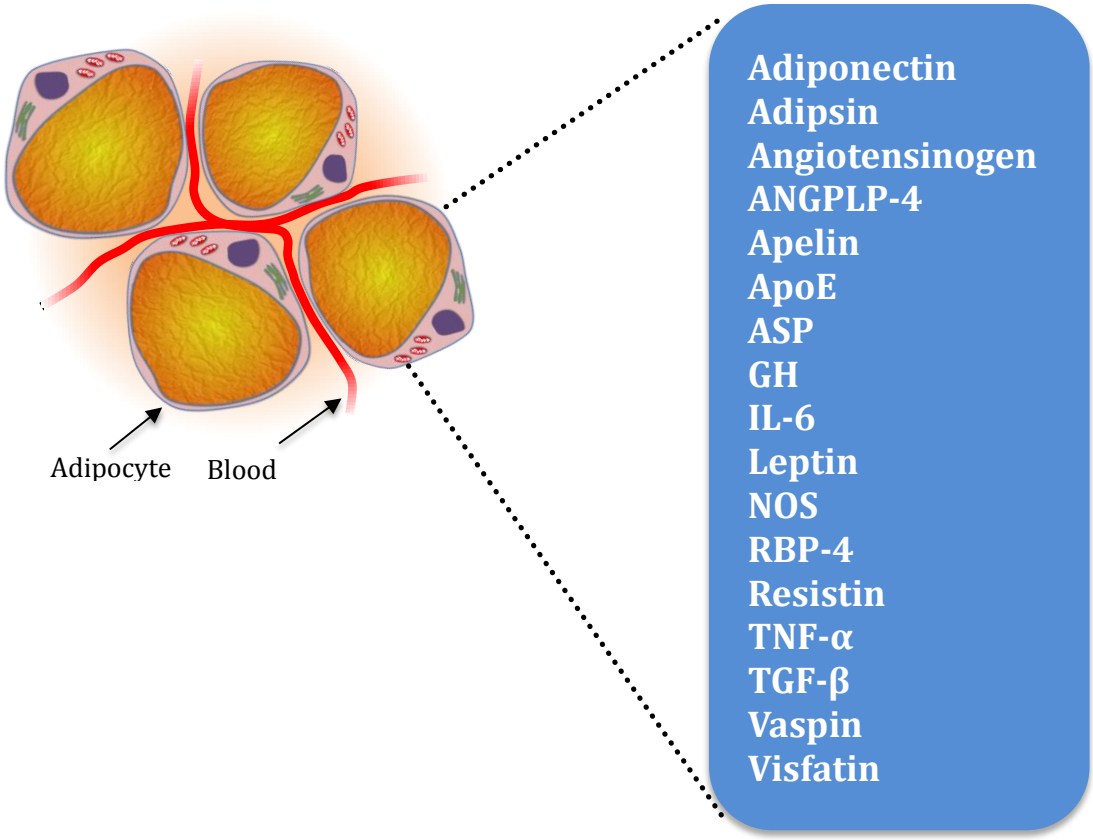

Figure 1.2: Adipocyte secretory products.

Adipocytes are known to secrete a large number of cytokines which in turn affect many physiological processes including, metabolism, inflammation and cardiovascular regulation. Abbreviations: ANGPLG-4; angiopoetin like protein 4, ApoE; apolipoprotein E, ASP; acylation stimulating protein, GH; growth hormone, IL-6; Interleukin 6, NOS; nitric oxide synthase, RBP-4; retinol binding protein 4, TNF-α; tumor necrosis factor alpha, TGF-β; transforming growth factor beta (Redrawn from Ahima & Lazar, 2008; Fruhbeck et al., 2001; Vlasova et al., 2010).

1.3.2.1 Adiponectin

Adiponectin is secreted almost exclusively by adipose tissue and is a considerable homeostatic agent in both lean and obese individuals (Brochu-Gaudreau *et al.*, 2010). Owing to its anti-atherogenic and anti-inflammatory properties, as well as its ability to improve endothelial dysfunction and insulin sensitisation, adiponectin is largely considered to be protective of obesity and its associated co-morbidities (Ahima & Lazar, 2008; Ouchi et al., 2000; Yamauchi et al., 2001). Curiously, circulating plasma levels of adiponectin are inversely proportional to BMI and visceral adiposity (Arita *et al.*, 1999; Brochu-Gaudreau *et al.*, 2010). This suggests both synthesis and release of adiponectin may be negatively impacted in obesity further exacerbating the condition (Yamauchi *et al.*, 2001). Adiponectin signals are mediated via three receptor subtypes AdipoR1, AdipoR2 and T-Cadherin (Brochu-Gaudreau *et al.*, 2010). Both AdipoR1 and 2 subtypes are intimately involved with energy homeostasis although they have contrasting impacts. AdipoR1$^{-/-}$ mice are obese and insulin resistant whilst AdipoR2$^{-/-}$ mice appear immune to obesity even when maintained on a high fat diet (HFD) and also display improved insulin sensitivity (Bjursell *et al.*, 2007).

Adiponectin likely acts centrally as both AdipoR1 and AdipoR2 receptors are also expressed in the brain (Kubota *et al.*, 2007). Acute infusions of adiponectin applied both systemically and centrally caused a similar decrease in blood pressure in adiponectin knockout mice (Tanida *et al.*, 2007). Interestingly, the hypotensive effect is not observed following chronic central infusions, suggesting long-term regulation of blood pressure by adiponectin may be mediated by its peripheral effects (Bassi et al., 2012; Kim et al., 2013). Indeed adiponectin has been shown to be a potent mediator of nitric oxide dependent vasodilation (Wang & Scherer, 2008). Adiponectin knockout mice have higher blood pressure than controls and exhibit impaired endothelium-mediated vasodilation (Ouchi *et al.*, 2003). Hypoadiponectinemia is associated with greater risk of developing hypertension among normotensive individuals within a relatively short period of time (Chow *et al.*, 2007). Perhaps most interesting is the relationship between adiponectin and the sympathetic nervous system, especially given the association between the latter and hypertension. Chronic, but not acute, exposure to cold resulted in decreased circulating adiponectin levels whilst sympathetic inhibition reversed the decrease in expression (Imai et al., 2006;

Puerta et al., 2002). Moreover, in patients with essential hypertension treated with rilmenidine, a centrally acting sympatholytic agent, there is a 26% increase in adiponectin levels (Nowak *et al.*, 2005). More specifically, renal sympathetic nerve activity (RSNA) is reduced by greater circulating adiponectin levels (Kim et al., 2013). Combined, these data suggest decreased adiponectin levels may comprise a mechanism by which metabolic and endothelial dysfunction as well as hypertension develop in obesity. More importantly, a better understanding of how adiponectin exerts its beneficial effects may aide in the long term treatment of obesity.

1.3.2.2 Resistin

So named for its role in promoting insulin resistance, resistin ('resist – insulin') is a peptide implicated in the pathophysiology of obesity. High circulating resistin levels are known to occur in obesity and are believed to increase the likelihood of type 2 diabetes (T2D), heart failure and myocardial infarction (Frankel et al., 2009; Steppan et al., 2001; Weikert et al., 2008). The target receptor for resistin has yet to be identified making the molecular mechanisms of its actions a challenge to characterise. Despite this, the association between resistin and the development of several pathologies is strong. A potent mediator of inflammation, resistin has been demonstrated to promote the expression of pro-inflammatory cytokines TNF-α and IL-12 in both mice and human macrophages (Silswal *et al.*, 2005) thus contributing to the chronic inflammation often associated with obesity. These findings are important given T2D patients are known to have high TNF-α circulating levels, which strongly correlate with insulin resistance (Hotamisligil *et al.*, 1995) whilst IL-12 has been linked to the susceptibility to autoimmune diabetes (Taylor-Fishwick *et al.*, 2013). Interestingly, the relationship between resistin expression and pro-inflammatory cytokines appears to be mutual with IL-6, TNF-α and interleukin beta (IL-β) inducing resistin mRNA expression in human mononuclear cells (Kaser *et al.*, 2003).

Resistin has also been implicated in feeding and energy homeostasis. Vazquez et al (2008) reported that acute central infusions of the peptide attenuated food intake in both fed and fasted rats. The diminution in food intake was concomitant with reduced expression of the potent orexigenic peptides neuropeptide Y (NPY) and Agouti Related Protein (AgRP) in the ARC, a major site of energy homeostasis (Vazquez *et al.*, 2008). Given that fasting is associated with increased NPY

expression and the propensity to consume more food (Mizuno et al., 1999; Sahu et al., 1988; 1995), it is intriguing that resistin is capable of overriding such a powerful regulator of appetite. By contrast, reports by Singhal and colleagues (2007) suggest central infusions of resistin in mice increase hypothalamic NPY expression. The authors reported that increased NPY expression is a likely mechanism by which resistin triggers hepatic insulin resistance. Equally puzzling are reports that resistin cerebrospinal fluid concentrations do not correlate with either BMI or T2D in humans (Kos *et al.*, 2007). In the absence of a clear resistin target receptor these conflicting reports are difficult to consolidate. It is possible that resistin acts on discrete subsets of NPY containing neurons in different ways. Importantly, both studies establish the ability of resistin to interact with NPY containing neurons.

Most pertinent to obesity are studies demonstrating that resistin directly modulates sympathetic output. Kosari and colleagues have shown that resistin decreases sympathetic output to brown adipose tissue (BAT) as well as augment RSNA (Kosari et al., 2012; Kosari et al., 2011). Such effects directly impact energy and blood pressure homeostasis because reduced sympathetic output to BAT attenuates energy expenditure whilst prolonged activation of RSNA is associated with hypertension (Armitage et al., 2012; Bartness et al., 2010; Prior et al., 2014; Prior et al., 2010).

1.3.2.3 Apelin

Expressed in several tissue types, including WAT and the hypothalamus, apelin is a potent mediator of nitric oxide (NO)-dependent vasodilation (Beltowski, 2006; Vlasova *et al.*, 2010). Exogenous apelin has been shown to decrease blood pressure in the rat and increase nitrite/nitrate plasma concentrations whilst pre-treatment with the NO inhibitor, L-NAME, prevented this depressor response (Tatemoto *et al.*, 2001). Similarly, NO-dependent vasodilation was observed in forearm resistance vessels of non-obese humans following administration of intravenous apelin (Japp *et al.*, 2008). Apelin has also been suggested to play a critical role in glucose homeostasis. Yue and colleagues (2010) reported that apelin knockout mice treated with exogenous apelin exhibited complete restoration of insulin sensitivity, becoming identical in nature to age-matched wild type littermates. Interestingly, the central actions of apelin appear to contradict its peripheral effects.

Kagiyama *et al* (2005), reported that intracerebroventricular (ICV) injections of apelin in the rat increased both mean arterial pressure (MAP) and heart rate (HR) in a dose-dependent manner and were associated with increased c-Fos expression, a protein released following depolarization and used as a marker of recent neuronal activation (Bullitt, 1990), in the paraventricular hypothalamus (PVH). Microinjections of apelin into the PVH increased sympathetic output to BAT (Masaki *et al.*, 2012). Notably, the central effects of apelin are not restricted to the PVH given that microinjections into the rostral ventrolateral medulla (RVLM; a key region of blood pressure regulation) have produced considerable increases in MAP and RSNA in anesthetised rats (Zhang *et al.*, 2009). Furthermore, induction of endogenous apelin in the RVLM by means of virus mediated gene transfer produced sustained increases in MAP lasting for several weeks (Zhang *et al.*, 2009). Whilst apelin plasma levels are higher in obese patients and correlate with BMI (Heinonen *et al.*, 2005), this does not appear to result in an anti-hypertensive effect. It is possible that the increased circulating levels cross the blood-brain barrier and exert a pressor response via the hypothalamus (Vlasova *et al.*, 2010). Reaux-Le Goazigo and colleagues (2011) extensively characterised apelin's function in the CNS. Apelin and its receptor are expressed in hypothalamic proopiomelanocortin (POMC) neurons and apelin directly modulates the release of α-MSH, a key effector peptide of the POMC system (Reaux-Le Goazigo *et al.*, 2011). The close association between apelin and the melanocortin system is a likely mechanism through which apelin's sympathoexcitatory and anorexic functions occur.

Table 1.1: Adipokines and their physiological actions in health and obesity.

Adipokine	Source	Physiological Effects in Health	Effect of Obesity on Adipokine	Physiological Effect in Obesity	References
Leptin	WAT	↓ Appetite ↑ Blood Pressure ↑ SNA	↑ Expression in WAT ↑ Circulating levels	↔ Appetite ↑ Blood Pressure ↑ SNA	Mark *et al*, 2003; Zhang *et al*, 1994; Eikelis *et al*, 2005.
Adiponectin	Mainly from WAT. Also, liver, pituitary gland, skeletal muscle, bone marrow, salivary glands and kidneys.	↑ Insulin Sensitivity ↑ Endothelial NO synthesis	↓ Expression in WAT ↓ Circulating levels	Loss of hypotensive and anti-insulin resistant properties due to decrease in expression.	Brochu-Gaudreau *et al*, 2010; Wang and Scherer, 2008.
Resistin	WAT and CNS	↑ Inflammation ↑ Cardiovascular Risk	↑ Circulating levels	↓ Food Intake ↓ SNA to BAT ↑ RSNA ↑ Inflammation	Kosari *et al*, 2011; Kosari *et al*, 2012; Silswal *et al*, 2005; Vazquez *et al*, 2008; Wilkinson *et al*, 2007.
Apelin	WAT, CNS	**Peripheral Actions**: ↑ NO Mediated vasodilation ↑ Insulin Sensitivity **Central Actions**: • Appetite ↑ RSNA, Blood Pressure	↑ Circulating levels		Beltowski *et al*, 2006; Vlasova *et al*, 2010.

1.3.2.4 Leptin

The discovery of leptin by Zhang *et al* (1994) considerably advanced our understanding of energy homeostasis in the field of obesity research. A product of the *ob* gene, leptin is a 16 kDa peptide primarily synthesised in, and secreted by, WAT (Zhang *et al.*, 1994) with plasma concentrations of the peptide remaining proportional to the level of adiposity in an individual (Considine *et al.*, 1996; Eikelis & Esler, 2005). Leptin has been implicated in numerous physiological functions including wound healing, reproduction, immune response and glucose homeostasis (Frühbeck, 2002l; Haque et al., 1999). Nevertheless, in the context of obesity-related investigations, its anorectic and sympathoexcitatory effects have been at the forefront of research (Mark, 2013).

Initial observations that plasma leptin levels were low prior to a meal and increased a few hours post-prandially led to the presumption that leptin may be involved in appetite regulation (Saladin *et al.*, 1995). Halaas and colleagues (1998) confirmed the metabolic function of leptin by demonstrating that leptin infusion in rodents, both systemically and centrally, produced a marked decrease in food consumption. Those findings were confirmed in studies using transgenic mice. Chua *et al* (1996) demonstrated that mice lacking the *ob* gene and which are therefore leptin deficient, become hyperphagic and obese. In addition, mice lacking the *db/db* gene which is required for expression of the leptin receptor, manifested similar feeding obesogenic behaviour and weight gain. Interestingly, ICV administration of leptin was considerably more effective in suppressing appetite than peripheral administration (Halaas *et al.*, 1997) suggesting that the CNS is the primary target of leptin. Indeed, areas of the hypothalamus devoid of an effective blood brain barrier (BBB) or lying adjacent to the ventricles possess high affinity uptake systems which are crucial mediators of leptin's effects (Friedman & Halaas, 1998). There are six known leptin receptor (LepR) variants (*a-f*), of which a single isotope (LepR*b*) is distinguishable due to its longer intracellular domain (Hegyi *et al.*, 2004). The longer variant is capable of activating several downstream intracellular signalling pathways (such as Janus tyrosine kinase/signal transducer and activators of transcription (JAK/STAT, Figure 1.3) and is believed to be the main receptor subtype through which the functional effects of leptin, namely those related to energy homeostasis, are mediated (Signore *et al.*, 2008). The remaining receptor variants can be classified as

either 'short' (LepR*a,c,d,f*), possessing a truncated intracellular domain, or 'soluble' (LepR*e*) lacking one altogether (Signore *et al.*, 2008). The short variants possess a limited capability to activate downstream intracellular pathways, and both short and soluble receptor subtypes appear to be more critical in active transport (Bjorbaek et al., 1997; Kastin et al., 1999; Murakami et al., 1997). Leptin is transported across the BBB against a concentration gradient and in a saturable fashion (Banks *et al.*, 1996) thus maintaining a considerably lower concentration in the CNS compared with circulation. Both short and soluble leptin receptors are expressed around the third cerebral ventricle (3V) and median eminence (Hakansson *et al.*, 1998). Pharmacological and lesion studies have demarcated the hypothalamus, and in particular the ARC which surrounds the 3V, as key regulators of appetite and energy expenditure (For an in-depth review, see Gao & Horvath, 2007). Indeed, leptin receptor mRNA is found at a high density in many hypothalamic nuclei (Elmquist *et al.*, 1998b).

Shortly after the discovery of leptin, Halaas and colleagues (1997) observed that diet-induced obese mice were less responsive to the anorexigenic effects of peripherally administered leptin despite maintaining the ability to respond to leptin centrally. Given reduced sensitivity to leptin occurred with increased circulating concentrations, Halaas and colleagues suggested that the likely explanation was a defect in the transport of leptin across the BBB. In an elegant set of experiments Aizawe-Abe and colleagues made use of three animal models to better characterise the effects of hyperleptinemia. They found that transgenic mice that were overexpressing leptin were lean, hypertensive and had increased urinary catecholamine excretion suggesting enhanced sympathetic output (Aizawa-Abe *et al.*, 2000). Agouti mice, which overexpress AgRP resulting in chronic inhibition of the melanocortin system, present with late-onset obesity due to resistance to the anorexigenic effects of leptin. In addition, these mice are hyperleptinemic, hypertensive and have increased urinary catecholamine excretion (Aizawa-Abe *et al.*, 2000). Lastly, leptin deficient *ob/ob* mice were normotensive but exhibited a considerable increase in arterial pressure following a 3-day infusion of leptin (Aizawa-Abe *et al.*, 2000). These data suggest hyperleptinemia promotes a state of leptin resistance whereby the appetite-regulating effects of leptin are lost whilst the pressor and sympathoexcitatory effects are preserved. Rahmouni (2002) highlighted

the selective nature of leptin resistance by examining the effect of ICV leptin in agouti mice and their controls. Central infusion of leptin reduced food intake and BAT mass and increased RSNA in control mice. Agouti mice exhibited the same increase in RSNA following ICV leptin but did not manifest any of the metabolic effects (Rahmouni *et al.*, 2002). Likewise, DIO mice, resistant to the anorectic properties of leptin, increased sympathetic output to BAT following central infusions of leptin (Enriori *et al.*, 2011). Similarly, obese humans present with greater circulating leptin levels (Lambert *et al.*, 2007a) and appear to be resistant to its metabolic effects (Considine *et al.*, 1996).

Whilst the exact mechanism by which leptin resistance develops remains unclear, it is likely that insufficient or lack of leptin transport across the BBB may contribute to the condition. Similarly, a failure of cellular signalling cascades to activate following leptin binding to its receptor may also lead to leptin resistance. Given the requirement for active transport across the BBB, any impairment in Lep*a*, *c-f* receptor subtypes may result in leptin not reaching its target sites in the CNS (Banks, 2012). Indeed the ratio between cerebrospinal fluid (CSF) and plasma leptin is about 4 times greater in lean individuals than in the obese despite circulating leptin being 318 % greater in the latter group (Caro *et al.*, 1996). Further evidence of impaired leptin transport occurring in obesity can also be found in several animal models. Van Heek and colleagues (1997) found that DIO in mice is associated with reduced leptin efficacy if the leptin is administered peripherally but not when administered centrally. Furthermore, age-related weight gain versus aging alone was associated with decreased leptin transport across the BBB of mice and was reversible by modest weight loss (Banks & Farrell, 2003). In the ovine model, obese animals exhibited no attenuation in food intake despite being hyperleptinemic and, in a manner comparable to human obesity, exhibited a lower CSF to plasma concentration ratio than controls (Adam & Findlay, 2010). Nevertheless, 3V injections of leptin resulted in sustained reductions in food intake which suggested that hypothalamic sensitivity to leptin was normal and transfer of peripheral leptin across the BBB was impaired (Adam & Findlay, 2010). Interestingly, leptin transport impairment was not reversed by weight loss in these animals (Adam & Findlay, 2010).

Given the complexity of the post-receptor signalling cascade (Figure 1.3) leptin resistance may also stem from faulty intracellular signalling pathways downstream of the leptin receptor. Indeed, naturally occurring precedents of downgraded intracellular sensitivity to leptin have been observed in the wild. In the Siberian hamster, the ability of leptin to activate post-receptor signalling cascades is seasonal (Tups *et al.*, 2006) whilst in the rat, leptin 'resistance' develops during pregnancy (Ladyman, 2008).

Signal transducer and activator of transcription 3 (STAT3) is a key component of the intracellular machinery activated by leptin (Bates et al., 2004; Bates et al., 2003) and is inhibited by suppressor of cytokine signalling 3 (SOCS3) in a negative-feedback manner (Bjorbak *et al.*, 2000). Pregnant rats exhibit hyperleptinemia that is concomitant with increased food intake and body weight which suggests the presence of leptin resistance (Trujillo *et al.*, 2011). Contrary to obesity, leptin resistance in this model appears to be both peripheral and central, with ICV injections of leptin failing to attenuate food intake or reduce body weight (Trujillo *et al.*, 2011). Furthermore, SOCS3 expression is increased in pregnant rats and subsequently inhibits STAT3 phosphorylation (Trujillo *et al.*, 2011). Recently, phosphoinositide 3-kinase (PI3K) has been identified as an additional signalling pathway through which leptin may exert its anorectic effects (Niswender *et al.*, 2001). This STAT3 independent pathway has also been shown to be down-regulated in pregnant rats (Trujillo *et al.*, 2011). These observations suggest modulation of leptin-dependent intracellular signalling cascades is not only possible but may be an adaptation to environmental changes allowing for the 'reset' of optimal body weight (Tups, 2009).

Impairment of the same intracellular mechanisms is believed to result in obesity-related leptin resistance (Mark, 2013). Mice with a targeted disruption of STAT3 signalling (s/s) are phenotypically analogous to *db/db* mice which lack a functional LepR*b* (Bates *et al.*, 2003). Importantly, both genetic strains exhibit reduced sympathetic output to BAT (Mark, 2013). Nevertheless, in response to leptin, s/s mice have greater renal sympathoexcitation whilst sympathetic output to renal vasculature is absent in *db/db* mice (Bates *et al.*, 2003). The discrete effects of STAT3 signalling on sympathetic activity demonstrate how aberrant molecular mechanisms inhibit specific leptin functions (Mark, 2013). Further evidence for the molecular

basis of selective leptin resistance arises from observations that s/s mice are normotensive suggesting STAT3 signalling is required for the pressor effects of leptin to take effect (Bodary *et al.*, 2007). Activation of hypothalamic proopiomelanocortin (POMC) containing neurons is leptin dependent and is a key step in the cardiovascular and metabolic actions of leptin. Targeted inhibition of STAT3 signalling in POMC neurons alone abolishes the pressor response to chronic leptin infusion but does not impede the long-term metabolic effects of leptin (Dubinion *et al.*, 2013). Importantly, changes to cellular mechanism have been observed in obesity with prolonged high-fat feeding in mice greatly diminishing leptin mediated STAT3 signalling in the hypothalamus (El Haschimi *et al.*, 2000). Despite this, the mechanisms underlying changes in the cellular response to leptin in obesity have yet to be fully defined.

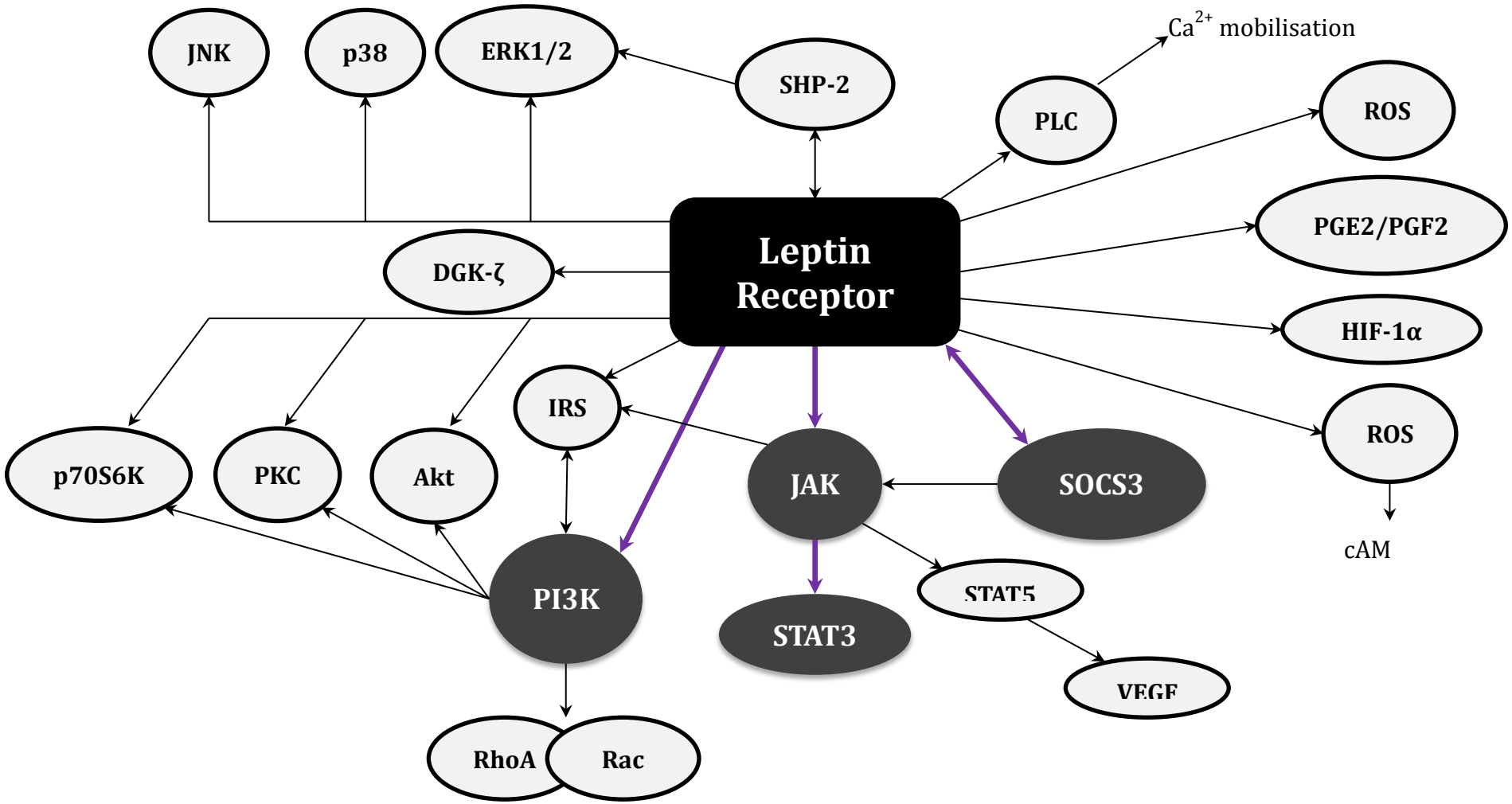

Figure 1.3: Leptin mediated cellular signalling pathways.

In dark grey are pathways important in metabolic and cardiovascular homeostasis (Redrawn from Fruhbeck, 2006).

1.4 Blood Pressure Homeostasis and Contributing Mechanisms

Prior to discussing the development of obesity related hypertension, it is imperative to appreciate the short, medium and long-term homeostatic mechanisms that regulate blood pressure and heart rate under normal conditions.

1.4.1 Regulation of Blood Pressure by Baroreflexes

Blood pressure is a function of vascular (total peripheral) resistance and cardiac output (Guyenet, 2006). Cardiac output is itself the outcome of two adjustable variables: stroke volume (the subtraction of end-systolic from end-diastolic volume) and heart rate (Vatner & Boettcher, 1978). Notably, heart rate and stroke volume are influenced by both the sympathetic and parasympathetic nervous systems (Guyton, 1968). Neural regulatory mechanisms offer an effective buffering system against transient stimuli such as posture, exercise, temperature changes and feeding and are the first to take effect in response to a change in blood pressure (Cowley *et al.*, 1973). This prompt response is mediated by arterial baroreceptors that have their cell bodies in the nodose and petrosal ganglia with nerve endings located in the adventitia of the aortic arch (via the vagus nerve) and carotid sinus (via the glossopharyngeal nerve, Benarroch, 2008; Silveira *et al.*, 2008). These termini function as mechanical stretch-receptors and are capable of registering minute deformations of the vessel wall that occur as a result of changes in pressure (Brown, 1980). A surge in blood pressure results in depolarisation of mechanosensitive cation channels, subsequently depolarising K^+ and Na^+ voltage-gated ion channels and increasing afferent action-potential firing rate (Benarroch, 2008; Chapleau et al., 2001). This complex mechanism is a defining characteristic of baroreceptors and allows for a suitable response to varying degrees and durations of changes in pressure (Chapleau *et al.*, 2001).

The axons from baroreceptors terminate on second-order neurons in the nucleus tractus solitarii (NTS) of the hindbrain (Benarroch, 2008). Projections arising from the NTS neurones activate GABAergic cells in the caudal ventrolateral medulla (CVLM), which in turn inhibit sympathoexcitatory neurons in the RVLM (Schreihofer & Guyenet, 2002). The RVLM is essential for tonic maintenance of blood pressure and sympathetic activity, removal or inhibition of which result in a

substantial drop in pressure (Horiuchi & Dampney, 1998; Varner et al., 1994; Willette et al., 1983). Additionally, GABAergic signals inhibit noradrenergic A1 neurons of the CVLM (Benarroch, 2008). Noradrenergic A1 neurons constitute a key link between the autonomic centres of the hindbrain and the supraoptic (SON) and PVH nuclei of the hypothalamus (Swanson *et al.*, 1981), both of which are sites of vasopressin release and sympathetic preganglionic activation (Schramm et al., 1993; Swaab et al., 1975). A rise in blood pressure results in a reflex withdrawal of sympathetic tone from the heart and vasculature as well as an increase in cardiac parasympathetic input (Fadel, 2008). This results in dilatation of peripheral blood vessels and a reduction in heart rate which further reduces mean arterial pressure (Guyton, 1991). The reverse occurs when blood pressure is decreased.

There is general agreement that baroreceptors are critical for blood pressure homeostasis although the time frame over which they contribute to blood pressure regulation remains controversial. Sino-atrial denervation has no long-term effect on baseline MAP (Cowley *et al.*, 1973) whilst baroreceptors reset to higher operating pressures (Potts & Mitchell, 1998; Walgenbach & Donald, 1983) suggesting the contribution of baroreflexes to blood pressure regulation is only relevant in the short term (seconds to minute). This is not entirely accurate as there are distinct subpopulations of baroreceptors with different resetting characteristics (Seagard *et al.*, 1992). Indeed studies using SHR suggest that baroreceptors take many months to reset completely (Thorén *et al.*, 1983). Lohmeier (2002) and colleagues assessed the effect of acute and chronic angiotensin II (AngII) infusions on NTS and CVLM activation patterns in the dog by means of c-Fos immunohistochemistry. Both experimental groups exhibited an increase in arterial pressure and a threefold increase in Fos immunohistochemistry compared with controls. The authors concluded that baroreceptor resetting was incomplete in response to AngII, suggesting that baroreceptors may participate in long-term regulation of arterial pressure (Lohmeier *et al.*, 2002). Importantly, the response of NTS neurons to angiotensin lessens over time and is completely absent by day 14 which is in keeping with the resetting of barosensitive neurons found in this region (Davern & Head, 2007). Therefore, long-term regulation of blood pressure by baroreceptors remains to be established.

1.4.2 The Renin-Angiotensin-Aldosterone System

Regulation of blood pressure over long periods of time involves mechanisms other than baroreceptors and these typically take effect a few minutes after neural mechanisms are initiated (Guyton *et al.*, 1972). The renin-angiotensin-aldosterone system is a highly effective mechanism for the maintenance of sodium balance, body fluid volume, vascular resistance and ultimately blood pressure homeostasis (Harrison-Bernard, 2009). The ability of the system to regulate blood pressure is evident by the success of pharmacological manipulation of the system in treating hypertension (Chrysant *et al.*, 2005).

AngII is the product of two proteolytic pathways and is the main metabolite of the renin-angiotensin system (Harrison-Bernard, 2009). In the liver, and to some degree in the kidneys, the α_2-globulin angiotensinogen is cleaved by the enzyme renin to form angiotensin I (Harrison-Bernard, 2009). Shortly thereafter, Angiotensin I is rapidly converted to AngII by angiotensin converting enzyme (ACE), found in renal epithelial cells (Atlas, 2007; Vio & Jeanneret, 2003). The effector properties of AngII are mediated by two G protein-coupled angiotensin receptors, AT_1R and AT_2R (Harrison-Bernard, 2009). The AT_1R subtype is most widespread in adulthood and is currently believed to mediate most of the known effects of AngII (Ardaillou, 1999; Harrison-Bernard, 2009). Synthesis of AngII is dependent on renin activity due to the latter's rate-limiting role in the conversion of angiotensinogen. Thus, availability of renin governs the degree of activation of the renin-angiotensin system (Harrison-Bernard, 2009). Release of renin from renal juxtaglomerular cells into the blood stream occurs when pressure sensing receptors in the afferent tubules register a drop in renal perfusion volume (Reudelhuber, 2005). It may also occur in response to altered NaCl delivery to the mucosa cells and sympathetic nerve activity to renal beds (Harrison-Bernard, 2009). Renin release produces vasoconstriction and augments sodium retention (Reudelhuber, 2005). These functions are mediated by AngII and aldosterone with the secretion of the latter being modulated by AngII (Sandberg & Ji, 2003).

1.4.3 Long-Term Regulation of Mean Arterial Pressure

Guyton (1972) first discussed the existence of a linear kidney-fluid system characterised by infinite gain. By balancing the amount of fluid and salt intake, against the amount of water and salt excretion, the system can alter blood volume and thus act to increase or decrease cardiac output and blood pressure (Guyton, 1991). A considerable limitation of this model was that the contribution of the sympathetic nerve system was restricted to baroreceptor function (Korner, 2007) effectively excluding the hypothalamus in the regulation of blood pressure. It is now well recognised that hypothalamic nuclei are essential to the maintenance of blood pressure and play a crucial role in the development of obesity related hypertension.

A more complete view of CNS regulation of blood pressure is that of an adaptive system able to detect and respond to external and internal stimuli in a number of ways (Korner, 2007). An array of sensory receptors transmit vital information about the external and internal environments to the brain where it is processed by several cortical regions prior to being referenced against memory and the appropriate response initiated by the hypothalamus (Korner, 2007). In addition, the CNS is able to anticipate external stimuli and respond accordingly (Korner, 2007). Thus the mere anticipation of physical demand is capable of increasing blood pressure in the absence of a physical stimulus (Herman *et al.*, 2003; Ulrich-Lai & Herman, 2009). It is important to remember that blood pressure is also under the long term influence of external cues, such as circadian rhythms. The 24-hour pattern of blood pressure is cyclical and occurs independently of acute physiological demands, although feeding behaviours and exposure to stress also have a considerable effect on the circadian pattern of blood pressure. The hypothalamus of the brain is where feeding, stress and circadian rhythmicity converge. There is a high degree of interaction between these systems and it is therefore imperative their mechanisms are well understood if obesity related hypertension is to be treated successfully. In the following sections, each of these functions will be discussed.

1.5 Influences of Circadian Rhythms, Feeding and Stress on Blood Pressure and Sympathetic Nerve Activity

1.5.1 Circadian Rhythms

The rotation of the earth about its axis results in predictable patterns of light and darkness over a period of 24 hours. These patterns govern several physiological processes in the body, including metabolism, sleep-wake cycles, locomotor activity and hormonal synthesis and release. Circadian (Latin: *circa* meaning 'about' and *dies* meaning 'day') clocks are robust cellular mechanisms that ensure the expression of physiological and behavioural patterns virtually match a 24-hour period (Reppert & Weaver, 2001). The ubiquitous nature of these clocks in eukaryotic organisms ranging from single-celled bacteria to humans is believed to confer an adaptive advantage in possessing such a mechanism (Ramsey *et al.*, 2007). To that end, the ability to predict periodic changes, rather than merely respond to them, allows organisms to optimise behaviour and function relative to time of day and, by extension, season (Hastings *et al.*, 2008). Circadian mechanisms are remarkably autonomous, as exemplified by temporally isolated organisms (Balsalobre et al., 1998; Hastings et al., 2008; Sack et al., 1992) yet maintain accuracy by responding to external cues (zeitgebers) such as light and temperature as well as behavioural cues such as feeding (Hastings *et al.*, 2008). Thus entirely-blind individuals display circadian rhythms that approach 24-hour cycles but these are not synchronised with their environment (Sack *et al.*, 1992). These 'free-running' circadian rhythms occur despite individuals having access to non-photic cues such as meals and knowledge of time suggesting that light is a pervasive synchroniser of circadian rhythms (Sack *et al.*, 1992).

In humans, the suprachiasmatic nucleus (SCN) of the hypothalamus receives direct visual cues from the eye, via the retinohypothalamic tract, as well as information pertaining to food intake (Froy, 2010). This 'awareness' of the environment and physiological processes enable it to function as the master clock through which circadian rhythmicity is coordinated (Froy, 2007). Similar circadian centres have also been described in hepatic, renal and adipose tissue (Schibler *et al.*, 2003). The circadian clock machinery is identical between central and peripheral centres with transcription-translation feedback loops comprising a major component

of the system (Dunlap, 1999; Liu & Reppert, 2000). These feedback loops give rise to protein products that in turn self-modulate their own expression (Hastings et al., 2008; Kume et al., 1999). Despite the mechanism remaining unclear it is believed that direct neural input from the SCN as well as specific cytokine activity aids the maintenance of synchrony between the master clock and all peripheral centres (Schibler *et al.*, 2003).

Central metabolic and circadian circuits overlap and affect one another in a reciprocal manner. Neural projections from the SCN synapse at key hypothalamic feeding and satiety centres, including the ARC and PVH, whilst SCN receives direct input from the ventromedial hypothalamus (VMH) (Froy, 2010). Indeed mice lacking the *Clock* gene, a key transcription factor required for normal circadian rhythmicity, are known to be hyperphagic and obese (Turek *et al.*, 2005). Recently, orexigenic NPY containing neurons synapse at the SCN whilst the activity of anorexigenic POMC containing ARC neurons is influenced by direct SCN projections (Abrahamson & Moore, 2001; Guzman-Ruiz *et al.*, 2014). In addition, leptin is secreted in a circadian fashion despite changing meal times (Ramsey *et al.*, 2007) whilst a subset of neurons in the dorsomedial hypothalamus (DMH) respond to food restriction by generating circadian gene oscillations (Mieda *et al.*, 2006). In light of the relationship between circadian and appetite regulatory pathways it is not surprising that perturbations to circadian rhythms such as shift work, jet lag and sleep deficit result in metabolic syndrome and obesity (Froy, 2010).

Of particular relevance to the study of obesity related hypertension is the influence circadian rhythms exert over blood pressure and heart rate (Van den Buuse, 1999). In humans, normotensive individuals display a 24-hour blood pressure pattern characterised by a diurnal peak and nocturnal low with pressure increasing just prior to waking up and steadily decreasing throughout the day (Millar-Craig *et al.*, 1978). By contrast, nocturnal dipping is blunted in hypertensive patients with the severity increasing in obesity (de la Sierra *et al.*, 2009; Raspopow *et al.*, 2014). Increased MAP variability is associated with greater cardiovascular risk and is now considered an effective predictor of disease (Eguchi et al., 2009; Parati, 2005).

Complex haemodynamic changes are also intrinsic to the physiological response to feeding. Cox et al (1995) have shown in humans that, in response to a large meal, MAP remained unchanged whilst blood flow to the gut was elevated along with increased renal and muscular sympathetic nerve activity. By contrast, a single daily meal elicited a marked increase in blood pressure and heart rate in the rabbit (Van den Buuse & Malpas, 1997) with comparable effects observed in the dog (Fronek & Stahlgren, 1968). It has been suggested that increased arousal may initiate the rise in MAP and heart rate just prior to consumption of a meal (Van den Buuse & Malpas, 1997). Combined, these studies indicate that the haemodynamic responses to feeding can involve behavioural as well as autonomic responses. It is worth noting that hyperphagia and consumption of a HFD (due to palatability) often present together in the setting of obesity (Corbit & Stellar, 1964). However, the separate impact of each factor on cardiovascular rhythms remains poorly characterised and is examined in depth in Chapter 4. Crucially, the integration of the autonomic responses to food intake, arousal and circadian rhythmicity involve hypothalamic signalling pathways (De Matteo et al., 2006; Elmquist et al., 1999; Sutton et al., 2008).

1.5.2 The Haemodynamics and Sympathoexcitation of Stress

1.5.2.1 Defining stress

Introduced almost 100 years ago, the concept of homeostasis is a dominant model in physiology and medicine (Sterling, 2004). Eloquently defined by Cannon (1929), the model outlines an organism's ability to maintain relative physiological stability despite environmental fluctuations. Decades later allostasis, a competing model, was introduced by Sterling and Eyer (1988). Subtly distinct from homeostasis, in which a 'set point' is defended (stability through constancy), Sterling argued allostatic systems in the body predict demands to be made and adjust physiological variables accordingly; stability through change (Sterling, 2004). Proponents of allostasis suggest stress can be easily explained if the emphasis is not placed on the change a stimulus causes but rather on inefficient management of mechanisms following a demand for change; this was termed allostatic load (McEwen & Wingfield, 2003). This new paradigm of physiological regulation and stress has not gone uncriticised. Day (2005) argues allostasis is a complete misunderstanding of the term homeostasis, which is in line with Cannon's (1929) explicit definition of the concept. Furthermore, Cannon (1929) proposed homeostasis is the result of cooperation amongst a wide range of organs with the goal of maintaining disturbances within narrow limits. A refined definition proposed by Day (2005) is that homeostasis does not require the stability of all parameters in the body, but rather the stability of particular parameters that are critical for cell survival. He further suggested the term 'stress' could reasonably encapsulate homeostasis by defining stress as the body's multi-system response to any challenge that overwhelms, or is judged likely to overwhelm, selective homeostatic processes (Day, 2005). Whilst logical, this approach may be incomplete as most ordinary daily functions result in a disturbance to homeostasis; thus 'a perceived or actual threat to homeostasis' is too broad a condition under which stress is to be defined (Koolhaas *et al.*, 2011). The task of delineating stress may be better served by understanding the varied forms of stress as well as the physiological responses they evoke.

1.5.3 Physiological Responses to Stress

Empirical evidence has delineated two distinct categories of stress: physical, pertaining to an injury to the body and psychological, relating to the perceived threat of injury (Ulrich-Lai & Herman, 2009). Importantly, both appear to induce a similar physiological response (Dayas & Day, 2001) and both are through to play a role in the development of obesity related hypertension (Korner, 2007).

1.5.3.1 Central processing of "stressful" stimuli

The response of peripheral organs to an aversive stimulus is mediated by the limbic-hypothalamic-pituitary-adrenal (LHPA) axis and sympathomedullary system (Chrousos, 2007; Whitnall, 1993). These two effector mechanisms are crucial in physically preparing an organism to respond appropriately to a challenge (Chrousos, 2009). The PVH acts as initiator of the LHPA cascade by releasing corticotrophin releasing hormone (CRH) and vasopressin from parvocellular neuronal populations (Fellmann et al., 1984; Swaab et al., 1975). Projections from PVH neurons terminate at the median eminence (Merchenthaler *et al.*, 1984), a functionally important anatomical feature which allows for direct transfer of hormonal signals between the brain and the pituitary gland (Knigge & Scott, 1970). Interestingly, the release of CRH and vasopressin appear to be mutually reinforced by one another (Bernardini *et al.*, 1994). Secretion of CRH is a key step in the LHPA cascade as it is the main regulator of adrenocorticotropic hormone (ACTH) which in turn acts on the adrenal cortex to stimulate the synthesis and release of glucocorticoids (Whitnall, 1993). Cortisol, the principal glucocorticoid in humans, facilitates energy utilisation, cardiovascular regulation and inhibits several immune system functions throughout the body (Whitnall, 1993). Cortisol also exerts an essential inhibitory action on the release of CRH and vasopressin from PVH neurons via a negative feedback loop (Di et al., 2003; Fleischer & Vale, 1968). While the peripheral response to stress was thought to be similar irrespective of the stressor (Lopez *et al.*, 1999), a more appropriate view suggests that both the peripheral response to a stressful stimulus and the central processing are heterogeneous and appropriate for the specific stressor (Pacak & Palkovits, 2001).

The primary function of central mechanisms is the identification and categorisation of stimuli which facilitates appropriate responses such as the inhibition of vegetative function (e.g feeding or reproduction) and enhancement of specific behaviours (e.g arousal, alertness, aggression and cognition) as required (Chrousos, 2009; McEwen & Chattarji, 2007). Central pathways integrating the response to stress are known to involve robust activation of A1 and A2 noradrenaline producing cells in the ventrolateral medulla and NTS respectively (Dayas & Day, 2002). The elegant studies of Dayas and Day (2001) demonstrated how the pattern of neuronal activation in the amygdala contributes to the categorisation of stress. Wistar rats were presented with one of five stressors, of which two were physical, two were psychological and the fifth being a forced swim test – an ambiguous stressor. All stressors engaged the LHPA axis, and activation of CRH cells was taken as a sign of LHPA activation. Importantly, the response of the amygdala was highly specific (Dayas *et al.*, 2001). Physical stressors elicited a response from the central amygdala whilst a greater response was observed in the medial amygdala following psychological stressors (Dayas *et al.*, 2001). Most importantly, the forced swimming produced an identical pattern of neuronal activation to that observed in psychological stress suggesting experimental ambiguity of a stressor may be clarified by the accompanying central responses (Dayas *et al.*, 2001).

Contrasting patterns of neuronal activation have also been identified in other brain regions. For example, the midbrain periaqueductal grey consists of parallel columnar circuits, each responding to distinct physical and psychological stressors (Keay & Bandler, 2001). Additionally, greater activation is observed in the PVH following presentation of a physical compared with a psychological stressor (Kwon *et al.*, 2008). Thus, not only do stressor classifications have neuroanatomical foundations, with differing patterns of neuronal activation dependent upon the type of stress presented (Dayas & Day, 2001), central processing of stressors may be an easier way of deriving a definition of stress. In the context of this thesis the term ‘stress’ relates to an acute emotional stressor which has been previously shown to activate somatosensory central pathways (Burke *et al.*, 1998).

1.5.4 The Consequence of Exposure to Stress

There is wide agreement that mental stress in humans results in increased blood pressure and heart rate (Carter et al., 2008; Carter et al., 2005; Kamiya et al., 2000) though the effect of mental stress on sympathetic nerve activity is less clear (Carter *et al.*, 2008). In humans, the Stroop word-colour conflict test decreased sympathetic nerve activity to the upper arm (Halliwill *et al.*, 1997). By contrast, a mental arithmetic task failed to elicit any change in sympathetic activity to the arm but did increase sympathetic output to the leg (Anderson *et al.*, 1987). Despite the divergent sympathetic nerve response to mental stress, the magnitude of arterial pressure and heart rate rise was comparable between the studies. Similarly, Esler and co-workers (1989) reported elevated blood pressure, heart rate and cardiac noradrenaline spillover in human subjects following exposure to mental stress. These findings suggest the effects of mental stress may initially affect cardiac function, rather than vascular function (Esler *et al.*, 1989). In addition, the lack of sympathetic response might be explained by timing. Carter and colleagues (2005) observed that muscle sympathetic nerve activity to the forearm and leg may increase after, but not during, exposure to mental stress, suggesting cardiovascular implications may arise at a later time point. Indeed Anderson and colleagues (1987) reported arterial pressure and sympathetic activity to the leg remain elevated during the recovery phase whilst heart rate returned to baseline.

The long-term cardiovascular implications of stress are not trivial and are epitomized in a comprehensive follow-up study by Ming and colleagues (2004). Air traffic controllers were used as a model of a high stress environment and displayed a positive association between large cardiovascular reactivity and long-term risk of developing hypertension, defined as large frequent increases in blood pressure and heart rate in response to psychological stress. Individuals in the highest quartile of blood pressure reactivity between 1974 and 1978 had a 3.5 relative odds ratio of being hypertensive twenty years later (Ming *et al.*, 2004). These findings echo previous observations made by Carter *et al* (2005) and further suggest that the cardiovascular consequences of stress may take some time to develop.

