Dietary, Lifestyle and Pharmaceutical Interventions for the Treatment of Metabolic Diseases

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ABSTRACT

The global prevalence of obesity and type 2 diabetes increases and current treatment options are not satisfactory. This thesis will investigate the efficacy of novel dietary, lifestyle, and pharmaceutical interventions to treat these metabolic disorders.

In rodents, acute-isoleucine treatment lowers blood glucose levels and protects from diet-induced weight gain, suggesting chronic-isoleucine may be beneficial in treating obesity and type 2 diabetes. This study aimed to investigate chronic-isoleucine treatment on body weight and glucose tolerance in lean and obese mice. Male C57BL/6 mice, fed a standard-laboratory-diet (SLD) or high-fat-diet (HFD) for 12-weeks, were randomly allocated to 1) Control (water); 2) Acute (0.3 g/ kg isoleucine); 3) Chronic (1.5% isoleucine in drinking water for 6-weeks). At 17-weeks, mice underwent a glucose tolerance test (GTT). In SLD- and HFD-mice, there was no difference in body weight between treatment groups. Acute-isoleucine did not improve glucose tolerance in SLD- or HFD-mice. Chronic-isoleucine impaired glucose tolerance in SLD-mice. In conclusion, chronic-isoleucine supplementation was not effective for weight loss and glucose intolerance in mice.

Shift-work increases obesity risk partly through circadian desynchrony. In rodents, time-restricted feeding (TRF) during the dark-phase (DP) reduces weight gain and entrains the circadian rhythms of metabolic genes. Whether TRF during the light-phase (LP) is also effective is unknown. This study aimed to compare TRF-LP and TRF-DP on body weight and circadian rhythmicity of hepatic glucose (Slc2a2, Irβ & Gys2) and lipid (Acc1α) metabolic markers in lean and obese mice. Male C57BL/6 mice were fed a SLD or HFD for 12-weeks. After 4-weeks, mice were randomly fed to 1) *ad libitum* (AL); 2) during the LP (Zeitgeber (ZT)0-12); 3) during the DP (ZT12-24). In HFD-AL mice, energy intake, weight gain, fat mass, plasma lipids and mean blood glucose levels were elevated compared to SLD-mice. These parameters of obesity were similarly reduced in HFD-LP and HFD-DP mice compared to HFD-AL mice. In general, TRF during the LP or DP aligned circadian rhythms of hepatic markers of glucose and lipid metabolism to the timing of food intake.

Human glucagon-like-peptide-1 (GLP-1) is a short-acting, blood glucose-lowering hormone. Comparatively, monotreme GLP-1s are potent, long-acting GLP-1 receptor agonists, resistant to degradation in humans and mice, suggesting monotreme GLP-1s may be beneficial in treating type 2 diabetes. This study aimed to investigate monotreme GLP-1 treatment on glucose tolerance in lean and obese mice. Male C57BL/6 mice were fed a SLD or HFD for 14-

weeks. At 13-weeks, mice were randomly allocated to 7-groups and injected twice-daily with either 1) phosphate buffer (PB); 2) exendin-4 (Ex-4); 3) echidna GLP-1 (eGLP-1); 4) F8S; 5) platypus GLP-1 (pGLP-1); 6) N14S; or 7) S26K for 7-days. At 14-weeks, mice underwent a GTT. In SLD-mice, the glucose AUC was reduced in all treatment groups compared to PB controls. In HFD-mice, treatment with Ex-4, pGLP-1, N14S, and S26K reduced the glucose AUC compared to PB controls. In conclusion, monotreme GLP-1s may be effective in treating type 2 diabetes.

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PUBLICATIONS

Publications related to this thesis

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CHAPTER 1. General Introduction

LIST OF ABBREVIATIONS

In alphabetical order

AgRP	Agouti-related peptide
AMP	Adenosine monophosphate
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acid
BMAL1	Brain and muscle ARNT-like 1
BMI	Body mass index
CCK	Cholecystokinin
CLOCK	Circadian locomotor output cycles kaput
CPT1	Carnitine palmitoyl transferase 1
CRY	Cryptochrome
CVD	Cardiovascular disease
DALY	Disability-adjusted life year
DP	Dark phase
DPP4	Dipeptidyl peptidase 4
eGLP-1	Echidna GLP-1
Ex-4	Exendin-4
GABA	Gamma aminobutyric acid
GHSR	Growth hormone secretagogue receptor
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter

HbA1c	Glycated haemoglobin
HFD	High-fat diet
IRS1	Insulin receptor substrate 1
LH	Lateral hypothalamus
LP	Light phase
MC3/4R	Melanocortin 3 and 4 receptors
NAFLD	Non-alcoholic fatty liver disease
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OGTT	Oral glucose tolerance test
PER	Period
pGLP-1	Platypus GLP-1
PI3K	Phosphatidylinositol 3 kinase
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
SCN	Suprachiasmatic nucleus
SGLT1	Sodium-dependent glucose cotransporter 1
SLD	Standard laboratory diet
SOC3	Suppressor of cytokine signalling 3
STAT3	Signal transducer and activator of transcription 3
TRF	Time-restricted feeding

Nutrient metabolism is the process of converting food into the energy the body needs to sustain life. Dysregulation of this process can lead to a number of metabolic diseases, including obesity, diabetes, cardiovascular diseases (CVDs), liver and kidney diseases, as well as some cancers (1). Among them, obesity and type 2 diabetes are two of the most highly prevalent metabolic disorders, affecting around 39.1 % (2) and 8.5 % (3), respectively, of the global population. The current treatment strategies for these disorders are suboptimal due to the lack of efficacy or undesired side effects (4). Therefore, the development of new approaches to combat these diseases is required. This thesis investigated the effectiveness of three novel dietary, lifestyle and pharmaceutical interventions on obesity and type 2 diabetes.

1.1 Obesity

Obesity is caused by a number of factors, for example, a sedentary lifestyle and genetic susceptibility (4) in combination with chronic consumption of high-energy, low-nutrient foods, generates an energy imbalance, where energy intake outweighs energy expenditure (1,5). This, in turn, promotes weight gain and obesity (1,5). Obesity is often described as having an excessive amount of body fat (6). However, living with overweight or obesity is not just a cosmetic issue. Instead, obesity is associated with a variety of health issues (7), including back pain and arthritis (8). It also increases the risk of developing metabolic diseases, such as CVDs (9), non-alcoholic fatty liver disease (NAFLD) (10,11) and type 2 diabetes (9). In response to this information, and other evidence demonstrating the complex, progressive and relapsing nature of obesity (4,12), in 2013 obesity was officially recognised by the American Medical Association as a metabolic disease (13). A decision that has since been supported by many other medical associations (4,12).

Prevalence and burden of disease

According to the most recent data from the World Health Organisation, in 2016, the global prevalence of overweight and obesity in adults was 39.1 % (2). However, in upper-middle and high-income economies, as categorised by World Bank income, the prevalence of individuals with a BMI > 25 (overweight or obese) is much greater, at 44.3 % and 60.3 % respectively (2). Further, in Australia, ~67 % of adults were overweight or obese (35.6 % overweight and 31.3 % obese) in 2017-18 (14), and only 31.7 % had a healthy body weight (14). Obesity was previously considered an adult-onset disease (15). However, recent evidence has demonstrated that the prevalence of obesity in children and adolescents is rapidly rising. In 2016, ~124 million or 1 in 5 children or adolescents worldwide were overweight or obese (16), a number

which has rapidly risen from ~11 million in 1975 (16). This is particularly concerning as childhood obesity is a leading risk factor for developing non-communicable diseases later in life (16).

Obesity is associated with considerable health, economic and social burdens. In fact, obesity is considered one of the leading burdens to global health, behind war and terrorism, armed violence and smoking (17). In a 2014 report by the McKinsey Global Institute ('Overcoming obesity: an initial economic analysis'), the global financial impact of obesity was estimated at ~2 trillion dollars or ~2.8 % of the gross domestic product that year (a measure of the global economy representing the dollar value of all goods and services) (17). In 2005, in Australia, the annual health care cost was estimated at ~$1472 for individuals with a healthy body weight compared to ~$2788 for individuals with obesity (18). Overall, in 2005, the total direct cost of overweight and obesity to Australians was ~$18.8 billion (18). In addition to the increased financial burden, individuals living with obesity are at an increased risk of mortality and morbidity. In 2015, obesity was classified as the major underlying cause of death for ~4 million individuals globally, representing 7.1 % of all deaths that year (7). Further, in 2015, obesity contributed to ~120 million disability-adjusted life years (DALYs; a measure of the number of years lost due to ill health, disability or death), ~4.9 % of DALYs from any disease that year (7). In Australia, overweight and obesity attributed to ~312,500 DALYs in 2011, ~7 % of the total disease or injury burden in Australia that year (8).

Diagnosis of obesity

Body weight can be evaluated using many approaches, including absolute body weight, body fat percentage, waist-hip ratio, or social and aesthetic norms (6). However, obesity is most often diagnosed in clinical settings using the body mass index (BMI; body weight (kg)/ height (m^2)) (6). According to BMI, underweight and healthy body weight are classified by a score of less than 18.4 and between 18.5 to 24.9, respectively (19,20). On the other hand, overweight is classified as a BMI score of 25 to 29.9, and obesity is classified as a BMI score greater than 30 (19,20). Further, obesity is then subdivided into three distinct classes, class 1 refers to a BMI score of 30 to 34.9, class 2 or severe obesity is classified by a BMI score of 35.0 to 39.9, and class 3 or morbid obesity is classified by a BMI score greater than 40 (6,7,20).

Comorbidities of obesity

Obesity is associated with a wide variety of comorbidities. One study identified roughly 195 diseases or ailments related to obesity (7). A number of these comorbidities are non-fatal, such as back pain or arthritis (8). However, their impact on the quality of life is often significant (8). For example, a study investigating knee osteoarthritis in middle-aged women found that every 5 kg of body weight above a healthy BMI increased knee arthritis risk by 35 % (6,21). This was attributed to a ~60 % greater ground-reaction force experienced during walking in individuals with a high BMI than individuals with a healthy BMI (6).

Obesity is also strongly associated with an increased risk of developing other chronic metabolic diseases, including NAFLD (10,11), CVDs (9) and diabetes, specifically, type 2 diabetes (9). In particular, a high BMI score, waist circumference, or waist-to-hip ratio is strongly associated with an increased risk of type 2 diabetes (9,22). Similarly, obesity is also associated with NAFLD, the most common liver disease worldwide, impacting ~1 billion people (23), of which ~80 % had obesity (10,11). Further, several large-scale population studies (24,25) have identified an association between a high BMI score and CVD risk factors, including elevated blood pressure, elevated triglycerides, low high-density lipoprotein cholesterol and prediabetes.

One potential mechanism contributing to the link between obesity and other metabolic diseases is ectopic lipid deposition (Figure 1.1). Ectopic lipid deposition refers to the abnormal accumulation of lipid in non-adipose tissues leading to inflammation and impaired organ function (26). In humans, energy from food intake that is not immediately utilised as fuel for cellular processes is stored in energy reservoirs (adipose tissue) for later use (26). In response to a transient increase in energy intake, healthy adipose tissue expansion occurs where adipocytes grow in both size (hypertrophy) and number (hyperplasia), supported by an adequate blood supply, to accommodate increased energy storage (26,27). However, prolonged excessive energy intake significantly increases the amount and rate of adipocyte hypertrophy and hyperplasia (26). This results in the adipose tissue quickly outgrowing its blood supply, leading to tissue hypoxia and adipocyte injury (26,28,29). In turn, tissue injury encourages immune cell infiltration, such as macrophages, which secrete pro-inflammatory cytokines contributing to a chronic inflammatory state (28,30,31). This lipo-toxic environment promotes adipocyte mitochondrial stress, impairing mitochondrial glucose and lipid metabolism and consequently generates toxic metabolites, such as reactive oxygen species, which are linked to tissue-specific insulin resistance (32-34). The pathophysiology of insulin resistance in

peripheral tissues will be discussed in detail in later sections of this chapter. In adipose tissue, insulin resistance is linked with an increased release of free fatty acids and adipokines from adipocytes into the circulation (32). Non-adipose tissues, such as skeletal muscle and the liver, take up these fatty acids, accumulating as ectopic lipid depositions (32). In these tissues, ectopic lipid deposition promotes lipid-induced insulin resistance (33), and in the liver leads to hepatic steatosis (35,36) and NAFLD (10,11,37).

To summarise, there is a plethora of evidence that demonstrates obesity is closely linked with an increased risk of developing other metabolic diseases, such as type 2 diabetes, CVDs and liver diseases (22,24,25). In one systematic review, a meta-analysis of findings from ~432 publications, reports that for every standard deviation increase in BMI, the relative risk of incident diabetes is ~1.9 (95 % confidence interval: 1.6, 2.1) (22). However, this review also highlights that a relatively lower BMI (i.e. a healthy body weight) is associated with a relatively lower risk of diabetes (22). Similarly, another review demonstrates that consumption of a healthy diet in individuals with type 2 diabetes, reduces metabolic disease risk factors such as high plasma lipids, blood pressure and blood glucose (38). This evidence suggests interventions for obesity, such as dietary and lifestyle interventions, may also reduce the risk of developing the co-morbidities associated with obesity. This thesis will focus on obesity and type 2 diabetes, a major obesity-linked metabolic disease, and investigate new or improved strategies for treating these diseases.

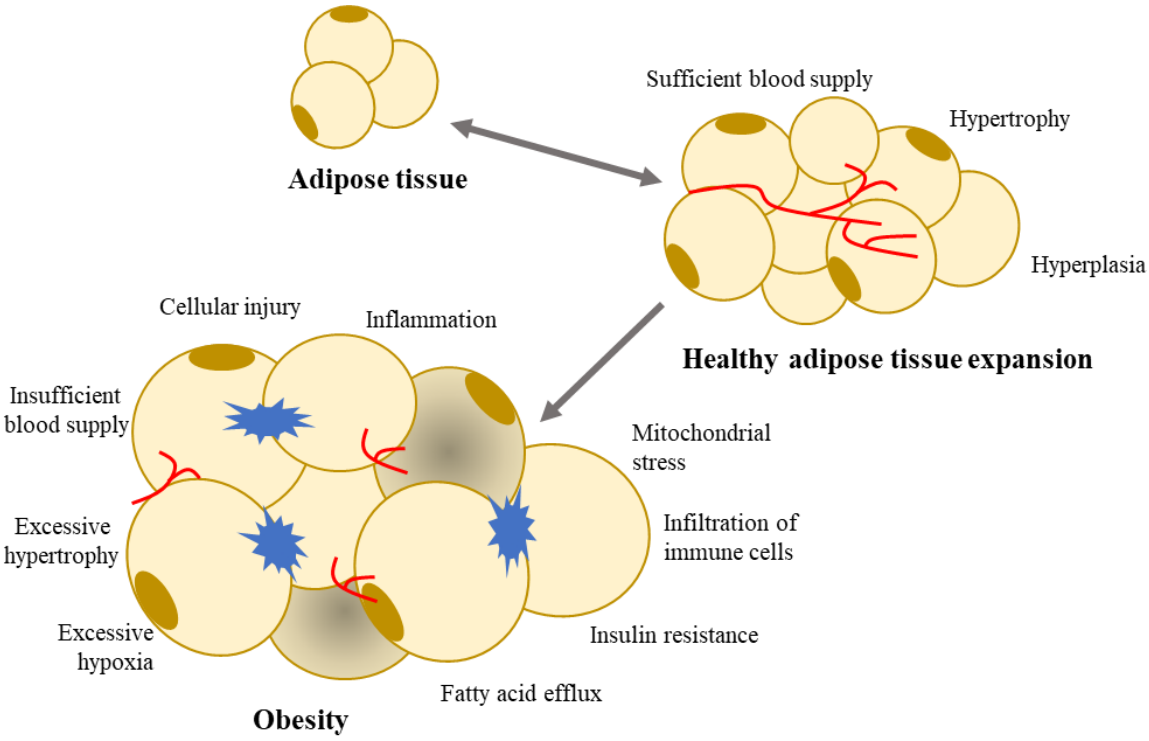

Figure 1.1 Altering adipocyte morphology in the progressive development of obesity. A transient increase in energy intake leads to healthy adipose tissue expansion, namely adipocyte hypertrophy and hyperplasia, supported by a sufficient blood supply (26,27). Constant high energy intake promotes excessive adipocyte hypertrophy and hyperplasia (26). Insufficient blood supply causes tissue hypoxia and adipocyte injury (26,28,29), promoting immune cells infiltration, such as macrophages, which secrete pro-inflammatory cytokines contributing to the progression of chronic inflammation (28,30,31). This lipo-toxic environment leads to adipocyte mitochondrial stress, local insulin resistance and release of free fatty acids into circulation (32,39). High plasma lipid concentrations stimulate free fatty acid flux into non-adipose peripheral tissues, including the liver and pancreas, ensuing obesity-induced inflammation, ectopic lipid deposition and progressive systemic insulin resistance (32).

1.1.1 Regulation of energy homeostasis in health and obesity

In humans, systemic energy homeostasis is a biological process that regulates long-term body weight and body composition by balancing energy intake and energy expenditure (1). Body weight is generally stable despite random daily fluctuations in food intake and exercise. However, chronic disruption of energy balance, where energy intake exceeds energy expenditure, can lead to obesity (1). For this reason, consumption of a high-calorie, low-nutrient dense diet and a sedentary lifestyle are critical risk factors for the development of obesity (4,16). There is also a wide variety of other factors, which influence energy intake and energy expenditure. For example, food intake may be influenced by mood, appetite, palatability, availability, access to cooking resources, and environmental or lifestyle factors, including season, culture or religion. Total energy expenditure involves 1) the energy expended to maintain bodily functions under resting conditions (basal metabolic rate); 2) the energy expended during physical activity; and 3) the energy required for adaptive thermogenesis, induced by nutrient digestion, absorption and metabolism (diet-induced thermogenesis) or by maintaining core body temperature during cold temperatures (cold-induced thermogenesis) (40-42). These energy expenditure parameters may also be influenced by mood, habituation, social cues or environmental factors such as the weather (40). Further, inside the body, there are a plethora of neural and hormonal signals, which influence energy metabolism, originating from peripheral metabolic tissues, including adipose tissue, the pancreas, the liver, skeletal muscle and the gastrointestinal tract. The relationship between these factors is summarised in Figure 1.2.

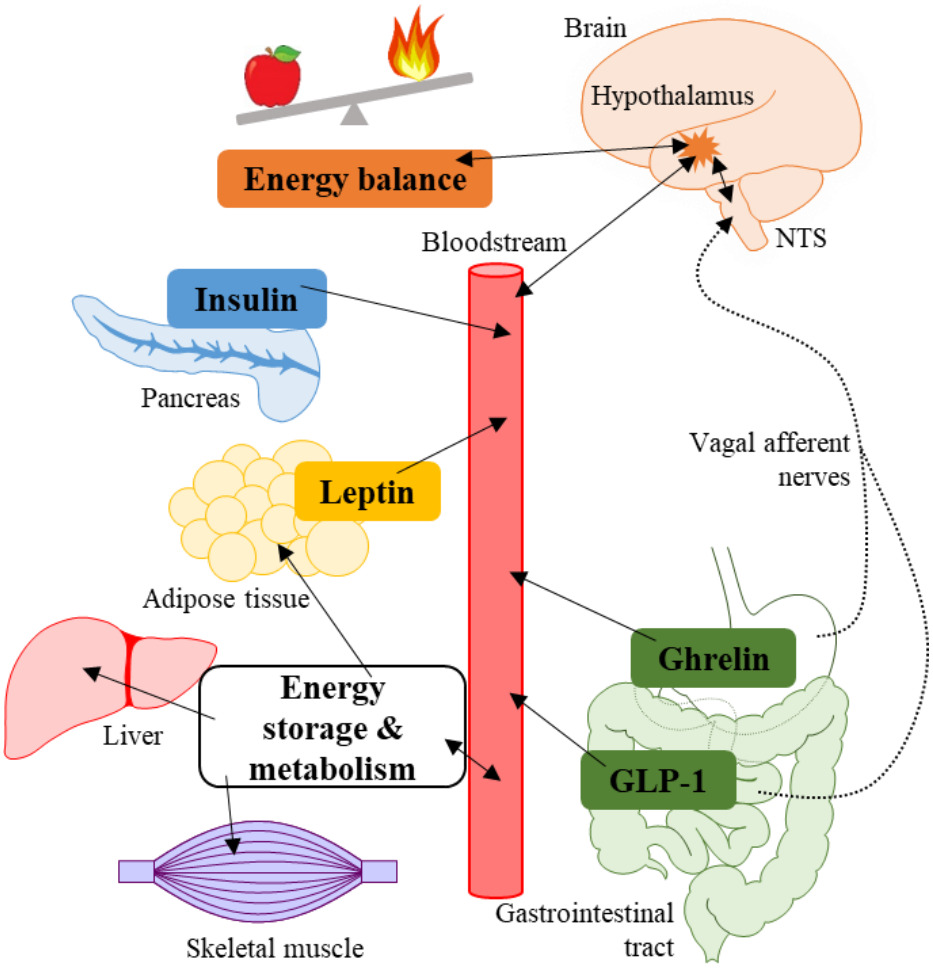

Figure 1.2 Regulation of energy balance via communication between the central nervous system and peripheral metabolic tissues. The hypothalamus receives, integrates and processes a wide variety of neural and hormonal signals originating from peripheral organs to regulate energy intake and energy expenditure (43). Neural signals include satiety signals via stimulation of vagal afferent nerves within the gastrointestinal tract (44). These vagal afferent nerves project to the nucleus of the solitary tract (NTS) located in the brainstem, which then communicates via neural circuits to the hypothalamus (43-45). Hormonal signals include appetite-regulatory hormones secreted from the gastrointestinal tract (e.g. ghrelin (46) and glucagon-like peptide-1 (GLP-1) (47)), insulin secreted from the pancreas (48) and leptin secreted from adipose tissue (49). Other peripheral tissues, such as the liver (50) and skeletal muscle (51), also contribute to systemic regulation of energy balance through energy storage and metabolism.

1.1.1.1 Central regulation of energy homeostasis

The influence of the central nervous system in the regulation of energy homeostasis is not the focus of this thesis. However, an appreciation of the central mechanisms involved in regulating energy homeostasis is important to understand whole-body energy homeostasis in health and in obesity. These concepts, therefore, will be briefly discussed in the section below.

Within the central nervous system, the hypothalamus is the primary site for the regulation of energy homeostasis. The hypothalamus receives, integrates, and processes various neural and hormonal signals originating from other regions in the central nervous system and periphery to regulate energy intake and energy expenditure (43). In particular, the hypothalamic areas associated with energy homeostasis include the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the dorsomedial nucleus, the ventromedial nucleus and the lateral hypothalamus (LH) (Figure 1.3) (43). This neural network also connects to other brain and spinal cord areas implicated in energy balance, including the nucleus of the solitary tract (NTS) located in the brainstem (43-45). These hypothalamic and associated regions form interconnected neuronal networks that sense fluctuations in energy intake and expenditure through neuronal, hormonal and nutrient signals (43). Overall, regulation of energy homeostasis is achieved by feedback communication between the central nervous system and the periphery through modulation of neuropeptide expression, circulatory hormone secretion and motor neuronal pathways (52).

The ARC is the most well-researched region of the hypothalamus (reviewed in (43,53-57)) and is located close to the median eminence, a neurohemal organ containing a capillary bed, which allows circulating hormones, including orexigenic (i.e. ghrelin) and anorexigenic (i.e. leptin or insulin) factors, to signal via activation of their receptors located within the hypothalamus (55). Within the ARC, two neuronal populations primarily govern the regulation of energy balance; the proopiomelanocortin (POMC)-expressing neurons and the agouti-related peptide/neuropeptide Y (AgRP/NPY)–expressing neurons (58,59). Evidence indicates activation of POMC-expressing neurons decreases food intake and increases energy expenditure, whereas activation of AgRP/NPY-expressing neurons increases food intake and decreases energy expenditure (59). These contrasting effects are achieved by neuropeptide-induced activation of receptors, such as melanocortin 3 and 4 receptors (MC3/4R), expressed in various regions of the hypothalamus and brainstem (59). For example, in response to anorexigenic signals, such as leptin and insulin, POMC-expressing neurons release α-

melanocyte-stimulating hormone, which targets MC3/4Rs located in the PVN and brainstem (59). In rats, stimulation of MC3/4R- containing neuronal terminals in the PVN and NTS via ventricular delivery of an MC3/4R agonist decreased food intake and elevated parameters of total energy expenditure, including core temperature, heart rate and activity levels (60). In rodent (61) and human studies (62,63), deletion of the MC3/4R or mutation of the POMC or MC3/4R genes resulted in severe hyperphagia and morbid obesity. In the case of NPY/AgRP-expressing neurons, stimulation by orexigenic signals, such as ghrelin, triggers the release of the neuropeptides NPY, AgRP and γ-aminobutyric acid (GABA). AgRP acts as an antagonist at MC3/4Rs in the PVN, blocking the action of α-melanocyte-stimulating hormone and increasing food intake (64). Similarly, the neurotransmitter NPY stimulates feeding behaviours in rodents by binding with Y1 and Y5 receptors in various hypothalamic and brainstem regions, including the PVN (64). In rodent studies, electrical activation of NPY/AgRP-expressing neurons in the ARC rapidly promotes food-seeking and eating behaviour, whereas ablation of these neurons or reduced electrical activity reduces food intake (65-67). There is also evidence of an inhibitory connection from NPY/AgRP-expressing neurons to POMC-expressing neurons in the ARC (59,65). In mouse brain slices, synaptic mapping revealed a direct connection from AgRP-expressing neurons and their axons to POMC-expressing neurons (65). In one study, selective activation of AgRP neurons strongly inhibited POMC neuron activity through release of the neuropeptide GABA (65). Chronic suppression of POMC neurons resulted in increased food intake in mice (65). However, it was also reported that direct excitation of POMC neurons was sufficient to overcome this AgRP neuron mediated inhibition (65). Overall, this evidence demonstrates the complexity of the counter-regulatory mechanisms employed to regulate feeding and maintain energy homeostasis.

Nutrient signals are also involved in the central regulation of energy homeostasis (56). In the brain, nutrients such as glucose and fatty acids are indicators of energy supply. For example, ARC AgRP-expressing neurons are inhibited by high glucose levels and activated by low glucose levels (68). Therefore, during periods of low energy, i.e. low glucose, AgRP-expressing neurons are activated, which stimulates appetite and food intake to restore energy homeostasis (68). This counter-regulatory mechanism is exemplified in a rat study where a central infusion of 2-deoxy D-glucose, a non-metabolisable glucose analogue that blocks central glucose utilisation, increased food intake through glucoprivation (69). Comparatively, the hypothalamic ARC region also contains glucose-excited cells, such as the POMC-expressing neurons (68). Glucose-excited cells sense high glucose levels and, in response,

suppress appetite and promote energy expenditure (68). There is also evidence that central fatty acid levels influence feeding behaviour (56,70). For example, in a rat study, the central infusion of oleic acid, a long-chain monounsaturated fatty acid, was demonstrated to suppress appetite and reduce hypothalamic NPY expression (70). This resulted in reduced food intake, limiting the additional delivery of fatty acids into the circulation (70).

In obesity, elevated central fatty acid levels are shown to impair hypothalamic signalling. Following chronic consumption of an obesogenic diet, high blood lipid levels contribute to an elevated amount of saturated fatty acids crossing the blood-brain barrier into the brain (57). Evidence demonstrates that high central fatty acids levels may stimulate reactive gliosis, the hypertrophy and proliferation of active tissue-resident macrophages, such as microglia. These accumulated immune cells secrete high levels of pro-inflammatory cytokines, which contribute to chronic low-grade inflammation in the brain, leading to endoplasmic reticulum stress (71) and dysregulated neuronal signalling pathways, including dysregulated central leptin and insulin signalling (57). For example, in mice, high-fat diet (HFD) feeding is demonstrated to elevate basal signal transducer and activator of transcription 3 (STAT3) protein activation in hypothalamic neurons (72). STAT3 is an essential signalling protein in the leptin-induced activation of POMC-expressing neurons (72). However, STAT3 also signals suppressor of cytokine signalling 3 (SOC3) expression, a negative feedback inhibitor of the leptin receptor (72). In obesity, enhanced basal STAT3 levels lead to upregulated SOC3 expression, inhibiting leptin-induced stimulation of POMC-expressing neurons and inducing central leptin resistance (72). This is consistent with reduced POMC gene expression observed in leptin-deficient (*ob/ob)* and leptin receptor-deficient (*db/db)* mice (73,74). In addition, SOC3 is also shown to inhibit insulin-induced activation of POMC-expressing neurons through the inhibition of the insulin receptor substrate 1 (IRS1) and phosphatidylinositol 3 kinase (PI3K) signalling pathway, thus contributing to central insulin resistance (75,76).

Obesity is also associated with the development of central ghrelin resistance (77). In obesity, central ghrelin resistance develops via a number of impaired mechanisms, including impaired ghrelin transport through the blood-brain barrier into cerebral circulation, reduced ghrelin receptor expression in hypothalamic neurons and inflammation-induced impairment of ghrelin intracellular signalling pathways (77,78). Together this leads to reduced ghrelin-induced activation of AgRP/NPY-expressing neurons and, therefore, reduced hunger sensations and the reduced orexigenic action of ghrelin (77). Paradoxically, there is also evidence that central

inflammation leads to spontaneous firing of AgRP/NYP-expressing neurons, stimulating hyperphagia and contributing to weight gain (57).

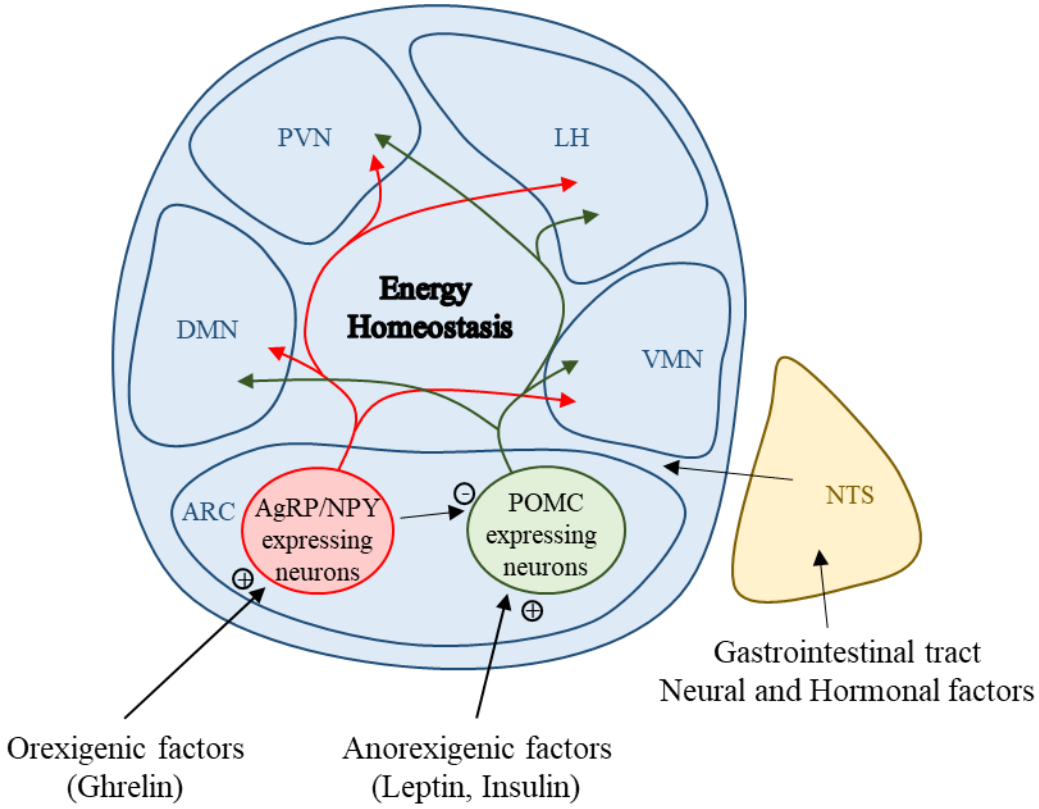

Figure 1.3 The major hypothalamic regions involved in energy homeostasis. The major hypothalamic areas associated with energy homeostasis include the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the dorsomedial nucleus (DMN), the ventromedial nucleus (VMN) and the lateral hypothalamus (LH) (43). This forebrain neural network also connects to other areas of the brain, including the nucleus of the solitary tract (NTS) located in the brainstem, which receives input from the gastrointestinal tract (43-45). Within the ARC, two neuronal populations primarily govern the regulation of energy balance; the pro-opiomelanocortin (POMC)-expressing neurons and the agouti-related peptide/neuropeptide Y (AgRP/NPY)-expressing neurons (58,59). Activation of POMC-expressing neurons by anorexigenic factors is demonstrated to activate receptors in various hypothalamic regions and the NTS, leading to decreased food intake and increased energy expenditure (59). Conversely, activation of AgRP/NPY-expressing neurons by orexigenic factors leads to increased food intake and decreased energy expenditure through simultaneous AgRP/NPY receptor activation and inhibition of POMC-expressing neurons (59).