1.5.5 Stress and Obesity

The physiological and psychological responses to stress are reportedly augmented in obese individuals (Epel et al., 2000; Pasquali et al., 1996). Pasquali and colleagues (1996) examined the response to a mental arithmetic challenge in three groups of women; those with visceral adiposity, those with subcutaneous adiposity and those with normal weight. They reported an enhanced heart rate and blood pressure response to the test in women with increased visceral adiposity compared with the other groups. Pasquali *et al* (1996) further reported that maximal stimulation of the LHPA axis, achieved by means of combined arginine-vasopressin and ACTH infusion, resulted in considerably greater cortisol release in women with visceral adiposity which suggests that they have a hyper-responsive LHPA axis. Similarly, lean women with greater visceral adiposity were more vulnerable to the physiological and psychological effects of stress as they released more cortisol and rated the experience as more severe compared with lean women with predominantly subcutaneous adiposity (Epel *et al.*, 2000). Although both studies reveal a strong association between visceral adiposity and the response to stress, they do not shed any light on whether obesity exacerbates the response to stress or whether it is itself the product of greater reactivity to stress. Indeed conflicting evidence is found in medical conditions not linked to obesity. Patients with Cushing's syndrome, a medical condition characterised by over-secretion of cortisol, often manifest with metabolic syndrome and visceral adiposity (Arnaldi *et al.*, 2003). Conversely, anorexia nervosa is often associated with an overactive LHPA axis alongside enhanced cortisol secretions.

Neural pathways involved in the modulation of stress responses overlap with those involved in energy homeostasis, suggesting hyper-reactivity to stress may lead to obesity and vice versa. De Matteo and colleagues (2006) have shown that the DMH is involved in mediating both feeding and stress related cardiovascular arousal in rabbits. Likewise, hypothalamic arcuate and PVH nuclei, required for appropriate LHPA-axis function, are also major sites of feeding homeostasis (Elmquist, 2001; McDougall *et al.*, 2005). Thus, an enhanced cardiovascular response to stress in obese individuals may contribute to the long-term cardiovascular burden in obesity.

1.6 Obesity Related Increases in Blood Pressure.

The relationship between bodyweight and blood pressure is such that relatively small increases in body weight are associated with increased blood pressure (Doll *et al.*, 2002; Israeli *et al.*, 2006). Equally, weight-loss is accompanied by a decrease in blood pressure (Stevens *et al.*, 2001) and obese individuals are five times more likely to develop hypertension than lean individuals (Haslam & James, 2005). Similarly, diet induced obesity has been shown in numerous animal models to result in greater adiposity and blood pressure (Dobrian et al., 2000; Prior et al., 2010; Rahmouni et al., 2005b; Verwaerde et al., 1999). Whilst the association between elevated blood pressure and obesity is unequivocal, the mechanisms by which obesity-related hypertension may develop remain less so. The following sections will explore the mechanistic links between obesity and hypertension.

1.6.1 Renal and Other Mechanisms Contributing to Obesity Hypertension

There are several means by which blood pressure may rise in obese individuals. In studies conducted in both dogs and rabbits, renal dysfunction has been implicated as a plausible mechanism (Montani *et al.*, 2002). Hall and colleagues (1993) reported weight-gain, hypertension and increased renal sodium retention in dogs maintained on a high fat diet. Additionally, Fujiwara (1999) reported impaired pressure-natriuresis in obese Zucker rats. Evidence from both dogs and humans suggests aberrant renal function is secondary to morphological distortions at the level of the renal medulla (Henegar *et al.*, 2001; Rea *et al.*, 2006). Structural changes to the kidney have been shown to occur following increased extrarenal pressure. Harman and colleagues (1982) studied the effect of increased abdominal pressure in vivo by placing an inflatable bag in the peritoneal cavity of dogs. They demonstrated that increased extrarenal pressure resulted in a considerable decrease in renal blood flow and glomerular filtration rate (Harman *et al.*, 1982). Indeed obese patients with greater visceral adiposity display greater intra-abdominal pressure than lean individuals (Sugerman *et al.*, 1997). Combined, these findings suggest a physical mechanism by which increased visceral adiposity may negatively impact on renal function, resulting in hypertension.

Blood pressure may also increase in response to changes in cardiac function. In the obese rabbit model established by Antic and colleagues (1999) marked increases in heart rate and arterial pressure follow consumption of a high fat diet. Comparably, cardiac output was enhanced in the dog model of diet-induced obesity, resulting in considerable increases in arterial pressure (Hall *et al.*, 1993).

Vascular function is yet another mechanism affecting systemic blood pressure. A known consequence of endothelial dysfunction is impaired NO release which leads to an increase in total peripheral resistance and hence arterial pressure (Montani *et al.*, 2002). Certainly, evidence of impaired endothelium-dependant vasodilation can be found in hypertensive individuals (Yoshida *et al.*, 1991). On the other hand, endothelial impairment is not present in all hypertensive individuals which suggests that it is not an essential factor in the development of hypertension (Cockcroft *et al.*, 1994) and may occur secondary to the blood pressure rise under certain circumstances. Insulin is a potent mediator of nitric oxide dependent vasodilation yet insulin resistance prevents this process (Steinberg *et al.*, 1996).

Whilst the aforementioned pathologies are observed in many obese subjects and influence blood pressure, they are likely accompanying characteristics rather than instigating factors through which obesity related hypertension might develop.

1.6.2 Sympathetic Nerve Activity

The sympathetic nervous system has long been associated with obesity since weight-gain was initially proposed to occur due to the lack of sympathetically regulated thermogenesis thus signifying that sympathetic output was reduced (Eikelis & Esler, 2005). Vastly enhanced experimental methodology, including accurate measurements of noradrenaline spillover from sympathetic nerves and direct recording of sympathetic nerve activity *in vivo*, have promoted a re-think of the conventional theory. A prominent challenge to this theory was put forward by Young and Landsberg (1977a) who observed greater cardiac sympathetic nerve activity in rats following two days of fasting. Importantly, ganglionic blockade failed to elicit a reduction in fasted animals whilst initiation of feeding completely reversed the sympatho-inhibitory effect of fasting (Young & Landsberg, 1977b). These data established a link between food consumption and sympathetic drive. The authors

hypothesised that sympathetic activity increases in order to facilitate greater energy expenditure (Young & Landsberg, 1977a).

There is a significant body of evidence in support of the hypothesis that sympathetic output is enhanced in obesity (Eikelis & Esler, 2005; Korner, 2007). For instance, ganglionic blockade is an effective means of lowering blood pressure in several animal species (Figure 1.4). Moreover, urinary noradrenaline concentrations are greater in the obese compared with lean subjects (Lee et al., 2001; Troisi et al., 1991). Crucially, urinary noradrenaline is considered to be a crude assessment of sympathetic tone due to the fact that rates of noradrenaline synthesis and release do not always coincide (Esler *et al.*, 1988).

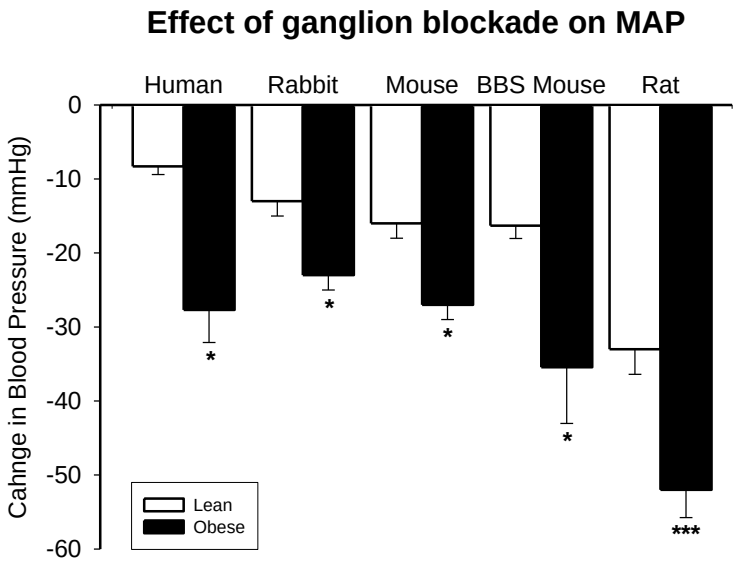

Figure 1.4: Ganglionic blockade in obesity.

Ganglion blockade reduces blood pressure more in the obese rabbit, mouse, rat and human compared to their lean counterparts. Abbreviations: BBS, Bardet-Biedl syndrome; MAP, mean arterial pressure. (Data taken from Armitage et al., 2012; Carlson et al., 2000; Rajapakse et al., 2013; Rocchini et al., 1999; Shibao et al., 2007)**.**

Plasma norepinephrine spillover, the rate at which norepinephrine enters the plasma, is a more accurate measure of sympathetic tone than urinary spillover techniques (Esler *et al.*, 1988) and is indeed elevated in essential hypertension as well as obesity (Esler, 1982). It is important to note that normotensive obese subjects do not have greater noradrenaline spillover rates, confirming that sympathetic output is a critical mediator of hypertension (Esler *et al.*, 2001). Nevertheless, single measurements refer to a point in time and are not appropriate for long-term characterisation of sympathetic output in obesity (Burke *et al.*, 2011). While greater muscle sympathetic nerve activity has been observed in obese individuals by means of direct, electrode recordings, muscle may not reflect renal sympathetic activity. In the rat model of Barnes and colleagues (2003), 12-week fat feeding resulted in greater bodyweight, adiposity and blood pressure compared with regular chow-fed rats. Notably, recordings of RSNA in these animals, conducted under general anaesthesia, revealed that fat-fed rats had greater sympathetic activity than controls (Barnes *et al.*, 2003). General anaesthesia has a pronounced effect on sympathetic activity and is therefore a considerable confounder (Sellgren *et al.*, 1992). Additionally, sympathetic recordings vary greatly according to quality of preparation (Barnes *et al.*, 2003; Burke *et al.*, 2011). Furthermore, sympathetic recordings in animals often involve the presentation of voltage as percentage of the maximum amplitude detected during rest, allowing for the elimination of some confounding influences, but hindering subject-to-subject comparisons (Burke et al., 2011; Sundlof & Wallin, 1977). Thus, nerve recordings in animals cannot be compared easily.

In humans, microneurography studies have indicated obese normotensive individuals had greater efferent sympathetic activity to skeletal muscle beds compared with lean normotensives (Grassi *et al.*, 1998). Pioneered in the 1960s, the technique employs the use of a fine tungsten electrode to record sympathetic activity in conscious human subjects (Vallbo & Hagbarth, 1968). A major limitation of the technique is the inability to compare recruitment between subjects, as determined from the amplitude of multi-fibre activity (Burke *et al.*, 2011). Thus, signal amplitude, which heavily depends on the position of the electrode in reference to the nerve fibre, is affected and cannot be reproduced. Nonetheless, chronic measurement of sympathetic burst frequency is possible in humans but successive re-implantation of the electrode per visit is required. Refinement of the technique allowed for

sympathetic output to be defined in terms of firing frequency, probability and number of spikes generated by a single fibre (Macefield *et al.*, 1994). This in turn allowed for the discovery that the pattern of sympathetic output in obesity-related hypertension is substantially different to that observed in non-obese essential hypertension (Lambert *et al.*, 2007b). In lean hypertensive individuals, burst frequency is increased whilst in obese hypertensives, firing frequency is normal despite increased sympathetic output, suggesting increased fibre recruitment takes place (Lambert *et al.*, 2007b). Importantly, the finding that lean hypertensive individuals have increased sympathetic tone bolsters the argument that changes to sympathetic nerve activity are a mechanism by which obesity-related hypertension may develop. Further strengthening the link between obesity and augmented sympathetic output is the observation that weight-loss produces a concomitant reduction in total body noradrenaline spillover and sympathetic output to skeletal muscle (Straznicky *et al.*, 2005).

In his characterisation of sympathetic output in obesity, Grassi (1998) noted that whilst sympathetic activity to skeletal muscle was increased, skin sympathetic tone was not. This subtle observation indicates that augmented sympathetic output in obesity is not global but rather it is organ specific. Certainly, studies in which organ-derived noradrenaline spillover measurements were obtained showed a reduction in cardiac noradrenaline spillover concomitant with an upsurge of renal noradrenaline spillover rates in obese normotensive subjects (Rumantir et al., 1999). Importantly, renal noradrenaline spillover is increased in obesity and correlates strongly with BMI (Vaz *et al.*, 1997). In rabbits, conscious recordings reveal fat-feeding augments RSNA (Prior *et al.*, 2010). Of note, increased RSNA takes place within the first few days of consuming a HFD and precedes changes in blood pressure (Burke *et al.*, 2013).

1.6.3 Renal Sympathetic Nerve Activity

Renal vasculature is particularly well innervated by sympathetic fibres. These fibres form junctions with smooth muscle cells of the afferent and efferent tubules as well as juxtaglomerular apparatus (Barajas *et al.*, 1992; DiBona, 2000). Since sympathetic input is present in all organs involved in the regulation of blood pressure (Schlaich *et al.*, 2011), the precise contribution of renal sympathetic drive to

hypertension remains unclear. Bilateral renal denervation in spontaneously hypertensive Okamoto rats delayed the onset but did not reduce the magnitude of the hypertension (Kline *et al.*, 1978). The slower onset but complete hypertension in the denervated group could not be explained by the authors though it is not likely to be due to reinervation given regrowth of renal sympathetic nerves in the rat has been shown to take at least 9 months (Grisk *et al.*, 2001). By contrast, renal denervation in the dog prevented the reduction in sodium excretion and increase in arterial pressure observed in controls following 5 week fat-feeding (Kassab *et al.*, 1995). In humans, radio-frequency ablation of renal nerves has been reported to reduce blood pressure in resistant hypertensive individuals (Krum *et al.*, 2009). Notably, the depressor effect was reported to last 2 years following the procedure (Schlaich *et al.*, 2011). Given these findings were made in uncontrolled studies they must be viewed with caution. To that end, a refined study in which a sham procedure was included, failed to detect a difference in arterial pressure between treatment and sham groups 6 months following the procedure (Bhatt *et al.*, 2014). The disparity between the earlier studies may be explained by the inability quantify nerve ablation or measure RSNA prior to the procedure being carried out. Thus renal denervation may not prove useful in all cases. In any event, these findings highlight the complex nature of the central mechanism related to hypertension.

Further evidence arises from mechanisms by which renal sympathetic activity regulates blood pressure. In the late 70s, Bello-Reuss (1976) and colleagues confirmed that direct stimulation of renal sympathetic nerves increased sodium and water reabsorption although they could not elucidate whether the effects were due directly to electrical stimulation and neurotransmitter release or indirectly via activation of the renin-angiotensin system. More recent studies have proven both to be correct. Increased renal sympathetic activity engages the renin-angiotensin system by increasing renin secretion (DiBona, 2000) whilst renal blood flow is decreased (Fink & Brody, 1978). These functional changes are frequency dependent (DiBona, 2000; DiBona & Sawin, 2002). The observation that sodium retention, and subsequent hypertension, are attenuated following renal denervation further suggests renal function is heavily influenced by sympathetic activity (Kassab *et al.*, 1995). It stands to reason that prolonged increases in RSNA, as observed in obesity, are likely to promote functional changes in renal vasculature. In support of that view, Michaels

(2009) *et al* showed rabbits fed a HFD exhibited greater reductions in glomerular filtration rate and sodium excretion in response to renal nerve stimulation compared with controls (Michaels *et al.*, 2009). Thus renal sympathetic activity is a likely contributor to the development of obesity related hypertension.

1.7 The Hypothalamus as a Site of Converging Physiological Functions - Implications for Obesity Related Hypertension

Hypothalamic nuclei are located at the centre of the limbic system and facilitate a myriad of physiological processes including reproduction and emotional drive (Hill et al., 2008; Swaab et al., 2005). Hypothalamic nuclei are also intimately involved in the regulation of feeding behaviours, energy homeostasis, blood pressure regulation and sympathetic output (Elmquist, 2001; Guyton, 1987; Korner, 2007). In view of this evidence the region has gained much attention in studies pertaining to obesity related hypertension. There is substantial evidence to support hypothalamic involvement in obesity and hypertension (Tables 1.2 and 1.3).

1.7.1 Hypothalamic Regulation of Appetite

Much of our current understanding of the function of hypothalamic nuclei in the context of feeding and energy homeostasis has come from early studies involving discrete lesions. Bilateral lesioning of the rat hypothalamus allowed Hetherington and Ranson (1940) to attribute specific roles to each nucleus, marking the lateral hypothalamus (LH) as the 'hunger centre' and the VMH as the 'satiety centre'. Later, more refined lesions studies conducted by Anand and Brobeck (1951) substantiated the findings made by Hetherington and Ranson, further demonstrating LH lesions could lead to a profound loss of feeding as well as death from starvation. Nevertheless, the satiety centre originally attributed to the VMH was found to be inaccurate. Perplexed by the observations that both lesioning and stimulation of the VMH produced a feeding response, Gold (1973) pursued further investigations into the matter. By demonstrating that discrete lesions made to the VMH were ineffective in producing hyperphagia, Gold concluded obesity resultant from destruction of the nearby ventral noradrenergic bundle was erroneously attributed to the VMH. These findings form the basis of our understanding of hypothalamic function and highlight the complex nature of neuronal circuits regulating feeding behaviour.

Table 1.2: Hypothalamic regulation of food intake and body weight.

Reference	Model	Treatment	Outcome
Lee *et al*, 2013.	Mice	Fed a HFD for up to 20 weeks. Electrophysiological studies conducted on isolated NPY neurons.	↑ NPY expression in DMH in DIO mice • NPY neurons in DMH are activated by leptin
de Backer *et al*, 2010.	Wistar Rat	Long-term inhibition of melanocortin receptors in specific hypothalamic nuclei	↑ Meal size in both light and dark phases following injections into PVH, VMH and LH ↑ Bodyweight and adiposity in PVH, VMH and LH
Tiesjema, *et al*, 2009.	Wistar Rat	Targeted overexpression of NPY in the PVH	↑ Food intake ↑ Bodyweight
Stanley, 1989.	Sprague-Dawley Rat	Chronic infusion of NPY into the PVH	↑ Bodyweight ↑ Total food intake
Shor-Posner et al, 1985.	Rat	Electrolytic lesion to the PVH	↑ Total food intake
Gold et al, 1973.	Albino Rat	Lesions to VMH and paraventricular projections.	↔ VMH lesions had no effect on food intake. ↑ Food intake following destruction of PVH projections.
Anand & Brobeck, 1951.	Rat	Lesions of the LH	↓ Feeding
Hetherington et al, 1940.	Albino Rat	Bilateral electrolytic lesions in the hypothalamus	↑ Body weight and lipid deposits

DIO; diet induced obesity, DMH; dorsomedial hypothalamus, HFD; high fat diet, NPY; neuropeptide Y, LH; lateral hypothalamus, PVH; paraventricular nucleus, VMH; ventromedial hypothalamus.

More recent studies, making use of immunohistochemical techniques, have further clarified the contribution of hypothalamic centres to feeding. Intravenous leptin enhanced c-Fos expression, a marker of recent neuronal activation, in the VMH, DMH and PVH either implying the existence of a secondary network of neurons which relay signals from the arcuate or that leptin can engage these nuclei independently of the ARC (Elmquist *et al.*, 1997; Sundquist & Nisenbaum, 2005). Both are now known to be true with leptin acting directly on various hypothalamic neuronal populations as the long form of the LepR is expressed in the VMH, DMH and PVH (Elias et al., 1999; Elmquist et al., 1998b; Elmquist et al., 1999). Moreover, there are several neuronal populations which transmit leptin signals from the ARC to other hypothalamic nuclei. These will be discussed in greater detail in section 1.8.

1.7.2 Hypothalamic Regulation of Blood Pressure

Lesion studies in which specific hypothalamic nuclei were destroyed have demonstrated a degree of commonality in the regulation of both appetite and blood pressure by these regions. Bastos *et al* (1997) reported a pressor effect in response to both AngII and clonidine injections into median-preoptic nucleus of the hypothalamus (MnPO). These pressor responses were considerably attenuated following electrolytic lesions to the VMH (Bastos *et al.*, 1997). These findings confirm those of Johnson *et al* (1981) who described a reduction in the pressor response to ICV AngII following lesions to the VMH. In addition, pathways originating at the anterior ventral wall of the 3rd ventricle (AV3V) of the brain pass through the VMH before ending at the primary cardiovascular control areas found in the medulla oblongata (Fink *et al.*, 1978). The VMH is not the only hypothalamic nucleus involved in blood pressure regulation. Furgeson *et al* (1984) showed that lesions of the PVH diminished the pressor response to electrical stimulation of the subfornical organ. Importantly, baseline blood pressure remained unaltered in PVH-lesioned rats implying central regulation of blood pressure at rest involves other areas of the brain (Ferguson & Renaud, 1984). Furthermore, discrete injections of leptin into the ARC produced increases in blood pressure which were also observed following ICV administration, leading the authors to conclude the ARC mediates the pressor effects of leptin (Rahmouni & Morgan, 2007). Similarly, injections of leptin directly into the VMH and DMH also increased blood pressure (Marsh *et al.*, 2003).

1.7.3 Hypothalamic Regulation of Sympathetic Activity

Microinjections of leptin into either the ARC or the VMH increase RSNA in anesthetised rats (Marsh *et al.*, 2003; Rahmouni & Morgan, 2007) whilst discrete injections of apelin into the PVH increase sympathetic output to BAT in rats (Masaki *et al.*, 2012). Similarly, application of the NO donor sodium nitroprusside into the PVH decreases RSNA (Zhang & Patel, 1998) whilst injection of the melanocortin agonist MT-II into the same nucleus enhanced RSNA (Li *et al.*, 2013a). Combined, these data imply sympathetic outflow is regulated by numerous neuropeptides acting at several hypothalamic nuclei. In view of the fact hypothalamic nuclei such as the DMH and PVH modulate appetite, cardiovascular regulation and sympathetic tone, it is possible to hypothesise that any change in feeding behaviour, resulting in obesity, may exact a change in sympathetic tone or cardiovascular regulation offering a mechanism by which hypertension may develop in obese individuals.

Table 1.3: Hypothalamic involvement in sympathetic output and blood pressure regulation.

Reference	Model	Methodology	Outcome	Effect On	
				MAP	SNA
Li, *et al*, 2013.	Sprague-Dawley Rats	Injection melanocortin agonists and antagonists into PVH Recording under anaesthesia	• Agonists – Increased SNA and MAP • Antagonists – Decreased SNA and MAP	↑ ↓	↑ ↓
Masaki *et al*, 2012.	Sprague-Dawley Rats	Apelin injections into the VMH, DMH, ARC and PVH Recording under anaesthesia	• PVH injections resulted in increased SNA to BAT • No response was observed in other targeted nuclei	Not Measured	↑
Ward, *et al* 2011.	Hyperinsu-linemic Rats	Injections of GABA antagonist muscimol into PVH	• Reduction in MAP and lumbar SNA	↓	↓
Purkayastha *et al*, 2011.	Chronically obese C57BL/6 mice	Acute inhibition of pro-inflammatory NF-κB pathways in the mediobasal hypothalamus	• Reduction in MAP, independent of changes in BW • Inflamed POMC neurons appear to mediate the hypotensive response.	↓	Not Measured
Prior *et al*, 2010.	Fat-Fed Rabbit	Infusion of leptin ICV. Conscious recording of RSNA	• Enhanced sensitivity to central leptin following HFD • Reduced c-Fos expression in ARC, PVH, and DMH suggestive of leptin resistance.	↑	↑
Rahmouni *et al*, 2007.	Sprague-Dawley Rats	Intra-arcuate and ICV infusions of leptin.	• ICV - Increased BAT SNA, RSNA and MAP. • Intra-arcuate – Increased MAP, BAT SNA and RSNA	↑	↑
Marsh *et al*, 2003.	Sprague-Dawley Rats	Leptin microinjection into the VMH, DMH and PVH. Recording under anaesthesia	• VMH - increased MAP and RSNA • DMH - increased MAP and HR • PVH - No significant effect on either parameter	↑	↑
Bastos *et al*, 1997.	Holtzman Rats	Electrolytic or sham lesions to VMH.	• Pressor response induced by angiotensin II attenuated • Pressor response induced by clonidine abolished	↑	Not Measured

MAP- Mean Arterial Pressure, HR- Heart Rate, SNA- Sympathetic Nerve Activity, RSNA- Renal Sympathetic Nerve Activity, VMH- Ventromedial Hypothalamus, DMH- Dorsomedial Hypothalamus, PVH- Paraventricular hypothalamus, Clonidine- an α2 adrenergic receptor antagonist.

1.8 Neuropeptides, Appetite and Blood pressure

Peripheral signals, principally leptin, regulate feeding behaviour by acting on specific neuronal populations contained within the hypothalamus (Havel, 2001; Matsumura *et al.*, 2003). This is enabled by anatomical features of the median eminence, characterized by a leaky BBB and proximity to peripheral circulation via the hypophyseal portal system (Broadwell & Sofroniew, 1993). In section 3.1.2.4 of this literature review, two possible mechanisms of leptin resistance were explored; impaired transport across the BBB and failure of leptin-dependent cellular signaling cascades. The involvement of vast regions of the CNS in response to central and peripheral administration of leptin (Jovanovic & Yeo, 2010) suggests impaired cellular signaling pathways, shown to contribute to the development of leptin resistance (Mark, 2013), are not the only mechanism by which leptin resistance may occur. Leptin directly modulates the activity of arcuate neurons including the POMC and NPY containing neurons (Elias et al., 1999; Sahu, 1998a). These neurons form a complex neurocircuitry that involves many regions of the CNS (Figure 1.5). Thus it is possible that normal leptin signaling at the ARC may be preserved in obesity with changes occurring in neurons downstream of leptin signaling (Head *et al.*, 2014). Importantly, this mechanistic view of leptin resistance still upholds the role of leptin as a link between obesity, hypertension and the hypothalamus. Whilst several other second order neurons exist, this thesis focuses on the role of NPY and POMC.

There are two distinct neuronal populations expressed in the ARC which exert complimentary effects on appetite. The first are NPY/AgRP containing neurons and the second are POMC and cocaine and amphetamine regulatory transcript (CART) containing neurons (Cowley et al., 2001; Hahn et al., 1998). The activity of these distinct neuronal populations is modulated by one another (Figure 1.6) as well as directly by leptin, which inhibits the former and stimulates the latter (Cowley *et al.*, 2001). These are of major interest to obesity and cardiovascular research and form the main focus of this thesis.

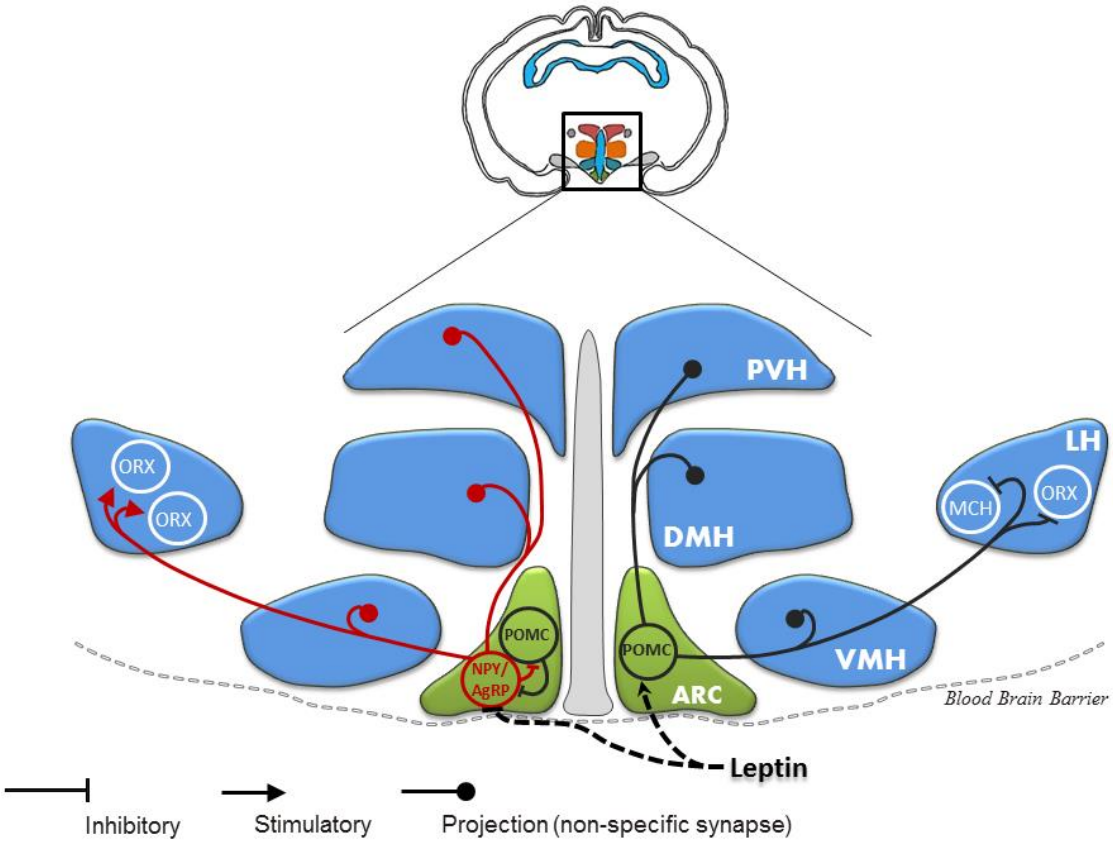

Figure 1.5: Coronal schema of the hypothalamus.

This representation of hypothalamic neurocircuitry highlights the major pathways responsible for the regulation of appetite, blood pressure and sympathetic output. Leptin-inhibited pathways (red lines) act to promote hunger and decrease energy expenditure. Leptin-activated pathways (black lines) promote satiety and increase energy expenditure. Projections of both neuropeptide Y (NPY)/agouti related protein (AgRP) and pro-opiomelanocortin (POMC) neurons ascend from the arcuate (ARC) nucleus and synapse at the same hypothalamic nuclei (indicated by closed circles) yet action contrasting effects. Orexigenic neurons containing orexin (ORX) and melanin concentrating hormone (MCH) are inhibited by POMC neurons. Conversely, ORX and MCH neurons are activated by NPY/AgRP containing neurons. Hence, appetite, blood pressure regulation and sympathetic tone are the outcome of the balance of activation of both neuronal populations. Anatomically, these nuclei are distributed in a rostral-caudal plane and are not all visible in a single section as shown.

Abbreviations: DMH – Dorsomedial Hypothalamic Nucleus, LH- Lateral Hypothalamus, PVH – Paraventricular Nucleus, VMH- Ventromedial Nucleus.

1.8.1 The Melanocortin System

The prohormone POMC gives rise to two classes of peptides, the melanocortins and β-endorphins (Cone, 2005). Melanocortin cleavage peptides are responsible for a range of physiological processes that are mediated by five G protein-coupled receptors, MC1-5R (Cone, 2005; da Silva *et al.*, 2014). In the periphery, melanocortin functions are varied and range from regulating skin and hair pigmentation to various exocrine functions (Gantz & Fong, 2003). Central melanocortin actions are propagated exclusively by the MC3R and MC4R, the only subtypes to be found in the CNS, and are closely linked to energy homeostasis (Cone, 2005; Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993). Several lines of evidence support this link. Firstly, both food restricted and leptin deficient mice exhibit low POMC expression in the ARC (Brady *et al.*, 1990; Mizuno *et al.*, 1998) whilst overfeeding and exogenous leptin have been demonstrated to increase central POMC expression (Hagan et al., 1999; Schwartz et al., 1997). It is worth noting that both mice and humans lacking the POMC gene are phenotypically obese suggesting central melanocortin function is exceptionally well preserved among species (Krude *et al.*, 1998; Yaswen *et al.*, 1999). Secondly, pharmacological blockade of both MC3R and MC4R by means of the antagonist SHU9199 results in hyperphagia and obesity (da Silva *et al.*, 2004). Lastly, acute central injections of α-MSH, a metabolite of the POMC gene with strong binding affinity to each of the MC3R and MC4R subtypes (Schioth *et al.*, 1995), attenuate food intake whilst chronic injections result in hypophagia and weight loss (McMinn *et al.*, 2000). These data, combined with the observation that POMC neurons express the LepR*b*, infer direct regulation of central melanocortin function by leptin at the level of the ARC (Cheung *et al.*, 1997).

Melanocortin neurons, chiefly located in the ARC, form a network of projections that synapse with several hypothalamic nuclei (Cone, 2005). As indicated by Figure 1.6, this comprehensive network is now known to mediate cardiovascular and sympathetic output as well as energy homeostasis (Cone, 2005; da Silva *et al.*, 2014). The cardiovascular ramifications of aberrant melanocortin function are evident in transgenic mice deficient in MC4R. Despite considerable adiposity, insulin resistance and greater circulating leptin levels, these mice are normotensive and bradycardic compared with control littermates (Tallam *et al.*, 2005). These findings are consistent with previous reports by the same group in which MC3/4R blockade

by SHU9119 attenuated HR and prevented hypertension in spite of increased food intake and obesity (Kuo *et al.*, 2003). Furthermore, ICV infusion of the same melanocortin antagonist completely abolishes hypertension in spontaneously hypertensive rats but does not affect basal blood pressure in control rats (da Silva *et al.*, 2008).

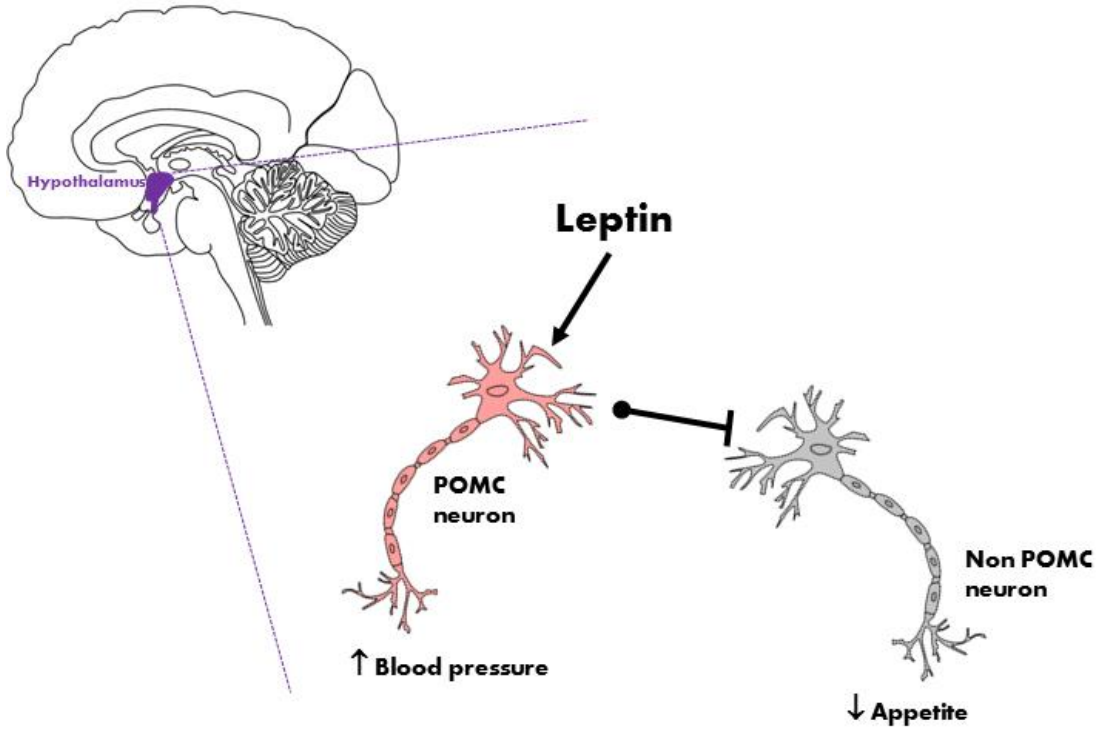

Figure 1.6: The hypothalamic melanocortin system

The divergent effects of MC3/4 receptor blockade can be explained by examining the interaction of proopiomelanocortin (POMC) neurons with leptin and other hypothalamic neurons. Leptin enters the hypothalamus and stimulates POMC neurons in the arcuate nucleus, producing an increase in blood pressure. Activated POMC neurons also inhibit appetite by stimulating neurons such as those containing neuropeptide Y (NPY), subsequently decreasing appetite. Treatment with an MC3/4 receptor antagonist, or removal of central melanocortin receptors, concomitantly prevents melanocortin-dependent hypertension and inhibition of orexigenic neurons in the hypothalamus.

The haemodynamic response to ICV SHU9119 is analogous to that observed following treatment with adrenergic blockers (Armitage et al., 2012; Carlson et al., 2000; da Silva et al., 2008; Shibao et al., 2007). Chronic pre-treatment with terazosin and propranolol, α1 and β1/β2 adrenergic receptor antagonists respectively, prevented the SHU9119 mediated depressor response previously observed in the hypertensive rats (da Silva *et al.*, 2008). Similarly, Kuo *et al* (2004) reported the pressor response to ICV MC3/4R agonist melanotan-II (MTII) was abolished by pre-treatment with adrenergic antagonists. Importantly, the anorectic actions of MTII remained unchanged following adrenergic blockade (Kuo *et al.*, 2004) suggesting highly specific pathways execute divergent melanocortin functions. Although sympathetic activity was not measured in either the da Silva or Kuo studies, the data provide further evidence of melanocortin-mediated sympathoexcitation as an important mechanism of hypertension, particularly in the context of obesity (Kuo *et al.*, 2004).

Direct recording of sympathetic output by Haynes *et al* (1999) confirmed the role of melanocortin neurons in influencing sympathetic tone. Anaesthetised rats injected with ICV MTII exhibited increased sympathetic activity to renal, lumbar and BAT which were abolished by ICV SHU9119 (Haynes *et al.*, 1999). Of note, treatment with ICV SHU9119 also prevented leptin-mediated increases in sympathetic flow to BAT and renal beds further suggesting the melanocortin system acts as a primary conduit for leptin signals (Haynes *et al.*, 1999). Similar findings were made by Dunbar *et al* (1999) who reported increased lumbar sympathetic activity in anesthetised rats following ICV α-MSH. Ye and colleagues (2011) used retrograde labelling to identify PVH neurons projecting to the RVLM, a site of pre-ganglionic sympathetic activity. Patch clamp recordings of labelled PVH neurons revealed comparable MTII and α-MSH-dependent increases in the firing rates of cells taken from both obese and lean rats (Ye & Li, 2011). These were reversed by application of SHU9119, leading the authors to draw similar conclusions to Haynes *et al* and state that melanocortin function was important in mediating central sympathetic output. Curiously, Ye and Haynes concluded that MC4R was the necessary receptor for sympathoexcitation to occur despite the two studies making use of agonists and antagonists of both the MC3R and MC4R subtypes.

The melanocortin system is known to influence appetite, cardiovascular regulation and sympathetic tone, yet the precise contribution of individual

melanocortin receptors to each of these functions remains contentious. In a recent review, da Silva (2014) suggests that MC3R function is more pertinent to appetite regulation whilst MC4R function is more critical in mediating cardiovascular and sympathoexcitatory responses. However, a recent publication by Li (2013a) clearly demonstrates the involvement of both MC3R and MC4R subtypes in pressor and sympathoexcitatory responses to MTII. Consistent with the findings of Ye (2011) and Haynes (1999), microinjection of MTII into the PVH elicited both pressor and renal sympathoexcitatory responses, both of which were inhibited by SHU9119 and the endogenous MC3/4R antagonist, AgRP (Li *et al.*, 2013a). Interestingly, administration of the highly selective MC4R antagonist HS204 attenuated but did not prevent the cardiovascular and sympathetic responses to MTII (Li *et al.*, 2013a). The authors concluded that activation of both receptor subtypes contributes to sympathetic and cardiovascular output with the possibility of MC3R subtypes exerting tonic control of blood pressure and sympathetic activity (Li *et al.*, 2013a). In the absence of a selective MC3R antagonist, this proposition is difficult to examine yet given the specificity of melanocortin pathways, as demonstrated by Kuo *et al* (2004), it is entirely possible the contribution of each receptor varies according to function. Song *et al* (2005) employed the retrograde tracer pseudorabies virus to provide evidence of MC4R-dependent sympathetic activity to WAT. The technique revealed a high degree of co-localization between MC4R expression and pre-sympathetic neurons projecting to the brain stem and hypothalamus (Song *et al.*, 2005). In a recent publication, the feeding response to ICV MTII was described in three separate cohorts of mice ($MC3R^{-/-}$, $MC4R^{-/-}$ and $MC3/4R^{-/-}$). Rowland and colleagues reported a lessening of the anorectic effect of MTII in both $MC3R^{-/-}$ and $MC4R^{-/-}$ groups whilst mice lacking both receptors showed no response (Rowland *et al.*, 2010). Surprisingly, when assessing c-Fos expression in response to MTII, $MC3R^{-/-}$ mice showed almost no activation of PVH neurons whilst the pattern of cellular activation in the PVH of $MC4R^{-/-}$ mice was similar to that observed in wild type mice (Rowland *et al.*, 2010). These findings suggest that despite reduced sensitivity in both knockout groups, each receptor is capable of mediating the anorectic response to MTII through diverse hypothalamic pathways.

Whilst the precise contribution of each melanocortin receptor subtype on appetite, cardiovascular and sympathetic regulation remains unclear, the involvement of melanocortins in these functions is evident (Cone, 2005; da Silva *et al.*, 2014). Melanocortins link appetite, haemodynamics and sympathetic output yet they only comprise part of the regulatory hypothalamic appetite circuitry.

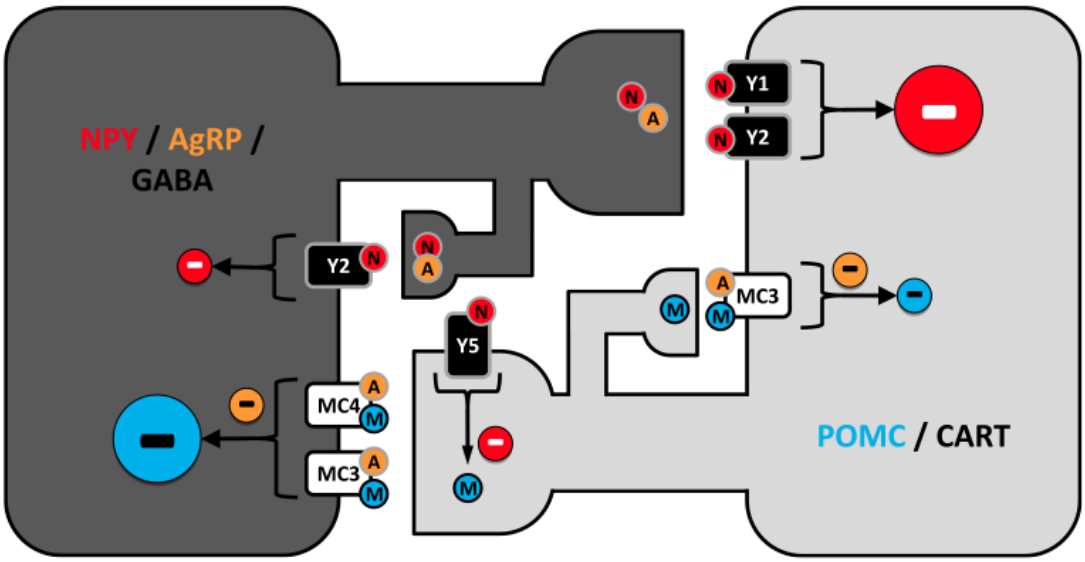

Figure 1.7: Interaction between NPY and POMC containing neurons.

The presence of melanocortin 3 and 4 receptors (MC3 and MC4) on neuropeptide Y (NPY) neurons and Y1 and Y2 neurons on proopiomelanocortin (POMC) and cocaine and amphetamine regulatory transcript (CART) neurons suggests the potential for mutual inhibition. Abbreviations: A; Agouti Related Protein, N; NPY, M, melanocortin. (Reproduced with permission from Mercer et al., 2011).

1.8.2 Neuropeptide Y.

NPY, a potent stimulant of appetite (Neary *et al.*, 2004) is chiefly expressed in ARC neurons (Berthoud & Morrison, 2008) although a high density of NPY-containing neurons has been observed in the PVH and DMH (Chronwall *et al.*, 1985). Concentrations of NPY in the PVH are augmented prior to mealtime and remain so in the fasted state (Neary et al., 2004; Sahu et al., 1988). Moreover, feeding decreases NPY expression whilst chronic infusion of NPY into the PVH produces hyperphagia and obesity (Sahu *et al.*, 1988; Stanley *et al.*, 1989). Furthermore, anterograde labelling of DMH neurons revealed extensive NPY projections to the PVH and LH in DIO mice (Lee *et al.*, 2013a) suggesting the two nuclei are linked. Additionally, NPY neurons express the LepRb (Mercer *et al.*, 1996) suggesting leptin modulates the activity of this potent orexigenic pathway though unlike the interaction of leptin with POMC neurons, NPY-containing neurons are inhibited, rather than stimulated, by leptin (Elmquist *et al.*, 1999). Indeed, *ob/ob* mice exhibit increased NPY mRNA expression in the ARC (Wilding *et al.*, 1993), which is reduced following treatment with exogenous leptin (Schwartz *et al.*, 1996a). In addition, fasting-induced NPY expression in the ARC is attenuated by ICV leptin (Schwartz *et al.*, 1996b).

Given NPY is a potent orexigenic molecule inhibited by leptin, greater circulating leptin associated with obesity suggests hyperphagia is either driven by another neuropeptide or leptin mediates the activity of NPY neurons in a nucleus specific manner. Indeed, Tritos and colleagues (1998) reported decreased NPY mRNA in the ARC of obese mice, compared with controls. Unexpectedly, NPY mRNA in the DMH of obese mice was considerably higher than in lean mice in which NPY expression was not observed in the DMH (Tritos *et al.*, 1998). It is therefore possible that in obesity, hyperphagia may be mediated directly by the NPY containing neurons of the DMH. Lee et al (2013b) characterised the induction of NPY mRNA in the DMH during obesity. Mice fed a HFD for 20 weeks had increased adiposity and circulating leptin levels. In addition, these mice exhibited diminished NPY signalling in the ARC and increased NPY expression in the DMH (Lee *et al.*, 2013b). Interestingly, NPY neurons in the DMH expressed the LepR*b* and were potentiated by leptin, which enters the hypothalamus via active transport (Banks et al., 1996; Lee et al., 2013b). Furthermore, NPY expression in the DMH peaked 10 weeks after maximum increase in leptin serum levels had occurred in response to a

high fat diet (Lee *et al.*, 2013b). These observations imply leptin may mediate hyperphagia via NPY DMH neurons at later stages of obesity but that this interaction between the two neuronal populations does not cause the condition (Lee *et al.*, 2013b). Interestingly, fasting results in decreased NPY expression in the DMH of both control and DIO despite NPY expression increasing in the ARC, suggesting this subset of neurons may be responding to nutrients or other hormonal signals (Lee *et al.*, 2013b). Indeed NPY activity and macronutrient content affect one another in a reciprocal manner (Beck, 2007).

NPY neurons are well conserved evolutionarily and whilst expression of NPY and its receptors in the cortex, hippocampus and hindbrain vary greatly between mammals, hypothalamic expression of Y1R,Y2R,Y4R, and Y5R remain similar (Dumont *et al.*, 1998), suggesting the function of NPY may also be preserved between species (Larhammar, 1996). To date six NPY target receptors have been identified in (Y1-6R) and although all subtypes participate in modulating feeding behaviour, the orexigenic properties of NPY are mediated principally by the Y1R and Y5R (Beck, 2007). Indeed, NPY exhibits strong binding affinity to these receptors (Beck, 2006) and chronic central administration of a Y5R specific agonist increases food intake and bodyweight in mice whilst a Y1R antagonist abolishes NPY-mediated food consumption in rats (Henry et al., 2005; Kanatani et al., 1996). More recently, chronic infusion of a Y2R specific agonist was reported to decrease food intake and resulted in transient weight-loss (Henry *et al.*, 2005). This finding is in agreement with previous reports of Y2R activation inhibiting NPY release from in vitro hypothalamic cells (King *et al.*, 2000). These data suggest that the Y2R may be involved in a feedback mechanism abating food consumption. Interestingly, the orexigenic effect of NPY is attenuated in $Y1R^{-/-}$ but not in $Y5R^{-/-}$ mice and Y5R agonists are capable of stimulating appetite in $Y5R^{-/-}$ mice, signifying a new, undiscovered receptor, may be involved in feeding regulation via the Y receptor family (Kanatani *et al.*, 2000).

Surprisingly, NPY knockout mice maintain normal food intake and bodyweight (Erickson *et al.*, 1996) challenging the convention that NPY is essential to food intake (Henry *et al.*, 2005) and suggesting the existence of an additional orexigenic signal. Shutter and colleagues (1997) identified the ancillary signal, the neurotransmitter AgRP, and described its co-expression in NPY containing neurons

(Figure 1.6). However, AgRP does not act via the Y receptor family and is an endogenous antagonist of the MC3/4R (Figure 1.7) thus, unlike NPY, the orexigenic function of AgRP is achieved by powerfully inhibiting the melanocortin system (Hahn et al., 1998; Ollmann et al., 1997). These findings offer an explanation for the perplexing observation made by Rahmouni (2003b) and Benoit (2000) who reported direct stimulation of MC4R in *db/db* mice produced attenuated sympathoexcitatory and anorectic responses compared with controls. At the time of publication both authors could not explain the results, given melanocortin function is considered downstream of leptin signalling (Rahmouni *et al.*, 2003b). However, the attenuation in melanocortin function observed in *db/db* mice is logical given leptin is not capable of inhibiting ARC neurons expressing NPY/AgRP. Thus decreased melanocortin function may be due to enhanced inhibition by AgRP at the level of the ARC exemplifying the dual functionality of NPY/AgRP neurons which simultaneously activate NPY receptors and inhibit the melanocortin system (Hahn *et al.*, 1998).

Aggregation of NPY containing neurons in nuclei such as the PVH and DMH (Chronwall *et al.*, 1985) implies it participates in cardiovascular and sympathetic regulation. Intracisternal administration of NPY decreases blood pressure and heart rate of the rat (Fuxe *et al.*, 1983). However, rats overexpressing NPY are similar to wild type littermates in both blood pressure and heart rate (Michalkiewicz *et al.*, 2001). Moreover, resting blood pressure is not different between either $Y1R^{-/-}$ (Pedrazzini *et al.*, 1998) and $Y2R^{-/-}$ mice and their respective controls (Naveilhan *et al.*, 1999). Nevertheless, removal of the Y1R in mice eliminates the pharmacological response to exogenous NPY (Pedrazzini *et al.*, 1998). This suggests that NPY neurons are not involved in cardiovascular regulation at rest yet basal blood pressure of mice lacking the Y4R is 25 % lower than controls (Smith-White *et al.*, 2002). These mice also show a reduced sensitivity to exogenous NPY compared with controls (Smith-White *et al.*, 2002). Thus, centrally-mediated cardiovascular effects of NPY require further characterisation.

NPY involvement in the development of hypertension also stems from its actions in the periphery. Noradrenaline and NPY are found in the same pre-synaptic vesicles of sympathetic nerve fibres innervating arteries, veins and the heart (Walker *et al.*, 1991). Co-localisation and release of these peptides is critical as NPY is a potent vasoconstrictor and reportedly enhances the effect of other vasoconstrictors,

including noradrenaline (Walker *et al.*, 1991). Indeed spontaneously hypertensive rats exhibit greater sensitivity to NPY-mediated arterial vasoconstriction (Gradin *et al.*, 2003). The observed vasoconstriction was markedly attenuated, but not abolished, by a selective Y1R antagonist suggesting Y receptors are directly involved in the process (Gradin *et al.*, 2003). Importantly, NPY-mediated vasoconstriction is independent of vascular endothelium and is mediated primarily by Y1R found on vascular smooth muscle cells (You *et al.*, 2001). Conversely, activation of Y2R, expressed in endothelial cells, results in vasodilation and appears to involve nitric oxide release as treatment with L-NAME prevents NPY-mediated vasodilation (You *et al.*, 2001). Thus despite the ubiquitous nature of NPY and its receptors, individual Y receptors subserve different functions depending on their location.

NPY has also been implicated in cardiac remodelling and disease and altered renal haemodynamics. The potent vasoconstrictor properties of NPY are presumed to instigate, or worsen, ischemia (McDermott & Bell, 2007). Indeed, NPY plasma levels are increased in patients with ischemic heart disease (Ullman *et al.*, 1990) whilst infusion of NPY into the coronary arteries of angina sufferers resulted in myocardial ischemia (Clarke *et al.*, 1987). Curiously, NPY has been shown to promote hypertrophy among adult ventricular cardiomyocytes (Millar *et al.*, 1994) yet left-ventricular hypertrophy is associated with withdrawal of NPY-containing sympathetic neurons. In addition to its effects on cardiac remodelling, NPY is known to act via Y1R and Y2R, expressed on cardiomyocytes, to directly influence the force and rate of contractions (McDermott & Bell, 2007). Co-expression of NPY in a distinct subpopulation of sympathetic fibres innervating the renal vasculature is also thought to be integral to hypertension (Denton *et al.*, 2004). Administration of NPY into the renal artery results in increased vascular resistance and decreased renal blood flow (Echtenkamp & Dandridge, 1989; Shin *et al.*, 2000). In addition, peripheral administration of exogenous NPY increases pressure natriuresis and diuresis independently of changes to renal blood flow (Bischoff *et al.*, 1996).Thus, enhanced sympathetic activity observed in obesity may be worsened by the actions of NPY on cardiac muscles and the vasculature.

The association between NPY, sympathetic nerve fibres and noradrenergic actions as well as the expression of NPY in hypothalamic nuclei that are known to modulate sympathetic tone are primary evidence that NPY is an important regulator

of sympathetic activity. Indeed Chao *et al* (2011) reported that targeted deletion of NPY mRNA in DMH neurons increases energy utilisation via BAT. Further characterisation of the neural mechanisms responsible for this effect was achieved by treating BAT depots with a neurotoxin, subsequently eliminating sympathetic innervation and decreasing BAT size (Chao *et al.*, 2011). Combined, these data suggest NPY in the DMH acts via the sympathetic system to regulate energy expenditure via BAT. Recent data also indicates central NPY regulates hepatic sympathetic activity (Bruinstroop et al., 2012). Despite evidence of NPY involvement in tissue-specific sympathetic output, there is no current data to suggest NPY is involved in modulating renal sympathetic nerve activity.

1.8.3 Other Hypothalamic Neuropeptides

Both NPY and α-MSH represent key signalling pathways linking leptin, obesity and hypertension; yet they are by no means the totality of hypothalamic signalling involved in the genesis of obesity and hypertension. Additional hypothalamic and gut-derived peptides implicated in appetite and cardiovascular regulation include orexin, CART, ghrelin and MCH. In the following section, the contribution of other neuropeptides will be presented.

1.8.3.1 Ghrelin

Ghrelin is a 28 amino acid peptide predominantly secreted in the stomach (Kojima *et al.*, 1999). Ghrelin acts via the growth hormone secretagogue receptor (GHSR), the strongest expression of which is found in the ARC (Zigman *et al.*, 2006), where it is believed to oppose the stimulatory actions of leptin by concurrently stimulating NPY neurons and inhibiting POMC neurons (Neary *et al.*, 2004). Not surprisingly, peripherally administered ghrelin potently increases food consumption in humans (Wren *et al.*, 2001) yet the actions of ghrelin are not limited to energy intake. Ghrelin is known to have several favourable effects on cardiac and vascular function. For instance, ghrelin was found to increase nitric oxide availability thereby reversing endothelial dysfunction in humans with metabolic syndrome (Tesauro *et al.*, 2005). Another example of the beneficial effects of ghrelin can be observed in the rat model of myocardial infarction. Cardiac sympathetic activity was greatly increased by ligation of the left anterior descending coronary artery, a response completely abolished by immediate treatment with ghrelin (Schwenke *et al.*, 2008).

Moreover, treatment with ghrelin 2 hours following infarction also decreased cardiac sympathetic activity although quicker treatment improved survival rates (Schwenke *et al.*, 2008).

Circulating ghrelin levels are reported to be low among patients with metabolic syndrome and obesity (Tesauro et al., 2005; Tschop et al., 2001) clarifying the loss of the cardio-protective effects of ghrelin. Moreover, oscillations in circulating ghrelin concentrations do not conform to patterns observed in healthy subjects. Lean individuals exhibit a steady increase in ghrelin concentrations prior to a meal, a response that fails to occur in obese patients (Yildiz *et al.*, 2004). Moreover, post-meal suppression of ghrelin is also abnormal in obese patients (Huda *et al.*, 2009). Interestingly, exogenous ghrelin increases hunger and food intake in morbidly obese and lean patients alike suggesting central sensitivity does not change (Huda *et al.*, 2009). Thus impaired release of ghrelin may underlie pathologies associated with obesity.

1.8.3.2 Orexins

The precursor peptide prepro-orexin gives rise to two orexin molecules, orexin A and orexin B which collectively act via the G protein-coupled receptors orexin 1 (OX1R) and orexin 2 (OX2R) although it is suggested orexin A acts via both receptor subtypes whilst the actions of orexin B are solely mediated by OX2R (Tsujino & Sakurai, 2009). Orexins are exclusively synthesised in LH neurons (Sakurai *et al.*, 1998) and these neurons have an extensive field of projection, reaching numerous brain regions and several hypothalamic nuclei (Nambu et al., 1999; Peyron et al., 1998) thus implicating the peptides in energy homeostasis. In support of this view, expression of orexin peptides has been noted to increase prior to a meal (Nakamachi *et al.*, 2006) whilst centrally administered orexins augment food consumption in several animal models (Edwards et al., 1999; Lubkin & Stricker-Krongrad, 1998; Nakamachi et al., 2006). Moreover, the activity of orexin neurons is inhibited by leptin and increased by ghrelin (Yamanaka *et al.*, 2003). Aside from regulating appetite, central orexins also act to increase energy expenditure by increasing metabolic rate in a manner resembling direct electrical stimulation of the LH (Lubkin & Stricker-Krongrad, 1998). Importantly, orexins have been implicated in central reward pathways (Harris *et al.*, 2005), sleep/wake cycles (Hara *et al.*, 2001)

and arousal mechanisms following food restriction (Yamanaka *et al.*, 2003), all functions that interrelate with energy homeostasis.

The involvement of orexins in sleep/wake cycles and arousal, both functions which require autonomic and cardiovascular adjustments, suggests these peptides regulate cardiovascular function (Carrive, 2013). Samson *et al* (1999) reported central administration of orexin A and B increased blood pressure and heart rate in conscious rats, with orexin A exerting a more potent effect. Similarly, Shirasaka and colleagues (1999) reported orexin-mediated increases in blood pressure and heart rate with a stronger effect following orexin A administration. In addition, Shirasaka *et al* (1999) also reported that centrally administered orexin A increased RSNA, a response which correlated strongly with increased blood pressure. These data suggest orexin-dependent increases in blood pressure are the result of increased renal sympathetic activity (Shirasaka *et al.*, 1999). However, ICV administration of orexin B did not elicit a sympathoexcitatory response despite increasing blood pressure suggesting blood pressure regulation by orexin may also be mediated by other mechanisms such as baroreceptors. More recent findings have indeed shown both orexin peptides to stimulate sympathetic pre-ganglionic neurons and RVLM neurons, a key region of blood pressure regulation (Chen *et al.*, 2000; Dun *et al.*, 2000). Most notably, orexin producing neurons synapse with both POMC and NPY-containing neurons in the ARC and VMH (Muroya *et al.*, 2004). Orexin neurons mirror the effects of leptin by inhibiting POMC neurons and depolarizing NPY neurons. Thus orexin neurons may well present an additional mechanism by which obesity and its associated cardiovascular diseases may develop.

1.8.3.3 Melanin Concentrating Hormone

Melanin concentrating hormone (MCH) is similar to orexin in three important respects; it is a key stimulator of food intake and, in the hypothalamus (Qu *et al.*, 1996), is solely expressed in LH neurons (Bittencourt *et al.*, 1992) and it is inhibited by leptin (Sahu, 1998b). Indeed, injections of MCH into the ARC, DMH and PVH each result in increased food intake (Abbott *et al.*, 2003) whilst mice lacking leptin are obese, hyperphagic and exhibit increased MCH expression in the hypothalamus (Qu *et al.*, 1996). MCH is also suggested to regulate the sympathetic and cardiovascular systems. Sympathetic fibres containing MCH have been traced from the LH to BAT, suggesting a direct link between MCH and energy expenditure

(Oldfield *et al.*, 2002). Indeed mice treated with exogenous MCH display reduced temperature and UCP1 expenditure in BAT, suggesting the neuropeptide attenuates energy expenditure (Ito *et al.*, 2003). Chronic treatment with MCH also produces bradychardia and decreases blood pressure (Messina & Overton, 2007). The evidence presented above supports the suggestion that MCH regulates the cardiovascular and sympathetic systems as well as energy homeostasis. However, in light of the limited nature of this evidence, more research is required to fully understand the contribution of MCH neurons to the development of obesity and hypertension.

1.8.3.4 Cocaine and amphetamine regulated transcript.

Named for its induction following cocaine or amphetamine (Douglass & Daoud, 1996), the CART system is more diffuse than the orexin and MCH neurocircuitry and can be found in several hypothalamic nuclei including the ARC, LH, VMH, DMH and PVH (Broberger, 1999; Elmquist *et al.*, 1999). In contrast to NPY, AgRP and orexins, CART neurons are potentiated by leptin and act to produce robust decreases in food intake (Elias et al., 1998; Lambert et al., 1997). Mice homozygous for the anorexia mutation (*anx*/*anx)* are characterised by decreased food consumption and premature death. These mice were also observed to have decreased CART expression in the ARC, LH and DMH, suggesting the down regulation of CART may occur in response to the low levels of energy available to the body and implicating CART neurons in energy homeostasis (Johansen *et al.*, 2000). To date there is a limited availability of evidence suggesting hypothalamic CART neurons directly regulate the cardiovascular system and sympathetic output. Matsumura and colleagues (2001) reported that conscious rabbits exhibited increased MAP, HR and RSNA in response to increasing doses of ICV CART. Importantly rabbits in the Matsumura study were not maintained on a HFD. Thus the contribution of the CART neurons to obesity related hypertension remains poorly understood.

1.9 The contribution of high fat fed rabbit studies to the understanding of obesity related hypertension.

The high fat-fed rabbit model confers several advantages to the study of obesity related hypertension. Primary among those are the physiological responses occurring in the rabbits following increased caloric intake from fat. These are analogous to those observed in obese humans and are summarised in Table 1.4. Carroll and colleagues were pioneers of the rabbit model and reported many of the physiological changes in the obese rabbit. In 1995, Carroll *et al* reported increased blood pressure, cardiac output and heart rate in fat fed rabbits compared with lean rabbits. Moreover, total peripheral resistance was reduced in obese animals whilst blood flow to non-adipose tissue was increased (Carroll *et al.*, 1995). Other groups have also reported comparable haemodynamic and neurohormonal changes between obese rabbits and obese humans. Antic et al (1999) have observed that Belgian rabbits fed a HFD are hypertensive and tachycardic compared with rabbits fed a control diet. Similarly, data from the Head laboratory found that New Zealand White (NZW) rabbits fed a HFD accumulate considerable adiposity, have greater circulating leptin and exhibit central leptin resistance compared with rabbits fed a control diet (Prior *et al.*, 2010). In spite of the rich characterization of the obese rabbit model, little attention has been given to the development of dyslipidaemia and its contribution to obesity related pathologies. Thus a lipidomic analysis of triacylglycerides, diacylglycerides, ceramides and cholesterols will be presented in Chapter 3. Importantly, the 3-week HFD increased RSNA which was closely correlated with adiposity and high blood pressure (Lim et al., 2013; Prior et al., 2010). The primary advantage of this model is conscious nerve recordings thus avoiding the confounding effect of general anaesthesia, enabling frequency and amplitude to be recorded under physiological conditions. Moreover, RSNA in the rabbit can be normalised to a nasopharyngeal response, which elicits maximal nerve activity (Burke & Head, 2003). This allows for between group comparisons that are not yet available for other species in which nerve activity is recorded. An additional advantage gained by the short-term duration of the diet is the absence of the numerous confounding variables often associated with ‘established’ obesity. Thus the short term fat fed rabbit model is well placed to identify primary changes that occur in response to increased dietary fat and that may contribute to the development of

obesity related hypertension. In the current thesis, this model was employed to assess the neurogenic origins of obesity related hypertension. An additional benefit gained from the use of rabbits is their size, allowing for easy instrumentation (e.g telemetric measurement of blood pressure, electrode assisted recording of RSNA). In addition, collection of blood as well as measurement of blood pressure by cannulating the median ear artery is also made easier.

Table 1.4: Comparisons between human and rabbit obesity.

Condition	Human	NZW Rabbit	References
Hypertension	✓	✓	Prior, 2010; Carroll, 1995; Lim, 2013.
Sympathetic Nerve Activity ↑	✓	✓	Prior, 2010; Lim, 2013.
Cardiac Output ↑	✓	✓	Carroll, 1995
Heart Rate ↑	✓	✓	Prior, 2010; Carroll, 1995; Lim, 2013.
Cardiac Hypertrophy	✓	✓	Carroll, 1996.
Diastolic dysfunction	✓	✓	Carroll, 1999.
Renal Blood Flow ↑	✓	✓	Carroll, 1995.
Glomerular Function Rate ↑	✓	✓	Antic, 1999.
Activated RAS	✓	✓	Carroll, 1996.
Hyperleptinemia	✓	✓	Prior, 2010.
Glucose ↑	✓	✓	Carroll, 1996.
Hyperinsulinemia	✓	✓	Prior, 2010.
Triglycerides ↑	✓	✓	Carroll, 1996.
Ceramides	✓	**?**	Torre-Villalvazo, 2009.
Cholesterol	✓	✓	Carroll, 2002.
Central Leptin Resistance	✓	✓	Prior, 2010.
% Fat in diet	**32 %**	**13 %**	Cordain, 2008.

1.10 Aims of this thesis

Obesity has been at the forefront of research for some decades, although an effective cure has yet to be established. Of particular concern is the association between obesity and cardiovascular diseases, including hypertension. The current appraisal of scientific literature has highlighted several mechanisms by which obesity related hypertension may develop. However, some aspects remain poorly defined. Thus, the broad aim of this thesis is to better characterise the establishment of obesity related hypertension in rabbits fed a HFD.

The aim of the first study was to characterise the metabolic and physiological responses that follow consumption of a HFD in the NZW rabbit. This is particularly relevant given metabolic changes such as altered plasma lipid profiles and insulin resistance are indirect mechanisms by which hypertension may develop. To complement the first study, the aim of the second study was to assess whether the phenotype associated with consumption of a HFD, in particular haemodynamic changes, was due to increased total caloric intake versus increased caloric intake from fat. Rabbits fed a HFD for 3 weeks exhibit central leptin resistance whereby the sympathoexcitatory effect of leptin is enhanced yet the anorectic properties of leptin appear lost. Thus the aim of the third study was to assess the contribution of NPY/AgRP and POMC signalling pathways in the hypothalamus to the development of leptin resistance. The aim of the fourth study was to localise the changes that are observed in hypothalamic signalling during consumption of a HFD.

Chapter II – General Methodology

2.1 Animals

Experimentation was carried out on male New Zealand White rabbits in accordance with the Australian Government code of practice for use of animals for scientific purposes (2004) and approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Rabbits were obtained from Nanowie Small Animal Production Unit, Bellbrae, Victoria, Australia, and were allowed to acclimatise to their new environment for a period of ten days. They were housed individually in a climate controlled room (22º ± 2) and maintained on 1.5 cups of control diet and free access to water.

2.2 Diet

Once committed to the experimental protocol, rabbits were fed either a control (normal diet; NFD) or a high fat diet (HFD) ad libitum for 3 weeks. The NFD consisted of standard rabbit and guinea pig chow (4.2% fat Rabbit and Guinea Pig Chow, Specialty Feeds, Glen Forest, Australia) whilst the HFD consisted of crushed NFD pellets mixed with lard and soy oil (13.3% fat, SF06-011, 10% Fat of which 5% was pork lard and 5% was soya oil, Rabbit and Guinea Pig chow, Specialty Feeds) to provide a range of saturated and polyunsaturated fatty acids. Of particular note to this thesis is the link between essential fatty acid (EFA) deficiency and the development of hypertension (Das, 2001). In order to ensure the experimental phenotype observed resulted from high fat consumption, not EFA deficiency, a wide spectrum of polyunsaturated lipids were present in the HFD. Similarly, micronutrient deficiency has been shown to play a role in the development of hypertension (Das, 2001). Thus vitamins and minerals were added to high fat diet pellets so that little micronutrient variability was present between the two groups. Table 2.1 specifies the nutritional components present in each diet.

Table 2.1: the ingredients, macronutrients and micronutrients of both the NFD and HFD diets.

Calculated Nutritional Parameters			Ingredients			Calculated Fat Composition			Calculated Vitamins		
Dietary Constituent	**NFD**	**HFD**	**NFD Dietary Constituents**	**Dietary Constituent**	**HFD**	**Dietary Constituent**	**NFD**	**HFD**	**Dietary Constituent**	**NFD**	**HFD**
Protein	18.2 %	17.5 %	Barley, Lupins, Oats, Wheat, Oaten Day, Lucerne, Soya meal, Canola meal, DL-Methionine, Magnesium oxide, Dicalcium phosphate, Cacium hydroxide, Calcium carbonate, Salt, Sodium bentonite, Mixed vegetable oils and Vitamin and Mineral premix.	Meat Free Guinea Pig and Rabbit (GPR) Diet	875 g/Kg	Myristic Acid 14:0	0.01 %	0.09 %	Vitamin A (Retinol)	37,000 IU / Kg	44,000 IU / Kg
Total Fat	4.2 %	13.3 %		Sodium Caseinate	22 g/Kg	Palmitic Acid 16:0	0.3 %	2.1 %	Vitamin D3 (Cholecalciferol)	No data	No data
Crude Fibre	14.4 %	13.1 %		Lard	50 g/Kg	Stearic Acid 18:0	0.1 %	1.2 %	Vitamin E (Tocopherol)	60 mg / Kg	60 mg / Kg
Acid Detergent Fibre	18.0 %	16.0 %		Soya Oil	50 g/Kg	Palmitoletic Acid 16:1	trace	0.02 %	Vitamin K (Menadione)	3 mg / Kg	3 mg / Kg
Digestible Energy	11 MJ/Kg	14 MJ/Kg		Dicalcium Phospahte	0.17 g/Kg	Oleic Acid 18:1	1.3 %	4.1 %	Vitamin C (Ascorbic Acid)	No data Available	No data Available
				Magnesium Oxide	0.58 g/Kg	Gadoleic Acid 20:1	0.02 %	0.06 %	Vitamin B1 (Thiamine)	7.0 mg / Kg	5.2 mg / Kg
				Calcium Carbonate	2 g/Kg	Linoleic Acid 18:2 n6	1.1 %	4.0 %	Vitamin B2 (Riboflavin)	7.0 mg / Kg	6.2 mg / Kg
				GPR trace mineral/ vitamin Px	875 g/Kg	a-Linolenic Acid 18:3 n3	0.1 %	0.5 %	Niacin (Nicotinic Acid)	55 mg / Kg	51 mg / Kg
						Arachidonic Acid 20:4 n6	trace	trace	Vitamin B6 (Pyridoxine)	6.0 mg / Kg	5.3 mg / Kg
						EPA 20:5 n3	0	0	Pantothenic Acid	22 mg / Kg	17 mg / Kg
						DHA 22:6 n3	0	0	Biotin	140 µg /Kg	120 µg / Kg
						Total Carotenoid	no data	no data	Folic Acid	0.5 mg / Kg	0.5 mg / Kg
						Total Phospholipid	no data	no data	Inositol	No data	No data
						Cholesterol	trace	trace	Vitamin B12 (Cyanocobalamin)	9 µg / Kg	7 mg / Kg
						Total saturated	no data	3.5 %	Choline	2,200 mg / Kg	2,000 mg / Kg
						Total monounsaturated	no data	4.3 %			
						Total polyunsaturated	no data	4.6 %			

Calculated Total Minerals			**Calculated Total Amino Acids**			**Added Trace Minerals**		
Dietary Constituent	**NFD**	**HFD**	**Dietary Constituent**	**NFD**	**HFD**	**Dietary Constituent**	**NFD**	**HFD**
Calcium	1.1 %	1.0 %	Valine	0.8 %	0.8 %	Iron	55 mg / Kg	55 mg / Kg
Phosphorous	0.7 %	0.7 %	Leucine	1.2 %	1.3 %	Copper	18 mg / Kg	18 mg / Kg
Magnesium	0.3 %	0.3 %	Isoleucine	0.7 %	0.7 %	Iodine	2.3 mg / Kg	2.3 mg / Kg
Sodium	0.2 %	0.2 %	Threonine	0.6 %	0.6 %	Manganese	120 mg / Kg	120 mg / Kg
Chloride	0.4 %	0.4 %	Methionine	0.2 %	0.2 %	Cobalt	0.9 mg / Kg	0.9 mg / Kg
Potassium	1.1 %	1.0 %	Cystine	0.2 %	0.2 %	Zinc	90 mg / Kg	90 mg / Kg
Sulphur	0.3 %	0.2 %	Lysine	0.8 %	0.9 %	Selenium	0.2 mg / Kg	0.2 mg / Kg
Iron	350 mg / Kg	320 mg / Kg	Phenylalanine	0.8 %	0.8 %			
Copper	27 mg / Kg	21 mg / Kg	Tyrosine	0.5 %	0.6 %			
Iodine	2.3 mg / Kg	1.7 mg / Kg	Tryptophan	0.2 %	0.2 %			
Manganese	160 mg / Kg	120 mg / Kg						
Cobalt	1.0 mg / Kg	0.7 mg / Kg						
Zinc	120 mg / Kg	90 mg / Kg						
Molybdenum	1.0 mg / Kg	0.9 mg / Kg						
Selenium	0.3 mg / Kg	0.3 mg / Kg						
Cadmium	0.1 mg / Kg	0.01 mg / Kg						
Chromium	No Data Available	No Data Available						

Abbreviation: NFD – Normal Fat Diet, HFD – High Fat Diet, EPA – Eicosapentaenoic Acid, DHA – Docosahexaenoic Acid.

2.3 Surgical Procedures

Rabbits underwent two surgical procedures prior to scheduled experimentation. Implantation of either an ICV or brain guide cannulae was the first surgical procedure and was carried out at least a week after arrival of rabbits into the institute and 10 days prior to commencement on a diet. Guide cannulae enabled the delivery of neuropeptides into the CNS. A renal nerve electrode was surgically placed onto the left renal nerve in the second week of the dietary protocol and 7 days prior to experimentation to enable the recording of RSNA. In a select cohort of animals a telemetry blood pressure transmitter was implanted a week after the cannula surgery and a week prior to commencement on a diet.

2.3.1 Manufacturing of Cannulae and Renal Nerve Electrodes

2.3.1.1 ICV Cannula

The ICV cannula was custom made in the Neuropharmacology Laboratory at the BakerIDI Heart & Diabetes Institute. A guide tube attached to a pedestal (22GA, Plastics One, Roanoke, Virginia, U.S.A) was affixed to the skull and served as a path to the lateral ventricle and was cut to a length of 5 mm. The injector (28GA, Plastics One) through which the various neuropeptides were administered was cut to a length of 6 mm, autoclaved and kept in a sterile environment until required. The cannula was plugged with a 5 mm long cap (Plastics One).

2.3.1.2 Brain Guide Cannula

The brain guide cannula was custom made in the Neuropharmacology Laboratory at the BakerIDI Heart & Diabetes Institute. Two guide tubes attached to a pedestal (22GA, Plastics One) were glued together (Araldite Epoxy Adhesive, Selleys, Padstow, Asutralia) so that they were parallel to one another and lumens precisely 2 mm apart. Once the glue dried, the guide tubes, serving as a direct path to the VMH, were cut to 14 mm in length so as to end 3.5 mm above the VMH and cause no damage to the nucleus during implantation. The injectors (28GA, Plastics One), through which neuropeptides were injected into the VMH, were cut to a length of 17.5 mm, autoclaved and kept in a sterile environment until required. The cannula was plugged using a 23GA needle that was bent at the tip. The smaller diameter served to alleviate the pressure formed during placement of the cap.

2.3.1.3 Renal Nerve Electrode

Two gold-coated pins were each attached to one end of a Teflon-coated stainless steel wire (Gore & Associates, GmbH, Nordring, Germany) and encased in Silastic tubing (Silastic 602-135 and Silastic 602-175 tubing, Amtron Australia, Mascot, Asutralia). The opposing end of the wire was stripped of its Teflon coating and coiled so as to allow easy attachment to the renal nerve. Prior to surgery, nerve electrodes were sterilised in a high level disinfectant (Cidex OPA, Ortho-Phthalarehyde 0.55 % w/w solution, Johnson & Johnson Medical, Tokyo, Japan) for 30 minutes.

2.3.2 Pre-surgical Preparation

The analgesic and anti-inflammatory agent Carprofen (3 mg/kg i.v., Rimadyl, Pfizer, North Ryde, NSW, Australia) was administered 1 hour prior to the commencement of all surgeries. During the implantation of renal nerve electrode and radiotelemetry transmitter surgery, a heat pad was used to keep rabbits warm and an infusion of Hartmann's solution (20 ml/h i.v., Baxter Viaflex, Old Toongabbie, NSW, Australia) was administered to maintain adequate fluid and electrolyte levels. In addition, the antibiotic metronidazole (6ml, subcutaneous, Claris Lifesciences, Burwood, NSW, Australia) was administered twice during anaesthesia, prior to commencement of surgery and following suturing to minimise the risk of post-operative infection.

2.3.3 Anaesthetic Induction and Maintenance

Anaesthesia was induced by propofol (10 mg/Kg, slow i.v, Fresofol, 200 mg/20 ml, Pharmatel Fresenius Kabi, Graz, Austria), a short acting sedative, following which a paediatric laryngoscope was used to insert an endotracheal tube (2.5 mm diameter) into the trachea. The tube was then connected to an open circuit inhalation anaesthetic apparatus (Komesaroff Anaesthetic Machine, Medical Developments Australia, Melbourne, Australia) delivering a mixture of oxygen, at 0.8 L/min, and Isoflurane (3.5-4 %, Forthane, inhalation anaesthetic, Abbott, Botany, Australia). During surgery the respiratory rate of the rabbit was monitored by an apAlert respiration monitor (model RMSD, MBM Enterprises, Clunes, NSW, Australia).

2.3.4 Intracerebroventricular and Brain Guide Cannulae

Cannulae were implanted according to the methods previously described (Head & Williams, 1992). Once anaesthetised, the rabbit was placed prone into a stereotaxic frame (David Kopf Instruments, CA, USA). The head was secured at the midpoint of the zygomatic arch with a modified David Kopf head holder. An incision was made from the base of lambda (a ridge in the caudal end of the skull) to approximately the midpoint between the eyes, the skin retracted and the periosteum cleared to expose bregma (the intersection of the rostrocaudal and sagittal sutures of the skull). The head was positioned so that bregma and lambda were on the same horizontal plane. The coordinates for cannula placement were taken from bregma (ICV, 3 mm lateral, 4 mm ventral; bilateral brain cannula, -2.2 mm caudal and ± 0.9 mm lateral to midline). Four small drill holes were made radially approximately 4 mm from bregma to allow the placement of small screws (Cheese Head Screws, Hargo Engineering, VIC, Australia) acting as anchors. The ICV cannula was lowered 4-5 mm into the left lateral ventricle using a micromanipulator equipped with a Vernier scale (David Kopf Instruments). Once in correct position, dental cement (Vertex, Zeist, The Netherlands) was applied to fasten the cannula and pedestal to the skull and anchor screws. At the end of the procedure a cap was placed into the cannula to prevent contamination and the skin was sutured using an absorbable polyglycolic acid suture (Dexon III, Tyco Healthcare, Norwalk, Connecticut, USA).

The location of the ICV cannula was confirmed using the pre-attached tubing, filled with Ringer's solution (Baxter) which was lowered and then elevated above the rabbit's head whilst the meniscus was observed. Correct placement was verified when CSF flowed freely out of the tube and Ringer's solution flowed into the ventricle under hydrostatic pressure.

2.3.5 Implantation of Renal Nerve Electrode

Once anaesthetised, rabbits were laid on the operating table with their left side up, allowing easy access to the left kidney, and the fur shaved. The bottom rib was located and the first incision through the skin was performed approximately two centimetres posterior and inferior to the rib line, in a vertical direction. The incision was pulled back, all three muscle layers were cut, and the opening retracted. The left kidney was exposed from underneath the retroperitoneal fat pads which were retracted

using damp gauze. The renal nerve, artery and vein were exposed and separated from one another. Using jeweller's forceps, the nerve bundle was cleared further from connective tissue, and the electrode (custom made in lab, section 2.3.1.3) put in place. The electrode was secured by sutures (Prolene 7-0, non-absorbable polypropylene suture, Ethicon, Livingston, U.K) placed through the tunica adventitia of the renal artery. The nerve was lifted into the coiled wire ends of the electrode. A silicon elastomer (Kwik-Sil, silicon elastomer, World Precision Instruments, Sarasota, Florida, USA) was poured onto the region of the nerve in contact with the electrode to further secure the electrode and isolate the area. Then, each muscle layer was sutured individually and prior to the skin being sutured, two small incisions were made, superior to the larger incision, allowing the externalisation of the electrode at a later date.

2.4 Measurement of Cardiovascular and Sympathetic Variables

Renal sympathetic nerve activity and cardiovascular variables were measured in conscious rabbits placed in a wooden boxes designed to hold one rabbit (length, width, height; 40, 14, 21 cm). Prior to experimentation, rabbits were acclimatised by being placed in the box for two hours on each of the two days prior to experimentation. Laboratory noise was kept to minimum throughout experiments so as to not startle the animals.

2.4.1 Recording of Mean Arterial Pressure and Heart Rate

Arterial blood pressure measurements were obtained from the central ear artery by means of a transcutaneous 22-gauge catheter (BD Insyte, Singapore) inserted under local anaesthesia (Xylocaine, Lignocaine Hydrochloride 200 mg/ml, AstraZeneca, North Ryde, Australia). A local anaesthetic cream (EMLA, Lidocaine 25 mg/g, Prilocaine 25 mg/g, AstraZeneca) was used to desensitise the area prior to use of Xylocaine. The arterial catheter was connected to a pressure transducer (Statham P23DG transducer, Hato Rey, Pueto Rico) via polyethylene tubing (SP65, single lumen, I.D 0.86 mm OD 1.52 mm, Dural Plastics & Engineering, NSW, Australia) filled with heparinised saline solution (12.5 IU/ml) to prevent clotting. Arterial pulse pressure from the transducer was amplified (DC amplifier NT218, Neomedix, NSW, Australia) and digitised and recorded by means of an acquisition card (PC Plus,

National Instruments, Austin, Texas, USA) and software written by Dr. Elena Lukoshkova, National Cardiology Research Centre, Moscow, Russia) written in LabView programming language (LABVIEW 8.2, National Instruments). Arterial pressure measurements presented in Chapters 4 and 5 were obtained via a blood pressure telemetry transmitter inserted under general anaesthesia as previously described (Figure 2.1 Lim *et al.*, 2012). To account for natural drift in the signal over time, telemetry transmitters were calibrated against arterial pressure taken from the ear artery. MAP (mmHg), was calculated on a beat-to-beat basis and instantaneous HR (bpm) was calculated from the pulse interval. Zero pressure was taken at the rabbit's mid-chest level. Prior to the start of each experiment the pressure transducer was calibrated to room air pressure and 100 mmHg against a mercury sphygmomanometer.

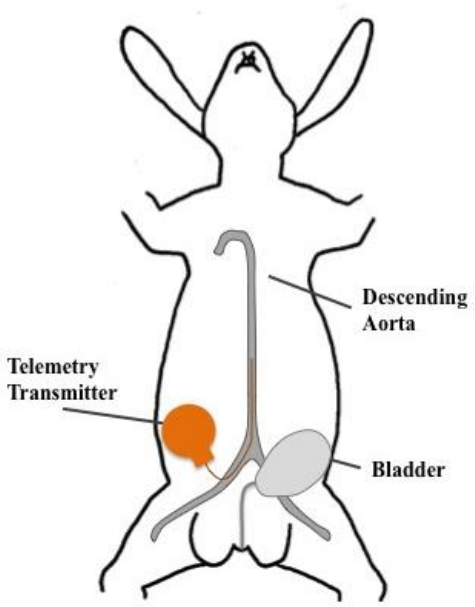

Figure 2.1: Blood Pressure Radiotelemetry transmitter surgery diagram.

The transmitter and battery pack (orange) were placed subcutaneously on the rabbit's flank whilst the catheter was inserted into the aorta via a small branch of the iliac artery.

2.4.2 Recording of Renal Sympathetic Nerve Activity

On the day of experiments, the electrode was externalised under local anaesthetic (Xylocaine) and connected to a low noise preamplifier and amplifier combination model (Baker IDI Heart and Diabetes Institute Models 187b and 190). Postganglionic RSNA was recorded with a bandwidth of 50 Hz to 1 kHz and amplified potentials were rectified and integrated using an integrator filtered with a 20-millisecond time constant. Using Data Acquisition, integrated RSNA was digitised and averaged over two-second periods. The software also recorded the frequency of bursts of synchronised sympathetic activity and their amplitude.

2.4.2.1 Normalisation of RSNA

Different physical conditions and manufacturing properties of renal nerve electrodes result in variations in the RSNA measured in μV (Dorward *et al.*, 1985). To enable comparisons between each experiment, RSNA was normalised to maximal nerve activity produced by a nasopharyngeal reflex following exposure to 60 ml of cigarette smoke drawn into a syringe and released into the recording box (Figure 2.2, Burke & Head, 2003). The peak response to smoke was taken as the greatest value of a burst during the nasopharyngeal reflex response. The normalisation procedure was done at the start of each experimental day following which rabbits were given 30 minutes to recover. Only rabbits with a RSNA nasopharyngeal response greater than 100 μV from bursts lasting longer than 100 ms were included in the RSNA analysis.

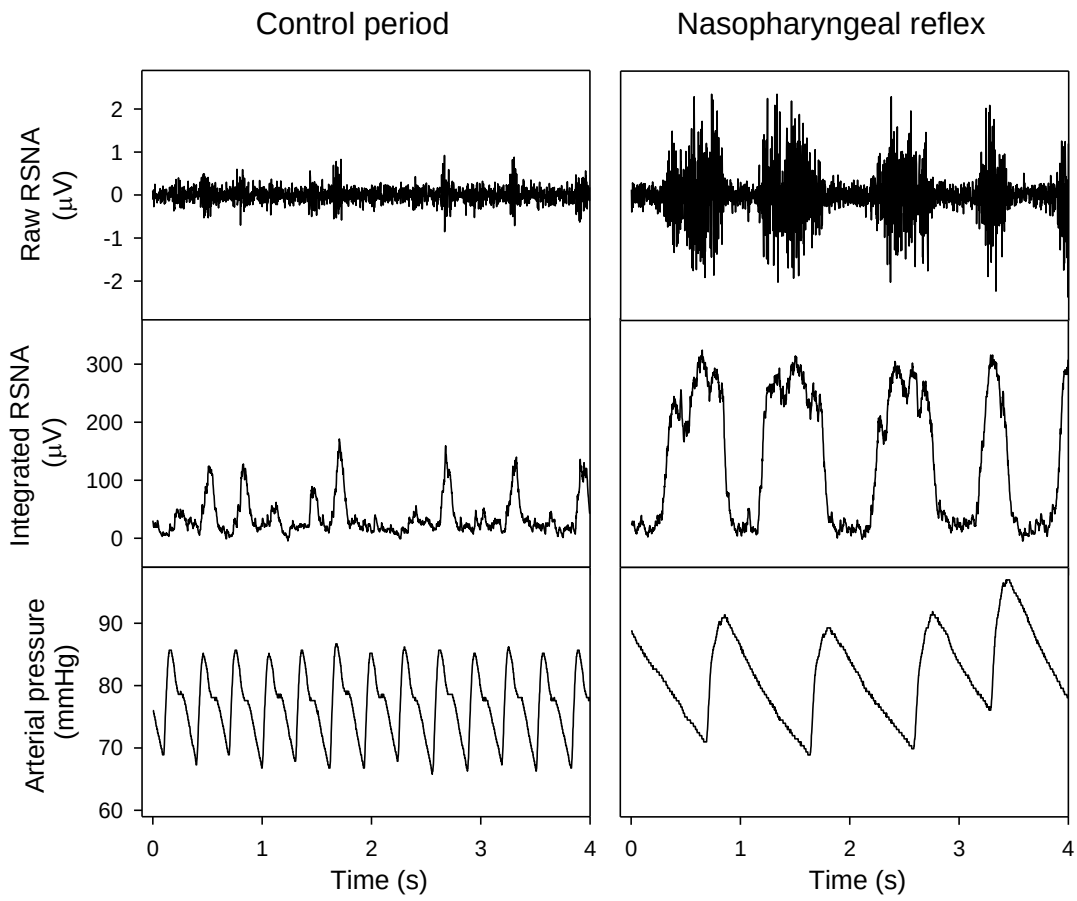

Figure 2.2: Recordings of renal sympathetic nerve activity and arterial pressure in the rabbit.

Sample renal sympathetic nerve activity (RSNA) and arterial pressure recordings obtained from a conscious rabbit at baseline (control period) and following exposure to cigarette smoke (nasopharyngeal reflex). RSNA was normalised between rabbits by expressing the integrated RSNA as a percentage of its maximum nerve response induced by the nasopharyngeal reflex.

2.5 Statistical Analysis

Values are expressed as mean ± standard error of the mean (SEM). Specific analyses used throughout the thesis are described in individual chapter methodology sections. For all statistical analyses, a Probability (*P*) equal to or less than 0.05 was considered significant.

Chapter III – Short Term Fat Feeding Rapidly Increases Plasma Insulin but Does Not Result in Dyslipidaemia.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception of experimental design; acquisition, analysis and interpretation of data; preparation of manuscript for publication (Frontiers Integrative Physiology).	**90**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Sandra L. Burke	Intellectual input to manuscript content and contribution to revision of manuscript
James A. Armitage	Conception of experimental design, collection of data, intellectual input to manuscript content and revision of manuscript.
Geoffrey A. Head	Conception of experimental design, collection and analysis of data, intellectual input to manuscript content and revision of manuscript.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date** **02.10.14**
Main Supervi Signatu	[redacted]	**Date** **02.10.14**

3.1 Abstract

Although the association between obesity and hypertension is well known, the underlying mechanism remains elusive. Previously, we have shown that 3 week fat feeding in rabbits produces greater visceral adiposity, hypertension, tachycardia and elevated renal sympathetic nerve activity. Because hyperinsulinaemia, hyperleptinemia and dyslipidaemia are independent cardiovascular risk factors associated with hypertension we now wish to compare plasma insulin, leptin and lipid profiles in male New Zealand White rabbits fed a normal fat diet (NFD 4.3 % fat, n = 10) or high fat diet (HFD 13.4 % fat, n = 13) at days 1, 2, 3 and weeks 1, 2, 3 of the diet. Plasma lipids were extracted by a modification of the Folch method. Concentrations of diacylglyceride (DAG), triacylglyceride (TAG), ceramide and cholesterol esters (CE) were obtained after analysis by liquid chromatography mass spectrometry. Plasma insulin and glucose were increased within the first 3 days of the diet in HFD rabbits (P <0.05) and remained elevated on week 1 (P <0.05). By contrast, in both groups plasma leptin levels remained unchanged on the first few days (P >0.05) increasing by week 3 in fat fed animals alone (P <0.05). Concentrations of total DAG, TAG, CE and Ceramides at week 3 did not differ between the groups (P >0.05). Thus our data show plasma insulin increases rapidly following consumption of a HFD and may play a role in the rapid rise of blood pressure observed in these animals. Dyslipidaemia does not appear to contribute to the hypertension in this animal model.

Key Words: Insulin, Leptin, Plasma lipids, Obesity, Hypertension.

3.2 Introduction

Obesity is often associated with increased mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA). Accumulating evidence suggests these changes are due to greater circulating concentrations of the adipokine leptin (Burke *et al.*, 2013; Lim *et al.*, 2013) which strongly correlate with RSNA and MAP in animal models of obesity (Burke et al., 2013; Prior et al., 2010). Consumption of a high fat diet (HFD) augments MAP and heart rate (HR) within the first few days of the diet, prior to any change in bodyweight (Burke *et al.*, 2013). However, levels of circulating leptin are proportional to adiposity (Considine *et al.*, 1996) and only begin to increase by the end of the first week of a HFD (Armitage *et al.*, 2012). Thus, rapid changes in cardiovascular parameters suggest that a separate, leptin independent, mechanism initiates the pressor response to a HFD. Plasma insulin concentrations increase within hours of meal consumption (Cummings *et al.*, 2001) and are greater in both obese animals and humans (Bagdade *et al.*, 1967; Lim *et al.*, 2013) as well as patients with essential hypertension (Sobotka *et al.*, 2011). Importantly, insulin is known to signal at the arcuate nucleus of the hypothalamus, the same nucleus at which a multitude of peripheral signals, including leptin, act to regulate energy and haemodynamic homeostasis (Benoit *et al.*, 2004). Central administration of insulin attenuates food intake and augments (Air *et al.*, 2002) sympathetic output (Muntzel *et al.*, 1994). We have previously shown that insulin signalling is one of the factors responsible for the development of obesity related hypertension which is later maintained by slowly rising circulating leptin concnetrations (Lim *et al.*, 2013).

The association between dyslipidaemia and obesity is important given several lipid species are associated with a number of cardiovascular risk factors (Siri-Tarino et al., 2010). In addition, a single high-fat meal has been shown to reduce endothelial-dependent vasodilation up to 4 hours post consumption in healthy normotensive individuals (Vogel *et al.*, 1997). It has been suggested that endothelial-mediated vasodilatory mechanisms are impaired by triacylglycerides and free fatty acids (Doi *et al.*, 1998; Lundman *et al.*, 2001). Thus it is possible that diet-induced changes in lipid profiles may play an early role in the development of obesity related hypertension. Lipid profiles have received scant attention in the fat-fed rabbit model of obesity related hypertension and only after several weeks of fat feeding (Eppel *et al.*, 2013). The contribution of dyslipidaemia to the progression of disease is well documented. Increased

circulating ceramide concentrations are known to increase in obesity and are inversely correlated with insulin resistance (Haus *et al.*, 2009). In addition, circulating levels of triacylglycerides (TAG) and cholesterol esters (CE) are also elevated in obesity and have been shown to affect fasting glucose and insulin sensitivity (Cameron et al., 2008; Sassolas et al., 1981). In the present study the effect of HFD consumption on plasma insulin, leptin and plasma lipid profiles was assessed in order to elucidate the contribution of each to the rapid rise in MAP observed within the first week of the diet.

3.3 Methods

3.3.1 Animals and diets

Experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals. Experiments were conducted in 24 conscious male New Zealand White rabbits (2.76-2.90 kg). Rabbits were housed under controlled light (6:00 to 18:00) and temperature (22° c ± 2°c) conditions. Rabbits were randomised into two dietary groups and given free access to either a normal-fat diet (NFD; 4.3 % total fat, 2.63 kcal/g, Specialty Feeds, Glen Forest, Australia) or a high-fat diet (HFD; 13.4 % total fat, 3.34 kcal/g, Specialty Feeds) for 3 weeks.

3.3.2 Plasma Collection and Analysis

In order to avoid the effects of recent food consumption, animals were fasted for 4 hours before blood samples were collected. Blood was collected before and on days 1, 2, 3, 7, 14 and 21 following the start of the HFD. Small samples of blood were used to measure blood glucose concentrations (Optium Xceed, Abbott, Doncaster, Victoria, Australia). Arterial blood (4 ml) was drawn into vacuum sealed cylinders containing K3EDTA (Vacuette Premium, Greiner bio-one, Wemmel, Belgium) and spun at 4 ºc for 10 minutes at 3,000 RPM. Plasma aliquots (100 µl) were snap frozen in liquid nitrogen and stored at -80 ºc until use. Plasma lipid species were extracted into chloroform/methanol according to a modified protocol of the Folch method and quantified using high performance liquid chromatography (Folch et al., 1957; Meikle et al., 2009). Lipid species identified were diacylglycerides (DAG), TAG, ceramides (Cer) and CE.