1.1.1.2 Peripheral regulation of energy homeostasis

Peripheral organs, including adipose tissue, the pancreas, liver, skeletal muscle, and the gastrointestinal tract, also play essential roles in maintaining energy homeostasis (43). For example, a variety of hormones are secreted from adipose tissue, the pancreas and the gastrointestinal tract into the circulation, communicating information about energy stores (79), blood glucose levels (43) or food intake (80) to different organs around the body, including the brain. Other peripheral organs such as the liver and skeletal muscle also contribute to the maintenance of energy homeostasis through the modulation of energy expenditure (i.e. macronutrient storage and oxidation) (51). This will be discussed in the following sections.

Adipose tissue

It is well established excess energy from food intake is stored as fatty acids, primarily in adipose tissue (26). However, adipose tissue is not merely an inert energy reservoir but rather an endocrine organ capable of synthesising and secreting hundreds of bioactive molecules, many of which are involved in energy homeostasis (81). In particular, leptin is a vital adipokine involved in modulating energy homeostatic pathways including appetite and energy expenditure (56,79).

Leptin was the first adipokine discovered in 1994 (80,82) and has a long-established role in regulating energy homeostasis and long-term body weight. In particular, circulating leptin levels inform the hypothalamus about stored energy supplies. In healthy humans, circulating leptin levels fluctuate according to daily feeding and fasting patterns (83). High circulating leptin levels act as a signal of abundant energy supply to the hypothalamus during fed conditions, promoting central anorexigenic actions (54,79). Conversely, circulating leptin levels are low during fasting conditions, signalling a drop in stored energy levels (54,79). Low circulating leptin levels, similar to high circulating ghrelin levels, promotes central anorexigenic actions (54,79). However, in obesity, the daily fluctuation in circulating leptin levels are blunted and mean leptin levels are elevated (83,84). This is mainly due to the increased synthesis and secretion of leptin in proportion to the increased amount of adipose tissue in the obese body (84).

Circulating leptin is known to act centrally as an anorexigenic signal (43). Passing through the blood-brain barrier, circulating leptin enters the hypothalamic capillary network and acts upon leptin receptors expressed in several hypothalamic regions, including the ARC (56,79). In

particular, leptin receptors are highly expressed in both AgRP/NYP- and POMC-expressing neurons in the ARC (56,79). Activation of leptin receptors on AgRP/NPY-expressing neurons inhibits their firing, whereas activation of leptin receptors on POMC-expressing neurons stimulates their firing (79). Overall, central leptin signalling leads to the suppression of appetite and the promotion of energy expenditure through the activation of the sympathetic nervous systems (79). The activation of the sympathetic nervous system has been demonstrated to increase heart rate and blood pressure and trigger the secretion of hormones such as adrenaline and noradrenaline, which encourages physical activity (85). Leptin-induced excitation of the sympathetic nervous system has also been shown to increase thermogenesis in brown adipose tissue (79) and fatty acid oxidation in skeletal muscle (86), further contributing to increased energy expenditure (56,86).

Pancreas

The pancreas is an exocrine and endocrine organ involved in many physiological processes, including macronutrient metabolism, energy balance and blood glucose homeostasis. The majority of the pancreas is made up of exocrine cells, which secrete pancreatic juice (containing digestive enzymes such as amylase lipase and trypsinogen) into the pancreatic duct, which leads into the intestinal tract to facilitate food digestion (48). In contrast, pancreatic endocrine cells secrete hormones, including insulin and glucagon, directly into the circulation (48). Pancreatic insulin and glucagon play vital roles in blood glucose homeostasis, which will be discussed in a later section of this chapter. However, insulin also plays another crucial role in modulating energy homeostasis, and this will be discussed below.

Circulating insulin levels fluctuate in line with feeding and fasting patterns in healthy humans, elevated under fed conditions and diminished in response to a fast (48). Circulating insulin levels are generally higher in obese compared to lean individuals (54). This is partly due to a greater volume of insulin needed to facilitate glucose uptake into insulin resistant tissue to maintain glucose and lipid homeostasis in obesity (54). In addition, adipocytes are known to secrete certain adipokines, including leptin and adiponectin, which influence pancreatic β-cell function by enhancing insulin release and promoting β-cell survival (87,88). Since adipokine secretion is enhanced in proportion to the amount of adipose tissue in the body (54), there is a potential route by which increased adipokine secretion in obesity increases insulin secretion (87). The increased circulating insulin levels in obesity may also indicate diet-induced central (54) or systemic (32) insulin resistance.

Centrally, insulin is an important peptide involved in regulating energy balance and long-term body weight (89). In the brain, glucose uptake occurs via the highly expressed glucose transporters (GLUT)-1 and GLUT3 and is not dependent on insulin signalling (68). Comparatively, in the hypothalamic ARC region, insulin is shown to act on receptors expressed on both AgRP/NYP- and POMC-expressing neurons (54,76). Insulin activates POMC-expressing neurons, promoting α-melanocyte-stimulating hormone expression (54). Conversely, insulin inhibits AgRP/NYP-expressing neurons, suppressing AgRP and NPY expression (54). Overall, central insulin signalling suppresses appetite and promotes energy expenditure (54).

Liver

The liver is a major metabolically active organ with a wide variety of physiological functions, including the synthesis of many biomolecules necessary for digestion and growth and the detoxification of potentially harmful chemicals (53). The liver is also the central hub for digested nutrients following absorption from the gut into the portal circulation, where macronutrients (i.e. glucose, fatty acids and amino acids) and micronutrients (i.e. vitamins and minerals) are metabolised into biomolecules necessary for whole-body energy homeostasis (53). This section will briefly discuss the role of the liver in amino acid and fatty acid metabolism in health and obesity. However, hepatic glucose metabolism will be discussed in a later section of this chapter.

Hepatic amino acid metabolism

Dietary protein is digested in the gastrointestinal tract and absorbed as amino acids into the portal circulation (90). Amino acids are then extracted from the blood via the portal vein or hepatic artery into the liver for metabolism (90). In the liver, amino acids are substrates for the synthesis of transport proteins, glutathione, anti-inflammatory molecules, blood-clotting factors and conjugates for bile acids (90). All of these pathways either require or generate energy and, therefore, influence hepatic energy homeostasis (90). The liver is also the primary organ for amino acids degradation with the exception of branched-chain amino acids (BCAAs), which are predominantly catabolised in skeletal muscle (91). The metabolism of BCAAs in skeletal muscle will be discussed in detail within the literature review entitled “Is there a Role for Branched-Chain Amino Acid Supplementation in the Treatment of Obesity and Diabetes?”, which is included in a later section of this chapter.

In the liver, amino acids undergo either deamination (removal of the amino group) or transamination (transfer of the amino group) in the synthesis of other non-essential amino acids, glucose, pyruvate, lactate, glycerol and fatty acids (90). Excess nitrogen from these pathways, or from the uptake of ammonia released from other peripheral organs, is converted to urea for excretion through the hepatic urea cycle (90). In obesity, there is evidence that hepatic amino acid metabolism, especially the urea cycle, is impaired. For example, a prolonged hyperlipidaemia diet in rats caused a blockage in the urea cycle, preventing the elimination of excess nitrogen as urea (92). These rats also exhibited increased plasma amino acid levels (92), which may be considered a biomarker for prediabetes, insulin resistance and future type 2 diabetes (93,94). In addition, in humans with NAFLD, impaired hepatic amino acid metabolism is considered a biomarker for an increased risk of advanced liver failure and liver cancer (90,95).

Hepatic fatty acid metabolism

In the gastrointestinal tract, dietary lipids are digested into fatty acids, monoglycerides and dietary cholesteryl esters, which are absorbed into enterocytes then packaged into chylomicrons for transportation into lymphatic capillaries (96). From the lymphatic system to the thoracic duct, the chylomicrons enter the systemic blood circulation (97). Here exposure to the extracellular enzyme lipoprotein lipase on vascular endothelial cells results in the degradation and liberation of fatty acids from within the chylomicron (97). The fatty acids are then taken up by tissues and utilised for fuel or stored (97). The remaining chylomicron remnants are then taken up and metabolised by the liver (97). Circulating non-esterified fatty acids can also enter hepatocytes along a concentration gradient through diffusion or via membranous fatty acid transporters, such as fatty acid translocase or fatty acid transport protein (98). Finally, within hepatocytes, fatty acids are covalently bound to an acyl-CoA protein and channelled towards either complex lipid synthesis and storage (i.e. *de novo* lipogenesis) or metabolised for energy (i.e. fatty acid β-oxidation) (98).

Hepatic *de novo* lipogenesis describes the heavily regulated processes whereby new fatty acids are manufactured primarily from non-lipid substrates, including carbohydrate-derived acetyl-CoA (98-100). New fatty acid molecules are synthesised in the cytosol via a series of decarboxylative condensation reactions that attach a carbon chain, two carbons at a time, to a starting molecule, usually acetyl-CoA (98). The rate-regulating step in this pathway is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (98). Malonyl-CoA

then undergoes further transformation into palmitic acyl-CoA, facilitated by fatty acid synthase (98,101). The newly synthesised fatty acids are then converted into di- and triacylglycerol, packaged into lipoprotein particles and exported for transportation to other tissues and for storage in adipose tissue (102). *De novo* lipogenesis intermediates can also be used to synthesise cholesterol, transporter lipoproteins and lipokines, converted to bile acids or utilised as substrates for protein palmitoylation (101). Substrate availability and hormones, such as pancreatic insulin (33,103), glucagon, and catecholamine (98), modulate this *de novo* lipogenesis process via activation and inactivation of acetyl-CoA carboxylase and fatty acid synthase. For example, *de novo* lipogeneses may be switched on (i.e. acetyl-CoA carboxylase and fatty acid synthase activation) under postprandial conditions (i.e. high circulating glucose and insulin levels) to promote the storage of excess energy (100). Similarly, *de novo* lipogenesis may be switched off (i.e. acetyl-CoA carboxylase and fatty acid synthase inactivation) by hepatic glucagon or catecholamine signalling during fasting conditions (98,101), to conserve energy (100).

Fatty acid β-oxidation is fundamentally opposite to *de novo* lipogenesis. Hepatic fatty acid β-oxidation is the process of metabolising fatty acids for energy. This process occurs within cytoplasmic peroxisomes and in the mitochondria. However, the greatest yield of energy is generated by mitochondria β-oxidation (98). As previously mentioned, upon entry into hepatocytes, fatty acids are converted into fatty acyl-CoA molecules (98). This process is necessary to 'activate' the molecule and facilitate transport into the mitochondria for oxidation (98). Transportation across the mitochondrial membrane is carried out by the enzyme carnitine palmitoyl transferase 1 (CPT1) and is the rate-regulating step in this pathway (98,101). Inside the mitochondria matrix, the β-oxidation process involves a series of enzymatic reactions that remove segments of the fatty acid chain two carbons at a time (98). Incomplete oxidation of fatty acids produces ketone bodies (β-hydroxybutyrate, acetoacetate and acetone), which are exported from the liver in response to low blood glucose levels, to provide a fuel source to energy-sensitive tissues, such as the brain, heart and skeletal muscle (100). Comparatively, complete fatty acid oxidation results in the formation of acetyl-CoA molecules that feed into the tricarboxylic acid cycle, fuelling the electron transport chain that drives energy (ATP) synthesis (98). Arguably, the main driver of hepatic fatty acid β-oxidation is substrate supply. Under fasting conditions, fatty acids released from adipose tissue through lipolysis are taken up into the liver, promoting β-oxidation (100). In addition, pancreatic glucagon signalling is demonstrated to activate transcriptional regulators of β-oxidation, including peroxisome

proliferator-activated receptor-α, which increases the mRNA expression of CPT1 (100,104). Conversely, the β-oxidation process is downregulated during the fed state. Specifically, CPT1 activity is allosterically inhibited by malonyl-CoA, an intermediate product of *de novo* lipogenesis (98). This fine regulation prevents a futile cycle of simultaneous synthesis and oxidation of fatty acids within hepatocytes (98,100).

In obesity, hepatic lipid metabolism is often impaired. Animal studies demonstrate that prolonged consumption of an obesogenic diet, such as *ad libitum* HFD feeding, leads to weight gain and fat accumulation in adipose and non-adipose tissues, including the liver (35,105,106). As previously mentioned, this abnormal and toxic hepatic lipid accumulation is a hallmark of NAFLD and precursor to liver cirrhosis and hepatocellular carcinoma (10,11). In a rodent study, HFD feeding for as little as 2.5 weeks was sufficient to induce hepatic lipid deposition and accumulation of toxic metabolites due to impaired mitochondrial lipid oxidation, including acylcarnitines and reactive oxygen species (106). In response to 25 weeks of HFD feeding, the rate of fatty acid β-oxidation was increased by ~60 % in these rodents, measured by respiratory quotient (106). However, this was insufficient to combat the dietary fatty acid overload, evidenced by the continued increase in hepatic lipid deposition (106). Consistent with this, other studies report significant increases in the expression of CPT1 and other β-oxidation enzymes in HFD fed mice and rats compared to SLD fed controls (107,108). In addition, the mRNA expression of lipid synthesis enzymes, namely acetyl-CoA carboxylase (108,109) and fatty acid synthase, are also reportedly elevated in HFD fed rodents compared to SLD fed controls (108). Further, in humans with obesity and type 2 diabetes, the rate of *de novo* lipogenesis is ~5 fold greater than in healthy individuals (101). Moreover, the rate of *de novo* lipogenesis is ~3 fold greater in individuals with NAFLD compared to BMI matched controls (110).

Skeletal Muscle

Skeletal muscle plays a vital role in energy homeostasis through energy expenditure, particularly the flexible utilisation of either glucose, fatty acids, or amino acids for energy to perform muscle contraction (53). Skeletal muscle and cardiac muscle are major energy-consuming peripheral organs, contributing to ~30 % of resting energy expenditure and ~100 % exercise-related energy expenditure (51). During the fed state, skeletal muscle predominantly oxidises glucose for energy, facilitated by insulin-stimulated glucose influx and activation of glycolysis enzymes (33). The mechanism of insulin signalling in skeletal muscle will be

discussed in detail in a later section of this thesis. Comparatively, during fasting, skeletal muscle predominantly oxidises fatty acids for energy (111,112). Thus, fatty acid availability is a prominent signal initiating β-oxidation in skeletal muscle (112). For example, circulating fatty acid levels increase when aerobic exercise is performed partly due to catecholamine-induced lipolysis (113). This increased substrate availability contributes to a ~60-75 % increase in whole-body fatty acid oxidation during exercise, revealed by increased respiratory quotient measurements in healthy individuals (112). This fatty acid oxidative capacity can be enhanced through exercise training in lean athletes (112). However, no beneficial change in the rate of skeletal muscle fatty acid oxidation is observed in individuals with obesity, despite increased lipid accumulation, suggesting signal transduction is impaired in obesity (112,114).

Amino acids, specifically surplus BCAAs, can also be oxidised for energy within skeletal muscle (115). Specifically, the BCAA are oxidised into metabolic intermediates that filter into the tricarboxylic acid cycle, a vital component of mitochondrial energy production (115,116). This process of BCAA oxidative catabolism in skeletal muscle will be discussed in the literature review entitled "Is there a Role for Branched-Chain Amino Acid Supplementation in the Treatment of Obesity and Diabetes?" included in a later section of this chapter.

Myofibril composition has also been shown to influence the pattern of nutrient oxidation in skeletal muscle and thus influence total energy consumption (51). Skeletal muscle is generally composed of both slow and fast-twitch myofibril types. Slow-twitch fibre types are slow to fatigue, have a high oxidative capacity and prefer fatty acids as a fuel source (51). Whereas fast-twitch fibre types have a greater contractile strength but fatigue more quickly, have a lower oxidative capacity and prefer glucose as a fuel source (51). In healthy but sedentary individuals, the ratio of fast to slow-twitch fibre types is approximately equal (117). However, there is evidence that performing endurance or aerobic exercise increases the metabolic demand on skeletal muscle resulting in increased muscle mass and a greater proportion of slow-to-fast-twitch myofibrils (118). This results in an increased glycolytic capacity of skeletal muscle and an increased basal metabolic rate (51). In individuals with obesity, who typically have low physical activity levels, fast-twitch myofibrils predominate, which have a relatively lower glycolytic capacity resulting in reduced resting energy expenditure (51).

Gastrointestinal tract

The gastrointestinal tract plays an important role in the regulation of food intake. This depends on the communication between the gut and brain (43-45), which involves neural innervation (119) and gut hormones (80).

Neural innervation

The gastrointestinal tract possesses an intrinsic nervous system, known as the enteric nervous system, which allows the gut autonomy over gut functions, including digestion, nutrient absorption and waste elimination (120). In addition, the central nervous system communicates with the gut, at least in part, via spinal and vagal neural pathways to oversee and modulate these gut functions (120). The major parasympathetic nerves innervating the gut are the vagal nerves (119,120). The vagus is also a conduit for vagal afferent nerves, which convey information from the gut to the brain and play a role in the short-term regulation of food intake (121). They have cell bodies located in the nodose ganglion, with axons projecting towards the gastrointestinal tract as well as the NTS in the brainstem (45,121). The peripheral endings of vagal afferents can detect food-related stimuli, including mechanical and chemical signals (44,121), and transfer these signals to the central nervous system, specifically the NTS, to regulate appetite and food intake (43,54). The vagal afferent endings densely innervate the proximal gut, namely the stomach and proximal small intestine and gradually diminish towards the distal small intestine and large intestine (45,121).

Mechanosensitive vagal afferents innervating the stomach and small intestine are particularly important in the control of food intake. In the stomach, there are two types of mechanosensitive vagal afferents, tension receptors and mucosal receptors (45,121-123). They have two types of afferent endings, intraganglionic laminar endings, located between circular and longitudinal muscular layers, observed throughout antrum, fundus and corpus (121), or intramuscular arrays, located along muscle layers, primarily near the pyloric antrum and cardiac sphincter (121). Tension receptors respond to food-induced gastric distension and transfer the information to the brain, generating feelings of satiety and fullness and leading to the termination of a meal (121,122). Mucosal receptors are responsive to gentle luminal stroking but not gastric distension. They are believed to recognize food particle size and delay gastric emptying in response to increased mechanical stimulation, thereby controlling the rate of gastric emptying (122). Gastric emptying refers to the rate at which chyme empties from the stomach into the duodenum (124). A slower rate of gastric emptying results in chyme remaining in the stomach longer (124), increasing the opportunity to stimulate vagal tension

receptors, contributing to increased satiety signals (45,80). Additionally, a slower rate of gastric emptying results in a slowed flow of luminal contents through to the intestines (124), optimising digestion and absorption of nutrients (80) and increasing the likelihood that specialised enteroendocrine cells within the intestinal mucosa will sense luminal nutrients and, in response, secrete appetite-regulatory hormones (80). In the small intestine, mechanosensitive vagal afferent receptors, both tension (located in intraganglionic laminar endings) and mucosal subtypes are observed in high density in the proximal small intestine, especially around the pylorus, but are also observed throughout the length of the small and large intestines (121). The mechanosensitive vagal afferents in the small intestine can sense food-related distension and sends satiety signals to the brain to inhibit food intake (121). In mice, optogenetic stimulation of tension receptors predominantly innervating the duodenum resulted in a significant reduction in food intake, which re-bounded shortly after termination of the stimulus (121).

Chemosensitive vagal afferents also play important roles in regulating food intake, especially in the small intestine, through their response to appetite-regulatory hormones secreted in response to nutrient activation of nutrient sensors on specialised cells within the gastrointestinal epithelium (121). Once secreted, these hormones, such as glucagon-like peptide-1 **(**GLP-1) and cholecystokinin (CCK), can directly act on adjacent vagal afferent endings transmitting information to the NTS on the nutrients consumed (121,125). The satiating effects of appetite-regulatory gut hormones will be discussed further in the section below.

Vagal afferents can also sense microbiota associated factors and transfer these signals to the brain to regulate food intake (126). Evidence demonstrates that the colonisation and survival of specific microbiota depends on the nutrient composition of food. For example, *Prevotella* prefers high-carbohydrate diets, *Bifidobacteria* prefers high-fibre diets, and *Bacteroidetes* prefers diets high in animal protein and fats (127,128). To optimise their own survival, gut microbiota are known to manipulate their host's eating behaviour (126). This can be achieved through a variety of mechanisms, including the secretion of factors, such as toxins, peptides and short-chain fatty acids that hijack the vagal nerve pathway and manipulate central mood, reward and appetite pathways to influence food intake (126). For example, in mice, dietary supplementation with *Lactobacillus* resulted in appetite suppression and reduced food intake, attributed to reduced AgRP and NPY expression in the hypothalamus (129). This effect was due to the increased manufacture of the short-chain fatty acid butyrate by the *Lactobacillus*

microbiota, which promoted the release of GLP-1 from intestinal L-cells (129). Subsequently, GLP-1 via the vagal afferent signalling pathway suppresses appetite (129).

In obesity, the vagal afferent satiety signals are disrupted (122,125,130). In HFD-induced obese mice, the mechanosensitivity of gastric tension receptors is reduced, leading to reduced satiety signals and increased energy intake (122,130,131). The mechanosensitivity of gastric tension receptors in mice displays circadian rhythmicity, with peak sensitivity during light phase (LP; the resting phase of mice), and nadir during dark phase (DP; the active phase of mice) (131). In obese mice, this circadian rhythm is disrupted, specifically during the LP consistent with the increase in food intake during the LP in these mice (131).

Gut hormones

More than 100 hormones have been shown to be expressed in the gastrointestinal tract (132). Many of these hormones are involved in regulating food intake, either via activation of vagal afferent endings or via the circulation to influence the brain's appetite regulatory centres (80). Some of these hormones and their effects on appetite regulation are summarised in Table 1.1.

Table 1.1 Gastrointestinal hormones involved in appetite regulation

Peptide	Food Intake	Mechanisms of action	References
Ghrelin	↑	Central orexigenic factor. Receptors in pituitary and hypothalamus. Stimulates hunger. Stimulates reward-associated feeding behaviour. Stimulates gastrointestinal motility.	(46,132-134)
Leptin	↓	Central anorexigenic factor. Receptors in hypothalamus and stomach. Suppresses appetite. Stimulates vagal afferent sensitivity. Promotes energy expenditure.	(132,135,136)
Glucagon-like peptide-1	↓	Central anorexigenic factor. Receptors in hypothalamus and peripheral organs. Suppresses appetite. Slows gastrointestinal motility. Stimulates insulin secretion.	(80,137-139)
Peptide Tyrosine Tyrosine	↓	Central anorexigenic factor. Receptors in hypothalamus. Suppresses appetite. Slows gastrointestinal motility.	(132,137,140-143)
Oxyntomodulin	↓	Central anorexigenic factor. Receptors in hypothalamus and peripheral organs. Suppresses appetite. Stimulates insulin secretion.	(80,144-146)
Cholecystokinin	↓	Central anorexigenic factor. Receptors in hypothalamus and peripheral organs Suppresses appetite. Slows gastrointestinal motility. Stimulates bile and pancreatic juice secretion.	(147-149)

↑ Designates an increased effect; ↓ designates an inhibitory effect.

Ghrelin

Ghrelin is the primary gut hormone that stimulates appetite (132). It is secreted from X/A like-cells in the stomach, predominantly located in the gastric fundus and antrum mucosa (150). In humans, circulating ghrelin levels peak during fasting conditions and diminish in response to food intake (46,132). Circulating ghrelin levels are also inversely associated with body fat; thus, circulating ghrelin levels are generally lower in individuals with obesity than lean (151). The receptor for ghrelin, growth hormone secretagogue receptor (GHSR), is expressed in the central nervous system and many peripheral tissues, including the stomach, intestines, pancreas and kidney (151). Acting upon GHSRs in the gastrointestinal tract, ghrelin enhances gastrointestinal motility and stimulates gastric juice secretion in preparation for food intake and to facilitate digestion and absorption of nutrients (152,153). In the hypothalamus, GHSRs are expressed on AgRP/NYP-expressing neurons (154). Their stimulation leads to increased AgRP and NYP expression, accompanied by increased food intake and resulting weight gain (133,134,155). Ghrelin is also shown to stimulate growth hormone-releasing hormone-expressing neurons in the pituitary (156). This leads to the release of growth hormone into the circulation, which promotes hepatic glucose production and fatty acid β-oxidation and suppresses glucose oxidation to conserve energy during an energy deficit (156). There is also evidence that central ghrelin signalling stimulates reward-associated feeding behaviour pathways, linking pleasure and other positive emotions to food intake, thus promoting food craving behaviour (53).

Cholecystokinin

CCK was one of the first appetite-regulatory gut hormones discovered and shown to suppress food intake (147,157). CCK is secreted from I-cells in the mucosa of the duodenum, jejunum, and proximal ileum (148), in response to digested protein and lipid, and to a lesser extent, carbohydrate (147). Receptors for CCK are expressed in the brain and peripheral tissues, including vagal afferents, stomach, intestine, gall bladder and the pancreas (147,148). In these peripheral organs, CCK is shown to slow gastric emptying (149,158) and promote the release of bile and pancreatic digestive enzymes into the duodenum to optimise nutrient digestion and absorption (147,159). In the brain, CCK has been shown to suppress appetite by activating receptors expressed on orexin-expressing neurons in the LH (148). Orexin (also known as hypocretin) is a potent neuropeptide usually expressed in a circadian manner to stimulate arousal, wakefulness, food-seeking behaviour and appetite during the active phase (160). In a

brain slice from orexin/ YC2.1 transgenic mice, calcium imaging revealed that infusion of CCK led to activation of orexin-expressing neurons via a CCK receptor-mediated mechanism (161). The CCK-induced stimulation of orexin-expressing neurons downregulates orexin release, therefore suppressing orexin-induced appetite and food-seeking behaviours (148,161).

Glucagon-like peptide-1

GLP-1 is another prominent appetite-regulatory gut hormone shown to suppress food intake (132). In humans, GLP-1 is cleaved from the proglucagon protein encoded by the preproglucagon gene, which is expressed in many tissues, including the pancreas and the brain. However, GLP-1 is predominantly produced in the gut (47,80). In response to digested carbohydrates, lipids (162,163) and proteins (164-166), GLP-1 is secreted from enteroendocrine L-cells located in the distal intestine, namely the ileum and colon (47,80). GLP-1 secretion can be directly induced via the detection of luminal nutrients by membranous chemoreceptors on L-cells (167). In addition, neuronal or hormonal signals can indirectly trigger secretion via vagal nerve signalling or the paracrine effects of other gut hormones (reviewed in (168)). Once secreted from L-cells, GLP-1 can either generate paracrine effects by acting upon receptors on nearby cells or vagal afferents endings in the gut (167) or enter the portal circulation to act upon receptors in other tissues (168). GLP-1 has been shown to slow gastric emptying in the gastrointestinal tract to optimise nutrient digestion and absorption (80). In the pancreas, GLP-1 stimulates insulin release from β-cells and promotes β-cell biogenesis (168). GLP-1 functions as an anorectic peptide in the brain, reducing feelings of hunger and inhibiting food intake (169,170). This satiety-inducing effect is due to the activation of GLP-1 receptors expressed on POMC-expressing neurons in the hypothalamic ARC, which leads to suppression of appetite and food intake (171). Activation of POMC-expressing neurons also triggers the release of the neurotransmitter GABA which inhibits the activity of AgRP/NPY-expressing neurons, further suppressing food intake (171). These direct central effects are likely accomplished by central GLP-1 as peripherally secreted GLP-1 is rapidly degraded within the circulation before reaching the cerebral circulation (168).

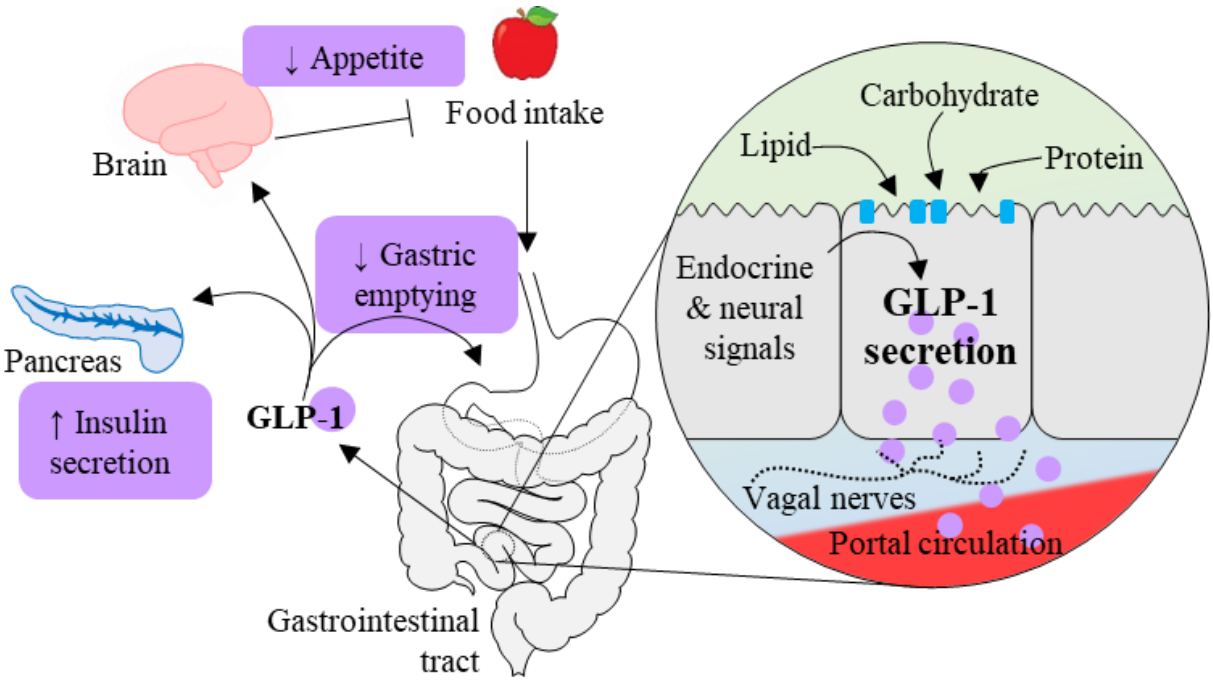

Figure 1.4 Food intake-induced GLP-1 secretion from the gastrointestinal tract and its physiological functions. The gastrointestinal hormone GLP-1 is secreted from enteroendocrine L-cells in the distal intestine in response to dietary carbohydrate, lipid (162,163) and protein (164-166), or via neural and hormonal feedforward signals (168). Once secreted, GLP-1 stimulates vagal afferent nerves (167) or enters the portal circulation to activate GLP-1 receptors expressed in the central nervous system and other peripheral tissues, including the stomach and pancreas (168). In the gastrointestinal tract, GLP-1 slows gastric emptying aiding nutrient digestion and slowing glucose absorption (80,168,170). In the pancreas, GLP-1 functions as an insulin secretagogue (167,168). GLP-1 also affects the brain directly via the blood circulation or indirectly via vagal afferents to generate satiety signals and inhibit food intake (169,170).

1.1.2 Influence of the circadian system on energy homeostasis in health and obesity

Through the evolutionary process, most organisms, including mammals, have developed an intrinsic regulatory system known as the central circadian clock, which entrains daily rhythms in physiology, behaviour and metabolic processes, such as the sleep-wake cycle with the 24 hr solar cycle (172-174). This master clock is located in the suprachiasmatic nucleus (SCN) and receives neural input from the retina to ensure alignment with the light-dark cycle (175). The rhythms in food intake are closely linked to the light-dark cycle, with the majority of food consumed in the natural active phase in humans and animals. For example, in nocturnal *ad libitum* fed standard laboratory diet (SLD) rats and mice, ~60-80 % of their daily food intake is consumed during the DP (176-179). The central clock also acts as a pacemaker, regulating the timing of secondary clocks located in almost all cell types in mammals (180), including peripheral organs, such as the liver, skeletal muscle, pancreas and adipose tissue, as depicted in Figure 1.5 (175,181). The synchrony between the central and peripheral clocks coordinates gene expression for opposing anabolic and catabolic energy metabolism pathways, preventing futile cycles (182,183). Furthermore, in peripheral organs, the diurnal rhythmicity of metabolic gene expression is also influenced by the timing of food intake (184). Evidence demonstrates that abnormal food intake patterns can lead to dysregulation of circadian rhythms in peripheral tissues causing metabolic disease (185-187).