Plasma insulin and leptin concentrations were assessed using an ultra-sensitive insulin ELISA (Crystal Chem, Chicago, USA) with rabbit insulin standard and a radio immunoassay multispecies kit (LINCO Research, St Charles, MO, USA), respectively.

3.3.3 Data Analysis

Data were analysed by split-plot repeated measures ANOVA allowing for between and within animal comparisons. Data collected at a single time point were analysed using a 1-way ANOVA. Bonferroni corrections were used to control for Type 1 error. A probability of $P<0.05$ was considered significant. For all statistics shown we refer to the main effect as a subscript, eg. $P_{baseline}$ pertains to comparisons between groups made prior to the consumption of either diet, P_{group}, refers to comparisons between HFD and NFD-fed rabbits during dietary intervention, P_{diet} refers to contrasts between baseline and dietary intervention within both NFD and HFD groups, P_{time}, refers to comparisons within each group made between baseline and week 3 time points, $P_{time\ x\ time}$ pertains to the interaction between diet and time.

3.4 Results

3.4.1 Effect of 3 week fat feeding on plasma insulin, glucose and leptin

Baseline plasma insulin concentrations were not different between the dietary groups and averaged 0.46 ± 0.03 ng/ml ($P_{baseline}$ >0.05; Figure 3.1). A 50% increase from baseline in plasma insulin was observed in both NFD and HFD rabbits over the first two days of the diet (Pdiet <0.05 for both groups; Figure 3.1). A further increase in plasma insulin concentrations on day 3 resulted in 65% greater insulin concentrations in HFD compared with NFD animals at both day 3 and week 1 time points (P_{group} < 0.05; Figure 3.1). By week 2, insulin concentrations in HFD rabbits had decreased to those observed in NFD rabbits (P_{group} >0.05; Figure 3.1). Plasma glucose concentrations at baseline were not different between the dietary groups and averaged and averaged 5.5 ± 0.12 mmol/L ($P_{baseline}$ >0.05; Figure 3.1). Plasma glucose concentrations followed a similar pattern to insulin, rising on days 1 and 2 of the diet in both NFD and HFD rabbits (P_{diet} <0.05 for both groups; Figure 3.1). However, HFD rabbits exhibited a 59% greater increase in plasma glucose concentrations than controls (P_{group} <0.05). By week 2, glucose concentrations returned to levels observed in NFD rabbits (P_{group} >0.05; Figure 3.1). By contrast, plasma leptin

concentrations, which were averaged 0.91 ± 0.13 ng/ml at baseline ($P_{baseline}$ >0.05; Figure 3.1), remained unchanged over the first 3 days of the diet in both dietary group (P_{diet} >0.05; Figure 3.1). However plasma leptin concentrations in HFD-fed rabbits increased on week 1 of the diet compared with baseline (P_{diet}>0.05; Figure 3.1) and were 60 % greater than controls by the end of week 3 (P_{group} <0.05; Figure 3.1).

3.4.2 Effect of HFD feeding on plasma lipid profiles

After 3 weeks of diet, total plasma DAG, TAG, Cer and CE concentrations were not different between the dietary group (P_{group} >0.05; Figure 3.2). Specific DAG, TAG and CE species did not change over the 3-week diet in either dietary group (P_{time} >0.05 for both NFD and HFD, Tables 3.2 – 3.4). By contrast, plasma Cer 16:0, 20:0 and 22:0 concentrations increased in HFD-fed rabbits over the 3 week period (P_{time} >0.05; Table 3.1) yet this was unlikely due to the consumption of the HFD (P_{diet} >0.05; Table 3.1) as the overall interaction between diet and time did not reach statistical significance ($P_{diet\ x\ time}$ >0.05; Table 3.1). Individual cholesterol ester species at week 3 were not different between the dietary groups (P_{group} >0.05; Table 3.2). Similarly, DAG (P_{group} >0.05; Table 3.3) and TAG (P_{group} >0.05; Table 3.4) lipid species were not different between the dietary groups.

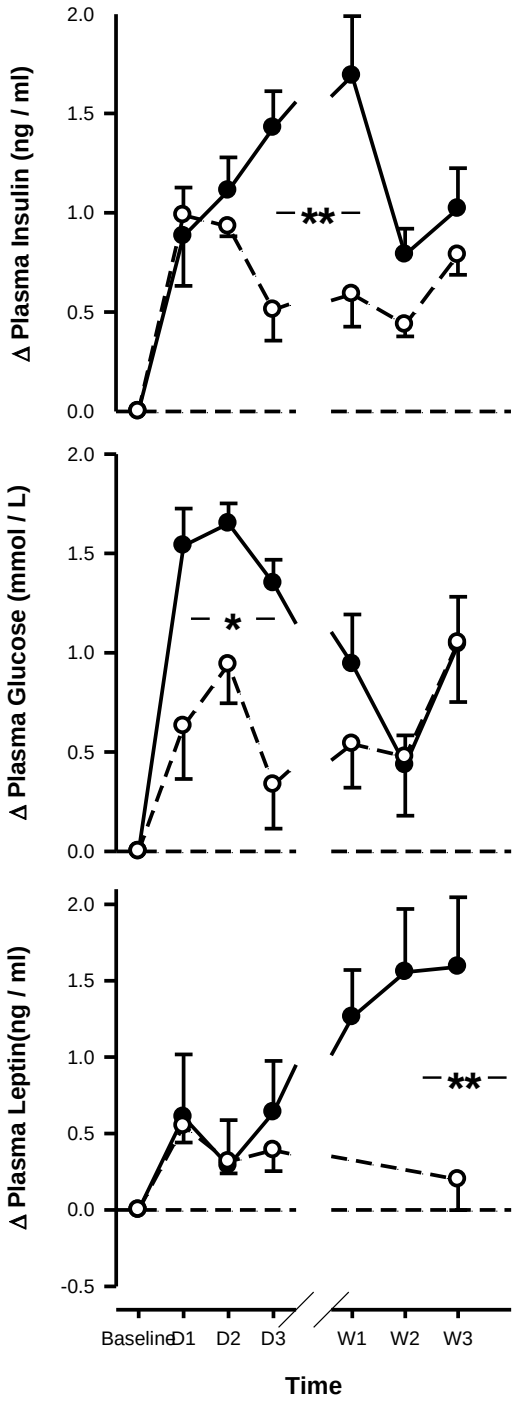

Figure 3.1: Plasma insulin and glucose levels in rabbits fed either a normal fat diet (NFD) or a high-fat diet (HFD) for 3 weeks.

Data are mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$for differences between dietary groups.

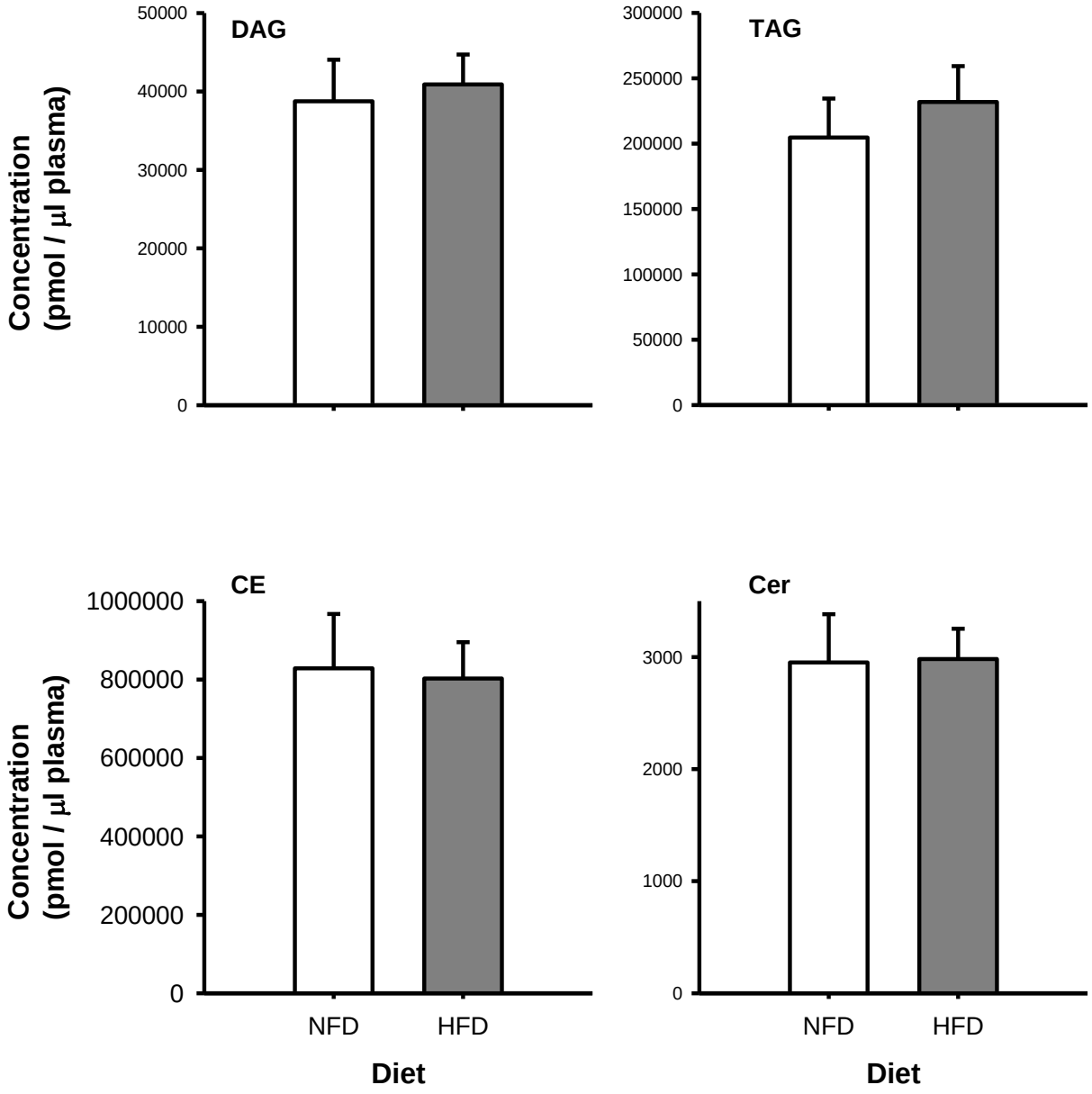

Figure 3.2: Total Plasma concentrations of triacylglycerides (TAG), diacylglycerides (DAG), cholesterol esters (CE) and ceramides (Cer) species in normal fat-fed (NFD; white bars) and high fat diet-fed (HFD; grey bars) after 3 weeks of diet.

Data are mean ± SEM.

Table 3.1: Ceramide species at baseline and week 3 in both NFD and HFD – fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3				
n	9		10		10		12				
Ceramide Species	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
Cer 16:0	189	28	253	28	189	16	287	21	1	0.01	1
Cer 18:0	131	18	136	17	139	13	179	26	1	1	1
Cer 20:0	168	22	206	27	159	12	239	21	1	0.05	1
Cer 22:0	608	93	754	108	550	47	882	84	1	0.05	1
Cer 24:1	440	71	633	94	395	43	510	50	1	0.21	1
Cer 24:0	833	141	971	174	665	56	885	95	1	1	1
Total Cer	2368	361	2952	430	2098	172	2983	271	1	0.18	1

Cer, Ceramides; NFD, normal fat diet; HFD, high fat diet

Table 3.2: Cholesterol esters at baseline and week 3 in both NFD and HFD – fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3				
n	9		10		11		13				
Cholesterol Esters	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
CE 14:0	7697	1061	8329	1187	6480	1096	5407	453	0.73	1	1
CE 15:0	14345	2862	11136	2272	10088	2064	6288	946	0.63	1	1
CE 16:2	481	102	517	101	344	86	621	70	1	1	1
CE 16:1	36966	6848	56509	14258	28942	6014	30401	4080	0.97	1	1
CE 16:0	166404	29325	153289	29209	127649	25054	134902	19118	1	1	1
CE 17:1	9896	1933	6440	1188	7643	1094	5736	805	1	0.95	1
CE 17:0	11718	2693	6294	1127	8364	1989	5420	892	1	0.50	1
CE 18:3	17329	3009	20249	5470	13419	2731	21319	3599	1	1	1
CE 18:2	253823	46115	224743	32202	197578	35925	273220	36054	1	1	1
CE 18:1	154389	27782	154154	30973	96569	19247	121237	14200	1	1	1
CE 18:0	22633	5433	12713	2946	14992	4003	13617	2003	1	1	1
CE 20:5	894	257	1211	327	946	221	1382	258	1	1	1
CE 20:3	1113	229	1374	238	805	153	1182	168	1	1	1
CE 20:4	24310	5865	21934	3782	17486	3941	24641	3141	1	1	1

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3				
Cholesterol Esters	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
CE 20:2	204	38	244	51	214	55	239	42	1	1	1
CE 20:1	367	73	444	120	3211	2937	289	43	1	1	1
CE 20:0	477	89	308	62	1812	1449	259	46	1	1	1
CE 22:5	901	212	1116	339	2859	2087	1227	235	1	1	1
CE 22:4	293	79	280	63	256	62	262	37	1	1	1
CE 22:1	91	22	117	30	76	26	80	14	1	1	1
CE 22:0	221	32	177	36	372	210	144	25	1	1	1
CE 24:0	171	40	90	20	312	175	137	27	1	1	1
COH	125399	20715	147173	25523	98990	17953	154609	16540	1	1	1
Total CE	849914	142086	828575	138733	639050	113456	802445	92730	1	1	1

CE; cholesterol esters, NFD; normal fat diet, HFD; high fat diet

Table 3.3: Diacylglycerides at baseline and week 3 in both NFD and HFD – fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3				
n	9		10		11		13				
DAG Species	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
DAG 14:0 14:0	28	4	34	5	23	4	25	5	1	1	1
DAG 14:0 16:0	378	52	448	54	343	47	375	42	1	1	1
DAG 14:1 16:0	57	9	109	11	74	10	68	14	1	1	0.61
DAG 16:0 16:0	1720	221	1753	201	1439	129	1689	213	1	1	1
DAG 14:0 18:1	670	139	888	121	632	113	618	72	1	1	1
DAG 14:0 18:2	436	70	402	80	386	59	518	46	1	1	1
DAG 16:0 18:0	993	110	871	109	837	81	1024	101	1	1	1
DAG 16:0 18:1	7054	1323	7679	932	6111	717	6785	739	1	1	1
DAG 16:0 18:2	5986	877	4836	976	4203	629	7382	999	1	1	0.36
DAG 16:1 18:1	1214	203	2012	227	1641	513	1223	147	1	1	1
DAG 18:0 18:0	212	16	185	42	277	119	255	23	1	1	1
DAG 18:0 18:1	1425	187	1444	151	1123	175	1384	110	1	1	1
DAG 18:0 18:2	1184	143	1001	189	895	114	1431	145	1	1	0.35
DAG 18:1 18:1	5021	767	6195	749	4079	691	4460	384	1	1	1
DAG 16:0 20:3	90	16	92	12	201	118	97	14	1	1	1

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3				
DAG Species	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
DAG 18:1 18:2	7275	1046	7253	1480	6040	711	8615	771	1	1	1
DAG 16:0 20:4	156	19	112	21	123	15	198	36	1	1	0.49
DAG 18:1 18:3	1069	159	1112	234	1648	767	1262	110	1	1	1
DAG 18:2 18:2	1702	253	1670	481	1274	193	2647	302	1	0.62	0.45
DAG 18:0 20:4	197	112	84	8	202	125	105	14	1	1	1
DAG 18:1 20:3	184	28	171	26	347	223	164	18	1	1	1
DAG 16:0 22:5	130	17	83	15	76	18	104	20	1	1	1
DAG 18:1 20:4	374	59	288	49	270	37	425	59	1	1	0.56
DAG 16:0 22:6	29	4	18	4	34	12	28	5	1	1	1
Total DAG	37583	5289	38739	5294	32277	3603	40884	3828	1	1	1

DAG; diacylglycerides, NFD; normal fat diet, HFD; high fat diet

Table 3.4: Triacylglycerides at baseline and week 3 in both NFD and HFD – fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3				
n	9		10		11		13				
TAG Species	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
TAG 14:0 16:0 18:2	3755	695	3729	521	2661	636	3812	495	1	1	1
TAG 14:0 16:1 18:1	1644	426	3188	562	1728	463	1548	226	1	1	1
TAG 14:0 16:1 18:2	432	94	557	74	585	202	600	76	1	1	1
TAG 14:0 18:0 18:1	344	58	301	56	365	108	304	49	1	1	1
TAG 14:0 18:2 18:2	514	90	493	114	729	313	767	110	1	1	1
TAG 14:1 16:0 18:1	569	148	1139	249	742	196	584	130	1	1	1
TAG 14:1 16:1 18:0	1798	450	3235	581	1729	375	1762	260	1	1	1
TAG 14:1 18:0 18:2	117	35	303	54	4881	4747	193	30	1	1	1
TAG 14:1 18:1 18:1	1378	299	1894	253	4834	3611	1644	186	1	1	1
TAG 15:0 18:1 16:0	2032	209	1417	309	1809	419	1072	138	1	0.92	1
TAG 15:0 18:1 18:1	1228	149	1075	216	2602	1586	754	93	1	1	1
TAG 16:0 16:0 16:0	3150	560	2154	491	2434	591	3199	697	1	1	1
TAG 16:0 16:0 18:0	2100	346	1811	675	1377	189	3107	616	1	1	1
TAG 16:0 16:0 18:1	25852	3856	19383	3561	15841	3018	22531	3518	1	1	1
TAG 16:0 16:0 18:2	12162	1992	7170	1940	7109	1269	15046	2945	1	1	0.23

TAG Species	NFD Week 0 Mean	SE	NFD Week 3 Mean	SE	HFD Week 0 Mean	SE	HFD Week 3 Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
TAG 16:0 16:1 18:1	12109	2080	16866	3160	10526	2406	11433	1657	1	1	1
TAG 16:0 18:0 18:1	7491	1216	4312	679	5718	807	5389	1143	1	1	1
TAG 16:0 18:1 18:1	50498	5980	41240	5833	34880	7887	38074	4161	1	1	1
TAG 16:0 18:1 18:2	35618	4652	23555	5079	24236	4482	36135	4229	1	1	0.56
TAG 16:0 18:2 18:2	11604	1763	8206	2297	8113	1453	16218	2561	1	1	0.30
TAG 16:1 16:1 16:1	173	41	284	39	291	130	191	25	1	1	1
TAG 16:1 16:1 18:0	521	66	430	55	1047	639	528	73	1	1	1
TAG 16:1 16:1 18:1	1723	299	1877	269	1293	302	1910	281	1	1	1
TAG 16:1 18:1 18:1	2441	552	3941	619	2040	489	2530	313	1	1	1
TAG 16:1 18:1 18:2	6301	1096	5597	980	5109	1078	7057	932	1	1	1
TAG 17:0 16:0 16:1	4652	557	2903	503	3843	829	2066	300	1	0.15	1
TAG 17:0 18:1 14:0	3653	450	2117	562	12151	9170	1141	203	1	1	1
TAG 17:0 18:1 16:0	2101	257	1443	337	4914	3326	1402	192	1	1	1
TAG 17:0 18:1 16:1	4237	499	3808	577	3463	941	2425	251	1	1	1
TAG 17:0 18:1 18:1	2622	603	2397	375	2664	572	1902	440	1	1	1

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3				
TAG Species	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P_{diet}	P_{time}	$P_{diet\ x\ time}$
TAG 17:0 18:2 16:0	3291	423	2115	287	2559	532	1921	262	1	1	1
TAG 18:0 18:0 18:0	71	26	31	7	1121	1084	55	11	1	1	1
TAG 18:0 18:0 18:1	555	92	440	93	15842	15377	734	120	1	1	1
TAG 18:0 18:1 18:1	5408	836	4963	817	31439	27087	6779	933	1	1	1
TAG 18:0 18:2 18:2	1713	227	1334	352	4942	3562	2033	532	1	1	1
TAG 18:1 14:0 16:0	4784	940	4858	781	3477	863	3857	641	1	1	1
TAG 18:1 18:1 18:1	8080	1312	9679	1041	6304	1327	8604	873	1	1	1
TAG 18:1 18:1 18:2	5822	917	6548	1358	4423	779	9515	1414	1	0.53	1
TAG 18:1 18:1 20:4	345	67	1053	803	352	123	2421	763	1	0.82	1
TAG 18:1 18:1 22:6	169	32	232	115	2570	2448	472	102	1	1	1
TAG 18:1 18:2 18:2	4289	798	5209	1225	3480	616	7900	1428	1	0.68	1
TAG 18:2 18:2 18:2	605	120	825	265	490	89	1440	341	1	0.72	1
TAG 18:2 18:2 20:4	314	164	509	197	229	74	797	352	1	1	1
Total TAG	238265	31727	204621	29850	246940	72879	231851	27372	1	1	1

TAG; triacylglycerides, NFD; normal fat diet, HFD; high fat diet

3.5 Discussion

The main findings of the present study were that plasma glucose and insulin concentrations were increased within the first 3 days of a HFD, remaining elevated for the first week of the diet and returning to control levels thereafter. Notably, circulating leptin concentrations were unaltered by a HFD at day 3 but were markedly increased by week 3 whilst in the same time period, no evidence of dyslipidaemia was found. Together, these data suggest hyperinsulinemia rapidly develops after the commencement of a HFD and is a likely mechanism by which haemodynamics and sympathetic tone may change rapidly in the fat-fed rabbit model of obesity related hypertension.

A considerable body of evidence suggests insulin acts centrally to increase both blood pressure and sympathetic tone (Landsberg, 1996; Lim et al., 2013; Straznicky et al., 2010; Ward et al., 2011). There is a strong association between obesity, hyperinsulinemia and, at a later stage, insulin resistance (Weyer *et al.*, 2001; Yuan *et al.*, 2001). Of note is the apparent delay between the engagement of sympathetic nerve activity in obesity and the development of insulin resistance (Flaa *et al.*, 2008) suggesting sympathetic overactivity may occur in response to very early changes in plasma insulin. Indeed central injections of insulin into the paraventricular nucleus of the hypothalamus produce large increases in lumbar sympathetic nerve activity (Ward *et al.*, 2011). In the present study we observed a near two-fold increase in plasma glucose and insulin concentrations within 3 days of starting the HFD. Importantly, we have previously shown that increased MAP, HR and RSNA in HFD-fed rabbits begins within the first few days of consumption (Armitage *et al.*, 2012; Burke *et al.*, 2013) suggesting that circulating insulin may be involved in augmenting MAP early in the diet. In support of this are the findings that central administration of an insulin antagonist attenuated MAP at 1 week of a HFD (Lim *et al.*, 2013). It is important to note that in the present study, plasma leptin concentrations in HFD-fed rabbits remained unchanged over the first 3 days of the diet but had increased by week 3. These results help explain our previous findings that central administration of a leptin antagonist to HFD-fed rabbits failed to elicit a reduction in either haemodynamic or sympathetic parameters at week 1 of the diet but produced large sympathoinhibitory and depressor responses at week 3 (Lim *et al.*, 2013). Combined, these observations imply plasma insulin is involved in the remodelling of haemodynamic and sympathetic tone within the first few days of consuming a HFD whilst leptin acts as a sympathoexcitatory signal later on in the diet, presumably once adiposity is increased. As

both plasma glucose and insulin concentrations normalised by week 2 of the diet the present observations point to sympathetic output preceding insulin resistance. Moreover, the apparent lack of effect of central administration of insulin on RSNA has been observed by others (Ward *et al.*, 2011) and may in part be due to the direct effect of insulin on baroreflex gain (Pricher *et al.*, 2008).

The present study also sought to establish the presence of dyslipidemia in our obese rabbit model and any subsequent contribution to the development of hypertension observed in these animals. In humans, dyslipidemia is a prominent feature of metabolic syndrome (Bays, 2009) and often appears in conjunction with hypertension (Nguyen *et al.*, 2008). An example of the consequences of dyslipidemia can be found in greater total plasma ceramide concentrations which are known to occur in obesity whilst specific ceramide species are strongly associated with insulin resistance (Haus *et al.*, 2009). In the present study plasma concentrations of 4 lipid classes (Cer, CE, DAG and TAG) remained unchanged after 3 weeks of HFD. Our findings are in agreement with those made by Eppel and colleagues who observed no change in total plasma cholesterol, and total plasma TAG in rabbits fed a HFD for 9 weeks (Eppel *et al.*, 2013) and suggests large changes in lipid profiles may take longer to develop in the rabbit model (Hamilton & Carroll, 1976). However, given the rapid haemodynamic and hormonal responses to dietary fat content, we expected to find changes in the expression of individual lipid species which would have been indicative of altered lipid metabolism. It is likely that our study was not powered to detect minute perturbations in the expression of specific plasma lipid species, contributing to our findings that plasma lipid profiles are unchanged by a diet high in fat. However, given that other parameters found in plasma, including insulin and leptin, can be measured accurately, our design is unlikely to be a confounding factor. Thus our findings discount dyslipidemia as a likely mechanism by which hypertension occurs during 3 weeks of a HFD.

In conclusion, our findings demonstrate plasma insulin is a likely mechanism by which rapid increases in MAP occur over the first few days of consumption of a HFD. In addition, dyslipidaemia does not appear to develop after 3 week of fat feeding suggesting plasma lipid profiles do not play a role in the genesis of hypertension in our animal model but may contribute to the development of comorbidities associated with obesity at a later stage.

3.6 References

See end of thesis for detailed bibliography.

Chapter IV - Specific Role of Dietary Fat in Modifying Cardiovascular and Locomotor Activity Circadian Rhythms

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception of experimental design; acquisition, analysis and interpretation of data; preparation of manuscript for publication (Chronobiology International).	**90**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Kyungjoon Lim	Collection of data
Sandra L. Burke	Intellectual input to manuscript content and contribution to revision of manuscript
James A. Armitage	Conception of experimental design, collection of data, surgical assistance with radiotelemetry probe, intellectual input to manuscript content and revision of manuscript.
Geoffrey A. Head	Conception of experimental design, collection and analysis of data, intellectual input to manuscript content and revision of manuscript.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date** **02.10.14**
Main Supervisor's Signature		**Date** **02.10.14**

4.1 Abstract

Meal fed conscious rabbits normally exhibit postprandial elevation in mean arterial pressure, heart rate and locomotor activity which is abolished by consumption of an *ad libitum* HFD. However, the contribution of the hyperphagia of the *ad libitum* diet or its higher fat content is unknown. Here, we assessed whether the cardiovascular changes were due to the increased caloric intake due to greater fat content or due to hyperphagia. Rabbits were meal-fed during the baseline period then maintained on either a normal fat (NFD) or high fat (HFD) diet *ad libitum* for 2 weeks. Blood pressure, heart rate and locomotor activity were measured daily by radio-telemetry alongside food intake and bodyweight. Caloric intake in rabbits given a NFD *ad libitum* rose 20% from baseline but did not cause any changes in cardiovascular parameters. By contrast, consumption of a HFD increased blood pressure (5%) and heart rate (10%) on the first day of the diet prior to any change in bodyweight, with blood pressure remaining elevated on day 13 of the diet ($p<0.05$). Baseline 24-hour patterns of blood pressure and heart rate were closely associated with meal time and were characterised by post-prandial peaks and pre-prandial troughs. Following 13 days on a HFD, pre-prandial values of heart rate and activity were increased by 17% and 61%, respectively. By contrast, only pre-prandial activity concentrations were increased in the NFD group ($p<0.05$). Increased caloric intake specifically from fat, but not as a result of hyperphagia, appears to directly modulate cardiovascular homeostasis and circadian patterns independently of white adipose tissue accumulation.

Keywords: 24-hour rhythm, blood pressure, heart rate, obesity, rabbits

4.2 Introduction

Obesity is the product of a consistent positive energy balance due to either a decrease in energy expenditure, a greater energy intake or a combination of both (Hill, 2006). The condition is associated with several comorbidities and its increasing prevalence places a considerable burden on global health and economy (Haslam & James, 2005; Thompson & Wolf, 2001). We have previously reported that consumption of a high fat diet (HFD) in rabbits for only a few weeks results in weight gain and increased adiposity as well as hypertension and tachycardia (Armitage et al., 2012; Prior et al., 2010). These animals also display aberrant cardiovascular and sympathetic meal associated rhythms characterised by a loss of pre-prandial dipping (Burke *et al.*, 2013). Whilst increased total caloric intake is an expedient gauge of the likelihood of developing obesity (Lichtman *et al.*, 1992), individual dietary constituents such as lipid and carbohydrate species are independently associated with varied risk profiles of cardiovascular disease (Siri-Tarino *et al.*, 2010). Importantly, dietary macronutrients have been shown to impact the pattern of meal associated rhythms. Consumption of a HFD has been demonstrated to abolish nocturnal dipping of mean arterial pressure (MAP) and heart rate (HR) observed at baseline in the canine model of obesity (Pelat *et al.*, 1999). In humans, high salt intake is associated with non-dipping in a subset of patients with essential hypertension (Uzu *et al.*, 2006).

Food consumption is a powerful synchroniser of circadian activity and is known to override signals from the 'master clock', the suprachiasmatic nucleus (Froy, 2010). Thus, changes in the time of meal presentation may affect peripheral circadian clocks such as the liver, heart and kidneys, in essence uncoupling central from peripheral circadian mechanisms (Damiola *et al.*, 2000). Time of consumption and dietary composition have been shown to alter circadian rhythms (Froy, 2010). For instance, diets high in fat, protein or carbohydrates affect the expression of SREBP-1protein, a key regulator of hepatic circadian homeostasis, to varying degrees (Matsumoto *et al.*, 2010). More specifically, mice fed a high fat diet exhibit a shift in 24-hour feeding rhythm characterised by increased caloric intake during the day (rest period) which was coupled with decreased amplitude the circadian transcription factors Bmal1 and Per2 expression in adipose tissue (Kohsaka *et al.*, 2007). Strikingly, these behavioural and cellular changes occur independently of body-weight gain (Kohsaka *et al.*, 2007). Concordant with these studies are observations in which the normal pre-prandial dipping in MAP and HR is abolished

by consumption of a HFD (Burke *et al.*, 2013). However, in that study the HFD-fed rabbits consumed more calories than those fed a normal fat diet (NFD) by virtue of being given a hypercaloric diet *ad libitum* whilst control animals were maintained on a restricted single meal of control feed. In rats, consumption of food *ad libitum* masks 24 hour water intake rhythms (Johnson & Johnson, 1991). Thus, provision of a HFD *ad libitum* in rabbits likely masks light-associated diurnal rhythms observed in NFD. In addition, consumption of a HFD is known to stimulate hyperphagia and thus increase total caloric intake when compared with a low-fat diet (Savastano & Covasa, 2005). Indeed 50% of the increased calories consumed in our previous studies were due to hyperphagia and 50% due to the higher fat content of the diet (Burke *et al.*, 2013). As these factors were combined, the separate contribution to the changes in the cardiovascular patterns of increased calories from fat compared to those due simply to hyperphagia could not be assessed. It is likely that these different sources of calories have quite different cardiovascular influences. Thus in the present study we compared the effect of consuming an *ad libitum* HFD with that of consuming an *ad libitum* NFD. Thus both groups will have increased caloric intake due to hyperphagia, and only one group will have the effect of the higher fat content. This design would allow us to ascertain whether the cardiovascular changes observed in HFD-fed rabbits were due to increased fat content or the hypercaloric nature of the diet.

4.3 Materials and Methods

4.3.1 Ethical Approval

Experiments were approved by the Alfred Medical Research and Education Precinct Ethics Committee and conducted in accordance with Australian Code of Practice for Scientific Use of Animals. Experiments were conducted on male New Zealand White rabbits (initial body weight 2.7-2.8 kg). Rabbits were housed in pens individually and were kept under controlled light (lights on 06:00 – 18:00) and temperature (18-22 °C) conditions with *ad libitum* access to water.

4.3.2 Experimental Procedures

Rabbits were fitted with radiotelemetry transmitters (model TA11PA-D70; Data Sciences International, St. Paul, MN, USA) under isoflurane anesthesia (3-4 % in 1 L/min Oxygen; Abbot, Botany, NSW, Australia) following induction with propofol (10 mg/Kg; Fresenius Kabi, Pymble,

NSW, Australia). The catheter of the transmitter was implanted into the aorta via a small branch arising from the left iliac artery. Analgesia was provided prior to and following surgery (Carprofen; 3 mg/Kg, Pfizer, North Ryde, NSW, Australia).

Following 9 days of recovery, baseline MAP and HR were measured in the laboratory by both telemetry and a catheter in the medial ear artery. The telemetry signal was calibrated to the ear artery signal and this adjustment was applied to MAP measured in the home cage in order to minimize the possibility of drift of the signal with time (Burke *et al.*, 2013). Baseline home cage MAP, HR, food intake and bodyweight (BWT) were measured over a 3 day period in which both dietary groups were given 150g of a control diet each day and the remaining food weighed the following day. Rabbits were then randomly assigned into either a normal fat diet (NFD; 4.2 % total fat, 2.63 kcal/g; Specialty Feeds, Glen Forrest, WA, Australia, n = 9) or a high fat diet (HFD; 13.3 % total fat, 3.34 kcal/Kg, SF06-011, Specialty Feeds; n = 10) group. Rabbits were given *ad libitum* access to food and were maintained on their respective diets for 2 weeks. During that period continuous MAP and HR measurements were made. In a subset of rabbits (n= 13), BWT and food intake were measured daily for the duration of the diet.

4.3.3 Data Analysis

Measurements of 24-hour MAP, HR and locomotor activity were performed as previously published (Burke *et al.*, 2013). To assess the effect of feeding, data from both preprandial and postprandial periods were compared. The former was designated as the 6 h period between 03:30 and 09:30 h (when the animals were quiet and values were stable) whilst the latter was defined as the 6 h period following feeding (13:00– 19:00 h). The influence of the light cycle over the 24-hour pattern of parameters was assessed by measuring the difference between values taken over the 12 h period when lights were on (06:00–18:00 h) and those taken over the 12 h dark period (18:00– 06:00 h). Values were expressed as mean ± SEM or mean difference ± SE of the difference (SED). Data were analysed by split plot repeated-measures analysis of variance, which is a mixed model allowing for within-animal and between-animal (between group) contrasts. Comparisons included, $p_{\text{at baseline}}$ referring to between groups made prior to the consumption of either diet, p_{lin}, refers to the linearity of changes due to diet over time, $p_{ad\ lib}$, refers to within group contrasts between the meal fed period and *ad libitum* period, p_{diet} refers to contrasts between HFD and NFD-fed rabbits during *ad libitum* feeding, p_{light} refers

to the effect of light on all measured parameters. Type 1 error was controlled using Bonferroni and the Greenhouse-Geisser corrections were used to correct for sphericity (Ludbrook, 1994). A probability of $p<0.05$ was considered significant.

4.4 Results

4.4.1 Effect of *Ad Libitum* NFD and HFD Consumption on 24-h averages of MAP, HR

Baseline home cage values averaged over 24-hours were 65.2 ± 1.2 mmHg (n=7), and 221 ± 4 b/min (n=7) for MAP and HR respectively. By day 2 of consuming a HFD, MAP and HR values increased by 6% and 10 % respectively (Figure 4.1). MAP remained elevated at 6 % of baseline for the duration of the 13 day period ($p_{lin}>0.05$; Figure 4.1). The effect of HFD consumption on 24-hour HR measurements was an initial increase of 8 % in the first 6 days of the diet which diminished over time and returned to baseline by day 13 ($p_{ad\ lib}>0.05$; Figure 4.1). By contrast, there was no detectable change in MAP or HR in rabbits with *ad libitum* access to a NFD ($p_{ad\ lib}>0.05$; Figure 4.1).

4.4.2 Effect of *Ad Libitum* NFD and HFD Consumption on Caloric Intake, Body Weight, Food Intake and locomotor activity

Baseline bodyweights, caloric intake food consumption and locomotor activity were 2.80 ± 0.05, 360 ± 6.4 kcal, 117.2 ± 3.0 g, 43.2 ± 2.1 au, respectively. Consumption of a NFD or HFD for 13 days produced an 18 % and 22 % increase in bodyweight respectively ($p_{ad\ lib}<0.05$; Figure 4.1). Caloric intake rose sharply following 1 day on a HFD but declined over the 13 day period in a pattern resembling that of HR ($p_{lin}>0.05$; Figure 4.1). However, for the duration of the diet caloric intake remained 49 % greater in fat-fed rabbits compared with controls ($p_{diet}<0.001$; Figure 4.1). A similar pattern of food intake was observed in both NFD and HFD rabbits with the marked hyperphagia observed on day 1 of the diet diminishing over the 13 day period ($p_{diet}>0.05$; Figure 4.1). A similar reduction in locomotor activity was observed in both dietary groups on the first 5 days (14 % and 18 %, respectively ($p_{ad\ lib}<0.05$ for both; Figure 4.1).

4.4.3 Effect of *Ad Libitum* NFD and HFD on 24-hour patterns of MAP, HR and Locomotor Activity

Variability in MAP, HR and locomotor activity over a 24-hour period show clear rhythms associated with meal presentation and consumption (Figure 4.2). During control period meal feeding, pre-prandial HR was lower than post-prandial values in rabbits planned to be fed either a NFD (209 ± 9 vs. 235 ± 4 b/min, $p_{\text{at baseline}}$ <0.01; Figure 4.2) or a HFD (201 ± 13 vs. 239 ± 4, $p_{\text{at baseline}}$ <0.001; Figure 4.2) fed rabbits. Following 6 days of *ad libitum* feeding, HR prior to consumption of a meal was higher than that observed during the baseline meal fed period (241 ± 6 vs. 201 ± 13 b/min, $p_{\text{ad lib}}$ <001; Figure 4.2) contributing to the loss of 'pre-prandial dipping' in HFD-fed rabbits, with a similar pattern of pre-prandial (224 ± 6 Vs. 201 ± 13 b/min, $p_{\text{ad lib}}$ <0.05; Figures 4.2) and post-prandial values (215 ± 2 b/min, $p_{\text{ad lib}}$ <0.05; Figures 4.2, 4.3) observed on day 13. By comparison, the 24-hour HR pattern of NFD-fed rabbits remained unchanged ($p_{\text{ad lib}}$>0.05; Figures 4.2, 4.4) with pre-prandial dipping observed just prior to meal time at every time point (Figures 4.2, 4.4). The between group contrast at baseline did not reveal differences in locomotor activity with presentation of food augmenting pre-prandial values (NFD; 28 ± 8 vs. 51 ± 11 au, HFD; 25 ± 4 vs. 55 ± 6 au, $p_{\text{at baseline}}$<0.05; Figure 4.2). The locomotor activity patterns of HFD rabbits closely resembled those of HR with day 6 pre-prandial activity values increasing above both baseline (61 ± 3 Vs. 25 ± 4 au, $p_{\text{ad lib}}$ <0.05; Figure 4.2) and post-prandial values (61 ± 3 Vs. 39 ± 7 au, $p_{\text{ad lib}}$ <0.05; Figures 4.2, 4.3). The pattern was still present on day 13 with locomotor activity pre-prandial values remaining +40 ± 18% greater than baseline ($p_{\text{ad lib}}$ <0.05; Figure 4.2). Whilst NFD-fed rabbits displayed elevated pre-prandial locomotor activity on day 6 of the diet compared with baseline levels (51 ± 9 au Vs. 28 ± 8 au, $p_{\text{ad lib}}$<0.05; Figure 4.2), these returned to baseline levels by day 13 ($p_{\text{ad lib}}$ >0.05; Figure 4.2). Presentation of a meal at baseline did not elicit an increase in post-prandial MAP in animals which were assigned to a dietary group but have not yet commenced on the diet ($p_{\text{at baseline}}$ >0.05; Figure 4.2). Similarly, post-prandial MAP remained unchanged relative to –pre-prandial values following consumption of a HFD although the net effect of the diet was such that pre- (70 ± 3 Vs. 63 ± 1 mmHg, $p_{\text{ad lib}}$ <0.01) and post- (71 ± 3 Vs. 66 ± 3 mmHg, $p_{\text{ad lib}}$ <0.05) prandial MAP values were higher in HFD-fed rabbits compared with baseline values (p_{diet} <0.05; Figures 4.2, 4.3). By contrast, MAP levels did not depart from baseline in rabbits fed a NFD *ad libitum* ($p_{\text{ad lib}}$ >0.05; Figures 4.1, 4.4).

4.4.4 Effect of HFD on light-related patterns

In order to characterise the relationship between 24-hour variability and the light cycle, we also measured the differences between the data collected during the 12 hour light and 12 hour dark periods. At baseline there were no differences in MAP between the light and dark periods in either dietary group (NFD; 64.6 ± 1.7 Vs. 66.8 ± 1.5 mm Hg, p_{light} >0.05, HFD; 64.7 ± 2.6 Vs. 64.7 ± 2.0 mmHg, p_{light} >0.05, Figures 4.3, 4.4). Consumption of a HFD, but not a NFD, over the 13-day period increased MAP in the dark period (2 ± 0.6 mmHg (p_{diet} <0.01; Figures 4.3, 4.4). Baseline levels of HR and locomotor activity were not influenced by the light cycle in either dietary group (p_{light} >0.05, Figures 4.3, 4.4). In both HFD and NFD rabbits, HR and locomotor activity did not change between the light and dark periods over the 13 day period (p_{light} >0.05; Figures 4.3, 4.4).

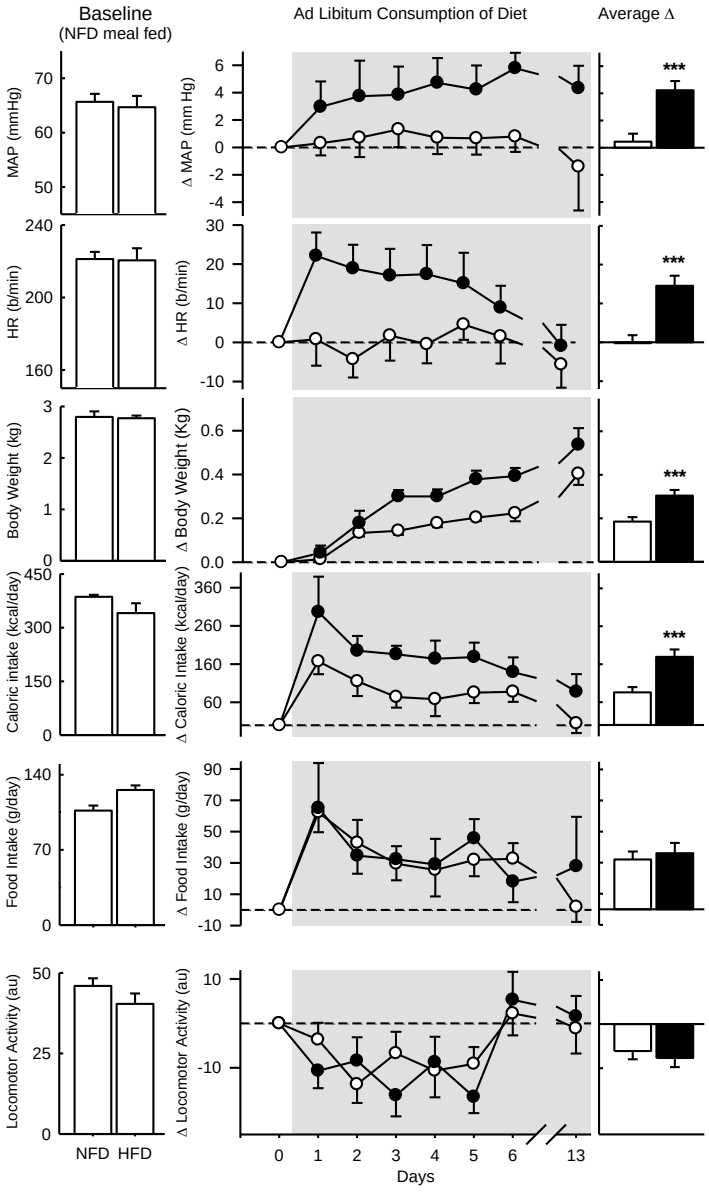

Figure 4.1: Baseline levels in meal fed rabbits and effect of 13 days of a high fat (HFD) or normal fat diet (NFD) fed ad libitum. Left panels: Baseline values averaged over 24 hours in meal fed rabbits before commencing ad libitum NFD or HFD. Values are mean ± SEM. Centre panels: Daily changes from baseline from the first until the 13th day of a NFD (open circles) or HFD (filled circles) fed ad libitum. Ad libitum feeding is indicated by grey panel. Values are mean difference ± SED indicating between animal variance. Right panels: Average change over the entire 13 day period in NFD (unfilled bars) and HFD (filled bars) animals. ***P*diet* <0.001 for HFD vs NFD (days 1-13). Mean arterial pressure (MAP), heart rate (HR, beats/min).

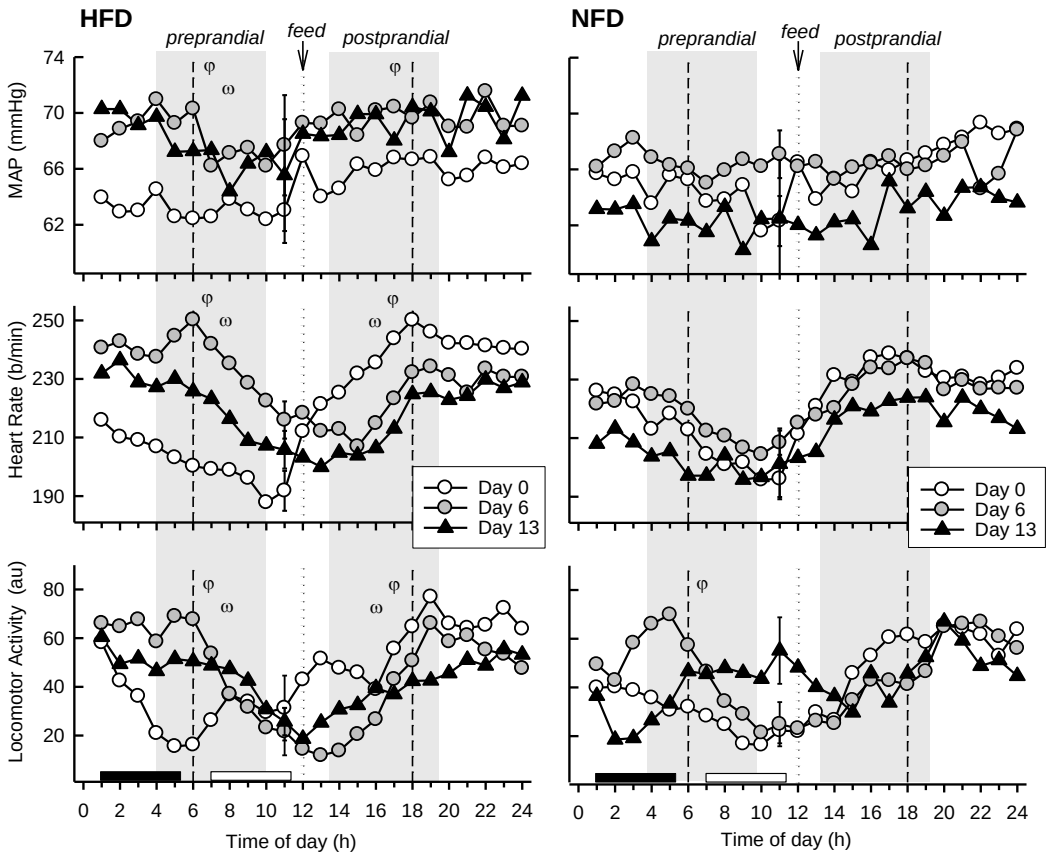

Figure 4.2: Left: Hourly averaged data showing the variation over 24 hours of mean arterial pressure (MAP), heart rate and activity (au, arbitrary units) in rabbits meal fed a normal fat diet on day 0 (open circles), day 6 (grey circles) and on day 13 (black triangles) after the start of a high fat diet fed *ad libitum*. Right: Hourly averaged data in rabbits meal fed a normal fat diet on day 0 (open circles), day 6 (grey circles) and day 13 (black triangles) after changing to *ad libitum* feeding of the same diet. Rabbits were fed at 12:00 h (dotted line) and the lights were on between 6:00 h and 18:00 h (dashed vertical lines). Values are mean ± SEM indicating between animal variance. The preprandial and postprandial periods (03:30 h - 09:30 h and 13:00 h-19:00 h) are shaded in grey; the preprandial dark and light periods (0:00 h-05:00 h and 06:00 h -11:00 h) are indicated by the black and white bars. $^{\varphi}P<0.05$ for Day 0 vs Day 6 during preprandial and postprandial periods. $^{\omega}P<0.05$ for Day 0 vs Day 13 during preprandial and postprandial periods. HFD, high fat diet, NFD, normal fat diet.

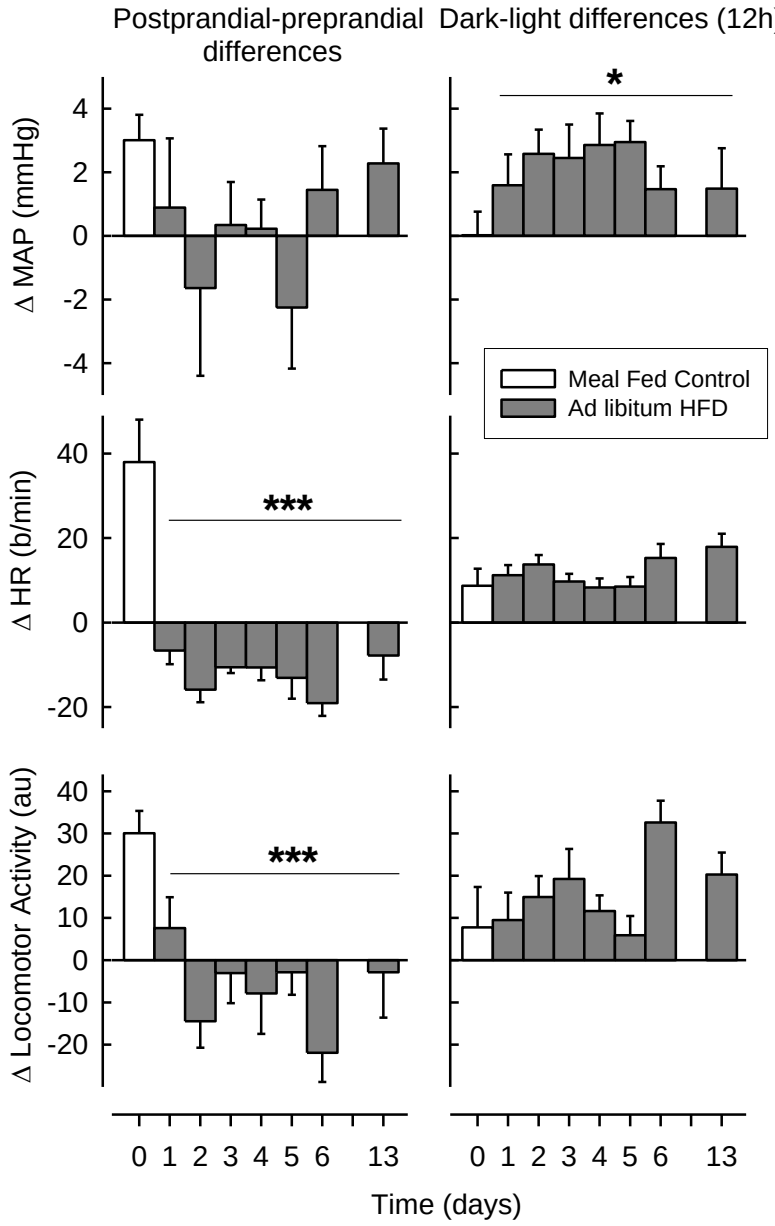

Figure 4.3: Left: Average differences between values collected during 6 hours preprandial (03:30 h - 09:30 h) and those during 6 hours postprandial (13:00 h -19:00 h) before (meal fed normal diet, open bars) and on days 1 - 13 (grey bars) of HFD fed *ad libitum*. Middle: Average differences between values collected during 12 hours of dark (18:00 h - 06:00 h) and 12 hours of light (06:00h - 18:00 h). Right: Average differences between values collected in the dark (0:00 h - 05:00 h) and the light (06:00 h - 11:00 h) of the preprandial period. Values are mean difference ± SED indicating between animal variance. $^{*}P<$ 0.05, $^{***}P<$ 0.001 for days 1-13 compared with day 0; HFD, high fat diet.

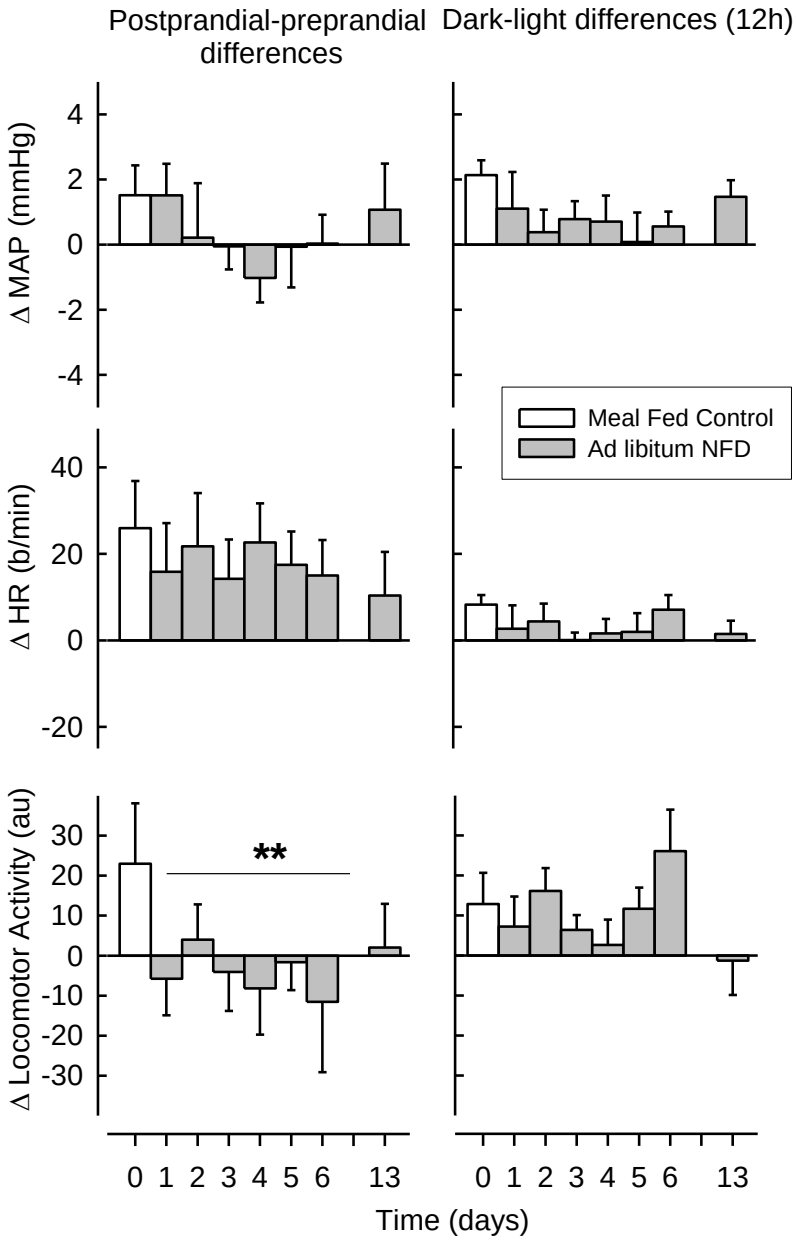

Figure 4.4: Left: Average differences between values collected during 6 hours preprandial (03:30 h - 09:30 h) and those during 6 hours postprandial (13:00 h -19:00 h) before (meal fed normal diet, open bars) and on days 1 - 6 (grey bars) of the same NFD fed *ad libitum*. Middle: Average differences between values collected during 12 hours of dark (18:00 h - 06:00 h) and 12 hours of light (06:00h - 18:00 h). Right: Average differences between values collected in the dark (0:00 h - 05:00 h) and the light (06:00 h - 11:00 h) of the preprandial period. Values are mean difference ± SED indicating between animal variance. $^{**}P< 0.01$ for days 1-6 compared with day 0; NFD, high fat diet.

4.5 Discussion

Dietary habits, including time of meal consumption and nutrients available, have a profound effect on haemodynamics and circadian rhythms (Damiola *et al.*, 2000; Uzu *et al.*, 2006). The major finding of this study is that greater dietary fat content, but not increased caloric intake due to hyperphagia, adversely affects haemodynamic variables with both MAP and heart rate increasing over the first 6 days of HFD consumption. The change in haemodynamics manifested both as a rise in daily average as well as a shift in the pattern of circadian rhythmicity. In addition, we reported a reduction in locomotor activity in both dietary groups concomitant with a reversal in the 24h pattern of locomotor activity circadian rhythm. Thus an increase in either total caloric intake or dietary fat content appears to affect different circadian rhythms.

4.5.1 Cardiovascular Circadian Rhythms are Influenced by HFD not Increased Caloric Intake

We have previously reported that meal-fed control rabbits exhibit a 24-h pattern heavily influenced by feeding with a preprandial low and a postprandial high (Burke *et al.*, 2013). In the current study, *ad libitum* consumption of the same diet did not change this pattern, despite a 30% increase in caloric intake and a 20% gain in body weight. By contrast, rabbits given free access to a diet rich in fat exhibited a loss of preprandial 'dipping' on the first day of the diet, contributing to the observed hypertension and tachycardia in these animals. In humans, greater body mass index (BMI) is associated with aberrant circadian periodicity characterised by a loss of diurnal dipping (Kotsis *et al.*, 2005) although the precise mechanism by which this occurs remains elusive. In the current study, a change in cardiovascular circadian pattern was linked to consumption of a HFD for 3 weeks whilst the circadian patterns of rabbits given free access to a NFD, subsequently increasing total caloric intake, did not depart from baseline. Despite this, a similar degree of bodyweight gain was noted in both dietary groups suggesting a specific effect on circadian patterns related to dietary fat. Indeed, consumption of a HFD in humans increases fasting plasma low-density lipoprotein (LDL) cholesterol and triglyceride levels (Kwiterovich *et al.*, 2003) and these correlate with blood pressure in 'non-dipping' obese patients (Kotsis *et al.*, 2005). Furthermore, experiments conducted by Puska (1983) in which a 6 week low fat diet

reduced systolic and diastolic pressures independently of salt intake and weight loss support our finding that dietary fat has a considerable impact on MAP.

4.5.2 Effects of Increasing Caloric Intake and Total Dietary Fat on Locomotor Activity

We have previously reported a reduction in locomotor activity in rabbits maintained on a HFD for 3 weeks (Burke *et al.*, 2013). Here we report a similar effect on locomotor activity in rabbits given free access to a NFD despite no observed change in cardiovascular parameters. In the present study, pre-prandial locomotor activity was increased in both dietary groups. Indeed, the 24-hour pattern of locomotor activity of HFD rabbits shifted along the time axis to the right so as to match that of heart rate. The observed increase in both groups is likely due to increased caloric intake and may indicate a compensatory response similar to that observed in mice (Butler *et al.*, 2001). Reduced activity in NFD rabbits from the current study stands in contrast with previous data from meal-fed animals given the same diet and which exhibit no change in locomotor activity (Burke *et al.*, 2013). Sedentary behaviour is associated with greater bodyweight gain in animals (Crews *et al.*, 1969) and greater obesity rates in humans (Epstein *et al.*, 2000). By day 13, rabbits fed a NFD *ad libitum* gained the same amount of weight as HFD-fed rabbits, likely due to decreased energy expenditure and increased caloric intake. Thus a pre-obese phenotype may be achieved by increasing either dietary fat content or total caloric intake.

4.6 Increased Calories from Fat Affect Cardiovascular Parameters

Here we report increased dietary fat augments the daily averages of MAP and HR yet increased caloric intake bears no impact on these parameters. Our observations suggest the haemodynamic changes observed in HFD rabbits are independent of BWT-gain given NFD animals in the current study increase body weight and move less, presumably decreasing energy expenditure. In fact, both dietary groups displayed similar increases in BWT over the 13 day period, albeit at different rates. Moreover, increased MAP in HFD rabbits precedes any change to BWT. Thus we may conclude haemodynamic changes occur in response to increased dietary fat. In animals, consumption of a HFD has been shown to augment MAP and HR (Boustany *et al.*, 2004; Cook *et al.*, 2004; Prior *et al.*, 2010; Yiannikouris *et al.*, 2012). Importantly, increased

calories from fat are known to induce hypertension and tachycardia in humans (Appel et al., 1997; Straznicky et al., 1993).

We have previously shown that the increase in MAP is present beyond the 13 day period and remains elevated following withdrawal of the HFD-diet (Armitage *et al.*, 2012; Burke *et al.*, 2013). Moreover, the pressor response to the diet occurs concomitantly with an increase in renal sympathetic nerve activity (Armitage et al). Of note is the fact that reintroduction of a NFD results in decreased HR but maintained MAP and augmented RSNA (Burke *et al.*, 2013). Thus a HFD appears to be an important instigator of hypertension but might not be required to maintain it over a long period of time. Additionally, the gradual attenuation in HR is likely due to calorie adjustment previously reported in these animals (Burke *et al.*, 2013). Considering rabbits given free access to a NFD do not show potentiation of RSNA (Chapter 5, Lim et al., 2013; Prior et al., 2010), increased dietary fat intake may play a central role in the genesis of obesity related hypertension via activation of sympathetic activity. Indeed, fatty acids have been shown to interact with hypothalamic neurons and alter the expression of key neuropeptides known to regulate energy and cardiovascular homeostasis as well as sympathetic tone (Obici *et al.*, 2002; Shimokawa *et al.*, 2002). Strikingly, consumption of a HFD over just 3 days significantly impairs the normal response of hypothalamic neurons to free fatty acids (Morgan *et al.*, 2004). Despite recognition from the WHO (WHO, 2003) that increased total fat intake is strongly associated with obesity related hypertension, specific fat species better correlate with relative risk of developing CVD. There is a strong association between CVD and trans fats, artificially altered unsaturated fatty acids (Mozaffarian *et al.*, 2006). In addition, saturated fatty acids (SFA) have been suggested to increase risk of developing CVD although the relationship remains controversial (Astrup *et al.*, 2011). Conversely, polyunsaturated fatty acid (PUFA) intake is known to lower the risk of coronary heart disease (Mozaffarian *et al.*, 2005) and has been shown to decrease blood pressure in children over a long period of time (Forsyth *et al.*, 2003). In addition, it is suggested that the ratio between lipid species is of particular relevance to risk of developing CVD (Mozaffarian *et al.*, 2010). In the present study, both dietary groups were not rich in saturated fatty acids and had a higher PUFA to SFA ratio.

A limitation of the current study is the difficulty in delineating the effect of calories from fat versus calories per se on MAP and HR given HFD rabbits consume more calories than controls. We have previously shown that consumption of a HFD induces fat accumulation and increases circulating leptin levels which strongly correlate with visceral adiposity and MAP (Burke et al., 2013; Prior et al., 2010). However, it is exceptionally difficult to distinguish between the effects of fat-derived calories and calories per se as fat is the most energy dense macronutrient and replacing it would necessitate greater amounts of either protein or carbohydrates. Neither macronutrients would adequately replace fat in the rabbit. On the other hand, caloric restriction would further complicate the interpretation of these experiments.

4.7 Summary and conclusion

We have shown that MAP and HR circadian rhythms are influenced by greater calories from fat but not increased total caloric intake per se. We have also demonstrated that locomotor activity appears to be more sensitive to total caloric intake, irrespective of fat content of the diet. Thus, despite only the HFD having adverse cardiovascular consequences, increased total caloric intake seems to abate energy expenditure by means of increasing sedentary behaviour. These diverging effects highlight the ways by which obesity, and associated hypertension, may develop.

4.8 References

See end of thesis for detailed bibliography.

Chapter V - Hypothalamic Pro-Opiomelanocortin and Neuropeptide Y Systems are Altered in the Development of Obesity Related Neurogenic Hypertension.

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception of experimental design; acquisition, analysis and interpretation of data; preparation of manuscript for publication (Hypertension Journal).	**90**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Sandra L. Burke	Surgical assistance with implantation of renal nerve electrode. Intellectual input to manuscript content and contribution to revision of manuscript
Pamela J. Davern	Confirmation of c-Fos immunohistochemistry counts and revision of manuscript.
Kyungjoon Lim	Revision of manuscript.
James A. Armitage	Conception of experimental design, collection of data, intellectual input to manuscript content and revision of manuscript.
Geoffrey A. Head	Conception of experimental design, collection, analysis and interpretation of data, intellectual input to manuscript content and revision of manuscript.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **02.10.14**
Main Supervisor's Signature		**Date** **02.10.14**

5.1 Abstract

Hypothalamic melanocortin and neuropeptide Y (NPY) neurons represent major signaling pathways through which leptin exerts its anorectic, cardiovascular and sympathoexcitatory effects. High fat diet (HFD) induced hypertension in rabbits is neurogenic and due to the central action of leptin, actions that are dependent on secondary neuronal activation in alpha-melanocortin stimulating hormone (α-MSH) and NPY positive cells. In the present study we assessed the contribution of α-MSH and NPY to the development of diet-induced neurogenic hypertension. Male New Zealand White rabbits were instrumented with an intracerebroventricular cannula, renal sympathetic nerve electrode and a blood pressure telemetry transmitter. After 3 weeks of a HFD (13.5 % fat, n=31) conscious rabbits had higher renal sympathetic nerve activity (+3.8nu), blood pressure (+8.6mmHg) and heart rate (+15 b/min) compared with control diet-fed (CD) animals (3.5% fat, n=32). Intracerebroventricular α-MSH increased sympathetic activity and heart rate further (P<0.01) but not blood pressure in HFD rabbits and had no effects in control rabbits. Central administration of the MC3/4 receptor antagonist SHU9119 reduced sympathetic activity (-3.0nu) and blood pressure (-6.8 mmHg) in HFD but not CD-fed rabbits thus reversing 80-90% of the effect of a HFD. Blocking central NPY Y1 receptors with BVD10 increased sympathetic activity but only in HFD rabbits. These findings suggest that obesity-induced hypertension and sympathetic activation is dependent on greater sensitivity in melanocortin signaling that is being opposed by greater activation of NPY signaling. While these effects may lie downstream from leptin signaling we cannot exclude an additional contribution from leptin.

Key Words: Obesity, Hypertension, Leptin, NPY, Renal Sympathetic Nerve Activity, α-MSH

5.2 Introduction

Obesity is a precursor to serious cardiovascular and metabolic diseases (Dalton et al., 2003) and relatively modest reductions in body weight are associated with reduced incidence of cardiovascular events (Wing et al., 2011). Evidence suggests that increased sympathetic nerve activity (SNA), particularly to the kidneys and skeletal muscle vasculature, occurs secondary to the accumulation of body fat and is a major mechanism of obesity induced hypertension (Eikelis & Esler, 2005; Esler et al., 2001; Mark, 2013; Prior et al., 2010). In animal studies, bilateral renal denervation in both dogs and rats reverses diet-induced hypertension (Huang et al., 1998; Kassab et al., 1995). Antic and colleagues abolished high fat diet (HFD) induced hypertension in conscious rabbits with α-adrenoceptor blockade (Antic et al., 2000). Increasingly the focus has been on the circulating adipokine leptin which has long been known to regulate appetite and hence energy intake and influence metabolism (Mark, 2013). Leptin is secreted primarily by adipocytes and is present in serum in direct proportion to the percentage of adipose tissue (Considine et al., 1996; Lambert et al., 2013). Chronic systemic or acute central infusions of leptin increase blood pressure in control rats or rabbits via stimulation of the SNS (Carlyle et al., 2002; Dunbar et al., 1997; Haynes et al., 1997; Matsumura et al., 2000; Shek et al., 1998). These effects are propagated by leptin receptors located on alpha-melanocortin stimulating hormone (α-MSH) and neuropeptide Y (NPY) containing neurons in the arcuate nucleus of the hypothalamus (ARC) (Rahmouni et al., 2005a). The interaction between the three involves simultaneous leptin mediated inhibition of NPY and stimulation of α-MSH positive cells as well as reciprocal connections between the two neuronal populations, resulting in a push-pull mechanism through which the effects of leptin ensue (Williams et al., 2001).

We have previously demonstrated that feeding a HFD for 3 weeks leads to increased mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) in conscious rabbits (Prior et al., 2010). Importantly we have shown that ganglion blockade completely abolishes the increase in blood pressure suggesting that this model of obesity hypertension is neurogenic (Armitage et al., 2012). Circulating plasma levels of leptin were increased in the first week of the diet and strongly correlated with increased visceral adiposity, MAP and RSNA (Armitage et al., 2012; Prior et al., 2010). The surprising discovery was that the central responses to leptin were augmented in HFD rabbits following only 3 weeks on the diet.

Other studies have suggested that the while the appetite inhibitory effects of leptin were reduced (leptin resistance), the SNA effects were preserved, a phenomenon termed selective leptin resistance (Correia et al., 2002). We suggested that the mechanism of the hypertension involved sympathetic activation and increased responsiveness to central sympathoexcitatory effects of leptin due to increased plasma leptin arising from visceral fat accumulation (Armitage et al., 2012; Prior et al., 2010). We recently investigated the contribution of insulin and leptin using specific peptide antagonists administered acutely after 1 or 3 weeks of a HFD in rabbits (Lim et al., 2013). The insulin antagonist had only a small effect on blood pressure and no effect on RSNA. By contrast the leptin antagonist after 3 weeks (and not after 1 week) reduced blood pressure and RSNA to levels close to those observed in rabbits on a normal fat control diet (CD) (Lim et al., 2013). The contemporaneous effects of both the leptin agonist and antagonist at week 3 suggest a leptin-dependent mechanism is likely involved in the development of obesity related hypertension. This HFD-induced change in leptin function could arise from either changes in leptin signalling and/or changes in the function of NPY and α-MSH neurons through which leptin signals are relayed downstream of the ARC.

In the present study we assessed whether a HFD results in a change to the cardiovascular effects of activating either the α-MSH or NPY receptors. If the diet-induced sensitization of the sympathoexcitatory effects of leptin occurs at the level of the leptin signalling, we would expect no change in the effects of α-MSH or NPY. If, however, the sensitization occurs downstream then we may see changes to the sensitivity to α-MSH or NPY.

5.3 Methods

5.3.1 Animals

Experiments were conducted in 63 conscious male New Zealand White rabbits (2.25-2.75kg). Rabbits were housed under controlled light (6:00 to 18:00) and temperature (22° c ± 2°c) conditions. Experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals.

5.3.2 Experimental Procedures and Protocol

Rabbits underwent preliminary surgery under isoflurane anaesthesia and carprofen (3 mg / kg) was given 24 hours before and after surgery for analgesia. Rabbits were implanted with an intracerebroventricular (icv) cannula into the lateral ventricle, as described previously (Head & Williams, 1992). Following 10 days recovery, a blood pressure telemetry transmitter (TA11PA-D70, Data Sciences International, St. Paul, MN, USA) was implanted into the aorta via a branch of the left iliac artery (Burke et al., 2010). Following a week of recovery, baseline MAP and HR were measured over a 1-hour period. Rabbits continued on a CD (4.2 % total fat) or were placed on a HFD (13.3 % total fat) *ad libitum* for 3 weeks (Prior et al., 2010).

Two weeks after the initiation of the diet, a recording electrode was implanted on the left renal nerve, under isoflurane anaesthesia (Dorward et al., 1985; Prior et al., 2010). One week later, after a 1-hour period for recovery from handling, resting MAP, HR and RSNA were recorded for 1 hour. This period is sufficient to provide stable data similar to that in the home cage (Lim et al., 2012). A 50 µl icv injection of the vehicle (Ringer's solution, Baxter, Toongabbie, NSW, Australia) was then given followed by increasing doses of either α-MSH or NPY (1, 3 and 10 nmol or 0.5, 1.5, 5 nmol, respectively, Tocris, Ellisville, USA) and their respective antagonists, SHU9119 (MC3/4 receptor antagonist; 0.038, 0.075, 0.188 nmol, Tocris) or BVD10 (Y1 receptor antagonist; 3, 10, 30 nmol, Tocris) delivered in 50 µl vehicle icv at 30 minute intervals. Experiments were conducted in randomised order on separate days. HS014 (Tocris) which has been shown to be an MC4 receptor partial agonist (Chai et al., 2003) was also given icv (3, 10, 30 nmol). A time control study involved the injection of 4 vehicle doses. Experiments were conducted in randomised order on separate days.

5.3.3 Assessment of Body, Fat, Organ Weights and c-Fos immunohistochemistry.

Rabbits were euthanized by anesthetic overdose (Lethabarb, 100 mg / Kg, i.v, Virbac Animal Health, Woolpit, UK). Lean body, total fat, total body mass, bone mineral density and bone mineral content at week 3 were measured using a dual energy absorptiometry X-ray (DEXA) machine (Discovery A-QDR series, Hologic Inc. MA, USA). White adipose tissue (WAT) pads from mesenteric viscera, retroperitoneal area, testes and bladder were dissected and weighed. Brains (CD, n = 4, HFD = 3) were perfusion fixed (4 % paraformaldehyde), sectioned in the coronal plane and processed for Fos immunohistochemistry (Davern & Head, 2007; Prior et al., 2010). Analysis of c-Fos immunoreactivity following the top dose of icv α-MSH was carried out as previously described (Prior et al., 2010).

5.3.4 Data Analysis

MAP, HR derived from the arterial pressure pulse, and integrated RSNA were digitized online at 500 Hz and averaged over 2 seconds. In order to allow for between animal comparisons, RSNA was normalized to the maximum RSNA recorded during the nasopharyngeal response evoked by smoke, taken to be 100 normalized units (Burke & Head, 2003; Lim et al., 2013; Prior et al., 2010). Values averaged over 30 minutes were expressed as mean ± SEM or mean difference ± SE of the difference (SED). Data were analyzed by split plot repeated-measures ANOVA, which allowed for within- animal and between-animal (group) contrasts and adjusted for multiple testing using the Bonferroni method (Burke et al., 2010). For all statistics shown we refer to the main effect as a subscript, e.g P_{diet} refers to the effect of diet. One-way ANOVA was used for data collected at a single time point. Type 1 error was controlled using Bonferoni and Greenhouse Geisser corrections. A probability of $P < 0.05$ was considered significant.

5.4 Results

5.4.1 Effect of 3 Week Fat-feeding on Body Weight, WAT and Organ Weights

Initial body weights were not different between the dietary groups (2.94 ± 0.06 kg before HFD and 2.91±0.06 kg before CD, P_{group} = 0.7). At the end of the 3 week feeding protocol, total body mass as measured by DEXA, was 9 % greater in HFD fed rabbits compared with CD fed rabbits due in the main to a 63 % greater total WAT mass (Table 5.1, P_{diet} < 0.01 for both). The retroperitoneal, visceral, cardiac and testicular fat pads were 31 – 40 % heavier in HFD rabbits compared with controls even after being expressed as percentage of body weight (Table 5.1, P_{diet} < 0.05). The weights of other organs such as liver, kidney, spleen and left ventricle heart were similar in both groups (P_{diet} > 0.3, Table 5.3).

5.4.2 Effect of HFD on Cardiovascular Variables and RSNA

At the end of 3 weeks of HFD, MAP had increased by +15 ± 1 % and HR had increased by +21 ± 2 % above baseline whilst CD animals showed no change in MAP from baseline and a smaller increase in HR (+9 ± 2 %, P_{diet} < 0.001, Table 5.2). After 3 weeks of HFD, normalized total RSNA was +30 ± 8 % higher in HFD rabbits compared with CD rabbits (P_{diet} = 0.02, Table 5.2) which was due to a +31 ± 6 % greater RSNA burst amplitude in HFD rabbits compared with controls (P_{diet} < 0.001, Table 5.2). RSNA frequency did not differ between the two groups even when the difference in HR was taken into account (P_{diet} = 0.1; Table 5.2). The average nasopharyngeal response (μV), which was used to normalize the RSNA signal, was similar between the CD and HFD groups (Table 5.2).

5.4.3 Effect of α-MSH and HS014 on Cardiovascular Variables and RSNA

ICV administration of α-MSH resulted in an increase in RSNA in HFD rabbits (+66 ± 11 % for 10 nmol dose, P_{drug} <0.001, n=11) that was dose dependent (P_{lin}= 0.02; Figure 5.1) but had little effect in CD rabbits (n= 11 thus P_{diet} = 0.006). HR also increased in a dose dependent manner (+24 ± 3 % for 10 nmol dose, P_{drug} < 0.001, P_{lin}< 0.001; Figure 5.1) in the HFD-fed rabbits whilst CD rabbits only responded to the top dose (+10 % for 10 nmol dose, P_{drug} = 0.01; Figure 5.1). These doses of α-MSH administered icv produced a small reduction in MAP in the

CD (-3.7±1.6 mmHg, P=0.003) and in the HFD group (-3.2±1.4 mmHg, P=0.002; P_{diet}=0.9, Figure 5.1).

Central administration of the MC4 receptor partial agonist HS014 had no effect at 3 and 10 nmol in either group but 30 nmol increased MAP and RSNA by +10 ± 3 % (P_{dose} =0.03) and +69 ± 11% (P_{dose} <0.001) respectively in the HFD group and not in the CD group (n=7-8, Figure 5.2). This was accompanied by a reduction in HR in both groups (P_{dose}< 0.01, Figure 5.2). The between group contrasts did not reveal differences for any parameter (P_{diet}>0.2) indicating that the observed effects of HS014 were independent of diet.

Central administration of the MC3/4 receptor antagonist SHU9119 attenuated RSNA and MAP by -26 ± 7 % (n=5, P_{drug} <0.01) and -9 ± 2 % (P_{drug} <0.001) respectively in HFD but not CD-fed rabbits (n=4, P_{drug} >0.05, Figure 5.3). By contrast, increasing doses of SHU9119 had no effect on HR in either dietary group (P_{drug} >0.05, Figure 5.3). Injection of vehicle had no effect on cardiovascular parameters or RSNA in either dietary group (P>0.05, Figure 5.7).

5.4.4 Effect of NPY and BVD10 on Cardiovascular Variables and RSNA

Administration of 0.5-5 nmol icv of NPY to HFD rabbits dose-dependently reduced MAP (P_{drug} and P_{lin}=0.05, n=11) but had no effect in CD rabbits (P_{drug}<0.9, n=11, Figure 5.4). NPY also increased RSNA at all doses (+41 ± 10 % for 5 nmol dose, P_{drug} = <0.001) in CD but not in HFD rabbits (P_{drug} >0.9, P_{diet}<0.001, Figure 5.4). HR was also increased very slightly by NPY (+6 ± 1 % for 5 nmol dose, P_{drug} <0.01, Figure 5.4) in CD rabbits but had no effect on the HR of HFD rabbits (P_{drug} >0.9, Figure 5.4). Central infusion of the NPY Y_1 receptor antagonist BVD10 (3-30 nmol) resulted in a marked elevation of RSNA in HFD animals alone (P_{diet}=0.03, n=7) that was dose dependent (P_{lin}=0.009; Figure 5.5). There were small non-significant effects on MAP and HR in both groups.

5.4.5 Effect of ICV α-MSH on Hypothalamic c-Fos Expression Levels.

Following administration of icv α-MSH to CD-fed rabbits, c-Fos expression was detected in all hypothalamic nuclei examined, with very high levels in the organum vasculosum of the lamina terminalis and strong activation in the medial preoptic nucleus, ARC, paraventricular hypothalamus (PVH), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH) and supraoptic nucleus (Figure 5.6). There was noticeably less activation of the median preoptic nucleus (Figure 5.6). By contrast, HFD rabbits had approximately 80 % fewer c-Fos positive cells compared with CD controls in the PVH, DMH and VMH nuclei (P_{diet}<0.001, Figure 5.6). In addition, c-Fos expression was 30-40 % lower in the ARC, medial preoptic, and organum vasculosum of the lamina terminalis nuclei of HFD-fed rabbits compared with controls (P<0.05 for all three, Figure 5.6). There was no effect of a HFD on the activation of the supraoptic and median preoptic nuclei (P_{diet}>0.05, Figure 5.6).

Table 5.1: Body Composition in NFD and HFD-fed rabbits after 3 weeks of diet.

Measurement	**NFD**	**HFD**	P_{diet}
Post-Mortem Body composition	n=22	n=24	
Bodyweight (Kg)	3.29 ± 0.06	3.48 ± 0.07	0.03
Retroperitoneal WAT(g)	53 ± 5.3	78.6 ± 6.4	0.004
Retroperitoneal WAT (% BWT)	1.6 ± 0.1	2.2 ± 0.2	0.004
Visceral WAT (g)	40.1 ± 6.3	67 ± 5.9	0.003
Visceral WAT (% BWT)	1.2 ± 0.2	1.9 ± 0.1	0.002
Cardiac WAT (g)	3.7 ± 0.6	5.4 ± 0.6	0.03
Cardiac WAT (% BWT)	0.11 ± 0.02	0.16 ± 0.02	0.04
Testicular & Bladder WAT (g)	5.1 ± 0.5	8.0 ± 0.7	0.003
Testicular & Bladder WAT (% BWT)	0.15 ± 0.01	0.22 ± 0.02	0.003
Total WAT (g)	97.7 ± 13.2	154.9 ± 12.4	0.003
Total WAT (% BWT)	2.9 ± 0.3	4.4 ± 0.3	0.002
DEXA Estimates	**n=8**	**n=9**	
Lean Body Mass (g)	3085 ± 41	3229 ± 50	0.04
Fat Mass (g)	103 ± 14	263 ± 26	< 0.001
Total Body Mass (g)	3193 ± 38	3492 ± 59	< 0.001
% Fat	3.2 ± 0.5	7.4 ± 0.7	< 0.001
Bone Mineral Content (g)	56.0 ± 0.95	60.5 ± 1.07	0.006
Bone Mineral Density (g/cm^2)	0.223 ± 0.004	0.230 ± 0.003	0.19

Values are mean ± SEM. BWT = bodyweight. P is the probability for the comparison between groups.

Table 5.2: Hemodynamics and sympathetic nerve activity from NFD and HFD-fed rabbits after 3 weeks of diet.

Measurement	**NFD**	**HFD**	P_{diet}
Hemodynamics and RSNA	n=11	n=12	
MAP (mmHg)	69.3 ± 1.3	77.9 ± 1.0	P<0.001
Δ MAP from baseline (mmHg)	-1.0 ± 1.6	11.6 ± 1.5	P<0.001
HR (bpm)	194.9 ± 4.8	209.2 ± 5.4	0.01
Δ HR from baseline (bpm)	17.8 ± 5.3	43.6 ± 3.7	P<0.001
RSNA (μV)	31.3 ± 5.6	30.3 ± 1.6	0.84
RSNA (nu)	9.0 ± 1.0	12.8 ± 1.0	0.02
RSNA Amplitude (nu)	24.3 ± 0	35.1 ± 2	P<0.001
RSNA Frequency (bursts per second)	7.1 ± 0.6	6.5 ± 0.3	0.30
RSNA Frequency (bursts per heart beat)	2.2 ± 0.2	1.9 ± 0.1	0.13
Nasopharyngeal Response (μV)	299 ± 38	256 ± 23	0.36

Values are mean ± SEM. P is the probability for the comparison between groups.

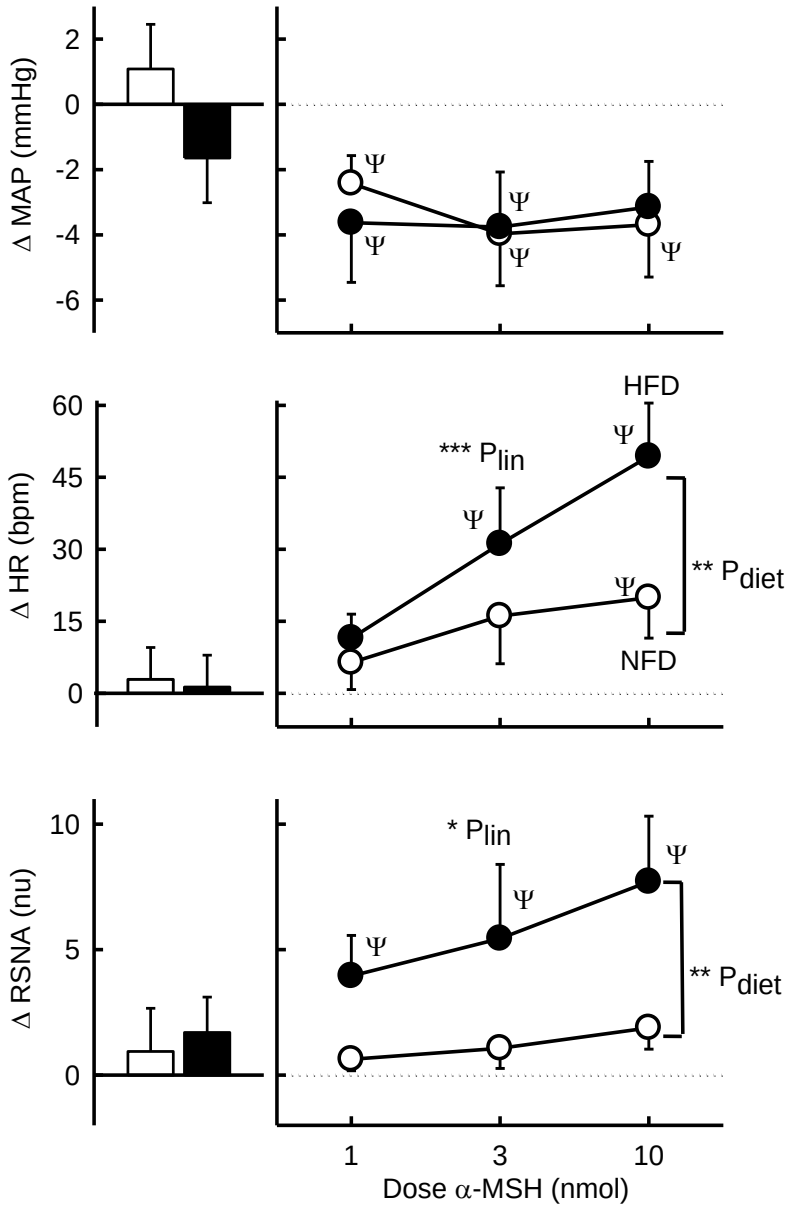

Figure 5.1:

Left: changes from baseline of mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) in response to vehicle injection (Ringer's Solution, 50 µl) in rabbits fed a control diet (NFD; unfilled bars; n=11) or a high fat diet (HFD;filled bars; n=11) for 3 weeks. Right: 30-minute averages of changes from vehicle in MAP, HR and RSNA in response to increasing doses of ICV α-MSH in both NFD (unfilled circles) and HFD (filled circles) rabbits. Data are mean ± SED indicating variance between animals. Ψ for effect of individual doses from baseline (P<0.05). **Pdiet<0.01 for for effect of diet. *Plin<0.05, ***Plin<0.001 for significance of linear trend effect of α-MSH.

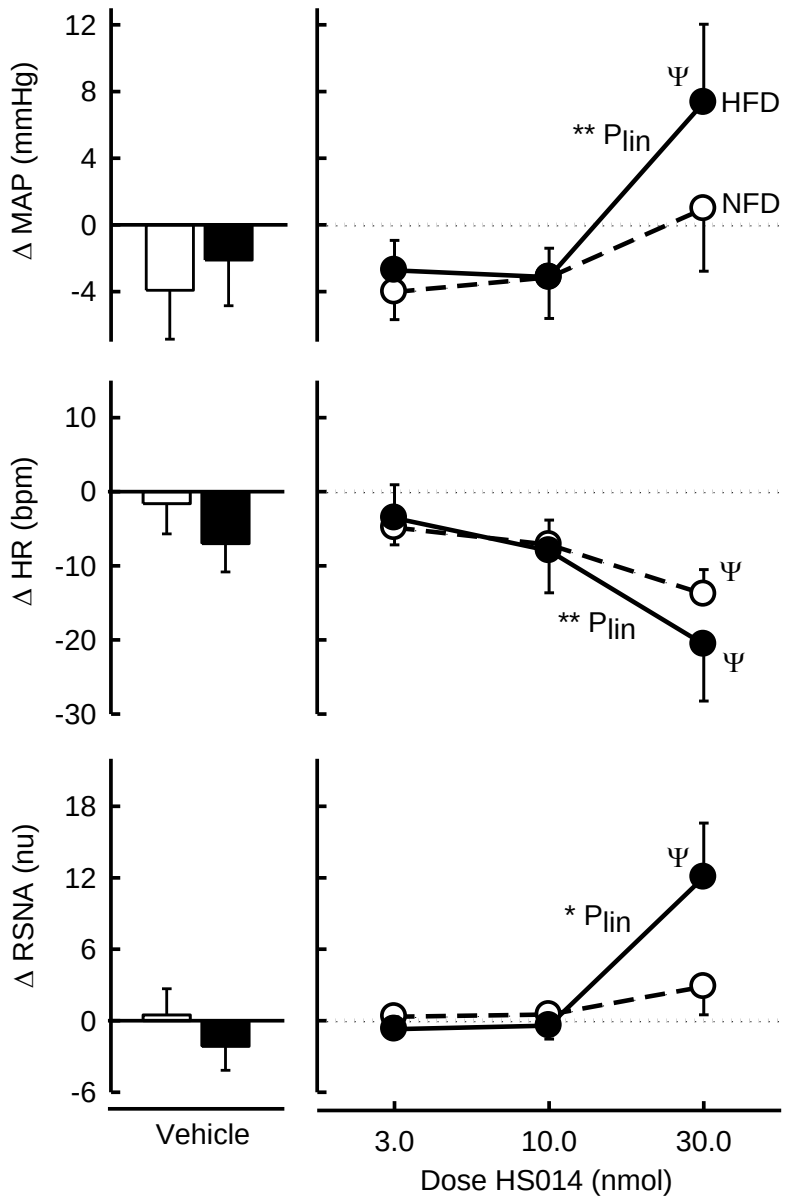

Figure 5.2: Left: changes from baseline of mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) in response to vehicle injection (Ringer's Solution, 50 µl) in rabbits fed a control diet (NFD; unfilled; n=7) or a high fat diet (HFD; filled; n=8) for 3 weeks. **Right**: 30-minute averages of changes from vehicle in MAP HR and RSNA in response to increasing doses of ICV HS014 in both NFD (unfilled circles) and HFD (filled circles) rabbits. Data are mean ± SED indicating variance between animals. Ψ for effect of individual doses from baseline ($P<0.05$). *Plin<0.05, **Plin<0.01for significance of linear trend effect of HS014.

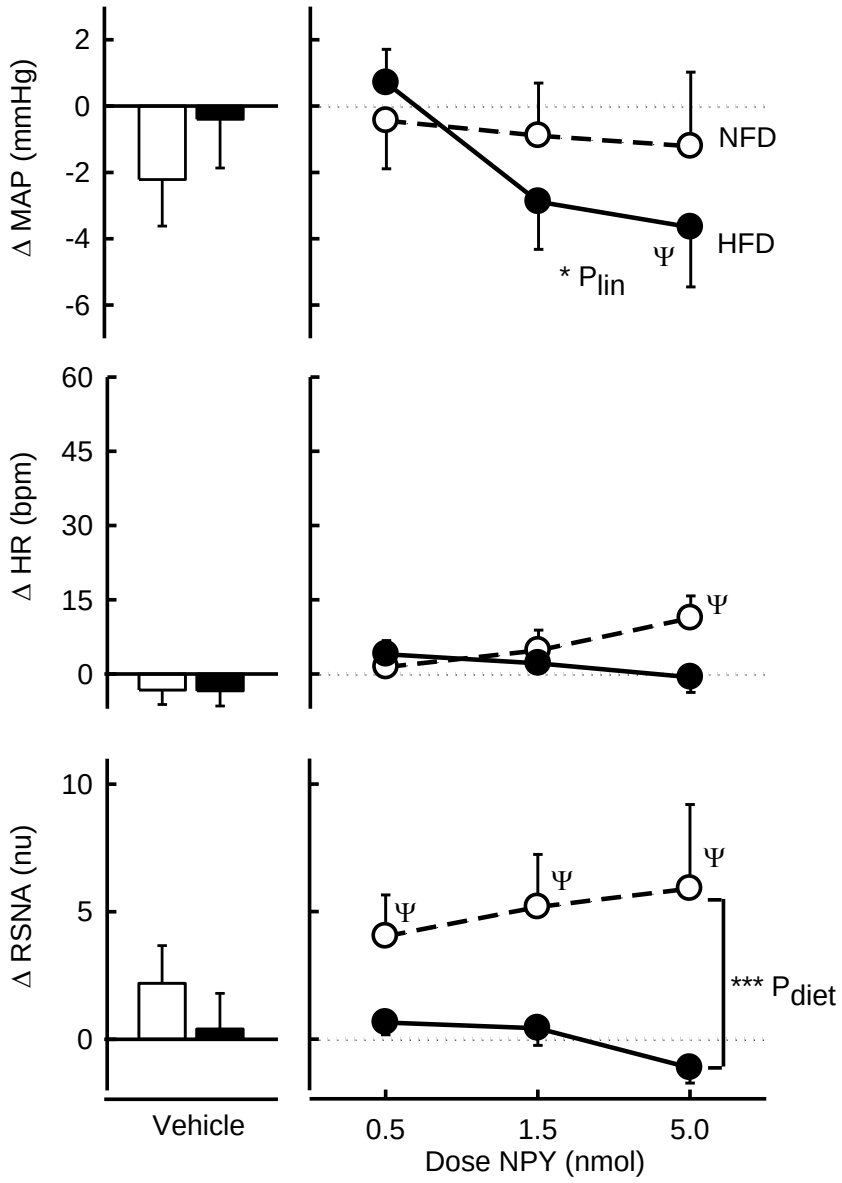

Figure 5.3: Left: changes from baseline of mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) in response to vehicle injection (Ringer's Solution, 50 µl) in rabbits fed a control diet (NFD; unfilled; n=10) or a high fat diet (HFD; filled; n=11) for 3 weeks. **Right**: 30-minute averages of changes from vehicle in MAP HR and RSNA in response to increasing doses of ICV NPY in both NFD (unfilled circles) and HFD (filled circles) rabbits. Data are mean ± SED indicating variance between animals. Ψ for effect of individual doses from baseline (P<0.05). $^{***}P_{diet}$<0.001 for effect of diet. $^{*}P_{lin}$<0.05, for significance of linear trend effect of NPY.

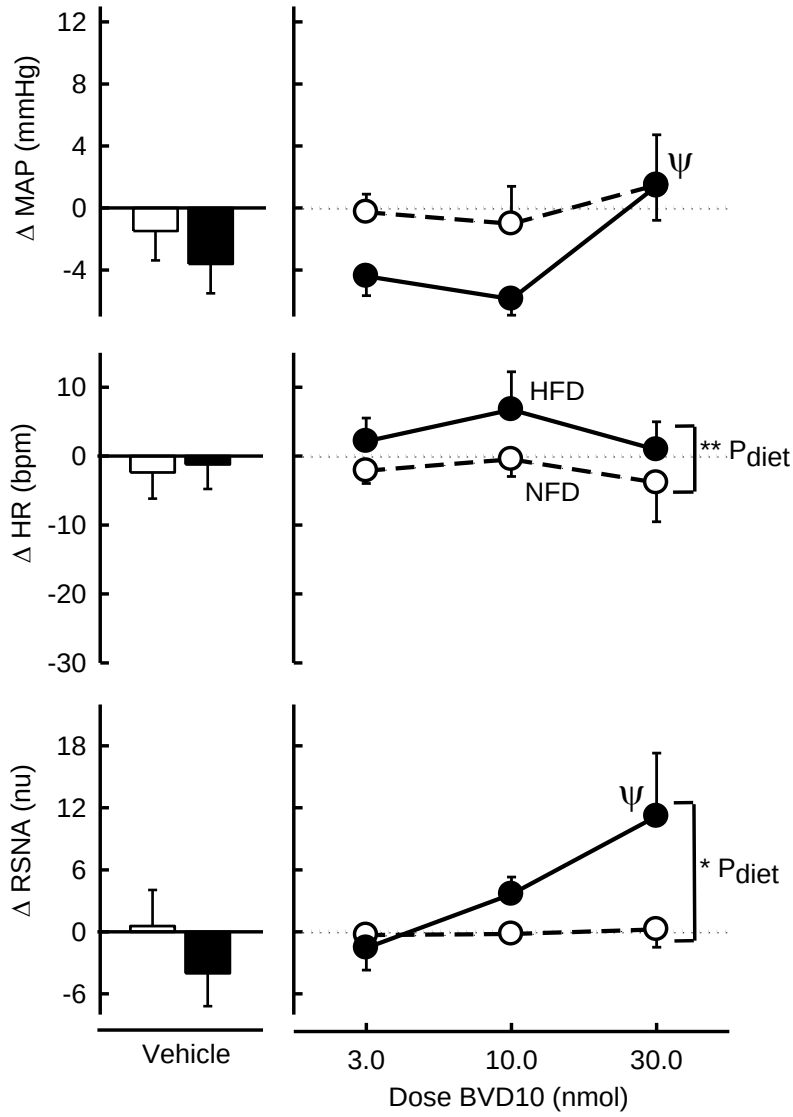

Figure 5.4: Left: changes from baseline of mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) in response to vehicle injection (Ringer's Solution, 50 µl) in rabbits fed a control diet (NFD; unfilled; n=7) or a high fat diet (HFD; filled; n=7) for 3 weeks. **Right**: 30-minute averages of changes from vehicle in MAP HR and RSNA in response to increasing doses of ICV BVD10 in both NFD (unfilled circles) and HFD (filled circles) rabbits. Data are mean ± SED indicating variance between animals. Ψ for effect of individual doses from baseline ($P<0.05$). *Pdiet<0.05, **Pdiet<0.01 for effect of diet.