Molecular components of the circadian clock

The circadian clock is a network of auto-regulatory gene transcriptional–translational feedback loops, driving the rhythmic expression of core-clock components (Figure 1.5) (188). The protein products of which, in turn, regulate the rhythmic gene expression of proteins involved in metabolic homeostasis, endocrine activity and neural excitability (180,188). In brief, the positive arm of the circadian clock involves the transcription of the prominent clock genes circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-Like 1 (BMAL1) (189). The protein products of CLOCK and BMAL1 heterodimerise and promote the transcription of clock genes within the negative arm of the circadian clock, such as cryptochrome (CRY) 1 and 2 and period (PER) 1 and 2 (189). Over the course of a day, the CRY and PER protein levels accumulate in the cytoplasm until they translocate into the nucleus and suppress transcription of CLOCK and BMAL1 in a negative feedback loop (180,189). E3 ubiquitin ligase complexes regulate the degradation of CRY and PER proteins, and once complete, the positive arm is free to restart the cycle (175,180). This core CLOCK-

BMAL1/PER-CRY loop takes about 24 hr to complete and is interlocked with numerous other feedback loops regulating the oscillations of metabolic genes (175).

Circadian control of energy homeostasis

Circadian control of energy homeostasis is influenced by neural projections between the SCN and the hypothalamic ARC (190). In rats, a distinct circadian rhythm was observed in the firing activity of appetite-inhibitory POMC-expressing neurons, with peak levels at the end of the DP (active phase) to suppress food intake during the LP (rest phase) (191). Bilateral SCN lesion in these rats abolished the diurnal rhythmicity of POMC neurons activity and food intake, demonstrating time-dependent appetite regulation by the central circadian clock (190). Similarly, the diurnal rhythms in food intake were dysregulated by constant light exposure in rats due to persistent SCN-derived GABAergic inhibition of POMC neurons leading to increased food intake, reduced energy expenditure and increased body mass (190,191).

Circadian feeding and locomotor behaviour patterns are also influenced by a link between the central circadian clock and orexin-expressing neurons in the LH (160,174). The LH is involved in various physiological functions such as arousal, stress, reward and motivated behaviours (i.e. food-, drugs- and sex-seeking behaviours) through the release of the neurotransmitter orexin (160). There is no direct synaptic link between orexin-expressing neurons in the LH and the SCN (160). However, there is evidence orexin is released rhythmically and is linked with the promotion of appetite and food-craving behaviour during the active phase (160). In mice, exposure to darkness during the LP caused a lack of orexin inhibition leading to increased food-seeking activity during the LP (192). In another mouse cohort, constant light exposure across 24 hr resulted in reduced total orexin expression in the LH and subsequently arrhythmic locomotor activity patterns (192). This evidence demonstrates that dysregulation of the central clock via altered photo-entrainment leads to a dysregulated feeding behaviour pattern (160).

Metabolic consequences of circadian desynchrony

Dysregulation of circadian rhythms (also referred to as circadian desynchrony) is associated with metabolic disease and can be caused by various dietary and lifestyle factors. For example, circadian desynchrony was induced in rodents by *ad libitum* HFD feeding (179,193), forced activity or feeding during the rest phase (186,194), or by exposure to altered light-dark cycles (195,196) leading to arrhythmic feeding behaviour where rodents ‘graze’ throughout the LP and DP. In the liver, desynchronised activity and food intake patterns cause dysregulation of

the daily oscillations of core clock components. In rats and mice, forced activity and feeding during the LP, modelling night-shift work, caused the circadian rhythm of the hepatic core clock components PER, CLOCK and BMAL1 to invert their peak expression phase (186,197). Circadian desynchrony was accompanied by increased total body weight and percentage fat in these rodents (186,197). Furthermore, desynchronised activity and food intake patterns cause circadian desynchrony of hepatic genes involved in nutrient metabolism, thereby dysregulating hepatic energy homeostasis. In SLD- and HFD-mice, exposure to an altered light-dark cycle caused dysregulation of hepatic lipid metabolism, i.e. increased mean expression and lost circadian rhythmicity of the *de novo* lipogenesis enzyme hepatic acetyl-CoA carboxylase (198). Impaired hepatic lipid metabolism in these mice led to obesity and increased hepatic and plasma lipid levels; biomarkers of NAFLD (198).

Circadian desynchrony has also been observed in humans. In current lifestyles, food intake and physical activity often occur across the 24 hr solar light cycle (199). This is particularly true for shift workers (200) who, despite having similar energy intake compared to individuals working 'normal' daylight hours (200), are at an increased risk of obesity and metabolic diseases (185,187,201). Furthermore, the risk of obesity and metabolic disease is higher in permanent night shift workers than individuals exposed to casual or rotating shift work (187,201).

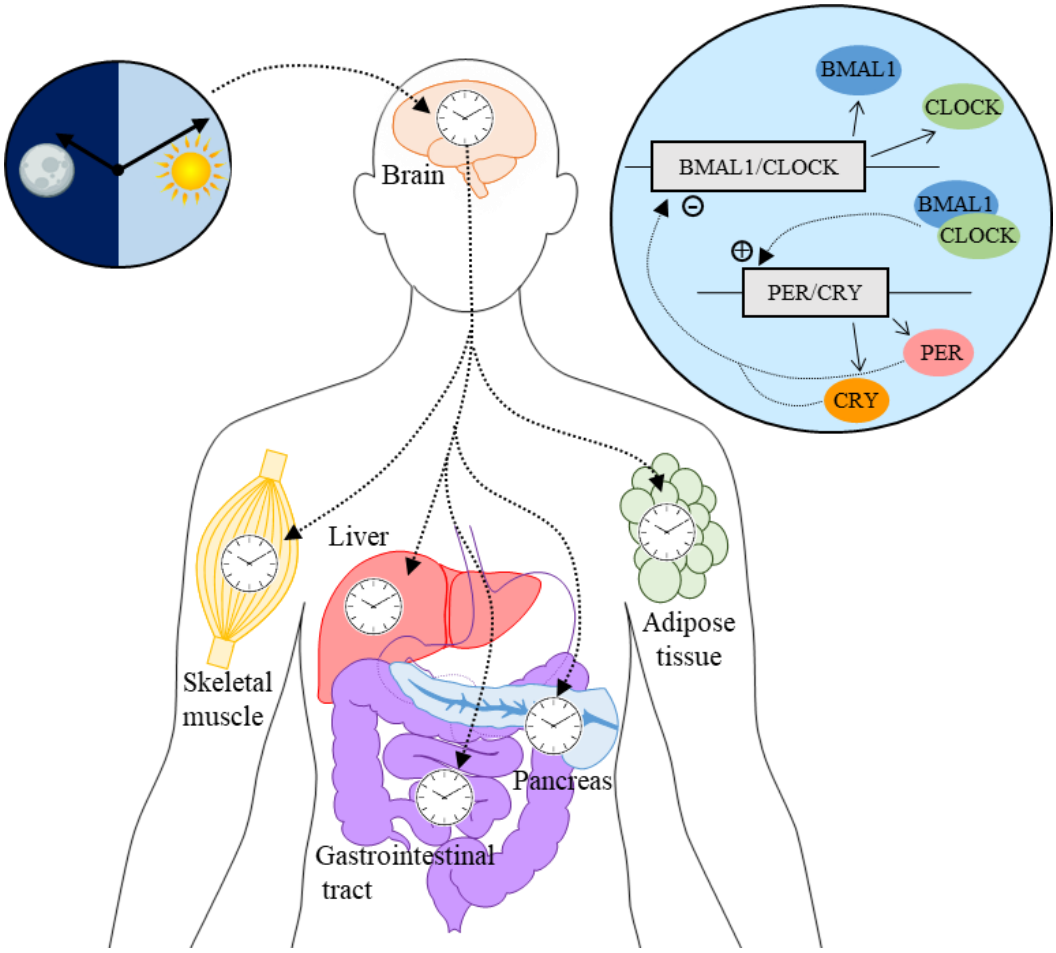

Figure 1.5 Photo-entrainment of the central circadian clock coordinates the timing of secondary clocks in peripheral organs through the rhythmic oscillations of core clock genes. The circadian clock is an intrinsic regulatory system that entrains diurnal changes in physiology, behaviour and metabolic processes, such as the sleep-wake cycle with the time of day (172-174). The central clock is located in the brain and receives neural input from the retina to align the clock with the 24 hr solar cycle (175,188). The central clock then acts as a pacemaker, regulating the timing of secondary clocks located in peripheral organs, including the liver, skeletal muscle, pancreas, adipose tissue and the gastrointestinal tract (175,181). The circadian clock is a network of auto-regulatory gene transcriptional–translational feedback loops, driving rhythmic oscillations of core-clock components (188). The protein products, in turn, regulate rhythmic gene expression of proteins, involved metabolic homeostasis, endocrine activity and neural excitability (180,188). This mechanism consists of a positive arm that drives gene expression and an opposing arm that, through feedback, inhibits the positive

arm's activity (180). 1) Transcription of circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-Like 1 (BMAL1) (189). 2) The protein products of CLOCK and BMAL1 heterodimerise and promote the transcription of cryptochrome (CRY) and period (PER) (189). 3) The protein products of CRY and PER, through a negative feedback loop, repress transcription of CLOCK and BMAL1 (189). 4) The protein products of CRY and PER are degraded and once complete, the positive arm is free to restart the cycle (175,180). This core CLOCK-BMAL1/PER-CRY loop takes ~24 hr to complete and is interlocked with additional feedback loops, which directly influence cyclic oscillations of metabolic genes (175).

1.2 Type 2 Diabetes

Diabetes mellitus refers to a group of chronic metabolic diseases characterised by elevated blood glucose levels (hyperglycaemia), including type 1 and type 2 diabetes. The global total prevalence of diabetes mellitus (type 1 and 2 diabetes) in 2019 was estimated at 9.3 % (~463 million people) (202). This prevalence rate has steadily increased from ~4.7 % (~108 million people) in the 1980s (3) and is projected to increase by 25 % to ~10.2 % (~578 million) by 2030 (202).

A genetic predisposition is a mutual risk factor for developing type 1 and type 2 diabetes (203). However, there are differences in the pathophysiology between these sub types of diabetes mellitus. Type 1 diabetes develops as an autoimmune disease that destroys the insulin-producing cells of the pancreas and, therefore, the pancreas produces little-to-no insulin itself (3,9). Comparatively, type 2 diabetes is a metabolic condition associated with largely modifiable health determinants, such as poor diet and physical inactivity, which contribute to the progressive development of insulin resistance (3). Therefore, this thesis will focus on type 2 diabetes and investigate novel dietary/lifestyle and pharmaceutical interventions for treating this metabolic disease.

Type 2 diabetes is the most common form of diabetes mellitus, being attributed to ~90 % of all diabetes cases (3). It is characterised by hyperglycaemia and insulin resistance. Specifically, the cells in the body gradually become resistant to the normal effects of insulin, and the pancreas gradually loses the capacity to produce enough insulin to maintain normal blood glucose homeostasis (3). The symptoms of type 2 diabetes include excessive urination, thirst, hunger, changes in vision and fatigue (9). However, these symptoms may be mild and, therefore, may go unnoticed for years. For this reason, type 2 diabetes is often undiagnosed and untreated until the onset of more severe complications, including heart disease, stroke and other CVDs (9), kidney and liver diseases (10,204), as well as retinopathy and neuropathy (3).

Prevalence and burden of type 2 diabetes

Type 2 diabetes was shown to affect ~462 million individuals worldwide in 2017, ~6.3 % of the population (205). The global prevalence is projected to reach ~7079 individuals per 100,000 by 2030 (205). Further, this value is widely believed to be underestimated due to a high proportion of undiagnosed and, therefore, unreported cases (3). In Australia, 1 in 20 adults (~1.2 million, 4.9 %) were reported to have diabetes mellitus in 2017-18 (14), and ~1 million

(4.1 %) of these individuals had type 2 diabetes (14). In addition, nearly 400 new cases of type 2 diabetes are reported in Australian children and youths per year, despite being typically considered an adult-onset disease (206).

In addition to this high prevalence rate, diabetes mellitus, specifically, type 2 diabetes, is associated with significant mortality, morbidity and financial burdens. In 2016, the estimated global deaths caused by diabetes mellitus was ~1.6 million, ~4 % of the total deaths that year (16). Type 2 diabetes alone contributed to more than 1 million deaths in 2017, ranking it within the top ten causes of death globally (205). According to the Australian Institute of Health and Welfare's national mortality database, diabetes mellitus contributed to ~16,700 deaths in Australia in 2018 and ~10.5 % of the total deaths that year (207), with type 2 diabetes contributing to ~9,500 of these deaths (56 %) (207), ranking it as the seventh leading cause of death (14). In terms of human suffering, diabetes mellitus contributed to ~67.9 million DALYs worldwide in 2017 (208). This is projected to 79.3 million by 2025 (208). Another study estimated type 2 diabetes alone caused 751 per 100,000 DALYs worldwide in 2017 (205). Further, findings from a recent systematic review estimated the global financial cost of diabetes mellitus was more than 827 billion US dollars in 2015 (209) and is predicted to reach 1.7 trillion US dollars by 2030 (3). In Australia, the annual direct health-related cost for individuals with high blood glucose levels was $4390 in 2005, compared to $1898 for healthy individuals with normal blood glucose levels (210). This equated to $10.6 billion ($4.4 billion in direct costs; $6.2 billion in government subsidies) in that year (210).

Risk factors of type 2 diabetes

Risk factors for developing type 2 diabetes include obesity, tobacco smoking, high blood pressure, high blood lipids, and non-modifiable determinants, such as age, family history and ethnicity (3,6,203). Arguably, the most prominent of these risk factors is obesity. In Australia, adults with an overweight BMI are twice as likely to have type 2 diabetes (~4.6 %), and adults with obesity are five times as likely to have type 2 diabetes (~9.8 %) than Australian adults with a healthy BMI score (~2.0 %) (14). In addition, individuals with normal glucose tolerance but are overweight or obese have an increased risk of insulin resistance in skeletal muscle compared to age- and sex-matched, lean individuals (211). In particular, increased visceral fat mass, as evidenced by an elevated waist circumference or waist-to-hip ratio, is tightly linked with insulin resistance (211,212). Comparatively, weight loss in individuals with obesity is

linked with improvements in insulin sensitivity and thus reduces the risk of type 2 diabetes (211).

Diagnosis of type 2 diabetes

Type 2 diabetes is diagnosed via the evaluation of blood glucose levels. In healthy adults, normal fasting blood glucose levels range between 4 to 6 mmol/ L, fluctuating in line with fed-fasting cycles (213). Slightly elevated fasting blood glucose levels in non-diabetic individuals, i.e. above 6 mmol/ L but below 7 mmol/ L, suggest impaired glucose tolerance (also known as prediabetes), which increases the risk of type 2 diabetes (214). Comparatively, high fasting blood glucose levels, i.e. equal to or greater than 7.0 mmol/ L, suggest diabetes. An oral glucose tolerance test (OGTT) is commonly performed to support this diagnosis, where 2 hr postprandial blood glucose levels equal to or greater than 11.1 mmol/ L are considered diabetic levels (214). Medical professionals may also utilise the glycated haemoglobin (HbA1c) test as a diagnostic tool for diabetes (214). The HbA1c test measures the percentage of blood sugar molecules attached to oxygen-carrying proteins (haemoglobin) in red blood cells (215). Using this method, a score equal to or greater than 6.5 % is considered diabetic (214). An increased percentage of these glycated proteins in the circulation also indicate an increased risk of atherosclerotic plaques and CVDs (216). The HbA1c test is a sophisticated type 2 diabetes diagnosis method because it reflects long-term glucose control (over the past 3 months) rather than in a single moment in time (214). However, this test is more costly than a fasting or postprandial blood glucose test, and resources are often unavailable in some developing countries (9,214).

Pathophysiology of type 2 diabetes

Insulin resistance is arguably the early prominent hallmark of type 2 diabetes (217). In healthy humans, insulin sensitivity naturally fluctuates throughout the life course; during puberty (218) or late pregnancy (219), insulin sensitivity is decreased, whereas physical activity (220) can enhance insulin sensitivity. In type 2 diabetes, impaired sensitivity of cells to insulin-mediated actions, i.e. insulin resistance, develops progressively (89). The development of insulin resistance has been attributed to a number of mechanisms, including genetic mutations, ageing, endocrine and metabolic factors (32). For example, the oversupply of fatty acid to skeletal muscle in obesity is demonstrated to promote the intracellular accumulation of diacylglycerol, ceramides and acylcarnitines (33). In general, these toxic metabolites impair insulin receptor

signalling and induce multiple post-receptor signalling defects, influencing glucose transport (i.e. reduced GLUT4 recruitment) and glucose metabolism (i.e. reduced glucose phosphorylation, oxidation and glycogen synthesis) (211). The pathophysiological mechanisms attributed to skeletal muscle insulin resistance will be discussed further in a section below.

Over time, increasing insulin resistance places an increasing demand on the pancreas to produce greater amounts of insulin (hyperinsulinemia) to maintain blood glucose homeostasis (89). This leads to pancreatic β-cells dysfunction and exhaustion, and thus lack of insulin secretion in some individuals (89,221). Increased glucagon release (hyperglucagonemia) is also evident, which further elevates blood glucose levels through stimulation of hepatic glucose production (222). The role of the pancreatic hormones insulin and glucagon in regulating blood glucose homeostasis in health and type 2 diabetes will be discussed in detail in a section below.

1.2.1 Regulation of blood glucose homeostasis in health and type 2 diabetes

Glucose is one of the primary sources of fuel for all cells in the body. (223). To prevent complications associated with inadequate or excessive glucose supply, i.e. hypoglycaemia and hyperglycaemia, respectively, fasting blood glucose levels are maintained within a relatively narrow range (between 4 and 6 mmol/ L in healthy humans) (213,224). Homeostasis of blood glucose levels is achieved through the coordinated efforts of many central and peripheral regulatory mechanisms, as displayed in Figure 1.6. In individuals with type 2 diabetes, these blood glucose homeostatic mechanisms are often impaired or dysregulated, resulting in insulin resistance and hyperglycaemia (124).

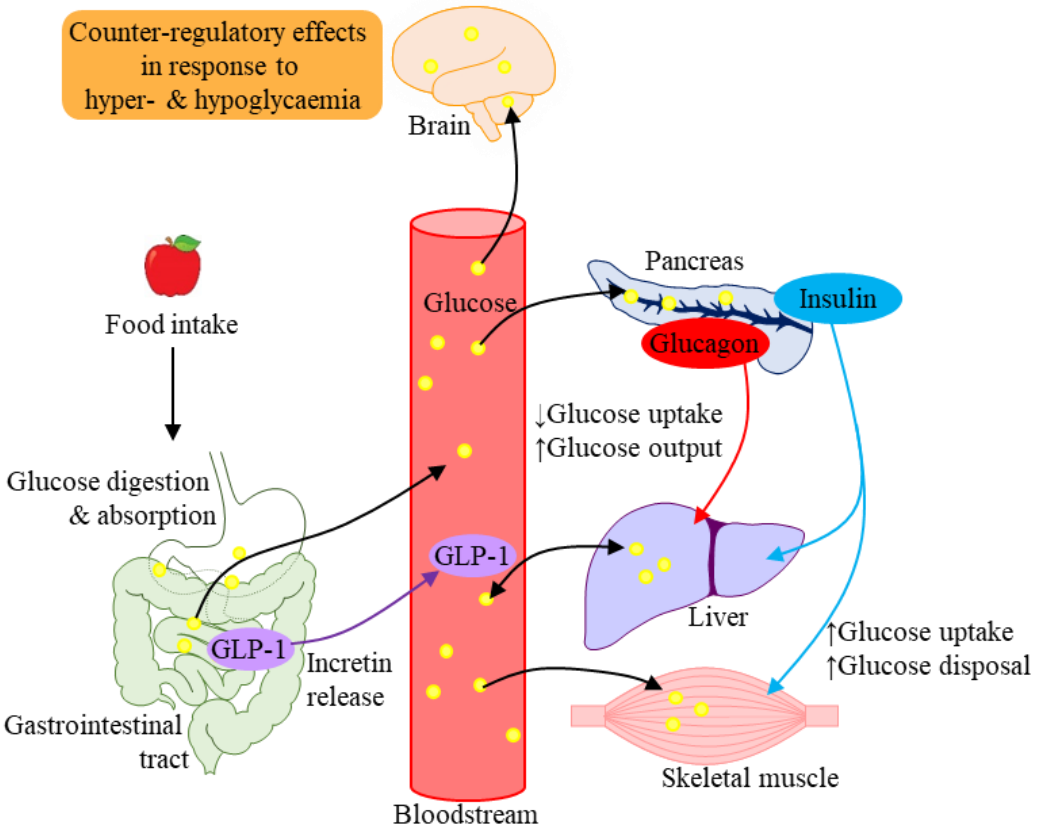

Figure 1.6 Mechanisms in the regulation of blood glucose homeostasis. In humans, blood glucose levels are maintained within a normal range to support energy metabolism in tissues and prevent harmful complications associated with hypo- or hyperglycaemia (224). Food intake leads to glucose absorption in the gastrointestinal tract and a subsequent increase in blood glucose levels (124,225). In the gastrointestinal tract, incretin hormones, such as glucagon-like-peptide-1 (GLP-1), are released in response to food intake and stimulate pancreatic insulin secretion to limit postprandial hyperglycaemia (168,226). The sensing of glucose levels in the brain allows the hypothalamus to induce counter-regulatory mechanisms, such as modulating pancreatic endocrine secretions, to maintain blood glucose homeostasis (68,213). Direct glucose sensing in the pancreas also helps maintain blood glucose homeostasis through the stimulated release of insulin and glucagon in response to high and low blood glucose levels, respectively (89,227). In other peripheral tissues such as the liver and skeletal muscle, insulin promotes glucose uptake and disposal pathways (89), whereas glucagon promotes hepatic glucose production (227).

1.2.1.1 Central regulation of blood glucose homeostasis

The central nervous system critically relies upon glucose to fuel its high-energy demands. The glucose utilised by the brain accounts for ~60-80 % of total glucose uptake from the circulation into tissues under post-absorptive conditions (213). Therefore, the brain is extremely sensitive to dysregulated blood glucose levels, especially hypoglycaemia. Unlike the complications associated with hyperglycaemia (i.e. diabetes mellitus) which may take years to develop, hypoglycaemia-induced complications in the brain occur much more rapidly (223). For example, hunger, fatigue, sweating and anxiety can be felt within minutes of blood glucose levels falling below 4 mmol/ L, and severe consequences, such as massive cerebral failure and seizures, can develop within a few hours (223).

The influence of the central nervous system in the regulation of blood glucose homeostasis is not the focus of this thesis. However, a fundamental understanding of the blood glucose sensing capability of the brain and the neural/hormonal counter-regulatory mechanisms of the central nervous system in response to hyper- and hypoglycaemia are necessary to appreciate whole-body blood glucose homeostasis in health and type 2 diabetes. Therefore, these concepts will be briefly discussed in the section below.

The central nervous system continuously monitors blood glucose levels through a network of glucose sensors located throughout the brain and the periphery to regulate blood glucose homeostasis (68). In the brain, glucose sensing is achieved by various glucose-sensing neurons, including both glucose-excited and glucose-inhibited neurons (213), located in several regions, including the brainstem, corticolimbic areas, and the hypothalamic PVN, ventromedial nucleus, ARC and LH regions (68,213). In glucose-excited neurons, high extracellular glucose levels promote glucose uptake, glucose oxidation and a corresponding increase in intracellular adenosine triphosphate (ATP) levels (213). This leads to the opening of membranous ATP-dependent ion channels, eliciting an action potential, which increases the neuron-firing rate (213). Conversely, glucose-inhibited neurons are activated by low extracellular glucose levels via a mechanism that involves adenosine monophosphate (AMP) activated protein kinase-induced closure of ion channels, causing depolarization and an ensuing action potential (68,228). Disruption of the functionality of these glucose-sensing neurons is associated with dysregulated blood glucose homeostasis. For example, in individuals with insulin-deficient (type 1 and advanced type 2) diabetes, hypoglycaemia-associated autonomic failure is a common complication where the brain cannot detect hypoglycaemia (229). This impaired

responsiveness of glucose-inhibited neurons leads to impaired stimulation of counter-regulatory mechanisms to restore normal blood glucose levels (229). There is also evidence that hyperactivity of glucose-inhibited neurons and ensuing hyper-stimulation of blood glucose elevating mechanism may contribute to hyperglycaemia in type 2 diabetes (228).

The hypothalamus is responsible for integrating input signals from central glucose-sensing neurons and generating the appropriate counter-regulatory mechanism to restore normal blood glucose levels (213). These counter-regulatory mechanisms involve various neural-endocrine pathways, including autonomic nervous system activation and hormone release (213). The sympathetic and parasympathetic nervous systems are known to, directly and indirectly, influence many peripheral tissues involved in blood glucose homeostasis, including the liver, gastrointestinal tract, and pancreas (68,213,230). The physiological effects of autonomic nervous system activation in specific peripheral organs, namely the liver and skeletal muscle, to modulate blood glucose homeostasis will be detailed in the sections below.

1.2.1.2 Peripheral regulation of blood glucose homeostasis

Many peripheral organs are also involved in maintaining blood glucose homeostasis. In particular, the pancreas releases endocrine hormones, including insulin and glucagon (48). Insulin and glucagon have opposing effects on peripheral organs, such as the liver (224) and skeletal muscle (89), to maintain normal blood glucose levels. The gastrointestinal tract is also heavily involved in modulating postprandial blood glucose levels (124). In individuals with type 2 diabetes, these blood glucose regulatory mechanisms are often impaired or dysregulated (231), resulting in hyperglycaemia and other diabetic complications (232).

Pancreas

The pancreas is a peripheral organ involved in digestion, energy balance and blood glucose homeostasis. The pancreas is comprised of both endocrine and exocrine cells. The endocrine cells account for only 1-2 % of the entire organ and are clustered together in formations known as the islets of Langerhans (233). There are five different types of endocrine cells, namely α-cells, β-cells, γ-cells, δ-cells and ε-cells (48). The glucagon-secreting α-cells and insulin-secreting β-cells (48) account for ~15-20 % and ~65-80 % of the total endocrine cell populations, respectively (233), and will be the focus of discussion in the sections below.

Insulin

Insulin is released under conditions of high blood glucose levels (48). In brief, glucose is taken up into β-cells via the cell surface GLUT2 (234). Within β-cells, glucose undergoes glycolysis and mitochondrial oxidation, driving ATP synthesis (227). Elevated intracellular ATP levels then close ATP-sensitive potassium channels, causing membrane depolarization and the opening of voltage-dependant calcium channels (227). This calcium influx then triggers the exocytosis of insulin-containing granules (227). Elevated plasma fatty acid levels (with mono-unsaturated free fatty acids being more powerful than short-chain fatty acids) (235) and several amino acids, including glutamine, alanine, and leucine, have also been shown to stimulate insulin secretion (236,237). This effect is partly due to increased mitochondrial oxidation and ATP synthesis and partly due to the initiation of mitochondria-independent signalling cascades (235). In addition, several gastrointestinal hormones, such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), potentiate insulin release through pancreatic receptor activation and the subsequent initiation of an intracellular signalling cascade (168,226). Comparatively, low blood glucose levels inhibit insulin secretion (89,238) due to low glucose oxidation, membrane hyperpolarization and inhibition of insulin-containing granule exocytosis (227).

Pancreatic insulin secretion is also modulated by the sympathetic and parasympathetic nervous systems (230,239). Through direct innervation of pancreatic β-cells, feeding-induced parasympathetic nervous system activity stimulates insulin secretion (230,239), whereas stress or exercise-induced sympathetic nervous system activity inhibits insulin release (230,239). Sympathetic nervous system activity also leads to the secretion of catecholamines (e.g. noradrenaline and adrenaline) from the adrenal glands, which have been shown to inhibit insulin secretion via β-cell α-adrenergic receptor activation (89,240).

In peripheral organs, such as the liver and skeletal muscle, insulin is well-known to promote glucose uptake, oxidation and storage, thereby reducing circulating glucose levels (224). These physiological effects will be discussed in detail in the sections below.

Glucagon

Pancreatic glucagon is released in response to low blood glucose levels (48). Briefly, low glucose uptake into α-cells reduces the rate of glucose oxidation and ATP synthesis (227,241). This leads to the partial closure of ATP-sensitive potassium channels resulting in membrane depolarization (227,241). Membrane depolarization then leads to the opening of voltage-

dependent calcium channels, allowing calcium entry into α-cells and triggering the exocytosis of glucagon-containing granules (227,241). Under conditions of high blood glucose levels, elevated glucose oxidation and ATP generation cause complete closure of ATP-sensitive potassium channels, thus, greater membrane depolarization (227,241). This leads to the inactivation of voltage-dependant calcium channels, suppressing calcium entry and inhibiting glucagon release (227,241). Elevated blood glucose levels also lead to the release of somatostatin from pancreatic δ-cells, and insulin, GABA and zinc ion release from β-cells (227). These endocrine secretions are shown to have paracrine inhibitory effects on glucagon release (227). In individuals with type 2 diabetes, the release of somatostatin, insulin, GABA and zinc is impaired due to δ-cell and β-cell dysfunction, resulting in unsuppressed glucagon release and ~50-100 % elevated plasma glucagon levels (hyperglucagonemia) compared to healthy individuals (222,227).

Glucagon secretion is also stimulated by the sympathetic nervous system through direct innervation of pancreatic α-cells (239). Central nervous system-induced activation of the sympathetic nervous system also leads to the production and secretion of catecholamines into the circulation, which activates β-adrenoceptors on α-cells in the pancreas, promoting glucagon release (242).

Circulating glucagon predominantly signals glucagon receptors highly expressed in the liver (243), where it promotes glycogenolysis and gluconeogenesis to elevate blood glucose levels (224). These physiological effects will be discussed in detail in the section below.

Liver

The liver is a major metabolically active organ (53). Through fine control of glucose disposal and glucose production pathways, regulated by pancreatic insulin and glucagon signalling, respectively, the liver is highly involved in blood glucose homeostasis (50,222,244).

In the liver, insulin is demonstrated to stimulate a wide variety of intracellular signalling cascades, ultimately causing enhanced glucose uptake into hepatocytes and enhanced glucose disposal through oxidation or storage in the form of glycogen or conversion into fatty acids (33). Under basal conditions, glucose entry and exit occurs passively along a concentration gradient through GLUT1 and GLUT2 and is insulin in-dependant (234). However, in response to the activation of membranous insulin receptors by circulating insulin, glucose uptake into

hepatocytes is enhanced (33). Specifically, insulin receptor activation triggers a signalling cascade involving IRS1, PI3K and AKT (also known as protein kinase B) (89). This signalling cascade leads to increased mRNA expression and activation of the enzyme glucokinase, responsible for converting glucose to glucose-6-phosphate and thereby trapping it within the liver (245). Insulin signalling also enhances glucose oxidation pathways such as glycolysis via the activation of phosphofructokinase-2 (also known as fructose bisphosphatase-2) (244). In addition, insulin signalling activates glycogen synthase, which converts glucose-6-phosphate to glycogen for temporary storage within hepatocytes (244). Further, insulin also activates vital enzymes involved in *de novo* lipogenesis, namely acetyl-CoA carboxylase, promoting conversion of excess glucose metabolites (i.e. acetyl-CoA) into fatty acids for long-term energy storage in adipocytes (33,103). Overall, insulin stimulated glucose uptake and disposal in the liver reduces blood glucose levels and preventing complications associated with hyperglycaemia (33).

Opposite to insulin, circulating glucagon is demonstrated to promote hepatic glucose production, the manufacture and release of glucose molecules from the liver into the circulation to maintain blood glucose homeostasis during fasting conditions (246). Specifically, activation of the hepatic glucagon receptor triggers a signalling cascade involving adenylate cyclase, cyclic AMP formation and protein kinase A activation (247). This signalling pathway leads to the suppression of glucokinase (245) and phosphofructokinase-2 (247) mRNA expression and enzyme activity, thereby inhibiting hepatic glucose oxidation. In addition, glucagon signalling leads to the deactivation of glycogen synthase, thereby suppressing glucose storage as glycogen (247). Instead, glucagon signalling activates phosphorylase kinase, the rate-regulating enzyme in glycogenolysis, the breakdown of glycogen into glucose 6-phosphate (247). Glycogenolysis initially contributes to ~40 % of hepatic glucose production (33). However, hepatic glycogen stores are finite and generally deplete after ~12 hrs of fasting in rodents and after ~48 hrs in humans (33,50). In addition, glucagon signalling activates the hepatic gluconeogenesis pathway, the synthesis of new glucose 6-phosphate molecules from glucogenic substrates such as lactate, glycerol, and glucogenic amino acids. Specifically, glucagon receptor activation leads to stimulation of the rate-regulating enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (50,247). Activation of phosphoenolpyruvate carboxykinase facilitates the conversion of glucogenic substrates into glucose 6-phosphate. Then, activated glucose-6-phosphatase facilitates the conversion of glucose 6-phosphate into glucose molecules (247), which can then exit the liver along a concentration gradient via GLUT1 and GLUT2 and

thereby elevate blood glucose levels (234). The rate of hepatic gluconeogenesis remains relatively constant during fasting, depending on substrate availability, and in glucagon-depleted rodents and humans, gluconeogenesis is the primary contributor to hepatic glucose production (33,50). However, upon return to the fed state, hepatic glucose production is rapidly suppressed by the direct and indirect action of insulin. Direct hepatic insulin signalling suppresses phosphoenolpyruvate carboxykinase and glucose-6-phosphatase activity (244), and suppression of lipolysis in adipose tissue indirectly suppresses gluconeogenesis by limiting substrate (e.g. glycerol) availability (33).

Hepatic glucose metabolism is also influenced by the autonomic nervous system (248,249). In response to hypoglycaemia, the hypothalamus promotes hepatic gluconeogenesis and glycogenolysis via activation of the sympathetic nervous system, restoring normal blood glucose levels (248-250). Conversely, in response to hyperglycaemia, the hypothalamus activates the parasympathetic nervous system, which leads to increased glucose oxidation and glycogenesis and a subsequent reduction in blood glucose levels (249,250).

In type 2 diabetes, hepatic glucose metabolism is primarily impaired by three major defects (245). Firstly, hepatocytes display reduced sensitivity to insulin, resulting in a diminished signalling cascade and reduced induction of insulin-stimulated glycogenesis and glycolysis (245). Secondly, abnormally elevated plasma glucagon levels continually stimulate hepatic glycogenolysis and gluconeogenesis, increasing hepatic glucose production and export despite the elevated high blood glucose levels (245). Thirdly, hepatocytes display defective glucokinase activity causing impaired glucose uptake and increased glucose export, further contributing to chronic hyperglycaemia (245). In fact, evidence suggests that deficient glucokinase activity alone is sufficient to induce type 2 diabetes in rodents (251) and humans (252,253).

Skeletal muscle

Skeletal muscle is another major energy-consuming organ that primarily utilises glucose as an energy source to perform muscle contraction and movement (211). Through insulin-mediated glucose uptake, storage and oxidation pathways, skeletal muscle also contributes to whole-body blood glucose homeostasis (33).

In skeletal muscle, glucose uptake is facilitated by GLUT1 and GLUT4 (211,234). Under basal or fasting conditions, glucose uptake into myocytes is low and primarily facilitated by GLUT1,

which is insulin-independent (211). This is because the skeletal muscle relies predominantly on fatty acid oxidation during fasting to fulfil its energy demands (211). Conversely, under postprandial conditions, glucose uptake into skeletal muscle is high and primarily occurs through insulin-dependent GLUT4 translocation to the cell surface (234). Similar to hepatic insulin signalling, circulating insulin activates the insulin receptor on the membrane of myocytes, triggering an IRS/PI3K/AKT signalling cascade (211). Additional GLUT4 proteins are recruited from intracellular storage vesicles to the cell membrane through this signalling pathway, facilitating a 10 to 20-fold increase in glucose uptake into myocytes along a concentration gradient (234,254). Upon entry into myocytes, glucose is immediately phosphorylated by hexokinase and converted to glucose-6-phosphate, trapping it within the cell (33). Similar to hepatic glucokinase, myocyte hexokinase activity is activated by insulin signalling (33,89). Then glucose-6-phosphate either enters the insulin-stimulated glycogen storage pathway or enters the glycolysis pathway (33,89). Of the glucose-6-phosphate that enters glycolysis, ~90 % is oxidised in the mitochondria for energy production and ~10 % is released as lactate (211). Lactate and other molecules released from skeletal muscle, such as the amino acid alanine, may be utilised by the liver as glycogenic substrates (53).

Insulin resistance in skeletal muscle is a prominent hallmark of type 2 diabetes (211). The literature describes a variety of mechanisms attributed to the development of skeletal muscle insulin resistance, including genetic mutations, ageing, endocrine and metabolic factors, such as intramyocellular accumulation of fatty acids (e.g. diacylglycerol, ceramides or acylcarnitines) (32,33,211,255). In general, the accumulation of these toxic metabolic factors can be attributed to the oversupply of lipids to the muscle (i.e. in obesity) (32,33). Briefly, diacylglycerol accumulation is proposed to cause insulin resistance through activation of protein kinase Cε, causing inhibitory phosphorylation of the insulin receptor, impairing insulin signal transduction to the IRS and other subsequent proteins (33,111). Ceramide accumulation is suggested to cause insulin resistance by decreasing AKT activity, another vital step in the insulin-signalling cascade, impairing downstream action (33,111). Acylcarnitine accumulation is demonstrated to cause insulin resistance through the production of reactive oxygen species, which impairs various cellular processes, including mitochondrial function and energy synthesis (33). There is a lot of debate over which upstream defect is the most important mechanism of action for skeletal muscle insulin resistance in the literature. In addition, there is likely to be differences in the mechanism of action between different species (33). Overall these, and other mechanisms of skeletal muscle insulin resistance result in impaired insulin

receptor signalling and multiple post-receptor signalling defects influencing glucose transport (i.e. reduced GLUT4 recruitment) and glucose metabolism (i.e. reduced glucose phosphorylation, oxidation and glycogen synthesis) (211). In individuals with type 2 diabetes, skeletal muscle insulin resistance results in a significant reduction of glucose uptake into myocytes under postprandial conditions compared to healthy individuals (256). This limits the body's capacity to lower blood glucose levels under hyperglycaemic conditions, increasing the risk of diabetic complications, such as nephropathy and retinopathy (3).

Gastrointestinal tract

The gastrointestinal tract also plays a vital role in maintaining blood glucose homeostasis, especially postprandial glycaemic control. Postprandial glucose levels refer to blood glucose levels after food intake, including a peak, which diminishes over several hours until reaching fasting levels (224). The rate at which glucose is absorbed from the gastrointestinal lumen into the circulation is a principal modulator of postprandial blood glucose levels (124,225). This process is regulated by several factors, including meal composition (257), gastrointestinal motility (124), and the activity of glucose digestion enzymes and absorption transporters (124). Further, several hormones are secreted from enteroendocrine cells within the gastrointestinal tract (80). Several of these gut hormones (known as incretin hormones), including GIP (also known as gastric inhibitory polypeptide) and GLP-1, have insulinotropic properties and assist in regulating postprandial blood glucose levels (80,168,217). In individuals with type 2 diabetes, these mechanisms of gastrointestinal glucose absorption are often impaired, dysregulating blood glucose homeostasis and causing postprandial hyperglycaemia (124,258).

Meal composition

The macronutrient composition of a meal strongly influences the postprandial blood glucose response. This is due to the different digestion and absorption rates of the different macronutrients (89). For example, in animals [258] and humans [165, 259], a high carbohydrate diet has been shown to induce a ~10-30 % higher postprandial glycaemic response than a high-protein diet or HFD.

The type and physical form of carbohydrates also affect the rate of gastrointestinal glucose absorption, thus influencing postprandial glycaemic control (89). For example, carbohydrates derived from fruits or vegetables must first be freed from within the plant matter before absorption, a process facilitated by intestinal microbiota (124). This process contributes to a

slower absorption rate for complex carbohydrates (i.e. contain fibre and starch) compared to simple carbohydrates (i.e. containing sugar). This difference in the rate of carbohydrate absorption and thereby how quickly blood glucose levels are elevated in response is referred to as glycaemic index (89). For example, in rats, consuming a high-fibre diet (low-glycaemic index) leads to significantly lower postprandial blood glucose levels in response to an OGTT compared to a control diet (257). Similarly, in humans with type 1 diabetes, consumption of a low glycaemic index meal reduced postprandial glucose levels by ~20 % compared to a high glycaemic index meal, despite identical macronutrient compositions (259).

Gastrointestinal motility

Gastrointestinal motility also directly influences the rate of gastrointestinal glucose absorption and thereby affects postprandial blood glucose levels. In healthy humans, the average rate of gastric emptying, the rate at which food empties from the stomach into the small intestine, is ~1-4 kcal/ min (80,124,225). Typically, liquids drain first, and there is a lag time of about ~20-30mins before emptying of solid foods (231). Overall, complete emptying of a meal into the intestines takes ~3-5 hr (231). In the small intestines, glucose absorption occurs at a rate of ~0.5 g/ min/ 30cm in healthy individuals, limited by the saturation of available digestive enzymes and glucose transporters (124,260). Fluctuations in intestinal motility alter the spread of nutrients across the length of the small intestine and, thus, reduce or increase the exposure of nutrients to available enzymes and transporters, consequently impacting the rate of glucose absorption (124). In individuals with normal glucose tolerance, optimised gut motility leads to a relatively quick intestinal glucose absorption resulting in a rapid postprandial peak in blood glucose levels (124,225).

Comparatively, individuals with type 2 diabetes exhibit a delayed peak in postprandial blood glucose levels by ~35 % (225,261). This delay may be due to gastrointestinal dysmotility due to the development of diabetic autonomic neuropathy (225). For example, in individuals with diabetic autonomic neuropathy, damage to the nerves innervating the stomach and intestine results in gastrointestinal dysmotility, including a delay in gastric emptying by as much as ~30-50 % (225), which subsequently prolongs the transit of chyme through the intestinal tract (231,262). Diabetic autonomic neuropathy can also manifest as oesophageal dysmotility, gastroparesis, and diabetic enteropathies and may include symptoms such as early satiety, acid reflux, bloating, abdominal pain, nausea, vomiting or faecal incontinence (262). Treatment of these symptoms involves the management of the underlying causes of diabetes (261).

The autonomic nervous system also influences gastrointestinal motility (120). For example, in response to hypoglycaemia, activation of the sympathetic nervous system enhances gastrointestinal motility by increasing the gastric emptying rate and improving intestinal motility (231,263). These effects accelerate the delivery of nutrients to the small intestine for absorption, elevating blood glucose levels (120,225). This counter-regulatory response to hypoglycaemia is observed in health and individuals with type 1 diabetes, diabetic autonomic neuropathy and diabetic gastroparesis (225). Conversely, parasympathetic nervous system activation leads to a slowing of gastric emptying and intestinal motility (231). This action prolongs nutrient delivery to the small intestine and thus postpones further glucose absorption (120).

Carbohydrate digestion and absorption

In humans, carbohydrate digestion and absorption are primarily facilitated by epithelium cells within the small intestine. In general, the small intestinal epithelium consists of various cell types, including mucus-secreting goblet cells, sensory enteroendocrine cells and absorptive enterocytes (264). These cells are arranged in brush-like villus folds, known as the brush border, which encompass various digestive enzymes (264). To facilitate carbohydrate digestion, luminal complex sugars (i.e. poly- and disaccharides) come in contact with glycoside hydrolase enzymes in the brush border (264) and are converted into simple sugars (i.e. glucose and other monosaccharides) (264). The transportation of these simple sugars across the intestinal epithelium into the portal circulation is carried out by absorptive enterocytes (265). Enterocytes account for ~90 % of all intestinal epithelial cells and are comprised of an apical surface (facing the lumen) and basolateral membrane domain (265). In enterocytes, glucose absorption is facilitated by membranous transporter proteins, namely sodium-dependent glucose cotransporter 1 (SGLT1) at the apical surface and GLUT2 at the basolateral membrane surface (124,234,266). SGLT1 co-transports simple sugars and sodium-ions along an electrochemical gradient, generated by an ATP-dependent sodium-potassium exchange transporter at the basolateral membrane surface (234). Comparatively, GLUT2 is bidirectional and facilitates glucose movement out of and into the portal circulation depending on the glucose concentration gradient (234).

The carbohydrate digestion and absorption rate in the small intestine is also influenced by the activity of gastrointestinal sweet taste receptors. In humans, broad-spectrum sweet taste receptors are expressed on enteroendocrine intestinal epithelium cells (267). These ‘sugar

sensors' are stimulated by various sweet-tasting molecules in the lumen, including simple sugars, D-amino acids and artificial low-calorie sweeteners (266,267) and in response upregulate the expression and activity of SGLT1 (266) and enhance the rate of intestinal glucose absorption. In health, duodenal sweet taste receptor mRNA expression is reduced under hyperglycaemic conditions to reduce intestinal glucose absorption rate (268). However, in individuals with type 2 diabetes, this counter-regulatory mechanism to maintain postprandial blood glucose homeostasis is not observed (268). Thus, intestinal glucose absorption remains elevated, exacerbating hyperglycaemia (268).

In humans and animal models of type 2 diabetes, a number of structural and functional changes are observed in the small intestine that also impact carbohydrate digestion and absorption. For example, hyperplasia of the intestinal epithelium and a 2-fold increase in glycoside hydrolase enzyme activity are observed in models of type 2 diabetes compared to healthy controls (269,270). These changes may contribute to an increased rate of carbohydrate digestion and absorption in the small intestine (269). In addition, there is also evidence of a 4-fold increase in jejunal SGLT1 and GLUT2 mRNA content, observed in individuals with type 2 diabetes compared to healthy individuals (270,271). Further, in individuals with morbid obesity and animal models of type 2 diabetes, jejunal GLUT2 was observed at both the apical and basolateral membrane surfaces, compared to just the basolateral membrane surfaces in healthy controls (272). This contributes to an increased rate of carbohydrate absorption in the small intestine. Overall, these structural and functional changes observed in carbohydrate digestion and absorption in type 2 diabetes contributes to postprandial hyperglycaemia.

Incretin hormones

A wide variety of hormones are secreted from the gastrointestinal tract and contribute to nutrient digestion and absorption, appetite regulation and energy homeostasis (80). In addition, there are certain gut hormones, known as incretin hormones, that are involved in blood glucose homeostasis (226). There are two main types of incretin hormones in humans: GIP secreted from K-cells in the proximal small intestine (duodenum and upper jejunum) (273), and GLP-1, secreted from L-cells in the distal small intestine (ileum) and bowel (47). In response to food intake, these hormones are rapidly released into the plasma and circulate throughout the body (226). Acting upon receptors in various tissues around the body, these incretin hormones assist in modulating the postprandial glycaemic response (168). In particular, GIP and GLP-1 exhibit insulinotropic properties, stimulating insulin secretion to lower the peak in postprandial blood

glucose levels in humans (274-276) and animals (277-279). However, this incretin effect is diminished in individuals with type 2 diabetes, dysregulating postprandial blood glucose homeostasis (226).

In healthy individuals, blood glucose levels peak at ~30 mins during an OGTT, coinciding with rapidly elevated plasma insulin and C-peptide levels (226). Intravenous glucose infusion results in similar peak blood glucose levels but with a substantially blunted plasma insulin and C-peptide response (226). This ~50-70 % difference in insulin release under postprandial conditions is attributed to the incretin effect (217). In the pancreas, insulin-secreting β-cells express receptors for both GIP and GLP-1 (226), and receptor binding initiates a signalling cascade involving adenylate cyclase, cyclic AMP production and protein kinase A activation (226). In turn, protein kinase A activation stimulates insulin biosynthesis promotes β-cell proliferation and reduces β-cell apoptosis (168). Overall, pancreatic incretin signalling augments insulin secretion. However, the actual release of insulin secretory granules from ß-cells requires cellular depolarization and a calcium ion influx triggered by high blood glucose levels (226).

In individuals with type 2 diabetes, blood glucose levels peak at ~60 to 90 mins during an OGTT, coinciding with a delay in the elevation in plasma insulin and C-peptide levels (226). These peaks in diurnal plasma glucose and insulin levels occur much later than healthy individuals, indicating impaired management of postprandial blood glucose homeostasis, possibly due to a diminished incretin effect. A number of studies have compared the impact of an oral glucose load on GIP and GLP-1 secretion and insulin release in health compared to type 2 diabetes. Meta-analysis of these clinical studies reported that gastrointestinal incretin hormone secretion is generally not different in type 2 diabetes (280,281). However, it appears that the insulinotropic effect of GIP and GLP-1 is markedly reduced in type 2 diabetes (282-284).

1.2.2 Influence of the circadian system on blood glucose homeostasis in health and type 2 diabetes

In most organisms, including humans, the circadian clock influences blood glucose homeostasis. Specifically, the circadian clock synchronises the transcription of many metabolic genes involved in regulating blood glucose levels, in line with daily reoccurring events such as food intake (182,183). However, evidence demonstrates that food consumption outside of the

normal active phase leads to circadian dysregulation and subsequent disruption of blood glucose homeostasis (285). This is particularly true for shift workers, who frequently consume food during the rest phase and are at an increased risk of glucose intolerance and type 2 diabetes (285). In fact, a meta-analysis of human studies reported that individuals exposed to shift work have a 9 % increased risk of type 2 diabetes compared to those not exposed to shift work (286).

In healthy humans, daily blood glucose levels exhibit circadian rhythmicity. Due to the influence of behavioural factors such as food intake, fasting duration and activity, daily blood glucose levels are generally elevated during the day and reduced at night (285,287). Furthermore, this daily rhythm in blood glucose levels is associated with rhythms in pancreatic insulin secretion (176,288). Similarly, insulin sensitivity and glucose tolerance, following an OGTT, is reportedly elevated in the morning and decreased in the evening (289-291). Further, in rodent studies, surgical lesion of the SCN, the location of the central circadian clock, leads to complete abolishment of diurnal variations in blood glucose levels, demonstrating the importance of the circadian clock in the homeostasis of blood glucose levels (292-294).

In individuals with type 2 diabetes, daily blood glucose levels also exhibit circadian rhythmicity (285). However, daily changes are more pronounced with significantly elevated levels during the early morning (morning hyperglycaemia), despite the overnight fast, indicating impaired blood glucose homeostasis (285,295). This effect is known as the dawn phenomenon and is observed in individuals with type 1 and type 2 diabetes (296). Animal studies demonstrate that this dawn phenomenon (morning hyperglycaemia) is likely due to an inappropriate increase in mRNA expression of gluconeogenesis enzymes, including phosphoenolpyruvate carboxykinase and glucose-6-phosphorylase (181,296). Suggesting dysregulation of blood glucose homeostasis in type 2 diabetes, may, at least in part, be caused by circadian desynchrony of hepatic glucose metabolism (285).

Circadian desynchrony can occur due to misalignment between the central clock and the environment (i.e. light-exposure during the night (196)), or due to altered behavioural cycles (i.e. eating or activity during the rest-phase (195,297,298)), or due to misalignment between the central and peripheral clocks (i.e. SCN lesion (292-294)). In human (299,300) and animal studies (186), evidence demonstrates that shift work, particularly night shift work, is linked with adverse metabolic health outcomes (200) through desynchrony of circadian systems (285). For example, simulated night shift work in healthy humans is demonstrated to increase plasma HbA1c levels (299) and reduce insulin sensitivity (300). In a controlled study, simulated night

shift working healthy participants, including night time activity, night time meals and day time sleep, resulted in increased area under curves for plasma glucose and insulin levels following a meal, suggesting worsened glucose tolerance and an increase risk of type 2 diabetes (300). Similarly, simulated shift work in rats is shown to adversely elevate blood glucose levels in response to an intraperitoneal glucose tolerance test (186).

1.3 Interventions

General guidelines for treating obesity include reducing intake of low-nutrient, high-energy foods and increasing total energy expenditure by engaging in regular exercise (3,16). There is also strong evidence to suggest that reducing body weight is beneficial for treating type 2 diabetes (20,301). In particular, insulin resistance and glucose intolerance are associated with a high BMI, and a reduction in BMI reduces the risk of type 2 diabetes (211,212).

A wide range of surgical, dietary/lifestyle and pharmaceutical interventions are currently available for the treatment of obesity or type 2 diabetes. The potential advantages and disadvantages of these treatment strategies will be discussed in the sections below.

1.3.1 Current surgical interventions

Currently, the most effective treatment for obesity is bariatric surgery (302-304). Bariatric surgery refers to a group of surgical strategies to reduce obesity, including the Roux-en-Y gastric bypass, sleeve gastrectomy and adjustable gastric band surgery (302). These techniques aim to reduce the stomach's storage capacity and/or bypass some areas of the small intestine, leading to early meal satiation, decreased food intake, and reduced nutrient absorption (302). Overall, evidence demonstrates that bariatric surgeries are effective strategies for significant and sustainable weight loss (302-304). In a 5 year study that followed patients who received either bariatric surgery or medical therapy (i.e. insulin or other medications), participants who received bariatric surgery sustained ~20 % weight loss compared to ~5 % in medical therapy groups (305). In addition, bariatric surgery patients had more significant reductions in plasma HbA1c levels and reported a better score in the quality of life questionnaire compared to medical therapy groups (305). This evidence suggests that bariatric surgery not only treated obesity but also improved other aspects of metabolic and mental health.

Unfortunately, due to the expensive medical costs, the highly invasive nature of the surgery, and the risk of surgical complications, bariatric surgery is not suitable for every overweight or

obese individual (302). The current criteria necessary to qualify for bariatric surgery is a BMI score greater than 40 or a BMI score greater than 35 but with significant diagnosed co-morbidities (302,306). Suitable candidates must also demonstrate weight loss failure after following more conventional dietary/lifestyle treatment strategies (302). Further, bariatric surgery is not without potential for procedure-related morbidities or side effects (302). The most frequent postoperative side effects include nausea, vomiting, abdominal pain, dysphagia and dehydration (302,307). Other more serious complications of bariatric surgery include anastomotic leaks, leakage of luminal contents from a surgical join potentially resulting in infection and sepsis (307). In a retrospective review of all patients that underwent bariatric surgery between 2001 and 2005 at a single hospital in America, the frequency of gastrointestinal leaks was ~0.3-0.5 %, rare but not negligible (307). Further, during this period, the frequency of patients requiring reoperations due to complications or failures was ~1.8-14.6 % (307). Emerging evidence also suggests that bariatric surgery, with removal or alteration of functional segments of the gastrointestinal tract, may impair gastrointestinal mechanisms involved in energy balance and blood glucose homeostasis (308). The long-term implications of this gastrointestinal impairment are still being investigated (308). Overall, these common surgical complications and side effects of gastrointestinal impairment or distress are significant detrimental factors when considering bariatric surgery as a treatment option.

This thesis will focus on dietary/lifestyle and pharmaceutical interventions used to treat obesity and type 2 diabetes, recommended to individuals before resorting to bariatric surgery.

1.3.2 Current dietary and lifestyle interventions

Generally, dietary and lifestyle interventions aim to change human behaviours that are detrimental to health and thereby prevent, treat or delay the progression of metabolic diseases (309). It is well-known that high energy intake and low physical activity generates an energy imbalance, leading to weight gain and obesity (1,5). Currently, a number of dietary/ lifestyle interventions exist, which aim to rebalance this scale by restricting food intake or increasing exercise, therefore promoting weight loss.

A common dietary intervention for treating obesity is calorie restriction. Calorie restriction involves consuming a diet that is low in calories but maintains an adequate nutritional value (310). In a wide range of species, including yeast, worms, spiders, fish, mice, rats and non-human primates, total calorie restriction has been demonstrated to prolong lifespan and healthy

aging (reviewed in (310)). Similarly, in overweight humans, 25 % calorie restriction was shown to improve specific biomarkers of longevity, including reduced fasting plasma insulin levels and lowered core body temperature (311). Participants also exhibited reduced total body weight and fat mass in this study, demonstrating calorie restriction is an effective treatment strategy for obesity (311). However, calorie restriction may also involve limiting the intake of certain discretionary foods, a challenge some individuals find difficult to sustain (312). There is also evidence that very-low-calorie diets, while promoting significant weight loss, may also cause fatigue, dehydration, dizziness, cramps and constipation (312).

Evidence also suggests increasing total energy expenditure by undertaking regular exercise may be somewhat effective in promoting weight loss. Generally, exercise is demonstrated to modestly reduce body weight (313,314), improve cardiovascular health (6,315) and improve mental health by reducing anxiety and symptoms of depression and supporting a positive self-image (6). However, there is evidence that the rate of exercise-induced weight loss generally declines and body weight plateaus after ~6 months (6). In addition, there is evidence that undertaking high-intensity, long-duration exercise promotes food intake in humans by stimulating appetite (6,316). This effect is partly due to a central nervous system-induced counter-regulatory mechanism to conserve and restore energy reservoirs after extreme exercise-induced depletion (6,316). Nevertheless, appetite stimulation may result in increased food intake, inhibiting weight loss.

Despite the initial apparent effectiveness of current lifestyle/dietary interventions for treating obesity, maintaining weight loss after initial success is challenging. For example, in a follow-up study of a diabetes prevention program, participants regained ~70 % of their initial weight loss after ~10 years, despite continued diet and exercise (4,317). This is consistent with a systematic review of follow-up studies, in which almost half of the initial weight loss reported across 33 different dietary/ lifestyle intervention studies was regained after 1 year (318). This relapse in obesity may be due to a variety of personal and environmental factors that impede weight loss and weight loss maintenance, including dysregulation of circulating energy balance-related hormones (4,319) and emotion-driven food intake (320). For example, in a clinical study, participants following a very-low energy diet for 10 weeks, exhibited significant reductions in circulating appetite-suppressing hormones, including leptin, peptide YY, CCK and insulin, and significant increases in the circulating appetite-stimulatory hormone ghrelin, despite weight loss (319). These participants also reported a significant increase in hunger

levels after the 10 week program, suggesting a hormone-related upregulation in appetite, contributing to the obesity relapse (319). In addition, in humans, food intake often involves an emotional component (320). Negative emotions such as stress, sadness, conflict and fear are commonly associated with a loss of dietary restraint, undermining a person's self-control or self-imposed restrictions to food intake (320). The outcome is that dieters may abandon their diet or even consume more food than they would previously, i.e. binge eating (320). This may, at least in part, contribute to the obesity relapse after dietary/lifestyle interventions.

Overall, this evidence suggests current dietary/lifestyle strategies for treating obesity are associated with many obstacles in losing weight and maintaining weight loss. Therefore, novel or improved intervention options are needed. The sections below will discuss the potential benefits of two promising dietary/lifestyle strategies for treating obesity and type 2 diabetes, namely time-restricted feeding (TRF) and chronic isoleucine supplementation.

1.3.3 Time-restricted feeding

TRF is a form of intermittent fasting, a type of dietary/lifestyle intervention for treating obesity and metabolic disease. Specifically, TRF involves eating only during a predetermined period, typically 8-12 hrs during the day in humans, then fasting for the remainder of the 24 hr solar cycle (321-323). Importantly, TRF does not explicitly aim to restrict calorie intake or increase exercise levels but rather aims to control the time of day food is consumed since evidence demonstrates that abnormal timing of food intake is a risk factor for obesity (175,183,324).

Several studies have shown that TRF for 8-12 hr during the active phase (that is, during the DP in nocturnal animals, i.e. rodents (325,326) and the LP in diurnal animals, i.e. humans (327)) is an effective intervention for the treatment of obesity and metabolic disease. For example, in rodent studies, 8-12 hr TRF during the DP reduced total body weight, fat mass and liver lipid deposition, reversing the progression of obesity and metabolic diseases such as NAFLD (108,109,326-329). In addition, TRF during the DP improved glucose tolerance and insulin sensitivity in rodents, reducing the risk of type 2 diabetes (295,328). Further, in drosophila flies, TRF during the LP (active phase) was shown to improve cardiovascular health, sleep duration and sleep quality (reviewed in (322)). Finally, in human studies, TRF during the LP was shown to reduce body weight and reduce fasting blood glucose and lipid levels (330-334). This evidence suggests that TRF during the active phase is effective for treating obesity and type 2 diabetes.

In the modern era, eating patterns in humans span a large portion of the 24 hr solar cycle, often beyond the LP. A population study with dietary data entered into a smartphone application revealed that ~35 % of food intake occurs after 6 pm in young adults (199). These 'abnormal' feeding patterns are associated with circadian desynchrony, which is demonstrated to increase the risk of many metabolic diseases, such as obesity, NAFLD, CVDs and type 2 diabetes (109,189,198,335-337). This is particularly true for shift workers who frequently perform physical activity and distribute food intake across the 24 hr period (200). In several systematic reviews, shift workers, especially night shift workers, were shown to be at increased risk of obesity, metabolic syndrome and type 2 diabetes compared to other daytime occupations (187,201,286).

For individuals whose lifestyle impedes food intake during the active phase, such as night-shift workers, it is important to investigate the potential benefit of TRF during the inactive phase for treating obesity and type 2 diabetes. Currently, evidence investigating the effect of TRF during the inactive phase is limited and unclear. In one mouse study, 6 weeks of TRF for 12 hr during the LP resulted in increased body weight compared to mice fed during the active phase (194). Conversely, two other studies showed that TRF during the LP significantly reduced weight gain and adiposity in HFD fed mice (338,339). However, the TRF protocol in these studies consisted of a 3-4 hr restricted feeding window. Therefore, this study cannot explain whether the metabolic benefits were due to TRF or the concurrent caloric restriction of food intake. Nevertheless, this evidence suggests that TRF during the inactive phase may be as effective as TRF during the active phase in treating obesity and metabolic disease. Chapter 3 in this thesis will investigate TRF as a lifestyle intervention for treating these metabolic diseases. In particular, chapter 3 will investigate the metabolic outcomes of TRF (12 hrs for 8 weeks) during the LP or DP in mice.

1.3.4 Dietary supplementation with isoleucine

High-protein, low-carbohydrate diets are generally considered to be effective interventions for treating obesity. For example, in individuals with obesity (340) or type 2 diabetes (341,342), consuming a high-protein diet at the expense of carbohydrates is demonstrated to reduce body weight, fat accumulation, and plasma lipid levels as well as improve glucose tolerance. These beneficial effects are partly attributed to reduced energy intake through a sustained reduction of appetite (343). However, chronic consumption of a high-protein diet is also associated with adverse health outcomes, including reduced longevity (344) and an increased risk of end-stage

liver disease in individuals with impaired kidney function (345). Therefore, recent evidence suggests chronic amino acid supplementation, especially BCAA supplementation, may be an effective alternative to a high-protein diet.

There is evidence from animal studies demonstrating dietary intake of the BCAAs leucine and isoleucine protects from HFD-induced weight gain and hyperglycaemia, suggesting a possible beneficial role of leucine or isoleucine supplementation in the treatment of obesity and type 2 diabetes. In a small number of mice studies, dietary supplementation of leucine (346-348) or isoleucine (347,349) in mice fed a HFD, prevented total weight gain and accumulation of adipose tissue. In another animal study, acute oral administration of leucine and isoleucine in lean rats reduced blood glucose levels in response to an OGTT (350). However, in this study the blood glucose lowering effect of isoleucine was significantly greater than leucine (350). Isoleucine has also been demonstrated to significantly reduce blood glucose levels in response to an GTT in obese and glucose-intolerant mice (351). This evidence, albeit limited, suggests that chronic dietary supplementation with BCAAs, particularly isoleucine, may be beneficial in the treatment of obesity and type 2 diabetes. However, chronic isoleucine supplementation in models of established obesity and glucose intolerance remains to be thoroughly investigated. Therefore, chapter 2 in this thesis will investigate isoleucine supplementation as a dietary intervention for treating these metabolic diseases. In particular, chapter 2 will investigate the effect of acute and chronic isoleucine supplementation on body weight and glucose tolerance in lean and diet-induced obese mice."

The literature review below entitled "Is there a role for branched-chain amino acid supplementation in the treatment of obesity and diabetes?" was conducted to investigate the physiological role and underlying mechanisms of BCAA supplementation on energy balance and glycaemic control.