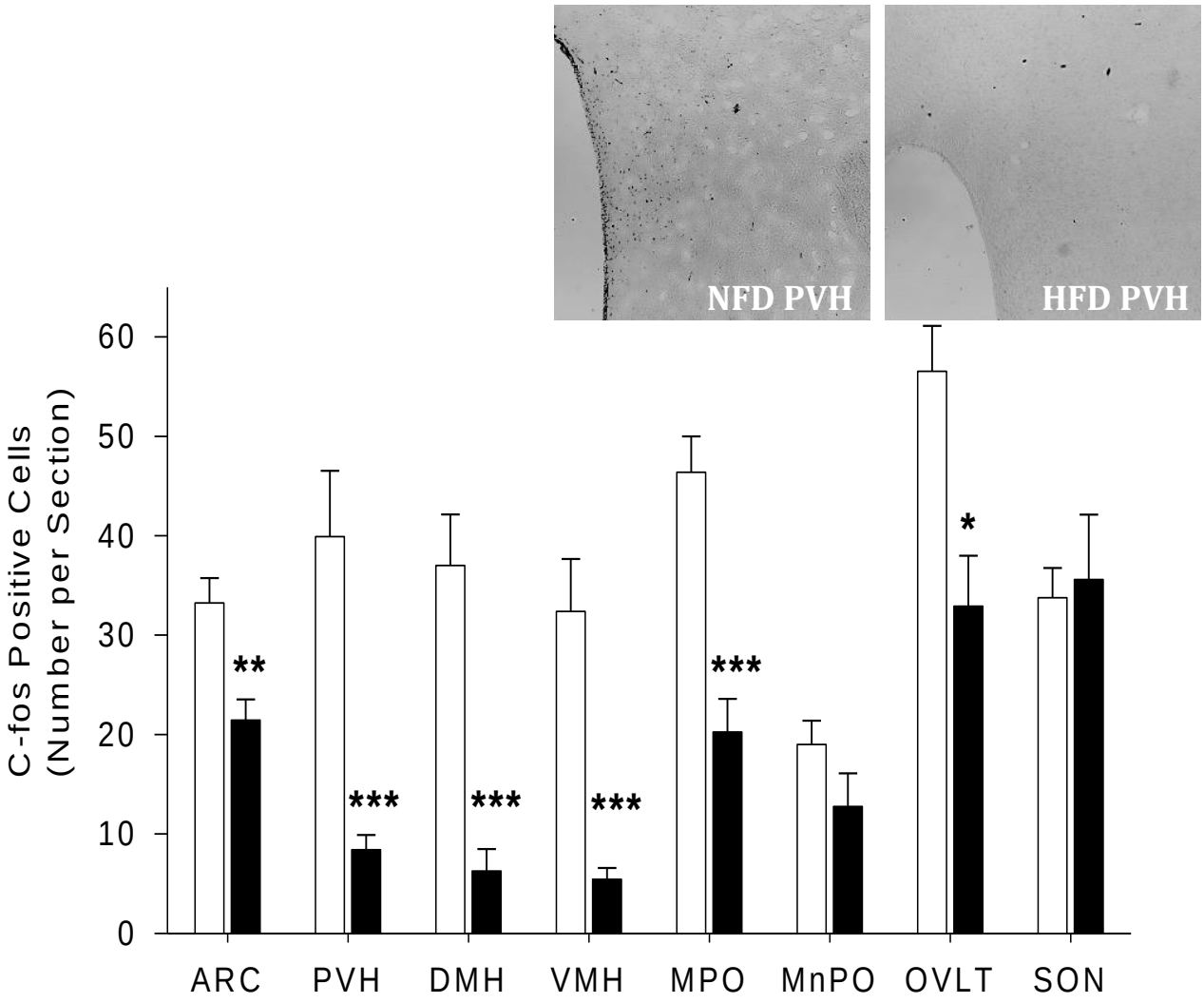

Figure 5.6: Mean number of c-Fos positive neurons in the hypothalamus of normal fat diet (NFD; n=4; unfilled bars) and high fat diet (HFD; n=3; filled bars) fed rabbits as detected by c-Fos immunoreactivity induced by ICV α-MSH. Top right corner, coronal micrographs of the PVH in NFD and HFD rabbits used as representative images of Fos immunoreactivity. Data are mean ± SEM indicating variance between animals. *Pdiet< 0.05, **Pdiet<0.01, ***Pdiet<0.001 for NFD vs HFD. ARC, arcuate nucleus; PVH, paraventricular hypothalamus; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; MPO, medial preoptic nucleus; MnPO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis; SON, supraoptic nucleus.

Table 5.3: Body and organ weights following 3 weeks on a diet in NFD and HFD-fed rabbits.

Measurement	**NFD**	**HFD**	P_{diet}
n	20-25	21-24	
Bodyweight (Kg)	3.29 ± 0.06	3.48 ± 0.07	0.03
Liver (g)	102 ± 7	99 ± 5	0.7
Liver (% BWT)	3.04 ± 0.16	2.84 ± 0.12	0.3
Left Kidney (g)	10.3 ± 0.3	10.5 ± 0.3	0.6
Left Kidney (% BWT)	0.31 ± 0.01	0.30 ± 0.01	0.6
Right Kidney (g)	9.8 ± 0.4	10.0 ± 0.3	0.8
Right Kidney (% BWT)	0.30 ± 0.01	0.29 ± 0.01	0.5
Left Cardiac Ventricle (g)	5.08 ± 0.24	5.08 ± 0.15	0.9
Left Cardiac Ventricle (% BWT)	0.15 ± 0.01	0.15 ± 0.01	0.4
Adrenal Glands (mg)	312.6 ± 32.4	325.6 ± 25.0	0.8
Adrenal Glands (% BWT)	0.01 ± 0.001	0.009 ± 0.001	0.8
Spleen (g)	1.28 ± 0.09	1.4 ± 0.07	0.3
Spleen (%BWT)	0.038 ± 0.003	0.041 ± 0.002	0.5

Values are mean ± SEM. BWT = bodyweight.

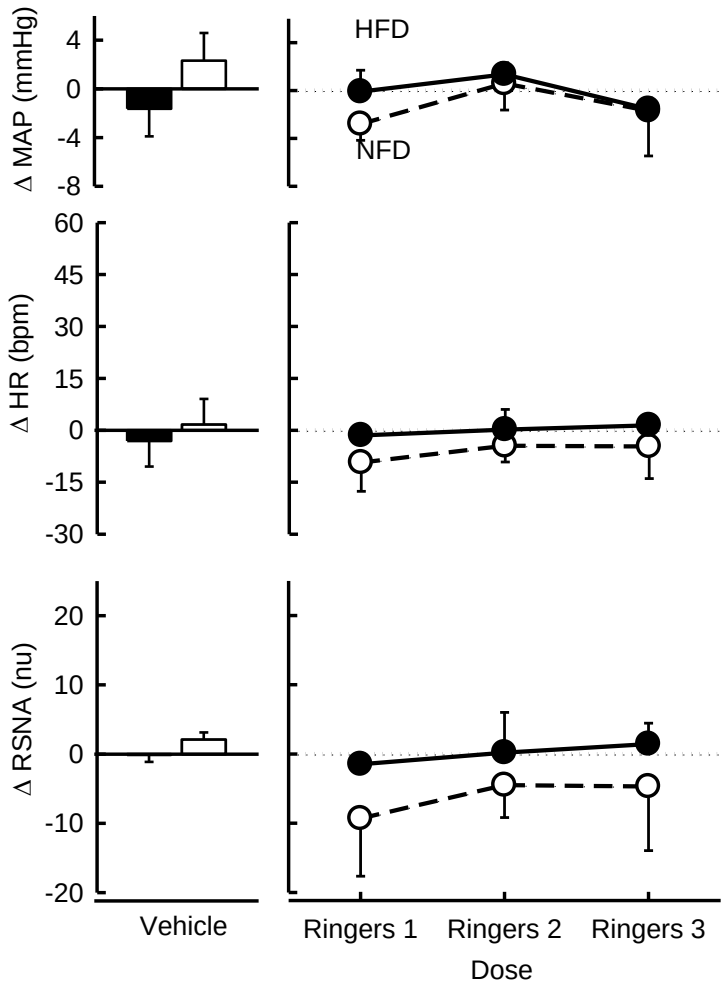

Figure 5.7: Left changes from baseline of mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) in response to vehicle injection (Ringer's Solution, 50 µl) in rabbits fed a control diet (NFD; open bars; n=5) or high fat diet (HFD; filled bars; n=5) for 3 weeks. **Right**, 30-minute averages of changes from vehicle in MAP HR and RSNA in response to consecutive doses of Ringers in both NFD (unfilled circles) and HFD (filled circles) rabbits. Data are mean ± SED indicating variance between animals.

5.5 Discussion

The hypothalamic projections from the ARC that release α-MSH and NPY are thought to be downstream from the arcuate receptors activated by circulating leptin. We have previously reported that short term exposure to a HFD results in increased sympathetic activation as well as greater HR and renal sympathetic nerve responses to central leptin (Prior et al., 2010). The major finding of the current study is that a 3 week exposure of rabbits to a HFD results in a greater activation of RSNA and an increase in HR to central α-MSH. Importantly, administration of the MC3/4 receptor antagonist, SHU9119, produces marked sympathoinhibition and a depressor response in the same animals. In addition, ICV NPY produced a hypotensive response in HFD rabbits compared with those on a CD, accompanied by an inhibition of the activation of RSNA (i.e. reduced RSNA levels). Blocking central NPY Y1 receptors with BVD10 had no effect in CD rabbits but increased RSNA in HFD rabbits. Our data suggest the melanocortin/NPY system of the hypothalamus becomes sensitised within 3 weeks of exposure to an obesogenic diet. Thus the greater sensitivity to leptin that we have previously reported is likely to be mediated downstream by the dominance of the sympatho-excitatory actions of melanocortin rather than any diminution in the sympatho-inhibitory effects of NPY signalling. However, these finding do not preclude the possibility that there is an additional contribution of increased leptin signalling.

5.5.1 Central α-MSH regulates specific sympathoexcitatory responses

Da Silva and colleagues (da Silva et al., 2006) have shown that the pressor, tachycardic and anorexic effects of chronic infusion of an MC3/4 agonist are maintained in HFD rats. Furthermore, chronic infusion of an MC3/4 antagonist lowered blood pressure in Zucker obese rats that have a defective leptin receptor but had no effect in normal rats (do Carmo et al., 2012). The same MC3/4 antagonist infused chronically for several days decreased blood pressure to a greater extent in diet-induced obese rats than rats on a normal fat diet and completely abolished the diet-induced hypertension (Dubinion et al., 2010). Our findings are consistent with the above studies above except that we observed a reduction in blood pressure with acute ICV administration of SHU9119. Our findings are therefore unlikely to be influenced by down or up-regulation of receptor signalling which may occur with chronic administration. Further, we measured RSNA in conscious animals reflecting the direct output from the central nervous system independent of any diet induced changes in neuroeffector function (Michaels et al.,

2009). Our observation of α-MSH administration producing a marked facilitation of RSNA and heart rate, parallels the previously reported augmented RSNA and tachycardia responses to ICV leptin in HFD rabbits (Prior et al., 2010). These findings suggest that the α-MSH signalling which is down-stream from the leptin receptor is not only intact but is tonically activated to the extent of maintaining the obesity-induced hypertension. The increased blood pressure is likely due to the pressor effects of leptin and also insulin as we have shown that an acute injection of specific antagonists reverses the hypertension (Lim et al., 2013). We suggest that increased circulating leptin (Eikelis et al., 2007) activates leptin receptors at the level of the ARC nucleus which are then amplified by the α-MSH signalling pathway which acts as a second order pathway to leptin. In support of this we have observed (in the present study) a very similar pattern of activation of specific hypothalamic nuclei by icv leptin (Prior et al., 2010) and α-MSH in CD rabbits. By contrast, HFD-fed rabbits exhibited reduced Fos protein accumulation, likely due to neurons already being chronically activated by the HFD (Xin et al., 2000). Thus, HFD induced activation of sympathetic output to renal beds might have its origins in augmented α-MSH activity. However, we cannot rule out a contribution of increased leptin signalling as well.

5.5.2 Lack of pressor effect to ICV α-MSH

One unexpected result was the lack of pressor response to α-MSH in either dietary group. Hypothalamic α-MSH is a known modulator of sympathetic outflow to skeletal muscle, brown adipose tissue and renal beds as these are increased with the administration of an MC3/4R agonist (Goldsmith et al., 2010; Haynes et al., 1999). Matsumura and colleagues have been able to demonstrate pressor effects of α-MSH and increases in RSNA given ICV to Japanese white rabbits on a normal diet at doses as low as 0.1 nmol (Matsumura et al., 2002). Also α-MSH increases blood pressure in fasted normal and obese female anaesthetised rats (Lu et al., 2000). Thus it is surprising in our study that α-MSH had little effect on blood pressure also given the MC3/4R antagonist SHU9119 markedly reduced both blood pressure and also RSNA. In the present study we also included HS014 which was developed as a MC4 antagonist (Schioth et al., 1998) but has been found to be a partial agonist (Chai et al., 2003). We observed that a 30 nmol dose of HS014 increased blood pressure as well as RSNA but had no effect at lower doses. Presumably the high dose effects are due to its partial agonist activity at MC4 receptors. We have previously shown that ICV leptin causes a pressor effect in 3 week HFD rabbits (Lim et al.,

2013) which we suggest is upstream from α-MSH effect. Taken together we can see little reason for a lack of pressor effect of α-MSH in the present study and in this regard α-MSH stands alone. We are very confident of our finding as none of the 22 rabbits given the drug increased blood pressure by more than 2 mmHg. One possibility is that we have used subpressor low doses of the drug and that α-MSH is particularly potent at increasing RSNA compared to other non-renal beds. We initially determined the threshold dose required to increase RSNA in a pilot study. Perhaps at higher doses α-MSH may well increase blood pressure.

5.5.3 Response to Central NPY

In the current study, rabbits fed a HFD for 3 weeks showed a modest reduction in MAP of approximately 4 mmHg following icv NPY administration representing a 50 % reduction from post-diet values but no change in RSNA. The same doses in CD rabbits had no effect on blood pressure but increased RSNA. Thus a HFD amplifies the hypotensive and sympatho-inhibitory effects of NPY. Further, icv administration of the NPY antagonist BVD10 increased MAP and RSNA in fat-fed rabbits suggesting that tonic NPY activity is increased. Indeed, NPY mRNA is known to increase in the DMH and VMH following high-fat feeding (Guan et al., 1998). Despite the functional relevance of increased NPY expression remaining unclear, it is likely to be pivotal in maintaining a positive energy balance (Chao et al., 2011). Combined, these observations suggest NPY signalling may be increased in our HFD rabbit model and may also have driven the observed weight-gain and increase in WAT observed in HFD rabbits. Given leptin directly inhibits NPY at the ARC (Schwartz et al., 1996a) and NPY activity is increased in the DMH and VMH (Guan et al., 1998), it is likely that in obesity the hyperphagic and cardiovascular effects of NPY are region-specific. POMC neurons are known to express the Y1 receptor, through which they may be inhibited by NPY (Roseberry et al., 2004). Thus, the observed increase in RSNA following administration of BVD10 may reflect decreased inhibition of the POMC system and subsequent increase in RSNA.

The strength of the current study is in our ability to quantify sympathetic output to renal vasculature following central infusion of α-MSH. This parameter is direct output from the central nervous system and is a more accurate measure of hypothalamic function. We conclude that the enhanced responsiveness to central α-MSH observed in HFD-fed rabbits is indicative of a change in the activity of the POMC system. Importantly, this change involves NPY containing neurons

as well and may underlie the hypersensitivity to leptin previously observed in this model. At present we don't know exactly where the sites of action are located. We have used ICV administration which is known to preferentially affect sites close to the ventricle but we do know from previous studies using peptides that the doses given into the cerebrospinal fluid (CSF) are about 5 times higher than those required when injecting into the brain site of action (Saigusa et al., 2003).

5.6 Perspectives

It has been assumed that impaired leptin signaling in the CNS is the cause of selective leptin resistance; a state in which the sympathoexcitatory effect of leptin is maintained despite a loss of its anorectic property. Indeed, we have shown that both inulin and leptinergic pathways are dysregulated early on in high fat feeding. Irrespective of the ligand, ARC cells transduce their information via secondary messenger signalling pathways and constitute a likely mechanism through which obesity related hypertension may occur. Here we demonstrate that consumption of a HFD for a relatively short period of time results in hypersensitivity of both the NPY and α-MSH signalling pathways. These results suggest leptin resistance may be a consequence of the impaired function of second order neurons in the hypothalamus. It is thus reasonable to suggest leptin signalling *per se* may not the primary cause of neurogenic hypertension. If our conclusion is correct, the cellular mechanisms of NPY and α-MSH containing neurons would respond differently to leptin. Indeed, there is evidence to suggest that PI3K activity, a major cellular mechanism of leptin signalling, is augmented by exposure to a HFD. In α-MSH and NPY positive cells, a change in cellular machinery could lead to physiological perturbations causing neurogenic hypertension.

5.7 References

See end of thesis for detailed bibliography.

Chapter VI - The Ventromedial Hypothalamus as the origin of aberrant blood pressure and sympathetic regulation in diet induced obesity.

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception of experimental design; acquisition, analysis and interpretation of data; preparation of manuscript for publication (Hypertension Journal).	**90**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Sandra L. Burke	Surgical assistance with implantation of renal nerve electrode. Intellectual input to manuscript content and contribution to revision of manuscript.
James A. Armitage	Conception of experimental design, collection of data, intellectual input to manuscript content and revision of manuscript.
Geoffrey A. Head	Conception of experimental design, collection, analysis and interpretation of data, intellectual input to manuscript content and revision of manuscript.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date** **02.10.14**
Main Supervisor's Signature		**Date** **02.10.14**

6.1 Abstract

High fat diet (HFD) induced hypertension in rabbits is neurogenic and due to the central action of leptin. This action is dependent on secondary neuronal activation in alpha-melanocortin stimulating hormone (α-MSH) and neuropeptide Y (NPY) positive cells. Neurons in the ventromedial hypothalamus (VMH) are innervated by both neuronal populations and transduce leptin signalling from the ARC to other hypothalamic and hindbrain nuclei. The VMH is also capable of responding to leptin signals directly, independent of NPY or α-MSH neurons. In the present study we assessed the contribution of leptin, α-MSH and NPY neurons in the VMH on development of diet-induced neurogenic hypertension. Male New Zealand White rabbits were instrumented with a VMH cannula and a renal sympathetic nerve electrode. Blood pressure was measured by means of an intra-arterial catheter. Following 3 weeks of a HFD (13.5 % fat, n=10) conscious rabbits had higher renal sympathetic nerve activity (RSNA), blood pressure and heart rate compared with control diet-fed animals (3.5 % fat, n=10). Microinjections of α-MSH into the VMH increased RSNA, heart rate and blood pressure ($P<0.05$) in HFD rabbits. By contrast only heart rate was increased in control rabbits. Microinjections of NPY had a hypotensive effect in controls ($P<0.05$) and a sympathoexcitatory effect in HFD rabbits ($P<0.05$). Microinjections of a leptin receptor antagonist decreased blood pressure, heart rate and RSNA in HFD compared with controls ($P<0.05$). We conclude that the VMH is the likely origin of leptin-mediated sympathoexcitation, α-MSH hypersensitivity and altered central responsiveness to NPY.

Key Words: Obesity, Hypertension, Leptin, NPY, Renal Sympathetic Nerve Activity, α-MSH, Ventromedial Hypothalamus

6.2 Introduction

Obesity is closely associated with a greater risk of developing hypertension with both human and animal studies presenting a strong link between bodyweight gain and greater blood pressure (Dalton et al., 2003; Jones et al., 1994; Prior et al., 2010). Inappropriate increases in sympathetic tone are believed to be the major cause of hypertension (Head *et al.*, 2014). Indeed, noradrenaline spillover rates are greater among the obese whilst muscle sympathetic nerve activity is greater among hypertensive individuals, irrespective of obesity (Lambert *et al.*, 2007b). Importantly, obesity is associated with a greater degree of sympathetic activation, particularly renal sympathetic nerve activity (RSNA), even in the absence of hypertension (Esler, 2000; Grassi et al., 1995). Similar observations can be observed in animal models of obesity where bilateral renal denervation in both the dog and rat models attenuates hypertension (Huang et al., 1998; Kassab et al., 1995). Equally, combined α and β-adrenergic blockade prevents high fat diet (HFD) induced hypertension in conscious rabbits (Antic *et al.*, 2000). A key focus in the field remains the identification of mechanisms underlying sympathetic overdrive in obesity (da Silva *et al.*, 2009; Davy & Orr, 2009).

The adipokine leptin is secreted primarily by white adipose tissue and found in serum in direct proportion to adiposity. Plasma leptin levels correlate strongly with blood pressure and RSNA (Prior *et al.*, 2010) whilst central administration of leptin increases mean arterial pressure (MAP) in both rats and rabbits via stimulation of the sympathetic nervous system (SNS) (Carlyle et al., 2002; Dunbar et al., 1997; Haynes et al., 1997; Matsumura et al., 2000; Shek et al., 1998). Thus leptin is considered a primary link between excess adiposity and increased sympathetic nerve activity (Considine *et al.*, 1996; Lambert *et al.*, 2013; Mark, 2013). The central effects of leptin are mediated primarily through the arcuate (ARC) nucleus of the hypothalamus from which peripheral signals are relayed downstream, to other hypothalamic nuclei, via a network of second order neurons. Alpha-melanocortin stimulating hormone (α-MSH) and neuropeptide Y (NPY) containing neurons in the ARC form the main population of neurons expressing the leptin receptor which propagate leptin signals throughout the hypothalamus (Rahmouni *et al.*, 2005a). Concomitant leptin mediated inhibition of NPY, stimulation of α-MSH neurons and mutual inhibition between the two neuronal populations form

a complex mechanism by which the metabolic, cardiovascular and sympathoexcitatory effects of leptin arise (Cowley *et al.*, 2001; Williams *et al.*, 2001).

Dense projections of both NPY and α-MSH containing neurons ascend from the ARC and terminate in several hypothalamic nuclei including the paraventricular (PVH), dorsomedial (DMH) and ventromedial (VMH) (Cone, 2005; Mercer *et al.*, 2011), key centers of energy homeostasis, hemodynamics and sympathetic tone to brown adipose and renal vasculature (Li et al., 2013a; Marsh et al., 2003; Williams et al., 2001). Moreover, leptin receptor expression in these nuclei is high (Elmquist *et al.*, 1998b) suggesting leptin is capable of bypassing first order neurons and stimulating neuronal populations downstream of the ARC thus exerting its anorectic, cardiovascular and sympathoexcitatory responses directly (Enriori et al., 2011; Marsh et al., 2001). Thus the actions of leptin may occur in several hypothalamic areas. Injections of leptin into the DMH and VMH increases MAP and heart rate yet the VMH was identified as the only nucleus capable of mediating leptin-dependent sympathoexcitation (Marsh *et al.*, 2003). Ablation of the VMH in rats reduces sympathetic tone to several target organs, including cardiac muscle, brown adipose tissue (BAT) and white adipose tissue (Vander Tuig *et al.*, 1982). Moreover, targeted deletion of the leptin receptor in the ARC abolishes leptin-induced STAT3 signalling in the ARC yet STAT3 signalling is still detected in the VMH (Harlan *et al.*, 2011).

It is therefore possible that a change in the activity of either POMC or, NPY neurons or both may underlie the mechanism by which sympathetic output is increased in obesity, leading to hypertension. To test this hypothesis, we determined the effects of bilateral injections of α-MSH, NPY and a leptin receptor antagonist into the VMH on blood pressure, heart rate and RSNA in conscious rabbits fed a HFD for 3 weeks.

6.3 Methods

6.3.1 Animals

Experiments were conducted in 20 conscious male New Zealand White rabbits (2.76-2.90 kg). Rabbits were housed under controlled light (6:00 to 18:00) and temperature (22° C ± 2°C) conditions. Experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals.

6.3.2 Experimental Procedures and Protocol

Under isoflurane anesthesia, rabbits were implanted with a bilateral brain cannula (22 gauge, Plastics One, Roanoke, Virginia, USA) into the VMH (coordinates from bregma: -2.20 mm caudal, ±0.9 mm lateral to midline and at a depth of 15 mm from the skull) and carprofen (3 mg/kg SC, Pfizer, North Ryde, NSW, Australia) was given 24 hours before and after surgery for analgesia. The guide cannula was situated 2 mm above the VMH, thus ensuring the nucleus was intact prior to experimentation. Following 10 days recovery, rabbits were then placed on a NFD (4.2 % total fat) or a HFD (13.3 % total fat) *ad libitum* for 3 weeks (Prior *et al.*, 2010). On day 14 of the diet, a recording electrode was implanted on the left renal nerve, under isoflurane anaesthesia and as previously described (Dorward *et al.*, 1985; Prior *et al.*, 2010).

6.3.3 Main Experiment

Each rabbit received 3 drugs, in random order and on separate days. Following a 1-hour baseline recording period, increasing doses of either α-MSH (0.3, 1 nmol in 400 nL, Tocris, Ellisville, USA), NPY (0.1, 0.5 nmol in 200 nL, Tocris) or leptin receptor antagonist (5, 10 µg in 400 nL, Protein Laboratories Rehoboth Ltd, Rehovot, Israel) were injected, 60 minutes apart using an injector (28 gauge, Plastics One and 2 mm longer than the guide cannula) and were calculated as a change from baseline. A time control study, conducted on a separate day, involved the injection of 2 vehicle (200 nL of Ringer's solution, Baxter, Toongabbie, NSW, Australia) doses.

6.3.4 Data Analysis

MAP and heart rate derived from the arterial pressure pulse were digitized online at 500 Hz and averaged over 2 seconds. To allow for between animal comparisons, RSNA was normalized to maximum RSNA recorded during a nasopharyngeal reflex response, evoked by smoke, and was expressed as a scale of 100 normalized units (Burke & Head, 2003). The blood pressure, heart rate and RSNA responses to each dose were averaged over the peak 30 minutes and were expressed as mean ± SEM or mean difference ± SE of the difference (SED) from baseline. Cannula placements were verified by injection of the methylene blue dye prior to perfusion fixation of the brain and sectioning in a cryostat. Injection sites outside of the VMH were not included in the study. Data were analyzed by split plot repeated-measures ANOVA, which allowed for within- animal and between-animal (group) contrasts and adjusted for multiple testing using the Bonferroni method (Burke *et al.*, 2010). One-way ANOVA was used for data collected at a single time point. Type 1 error was controlled using Bonferoni and the Greenhouse Geisser correction was used to correct for sphericity. A probability of $P < 0.05$ was considered significant.

6.4 Results

6.4.1 Effect of 3 week fat feeding on bodyweight, cardiovascular variables and RSNA

At the end of the 3-week feeding protocol there was no difference in bodyweight between the dietary groups ($P_{diet}>0.05$; Table 6.1). By contrast, blood pressure and heart rate were greater (6 ± 2 %, $P_{diet}= 0.004$, 8 ± 2 %, $P_{diet}=0.02$, respectively; Table 6.1) in rabbits fed a HFD compared with rabbits fed a NFD. Similarly, RSNA, expressed relative to the nasopharyngeal response (normalized units), was 28 ± 10 % ($P_{diet}=0.02$) greater in fat-fed rabbits compared with controls and was due to a 19 ± 7 ($P_{diet}=0.04$) greater burst amplitude in rabbits fed a HFD compared with NFD rabbits (Table 6.1). There was no difference in RSNA frequency between the dietary groups, even when adjusted to heart rate ($P_{diet}>0.05$; Table 6.1). Moreover, there was no difference in the nasopharyngeal response between dietary groups ($P_{diet}>0.05$; Table 6.1).

6.4.2 Effect of α-MSH

Administration of α-MSH into the VMH resulted in a marked increase in RSNA (39 ± 13 % for 1 nmol, $P<0.001$; Figure 6.1) in rabbits fed a HFD, compared with NFD-fed rabbits ($P>0.05$; Figure 6.1). Similarly, an increase in blood pressure (6 ± 3 % for 1 nmol, $P<0.01$; Figure 1) was observed in the HFD group but not in the NFD group ($P>0.05$; Figure 6.1) following treatment with α-MSH. By contrast, an increase in heart rate, of similar magnitude, occurred in both NFD (21 ± 3 % for 1 nmol, $P<0.001$; Figure 6.1) and HFD-fed rabbits (15 ± 4 % for 1 nmol, $P<0.001$; Figure 6.1).

6.4.3 Effect of NPY

Microinjections of NPY into the VMH resulted in a depressor response in NFD-fed rabbits (-6 ± 3 % for 0.5 nmol, $P=0.01$; Figure 6.2) but had no effect on HFD-fed rabbits ($P>0.05$, Figure 6.2). Rabbits fed a HFD exhibited a small increase in RSNA but only in response to the second dose of NPY (15 ± 5 % to 0.5 nmol, P = 0.03; Figure 6.2) whilst no change in RSNA was observed in NFD rabbits ($P>0.05$; Figure 6.2). There was no effect on heart rate in either dietary group following injections of NPY into the VMH (P>0.05; Figure 6.2).

6.4.4 Effect of leptin receptor antagonist

Leptin antagonist administration into the VMH resulted in a decrease in blood pressure in animals fed a HFD (-8 ± 2 % for 10 µg, $P<0.001$; Figure 6.3). Similarly, increasing doses of the leptin receptor antagonist resulted in mild bradycardia in the HFD group (-6 ± 3 % for 10 µg, $P < 0.001$; Figure 6.3). In the same group, a small degree of sympathoinhibition was observed in response to leptin receptor antagonism (-11 ± 9 %, $P=0.05$ for 10 µg; Figure 6.3). Injection of vehicle had no effect on MAP or RSNA in either dietary group ($P>0.05$, Figure 6.5). However, a small decrease in HR was observed in NFD rabbits following vehicle injections ($P>0.05$, Figure 6.5).

6.4.5 Distribution of injections sites

The VMH was successfully targeted in 15 of 20 animals (Figure 6.4). In 4 animals (2 HFD, 2 NFD) injections were performed in the DMH. In a third NFD animal, injections occurred in the ARC. All successful injections were within 1 mm of the target nucleus. Only VMH located injection sites were included in the analysis.

Table 6.1: Hemodynamics and sympathetic nerve activity from NFD and HFD-fed rabbits after 3 weeks of diet.

Measurement	**NFD**	**HFD**	P_{diet}
	n= 10	n= 10	
Bodyweight (Kg)	3.17 ± 0.07	3.13 ± 0.03	1.0
Hemodynamics and RSNA			
MAP (mmHg)	71.1 ± 0.9	75.9 ± 1.2	***0.004***
HR (b/min)	189 ± 4	205 ± 5	***0.02***
RSNA (µV)	33.2 ± 4.1	41.1 ± 3.8	0.2
RSNA (nu)	8.0 ± 0.6	11.1 ± 1.1	***0.02***
RSNA Amplitude (nu)	24.6 ± 1.6	30.2 ± 2.1	**0.04**
RSNA Frequency (bursts per second)	5.8 ± 0.3	5.9 ± 0.3	0.8
RSNA Frequency (bursts per heart beat)	1.7 ± 0.08	1.7 ± 0.1	0.9
Nasopharyngeal Response (µV)	464.5 ± 43.9	394.8 ± 24.6	0.2

Values are mean ± SEM. P is the probability for the comparison between groups.

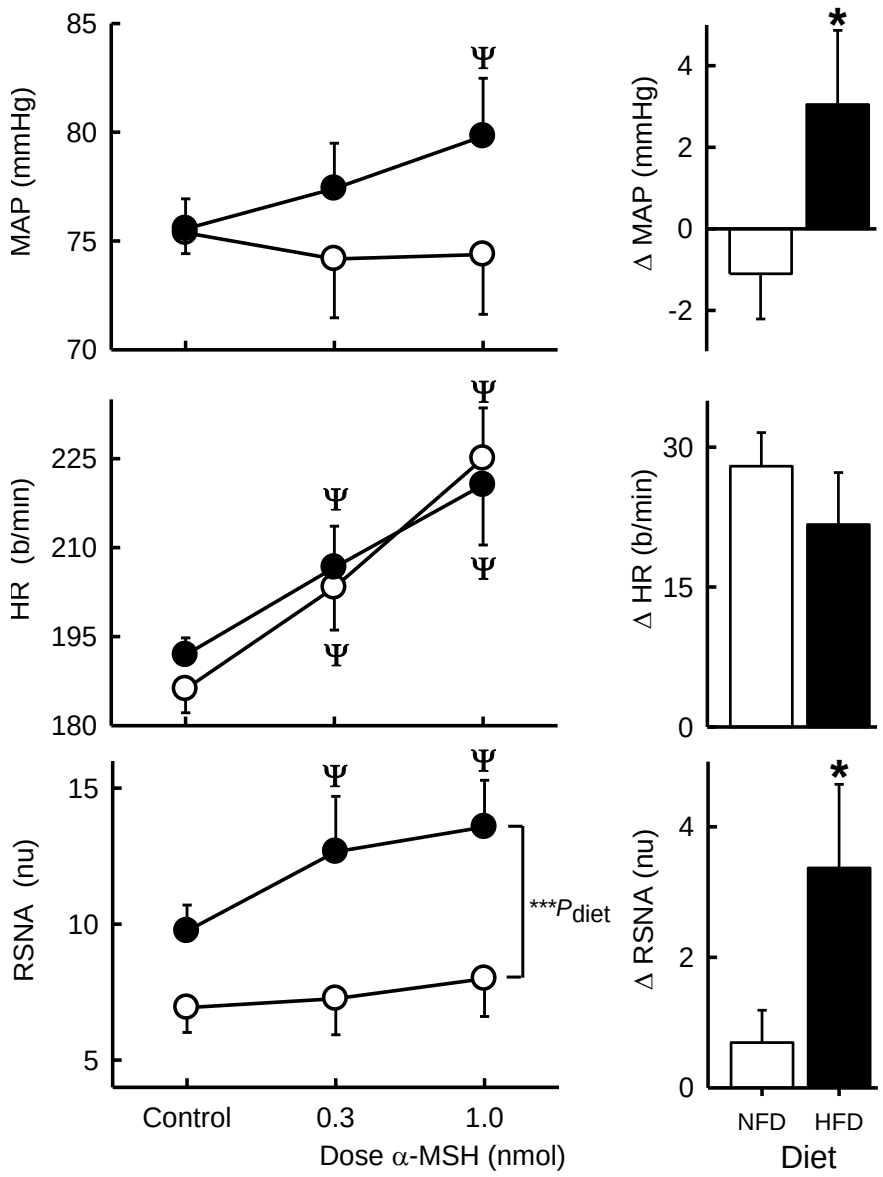

Figure 6.1: Left: 30-minute averages of MAP, HR and RSNA in response to increasing doses of intra-VMH injections of α-MSH in rabbits fed a control diet (NFD; open bars; n=11) or a high fat diet (HFD; filled bars; n=11) for 3 weeks. Data are mean ± SEM indicating variance between animals. $^{\Psi}P<0.05$ for effect of individual doses compared with control. **Right**: Average changes from control in MAP, HR and RSNA. Data are mean ± SED indicating variance between animals. $^{*}P<0.05$ for effect of diet. Abbreviations: MAP, mean arterial pressure, HR, heart rate, RSNA, renal sympathetic nerve activity (normalized units).

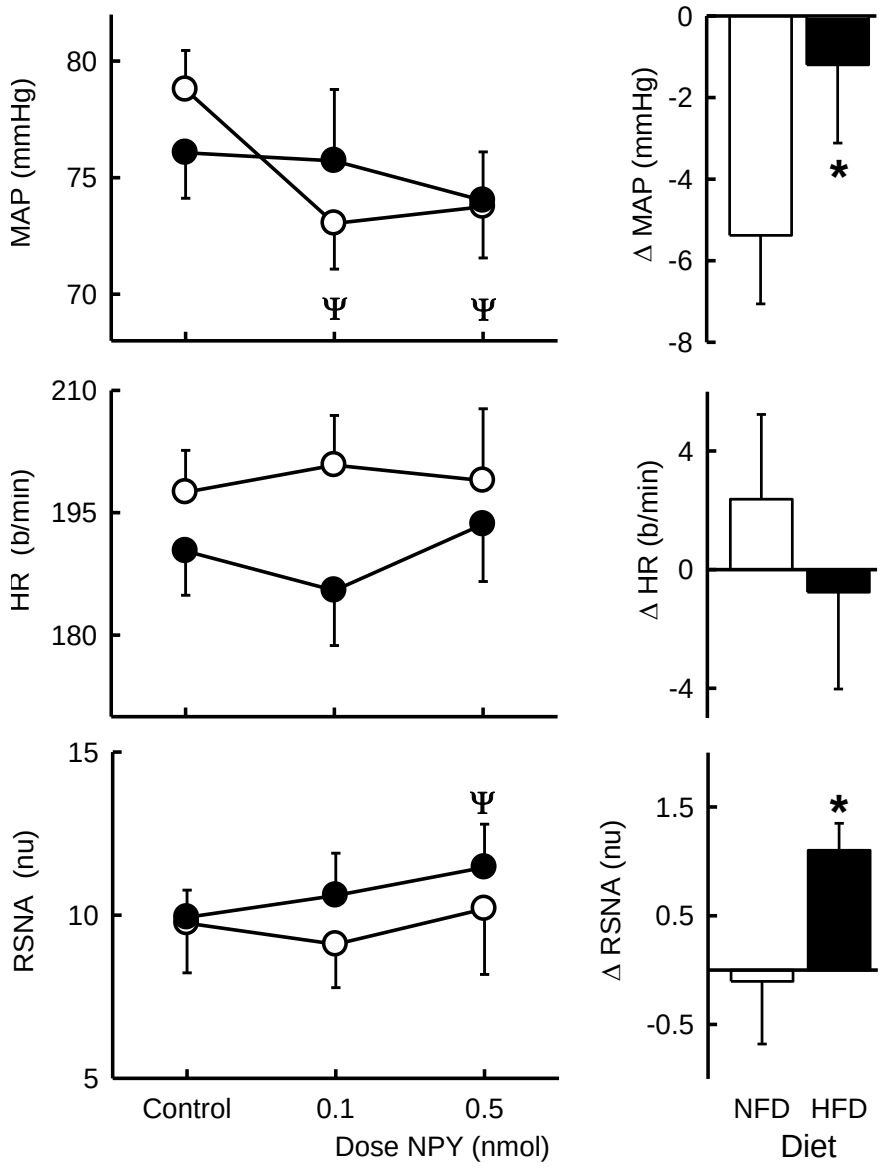

Figure 6.2: Left: 30-minute averages of MAP, HR and RSNA in response to increasing doses of intra-VMH injections of NPY in rabbits fed a control diet (NFD; open bars; n=11) or a high fat diet (HFD; filled bars; n=11) for 3 weeks. Data are mean ± SEM indicating variance between animals. $^{\Psi}P<0.05$ for effect of individual doses compared with control. **Right**: Average changes from control in MAP, HR and RSNA. Data are mean ± SED indicating variance between animals. $^{*}P<0.05$ for effect of diet. Abbreviations: MAP, mean arterial pressure, HR, heart rate, RSNA, renal sympathetic nerve activity (normalized units).

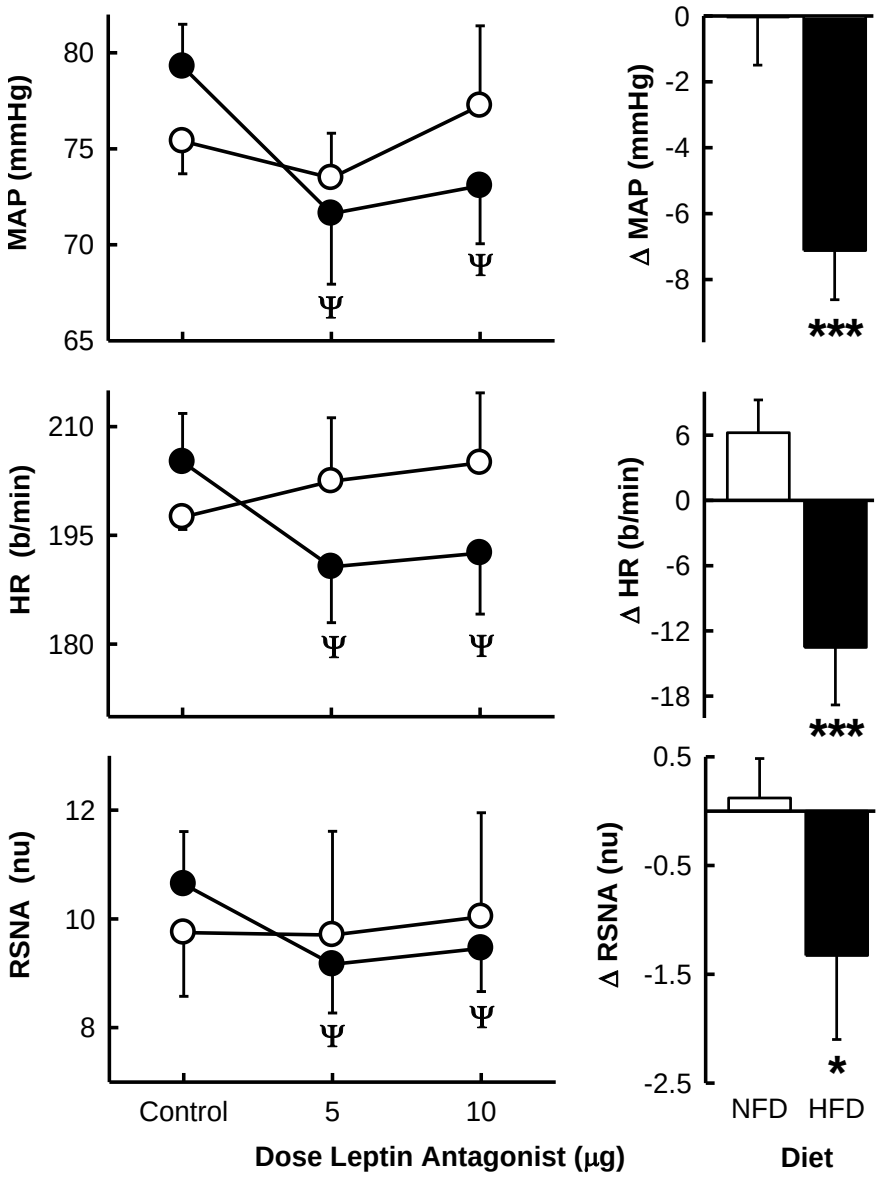

Figure 6.3: Left: 30-minute averages of MAP, HR and RSNA in response to increasing doses of intra-VMH injections of a leptin receptor antagonist in rabbits fed a control diet (NFD; open bars; n=11) or a high fat diet (HFD; filled bars; n=11) for 3 weeks. Data are mean ± SEM indicating variance between animals. $^{\Psi}P<0.05$ for effect of individual doses compared with control. **Right**: Average changes from control in MAP, HR and RSNA. Data are mean ± SED indicating variance between animals. $^{*}P<0.05$ for effect of diet. Abbreviations: MAP, mean arterial pressure, HR, heart rate, RSNA, renal sympathetic nerve activity (normalized units).

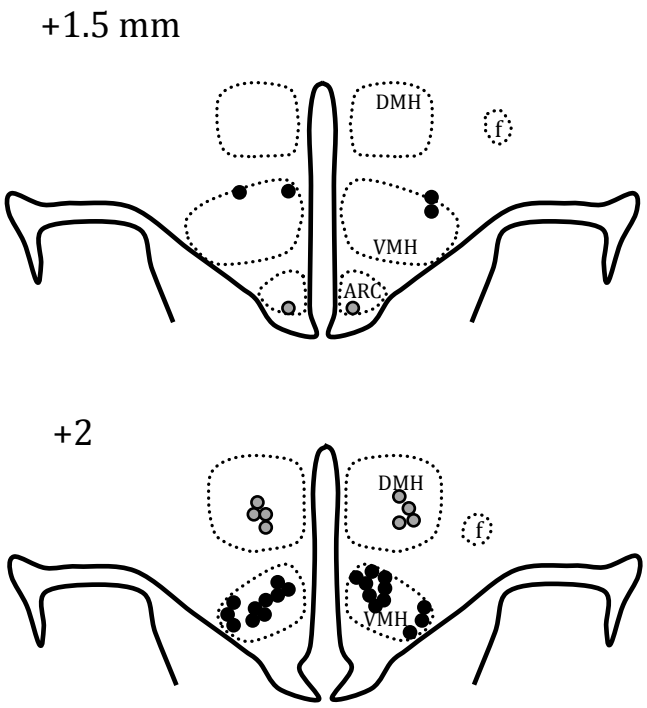

Figure 6.4:

An illustration of 2 coronal sections of the hypothalamus, at 1.5 and 2 mm rostral/caudal to Bregma. The location of injection sites within the VMH is indicated by black circles, injections outside the VMH indicated by gray circles.

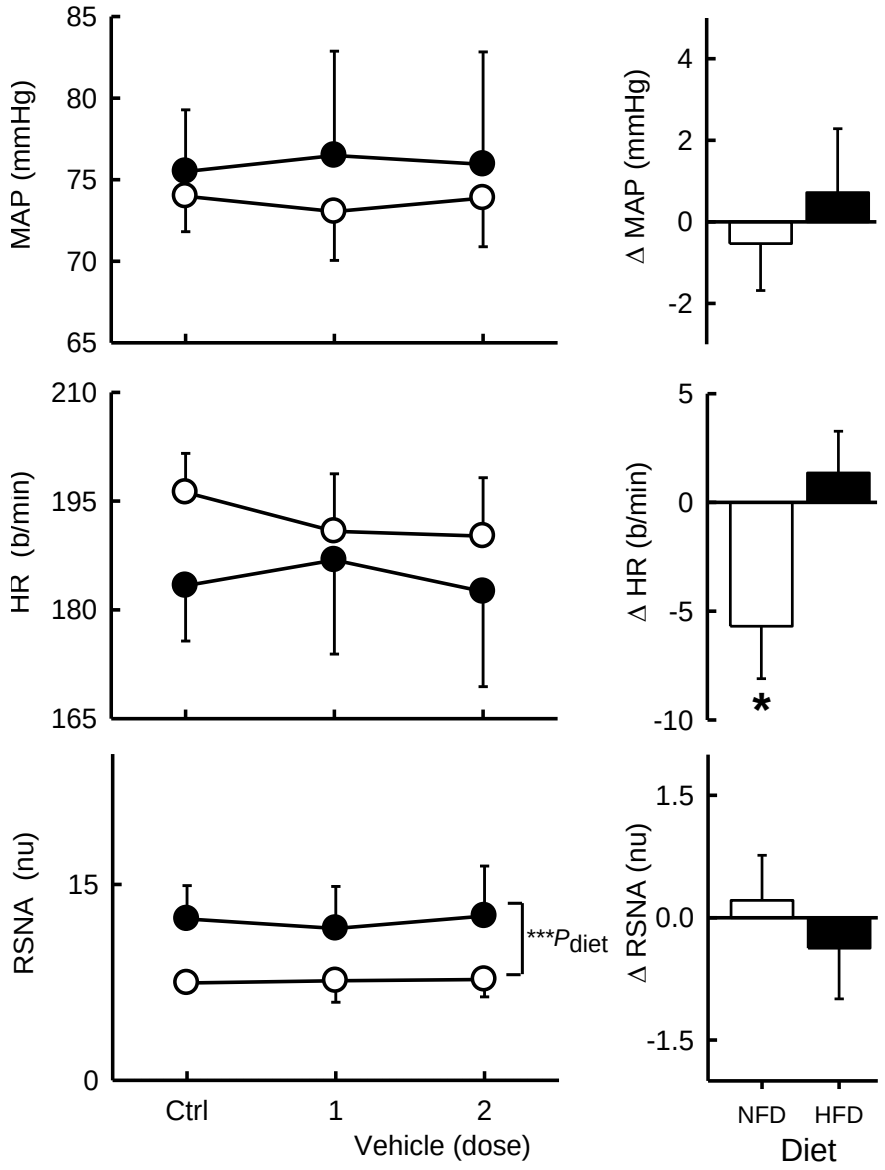

Figure 6.5: Left: 30-minute averages of MAP, HR and RSNA in response to increasing doses of intra-VMH vehicle injections in rabbits fed a control diet (NFD; open bars; n=11) or a high fat diet (HFD; filled bars; n=11) for 3 weeks. Data are mean ± SEM indicating variance between animals. $^{\Psi}P<0.05$ for effect of individual doses compared with control. **Right**: Average changes from control in MAP, HR and RSNA. Data are mean ± SED indicating variance between animals. $^{*}P<0.05$ for effect of diet, $^{***}P_{diet}$ <0.001 for effect of diet. Abbreviations: MAP, mean arterial pressure, HR, heart rate, RSNA, renal sympathetic nerve activity (normalized units).

6.5 Discussion

The main finding of the current study was that injection of the leptin receptor antagonist into the VMH normalised diet induced hypertension observed in HFD-fed rabbits suggesting a considerable proportion of the sympathoexcitatory and hypertensive effects of leptin may originate from the VMH. In addition, fat-fed rabbits exhibited a greater degree of sensitivity to intra-VMH injections of exogenous α-MSH and a concomitant desensitization to the hypotensive effects of NPY. The haemodynamic and sympathoexcitatory effects of α-MSH closely resemble those of leptin (Chapter 5) and appear to converge at the level of the VMH. We therefore propose that in obesity, increased sympathetic tone and blood pressure are the result of altered sensitization to leptin, α-MSH and NPY at the level of the VMH.

Central administration of leptin is known to evoke pressor and sympathoexcitatory responses (Lim et al., 2013; Prior et al., 2010; Rahmouni et al., 2003a; Rahmouni & Morgan, 2007) but the nuclei involved in these responses remain unclear. Importantly, leptin is capable of activating VMH neurons independently of the ARC nucleus (Elmquist *et al.*, 1997), the only hypothalamic site through which leptin-dependent renal sympathoexcitation can be achieved directly (Marsh *et al.*, 2003). We have previously demonstrated an exaggerated RSNA response to exogenous leptin in animals fed a HFD (Prior *et al.*, 2010) whilst in the present study administration of the leptin receptor antagonist potently attenuated MAP and RSNA. Interestingly, the sympathoexcitatory effect of leptin is observed at week 3 of a HFD despite slow increases in circulating levels occurring earlier (Antic *et al.*, 2000; Lim *et al.*, 2012) and is comparable to that observed following administration of α-MSH (Chapter 5). Thus, consumption of a HFD may produce hypersensitivity to α-MSH which in turn affects the central response to leptin. Moreover, since signs of hypersensitivity are observed in the current study it is likely that both leptin and α-MSH act via the same neuronal population of the VMH, resulting in marked sympathoexcitation.

In support of our view, Montanaro and colleagues reported microinjections of leptin into the ARC and VMH, but not PVH, increased lumbar sympathetic tone (Montanaro *et al.*, 2005). Notably, the sympathoexcitatory effect of leptin in the VMH was double that observed in the ARC further suggesting that the symapthoexcitatory actions of leptin are mediated chiefly via the VMH.(Montanaro *et al.*, 2005). Rahmouni and colleagues reported that intracerebroventricular

(ICV) and intra-ARC injections in anesthetized rats comparably increased MAP, HR and RSNA, concluding that the ARC is a major site of leptin-mediated cardiovascular and sympathetic control (Rahmouni & Morgan, 2007). This is not surprising given the ARC acts as a key receiver of peripheral leptin signals which are then relayed to other hypothalamic nuclei (Elmquist *et al.*, 1999). In addition, the observations from the Rahmouni study occurred three hours post injection whilst observations reported in the current manuscript occurred three times faster, suggesting the ARC may not be the site of direct modulation of RSNA and the effects observed may be mediated via other nuclei. The findings of Rahmouni are consistent with the contribution of second order neurons in propagating the cardiovascular and sympathoexcitatory actions of leptin as suggested by Cowley (2001). Indeed discrete injections of leptin into the VMH augment POMC expression in the same nucleus (Ambati *et al.*, 2009). We have previously reported that rabbits fed a HFD exhibit increased sensitivity to exogenous α-MSH which manifests as a marked increase in HR and RSNA (Chapter 5). In the current study, bilateral injections of α-MSH into the VMH increased MAP and RSNA in fat-fed rabbits compared with controls. Indeed, the sympathoexcitatory response following intra-VMH injections of α-MSH was three times greater than the response observed following ICV injections of α-MSH at the same dose whilst the pressor response following intra-VMH injections of α-MSH was comparable to that following ICV leptin (Prior *et al.*, 2010). These observations suggest the VMH may play a dual role as the site of α-MSH hypersensitivity and amplification of leptinergic signalling.

We have previously reported that ICV administration of NPY increases RSNA in control animals and decreases MAP in HFD-rabbits (Chapter 5). One possible explanation is that NPY signals are not sufficient enough to counteract an amplified α-MSH system, resulting in greater sympathoexcitation in fat-fed rabbits. We also suggested the presence of distinct NPY pathways controlling MAP and RSNA. Data from the present study confirm the latter, as injections of NPY into the VMH resulted in a depressor response in control but not HFD rabbits. The same doses of NPY produced a small degree of sympathoexcitation in fat-fed rabbits but not in controls. These findings imply high fat feeding may induce pathway-specific changes in the VMH.

Whilst the exact contribution of NPY neurons in the VMH to blood pressure regulation is not well characterised, our findings suggest NPY exerts a hypotensive effect in the VMH.

Importantly, this effect is constrained by consumption of a HFD. Indeed, central administration of NPY has been demonstrated to reduce blood pressure and sympathetic nerve activity (Fuxe *et al.*, 1983; Grundemar *et al.*, 1991; Shi *et al.*, 2013). In the obese Zucker rat, the presynaptic effects of NPY are attenuated compared with lean littermates (Pronchuk & Colmers, 2004). In addition, obese Zucker rats also exhibit down-regulation of both the Y1 and Y5 receptors which appears to be site-specific down-regulation of Y receptors (Beck et al., 2001; Widdowson, 1997). These findings present molecular mechanisms by which NPY desensitization may occur in obesity.

Central administration of NPY in anesthetized rats has been shown to inhibit sympathetic nerve activity to BAT (Egawa *et al.*, 1990; Egawa *et al.*, 1991) whilst direct activation of ARC NPY neurons produces sympathoinhibition to splanchnic beds (Shil & Brooks, 2014). By contrast, data from the present study shows microinjections of NPY into the VMH caused a small increase in RSNA in HFD-fed rabbits compared with controls. This suggests that the function of NPY neurons is changed by consumption of a HFD and that aberrant signalling via this population of neurons may comprise an additional means by which RSNA increases in obesity. Recently, Dhillon reported leptin's ability to differentially regulate intracellular pathways in two distinct NPY containing neurons (Dhillon & Belsham, 2011). Such observations present a precedent in which seemingly homogenous hypothalamic neurons respond differently to key neuropeptides. Thus the unexplained increase in RSNA following microinjections of NPY into the VMH may reflect a change in behaviour of a discrete population of NPY neurons.

6.6 Summary and Conclusion

Our study shows that the VMH plays a considerable part in leptin-mediated renal sympathoexcitatory pathways. This is evidenced by the response to injections of a leptin receptor antagonist, which returned blood pressure, heart rate and RSNA, in HFD rabbits to pre-diet values. Furthermore, the hypersensitivity to VMH injections of α-MSH manifest as increased blood pressure and RSNA and closely resemble the haemodynamic and RSNA responses to leptin. Thus, both leptin and α-MSH likely engage the same pressor and sympathoexcitatory pathways in the VMH resulting in obesity-related hypertension.

6.7 References

See end of thesis for detailed bibliography.

Chapter VII – General Discussion

There is an inherent difficulty in studying the mechanisms of obesity related hypertension as obesogenic diets are characterised by multiple factors such as macronutrients and caloric value. These factors are further compounded by different rates and durations of exposure and subsequent influence on meal-related patterns. The findings presented in this thesis define more clearly the dietary stimuli which initiate hypertension through specific signals that over time amplify sympathetic activity to the kidney. The results presented in Chapter 3 reveal the importance of the initial contribution from insulin to a HFD induced hypertension and the subsequent transition to the predominance of leptin signalling. The work described in Chapter 4 indicates the importance of the source of calories for altering meal-associated haemodynamic patterns. Finally, the findings described in Chapters 5 and 6 pinpoint the CNS mechanisms involved which has led to a novel concept in the understanding of genesis of obesity related hypertension.

7.1 Rapid Increases in Cardiovascular Variables in HFD Rabbits are Dependent on Dietary Fat and Plasma Insulin

Consumption of a HFD by NZW rabbits for as little as three weeks produces increased visceral adiposity, hypertension, tachycardia and increased RSNA (Armitage et al., 2012; Burke et al., 2013; Lim et al., 2013; Prior et al., 2010). Importantly, the cardiovascular effects occur within the first few days of a HFD before there has been sufficient time for any marked increase in adiposity (Armitage *et al.*, 2012; Burke *et al.*, 2013). Thus the early cardiovascular changes are unlikely to be due to hyperleptinemia as circulating leptin levels are proportional to the level of adiposity in the body and take time to accumulate (Considine *et al.*, 1996; Eikelis & Esler, 2005). Indeed the rapid increases in MAP and HR that precede any bodyweight gain appear to coincide with greater caloric intake and higher circulating insulin levels as described in Chapter 3. Moreover, central administration of an insulin receptor antagonist at week 1 of a HFD reduced MAP whilst central administration of the leptin receptor antagonist at the same time point had no effect (Lim *et al.*, 2013). By week 3 of the HFD, central administration of a leptin receptor antagonist decreased MAP coinciding with high circulating leptin levels whilst the haemodynamic response to the insulin receptor antagonist was maintained. Importantly, sympathoinhibition is only observed following injection of the leptin

receptor antagonist. Thus the pressor effects of leptin and insulin are mechanistically and temporally distinct from one another. The observation that central administration of insulin increases MAP in several animal species (Lim et al., 2013; Nakata et al., 1998), further highlights insulin is involved in the early development of obesity related hypertension.

The absence of dyslipidaemia in HFD rabbits as described in Chapter 3 has been useful from the point of view of removing a potential confounding variable from the studies. However, there is a well-known association between dyslipidaemia, cardiovascular disease (Siri-Tarino et al., 2010) and impairment of endothelial-mediated vasodilation (Doi *et al.*, 1998; Lundman *et al.*, 2001) both of which actively contribute to hypertension. The lack of a detectable increase in any plasma lipids in the rabbit stands in contrast with human obesity in which only 30 % of obese individuals are metabolically normal (Fabbrini *et al.*, 2010). However, longer exposure does result in dyslipidaemia in the rabbit (Carroll & Kyser, 2002; Eppel *et al.*, 2013). Clearly in studies characterising human obesity it may be possible to dissociate the dyslipidaemia from other factors if the length of exposure to an obesogenic diet is taken into consideration and new onset obesity is considered. The importance of this distinction is that dyslipidaemia may well contribute to the overall state of disease in obesity but is unlikely to be involved in the early development of hypertension.

Underlying the rapid elevation in MAP and HR observed within the first week of the HFD is a marked change to the daily rhythm of these variables. Cardiovascular rhythms in meal-fed rabbit are known to be influenced by food intake and are typically characterised by pre-meal troughs and post-meal peaks (Lim et al., 2012; Van den Buuse & Malpas). Studies in Chapter 4 clearly show increased caloric consumption from fat abolishes pre-meal ‘dipping’ such that both MAP and HR remain elevated throughout the day. Similar non-dipping status is also observed in humans and is thought to contribute to the general state of hypertension and disease (Pickering & Kario, 2001). Importantly, the association between ‘non-dipping’ status and enhanced cardiovascular risk among hypertensives remains strong irrespective of treatment (de la Sierra *et al.*, 2009) suggesting the ramifications of a shift in circadian patterns are considerable. Cardiovascular variables are not the only ones affected by

increased dietary fat with reduced nocturnal locomotor activity observed in rabbits fed a HFD (Burke *et al.*, 2013). Interestingly, the same reduced activity was observed in rabbits given free access to a NFD but there was no change in MAP or HR. Thus hyperphagia itself promotes a state of positive energy balance which may indirectly affect cardiovascular health through reduced activity rather than a direct effect on MAP (Chapter 4). Combined, the meal-associated differences in NFD and HFD fed rabbits suggest that the higher fat content rather than increased calories from another source is critical for the development of hypertension. At this stage, it is not clear whether an isocaloric diet high in fat would result in hypertension.

7.2 Hypothalamic Mechanism of Obesity Related Hypertension.

Evidence that hypertension in the 3-week HFD rabbit model is neurogenic stems from reductions in arterial pressure following ganglionic blockade (Armitage *et al.*, 2012). Similar effects have also been observed in other animal models (Figure 1.4). Increased sympathetic tone in obesity is a likely mechanism through which hypertension develops, although the precise mechanism by which sympathoexcitation develops remains controversial (Head *et al.*, 2014). Leptin appears to mediate much of the sympathoexcitation observed in HFD rabbits as increased circulating leptin levels strongly correlate with MAP and RSNA (Prior *et al.*, 2010) whilst central administration of a leptin receptor antagonist attenuates the hypertension at week three. Importantly, the relationship between leptin and hypertension is well established by week three of the diet suggesting leptin is a key mechanism by which obesity related hypertension develops. Consequently a large portion of the work presented in the present thesis examines the role of hypothalamic neuropeptides downstream from leptin signals in the genesis of obesity related hypertension. More specifically, in light of the fact leptin signals at the ARC are relayed by second order α-MSH and NPY containing neurons, it is likely hypertension arises from a functional change in both neuronal populations.

The primary finding outlined in Chapter 5 was that HFD-fed rabbits developed marked hypersensitivity to central α-MSH as evident by the large increases in RSNA. It is worth noting that the effect of α-MSH on HFD rabbits was comparable to that of leptin (Prior *et al.*, 2010) suggesting the amplification of leptin signals and subsequent sympathoexcitation were α-MSH-dependent. Whilst the

concept that central melanocortin pathways are major contributors to increased sympathetic output in obesity is not new (da Silva *et al.*, 2014), the current thesis demonstrates the rapid engagement of the melanocortin system following small perturbations to bodyweight. The additional finding that centrally applied exogenous NPY reduced MAP in fat-fed rabbits suggests NPY-mediated tonic inhibition of α-MSH neurons fails to occur, providing a mechanism by which over-activation of α-MSH may be achieved.

7.2.1 Localisation of α-MSH Hypersensitivity in the Hypothalamus

Central administration of neuropeptides is an effective way to assess physiological effects in conscious animals yet it does not reveal the nuclei through which the signal is transduced. In light of the marked RSNA response in HFD-rabbits following α-MSH administration, c-Fos immunohistochemistry was employed to determine the hypothalamic nuclei activated by α-MSH. The pattern of neuronal activation in fat-fed rabbits was similar across all major hypothalamic nuclei with around 80% less active neurons in the DMH, PVH, VMH, and ARC, indicative of chronic activation of those neurons (Chapter 5). Again, the effect of α-MSH on the pattern hypothalamic neuronal activation was similar to that of leptin with a general attenuation in the number of c-Fos positive cells in HFD rabbits (Prior *et al.*, 2010). One exception is the similar pattern of activation in the VMH following ICV leptin (Prior *et al.*, 2010) whilst in the present thesis, HFD rabbits had less neuronal activation in the VMH following ICV α-MSH compared with control rabbits (Chapter 5). Bearing in mind the dense α-MSH and NPY projections which arise from the ARC and terminate in the VMH (Mercer *et al.*, 2011) as well as the excitatory input from the VMH which potentiates ARC POMC neurons directly (Sternson *et al.*, 2005), the VMH is the likely source of α-MSH hypersensitivity. Perhaps most convincingly, the VMH is the only hypothalamic nucleus found to enable leptin-mediated renal sympathoexcitation (Marsh *et al.*, 2003).

7.2.2 The Contribution of VMH Neurons to Obesity Related Hypertension

The finding that targeted microinjections of α-MSH into the VMH produced near identical increases in RSNA in fat-fed rabbits as compared with ICV doses (Chapters 5 and 6) was further evidence that the VMH was the likely site of α-MSH

hypersensitivity and a major contributor to augmented sympathetic tone in HFD animals. Moreover, the small increase in MAP, representative of about 60 % of the difference between the dietary groups at week 3, suggested α-MSH acts directly via the VMH to regulate blood pressure though this function likely involves other hypothalamic nuclei. Importantly, The VMH is a major site of leptin receptor expression, suggesting leptin can bypass the ARC nucleus and regulate feeding and cardiovascular homeostasis directly via the VMH (Elmquist et al., 1998a; Elmquist et al., 1997). In the present thesis, microinjections of the leptin receptor antagonist into the VMH produced marked reductions in MAP, HR and RSNA in HFD rabbits yet had little effect on recorded variables in NFD rabbits. Thus blockade of leptin receptors in the VMH essentially reversed the cardiovascular and sympathoexcitatory effects of high-fat feeding. Taken together, these findings suggest greater sympathoexcitation observed in fat-fed rabbits is produced by amplification of both leptin and α-MSH signalling at the VMH.

One limitation of the current thesis is that the cellular mechanism underlying the amplification in the VMH remains uncharacterised. Although it is likely both MC3/4R are involved, the degree of contribution of each receptor subtype in the VMH can only be examined following use of selective receptor antagonists. Moreover, data from the current thesis does not shed light on which neuronal populations found in the VMH amplify leptin and α-MSH signalling to pre-ganglionic centres. Intra-VMH administration of α-MSH following treatment of the nucleus with a leptin receptor antagonist could confirm whether the two neuropeptides act on the same neuronal population. Another aspect which could be expanded upon is the temporal characterisation of VMH-mediated amplification. Repeating the same experiments on days 3, 7 and 14 of a HFD should pinpoint when VMH neurons become sensitive to exogenous leptin and α-MSH. The time point at which VMH neurons become hypersensitive is interesting given the changes in the hormonal milieu during the course of the HFD (Chapter 3) and would reveal the specific stimulus responsible for functional changes of VMH neurons. Lastly, the contribution of other hypothalamic nuclei, specifically the ARC, should be examined. Lesioning of the ARC would shed light on whether amplification of α-MSH signalling involves projections of POMC and NPY neurons from the ARC or whether circulating leptin level act directly at the VMH. ICV injections of leptin produced

similar counts of c-Fos in the VMH (Prior *et al.*, 2010) to those observed following ICV α-MSH (Chapter 6). One explanation is that greater circulating leptin in HFD rabbits already activates VMH neurons, subsequently masking the response to ICV α-MSH. FRA immunohistochemistry should reveal whether or not VMH neurons are chronically activated in HFD-fed rabbits at 3 weeks.

7.2.3 The Contribution of VMH NPY Neurons to Increased Sympathetic Tone

Based on the observation that microinjections of NPY attenuated MAP in NFD but not HFD rabbits, withdrawal of NPY inhibition appears to occur in fat feeding. This appears to be confirmed by the finding that the efficiency of presynaptic NPY is reduced in obese Zucker rats (Pronchuk & Colmers, 2004) concomitant with a down-regulation of Y1R and Y5R (Beck et al., 2001; Widdowson, 1997). A depressor response would seemingly go hand in hand with the suggestion that NPY exerts a sympathoinhibitory effect in the hypothalamus (Egawa *et al.*, 1990; Egawa *et al.*, 1991; Shil & Brooks, 2014) yet interestingly, microinjections of NPY into the VMH produced small increases in RSNA in HFD rabbits. It appears NPY sub populations in the VMH may exert different functions. Discrete subpopulations of NPY neurons have been described previously (Horvath *et al.*, 1997; Marston *et al.*, 2011) yet crucially subpopulations of NPY have been recently shown to respond differently to leptin (Dhillon & Belsham, 2011). Thus, in HFD rabbits both the lack of depressor response and the observed sympathoexcitation following intra-VMH injections of NPY could be explained by a functional change in a specific subpopulations of NPY neurons.

7.3 A New Concept in Obesity Related Neurogenic Hypertension

Much of the current understanding of hypothalamic function in obesity is based on appetite neurocircuitry whereby POMC and NPY containing neurons exert opposing effects to maintain energy homeostasis (Fehm *et al.*, 2006). The findings outlined in this thesis, particularly in Chapters 5 and 6, suggest a re-evaluation of the manner in which these systems contribute to the development of obesity related hypertension is required. Accumulating evidence on the heterogeneity of these neuronal populations (Dhillon & Belsham, 2011; Hu et al., 2014; Lee et al., 2013b;

Sohn et al., 2011) implies cardiovascular perturbations in obesity are unlikely to result from global changes in the function of these neurons. Instead, discrete nuclei are more likely to change in response to peripheral signal as has been observed in the VMH (Chapter 6). The findings presented in this thesis offer several mechanisms by which consumption of a HFD may elevate blood pressure in a relatively short period of time. Importantly, all of the mechanisms engage the hypothalamus as both leptin and insulin are known to signal at the ARC. The involvement of the hypothalamus in the development of obesity related hypertension has been heavily linked to selective leptin resistance although a precise mechanism has yet to be clearly defined. The major findings of this thesis lend themselves to a new mechanism of selective leptin resistance which centres on the functional changes of NPY and α-MSH containing neurons. The most prominent of these changes is the amplification of leptin and α-MSH signals at the VMH, resulting in a pressor response and a marked sympathoexcitation to the renal vasculature. A better characterisation of the neurons involved as well as the instigating stimulus, be it hormonal or dietary, remain to be established.

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Appendices

Appendix I – Published Version of Chapter III

Barzel B, Weir JM, Meikle PJ, Burke SL, Armitage JA, Head GA. (2014). Short term fat feeding rapidly increases plasma insulin but does not result in dyslipidaemia. *Front Physiol*. 5, 1-8.

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Short term fat feeding rapidly increases plasma insulin but does not result in dyslipidaemia

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Although the association between obesity and hypertension is well-known, the underlying mechanism remains elusive. Previously, we have shown that 3 week fat feeding in rabbits produces greater visceral adiposity, hypertension, tachycardia and elevated renal sympathetic nerve activity (RSNA) compared to rabbits on a normal diet. Because hyperinsulinaemia, hyperleptinemia, and dyslipidaemia are independent cardiovascular risk factors associated with hypertension we compared plasma insulin, leptin, and lipid profiles in male New Zealand White rabbits fed a normal fat diet (NFD 4.3% fat, $n = 11$) or high fat diet (HFD 13.4% fat, $n = 13$) at days 1, 2, 3 and weeks 1, 2, 3 of the diet. Plasma concentrations of diacylglyceride (DG), triacylglyceride (TG), ceramide and cholesteryl esters (CE) were obtained after analysis by liquid chromatography mass spectrometry. Plasma insulin and glucose increased within the first 3 days of the diet in HFD rabbits ($P < 0.05$) and remained elevated at week 1 ($P < 0.05$). Blood pressure and heart rate (HR) followed a similar pattern. By contrast, in both groups, plasma leptin levels remained unchanged during the first few days ($P > 0.05$), increasing by week 3 in fat fed animals alone ($P < 0.05$). Concentrations of total DG, TG, CE, and Ceramide at week 3 did not differ between groups ($P > 0.05$). Our data show plasma insulin increases rapidly following consumption of a HFD and suggests that it may play a role in the rapid rise of blood pressure. Dyslipidaemia does not appear to contribute to the hypertension in this animal model.

Keywords: insulin, leptin, plasma lipids, obesity, hypertension

INTRODUCTION

Obesity is associated with increased mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA). Accumulating evidence suggests these changes are due to greater circulating concentrations of the adipokine leptin (Burke et al., 2013; Lim et al., 2013) which strongly correlate with RSNA and MAP in animal models of obesity (Prior et al., 2010; Burke et al., 2013). Consumption of a high fat diet (HFD) augments MAP and heart rate (HR) within the first few days of the diet, prior to any change in bodyweight (Burke et al., 2013). However, levels of circulating leptin are proportional to adiposity (Considine et al., 1996) and only begin to increase by the end of the first week of a HFD (Armitage et al., 2012). Thus, rapid changes in cardiovascular parameters suggest that a separate, leptin independent mechanism initiates the pressor response to a HFD. Plasma insulin concentrations increase within hours of meal consumption (Cummings et al., 2001) and are greater in both obese animals and humans (Bagdade et al., 1967; Lim et al., 2013) as well as patients with essential hypertension (Sobotka et al., 2011). Importantly, insulin is known to signal at the arcuate nucleus of the hypothalamus, the same nucleus at which a multitude of peripheral signals, including leptin, act to regulate energy and haemodynamic homeostasis (Benoit et al., 2004). Central administration of insulin attenuates food intake (Air et al., 2002) and augments sympathetic output (Muntzel et al., 1994). We have previously shown that insulin signaling is one of the factors responsible for the development of obesity related hypertension which is later maintained by slowly rising circulating leptin concentrations (Lim et al., 2013).

The association between dyslipidaemia and obesity is important given several lipid species are associated with a number of cardiovascular risk factors (Siri-Tarino et al., 2010). In addition, a single high-fat meal has been shown to reduce endothelial-dependent vasodilation up to 4 h post consumption in healthy normotensive individuals (Vogel et al., 1997). It has been suggested that endothelial-mediated vasodilatory mechanisms are impaired by triacylglycerides (TG) and free fatty acids (Doi et al., 1998; Lundman et al., 2001). Thus, it is possible that diet-induced changes in lipid profiles may play an early role in the development of obesity related hypertension. Lipid profiles have received scant attention in the fat-fed rabbit model of obesity related hypertension and only after several weeks of fat feeding (Eppel et al., 2013).

The contribution of dyslipidaemia to the progression of disease is well-documented. Increased circulating ceramide concentrations are known to increase in obesity and are inversely correlated with insulin resistance (Haus et al., 2009). In addition, circulating levels of TG and cholesteryl esters (CE) are also elevated in obesity and have been shown to affect fasting glucose and insulin sensitivity (Sassolas et al., 1981; Cameron et al., 2008). In the present study the effect of HFD consumption on plasma insulin, leptin, and plasma lipid profiles was assessed in order to elucidate the contribution of each to the rapid rise in MAP observed within the first week of the diet.

MATERIALS AND METHODS

ANIMALS AND DIETS

Experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals. Experiments were conducted in 24 conscious male New Zealand White rabbits (2.76–2.90 kg). Rabbits were housed in individual cages with a telemetry blood pressure receiver (model RLA 1020, Data Sciences International, St. Paul, MN, U.S.A) fitted to the door, under controlled light (6:00–18:00) and temperature (22°C ± 2°C) conditions. Rabbits were initially fed a restricted (150 g daily) normal-fat diet (NFD; 4.3 % total fat, 2.63 kcal/g, Specialty Feeds, Glen Forest, Australia) but after baseline recordings were randomized into two dietary groups and given free access to either a NFD or a high-fat diet (HFD; 13.4 % total fat, 3.34 kcal/g, Specialty Feeds) for 3 weeks. Daily food intake was determined by weighing the contents of the food hopper daily as well as weighing the food added.

EXPERIMENTAL PROCEDURES

A subset of rabbits underwent a preliminary operation under isoflurane anesthesia (3–4% in 1L/min oxygen; Abbot, Botany, NSW, Australia) following induction with propofol (10 mg/kg, Fresenius Kabi, Pymble, NSW, Australia). A radiotelemetry transmitter (model TA11PA-D70, Data Sciences) and catheter was implanted in the aorta via a small branch of the left iliac artery. Carprofen (3 mg/kg, Pfizer, Noth Ryde, NSW, Australia) was given before and 24 h after surgery for analgesia. After 1 week recovery, baseline MAP and HR were measured in the laboratory both by telemetry and by a catheter in the central ear artery. The telemetry signal was calibrated to the ear artery signal and this adjustment was applied to MAP measured in the home cage to minimize the possibility of drift of the signal with time. Baseline home cage MAP and HR were recorded for 1–2 days before rabbits were allocated to a group to receive either NFD or HFD. Home cage measurements were made continuously over 2 weeks.

PLASMA COLLECTION AND ANALYSIS

In order to avoid the effects of recent food consumption, animals were fasted for 4 h before blood samples were collected. Blood was collected before and on days 1, 2, 3, 7, 14, and 21 following the start of the HFD. Small samples of blood were used to measure blood glucose concentrations (Optium Xceed, Abbott, Doncaster, Victoria, Australia). Arterial blood (4 ml) was drawn into vacuum sealed cylinders containing K3EDTA (Vacuette Premium, Greiner bio-one, Wemmel, Belgium) and spun at 4°C for 10 min at 3000 RPM. Plasma aliquots (100 μl) were snap frozen in liquid nitrogen and stored at −80°C until use. Plasma lipid species were extracted into chloroform/methanol and quantified using high performance liquid chromatography-tandem mass spectrometry (Weir et al., 2013). Lipid species identified were diacylglycerides (DG), TG, ceramides (Cer), and CE. Total lipids within each class were calculated from the sum of the individual species. Plasma insulin and leptin concentrations were assessed using an ultrasensitive insulin ELISA kit (Crystal Chem, Chicago, USA) with rabbit insulin standard and a radio immunoassay multispecies kit (LINCO Research, St Charles, MO, USA), respectively.

DATA ANALYSIS

MAP and HR, derived from the pressure pulse, were digitized online at 500 Hz using an analog-to-digital data acquisition card (National Instruments 6024E, Austin, Texas, USA) and averaged over 2 s. MAP and HR were collected continuously over each 24 h period and averaged over one hourly intervals. Data were analyzed by split-plot repeated measures ANOVA allowing for between and within animal comparisons using excel version 2010 (Microsoft). MAP and HR were analyzed by repeated measures analysis of variance that allowed for within-animal contrasts. Data collected at a single time point were analyzed using a One-Way ANOVA. Bonferroni corrections were used to control for Type 1 error. A two sided probability of $P < 0.05$ was considered significant. For all statistics shown we refer to the main effect as a subscript, e.g., $P_{baseline}$ pertains to comparisons between groups made prior to the consumption of either diet, P_{group}, refers to comparisons between HFD and NFD-fed rabbits during dietary intervention, P_{diet} refers to contrasts between baseline and dietary intervention within both NFD and HFD groups, P_{time}, refers to comparisons within each group made between baseline and week 3 time points, $P_{dietxtime}$ pertains to the interaction between diet and time.

RESULTS

EFFECT OF 3 WEEK FAT FEEDING ON PLASMA INSULIN, GLUCOSE AND LEPTIN, FOOD INTAKE AND HAEMODYNAMICS

Baseline plasma insulin concentrations were not different between the dietary groups and averaged 0.46 ± 0.03 ng/ml ($P_{baseline} > 0.05$; **Figure 1**, **Table 1**). A 50% increase from baseline in plasma insulin was observed in both NFD and HFD rabbits over the first 2 days of the diet ($P_{diet} < 0.05$ for both groups; **Figure 1**). A further increase in plasma insulin concentrations on day 3 resulted in 65% greater insulin concentrations in HFD compared with NFD animals at both day 3 and week 1 time points ($P_{group} < 0.05$; **Figure 1**). By week 2, insulin concentrations in HFD rabbits had decreased to those observed in NFD rabbits ($P_{group} > 0.05$; **Figure 1**). Plasma glucose concentrations at baseline were not different between the dietary groups and averaged 5.5 ± 0.12 mmol/L ($P_{baseline} > 0.05$; **Figure 1**, **Table 1**). Plasma glucose concentrations followed a similar pattern to insulin, rising on days 1 and 2 of the diet in both NFD and HFD rabbits ($P_{diet} < 0.05$ for both groups; **Figure 1**). However, HFD rabbits exhibited a 59% greater increase in plasma glucose concentrations than controls ($P_{group} < 0.05$). By week 2, glucose concentrations

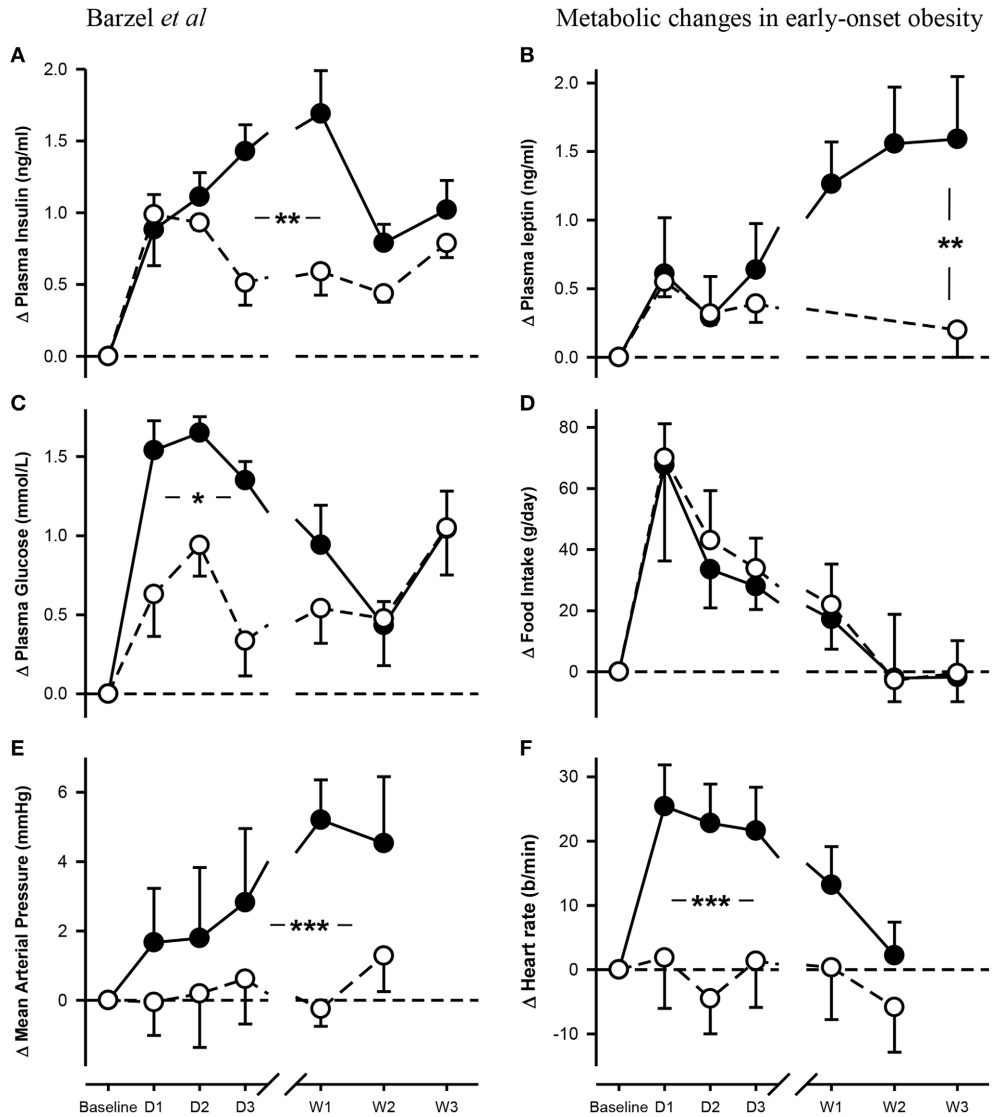

FIGURE 1 | Changes from baseline in levels of plasma insulin (A), leptin (B) and glucose (C) concentrations, food intake (D), mean arterial pressure (E) and heart rate (F) in rabbits fed either a normal fat diet (open circles) or a high-fat diet (closed circles) for 3 weeks. Data are mean difference ± SED, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for differences between dietary groups. Day, D; Week, W.

Table 1 | Baseline concentrations of insulin, glucose, and leptin.

	Pre-NFD	Pre-HFD	P_{group}
Insulin (ng/ml)	0.440 ± 0.036	0.472 ± 0.048	0.61
Glucose (mmol/l)	5.54 ± 0.20	5.42 ± 0.16	0.65
Leptin (ng/ml)	0.751 ± 0.058	0.964 ± 0.146	0.20

Values are mean ± SEM. P_{group} is comparison of normal fat diet (NFD) with high fat diet (HFD).

returned to levels observed in NFD rabbits ($P_{group} > 0.05$; **Figure 1**). By contrast, plasma leptin concentrations, which were averaged 0.91 ± 0.13 ng/ml at baseline ($P_{baseline} > 0.05$; **Figure 1**, **Table 1**), remained unchanged over the first 3 days of the diet in both dietary group ($P_{diet} > 0.05$; **Figure 1**). However, plasma leptin concentrations in HFD-fed rabbits increased on week 1 of the diet compared with baseline ($P_{diet} > 0.05$; **Figure 1**) and were 60 % greater than controls by the end of week 3 ($P_{group} < 0.05$; **Figure 1**). Food intake was similar in both groups with rabbits consuming 47–51% more food on the first day of both diets ($P_{diet} < 0.05$). Intake in both groups gradually diminished to baseline levels after the first week (**Figure 1**). HR also increased rapidly on the first day after the start of the HFD to a level 12% greater than baseline ($P_{diet} < 0.001$; **Figure 1**). HR remained elevated for the first week but had returned to control levels by week 2 ($P_{diet} > 0.05$). By contrast, MAP increased from baseline levels by the 3rd day of the HFD ($P_{diet} < 0.05$; **Figure 1**) and remained 7–8% elevated at 1–2 weeks ($P_{diet} < 0.01$; **Figure 1**). Both MAP and HR in HFD fed rabbits were markedly higher over the 2 weeks of measurements than those fed a NFD ($P_{group} < 0.001$; **Figure 1**).

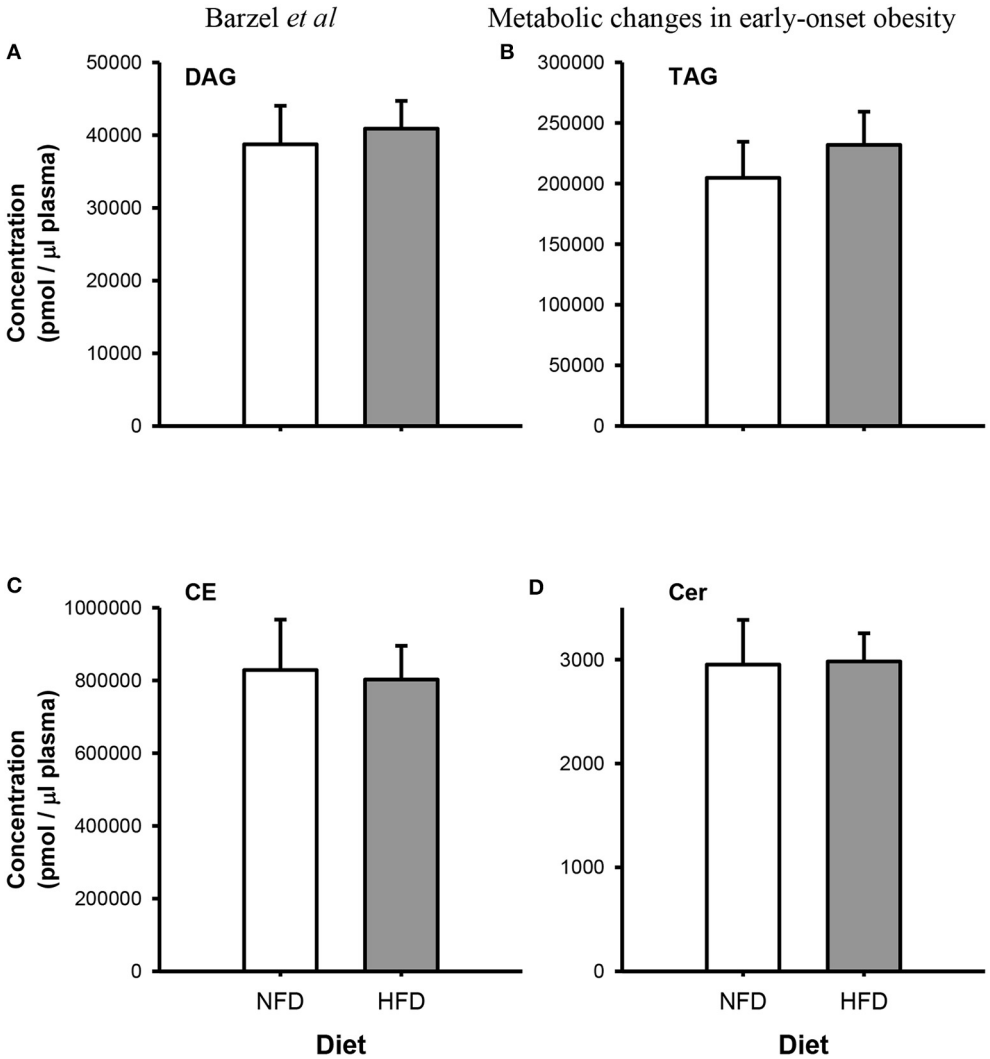

FIGURE 2 | Total Plasma concentrations of diacylglycerides (DG; A), triacylglycerides (TG; B), cholesteryl esters (CE; C) and ceramides (Cer; D) species in normal fat-fed (NFD; white bars) and high fat diet-fed (HFD; gray bars) after 3 weeks of diet. Data are mean ± SEM.

Table 2 | Ceramide species at baseline and week 3 in both NFD and HFD–fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3		P_{diet}	P_{time}	$P_{diet \times time}$
n	**9**		**10**		**10**		**12**				
Ceramide species	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***			
Cer 16:0	189	28	253	28	189	16	287	21	1	0.01	1
Cer 18:0	131	18	136	17	139	13	179	26	1	1	1
Cer 20:0	168	22	206	27	159	12	239	21	1	0.05	1
Cer 22:0	608	93	754	108	550	47	882	84	1	0.05	1
Cer 24:1	440	71	633	94	395	43	510	50	1	0.21	1
Cer 24:0	833	141	971	174	665	56	885	95	1	1	1
Total Cer	2368	361	2952	430	2098	172	2983	271	1	0.18	1

Cer, Ceramides; NFD, normal fat diet; HFD, high fat diet.

Table 3 | Cholesteryl esters at baseline and week 3 in both NFD and HFD–fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3		P_{diet}	P_{time}	$P_{diet \times time}$
n	**9**		**10**		**11**		**13**				
Cholesteryl esters	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***			
CE 14:0	7697	1061	8329	1187	6480	1096	5407	453	0.73	1	1
CE 15:0	14345	2862	11136	2272	10088	2064	6288	946	0.63	1	1
CE 16:2	481	102	517	101	344	86	621	70	1	1	1
CE 16:1	36966	6848	56509	14258	28942	6014	30401	4080	0.97	1	1
CE 16:0	166404	29325	153289	29209	127649	25054	134902	19118	1	1	1
CE 17:1	9896	1933	6440	1188	7643	1094	5736	805	1	0.95	1
CE 17:0	11718	2693	6294	1127	8364	1989	5420	892	1	0.50	1
CE 18:3	17329	3009	20249	5470	13419	2731	21319	3599	1	1	1
CE 18:2	253823	46115	224743	32202	197578	35925	273220	36054	1	1	1
CE 18:1	154389	27782	154154	30973	96569	19247	121237	14200	1	1	1
CE 18:0	22633	5433	12713	2946	14992	4003	13617	2003	1	1	1
CE 20:5	894	257	1211	327	946	221	1382	258	1	1	1
CE 20:3	1113	229	1374	238	805	153	1182	168	1	1	1
CE 20:4	24310	5865	21934	3782	17486	3941	24641	3141	1	1	1
CE 20:2	204	38	244	51	214	55	239	42	1	1	1
CE 20:1	367	73	444	120	3211	2937	289	43	1	1	1
CE 20:0	477	89	308	62	1812	1449	259	46	1	1	1
CE 22:5	901	212	1116	339	2859	2087	1227	235	1	1	1
CE 22:4	293	79	280	63	256	62	262	37	1	1	1
CE 22:1	91	22	117	30	76	26	80	14	1	1	1
CE 22:0	221	32	177	36	372	210	144	25	1	1	1
CE 24:0	171	40	90	20	312	175	137	27	1	1	1
COH	125399	20715	147173	25523	98990	17953	154609	16540	1	1	1
Total CE	849914	142086	828575	138733	639050	113456	802445	92730	1	1	1

CE, cholesteryl esters; NFD, normal fat diet; HFD, high fat diet.

EFFECT OF HFD FEEDING ON PLASMA LIPID PROFILES

After 3 weeks of diet, total plasma DG, TG, Cer, and CE concentrations were not different between the dietary groups ($P_{group} > 0.05$; **Figure 2**). Specific DG, TG, and CE species did not change over the 3-week diet in either dietary group ($P_{time} > 0.05$ for both NFD and HFD, **Tables 2–5**). By contrast, plasma Cer 16:0, 20:0, and 22:0 concentrations increased in HFD-fed rabbits over the 3 week period ($P_{time} > 0.05$; **Table 2**) yet this was unlikely due to the consumption of the HFD ($P_{diet} > 0.05$; **Table 2**) as the overall interaction between diet and time did not reach statistical significance ($P_{diet \times time} > 0.05$; **Table 2**). Individual cholesteryl ester species at week 3 were not different between the dietary groups ($P_{group} > 0.05$; **Table 3**). Similarly, DG ($P_{group} > 0.05$; **Table 4**) and TG ($P_{group} > 0.05$; **Table 5**) lipid species were not different between the dietary groups.

DISCUSSION

The main findings of the present study were that alongside elevations in blood pressure and HR, plasma glucose and insulin concentrations were increased within the first 3 days of a HFD, remaining elevated for the first week of the diet and returning to control levels thereafter. Notably, circulating leptin concentrations were unaltered by a HFD at day 3 but were markedly increased by week 3 whilst in the same time period, no evidence of dyslipidaemia was found. Together, these data suggest hyperinsulinemia rapidly develops after the commencement of a HFD and is a likely mechanism by which haemodynamics and sympathetic tone may change rapidly in the fat-fed rabbit model of obesity related hypertension.