Statement of Authorship

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Is there a Role for Branched-Chain Amino Acid Supplementation in the Treatment of Obesity and Diabetes?

Abstract

The branched-chain amino acids (BCAAs), leucine, isoleucine, and valine are essential amino acids involved in a variety of physiological pathways. In rodent studies, chronic BCAA supplementation has been demonstrated to prevent diet-induced weight gain. In other studies, BCAA supplementation improved insulin sensitivity and glucose tolerance, evident by a reduction in fasting and postprandial blood glucose levels. This evidence suggests a potential role of BCAA supplementation in the treatment of obesity and diabetes. However, there is also evidence BCAAs do not affect body weight or glycaemic control. Further, epidemiological studies have identified a link between high dietary BCAAs and type 2 diabetes. This review will evaluate the literature on the physiological role and underlying mechanisms of BCAA supplementation on energy balance and glycaemic control to elucidate the potential benefits of BCAA supplementation in treating obesity and diabetes.

LIST OF ABBREVIATIONS

In alphabetical order

BC acyl-CoA	Branched-chain acyl-coenzyme A
BCAA	Branched-chain amino acid
BCAT	Branched-chain aminotransferase
BCKA	Branched-chain alpha-keto acid
BCKD	Branched-chain alpha-keto acid dehydrogenase
BP	Binding protein
CCK	Cholecystokinin
eIF	Eukaryotic initiation factor
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
HFD	High-fat diet
mTOR	Mammalian target of rapamycin
PPARα	Peroxisome proliferator-activated receptor alpha
S6K1	Ribosomal protein S6 kinase 1

Introduction

Amino acids, the base components of protein, are essential nutrients for physical growth, development and metabolic health (352,353). In addition to their function as substrates in the synthesis of new proteins (354), amino acids, in particular the branched-chain amino acids (BCAAs), isoleucine, leucine and valine, are demonstrated to influence physiological pathways such as nutrient metabolism, energy balance, and glycaemic control (355,356). In rodent studies, chronic dietary supplementation with BCAAs has been shown to prevent diet-induced weight gain (349,357). Similarly, in human population studies, a high dietary intake of BCAAs is associated with a low prevalence of overweight and obesity (358,359). Further, BCAA supplementation supported lean muscle mass maintenance during weight loss in humans (360). This evidence suggests there may be a role for BCAA supplementation in the treatment of obesity. In addition, acute BCAA administration in rats has been demonstrated to reduce blood glucose levels in response to an oral glucose tolerance test (350), which suggests a possible role of BCAA supplementation in the treatment of diabetes. However, there is also evidence where BCAA supplementation had no beneficial effect on body weight or glycaemic control in rodents (361-363) or humans (364-366). In addition, high plasma BCAA levels are associated with an increased risk of type 2 diabetes in humans (367,368). Taken together, these conflicting findings highlight the need to fully understand the potential benefits or risks of BCAA supplementation in the treatment of obesity or diabetes. Therefore, this review will evaluate the literature on the physiological role and underlying mechanisms of BCAA supplementation on energy homeostasis and glycaemic control.

Branched-chain amino acid metabolism

The BCAAs are essential amino acids, which cannot be biosynthesized within the mammalian body (369). Therefore, they need to be absorbed from dietary protein sources such as red meat, poultry, bread, rice and beans (370). Following digestion, the BCAAs are absorbed into the circulation (371) and distributed according to the nutritional demands of select tissues (372). BCAA entry into cells is facilitated by a complex system of specialized amino acid transporters, which exchange non-essential amino acids for the BCAAs (369,373). Within cells, BCAAs can influence several signalling pathways (e.g. stimulation of the mammalian target of rapamycin (mTOR) pathway to enhance protein synthesis (115,354)) or are utilized as substrates in metabolic pathways such as protein synthesis, ketogenesis, gluconeogenesis or energy metabolism (374).

Oxidative catabolism of BCAAs is a well-established process for BCAA metabolism (Figure 1.**7**) (91,115,353,375-377). In brief, the BCAAs, leucine, isoleucine and valine, are first converted to the branched-chain α-keto acids (BCKAs) 2-keto-isocaproate, 2-keto-3methyl-glutarate and 2-keto-isovalerate, respectively (377). This reversible transamination of BCAAs to BCKAs is catalysed by the enzyme branched-chain aminotransferase (BCAT) (377). Facilitating this reaction, α-keto glutarate (also known as 2-oxoglutarate) accepts the transferred amino group and, in the process, is converted to glutamate (378). In mammals, two genes encode BCATs: BCAT1 is primarily expressed in the brain and encodes a cytoplasmic protein, whereas BCAT2 encodes a mitochondrial protein expressed throughout the body (115). In particular, BCAT2 expression is higher in skeletal muscle than in the liver (91), suggesting that the first step in BCAA catabolism primarily occurs in skeletal muscle, rather than the liver, where most other amino acids are metabolized. BCKAs can enter the circulation and be transported to other tissues where they are converted back into BCAAs (376) or further metabolised (377).

BCKAs are irreversibly decarboxylated into branched-chain acyl-coenzyme A (BC acyl-CoA) intermediates; namely isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA (377). This process is catalysed by the active branched-chain α-keto acid dehydrogenase (BCKD) complex and is the second and rate-limiting step of BCAA metabolism (115,377). The activity of the BCKD complex is tightly regulated by phosphorylation and dephosphorylation (115). The enzyme BCKD kinase adds a phosphate to the BCKD complex suppressing its activity (91,375,378), whereas dephosphorylation, and therefore activation, is carried out by the enzyme BCKD phosphatase (91,375). Further, the BCKD complex activity seems to be largely proportionate to the amount of intracellular BCKAs present (115). This is due to the allosteric suppression of BCKD kinase by BCKAs, especially 2-keto-isocaproate (highest affinity) (115). Therefore, high levels of BCAAs seem to promote their own oxidation. Following decarboxylation by the BCKD complex, the BC acyl-CoA metabolites undergo a series of enzymatic conversions, mostly unique to each BCAA, and are subsequently converted into their final-products, namely acetyl-CoA from leucine, acetyl-CoA and succinyl-CoA from isoleucine and succinyl-CoA from valine (355,377), which enter the tricarboxylic acid cycle, fuelling energy metabolism.

In humans, congenital deficiency of the BCKD complex (also known as maple syrup urine disease) leads to the toxic plasma accumulation of BCAAs and BCKAs, leading to seizures,

coma and death in severe cases (116,375,379). However, without impairment from mutation or disease, enzymatic regulation of BCAA metabolism allows a high BCAA intake to be generally well-tolerated (375).

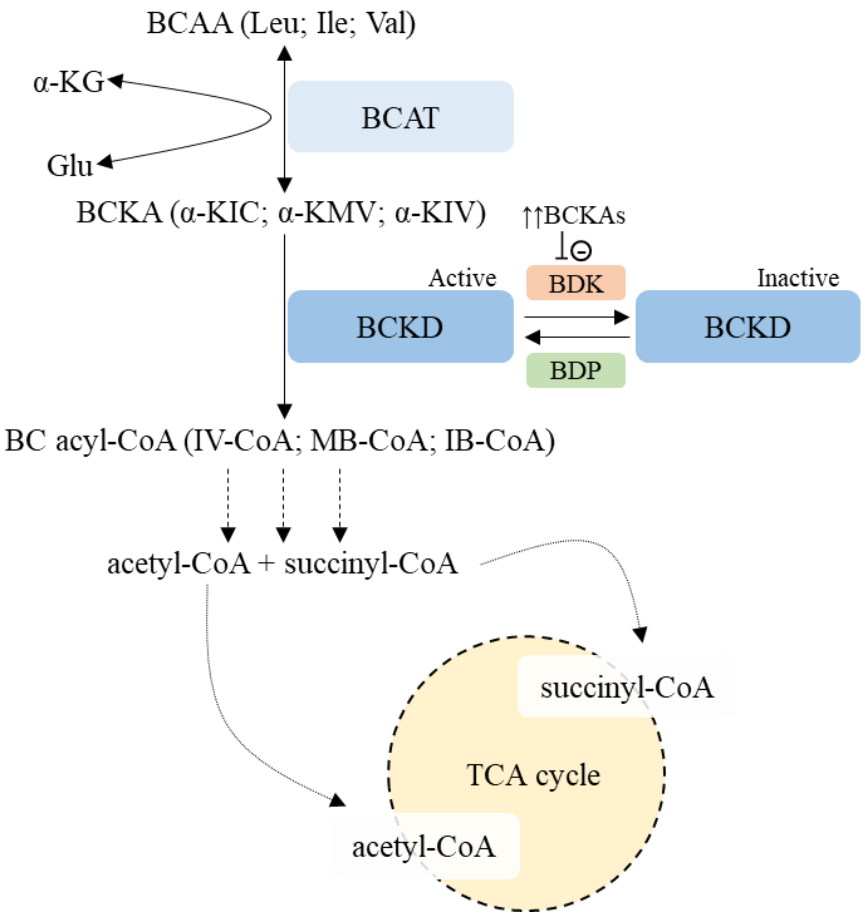

Figure 1.7. The branched-chain amino acid metabolism pathway. Within tissues, such as the skeletal muscle, the branched-chain aminotransferase (BCAT) enzyme reversibly converts the branched-chain amino acids (BCAAs), leucine (Leu), isoleucine (Ile) and valine (Val) to branched-chain α-keto acids (BCKAs), namely 2-keto-isocaproate (α-KIC), 2-keto-3methyl-glutarate (α-KMV) and 2-keto-isovalerate (α-KIV) respectively (377). Facilitating this reaction, α-ketoglutarate (α-KG) is reversibly converted to glutamate (Glu) (378). Then, the branched-chain α-keto acid dehydrogenase (BCKD) complex may irreversibly convert the BCKAs into branched-chain acyl-coenzyme As (BC acyl-CoAs), namely isovaleryl-CoA (IV-CoA), 2-methylbutyryl-CoA (MB-CoA) and isobutyryl-CoA (IB-CoA), respectively (377). The BCKD complex is activated by BCKD phosphatase (BDP) and inactivated by BCKD kinase (BDK) (115). The BC acyl-CoA metabolites are then further converted into their final-products, acetyl-CoA and succinyl-CoA, which enter the tricarboxylic acid (TCA) cycle, fuelling energy metabolism (355,377).

BCAA supplementation and Obesity

In two human epidemiological studies, a high dietary intake of BCAAs was associated with a low prevalence of overweight and obesity in adults, particularly central adiposity (358,359). Many rodent studies have also explored the role of BCAA supplementation in the regulation of body weight and obesity, as summarized in Table 1.2. Some of these studies suggest BCAA supplementation may be beneficial in the prevention of obesity. For example, chronic leucine supplementation in mice, at a dose of 1.5 % for 14 weeks, significantly reduced high-fat diet (HFD)-induced weight gain compared to control mice (357). Similarly, chronic isoleucine supplementation in male mice at a dose of 2.5 % for 6 weeks also reduced HFD-induced weight gain (349). However, there are studies where BCAA supplementation did not beneficially affect body weight. For example, at a dose of 1-2 % for 6 weeks, chronic isoleucine supplementation had no effect on body weight in female mice fed a high-fat, high-sucrose diet (351). It should be noted that in this study, the high-fat, high-sucrose diet did not induce obvious obesity in either the non-isoleucine control (26.1 ± 0.9 g) or isoleucine treatment groups (26.5 ± 1.3 g & 24.2 ± 0.8 g, respectively) (351). Consistent with this, there was no effect of chronic leucine supplementation on body weight in lean rodents (357,361). In another study, 6-weeks of leucine supplementation added to the HFD did not affect body weight in rats (363). However, in this study, food intake was restricted to only a few hours during the daytime (breakfast: 0700-0720 hr, lunch: 1300-1400 hr and dinner: 1800-1900 hr), consistent with a typical human mealtime feeding pattern (363). This eating pattern likely restricted total energy intake in both the control and leucine treatment groups, possibly confounding any direct leucine treatment effect on body weight. It is well established that rodents fed a HFD with restricted access to food for only a few hours per day cannot consume the same amount of calories compared to *ad libitum* fed rodents (176,338,380). In another study, 1.5 % leucine supplementation for 14-15 weeks did not affect body weight compared to controls (381). Comparatively, 24 weeks of chronic leucine supplementation, at doses ranging from 1.5 % to 4.5 %, in HFD-fed rats, actually increased body weight gain and fat mass compared to controls (361).

It is also possible that while BCAA supplementation may prevent obesity when initiated at the onset of HFD feeding (349), chronic BCAA supplementation may not be capable of reversing body weight gain in animal models of established obesity. For example, in mice fed a western diet for 12 weeks, BCAA supplementation for a further 14 weeks did not affect weight gain or

adiposity compared to the non-BCAA control group (382). In this study, supplementation did not affect energy intake or energy expenditure, suggesting that BCAAs or, more specifically, isoleucine supplementation may not be able to overcome the effect of a prolonged obesogenic diet on long-term energy homeostasis. Another possible explanation involves the susceptibility of inbred C57BL/6J mice to HFD-induced obesity (383) and, therefore, a possible resistance to the effects of BCAA supplementation on body weight (381).

Overall, the mechanisms underlying the contradictory results of BCAA supplementation on body weight are not clear. For example, several studies (349,363,381,382) have indicated that leucine or isoleucine supplementation combined with HFD feeding did not affect food intake. However, 13 weeks of mixed BCAA supplementation (an extra 150 % BCAA added to diet) in HFD-fed rats was demonstrated to reduce body weight through reduced food intake compared to controls (362). This conflicting evidence, for the effect of BCAAs supplementation on food intake, highlights the need for clarification in this area. It is also possible that any effect of BCAA supplementation on body weight may be due to changes in energy expenditure. Therefore, the influence of a single BCAA supplementation on food intake and energy expenditure will be investigated further below.

In summary, the existing evidence suggests BCAA supplementation may have a beneficial effect in preventing diet-induced obesity but not in reversing established obesity. Furthermore, different study designs, such as altered feeding patterns, may influence the effect of BCAAs on body weight. Therefore, more research is needed to exclude these biases in the link between BCAA supplementation and obesity.

Table 1.2. The reported effects of BCAA supplementation on body weight, food intake and energy expenditure.

BCAA	Dose & Duration	Species	Diet before BCAA	Diet during BCAA	Effect on Body Weight	Effect on Food Intake	Effect on Energy Expenditure	Reference
Leucine	14 weeks of 1.5% (wt/vol) in drinking water	Male C57BL/6J mice	SLD	HFD	↓	↔	↑	(357)
	14 weeks of 1.5% (wt/vol) in drinking water	Male C57BL/6J mice	SLD	SLD	↔	↑	N/R	(357)
	24 weeks of 1.5, 3.0, 4.5% (g/g) added to diet	Male Sprague–Dawley rats	SLD	HFD	↑	↔	N/R	(361)
	24 weeks of 1.5, 3.0, 4.5% (g/g) added to diet	Male Sprague–Dawley rats	SLD	SLD	↔	↔	N/R	(361)
	6 weeks of 0.054 g/g diet	Male Sprague-Dawley rats	SLD	HFD	↔	↔	N/R	(363)
	6 weeks of 0.048 g/g diet	Male Sprague-Dawley rats	SLD	SLD	↔	↔	N/R	(363)
	14-15 weeks of 1.5% (wt/vol) in drinking water	C57BL/6J mice	SLD	HFD	↔	↔	↔	(381)
Isoleucine	4 weeks 2.5% (wt/vol) + 0.5% methylcellulose (vol/vol) in drinking water	Male C57BL/6J mice	2 weeks HFD	HFD	↓	↔	N/R	(349)
	6 weeks of 1, 2% (wt/vol) + 20% (wt/vol) sucrose in drinking water	Female C57BL/6J mice	SLD	HFHSD	↔	N/R	N/R	(351)
Mixed BCAA	14 weeks extra BCAA (Leu 10.7, Ile 8.9, Val 10.7 g/kg)	Male C57BL/6J mice	12 weeks of WD	WD	↔	↔	↔	(382)
	13 weeks of 150% extra BCAAs added to diet	Male Wistar rats	SLD	HFD	↓	↓	↔	(362)

↑ Designates an increase; ↓ designates a decrease; ↔ designates no effect; N/R identifies the corresponding information on weight gain, food intake and energy expenditure was not reported. HFD, high-fat diet; HFHSD, high-fat high-sugar diet; Ile, isoleucine; Leu, leucine; SLD, Standard laboratory diet; Val, valine; WD, western diet.

Food intake

Dietary protein is a well-known appetite regulator, with a high-protein diet suppressing appetite in humans (343,384). This is, at least in part, due to the stimulated secretion of appetite-regulatory hormones, such as glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and cholecystokinin (CCK) from within the gastrointestinal tract (80). These appetite-regulatory hormones are secreted from specialised enteroendocrine cells located in the gastrointestinal epithelium in response to various stimuli, including protein, glucose, and fatty acid luminal nutrient sensing (167). Evidence demonstrates that upon secretion, these hormones stimulate nearby vagal afferent nerve endings or enter the portal circulation to induce various physiological effects in the central nervous system and peripheral organs (132,168). Through stimulation of the vagal afferent nerve pathway or entering the cerebrovascular system by crossing the blood-brain barrier, these appetite-regulatory hormones relay satiety signals to the appetite-regulatory centres of the brain and suppress appetite (132,168). There is also evidence that some of these hormones influence gastrointestinal motility, namely the rate of gastric emptying (80). Slowing gastric emptying aids nutrient digestion and absorption by slowing the delivery of nutrients to the small intestine (80). Slowing gastric emptying is also correlated with meal satiation, satiety and postprandial fullness in humans by increasing gastrointestinal transit time and maximising satiety signalling (385).

There is some evidence that acute BCAA supplementation leads to the secretion of gut hormones (386-388) and a delay in gastric emptying (365,389). Therefore, acute BCAA supplementation may enhance satiety signalling and suppress appetite (displayed in Figure 1.8). However, the appetite regulatory role of chronic BCAA supplementation is not clear, with the majority of existing studies showing that chronic BCAA supplementation does not affect food intake (Table 1.2).

Appetite-inhibitory hormones

Detection of nutrients, including protein, within the gastrointestinal tract induces the secretion of appetite-inhibitory hormones, such as GIP, GLP-1 and CCK (80). In health (165) and type 2 diabetes (166), oral consumption of a whey protein drink elevated plasma GLP-1, GIP and CCK levels in response to a meal. There is also evidence that acute BCAA supplementation increases GLP-1, GIP and CCK secretion. For example, in healthy humans, acute oral

consumption, but not intravenous administration, of a mixed BCAA solution at a dose of 0.4 g/ kg, elevated plasma GLP-1 and GIP levels compared to the placebo treatment (388). Similarly, in lean humans, an intra-duodenal infusion of leucine at doses of 3.3 g and 9.9 g elevated plasma CCK levels (386). Increased plasma CCK levels were also observed following an intra-duodenal isoleucine infusion at doses ranging from 10g to 30g in dairy heifers (387). Comparatively, some studies did not observe any effect of BCAA supplementation on plasma gut hormone levels. For example, an intra-gastric infusion of leucine at a dose of 1.56g did not affect plasma CCK or GLP-1 levels in lean or obese humans (366). In a similar study, an intra-gastric infusion of leucine or isoleucine at doses of 5g or 10g in lean humans did not affect plasma GLP-1, GIP or CCK levels (365). Given these contradictory findings, it is clear that further investigation is required to confirm the effects of BCAA supplementation on appetite-regulatory gut hormone secretion.

Overall, the differential effects of BCAA supplementation on plasma gut hormone levels observed in these studies might be attributed to the dose of BCAAs used. The differential effects may also be dependent on the specific BCAA used. For example, in lean humans, an intra-duodenal infusion of valine, at a dose of 4.6 g or 13.8 g, was shown not to affect plasma CCK levels (390). This suggests that valine, in particular, may not stimulate gastrointestinal hormone secretion. Nevertheless, even in the studies where isoleucine or leucine increased plasma hormone levels, there was no change in participant perceived appetite (365,366) and, therefore, further investigation is required.

Gastric emptying

In humans with type 2 diabetes, oral consumption of a whey protein drink (high in leucine and isoleucine) before a meal was shown to significantly slow gastric emptying (166). Similarly, in lean humans, an intra-duodenal infusion of isoleucine slowed gastric emptying (365). This effect was also observed following oral gavage of isoleucine in lean rats (389). Conversely, the reported effects of leucine or valine on gastric emptying are less consistent. In one study, oral gavage of leucine and valine in lean rats did not affect gastric emptying (389). Similarly, in humans, intra-duodenal administration of leucine and valine did not affect gastric emptying or intestinal motility (365,366,390). In another study, oral gavage of leucine or valine in lean rats significantly delayed gastric emptying, although the gastric emptying curves varied drastically depending on the specific BCAA administered (389). This suggests individual BCAAs regulate

gastric emptying through different mechanisms (389). Therefore, further studies are required to establish the mechanism of action of specific BCAAs on gastric emptying.

In humans, slowing of gastric emptying is correlated with elevated postprandial fullness through meal satiation and satiety signaling (385). However, in a human study, isoleucine was found to slow gastric emptying but had no effect on food intake or participant perceived appetite, including sensations of hunger, fullness, desire to eat, and nausea or bloating (365). Therefore, the delayed gastric emptying may not be a major factor controlling appetite after isoleucine supplementation.

Overall, acute studies indicate that certain BCAAs may slow gastric emptying and induce secretion of appetite-inhibitory gut hormones, which should increase satiety signals from the gastrointestinal tract. However, the majority of existing evidence suggests that chronic BCAA supplementation does not affect food intake.

In addition to energy intake, energy expenditure contributes to the long-term regulation of body weight (1). Many of the studies summarized in Table 1.2 did not report the effect of BCAA supplementation on parameters of energy expenditure. However, there is some evidence to indicate BCAA supplementation increases specific components of total energy expenditure. This evidence will be explored in the next section.

Total energy expenditure

There is some evidence that chronic BCAA supplementation increases energy expenditure as a mechanism of body weight regulation. In one study, chronic leucine supplementation increased food intake without affecting weight gain in lean rats and reduced body weight without affecting energy intake in HFD-fed obese rats (357). This suggests that the chronic leucine supplementation increased energy expenditure in these lean and obese rats (357). In another study, chronic isoleucine supplementation prevented diet-induced weight gain in mice without affecting food intake (349), again suggesting isoleucine supplementation increased energy expenditure (349). Comparatively, in humans, acute ingestion of a mixed BCAA drink did not affect mean energy expenditure (measured via indirect calorimetry) compared to a carbohydrate-based placebo drink (364). This lack of an observed effect may be due to acute rather than chronic supplementation. There is also evidence where chronic leucine supplementation increased total body weight in rats without affecting food intake, suggesting

leucine reduced total energy expenditure (361). In light of these conflicting findings, the effect of chronic BCAA supplementation on energy expenditure is uncertain.

Total energy expenditure is composed of the energy required for basal metabolic rate, diet-induced thermogenesis, cold-induced thermogenesis and the energy expended during exercise (40). The possible role of BCAA supplementation on these specific types of energy expenditure is discussed below.

Basal metabolic rate

Basal metabolic rate is the minimal amount of energy required, to perform vital bodily functions (e.g. respiration, cardiac function and the growth and repair of tissues (40,391)) for an individual to sustain life. Basal metabolic rate is the largest contributor to daily total energy expenditure (391) and is dependent on the degree of lean body mass, with a higher lean mass associated with a higher basal metabolic rate (313,392,393). Evidence indicates that BCAA supplementation, combined with moderate calorie restriction, reduces fat mass while maintaining lean muscle mass in healthy (394,395) and obese humans (360). BCAA supplementation has also been shown to promote muscle hypertrophy in athletes (396,397) and prevent disease- or age-related muscle wasting (398-400). This evidence suggests that BCAA supplementation, through increased lean mass and thereby increased basal metabolic rate, may elevate total energy expenditure as a mechanism of treating obesity.

Increased lean muscle mass following BCAA supplementation is due, at least in part, to BCAA-stimulated protein synthesis. It is well established that elevated amino acid availability promotes protein synthesis in humans (369). There is also evidence that BCAAs, particularly leucine, directly stimulate the protein synthesis pathway (Figure 1.8) (354). In food-deprived rats, leucine supplementation stimulated the mTOR signalling pathway in skeletal muscle (401,402). Activation of mTOR then leads to hyper-phosphorylation of proteins involved in protein translation initiation, namely ribosomal protein S6 kinase 1 (S6K1) and the binding protein (BP) 4E-BP1 (354,403). Briefly, activation of S6K1 results in the preferential translation of mRNAs for ribosomal proteins involved in mRNA translation, such as eukaryotic initiation factor (eIF)4G, enhancing the cell's capacity to synthesize proteins (354,403). Phosphorylation of 4E-BP1 results in the release of eIF4E from its inactive 4E-BP-eIF4E bound state (354,403). Available eIF4E then binds with other protein translation initiation factors, such as eIF4G, to form the necessary mRNA translation eIF4F complex, promoting

protein formation (354,403). Isoleucine and valine also contribute to elevated protein synthesis through increased substrate availability (402). However, they are much less effective than leucine in activating the mTOR pathway (402). In contrast to this evidence, a human study has shown that intravenous BCAA infusion leads to reduced total protein turn over since the rate of BCAA catabolism exceeds the rate of muscle protein synthesis *in vivo* (404).

Diet- and cold-induced thermogenesis

Diet-induced thermogenesis, also known as the thermic effect of food, is the energy required for nutrient processing and storage following a meal (41,405). Evidence indicates that meals with a high-protein or high-carbohydrate composition induce higher diet-induced thermogenesis than high-fat meals (406,407). There is no clear evidence to suggest different protein sources might stimulate different degrees of diet-induced thermogenesis. However, their varying amino acid compositions may promote an array of body weight and composition changes based on the stimulation of different metabolic pathways (406). In one study, chronic isoleucine supplementation in mice increased the mRNA expression of peroxisome proliferator-activated receptor-α (PPARα) and fatty acid translocase in the liver and skeletal muscle (349). Activation of PPARα accelerates fatty acid oxidation (408), while upregulation of fatty acid translocase increases fatty acid uptake in liver and skeletal muscle (409). This evidence suggests that BCAA supplementation, particularly isoleucine, may increase diet-induced thermogenesis through upregulation of lipid metabolism in liver and skeletal muscle (Figure 1.8). However, diet-induced thermogenesis only contributes ~5-15 % of total daily energy expenditure (41). Therefore, any BCAA-induced elevation in diet-induced thermogenesis is unlikely to impact total energy expenditure significantly.

There is also evidence that an infusion of BCAAs in patients under general anaesthesia prevents the lowering of core body temperature (410). This suggests there may be a role for BCAAs supplementation in elevating cold-induced thermogenesis. BCAA uptake and oxidation in humans and mice are elevated in brown adipose tissue for thermogenesis and heat production in response to cold exposure (411). However, whether BCAA supplementation can enhance thermogenesis in brown adipose tissue under thermo-neutral conditions is not clear. In mice housed at room temperature, chronic leucine (357) or isoleucine (349) supplementation did not affect mRNA expression of uncoupling protein 1, a key enzyme involved in brown adipose tissue thermogenesis (412). This evidence suggests there is no direct role of oral BCAA supplementation in enhancing cold-induced thermogenesis in brown adipose tissue.

Activity

Another component of total energy expenditure is the energy expended during physical activity, particularly exercise (40). In rats fed with a HFD, chronic leucine supplementation did not affect 24 hr locomotor activity compared to controls, despite a reduced total body weight and increased total energy expenditure in these animals (357). This evidence suggests activity-related energy expenditure does contribute to any BCAA induced increase in energy expenditure.

Overall, the evidence suggests BCAA supplementation may enhance basal metabolic rate by promoting lean mass and enhance diet-induced thermogenesis through stimulation of lipid metabolism. This increase in total energy expenditure may be responsible for the beneficial effects of chronic BCAA supplementation in preventing diet-induced obesity. However, future studies should aim to clarify this connection.

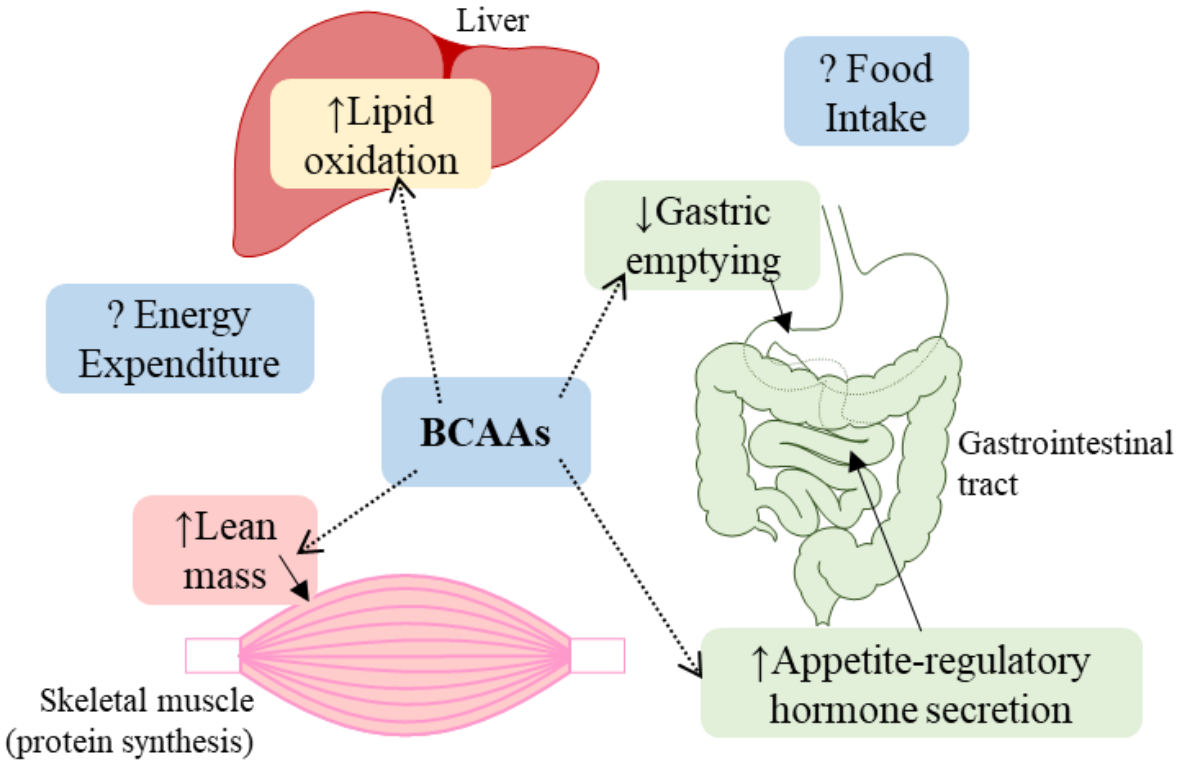

Figure 1.8. The possible mechanisms of branched-chain amino acid supplementation on food intake and energy expenditure. The roles of branch-chain amino acids (BCAAs) on food intake and energy expenditure are not clear. However, possible mechanisms underlying these roles include: 1) BCAAs stimulate the protein synthesis in skeletal muscle (401,402), preserving lean muscle mass during weight loss (360,394,395), and a high lean mass composition is associated with a high basal metabolic rate (313,392,393); 2) BCAAs elevate diet-induced thermogenesis through enhanced lipid oxidation in the liver (349); 3) BCAAs slow gastric emptying (365,389); 4) BCAAs stimulate secretion of gastrointestinal appetite-regulatory hormones (386-388).

BCAA supplementation and diabetes

Type 2 diabetes is a metabolic disease characterized by chronically elevated blood glucose levels, contributing to the progressive development of insulin resistance (301). Similar to their role in treating obesity, there is some evidence that BCAA supplementation may be an effective treatment for type 2 diabetes.

In several rodent (350,351,413) and human studies (365,414), acute administration of leucine or isoleucine, but not valine, has been demonstrated to dose-dependently reduce postprandial blood glucose levels following an oral glucose tolerance test. This glucose-lowering effect was more significant following isoleucine than leucine or valine administration in lean rats (350). A similar blood glucose-lowering effect was observed following acute isoleucine administration in leptin receptor-deficient (*db/db*) mice, a model of type 2 diabetes (351). Further, acute leucine and isoleucine supplementation also reduced postprandial blood glucose levels in a rat model of carbon tetrachloride-induced liver cirrhosis, which has a confirmed phenotype of glucose intolerance (413). This evidence suggests acute BCAA supplementation, specifically isoleucine or leucine, lowers postprandial blood glucose levels and, therefore, may be effective in improving glucose tolerance in individuals with type 2 diabetes.