A considerable body of evidence suggests insulin acts centrally to increase both blood pressure and sympathetic tone (Landsberg, 1996; Straznicky et al., 2010; Ward et al., 2011; Lim et al., 2013). There is a strong association between obesity, hyperinsulinemia and, at a later stage, insulin resistance (Weyer et al., 2001; Yuan et al., 2001). Of note is the apparent delay between the engagement of sympathetic nerve activity in obesity and the development of insulin resistance (Flaa et al., 2008) suggesting sympathetic overactivity may occur in response to very early changes in plasma insulin. Indeed central injections of insulin into the paraventricular nucleus of the hypothalamus produce large increases in lumbar sympathetic nerve activity (Ward et al., 2011). In the present study we observed a near two-fold increase in plasma glucose and insulin concentrations within 3 days of starting the HFD. Importantly, increases in MAP and HR in HFD-fed rabbits also began in the first few days of consumption as do increases in RSNA (Armitage et al., 2012; Burke et al., 2013) suggesting that circulating insulin may be involved in augmenting MAP early in the diet. In support of this are the findings

Table 4 | Diacylglycerides at baseline and week 3 in both NFD and HFD –fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3		P_{time}	P_{time}	$P_{diet \times time}$
n	**9**		**10**		**11**		**13**				
DG Species	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***			
DG 14:0 14:0	28	4	34	5	23	4	25	5	1	1	1
DG 14:0 16:0	378	52	448	54	343	47	375	42	1	1	1
DG 14:1 16:0	57	9	109	11	74	10	68	14	1	1	0.61
DG 16:0 16:0	1720	221	1753	201	1439	129	1689	213	1	1	1
DG 14:0 18:1	670	139	888	121	632	113	618	72	1	1	1
DG 14:0 18:2	436	70	402	80	386	59	518	46	1	1	1
DG 16:0 18:0	993	110	871	109	837	81	1024	101	1	1	1
DG 16:0 18:1	7054	1323	7679	932	6111	717	6785	739	1	1	1
DG 16:0 18:2	5986	877	4836	976	4203	629	7382	999	1	1	0.36
DG 16:1 18:1	1214	203	2012	227	1641	513	1223	147	1	1	1
DG 18:0 18:0	212	16	185	42	277	119	255	23	1	1	1
DG 18:0 18:1	1425	187	1444	151	1123	175	1384	110	1	1	1
DG 18:0 18:2	1184	143	1001	189	895	114	1431	145	1	1	0.35
DG 18:1 18:1	5021	767	6195	749	4079	691	4460	384	1	1	1
DG 16:0 20:3	90	16	92	12	201	118	97	14	1	1	1
DG 18:1 18:2	7275	1046	7253	1480	6040	711	8615	771	1	1	1
DG 16:0 20:4	156	19	112	21	123	15	198	36	1	1	0.49
DG 18:1 18:3	1069	159	1112	234	1648	767	1262	110	1	1	1
DG 18:2 18:2	1702	253	1670	481	1274	193	2647	302	1	0.62	0.45
DG 18:0 20:4	197	112	84	8	202	125	105	14	1	1	1
DG 18:1 20:3	184	28	171	26	347	223	164	18	1	1	1
DG 16:0 22:5	130	17	83	15	76	18	104	20	1	1	1
DG 18:1 20:4	374	59	288	49	270	37	425	59	1	1	0.56
DG 16:0 22:6	29	4	18	4	34	12	28	5	1	1	1
Total DG	37583	5289	38739	5294	32277	3603	40884	3828	1	1	1

DG, diacylglycerides, NFD, normal fat diet, HFD, high fat diet.

that central administration of an insulin antagonist attenuated MAP after 1 week of a HFD (Lim et al., 2013). It is important to note that in the present study, plasma leptin concentrations in HFD-fed rabbits remained unchanged over the first 3 days of the diet but had increased by week 3. These results help explain our previous findings that central administration of a leptin antagonist to HFD-fed rabbits failed to elicit a reduction in either haemodynamic or sympathetic parameters at week 1 of the diet but produced large sympathoinhibitory and depressor responses at week 3 (Lim et al., 2013). Combined, these observations imply plasma insulin is involved in the remodeling of sympathetic tone within the first few days of consuming a HFD whilst leptin acts as a sympathoexcitatory signal later on in the diet, presumably once adiposity is increased. As both plasma glucose and insulin concentrations normalized by week 2 of the diet, the present observations point to sympathetic output preceding insulin resistance. Moreover, the apparent lack of effect of central administration of insulin on RSNA has been observed by others (Ward et al., 2011) and may in part be due to the direct effect of insulin on baroreflex gain (Pricher et al., 2008).

The present study also sought to establish the presence of dyslipidemia in our obese rabbit model and any subsequent contribution to the development of hypertension observed in these animals. In humans, dyslipidemia is a prominent feature of metabolic syndrome (Bays, 2009) and often appears in conjunction with hypertension (Nguyen et al., 2008). An example of the consequences of dyslipidemia can be found in greater total plasma ceramide concentrations which are known to occur in obesity whilst specific ceramide species are strongly associated with insulin resistance (Haus et al., 2009). In the present study, plasma concentrations of 4 lipid classes (Cer, CE, DG, and TG) were unchanged after 3 weeks of HFD. Our findings are in agreement with those made by Eppel and colleagues who observed no change in total plasma cholesteryl, and total plasma TG in rabbits fed a HFD for 9 weeks (Eppel et al., 2013) and suggests large changes in lipid profiles may take longer to develop in the rabbit model (Hamilton and Carroll, 1976). However, given the rapid haemodynamic and hormonal responses to dietary fat content, we expected to find changes in the expression of individual lipid species which would have been indicative of altered lipid metabolism. It is likely that our study was not powered to detect minute perturbations in the expression of specific plasma lipid species, contributing to our findings that plasma lipid profiles are unchanged by a diet high in fat. However, given that other parameters found in plasma, including insulin and leptin, can be measured accurately, our design is unlikely to be a confounding factor.

Table 5 | Triacylglycerides at baseline and week 3 in both NFD and HFD–fed rabbits.

	NFD Week 0		NFD Week 3		HFD Week 0		HFD Week 3		P_{diet}	P_{time}	$P_{diet \times time}$
n	**9**		**10**		**11**		**13**				
TG Species	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***	**Mean**	***SE***			
TG 14:0 16:0 18:2	3755	695	3729	521	2661	636	3812	495	1	1	1
TG 14:0 16:1 18:1	1644	426	3188	562	1728	463	1548	226	1	1	1
TG 14:0 16:1 18:2	432	94	557	74	585	202	600	76	1	1	1
TG 14:0 18:0 18:1	344	58	301	56	365	108	304	49	1	1	1
TG 14:0 18:2 18:2	514	90	493	114	729	313	767	110	1	1	1
TG 14:1 16:0 18:1	569	148	1139	249	742	196	584	130	1	1	1
TG 14:1 16:1 18:0	1798	450	3235	581	1729	375	1762	260	1	1	1
TG 14:1 18:0 18:2	117	35	303	54	4881	4747	193	30	1	1	1
TG 14:1 18:1 18:1	1378	299	1894	253	4834	3611	1644	186	1	1	1
TG 15:0 18:1 16:0	2032	209	1417	309	1809	419	1072	138	1	0.92	1
TG 15:0 18:1 18:1	1228	149	1075	216	2602	1586	754	93	1	1	1
TG 16:0 16:0 16:0	3150	560	2154	491	2434	591	3199	697	1	1	1
TG 16:0 16:0 18:0	2100	346	1811	675	1377	189	3107	616	1	1	1
TG 16:0 16:0 18:1	25852	3856	19383	3561	15841	3018	22531	3518	1	1	1
TG 16:0 16:0 18:2	12162	1992	7170	1940	7109	1269	15046	2945	1	1	0.23
TG 16:0 16:1 18:1	12109	2080	16866	3160	10526	2406	11433	1657	1	1	1
TG 16:0 18:0 18:1	7491	1216	4312	679	5718	807	5389	1143	1	1	1
TG 16:0 18:1 18:1	50498	5980	41240	5833	34880	7887	38074	4161	1	1	1
TG 16:0 18:1 18:2	35618	4652	23555	5079	24236	4482	36135	4229	1	1	0.56
TG 16:0 18:2 18:2	11604	1763	8206	2297	8113	1453	16218	2561	1	1	0.30
TG 16:1 16:1 16:1	173	41	284	39	291	130	191	25	1	1	1
TG 16:1 16:1 18:0	521	66	430	55	1047	639	528	73	1	1	1
TG 16:1 16:1 18:1	1723	299	1877	269	1293	302	1910	281	1	1	1
TG 16:1 18:1 18:1	2441	552	3941	619	2040	489	2530	313	1	1	1
TG 16:1 18:1 18:2	6301	1096	5597	980	5109	1078	7057	932	1	1	1
TG 17:0 16:0 16:1	4652	557	2903	503	3843	829	2066	300	1	0.15	1
TG 17:0 18:1 14:0	3653	450	2117	562	12151	9170	1141	203	1	1	1
TG 17:0 18:1 16:0	2101	257	1443	337	4914	3326	1402	192	1	1	1
TG 17:0 18:1 16:1	4237	499	3808	577	3463	941	2425	251	1	1	1
TG 17:0 18:1 18:1	2622	603	2397	375	2664	572	1902	440	1	1	1
TG 17:0 18:2 16:0	3291	423	2115	287	2559	532	1921	262	1	1	1
TG 18:0 18:0 18:0	71	26	31	7	1121	1084	55	11	1	1	1
TG 18:0 18:0 18:1	555	92	440	93	15842	15377	734	120	1	1	1
TG 18:0 18:1 18:1	5408	836	4963	817	31439	27087	6779	933	1	1	1
TG 18:0 18:2 18:2	1713	227	1334	352	4942	3562	2033	532	1	1	1
TG 18:1 14:0 16:0	4784	940	4858	781	3477	863	3857	641	1	1	1
TG 18:1 18:1 18:1	8080	1312	9679	1041	6304	1327	8604	873	1	1	1
TG 18:1 18:1 18:2	5822	917	6548	1358	4423	779	9515	1414	1	0.53	1
TG 18:1 18:1 20:4	345	67	1053	803	352	123	2421	763	1	0.82	1
TG 18:1 18:1 22:6	169	32	232	115	2570	2448	472	102	1	1	1
TG 18:1 18:2 18:2	4289	798	5209	1225	3480	616	7900	1428	1	0.68	1
TG 18:2 18:2 18:2	605	120	825	265	490	89	1440	341	1	0.72	1
TG 18:2 18:2 20:4	314	164	509	197	229	74	797	352	1	1	1
Total TG	238265	31727	204621	29850	246940	72879	231851	27372	1	1	1

TG, triacylglycerides, NFD, normal fat diet, HFD, high fat diet.

Thus, our findings discount dyslipidemia as a likely mechanism by which hypertension occurs during 3 weeks of a HFD.

In conclusion, our findings demonstrate plasma insulin is a likely mechanism by which rapid increases in MAP occur over the first few days of consumption of a HFD. In addition, dyslipidaemia does not appear to develop after 3 weeks of fat feeding suggesting plasma lipid profiles do not play a role in the genesis of hypertension in our animal model but may contribute to the

development of comorbidities associated with obesity at a later stage.

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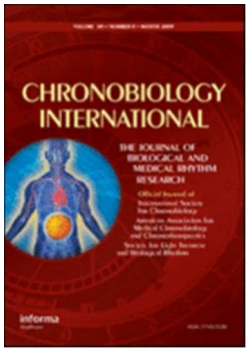

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Specific role of dietary fat in modifying cardiovascular and locomotor activity 24h rhythms

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Abstract

Meal fed conscious rabbits normally exhibit postprandial elevation in blood pressure, heart rate and locomotor activity which is abolished by consumption of a high fat diet (HFD). Here, we assessed whether the cardiovascular changes are attributable to the increased caloric intake due to greater fat content or to hyperphagia. Rabbits were meal-fed during the baseline period then maintained on either an *ad libitum* normal fat diet (NFD) or *ad libitum* HFD for 2 weeks. Blood pressure, heart rate and locomotor activity were measured daily by radio-telemetry alongside food intake and bodyweight. Caloric intake in rabbits given a NFD *ad libitum* rose 50% from baseline but there were no changes in cardiovascular parameters. By contrast, heart rate increased by 10% on the first day of the *ad libitum* HFD ($p<0.001$) prior to any change in bodyweight whilst blood pressure increased 7% after 4 days ($p<0.01$) and remained elevated. Baseline 24-hour patterns of blood pressure and heart rate were closely associated with mealtime, characterised by afternoon peaks and morning troughs. When the NFD was changed from meal fed to *ad libitum*, blood pressure and heart rate did not change but afternoon activity levels decreased ($p<0.05$). By contrast, after 13 days *ad libitum* HFD, morning heart rate, blood pressure and activity increased by 20%, 8% and 71%, respectively. Increased caloric intake specifically from fat, but not as a result of hyperphagia, appears to directly modulate cardiovascular homeostasis and circadian patterns independently of white adipose tissue accumulation.

Keywords: 24-hour rhythm, blood pressure, heart rate, obesity, rabbits, high-fat diet

Introduction

Obesity is the product of a consistent positive energy balance due to a decrease in energy expenditure, greater energy intake or a combination of both (Hill, 2006). The condition is associated with several comorbidities and its increasing prevalence places a considerable burden on global health and economy (Haslam & James, 2005; Thompson & Wolf, 2001). We have previously reported that consumption of a high fat diet (HFD) in rabbits for only a few weeks results in weight gain and increased adiposity as well as hypertension and tachycardia (Armitage et al., 2012; Prior et al., 2010). These animals also display aberrant cardiovascular and sympathetic meal associated rhythms characterised by a loss of pre-prandial dipping (Burke et al., 2013). Whilst increased total caloric intake is an expedient gauge of the likelihood of developing obesity (Lichtman et al., 1992), individual dietary constituents such as lipid and carbohydrate species are independently associated with varied risk profiles of cardiovascular disease (Siri-Tarino et al., 2010). Importantly, dietary macronutrients have been shown to impact the pattern of meal associated rhythms. Consumption of a HFD has been demonstrated to abolish nocturnal dipping of mean arterial pressure (MAP) and heart rate (HR) observed at baseline in the canine model of obesity (Pelat et al., 1999). In humans, high salt intake is associated with non-dipping in a subset of patients with essential hypertension (Uzu et al., 2006).

Food consumption is a powerful synchroniser of circadian activity and is known to override signals from the 'master clock', the suprachiasmatic nucleus (Froy, 2010). Thus, changes in the time of meal presentation may affect peripheral circadian clocks such as the liver, heart and kidneys, in essence uncoupling central from peripheral circadian mechanisms (Damiola et al., 2000). Time of consumption and dietary composition have been shown to alter circadian rhythms (Froy, 2010). For instance, diets high in fat, protein or carbohydrates affect the expression of SREBP-1protein, a key regulator of hepatic circadian homeostasis, to varying degrees (Matsumoto et al., 2010). More specifically, mice fed a HFD exhibit a shift in 24-hour feeding rhythm characterised by increased caloric intake during the day (rest period) which was coupled with decreased amplitude of the circadian transcription factor Bmal1 and Per2 expression in adipose tissue (Kohsaka et al., 2007). Strikingly, these behavioural and cellular changes occur independently of body-weight gain (Kohsaka et al., 2007). Concordant with these studies are observations in which the normal preprandial dipping in MAP and HR is abolished by consumption of a HFD (Burke et al., 2013). However, in that study the HFD-fed rabbits consumed more calories than those fed a normal

fat diet (NFD) by virtue of being given a hypercaloric diet whilst control animals were maintained on a restricted single meal of control feed. In rats, consumption of food *ad libitum* masks 24 hour water intake rhythms (Johnson & Johnson, 1991). Thus, provision of a HFD *ad libitum* in rabbits likely masks light-associated diurnal rhythms observed in NFD rabbits. In addition, consumption of a HFD is known to stimulate hyperphagia and thus increase total caloric intake when compared with a low-fat diet (Savastano & Covasa, 2005). Indeed 50% of the increased calories consumed in our previous studies were due to hyperphagia and 50% due to the higher fat content of the diet (Burke et al., 2013). As these factors were combined, the separate contribution to the changes in the cardiovascular patterns of increased calories from fat compared to those due simply to hyperphagia could not be assessed. It is likely that these different sources of calories have quite different cardiovascular influences. Thus in the present study we compared the effect of consuming an *ad libitum* HFD with that of consuming an *ad libitum* NFD. Thus both groups will have increased caloric intake due to hyperphagia, but only one group will have the effect of the higher fat content. This design would allow us to ascertain whether the cardiovascular changes observed in HFD-fed rabbits were due to increased fat content or the hypercaloric nature of the diet.

Materials and Methods

Ethical Approval

Experiments were approved by the Alfred Medical Research and Education Precinct Ethics Committee and conducted in accordance with Australian Code of Practice for Scientific Use of Animals. The study conforms to international ethical standards (Portaluppi et al., 2010). Experiments were conducted in male New Zealand White rabbits (initial body weight 2.7-2.8 kg). Rabbits were housed in pens individually and were kept under controlled light (lights on 06:00 – 18:00 h) and temperature (20-22 °C) conditions with *ad libitum* access to water.

Experimental Procedures

Rabbits were fitted with radiotelemetry transmitters (model TA11PA-D70; Data Sciences International, St. Paul, MN, USA) under isoflurane anesthesia (3-4 % in 1 L/min Oxygen; Abbot, Botany, NSW, Australia) following induction with propofol (10 mg/Kg; Fresenius Kabi, Pymble, NSW, Australia). The catheter of the transmitter was implanted into the aorta via a small branch arising from the left iliac artery. Analgesia was provided prior to and following surgery (Carprofen; 3 mg/Kg, Pfizer, North Ryde, NSW, Australia).

Following 9 days of recovery, baseline MAP and HR were measured in the laboratory by both telemetry and a catheter in the medial ear artery. The telemetry signal was calibrated to the ear artery signal and this adjustment was applied to MAP measured in the home cage in order to minimize the possibility of drift of the signal with time (Burke et al., 2013). Baseline home cage MAP, HR and locomotor activity as well as food intake and bodyweight were measured over a 3 day period in which all rabbits were meal fed 150g of a normal fat diet (NFD; 4.2 % total fat, 2.63 kcal/g; Specialty Feeds, Glen Forrest, WA, Australia) each day at 12:00 h with the remaining food weighed the following day. Following this baseline period, rabbits were given *ad libitum* access to food after being randomly assigned either to continue the NFD (n = 16) or to receive a high fat diet (HFD; 13.3 % total fat, 3.34 kcal/Kg, SF06-011, Specialty Feeds; n = 20). Supply of the diet was checked daily and rabbits were maintained on their respective diets for 2 weeks. During that period continuous MAP, HR and locomotor activity measurements were made (n = 14). Bodyweight and food intake were measured daily (n = 12) and hourly measurements of food intake over 24 h were recorded at baseline, day 6 and day 13 in another group of rabbits (n = 10).

Data Analysis

Measurements of 24-h MAP, HR and locomotor activity were performed as previously published (Burke et al., 2013). To assess the effect of feeding, data from morning and afternoon periods were compared. The former was designated as the 6-h period between 03:30 and 09:30 h (when, on the meal-fed days, the animals were quiet and values were stable) whilst the latter was defined as the 6-h period in the afternoon (13:00– 19:00 h, following feeding on the meal-fed days). The influence of the light cycle over the 24-h pattern of parameters was assessed by measuring the difference between values taken over the 12-h period when lights were on (06:00–18:00 h) and those taken over the 12-h dark period (18:00– 06:00 h). Values were expressed as mean ± SEM or mean difference ± SE of the difference (SED). Data were analysed by split plot repeated-measures analysis of variance, which is a mixed model allowing for within-animal and between-animal (between group) contrasts. Comparisons included p_{meal} referring to the effect of meal feeding during the baseline recording (both groups combined); p_{lin} refers to the linearity of changes due to diet over time, $p_{ad\ lib}$ refers to within group contrasts between the meal fed baseline period and the *ad libitum* period, p_{diet} refers to contrasts between HFD and NFD-fed rabbits during *ad libitum* feeding, p_{light} refers to the effect of light on all measured parameters. Type 1 error was controlled using a Bonferroni adjustment and Greenhouse-Geisser estimates were used to

correct for asphericity (Ludbrook, 1994). A probability of $p<0.05$ was considered significant. The study was well powered to detect differences within groups. For instance, the power to detect a 4.3 mmHg difference in MAP between NFD (n=7) and HFD (n=7) rabbits at an alpha level of 0.05 was 0.97.

Results

Effect of Ad Libitum NFD and HFD Consumption on 24-h averages of MAP, HR

Baseline home cage values averaged over 24 h were 65 ± 1 mmHg and 221 ± 4 b/min (n = 14) for MAP and HR respectively. When the NFD meal-fed diet was changed to NFD *ad libitum,* there were no detectable changes in MAP or HR over 13 days ($p_{\text{ad lib}}>0.05$; n = 7, Figure 1). However, in rabbits presented with a HFD *ad libitum*, HR increased by 10 % on the first day ($p_{\text{ad lib}}<0.001$, n = 7) and remained elevated for 5 days (average increase +8 % over days 1-5, $p_{\text{ad lib}}<0.001$; Figure 1). This effect subsequently diminished and HR was similar to baseline levels on day 6 - 13. By contrast, MAP increased more slowly and was not significantly greater than baseline until day 4 of the HFD (+7 %, $p_{\text{ad lib}} < 0.05$; Figure 1). However, unlike HR, MAP remained elevated at +7 % of baseline for the remainder of the 13 day protocol ($p_{\text{ad lib}}<0.01$).

Effect of Ad Libitum NFD and HFD Consumption on Caloric Intake, Body Weight, Food Intake and Locomotor Activity

When rabbits were meal-fed a NFD, baseline bodyweight, food consumption, caloric intake and locomotor activity averaged over 24 hours were 2.78 ± 0.05 kg, 134 ± 4 g, 360 ± 10 kcal, and 43 ± 2 au, respectively. Switching to an *ad libitum* NFD produced 50 % increases in food and caloric intake on the first day ($p_{\text{ad lib}}<0.001$; Figure 1). Both values decreased over the remainder of the measurement period and after 13 days were not different to baseline (Figure 1). Bodyweight increased linearly and was 14 % greater than at baseline after 13 days of NFD *ad libitum* ($p_{lin}<0.001$) but locomotor activity was reduced by 30 %, compared to baseline, over the first 5 days ($p_{\text{ad lib}}<0.01$; Figure 1). In rabbits fed an *ad libitum* HFD, there was a similar pattern of food intake as observed in the NFD rabbits fed *ad libitum* ($p_{\text{diet}}>0.05$) and caloric intake also rose sharply on the first day (+79 %) and declined over the 13 day period in a pattern resembling that of HR ($p_{\text{lin}} < 0.05$; Figure 1). However, for the duration of the *ad libitum* regimen, caloric intake in HFD fed rabbits remained more than double that of NFD fed rabbits ($p_{diet} < 0.001$; Figure 1). The greater caloric intake was associated with an

increase in bodyweight which over 13 days was 65 % greater than that observed in NFD *ad libitum* fed rabbits ($p_{diet} < 0.001$) but there was a similar reduction in locomotor activity ($p_{diet} > 0.05$; Figure 1).

Effect of Ad Libitum NFD and HFD on 24-h patterns of MAP, HR, Locomotor Activity and Food Intake

During the baseline measurement period when rabbits were meal fed a NFD, MAP, HR, locomotor activity and food intake over 24 h showed clear rhythms associated with meal presentation and consumption (Figure 2). The average morning levels of HR and MAP were 205 ± 7 b/min and 64 ± 1 mmHg, respectively (n = 14). Presentation of food was accompanied by an increase of 16 % in HR ($p_{meal} < 0.01$) and a doubling of locomotor activity but only a small but significant increase in MAP of 4 % ($p_{meal} < 0.01$) measured during the afternoon period (Figure 2). Food intake averaged 2.3 ± 0.3 g/h during the morning and rose to 6.2 ± 0.6 g/h during the afternoon measurement period ($p_{meal} < 0.01$).

When the NFD meal-fed diet was switched to NFD *ad libitum*, the 24-h HR and MAP patterns were unchanged ($p_{ad\ lib} > 0.05$) with HR morning values remaining lower than afternoon values at every time point (Figures 2 and 3). Locomotor activity, however, was 43 % lower in the afternoon during *ad libitum* feeding compared to the same period during meal feeding ($p_{ad\ lib} < 0.01$). Food consumption on day 6 of the *ad libitum* diet was similar to baseline but by day 13, food was equally consumed in the morning and afternoon ($p_{ad\ lib} < 0.05$, n = 4, Figures 2 and 3).

This pattern was markedly altered from the first day of *ad libitum* HFD feeding. Morning HR, averaged over 13 days, was 20 % higher than baseline morning HR ($p_{ad\ lib} < 0.001$) and afternoon HR was 4 % lower than afternoon baseline HR ($p_{ad\ lib} < 0.05$, Figures 2 and 4). Thus the increase in HR from morning to afternoon (+38 ± 10 b/min) at baseline was reduced to a decrease of -12 ± 1 b/min, averaged over 13 days of *ad libitum* HFD feeding ($p_{ad\ lib} < 0.001$, Figure 4). The locomotor activity patterns of HFD *ad libitum* fed rabbits closely resembled those of HR over 13 days, with morning activity levels 71 % above those at baseline and afternoon levels 34 % below baseline (both $p_{ad\ lib} < 0.01$; Figures 2 and 4). Similarly, with the change of feeding regimen to a HFD, morning MAP was 8% higher than morning baseline MAP ($p_{ad\ lib} < 0.01$) but afternoon MAP did not change, thus there was little overall change in MAP over the 24-h period (Figures 2 and 4). Food intake was altered by the

HFD so that there was no difference between consumption in the afternoon and that in the morning over both timepoints measured ($p_{ad\ lib}$ <0.01, n = 6).

Effect of Ad Libitum NFD and HFD on light-related patterns

In order to characterise the relationship between 24-h variability and the light cycle, we also measured the differences between the data collected during the 12-h light and 12-h dark periods. At baseline, when rabbits were meal fed a NFD, HR and MAP in the light period were 65 ±1 mmHg and 217 ± 3 b/min, respectively. MAP, HR, locomotor activity were not influenced by the light cycle under either of the NFD feeding regimens (meal or *ad libitum* feeding, p_{light} >0.05, Figure 3). Food consumption during the NFD meal-fed regimen was 31 % lower in the dark period than the light (p_{light} <0.05). Switching to an *ad libitum* NFD increased food intake during the dark period so that feeding occurred more uniformly over 24 h (Figure 3). Consumption of an *ad libitum* HFD produced slightly greater MAP in the dark period (+2.2 ± 0.3 mmHg, p_{light} <0.05; Figure 4). HR, locomotor activity and the pattern of food consumption did not change between the light and dark periods over the 13 day protocol (p_{light} >0.05; Figure 4).

Discussion

Dietary habits, including time of meal consumption and nutrients available, have a profound effect on haemodynamics and circadian rhythms (Damiola et al., 2000; Uzu et al., 2006). The major finding of this study is that greater dietary fat content, but not increased caloric intake due to hyperphagia, adversely affects haemodynamic variables with both MAP and heart rate increasing over the first 6 days of HFD consumption. The change in haemodynamics manifested both as a rise in the daily average as well as a change in the pattern of circadian rhythmicity. In addition, we report a reduction in locomotor activity in both dietary groups concomitant with a reversal in the 24-h pattern of locomotor activity circadian rhythm. Thus an increase in either total caloric intake or dietary fat content appears to affect different circadian rhythms.

Cardiovascular Circadian Rhythms are Influenced by HFD not Increased Caloric Intake

We have previously reported that meal-fed control rabbits exhibit a 24-h pattern heavily influenced by feeding with a preprandial low and a postprandial high (Burke et al., 2013). In the current study, *ad libitum* consumption of the same diet did not change this pattern, despite a 50% increase in caloric intake and a 14% gain in body weight. By contrast, rabbits given

free access to a diet rich in fat exhibited a loss of 'preprandial dipping' on the first day of the diet, contributing to the observed hypertension and tachycardia in these animals. In humans, greater body mass index is associated with aberrant circadian periodicity characterised by a loss of diurnal dipping (Kotsis et al., 2005) although the precise mechanism by which this occurs remains elusive. In the current study, a marked change in the cardiovascular circadian pattern was linked to consumption of a HFD for 2 weeks whilst the circadian patterns of rabbits given free access to a NFD, also increasing total caloric intake, did not depart from baseline. Consumption of a HFD in humans increases fasting plasma low-density lipoprotein cholesterol and triglyceride levels (Kwiterovich et al., 2003) and these correlate with blood pressure in 'non-dipping' obese patients (Kotsis et al., 2005). Furthermore, experiments conducted by Puska (1983) in which a 6 week low fat diet reduced systolic and diastolic pressures independently of salt intake and weight loss support our finding that dietary fat has a considerable impact on MAP.

Effects of Increasing Caloric Intake and Total Dietary Fat on Locomotor Activity

We have previously observed a switching of high locomotor activity in the postprandial period following meal feeding to high activity in the morning period when rabbits are given a HFD for 3 weeks (Burke et al., 2013). In the present study, locomotor activity in rabbits given free access to a NFD was also reduced in the afternoon. Indeed, afternoon locomotor activity was decreased in both dietary groups, although morning activity was elevated to a greater extent in the HFD fed group. The net reduction in activity over 24 h in both groups in the early phase of their respective diets is likely due to increased caloric intake and may relate to loss of central circadian clock regulation of sleep and activity (Froy, 2010). Locomotor activity also failed to increase in rats on a diet of progressively increasing calories or of high fat (Rupp & Maisch, 1999; Vaanholt et al., 2008). Sedentary behaviour is associated with greater bodyweight gain in animals (Crews et al., 1969) and greater obesity rates in humans (Epstein et al., 2000).

Increased Calories from Fat Affect Cardiovascular Parameters

Here we report that increased dietary fat augments the daily averages of MAP and HR yet an increase in caloric intake has no impact on these parameters. Our observations suggest the haemodynamic changes observed in HFD rabbits are independent of bodyweight gain given NFD animals in the current study increase bodyweight and move less, presumably decreasing energy expenditure. In fact, both dietary groups displayed increases in bodyweight over the 13 day period, albeit at different rates. Moreover, increased MAP in HFD rabbits preceded

any change to bodyweight. In animals, consumption of a HFD has been shown to augment MAP and HR (Boustany et al., 2004; Cook et al., 2004; Prior et al., 2010; Yiannikouris et al., 2012). Importantly, increased calories from fat are known to induce hypertension and tachycardia in humans (Appel et al., 1997; Straznicky et al., 1993). Thus it appears haemodynamic changes occur in response to increased dietary fat although at present we cannot differentiate between the effects of increased calories and increased dietary fat content.

We have previously shown that the increase in MAP is present beyond the 13 day period and remains elevated following withdrawal of the HFD (Armitage et al., 2012; Burke et al., 2013). Moreover, the pressor response to the diet occurs concomitantly with an increase in renal sympathetic nerve activity (RSNA) (Armitage et al., 2012). Of note is the fact that reintroduction of a NFD results in decreased HR but maintained MAP and augmented RSNA (Burke et al., 2013). Thus a HFD appears to be an important instigator of hypertension but might not be required to maintain it over a long period of time. Additionally, the gradual attenuation in HR is likely due to calorie adjustment previously reported in these animals (Burke et al., 2013). We have previously shown that increased dietary fat intake plays a central role in the genesis of obesity related hypertension via activation of sympathetic activity (Burke et al., 2013; Prior et al., 2010). Indeed, fatty acids have been shown to interact with hypothalamic neurons and alter the expression of key neuropeptides known to regulate energy and cardiovascular homeostasis as well as sympathetic tone (Obici et al., 2002; Shimokawa et al., 2002). Strikingly, consumption of a HFD over just 3 days significantly impairs the normal response of hypothalamic neurons to free fatty acids (Morgan et al., 2004). Despite recognition from the WHO (2003) that increased total fat intake is strongly associated with obesity related hypertension, specific fat species better correlate with relative risk of developing cardiovascular disease (CVD). There is a strong association between CVD and trans fats, artificially altered unsaturated fatty acids (Mozaffarian et al., 2006). In addition, saturated fatty acids have been suggested to increase risk of developing CVD although the relationship remains controversial (Astrup et al., 2011). Conversely, polyunsaturated fatty acid intake is known to lower the risk of coronary heart disease (Mozaffarian et al., 2005) and has been shown to decrease blood pressure in children over a long period of time (Forsyth et al., 2003). In addition, it is suggested that the ratio between lipid species is of particular relevance to risk of developing CVD (Mozaffarian et al., 2010).

In the present study, both dietary groups were not rich in saturated fatty acids and had a higher polyunsaturated fatty acid to saturated fatty acids ratio.

A limitation of the current study is the difficulty in delineating the effect of calories from fat versus calories per se on MAP and HR given HFD rabbits consume more calories than controls. We have previously shown that consumption of a HFD induces fat accumulation and increases circulating leptin levels which strongly correlate with visceral adiposity and MAP (Burke et al., 2013; Prior et al., 2010). However, it is exceptionally difficult to distinguish between the effects of fat-derived calories and calories per se as fat is the most energy dense macronutrient and replacing it would necessitate greater amounts of either protein or carbohydrates. Neither macronutrients would adequately replace fat in the rabbit. On the other hand, caloric restriction would further complicate the interpretation of these experiments.

Summary and conclusion

We have shown that MAP and HR circadian rhythms are influenced either by greater calories from fat or greater fat content but not hyperphagia. We have also demonstrated that locomotor activity appears to be more sensitive to total caloric intake, irrespective of the fat content of the diet. Thus, despite only the HFD having adverse cardiovascular consequences, increased total caloric intake seems to abate energy expenditure by means of increasing sedentary behaviour. These diverging effects highlight the ways by which obesity, and associated hypertension, may develop.

Declaration of interest

The study was supported by National Health and Medical Research Council of Australia project grant 526618, NHMRC Fellowship award 367631 (G.A.H.) and National Heart Foundation Post Doctoral Research Fellowship PF 06M 2766 (J.A.A.). The study was supported in part by the Victorian Government's Operational Infrastructure Support Program. There are no conflicts of interest.

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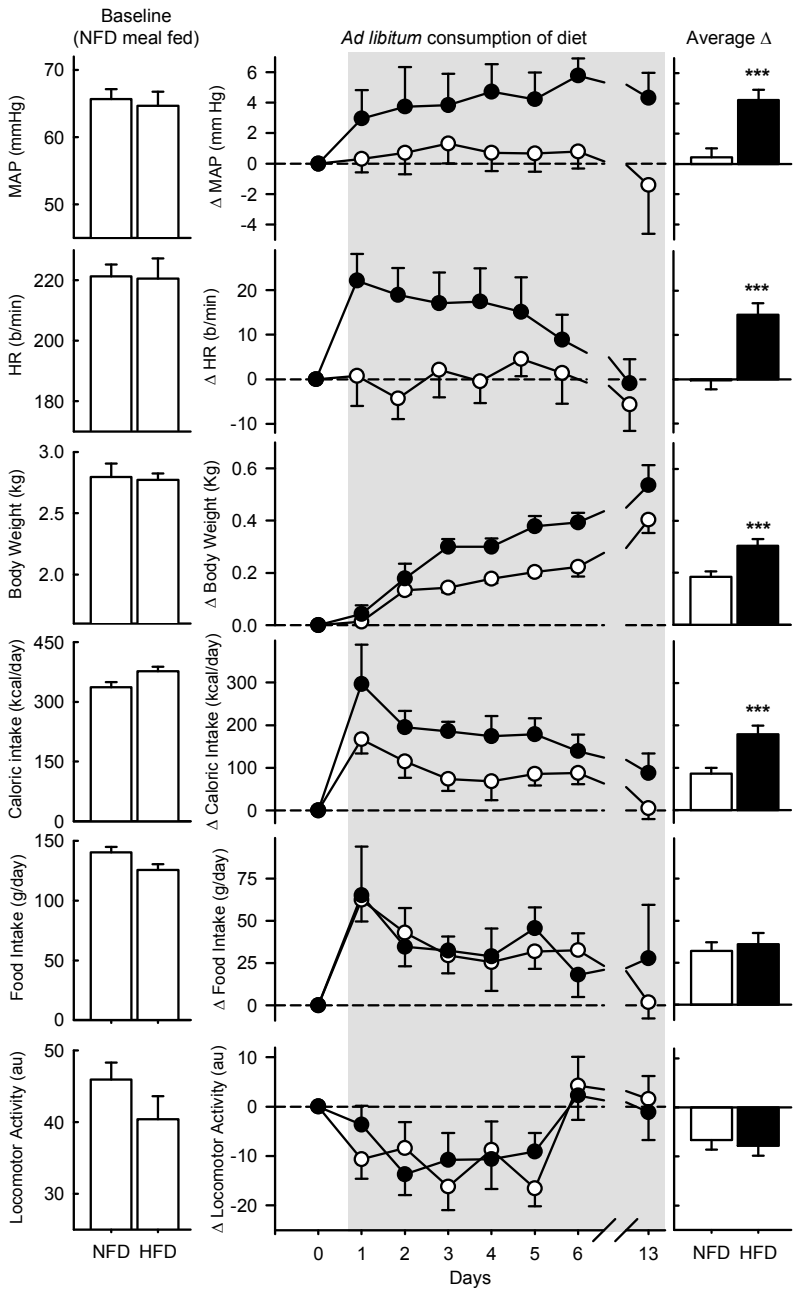
Figure Legends

Figure 1: Baseline levels in meal fed rabbits and effect of 13 days of a high fat (HFD) or normal fat diet (NFD) fed *ad libitum*. **Left** panels: Baseline values averaged over 24 hours in meal fed rabbits before commencing *ad libitum* NFD or HFD. Values are mean ± SEM. **Centre** panels: Daily changes from baseline from the first until the 13th day of a NFD (open circles) or HFD (filled circles) fed *ad libitum*. *Ad libitum* feeding is indicated by grey panel. Values are mean difference ± SED indicating between animal variance. **Right** panels: Average change from baseline over the entire 13 day period in NFD (unfilled bars) and HFD (filled bars) animals. $^{***}P_{diet}$ <0.001 for HFD vs NFD (days 1-13). Mean arterial pressure (MAP), heart rate (HR, beats/min).

Figure 2: Left: Hourly averaged data showing the variation over 24 hours of mean arterial pressure (MAP), heart rate, locomotor activity (au, arbitrary units) and food intake in rabbits meal fed a normal fat diet on day 0 (open circles), and on day 6 (grey circles) and day 13 (black triangles) after changing to *ad libitum* feeding of the same diet. **Right**: Hourly averaged data in rabbits meal fed a normal fat diet on day 0 (open circles), and day 6 (grey circles) and on day 13 (black triangles) after the start of a high fat diet fed *ad libitum*. Rabbits were fed at 12:00 h (baseline only; dotted line) and the lights were on between 6:00 h and 18:00 h (dashed vertical lines). Values are mean ± SEM indicating between animal variance. The morning and afternoon periods (03:30 h - 09:30 h and 13:00 h-19:00 h respectively) are shaded in grey. $^{\varphi}P$<0.05 for Day 0 vs Day 6 during morning and afternoon periods. $^{\omega}P$<0.05 for Day 0 vs Day 13 during morning and afternoon periods. NFD, normal fat diet, HFD, high fat diet.

Figure 3: Left: Average differences between values collected during 6 hours in the morning (03:30 h - 09:30 h) and those during 6 hours in the afternoon (13:00 h -19:00 h) at baseline (day 0, meal-fed normal fat diet, open bars) and on days 1 - 13 (grey bars) of the same normal fat diet fed *ad libitum*. **Right**: Average differences between values collected during 12 hours of dark (18:00 h - 06:00 h) and 12 hours of light (06:00 h - 18:00 h). Values are mean difference ± SED indicating between animal variance. $^{*}P$< 0.05 and $^{**}P$< 0.01 for days 1-13 compared with day 0; NFD, normal fat diet.

Figure 4: Left: Average differences between values collected during 6 hours in the morning (03:30 h - 09:30 h) and those during 6 hours in the afternoon (13:00 h -19:00 h) at baseline (day 0, meal-fed normal fat diet, open bars) and on days 1 - 13 (grey bars) of a HFD fed *ad libitum*. **Right**: Average differences between values collected during 12 hours of dark (18:00 h - 06:00 h) and 12 hours of light (06:00h - 18:00 h). Values are mean difference ± SED indicating between animal variance. $^{*}P$< 0.05, $^{***}P$< 0.001, $^{**}P$< 0.01 for days 1-13 compared with day 0; HFD, high fat diet.



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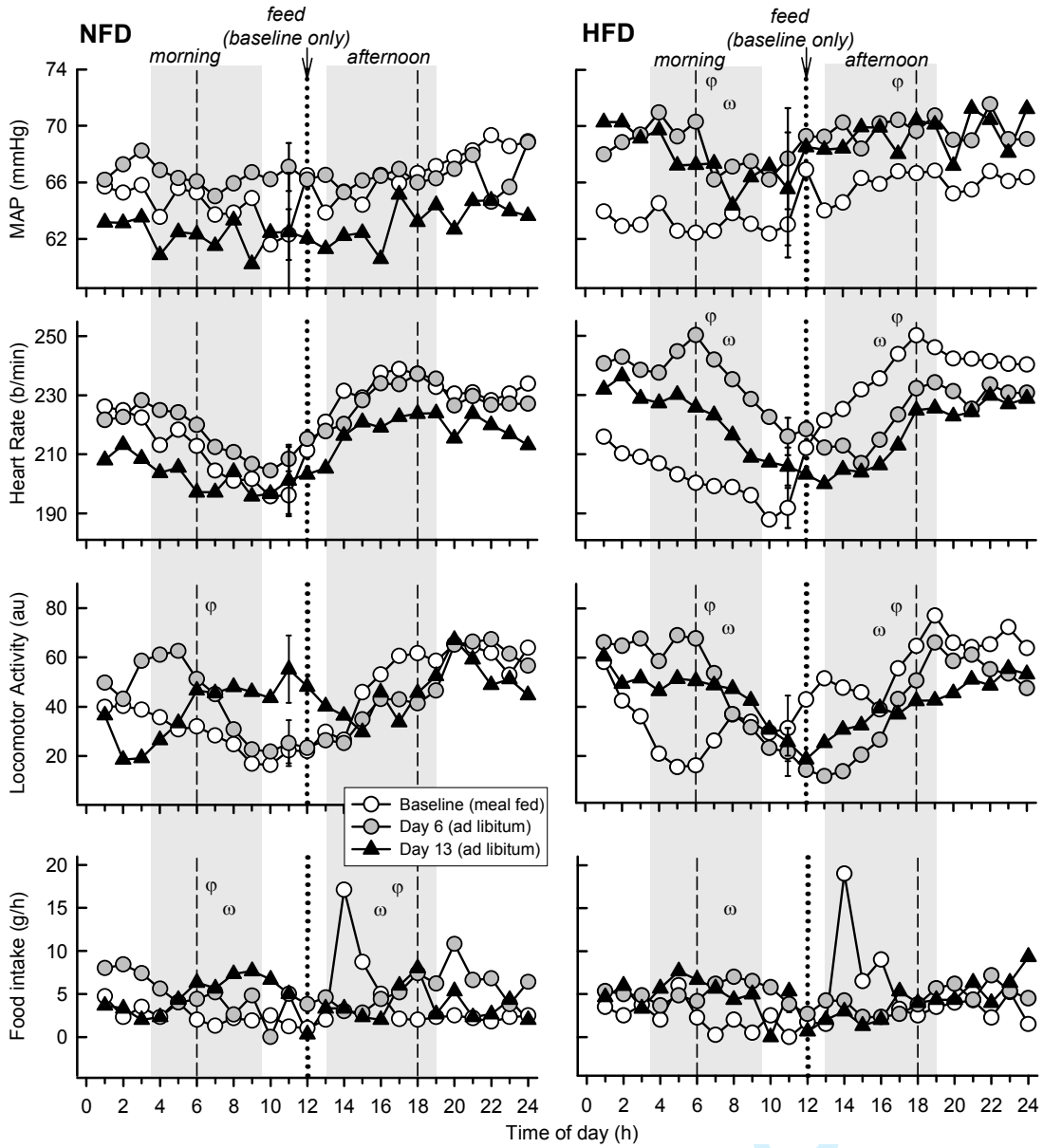

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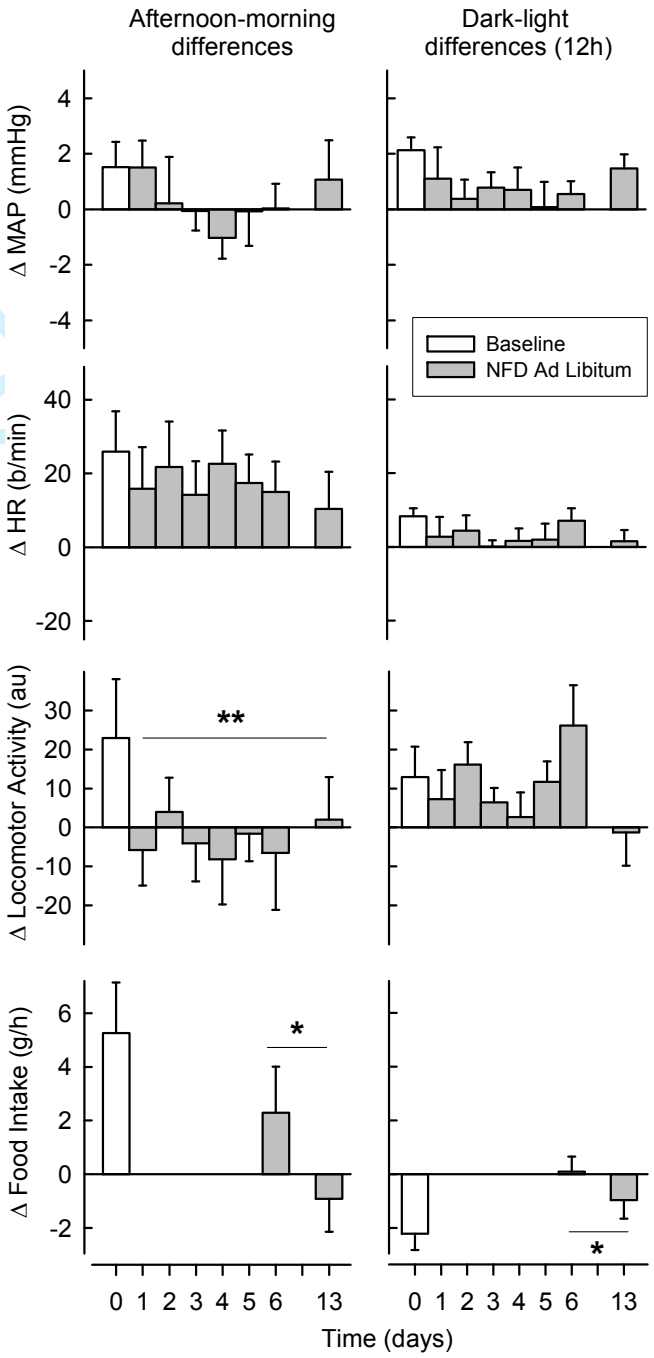

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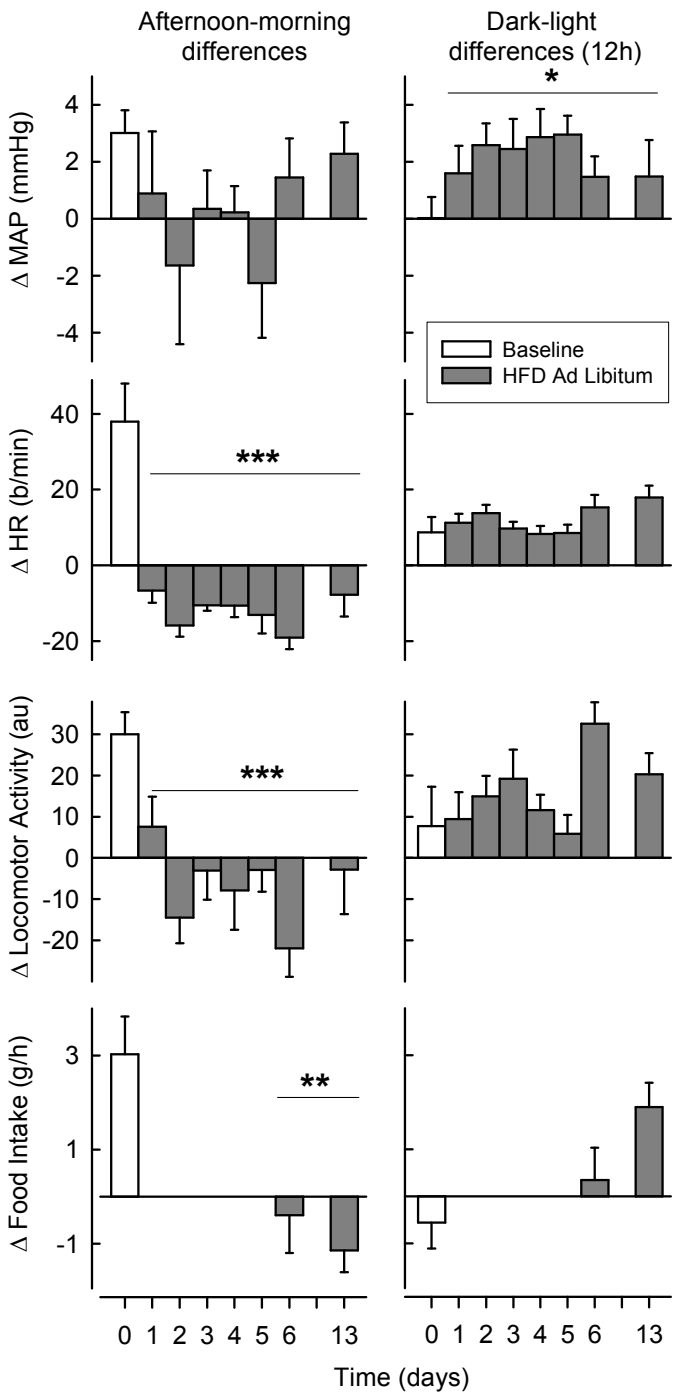

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