There is also evidence chronic BCAA supplementation may improve glucose tolerance. In high-fat high-sucrose fed mice, chronic isoleucine supplementation reduced fasting blood glucose levels compared to controls (351). Conversely, in lean rats and mice, 6 weeks of leucine or isoleucine supplementation was found to have no beneficial effect on postprandial blood glucose levels in response to a meal (363) or in response to an oral glucose tolerance test (351). These conflicting findings highlight the complicated relationship between postprandial blood glucose levels and long-term glycaemic control.

Recently, clinical studies have identified an association between high plasma BCAA levels and an increased risk of type 2 diabetes (362,415). The nature of the relationship between BCAAs and type 2 diabetes is currently unclear (367,368). Therefore, this review will examine the effects of individual BCAA supplementation on blood glucose control mechanisms.

It is well established that postprandial blood glucose levels are influenced by gastrointestinal motility, namely gastric emptying and intestinal motility (124,225). It is also known that certain hormones secreted from the gastrointestinal tract, such as GLP-1 and GIP, have insulinotropic properties, contributing to postprandial glycaemic control (168,225). In the previous section

(*Food intake*), evidence was provided on the possible effect of BCAA supplementation on gastric emptying and gastrointestinal hormone secretion. This section will therefore limit discussion to the evidence regarding the potential effects of BCAA supplementation on other aspects of glucose tolerance, such as insulin sensitivity, glucose uptake into skeletal muscle and hepatic glucose production.

Insulin sensitivity

Existing evidence indicates that BCAA supplementation, especially leucine, may improve insulin sensitivity. In diet-induced obese rats, chronic leucine supplementation improved insulin sensitivity and reduced the HOMA-IR score (homeostatic model of assessment for insulin resistance) (361,416). This was due to reduced blood insulin levels and reduced blood glucose levels in response to an insulin tolerance test (361,416). Improved insulin sensitivity in these rats was attributed to leucine-stimulated hyper-phosphorylation and upregulation of critical proteins within the insulin intracellular signalling cascade, including mTOR, protein kinase B or AKT, and the insulin receptor substrate tyrosine 632 residue, in the liver, skeletal muscle and adipose tissues (361).

In another study, chronic leucine supplementation was demonstrated to improve obesity-induced BCAA dysmetabolism and impaired fatty acid metabolism (416). In obesity, chronic inflammation is known to impair the mitochondrial oxidation of BCAAs/BCKAs and fatty acids (417,418). This leads to the accumulation of toxic incomplete fatty acid oxidation metabolites, namely acylcarnitines (419). Accumulated acylcarnitine species, in turn further impairs mitochondrial oxidation and contributes to the development of insulin resistance (418). In rats, chronic leucine supplementation reduced acylcarnitine accumulation and mitochondrial function (416). This suggests leucine supplementation, through the improvement of obesity-induced BCAA and fatty acid dysmetabolism, may prevent insulin resistance.

Conversely, there is also evidence that BCAA supplementation decreases insulin sensitivity. In humans, an acute infusion of a mixed BCAA solution was shown to reduce insulin sensitivity under hyperinsulinemic-euglycemic clamp conditions (420-422). In another clinical study, a high intake of dietary BCAAs, especially leucine and valine, was shown to increase the incidence of insulin resistance in adults by ~60 %, therefore increasing the risk of developing type 2 diabetes (423). However, this study also reported no significant association between dietary BCAAs, either total or individual, and risk of hyperinsulinemia, insulin sensitivity or

pancreatic β-cell dysfunction (423). In another study, 24 weeks of chronic leucine supplementation in lean rats did not beneficially affect insulin sensitivity (361). However, this report investigated insulin sensitivity in healthy lean rats, where insulin sensitivity was already well regulated. In light of these differential findings, recent studies hypothesize leucine supplementation may affect insulin sensitivity differently in lean subjects compared to models of diet-induced obesity with established insulin resistance (416). Future studies should also investigate the effects of valine or isoleucine supplementation on insulin sensitivity.

Glucose uptake into skeletal muscle

In skeletal muscle, glucose uptake is facilitated by insulin signaling and impaired by insulin resistance (224). In isolated skeletal muscle cells (differentiated C2C12 myotubes), infusion with either isoleucine or leucine, but not valine, stimulated glucose uptake into cells, with or without insulin (350,424). However, when isoleucine or leucine was incubated in combination with insulin, the rate of glucose uptake was significantly enhanced compared to insulin alone (424). This additive effect was attributed to BCAA-induced hyper-phosphorylation and activation of essential proteins within the insulin intracellular signaling cascade, namely phosphoinositide 3-kinase and protein kinase C (425). Enhanced activation of these signaling proteins leads to upregulated mRNA expression and recruitment of the glucose transporters GLUT1 and GLUT4 from intracellular storage sites to the cell membrane (254,413,425,426). Overall, the increased number of glucose transporters at the cell membrane results in increased glucose uptake into skeletal muscle and thereby reduced blood glucose levels (224). Similarly, increased glucose uptake into skeletal muscle and consequently reduced blood glucose levels has also been observed *in vivo* following acute administration of isoleucine or leucine in various rodent studies (296,350,427,428) (Figure 1.9). However, this effect is yet to be demonstrated following chronic BCAA supplementation in rodents or humans.

Hepatic glucose production

Reduced blood glucose levels following BCAA supplementation has also been attributed to suppressed hepatic glucose production. In type 2 diabetes, hyperglucagonemia results in the constant inappropriate stimulation of hepatic glucose production via glucagon-stimulated gluconeogenesis, contributing to hyperglycaemia (221,246). In isolated rat hepatocytes, acute incubation with isoleucine was shown to reduce the mRNA expression and protein content of critical enzymes involved in hepatic gluconeogenesis, namely phosphoenolpyruvate

carboxykinase and glucose-6-phosphatase (428,429). Similarly, chronic leucine supplementation reduced the mRNA expression of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase in the liver of HFD-fed rats, but not in lean rats (361). This evidence suggests there is a role for leucine or isoleucine supplementation in reducing hyperglycaemia via suppressing hepatic glucose production (Figure 1.9). However, whether this proves beneficial for the treatment of type 2 diabetes remains to be determined.

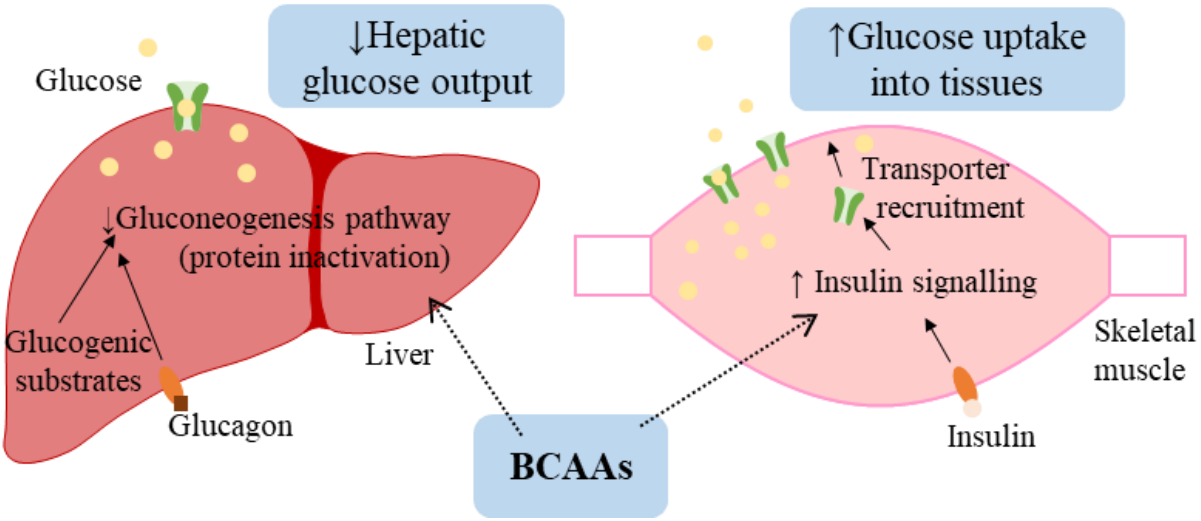

Figure 1.9. The possible effects of branched-chain amino acid supplementation on glycaemic control. Possible mechanisms include: 1) Branched-chain amino acids (BCAAs) suppress hepatic glucose output through the reduction in expression of critical gluconeogenesis enzymes (361,428,429); and 2) BCAAs increase glucose uptake into skeletal muscle (296,350) through enhanced insulin sensitivity (361,416).

Conclusion

In summary, the role of BCAA supplementation in preventing diet-induced obesity (349,357) is controversial, but it is unlikely to be an effective treatment option for individuals with established obesity (382). The possibility that BCAA-induced prevention of obesity is likely due to increased energy expenditure through increased basal metabolic rate (360,394,395) and diet-induced thermogenesis (349). Some evidence indicates BCAA supplementation may contribute to increased satiety signalling through the increased secretion of gastrointestinal appetite-inhibitory hormones (386,388,416) and reduced gastric emptying rate (365). However, there is no direct evidence to suggest that the inhibitory effect of BCAA supplementation on appetite leads to body weight regulation (365,366). Further, the evidence for BCAA supplementation in the management of glucose homeostasis is also controversial. There is some evidence to suggest BCAA treatment may improve glucose tolerance in rodents (350,351,413) through improved insulin sensitivity (361,416), increased glucose uptake into skeletal muscle (296,350), and suppressed hepatic glucose production (428,429). However, the beneficial effect of chronic BCAA supplementation on glycaemic control is yet to be confirmed in rodents or investigated in humans.

The following sections are not part of the above literature review.

1.3.5 Current pharmaceutical interventions

Health practitioners generally recommend pharmaceutical interventions for individuals who demonstrate little to no health improvements following conventional dietary/lifestyle interventions alone (430). For such patients, many pharmacotherapy options exist for the treatment of obesity and type 2 diabetes, including medications such as phentermine, orlistat, liraglutide, metformin, and exenatide. However, all of them are associated with side effects. The therapeutic effects and side effects of current anti-obesity and anti-diabetes medications are described in Table 1.3. These current anti-obesity and anti-diabetic medications have been approved for use by safety and efficacy regulatory bodies, such as the Therapeutic Goods Administration in Australia and the Food and Drug Administration in America (430-433). However, research continues in the pursuit to develop new and improved gold standard medications for the treatment of metabolic disease without side effects.

Table 1.3 The therapeutic effects and side effects of current anti-obesity and anti-diabetes medications.

	Therapeutic effects	Side effects	Reference
Anti-obesity medications			
Phentermine	Central acting noradrenergic agent. Appetite-suppressant.	Insomnia, tachycardia, hypertension, dry mouth, taste alterations, dizziness, tremors, headache, anxiety, nausea and gastrointestinal distress.	(430,431,434)
Orlistat	Lipase inhibitor. Inhibits pancreatic and gastric lipase release. Reduces intestinal fat absorption.	Gastrointestinal distress due to fat malabsorption including steatorrhea, flatulence, faecal incontinence, fat-soluble vitamin deficiencies and kidney stones.	(430,431,435)
Liraglutide	GLP-1 receptor agonist. Appetite-suppressant.	Nausea, diarrhoea, constipation and dyspepsia.	(430,435,436)
Anti-diabetes medications			
Insulin	Hormone. Stimulates glucose uptake and disposal in tissues. Lowers blood glucose levels.	Excess insulin or misuse causes hypoglycaemia.	(430,437)
Metformin	Biguanide. Suppresses hepatic glucose production. Lowers blood glucose levels.	Lethargy, nausea, diarrhoea, and abdominal pain.	(430,438)
Exenatide	GLP-1 receptor agonist. Lowers blood glucose levels. Appetite-suppressant.	Weight loss, nausea, diarrhoea and vomiting.	(168,430,439-441)

Current anti-obesity medications

Several medications are currently approved for the treatment of obesity, such as phentermine, orlistat and liraglutide (430,431). One of the most widely prescribed anti-obesity medications in Australia and America is phentermine (430,431). Phentermine was first approved for short-term use to treat obesity in America in 1959 (431). It belongs to a class of drugs called sympathomimetic amines, which act centrally to stimulate noradrenaline release, causing appetite suppression (431). In a systematic review, administration of 15 to 30 mg of phentermine in overweight individuals significantly reduced body weight (~6 kg) compared to a placebo (434). However, there is also evidence that phentermine causes side effects such as nausea, gastrointestinal distress, insomnia, anxiety, tachycardia and hypertension (430). Further, due to the potential severity of these side effects, phentermine is not recommended for long-term use or in patients with a history of CVD (430).

Current anti-diabetes medications

Several medications also exist for the treatment of type 2 diabetes, including insulin, metformin and GLP-1 receptor agonists such as exenatide. The therapeutic effects and side effects of these anti-diabetes medications will be discussed below.

Insulin

In type 1 diabetes, the pancreas is unable to produce insulin, and thus, exogenous insulin administration is needed for survival (437). Comparatively, in type 2 diabetes, the pancreas is still able to synthesise and secrete insulin. However, over time pancreatic insulin secretion is impaired in type 2 diabetes, and exogenous insulin administration may become necessary to maintain adequate blood glucose control (437). In a clinical study, 10 years of insulin therapy in individuals with type 2 diabetes significantly reduced plasma Hb1Ac levels and improved glucose tolerance (442). However, this study also demonstrated insulin therapy is not without unwanted side effects since participants also gained ~4 kg of body weight (442). Furthermore, insulin therapy is also associated with a risk of hypoglycaemia following inappropriate administration (437).

Metformin

Metformin is another effective treatment for type 2 diabetes. Metformin belongs to a class of drugs called biguanides, which lower blood glucose levels by suppressing hepatic glucose production (443). In individuals with obesity, metformin treatment has been shown to reduce the onset of type 2 diabetes (444). In addition, metformin combined with insulin therapy was shown to significantly reduce blood glucose levels and reduced exogenous insulin requirements in individuals with type 2 diabetes (445). Evidence also demonstrates metformin therapy improves cardiovascular health in individuals with type 2 diabetes by reducing plasma fatty acid and cholesterol levels (445). Further, a systematic review shows that 6 months of metformin therapy is sufficient to reduce BMI in adults and children with overweight and obesity (446). Overall, evidence suggests metformin therapy is beneficial for the treatment of obesity and type 2 diabetes. In fact, metformin is currently considered the gold standard medication for treating type 2 diabetes (438). However, metformin is associated with side effects, including lethargy, nausea, diarrhoea, and abdominal pain (447). In addition, controversy also exists over the safety of metformin use in individuals with impaired renal function due to the increased risk of lactic acidosis (448).

GLP-1 receptor agonists

Another type of anti-diabetes medication is GLP-1 receptor agonists, such as exenatide, liraglutide, lixisenatide, dulaglutide, albiglutide and semaglutide (433). These medications mimic the effects of native GLP-1 in humans but with synthetically developed changes to the protein-peptide sequences, which increase the potency and duration of their effects (433,449,450).

In humans, native GLP-1 plays a role in appetite regulation (47,80) and blood glucose homeostasis (168,226) via activation of GLP-1 receptors, 463 amino acid G-protein coupled receptors, which are expressed in a variety of tissues, including the brain, vagal afferents, pancreas, kidneys, stomach, heart, and adipose tissue (47,168). In a clinical study, continuous infusion of human GLP-1 for 6 weeks in individuals with type 2 diabetes significantly reduced plasma HbA1c levels, reduced fasting and postprandial blood glucose levels, improved insulin sensitivity (measured via a hyperinsulinaemic euglycaemic clamp) and improved pancreatic β-cell function (169). Continuous administration of native GLP-1 has also been shown to reduce total body weight and plasma lipid levels in individuals with type 2 diabetes by suppressing

appetite and subsequent food intake (169). Similar anti-obesity and anti-diabetic effects are also observed following the administration of human GLP-1 in mice (451).

Human GLP-1 is very short-acting. In humans, bioactive GLP-1 is derived from GLP-1(1–37) and exists in two molecular forms in the circulation, GLP-1(7–37) and GLP-1(7–36) amide, with GLP-1(7–36) amide being the predominant form (47,168). However, the majority of GLP-1 is degraded in the gut, with only an estimated 10-15 % reaching the systemic circulation, where it is progressively degraded with increasing distance from the site of secretion (47). Human GLP-1(7–37) and GLP-1(7–36) amide are 31 and 30 amino acids long, respectively, and both contain an alanine at position 2, enabling rapid cleavage and degradation by the enzyme dipeptidyl peptidase 4 (DPP4) (47,168). Therefore, the potentially therapeutic blood glucose-lowering and appetite inhibitory effects of human GLP-1 administration are generally short-lived (168). Instead, research interests have shifted towards other potential GLP-1 receptor agonists, such as exenatide and monotreme GLP-1s, which mimic the potent effects of human GLP-1 but are resistant to degradation by DPP4 (433).

In individuals with type 2 diabetes, treatment with GLP-1 receptor agonists are shown to improve glucose tolerance, specifically reducing fasting and postprandial blood glucose levels (452-454). For example, in a systematic review, GLP-1 receptor agonist therapy (e.g. exenatide, liraglutide or dulaglutide administration) in individuals with type 2 diabetes is shown to reduced fasting blood glucose levels and plasma HbA1c levels further than those accomplished with metformin therapy (455). Evidence also demonstrates GLP-1 receptor agonist therapy in individuals with type 2 diabetes promotes weight loss by suppressing appetite (436,456,457).

Exenatide

Exenatide is a highly potent, naturally occurring GLP-1 receptor agonist (168,458,459), approved for use in the treatment of type 2 diabetes in the USA in 2005 (433) and Australia in 2012 (432). Exenatide is a synthetic derivative of exendin-4 (Ex-4), originally isolated from the poisonous salivary secretions of the Gila monster (*Heloderma suspectum*) (460,461). The amino acid peptide sequence of Ex-4 is ~50 % similar to that of human GLP-1 (217,458), with a specific change in the amino acids at position 2 (alanine to glycine) (217), resulting in significant changes to the pharmacodynamic profile, providing resistance to cleavage by DPP4 and thus a longer half-life (217,458). The mean terminal half-life of Ex-4 is ~2.4 hr after a

single subcutaneous administration, and it is detectable in plasma for ~10 hr (433). Further, *in vitro* studies have demonstrated that Ex-4 binds to the human GLP-1 receptor with ~400 times greater affinity than human GLP-1 (462,463), suggesting that Ex-4 is likely to have a greater duration of effect compared to human GLP-1.

In diabetic animal models (439,464) and clinical studies (441,454,465), treatment with exenatide (or Ex-4) induces a dose-dependent reduction in fasting and postprandial blood glucose levels. In addition, in individuals with type 2 diabetes, exenatide induces greater reductions in plasma HbA1c levels and body weight compared to other GLP-1 receptor agonists (i.e. liraglutide) (457) and metformin (466). However, in clinical studies, exenatide therapy may also induce mild to moderate nausea (433,441,454,465). Other side effects include significant gastrointestinal distress, vomiting and diarrhoea, and an increased risk of mild to moderate hypoglycaemia when administered alongside insulin therapy (217,433). Therefore, there is a need for new pharmacological treatment options, with similar or greater efficacy but reduced side effects. One possibility is the monotreme GLP-1 analogues.

1.3.6. Monotreme GLP-1

Similar to Ex-4, monotreme GLP-1 results from an investigation into the evolution of incretin hormones in different species. During the platypus genome project (467), it was discovered that the modern platypus has lost or mutated many genes essential for gut function, including those encoding the gastrointestinal hormone GLP-1. Further investigation revealed that monotreme GLP-1, expressed in both the venom gland and digestive tract of the platypus (*Ornithorhynchus anatinus*) and short-beaked echidna (*Tachyglossus aculeatus*) (461,468), can bind and signal through the same GLP-1 receptor. Although the role of GLP-1 in the gastrointestinal tract is well-known in many species, the role of GLP-1 in monotreme venom is unclear (461).

The amino acid sequence of the platypus (pGLP-1) and echidna (eGLP-1) GLP-1s are similar, 63.3 % and 43.3 %, respectively, to that of human GLP-1 (468). While pGLP-1 and eGLP-1 are degraded in monotremes, the differences in the amino acid sequences correspond to changes in the DPP4 cleavage site, resulting in resistance to degradation in humans and mice, suggesting prolonged physiological effects (468). In fact, following incubation of human and monotreme GLP-1 peptide in human serum containing DPP4, pGLP-1 and eGLP-1 remained almost entirely intact after 4 hr, whereas human GLP-1 had degraded by ~50 % after 1 hr and

~80 % after 4 hr (468). However, unlike Ex-4, the pGLP-1 and eGLP-1 in cultured rat insulinoma (INS-1(832/13)) cells appeared to bind to the human GLP-1 receptor with lower affinity and are less potent in their stimulation of intracellular signalling, i.e. reduced elevations in intracellular calcium and cyclic AMP (468). This suggests the monotreme GLP-1s may elicit long-lasting physiological effects such as supporting blood glucose and energy homeostasis, with a low risk of causing side effects, such as hypoglycaemia.

For the first time *in vivo*, chapter 4 in this thesis will investigate the potential of the monotreme GLP-1s as effective, naturally occurring GLP-1 receptor agonists. In particular, chapter 4 will investigate the effect of monotreme GLP-1s on glucose tolerance in lean and HFD-induced obese mice.

1.4 Aims of the thesis

This thesis aims to explore potential treatment options for obesity and diabetes, including new or improved dietary, lifestyle and pharmaceutical interventions.

1.4.1 Aim 1

In rodents, chronic isoleucine supplementation prevents diet-induced obesity (349), and acute isoleucine supplementation improves glucose tolerance (351). However, little is known about the effects of chronic isoleucine supplementation on body weight and glycaemic control in mice with established obesity. Chapter 2 of this thesis details findings from the study entitled "The effect of dietary isoleucine supplementation on body weight and blood glucose response in lean and obese mice". This study aimed to determine whether chronic dietary supplementation with the BCAA isoleucine is an effective treatment for diet-induced obesity and glucose intolerance.

1.4.2 Aim 2

Shift-workers have a greater risk of obesity compared to individuals with daytime occupations (185), attributed to the misalignment of their circadian clock with external cues, such as the timing of food intake (201,324). TRF during the active phase, DP in rodents (325,326) and LP in humans (327), may be an effective treatment for diet-induced obesity and circadian desynchrony. However, lifestyles such as shift work often impede food intake during the LP in humans. Chapter 3 of this thesis details findings from the study "Metabolic benefits of light- and dark-phase time-restricted feeding in mice". This study aimed to investigate in lean and obese mice the impact of TRF during the LP compared to TRF during the DP.

1.4.3 Aim 3

Monotreme GLP1 is a novel, natural, long-acting GLP-1 receptor agonist with the potential, based on a previous *in vitro* study (468), to become an effective treatment for type 2 diabetes. However, very little is known about the efficacy of monotreme GLP-1 *in vivo*. Chapter 4 of this thesis details findings from the study entitled "The effect of monotreme GLP-1 analogues on glucose tolerance in lean and diet-induced obese mice". This study aimed to determine the potential effects of monotreme GLP-1 receptor agonists on glucose tolerance *in vivo*.

CHAPTER 2. Dietary Intervention; Isoleucine Supplementation

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Rebecca Jane O'Rielly
Contribution to the Paper	Planned the study, husbandary of animals, performed the experiments, collected and analysed the data, and drafted the manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 02/07/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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The Effect of Isoleucine Supplementation on Body Weight Gain and Blood Glucose Response in Lean and Obese Mice

2.1 Abstract

Chronic isoleucine supplementation prevents diet-induced weight gain in rodents. Acute-isoleucine administration improves glucose tolerance in rodents and reduces postprandial glucose levels in humans. However, the effect of chronic-isoleucine supplementation on body weight and glucose tolerance in obesity is unknown. This study aimed to investigate the impact of chronic isoleucine on body weight gain and glucose tolerance in lean and high-fat-diet (HFD)-induced obese mice. Male C57BL/6 mice, fed a standard-laboratory diet (SLD) or HFD for 12 weeks, were randomly allocated to: (1) Control: drinking water; (2) Acute: drinking water with a gavage of isoleucine (300 mg/ kg) prior to the oral glucose tolerance test (OGTT) or gastric emptying breath test (GEBT); (3) Chronic: drinking water with 1.5 % isoleucine, for a further 6 weeks. At 16 weeks, an OGTT and GEBT was performed and at 17 weeks metabolic monitoring. In SLD- and HFD-mice, there was no difference in body weight, fat mass, and plasma lipid profiles between isoleucine treatment groups. Acute-isoleucine did not improve glucose tolerance in SLD- or HFD-mice. Chronic-isoleucine impaired glucose tolerance in SLD-mice. There was no difference in gastric emptying between any groups. Chronic-isoleucine did not alter energy intake, energy expenditure, or respiratory quotient in SLD- or HFD-mice. In conclusion, chronic isoleucine supplementation may not be an effective treatment for obesity or glucose intolerance.

LIST OF ABBREVIATIONS

In alphabetical order

A	Acute
AUC	Area under the curve
BCAA	Branched-chain amino acid
C	Control
Ch	Chronic
GEBT	Gastric emptying breath test
HDL	High-density lipoprotein
HFD	High-fat diet
LDL	Low-density lipoprotein
OGTT	Oral glucose tolerance test
RQ	Respiratory quotient
SLD	Standard laboratory diet
UCP	Uncoupling protein

2.2. Introduction

The branched-chain amino acids (BCAAs), isoleucine, leucine and valine, are essential amino acids accounting for ~35 % of the essential amino acids comprising muscle proteins in humans and ~40 % of the pre-formed amino acids required by all mammals (116). In population studies, an elevated dietary intake of BCAAs was associated with a lower prevalence of overweight and obesity in adults (358,359). Further, BCAA supplementation was demonstrated to preserve lean muscle mass during weight loss (360,394,396). This evidence suggests a role for BCAA supplementation in the treatment of obesity. In particular, chronic isoleucine supplementation in rodents has been demonstrated to prevent high-fat diet (HFD)-induced obesity (349). However, whether chronic isoleucine supplementation is an effective approach to ameliorate weight gain and promote weight loss in established obesity, is unknown.

Acute administration of isoleucine and leucine in rats improved glucose tolerance, with isoleucine showing greater effectiveness than leucine (350). This was attributed to the synergistic action of isoleucine with endogenous insulin to enhance glucose uptake into tissues (254,413,426). In addition, acute isoleucine supplementation improved glucose tolerance in leptin receptor-deficient (*db/db)* mice, a model of morbid obesity and hyperglycaemia (351). This evidence suggests that isoleucine supplementation may be useful in the treatment of glucose intolerance. However, whether this glucose-lowering effect persists following a chronic supplementation regime is unknown.

It is known that postprandial blood glucose levels are influenced by the rate of gastric emptying (225). In participants with type 2 diabetes, consumption of whey protein before a meal slowed gastric emptying and was associated with lower postprandial blood glucose levels (166,469). Further, in healthy lean participants, acute intragastric administration of isoleucine lowered the blood glucose response to a mixed nutrient drink, which was attributed to a slowing of gastric emptying (365). Therefore, we hypothesised that chronic isoleucine supplementation will slow gastric emptying, improve glucose tolerance, and reduce body weight gain in mice.

The current study aimed to determine whether chronic dietary supplementation with the BCAA isoleucine, alters body weight gain, adiposity, glucose tolerance, and energy metabolism in mice with HFD-induced obesity.

2.3 Materials and Methods

Ethics Approval

This study was approved (Ethics approval: SAM237) by the South Australian Health and Medical Research Institute Animal Ethics Committee. All experimental protocols were performed in alignment with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Study Design

Eight week old male C57BL/6 mice (N = 54) were group-housed in a 12:12 hr light-dark cycle within a temperature (24 ± 1 °C) controlled facility. Mice were provided *ad libitum* access to either a standard laboratory diet (SLD; 12 %, 23 %, and 65 % of energy from fat, protein, and carbohydrates, respectively; Specialty Feeds, Western Australia, Australia; N = 30) or HFD (60 %, 20 %, and 20 % of energy from fat, protein, and carbohydrates, respectively; adapted from Research Diets Inc., New Brunswick, NJ, USA; N = 24). Consistent with the previous literature, the HFD-induced obese mouse model was chosen as both a model of obesity (470) and impaired glucose tolerance (471). After 12 weeks on their respective diets, a sub-group of SLD (N = 10; SLD-Chronic (Ch)) and HFD-mice (N = 8; HFD-Ch) received *ad libitum* isoleucine (1.5 % w/v; Purebulk Inc., Roseburg, OR, USA) supplemented in the drinking water. The remaining SLD (N = 20) and HFD-mice (N = 16) continued with *ad libitum* access to normal drinking water. At 16 weeks, all mice were singly housed and underwent an oral glucose tolerance test (OGTT) (at 1400 hr) and gastric emptying breath test (GEBT) (at 0900 hr) in random order with a 3 day recovery between tests. In each diet group, the mice provided normal drinking water were subdivided into 2 groups, receiving either an oral gavage of water (N = 10, SLD-Control (C); N = 8, HFD-C) or isoleucine (300 mg/ kg body weight; N = 10, SLD-Acute (A); N = 8, HFD-A) 30 min before the OGTT or GEBT. The SLD and HFD-Ch mice received an oral gavage of drinking water similar to the control groups. The doses for acute and chronic isoleucine treatments were chosen based on previous studies (350,351). At 17 weeks, all mice were placed in metabolic monitoring cages. The body weight of all the mice was measured weekly, except the final 2 weeks due to the different interventions.

Oral Glucose Tolerance Test

Consistent with previous studies (472,473), mice were fasted for six hours (0800–1400 hr) before receiving an oral gavage of either isoleucine (SLD/HFD-A groups) or water (SLD/HFD-

C and SLD/HFD-Ch groups). After 30 min, all mice received an oral gavage of 20 % D-glucose (1 g/ kg BW), a dose chosen to ensure the HFD-mice did not experience a severe hyperglycemic response with blood glucose levels beyond the range of the glucose monitor. Blood was collected from a tail prick before isoleucine/water administration, considered as the baseline, and again at 15, 30, 45, 60, and 120 min post glucose administration. Blood glucose levels were determined with an ACCU CHEK Performa monitor (ACCU CHEK, New South Wales, Australia).

Gastric Emptying Breath Test

Gastric emptying of a solid meal was determined using a non-invasive breath test as previously described (474,475). Mice were fasted overnight (1600-0900 hr) prior to an oral gavage of either isoleucine (SLD/HFD-A) or water (SLD/HFD-C and SLD/HFD-Ch). After 30 min, all mice were provided 0.1 g of baked egg yolk containing ^{13}C-octanoic acid (1 μL/ g; 99 % enrichment, Cambridge Isotope Laboratories, Andover, MA, USA) to consume voluntarily within 1 min. Breath samples were collected before isoleucine administration (baseline; 0 min) and again at regular intervals (5 min intervals from 5–30 min and 15 min intervals from 30–150 min) after egg consumption. Breath samples were analysed for the $^{13}CO_2$ content using an isotope ratio mass spectrometer (Europa Scientific, Crewe, UK). The $^{13}CO_2$ excretion data were analysed by non-linear regression analysis for curve fitting and for calculation of gastric half emptying time (t ½) (476). Gastric half emptying time was not measured in the HFD-mice due to sampling difficulties; the egg yolk was not consumed within 1 min which invalidates results.

Metabolic Monitoring

Mice were individually housed in Promethium metabolic cages (Sable Systems International, North Las Vegas, NV, USA) for 72 hr of continuous metabolic monitoring. Energy intake (kJ), energy expenditure (kJ/ lean mass), respiratory quotient (RQ; VCO_2/ VO_2), and total activity (meters, m) were measured and analysed using the ExpeData data analysis software (Sable Systems International, Nevada, USA).

Tissue Collection

Mice were fasted overnight (1600–0900 hr) then anaesthetised with isoflurane (5 % in medical oxygen). The nose-to-tail length and abdominal circumference of mice were measured. Blood was collected from the abdominal aorta and transferred to ethylenediaminetetraacetic acid (EDTA) tubes (Thermo Fisher Scientific, Victoria, Australia). Plasma was extracted by

centrifugation at 1000 g and 4 °C for 15 min, and snap-frozen in liquid nitrogen prior to storage at −80 °C until further analysis. Liver, gonadal fat pads, and inter-scapula brown fat pads were collected and weighed. Lean mass was determined by the final body weight minus the weight of collected fat pads. A section of the liver was fixed in 4 % paraformaldehyde for 4 hr, cryoprotected overnight in 30 % sucrose in a phosphate buffer, frozen in Tissue-Tek O.C.T. compound (Sakura Finetek USA Inc., Torrance, CA, USA) and stored at −80 °C before processing for histology.

Plasma Metabolites

Plasma total triglycerides, total cholesterol, and high-density lipoprotein (HDL)-cholesterol concentrations were measured using commercial enzymatic kits (OSR60118, OSR6116, and OSR6187, respectively (Beckman Coulter Inc., Georgia, USA)) on a Beckman AU480 clinical analyser (Beckman Coulter Inc., Atlanta, GA, USA). Plasma low-density lipoprotein (LDL)-cholesterol concentrations were estimated using the Friedewald equation (477):

$$\text{LDL-cholesterol} = \text{Total cholesterol} - (\text{total triglyceride}/2.2) - \text{HDL-cholesterol}$$

Liver Lipid Content

The histological lysochrome lipid stain Oil Red O was performed on liver sections using a standard protocol (478). Slides were imaged using a NanoZoomer digital slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan) and analysed for the average percentage of stained lipid per 1 mm^2 area using the ImageJ-win64 software.

Statistical Tests

Results are expressed as the mean ± SEM. A two-way ANOVA was performed to assess diet and isoleucine treatment effects with a Tukey's post hoc test for multiple comparisons, using the GraphPad Prism v8 software (GraphPad, California, USA). The $OGTT_{0–120\ min}$ blood glucose area under the curve (AUC) was generated using the IBM SPSS Statistics 26 software (IBM, New York, USA). A correlation between gastric half emptying time (t ½) and $OGTT_{0–120\ min}$ blood glucose AUC for SLD groups was performed in the GraphPad Prism v8 software. The coefficient of determination value (r^2) was considered significant at $p < 0.05$.

2.4 Results

Chronic Isoleucine Treatment Does Not Affect Weight Gain and Adiposity

At the beginning of week 12, prior to isoleucine supplementation, HFD-mice gained significantly more weight than SLD-mice ($p < 0.001$, unpaired *t*-test; Figure 2.1 A). There was no difference in body weight between different treatment groups in mice fed a SLD or HFD prior to chronic isoleucine treatment ($p < 0.0001$, F (1, 50) = 28.17, diet effect; $p = 0.1262$, F (1, 50) = 2.418, isoleucine effect; SLD-C/A; 34.8 ± 0.7 g (N = 20), SLD-Ch; 37.2 ± 1.3 g (N = 10), HFD-C/A; 42.3 ± 1.6 g (N = 16), and HFD-Ch; 44.1 ± 1.7 g (N = 8)).

In weeks 12-15, HFD-mice continued to gain more weight compared to SLD-mice ($p < 0.0001$, F (1, 50) = 19.21, diet effect; Figure 2.1 A), but there was no effect of chronic isoleucine treatment on weight gain (Figure 2.1 A).

At week 18, abdominal circumference was greater in HFD-mice than SLD-mice, but was not affected by chronic isoleucine treatment ($p < 0.0001$, F (1, 26) = 24.78, diet effect; SLD-C, 9.3 ± 0.2 cm, SLD-Ch, 9.9 ± 0.5 cm, HFD-C, 11.4 ± 0.3 cm, and HFD-Ch, 11.2 ± 0.3 cm). In addition, HFD-mice had heavier gonadal fat pads and brown fat pads than SLD-mice (both $p < 0.01$, F (1, 50) = 11.13 and F (1, 47) = 11.44, respectively, diet effect; Figure 2.1 Bi, ii), but these parameters were not affected by the chronic isoleucine treatment.

Chronic Isoleucine Treatment Does Not Affect Liver Lipid Content

HFD-mice had heavier livers than SLD-mice ($p < 0.0001$, F (1, 50) = 18.34, diet effect; Figure 2.2A). The liver lipid content was greater in HFD-mice than SLD-mice ($p < 0.0001$, F (1, 28) = 37.31, diet effect; Figure 2.2 B). There was no effect of the chronic isoleucine treatment on liver mass or lipid content (Figure 2.2 A, B).

Chronic Isoleucine Treatment Does Not Alter Energy Intake, Energy Expenditure, Activity, and Respiratory Quotient

HFD-mice consumed more energy across 24 hr ($p < 0.05$, F (1, 50) = 4.157, diet effect; Figure 2.3 Ai) compared to SLD-mice, predominantly due to increased energy intake during the light phase ($p < 0.05$, F (1, 50) = 5.715, diet effect; Figure 2.3 Aii). There was no effect of the chronic isoleucine treatment on total energy intake across 24 hr, during the light phase or dark phase (Figure 2.3 Ai–iii). HFD feeding and chronic isoleucine treatment had no effect on 24 hr of

total water intake (SLD-C; 3.5 ± 0.07 mL/day, SLD-Ch; 3.8 ± 0.17 mL/day, HFD-C; 3.5 ± 0.1 mL/day, and HFD-Ch; 3.6 ± 0.2 mL/day).

HFD-mice had a significantly lower energy expenditure (normalised to lean body mass) compared to SLD-mice across 24 hr, during the light phase or dark phase ($p < 0.01$, F (1, 50) = 9.151, $p < 0.05$, F (1, 50) = 6.499, $p < 0.01$, F (1, 50) = 11.25, respectively, diet effect; Figure 2.3 Bi–iii). A significant diet by the chronic isoleucine treatment interaction was observed in energy expenditure across 24 hr ($p < 0.05$, F (1, 50) = 5.416, interaction; Figure 2.3 Bi) and during the dark phase ($p < 0.05$, F (1, 50) = 6.468, interaction; Figure 2.3 Biii). During the dark phase, the chronic isoleucine treatment reduced energy expenditure in SLD-mice ($p < 0.05$, Sidak's post hoc test), but not in HFD-mice.

Total activity levels were not affected by HFD feeding or chronic isoleucine treatment across 24 hr or during the light or dark phase (Figure 2.3 Ci–iii).

HFD-mice had lower RQ values compared to SLD-mice across 24 hr ($p < 0.001$, F (1, 50) = 19.66, diet effect; Figure 2.3 Ci), during the light phase ($p < 0.05$, F (1, 50) = 4.082, diet effect; Figure 2.3 Cii) and dark phase ($p < 0.001$, F (1, 50) = 38.4, diet effect; Figure 2.3 Ciii). Average RQ values were not affected by the chronic isoleucine treatment across 24 hr, during the light phase or dark phase (Figure 2.3 Ci–iii).

Chronic Isoleucine Treatment Does Not Affect Plasma Lipid Metabolites

HFD-mice had elevated plasma total triglycerides ($p < 0.001$, F (1, 30) = 31.28, diet effect; Figure 2.4 A), total cholesterol ($p < 0.001$, F (1, 30) = 40.02, diet effect; Figure 2.4 B), HDL-cholesterol ($p < 0.001$, F (1, 30) = 40.16, diet effect; Figure 2.4 C), and LDL-cholesterol ($p < 0.0001$, F (1, 30) = 25.36, diet effect; Figure 2.4 D) compared to SLD-mice. There was no effect of chronic isoleucine treatment on these plasma lipid metabolites (Figure 2.4 A–D).

Acute and Chronic Isoleucine Treatment Differentially Affect Glucose Tolerance in SLD- and HFD-Mice

HFD-mice had higher fasting blood glucose levels than SLD-mice ($p < 0.0001$, F (1, 48) = 19.34, diet effect; Figure 2.5 Ai, Bi). There was no effect of the chronic isoleucine treatment on fasting blood glucose levels (Figure 2.5 Ai, Bi).

HFD-mice had a greater glucose AUC than SLD-mice ($p < 0.001$, F (1, 48) = 34.44, diet effect; Figure 2.5 Aii, Bii). In SLD groups, an elevated glucose AUC was observed in chronic isoleucine treated mice compared to control mice ($p < 0.05$, one-way ANOVA; Figure 2.5 Aii), but there was no difference between acute and chronic isoleucine treated mice. In HFD groups, there was no effect of acute or chronic isoleucine treatment on glucose AUC (Figure 2.5 Bii).

Acute and Chronic Isoleucine Treatment Do Not Affect Gastric Emptying

There was no significant difference in the gastric half emptying time (t ½) between different isoleucine groups in SLD-mice (SLD-C, 125.3 ± 13.2 min (N = 8); SLD-A, 144.4 ± 15.3 min (N = 8) and SLD-Ch, 126.7 ± 5.1 min (N = 7)).

There was no correlation between gastric half emptying time and blood glucose AUC in SLD-mice (correlation coefficient of determination value (r^2); SLD-C, $r^2 = 0.0197$, SLD-A, $r^2 = 0.2602$, and SLD-Ch, $r^2 = 0.004292$).

2.5 Discussion

Longitudinal population studies have demonstrated an inverse association between dietary BCAA consumption and the risk of obesity and diabetes (358,359), suggesting BCAA supplementation may be an effective dietary intervention to prevent obesity. In the current study, the acute isoleucine treatment had no beneficial effect on blood glucose levels in response to an OGTT. Further, chronic isoleucine supplementation was not an effective treatment for obesity and actually impaired glucose tolerance in SLD-mice.

In the current study, 6 weeks of chronic isoleucine treatment had no effect on body weight, gonadal fat pad mass, hepatic lipid content, and plasma lipid levels in SLD- and HFD-mice. Previously, 4 weeks of chronic isoleucine supplementation, protected mice from diet-induced weight gain and fat accumulation (349). In that study, isoleucine supplementation was initiated after only two weeks of HFD feeding (349), which is arguably an insufficient time-course to establish obesity in mice (106). Consistent with previous reports (131,198), 12 weeks of HFD feeding in the current study, led to significantly greater weight gain and adiposity compared to SLD-mice. Therefore, the findings suggest that isoleucine may be able to prevent diet-induced obesity, but may not be an effective treatment for reversing obesity.

Energy intake and energy expenditure are well-known effectors of body weight regulation (479). In the current study, the chronic isoleucine treatment did not alter total energy expenditure, total energy intake, average RQ, or total activity in SLD- or HFD-mice. Previously, chronic isoleucine or leucine supplementation have been demonstrated to protect mice from diet-induced weight gain without reducing energy intake or increasing total activity levels (349,357). Instead, leucine supplementation was observed to enhance total energy expenditure via an elevated 24 hr oxygen consumption rate, normalised to body weight (357). This elevated energy expenditure was attributed to an increased expression of uncoupling protein (UCP) 3 in thermogenic tissue (357). In the isoleucine supplemented mice, energy expenditure was not directly measured (349). However, expression of proteins involved in fatty acid uptake and oxidation were upregulated, namely fatty acid translocase, peroxisome proliferator-activated receptor and UCP2&3 in the liver and skeletal muscle (349). This evidence suggests that chronic isoleucine or leucine supplementation in the previous reports may have protected from diet-induced fat gain through elevating fatty acid oxidation. However, in the current study, the chronic isoleucine treatment did not reduce 24 hr RQ, suggesting no increase in lipid oxidation. Therefore, further investigation of lipid metabolism was not pursued. Indeed, the chronic supplementation of isoleucine in the current study, did not reverse the changes to energy expenditure observed in the HFD-induced obese mice.

In the current study, the chronic isoleucine treatment had no beneficial effect on fasting blood glucose levels in HFD-mice. This is consistent with a previous study where four weeks of isoleucine supplementation at a dose of 2.5 %, did not affect fasting blood glucose levels in male mice fed a 45 % HFD (349). In contrast, 6 weeks of 2 % chronic isoleucine supplementation significantly reduced fasting blood glucose levels compared to controls, in high-fat, high-sucrose fed female mice; a model of glucose intolerance (351). These findings suggest that chronic isoleucine supplementation may be more effective in ameliorating fasting blood glucose elevated by high sucrose diets rather than high fat diets. In addition, there may be a difference in treatment effects between male and female mice. Future studies should, therefore, investigate the mechanisms of the effect of chronic isoleucine supplementation on glycemic regulation, such as glucose uptake into tissues and hepatic glucose export. For example, acute administration of isoleucine to isolated myocytes stimulates the glucose uptake through enhanced recruitment of glucose transporters to the cell membrane (350). Further, acute isoleucine administration in mice, suppressed key enzymes in hepatic gluconeogenesis, namely phosphoenolpyruvate carboxykinase and glucose 6-phosphatase (428,429), contributing to lower fasting blood glucose levels.

Therefore, it is possible that the effect of isoleucine on these blood glucose regulatory mechanisms persists under a chronic supplementation regime. However, there was no difference in blood glucose levels in the OGTT after acute isoleucine treatment in either SLD- or HFD-mice and, therefore, this was not pursued in the current study but may warrant future investigation.

In the current study, acute isoleucine orally administered at a dose of 0.3 g/ kg, had no effect on postprandial blood glucose levels in SLD- or HFD-mice. Previously, acute administration of 0.3 g/ kg isoleucine in lean (350) and diet-induced obese mice (60 % HFD for eight weeks) (351) induced a dose-dependent reduction in postprandial blood glucose levels. Further, acute isoleucine administration in obese leptin receptor-deficient (*db/db)* mice, significantly reduced blood glucose levels in response to an OGTT, albeit at a higher dose of 0.5 g/ kg (351). Considering this observed dose-dependent effect, a larger dose may have been necessary to reduce the blood glucose AUC in the current more chronic (12 weeks) model of diet-induced obesity. However, this does not explain why no effect was observed in the SLD-mice.

Consistent with a previous report (351), the chronic isoleucine treatment had no beneficial effect on blood glucose levels in response to an OGTT in HFD-mice. In contrast, the chronic isoleucine supplementation impaired glucose tolerance in lean mice, suggesting an adverse effect of chronic isoleucine supplementation. It has been reported that a western diet low in BCAAs reduced the blood glucose AUC compared to mice fed a standard western diet (382). Furthermore, consistent with the current study a western diet supplemented with BCAAs had no effect on blood glucose AUC compared to mice fed a standard western diet (382). Further, there is some evidence that obesity-induced BCAA dysmetabolism may promote insulin resistance through mitochondria dysfunction and impaired fatty acid oxidation (221,353). Whether chronic isoleucine supplementation induces BCAA dysmetabolism, and promotes insulin resistance in lean mice, requires further investigation.

Slowing gastric emptying allows for efficient digestion and absorption of nutrients, including glucose (80,225,480). In the current study, the acute and chronic isoleucine treatment did not affect gastric emptying in SLD-mice. Further, there was no significant correlation between postprandial blood glucose levels and gastric emptying in any groups. Previously, the consumption of whey protein, rich in BCAAs, including isoleucine (481), before a meal has been demonstrated to slow gastric emptying and reduce postprandial hyperglycaemia in people with type 2 diabetes (166,469). Similarly, intragastric administration of isoleucine slowed gastric emptying and reduced postprandial glucose levels in lean participants (365). However,

in rodent studies, the effect of isoleucine administration on gastric emptying is less clear. For example, in rats, oral administration of isoleucine reduced the $^{13}CO_2$ content in the breath following a gastric emptying breath test, but did not significantly affect the $^{13}CO_2$ AUC, Cmax, or Tmax values (peak concentration and the time at which it occurred) compared to controls (389).

2.6 Conclusion

The acute and chronic isoleucine supplementation had no beneficial effect to limit HFD-induced body weight gain, adiposity, fasting blood glucose levels, and glucose tolerance. In contrast, the chronic isoleucine treatment impaired glucose tolerance in SLD-mice. Therefore, the chronic isoleucine supplementation is unlikely to be an effective dietary intervention for the treatment of obesity and type 2 diabetes.

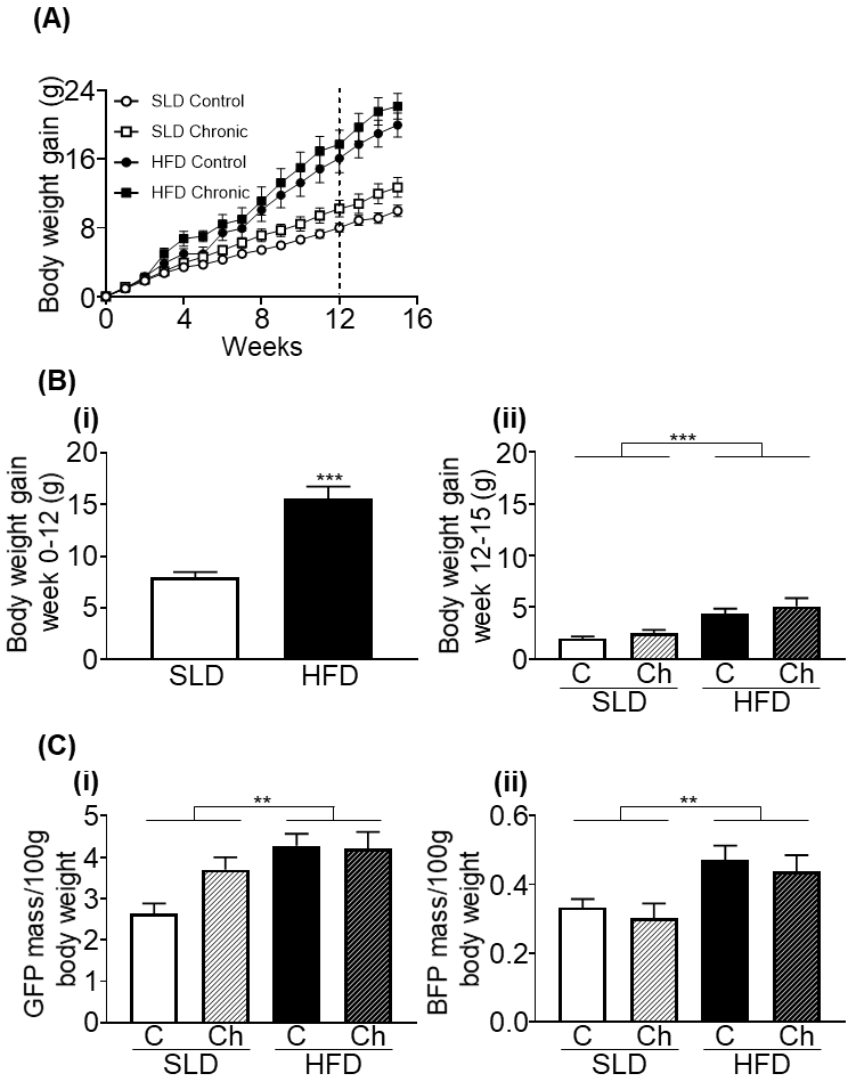

Figure 2.1. High fat diet (HFD) feeding but not the chronic isoleucine treatment increased weight gain and adiposity in mice. (A) Body weight gained during weeks 0–15 in mice fed a standard laboratory diet (SLD) or HFD (SLD/HFD-Control (C; control and acute groups pooled as no acute gavage of isoleucine had occurred at this point) N = 16–20; SLD/HFD-Chronic (Ch) N = 8–10). The dotted line indicates the onset of chronic isoleucine supplementation. (Bi) Body weight gained during week 0–12 in SLD or HFD (N = 24–30/group; all HFD and SLD groups pooled as no chronic or acute isoleucine treatment had occurred at this point); *** $p < 0.001$ unpaired t-test. (Bii) Body weight gained in week 12–15 (SLD/HFD-Control (C; control and acute groups pooled as no acute gavage of isoleucine had occurred at this point) N = 16–20; SLD/HFD-Chronic (Ch) N = 8–10). (Ci) Gonadal fat pad (GFP) mass and (Cii) brown fat pad (BFP) mass per 100 g of total body weight (N = 8–10/group). Values are mean ± SEM. ** $p < 0.01$, *** $p < 0.001$; diet effect, two-way ANOVA.

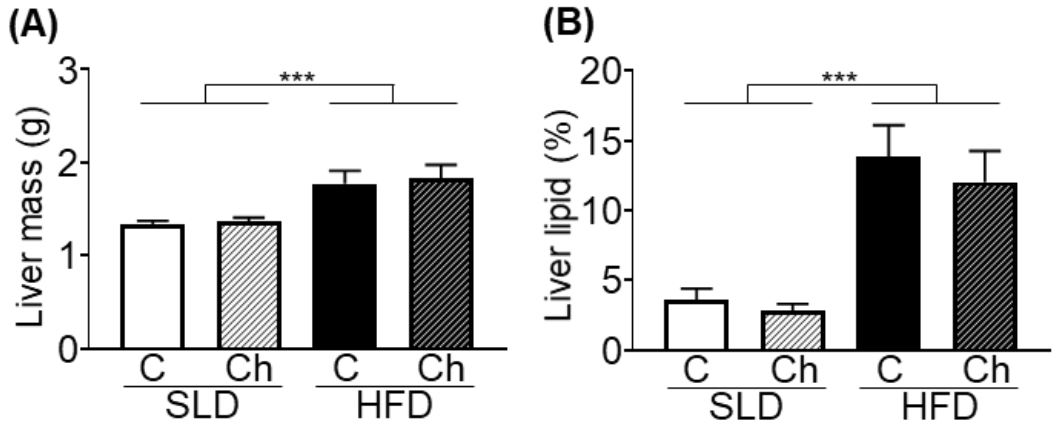

Figure 2.2. High fat diet (HFD) feeding but not chronic isoleucine treatment increased the liver mass and lipid content in mice. (A) Liver mass and (B) percentage lipid area per 1 mm^2 liver area of standard laboratory diet (SLD) and HFD-mice (SLD/HFD-Control (C) and SLD/HFD-Chronic (Ch), N = 8–10). Values are mean ± SEM. *** $p < 0.001$; diet effect, two-way ANOVA.

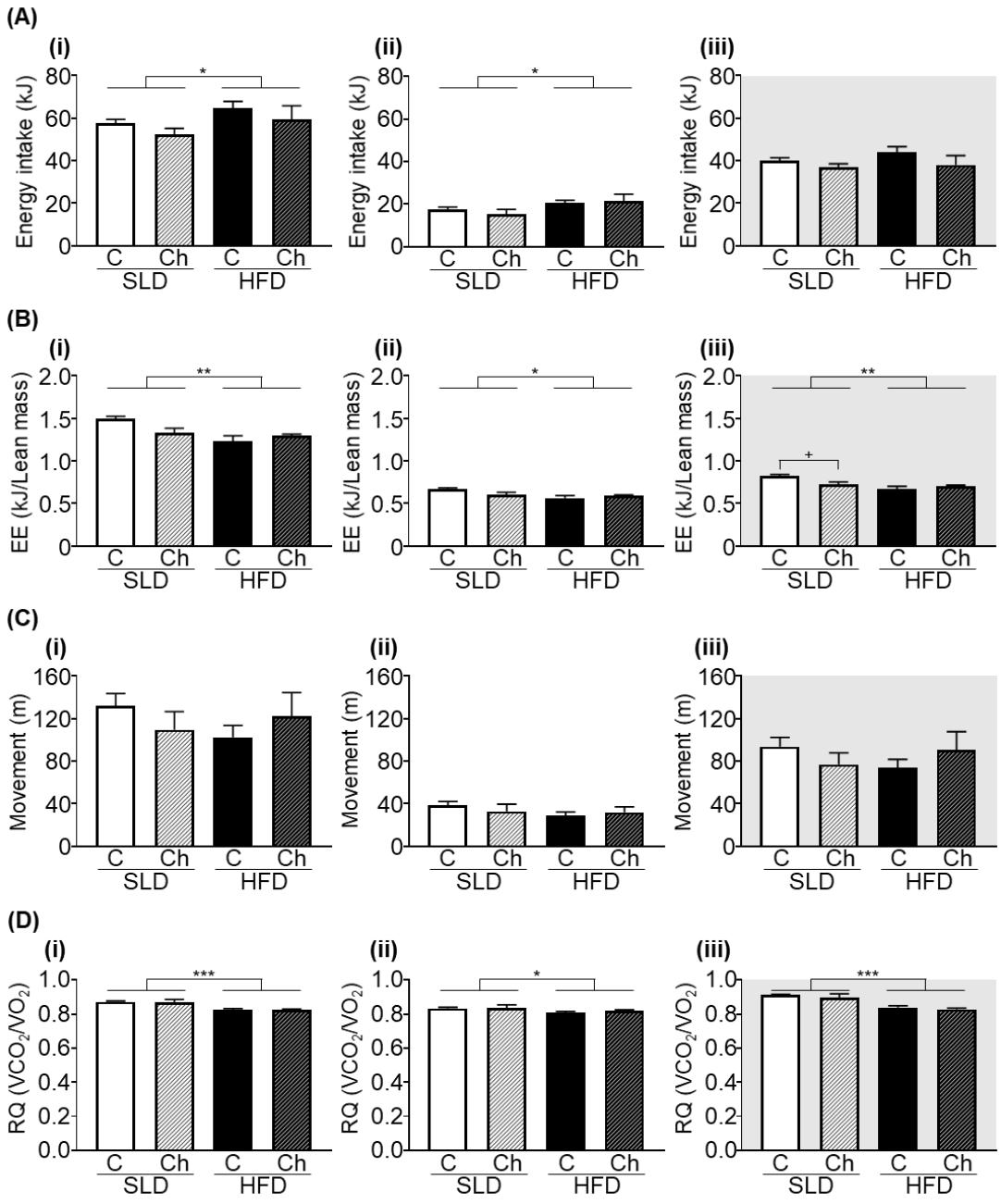

Figure 2.3. High fat diet (HFD) feeding but not the chronic isoleucine treatment affects energy balance in mice. (A) Energy intake, (B) energy expenditure (EE), (C) activity (distance of movement), and (D) respiratory quotients (RQ), across 24 h (i), 12 h of day (light phase) (ii), and 12 h of night (dark phase) (iii) in a standard laboratory diet (SLD) and HFD-mice (SLD/HFD-Control (C) N = 16–20, SLD/HFD-Chronic (Ch) N = 8–10). Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, diet effect; two-way ANOVA with Sidak's *post hoc* test, +$p < 0.05$.

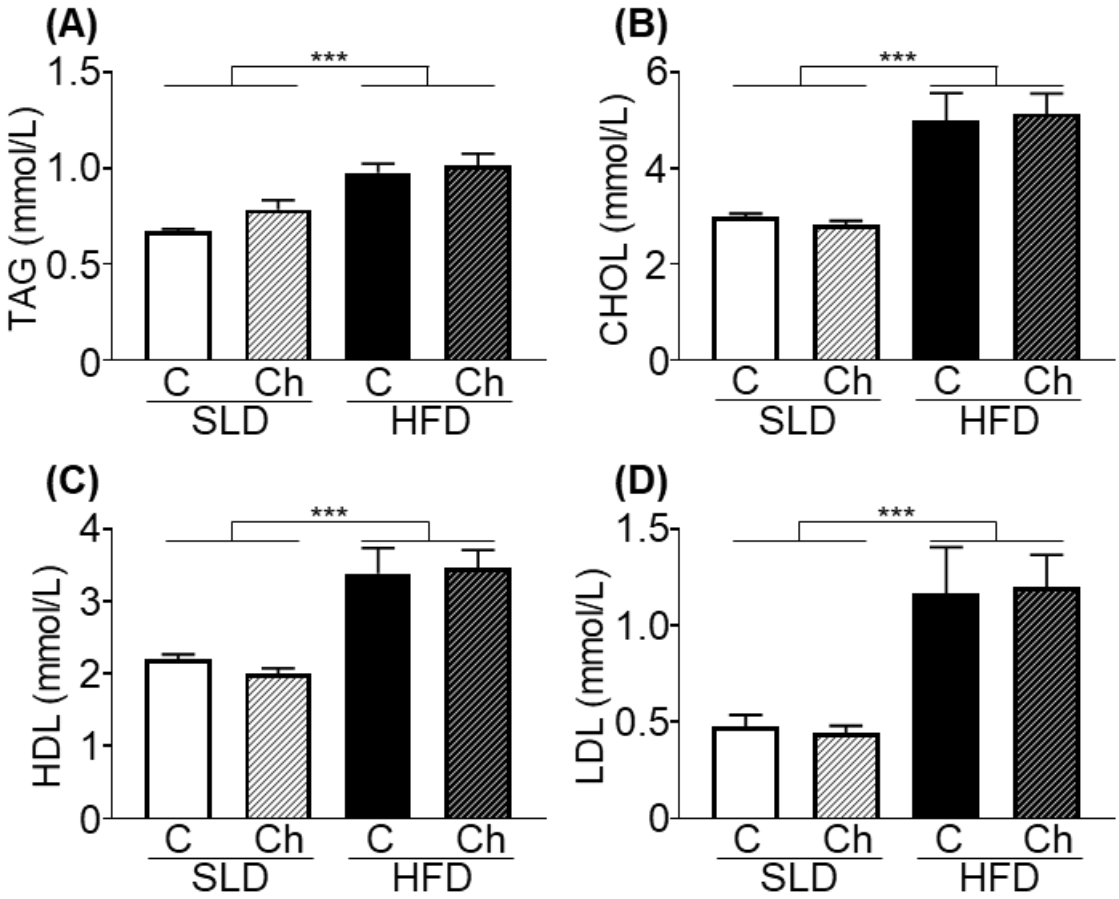

Figure 2.4. High fat diet (HFD) feeding but not the chronic isoleucine treatment elevated plasma lipid metabolites in mice. Plasma (A) total triglycerides (TAG), (B) total cholesterol (CHOL), (C) high density lipoprotein (HDL), and (D) low density lipoprotein (LDL) levels in a standard laboratory diet (SLD) and HFD-mice (SLD/HFD-Control (C) N = 8–10, SLD/HFD-Chronic (Ch) N = 8–10). Values are mean ± SEM *** $p < 0.001$, diet effect; two-way ANOVA.

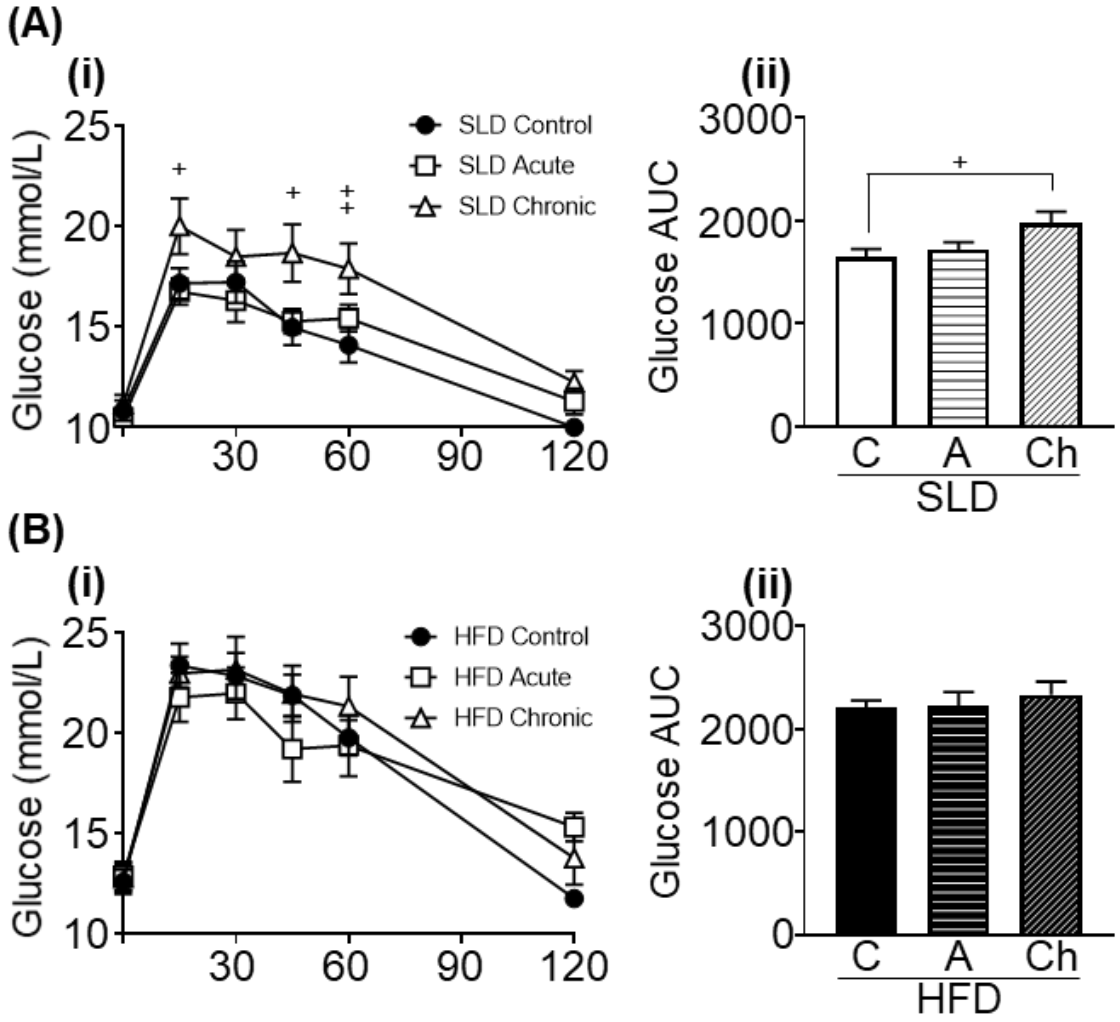

Figure 2.5. Acute (A) and chronic (Ch) isoleucine treatment differentially affects glucose tolerance. (i) Blood glucose levels in response to an oral glucose tolerance test and (ii) glucose area under curve (AUC) in (A) standard laboratory diet (SLD) and (B) high fat diet (HFD)-mice. (SLD/HFD-Control (C) N = 8–10, SLD/HFD-A N = 8–10 and SLD/HFD-Ch N = 8–10). Values are mean ± SEM. + $p < 0.05$, one-way ANOVA.

CHAPTER 3. Lifestyle Intervention; Time-Restricted Feeding

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Rebecca O'Rielly		
Contribution to the Paper	Contributed to planning of study. Husbandary of animals, performed the experiments, collected and analysed the data, and drafted the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	17/06/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Metabolic Benefits of Light- and Dark-Phase Time-Restricted Feeding in Mice.

3.1 Abstract

Background: Desynchrony of circadian rhythms (e.g. shift work) is a risk factor for obesity. In mice, restricting feeding to the active phase (dark phase, DP) reduces weight gain and entrains circadian rhythms. This study aimed to compare the effects of time-restricted feeding (TRF) in the DP and inactive light phase (LP) on body weight, energy balance and circadian rhythmicity of hepatic glucose and lipid metabolic markers, in lean and high-fat diet (HFD)-induced obese mice.

Methods: Male C57BL/6 mice were fed a standard laboratory diet (SLD) or HFD for 12 weeks. After 4 weeks diet acclimatisation, mice were assigned to 1 of 3 groups/diet and fed: i) *ad libitum* (AL); ii) LP (Zeitgeber (ZT) 0-12 hr); or iii) DP (ZT12-24 h) for the remaining 8 weeks. In week 12 the mice were placed into metabolic monitoring cages. Finally, blood and liver were collected every 3 hr for measurement of blood glucose and plasma triglyceride concentrations as well as gene expression markers of hepatic glucose uptake (glucose transporter solute carrier family 2 member 2 (Slc2a2); insulin receptor β-substrate (Irβ)) and storage (glycogen synthase (Gys2)), and lipid synthesis (acetyl-CoA carboxylase (Acc1α)).

Results: In HFD-mice, both forms of TRF reduced energy intake, weight gain, fat mass, plasma triglycerides and hepatic lipid content compared to HFD-AL. Mean blood glucose concentrations were elevated in all groups compared to SLD-AL mice. In general, gene expression of hepatic Slc2a2, Irβ and/or Gys2 was also elevated compared to SLD-AL mice. Circadian rhythmicity in blood glucose concentrations and gene expression of hepatic Slc2a2 and Gys2 were reversed by LP-TRF in SLD and HFD-mice. Plasma triglyceride levels were reduced in LP and DP-TRF in both SLD and HFD-mice compared to SLD-AL mice. This was associated with an increase in gene expression of hepatic Acc1α in LP and DP-TRF in both diet groups.

Conclusion: TRF during either the LP or DP reduced HFD-induced weight gain and aligned circadian rhythms of hepatic markers of glucose and lipid metabolism to the timing of food intake.

LIST OF ABBREVIATIONS

In alphabetical order

Acc1α	Acetyl-CoA carboxylase
AL	*Ad libitum*
DP	Dark phase
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Gys2	Glycogen synthase
HFD	High-fat diet
Irβ	Insulin receptor β-substrate
LP	Light phase
NAFLD	Non-alcoholic fatty liver disease
OD	Optical density
Ppia	Peptidylprolyl isomerase A
RQ	Respiratory quotient
SCN	Suprachiasmatic nucleus
Slc2a2	Glucose transporter
SLD	Standard laboratory diet
TRF	Time-restricted feeding
ZT	Zeitgeber

3.2 Introduction

Shift-workers have a 3-fold greater risk of obesity and related chronic diseases compared to individuals with daytime occupations (185). Increased risk is primarily attributed to the misalignment of the circadian clock with external cues, such as the timing of food intake (201,324).

The circadian clock is a fundamental regulatory system that entrains diurnal behavioural and metabolic processes to the 24 hr solar light-dark cycle (173). In brief, the central 'clock', located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, consists of a network of gene transcriptional–translational feedback loops, driving rhythmic oscillations of core-clock components (188). The SCN receives neural input from the retina to allow alignment with the light-dark cycle (188). This central clock then regulates other clocks located in peripheral tissues, such as the liver (181). Subsequently, these peripheral clocks regulate other homeostatic pathways including metabolic gene transcription, specifically the transcription of genes involved in hepatic glucose metabolism (e.g. glucose transporter (Slc2a2) (181,482,483), insulin receptor β-substrate (Irβ) (484) and glycogen synthase (Gys2) (483)) and lipid metabolism (e.g. acetyl-CoA carboxylase (Acc1α) (109,338,485)).

Peripheral clocks are strongly entrained by food intake (486-489). Under 'normal' physiological conditions, rhythms in food intake are closely linked to the light-dark cycle, with the majority of food consumed in the natural wake period in animals. Rats and mice fed a standard laboratory diet (SLD) *ad libitum* (AL) consume 60-80 % of their daily food intake during the active dark phase (DP) (176-179). However, in mice fed a high-fat diet (HFD)-AL (179,193), and/or exposed to simulated shift work conditions (195), or exposed to dim light during the DP (196) food intake is no longer in phase with the light-dark cycle. Rather, these mice tend to 'graze' throughout the light phase (LP) and DP, leading to increased weight gain and disrupted circadian rhythmicity of hepatic genes involved in glucose and lipid metabolism (173,176,193,490,491).

In the modern era, activity extends beyond the solar light cycle, and food intake in humans spans more than half of the 24 hr period (199). This is particularly true of shift workers who frequently distribute food intake across a 24 hr period (200). These disrupted feeding patterns are associated with circadian desynchrony of hepatic glucose and lipid metabolism in rodents,

increasing the risk of hyperglycaemia and obesity as well as linked metabolic diseases such as type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) (109,189,198,335-337).

Time-restricted feeding (TRF), involving a daily fed-fast cycle, entrains diurnal rhythms of metabolic genes in line with feeding behaviour and improves metabolic outcomes. For example, in HFD-fed rodents, TRF for 8-12 hr during the DP reduced weight gain and preserved the circadian rhythmicity of metabolic genes in a variety of tissues (325,326,492). Although studies are limited, human TRF trials indicate improvements in glucose control and lipid profiles (see review (493)). Therefore, TRF during the active-phase, DP in rodents (325,326) and LP in humans (327), has potential to be an effective lifestyle intervention for diet-induced obesity and metabolic circadian desynchrony. A number of rodent studies have investigated TRF during the LP, out of phase to 'normal' feeding behaviour (338,494-496), and shown entrainment of hepatic metabolic genes to the timing of food intake, out of phase with their 'normal' diurnal rhythms. However, in these studies the TRF protocol occurred over a relatively short duration (1-3 weeks) (494-496). Thus, the long-term metabolic impact is unknown. In a study of longer duration (20 weeks), the TRF protocol was limited to 4 h per day (338) and resulted in a significant reduction in weight gain. However, this study cannot separate whether the metabolic benefits were due to TRF or the concurrent caloric restriction of food intake. Therefore, the current study aimed to investigate in SLD- and HFD-mice, the impact of a 12 hr TRF regime during the LP for 8 weeks on mice metabolic profile, plasma and hepatic triglyceride content, and hepatic markers of glucose and lipid metabolism.

3.3 Materials and Methods

Ethics Approval

This study was approved (Ethics approval number: SAM-97) by the South Australian Health and Medical Research Institute Animal Ethics Committee. All experimental protocols were performed in alignment with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 8th Edition.

Study Design

This animal cohort has been previously described by Kentish *et al.* (2018) (380). Eight-week-old C57BL/6 male mice (N = 256), were housed in a 12:12 hr light-dark cycle (lights on at 0600 hr, Zeitgeber (ZT) 0) and temperature (24±1^{o}C) controlled facility. Mice were provided

AL access to either a SLD (N = 128; 12 %, 23 % and 65 % of energy from fat, protein and carbohydrate respectively; Specialty Feeds, Western Australia, Australia) or HFD (N = 128; 60 %, 20 % and 20 % of energy from fat, protein and carbohydrate respectively; adapted from Research Diets Inc., New Brunswick, USA), for 4 weeks. Mice were then randomly allocated into 3 groups per diet: 1) mice fed AL (SLD/HFD-AL, N = 48); 2) mice fed during the LP (SLD/HFD-LP, N = 40 (ZT0-12)) and 3) mice fed during the DP (SLD/HFD-DP, N = 40 (ZT12-24)) for the remaining 8 weeks.

Metabolic Monitoring

During week 12, a subgroup of mice, randomly allocated from each group (N = 8/group), were individually housed in Promethium metabolic monitoring cages (Sable Systems International, Nevada, USA) for 72 hr of continuous monitoring. Energy intake (kJ), energy expenditure normalised using lean mass (kJ/ lean mass), distance moved (activity; m) and respiratory quotient (RQ; VCO_2/VO_2) were quantified and analysed using ExpeData data analysis software (Sable Systems International).

Tissue Collection

At the end of week 12, mice were anaesthetised with isoflurane (5 % in medical oxygen) at 3 hr intervals starting at ZT0 (N = 5-6 per time point), as described by Kentish *et al.* (380). Blood was collected from the abdominal aorta, and blood glucose levels were determined using an ACCU CHEK Performa monitor (ACCU CHEK, New South Wales, Australia). The remaining blood collected was transferred to ethylenediaminetetraacetic acid tubes (ThermoFisher Scientific, Victoria, Australia) and centrifuged at 1000 g and 4°C for 15 min. The plasma was collected and immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Liver was collected, weighed and immediately frozen in liquid nitrogen prior to storage at -80°C until analysis. Gonadal fat pads were collected and weighed, and lean mass was approximated as final body weight minus the weight of gonadal fat pads as previously described (497).

Plasma Metabolites

Plasma triglyceride and total cholesterol levels were measured using commercial enzymatic kits (OSR60118 and OSR6116 respectively; Beckman Coulter Inc., Georgia, USA) following

the manufacturer's instructions, using a Beckman AU480 clinical analyser (Beckman Coulter Inc.).

Plasma C-peptide concentrations, a marker of pancreatic insulin secretion, were measured at ZT12, in anticipation of an insulin response to the onset of food intake during the DP in SLD-AL mice (176,224), using an ELISA kit (CAT#10114101, Mercodia, Sapphire Biosciences, New South Wales, Australia) following the manufacturer's instructions and reading at an optical density (OD) of 450 nm using a VersaMax microplate reader (Molecular Devices, California, USA).

Hepatic Glycogen, Citrate and Triglycerides

Hepatic glycogen content was measured during the DP at ZT21, anticipating replenished glycogen stores in SLD-AL mice (50). Tissue was analysed using a colorimetric glycogen assay kit (ab169558; Abcam, Victoria, Australia) following the manufacturer's instructions and read at OD 450 nm using a VersaMax microplate reader.

Hepatic triglyceride was measured during the LP, at ZT9, in anticipation of reduced lipid levels during fasting in SLD-AL mice (50,498). Tissue was analysed using a colorimetric triglyceride quantification assay kit (ab65336; Abcam) following the manufacturer's instructions and read at OD 570 nm using a VersaMax microplate reader.

Hepatic citrate content was measured at ZT18, in anticipation of elevated citrate levels stimulating de novo lipogenesis in SLD-AL mice (499). Tissue was analysed using a citrate assay kit (MAK057; Merck, Sigma-Aldrich, New South Wales, Australia) following the manufacturer's instructions and read at OD 570 nm with a VersaMax microplate reader.

RNA Extraction and Quantitative Real-Time PCR

Liver total RNA was extracted using a Pure LINK ® RNA Mini kit (12183018A; ThermoFisher Scientific) following the manufacturer's instructions for 50 mg of tissue and measured at 260nm absorbance (A260), using a Nanodrop Lite spectrophotometer (ThermoFisher Scientific). RNA quality was determined using the A260 nm: A280 nm ratio. A ratio of 1.9-2.1 was considered acceptable for use.

Relative mRNA content was determined using an EXPRESS One-Step SuperScript qRT PCR kit, Universal (11781200; Invitrogen, ThermoFisher Scientific, Victoria, Australia). A 7500

FAST PCR machine (Applied Biosystems, ThermoFisher Scientific, Massachusetts, USA) was used for detection of pre-designed TaqMan Primers (ThermoFisher Scientific) in Table 3.1. These primer assays were used under the following conditions: holding stage: 50°C for 15 min, holding stage: 95°C for 20 sec, cycling stage: (40 cycles) 95°C for 3 sec and 60°C for 30 sec. The amount of mRNA was calculated using the ΔCT method relative to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and peptidylprolyl isomerase A (Ppia). Gapdh and Ppia were chosen as housekeeping genes based on their stability across all time-points and diets as determined using NormFinder software (stability value 0.089; Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark).

Statistical analysis

Body composition and metabolic monitoring data were expressed as mean ± S.E.M. A two-way ANOVA was performed to assess diet and TRF treatment effects with a Tukey's *post hoc* test for multiple comparisons, using GraphPad Prism v8 software (GraphPad, California, USA).

For plasma metabolites and gene expression levels, daily mean, amplitude (peak height), peak (ZT at which peak expression/metabolite levels occurred), line of best fit and corresponding 95 % confidence intervals [95%CI] were estimated using a cosinor regression with outcomes log2 transformed. Significant mean diff., amplitude diff. and peak diff. [95%CI] compared to SLD-AL mice. The differences were considered significant at $p < 0.05$ with no adjustment for multiple testing. Rhythmic and non-rhythmic cycling variables over 24 hr (i.e. presence of circadian rhythmicity) were assessed using the JTK-Cycle algorithm and considered significant at $p < 0.05$ (500). These analyses were performed in R version 3.6.1 software with the Cosinor and MetaCycle packages (R Core Team, Vienna, Austria).

3.4. Results

Body weight, fat pad mass, hepatic lipid content and food intake

The effects of HFD feeding and TRF on mice body weight, gonadal fat pad mass and energy intake have been reported previously (380). The final body weight, fat pad mass, hepatic lipid content and energy intake are shown in Table 3.2. There was a diet by TRF interaction in final body weight, gonadal fat pad mass, hepatic lipid content and total energy intake; with no difference in body weight, gonadal fat pad mass and hepatic lipid content between the SLD

groups (Table 3.2). Further, there was no difference in total energy intake between the SLD-TRF groups and SLD-AL mice, although SLD-LP mice had lower energy intake than SLD-DP mice (Table 3.2). In HFD groups, HFD-LP or HFD-DP mice weighed less than the HFD-AL mice (Table 3.2). This was reflected in the gonadal fat pad mass, hepatic lipid content and total energy intake (Table 3.2). The HFD-LP mice had lower fat pad mass than HFD-DP mice (Table 3.2).

Energy expenditure

Total energy expenditure, total activity and average RQ are displayed in Figure 3.1 and statistics are shown in Table 3.3. There was no difference in total energy expenditure across 24 h or during the LP between SLD- and HFD-mice fed AL or under a TRF protocol (Figure 3.1 Ai and ii & Table 3.3). However, in both SLD and HFD mice during the DP there was a TRF effect due to a lower energy expenditure in LP-mice compared to AL- and DP-mice (Figure 3.1 Aiii & Table 3.3).

There was no diet by TRF interaction in locomotor activity (distance moved) between SLD- and HFD-mice (Figure 3.1 B & Table 3.3). However, there was a TRF effect due to increased activity across 24 h and during the DP in LP-mice as compared to AL-mice, and as compared to both AL- and DP-mice during the LP (Figure 3.1 B & Table 3.3), a pattern observed in SLD and HFD mice.

There was a diet and TRF effect on RQ. HFD-mice had lower average RQ values compared to the SLD-mice (Figure 3.1 C & Table 3.3). RQ values were also increased in LP-mice compared to AL-mice during the LP, and reduced RQ values in LP-mice compared to AL- and DP-mice during the DP (Figure 3.1 C & Table 3.3). This pattern was similar regardless of diet consumed. However, there was no diet by TRF interaction.

The effect of time-restricted feeding on blood glucose levels and hepatic glucose metabolism

The circadian profiles of BGLs and hepatic Slc2a2, Irβ and Gys2 mRNA content are displayed in Figure 3.2 and statistics are shown in Table 3.4.

In SLD-AL and SLD-LP mice but not SLD-DP mice, BGLs exhibited circadian rhythmicity (Figure 3.2 Ai & Table 3.4) albeit at a greater amplitude and phase-reversed in SLD-LP mice, compared to SLD-AL mice (Figure 3.2 Ai & Table 3.4). The BGL amplitudes were not different in HFD-mice groups compared to SLD-AL mice (Table 3.4). However, peak BGLs

were phase-reversed in HFD-LP mice compared to SLD-AL (Figure 3.2 Aii & Table 3.4). Mean BGLs were elevated in all HFD groups compared to SLD-AL mice (Figure 3.2 A & Table 3.4).

There was no difference in plasma C-peptide levels between SLD- and HFD-mice and there was no diet by TRF interaction (SLD-AL: 122.7 ± 1.2 mmol/ L, SLD-LP: 146.2 ± 2.9 mmol/ L, SLD-DP: 145.5 ± 1.8 mmol/ L, HFD-AL: 132.3 ± 0.9 mmol/ L, HFD-LP: 146.3 ± 1.8 mmol/ L and HFD-DP: 152.5 ± 6.3 mmol/ L). However, there was a TRF effect due to elevated plasma C-peptide levels in LP- and DP-mice compared to AL-mice ($p < 0.001$, F (2,20) = 21.96, TRF effect). A pattern observed in both SLD and HFD mice.

All groups exhibited circadian rhythmicity in hepatic Slc2a2 mRNA content (Figure 3.2 B & Table 3.4), although the rhythms were phase-reversed in SLD-LP and HFD-LP mice and phase-advanced in HFD-AL and HFD-DP mice compared to SLD-AL mice (Figure 3.2 B & Table 3.4). Mean hepatic Slc2a2 mRNA levels were elevated in SLD-LP, SLD-DP and HFD-DP mice compared to SLD-AL mice (Figure 3.2 B & Table 3.4). Further, the amplitude of the circadian rhythms in hepatic Slc2a2 was greater in HFD-AL compared to SLD-AL mice (Table 3.4).

With the exception of the SLD-AL and HFD-LP mice, all groups exhibited diurnal variation in hepatic Irβ mRNA content (Figure 3.2 C & Table 3.4), although rhythms were phase-advanced in SLD-DP and HFD-AL mice, and phase-reversed in HFD-DP mice compared to SLD-AL mice (Figure 3.2 C & Table 3.4). Mean hepatic Irβ mRNA content was elevated in SLD-DP and all HFD groups compared to SLD-AL mice (Figure 3.2 C & Table 3.4). There was no difference in amplitude of rhythms between groups (Table 3.4).

Hepatic glycogenesis and glycogen content

In all groups, hepatic Gys2 mRNA content exhibited circadian rhythmicity (Figure 3.2 D & Table 3.4), although phase-reversed in the SLD-LP and HFD-LP mice and phase-advanced in the HFD-AL mice compared to SLD-AL mice (Figure 3.2 D & Table 3.4). Mean levels of hepatic Gys2 mRNA content were elevated in all groups except HFD-LP mice compared to SLD-AL mice, and amplitude was reduced in HFD-DP mice compared to SLD-AL mice (Figure 3.2 D & Table 3.4).

Hepatic glycogen content was greater in HFD-AL mice compared to SLD-AL mice ($p < 0.01$, F (1,22) = 9.181, diet effect; SLD-AL: 3.9 ± 0.5 nM/ 10 mg, SLD-LP: 2.6 ± 0.4 nM/ 10 mg, SLD-DP: 2.8 ± 0.7 nM/ 10 mg, HFD-AL: 6.1 ± 0.4 nM/ 10 mg, HFD-LP: 3.8 ± 0.6 nM/ 10 mg and HFD-DP: 3.8 ± 0.7 nM/ 10 mg). There was no diet by TRF interaction. However, there was a TRF effect due to reduced glycogen content in LP- and DP-mice compared to AL-mice ($p < 0.01$, F (2,22) = 6.481, TRF effect), a pattern observed regardless of diet.

The effect of time-restricted feeding on plasma lipid profiles and hepatic lipid metabolism

The circadian profiles of plasma triglyceride levels and hepatic Acc1α mRNA content are displayed in Figure 3.3 and statistics are shown in Table 3.5.

Plasma lipid profile

Plasma triglyceride levels did not exhibit circadian rhythmicity in SLD groups or in HFD-AL mice (Figure 3.3 A & Table 3.5). However, in HFD-TRF groups circadian rhythmicity was observed (Figure 3.3 Aii & Table 3.5), albeit phase-reversed in HFD-LP mice compared to SLD-AL mice. There was no difference in the amplitude of rhythms between all groups (Table 3.5). Mean plasma triglyceride levels were elevated in HFD-AL mice and reduced in all other groups compared to SLD-AL mice (Figure 3.3 A & Table 3.5).

There was no difference in plasma total cholesterol levels between SLD- and HFD-mice and there was no diet by TRF interaction (SLD-AL: 2.57 ± 0.1 mmol/ L, SLD-LP: 3.24 ± 0.4 mmol/ L, SLD-DP: 3.01 ± 0.2 mmol/ L, HFD-AL: 2.14 ± 0.5 mmol/ L, HFD-LP: 3.56 ± 0.1 mmol/ L and HFD-DP: 3.66 ± 0.1 mmol/ L). However, there was a TRF effect due to elevated plasma total cholesterol levels in HFD-LP and HFD-DP mice compared to HFD-AL mice ($p < 0.001$, F (2,39) = 9.363, TRF effect).

Hepatic de novo lipogenesis and citrate content

In all groups except HFD-DP mice, hepatic Acc1α mRNA content exhibited circadian rhythmicity, although all phase-advanced compared to SLD-AL mice (Figure 3.3 B & Table 3.5). In HFD-DP mice, rhythm amplitude was reduced compared to SLD-AL mice (Table 3.5). Mean hepatic Acc1α mRNA content was elevated in HFD-AL and reduced in HFD-LP and HFD-DP mice on both diets compared to SLD-AL mice (Figure 3.3 B & Table 3.5).

There was no difference in hepatic citrate content between all groups (SLD-AL: 173.2 ± 15.9 nmol/ 100 ul, SLD-LP: 187.7 ± 28.9 nmol/ 100 ul, SLD-DP: 178.4 ± 16.3 nmol/ 100 ul, HFD-AL: 183.4 ± 9.4 nmol/ 100 ul, HFD-LP: 124.8 ± 19.2 nmol/ 100 ul and HFD-DP: 152.8 ± 19.1 nmol/ 100 ul).

3.5 Discussion

In the current study, TRF during either the LP or DP reduced HFD-induced weight gain and adiposity, likely due to a reduction in energy intake. DP-TRF phase advanced circadian rhythmicity of blood glucose concentrations in SLD-mice, reflected in the phase advance in circadian rhythmicity of Irβ. LP-TRF phase reversed circadian rhythmicity of blood glucose concentrations in SLD-mice, which was reflected in a reversal in the rhythmicity of Slc2a2 and Gys2. In HFD-mice, although blood glucose levels were elevated, the timing of the circadian rhythmicity was similar to SLD-AL mice with a reversal in rhythmicity in the HFD-LP mice, reflected in a reversal in the rhythmicity of Slc2a2 and Gys2. Plasma triglyceride concentrations did not exhibit circadian rhythmicity apart from in HFD-LP and -DP mice.

Consistent with previous studies (176,492,501), there was no difference in body weight or adiposity between SLD groups. SLD-AL mice typically consume 60-80 % of their daily food intake during the DP (176). Consistent with previous studies (176,492,501), TRF for 12 hr was sufficient for mice to consume approximately the same amount of energy as SLD-AL mice, predominantly through increased meal size and meal number (380). However, SLD-LP mice consumed less energy than SLD-DP mice and exhibited less energy expenditure than SLD-DP and SLD-AL mice during the DP, possibly the result of a reduction in diet-induced thermogenesis during the DP with no food availability (406,502). Similar to previous findings (339), SLD-LP mice also exhibited increased activity during the LP; however, there was no significant difference in total activity compared to SLD-DP mice across a 24 hr period.

In the current study, TRF during the DP or during the LP similarly reduced HFD-induced weight gain and adiposity. This was largely due to the reduced energy intake observed in both TRF groups compared to HFD-AL mice, as HFD-LP and HFD-DP mice were unable to fully compensate for the restricted window of food availability (380). However, HFD-LP mice also had less gonadal fat pad mass compared to HFD-DP mice, despite no difference in energy intake or total energy expenditure. In contrast, a previous report demonstrated that HFD-LP mice had increased weight gain and adiposity compared to HFD-DP mice, despite no difference

in energy intake or activity between groups (194). This discrepancy could be due to the lack of diet acclimatisation prior to commencement of the TRF protocol in the previous study (194) but, nonetheless, highlights the need for further investigation into the mechanisms driving these changes. In particular, future studies should include a more sophisticated measure of body fat composition, such as via dual-energy X-ray absorptiometry (503), rather than the simplistic measurement of gonadal fat pad mass, a limitation of the current study.

Consistent with previous studies (295,504,505), the blood glucose levels of SLD-AL mice exhibited circadian rhythmicity. This was also reflected in the circadian rhythmicity of hepatic markers for glucose uptake and glycogen storage, with gene expression peaking in anticipation of food intake during the DP (224). In SLD-TRF groups, mean blood glucose levels were elevated compared to SLD-AL mice. This is in contrast to a previous report showing an improvement in fasting blood glucose levels (109), likely reflecting measurement of fed, rather than fasted, blood glucose levels in the current study. Alternatively, it is possible that the observed elevated blood glucose levels are in response to stress, with stress-induced hyperglycaemia an adaptive response mobilising energy stores in response to threat (506). This requires further investigation. Plasma C-peptide and mean hepatic Slc2a2, Irβ and Gys2 mRNA content were also elevated in SLD-TRF groups compared to SLD-AL mice, possibly to compensate for the higher blood glucose levels (224). In the SLD-LP mice, circadian rhythmicity of blood glucose levels, and hepatic Slc2a2 and Gys2 mRNA levels was maintained but phase-reversed in line with the timing of food intake. This is consistent with a previous study (295), which also describes a phase-reversal of peak fasting blood glucose levels coinciding with shifted rhythmicity of mRNA expression of hepatic glucose export-related genes, in response to 2 weeks TRF during the LP.

Similar to previous reports (109,176), HFD-AL feeding was sufficient to induce circadian desynchrony. In the current study, the blood glucose levels of HFD-AL mice were elevated compared to SLD-AL mice, likely a consequence of the increased food intake during the LP in HFD-AL mice (380). Similarly, peak gene expression of hepatic Slc2a2, Irβ and Gys2 was phase-advanced in HFD-AL mice compared to SLD-AL mice, which further indicates circadian misalignment. Previously, TRF during the DP was shown to ameliorate high blood glucose levels in HFD-mice through normalisation of genes involved in hepatic glucose export, namely pyruvate carboxylase and glucose 6-phosphatase (109). However, in the current study, mean blood glucose levels were elevated in all HFD groups compared to SLD-AL mice. In

general, circadian rhythmicity of hepatic genes for glucose metabolism was similar between DP and AL mice, with the exception of a phase advance in Irβ in SLD-DP mice and Slc2a2 in HFD-DP mice. In contrast, circadian rhythmicity of many of the hepatic genes were phase-reversed in LP fed mice. This evidence suggests TRF during the LP or DP is synchronised to the timing of food intake and hepatic glucose uptake and storage. However, this was not always the case with, for example, circadian rhythms in Irβ phase-reversed in HFD-DP mice. This dis-synchrony in hepatic Irβ rhythms may impact on the homeostasis of hepatic glucose storage and production (507) and contribute to the elevated mean blood glucose levels in all groups compared to SLD-AL, however, this requires further investigation particularly in the context of the fed rather than fasted state.

Consistent with previous studies (338,339), there was a reduction in hepatic lipid content and plasma triglyceride, possibly due to the reduced energy intake in the HFD-LP and HFD-DP, compared to HFD-AL mice. However, previous studies have demonstrated improvements in these variables with TRF protocols that did not restrict food intake (109,327) and, therefore, the fasting period may play an important role in reducing hepatic lipid content. This requires further investigation.

In the current study, mean Acc1α mRNA content was significantly elevated in HFD-AL mice compared to SLD-AL mice. This is consistent with previous reports demonstrating the rate of hepatic lipid metabolism is enhanced, presumably in an attempt to compensate for HFD feeding (106). However, the increased hepatic lipid content and plasma triglyceride levels in HFD-AL mice compared to SLD-AL mice, suggests the enhanced rate of hepatic lipid clearance was insufficient to prevent ectopic lipid deposition, which is a contributor to the development of obesity linked metabolic diseases, such as NAFLD (35). Further, in HFD-AL mice, the circadian rhythmicity of Acc1α gene expression was phase-advanced compared to SLD-AL mice. This may lead to enhanced de novo lipogenesis during the fasting period (109,508), further contributing to obesity.

3.6 Conclusion

In conclusion, both LP- and DP-TRF had beneficial effects on weight gain and adiposity, and this is likely due to the reduced energy intake. In general, TRF entrained circadian rhythms of hepatic markers of glucose and lipid metabolism, although there were exceptions, such as Irβ

which may have contributed to the elevated blood glucose levels observed in all groups compared to SLD-AL control mice.

Table 3.1. Pre-designed TaqMan Primers used for Quantitative Real-Time PCR.

Protein Name	**Gene (Mus)**	**Primer Identification**
Glucose Transporter 2	Slc2a2	Mm00446229_m1
Insulin Receptor β	Irβ	Mm00501505_m1
Glycogen Synthase 2	Gys2	Mm00439564_m1
Acetyl-CoA Carboxylase 1 α	Acc1α	Mm01304257_m1
Carnitine Palmitoyltransferase 1 α	Cpt1α	Mm01231183_m1
Glyceraldehyde 3-Phosphate Dehydrogenase	Gapdh	Mm99999915_g1
Peptidylprolyl Isomerase A	Ppia	Mm02342430_g1

Purchased from ThermoFisher Scientific, Victoria, Australia

Table 3.2. The effect of time-restricted feeding on body weight, adiposity and energy intake parameters.

Diet	SLD			HFD			Two-way ANOVA
Food Availability	**AL**	**LP**	**DP**	**AL**	**LP**	**DP**	
Final Body Weight (g)	30.2±0.2 ^{a}	30.0±0.3 ^{a}	29.5±0.4 ^{a}	43.6±0.7 ^{c}	32.5±0.6 ^{b}	34.3±0.6 ^{b}	I $p < 0.0001$ D $p < 0.0001$ T $p < 0.0001$
Fat Pad Mass (g)	0.5±0.02 ^{a}	0.5±0.03 ^{a}	0.5±0.03 ^{a}	2.6±0.05 ^{b}	1.2±0.10 ^{c}	1.6±0.10 ^{d}	I $p < 0.0001$ D $p < 0.0001$ T $p < 0.0001$
Hepatic Lipid Content (nM/100mg)	0.8±0.03 ^{b,d}	2.2±0.6 ^{c,d}	1.5±0.2 ^{b,d}	5.2±0.2 ^{a}	1.8±0.2 ^{c,d}	3.2±0.7 ^{c}	I $p < 0.0001$ D $p < 0.0001$ T $p < 0.05$
Total Energy Intake (kJ)	41.5±3.2 ^{a,c}	33.8±3.1 ^{c}	48.5±1.2 ^{a}	64.2±3.3 ^{b}	47.2±2.8 ^{a,c}	49.7±3. 5 ^{a,c}	I $p < 0.01$ D $p < 0.0001$ T $p < 0.001$

Standard laboratory diet (SLD) and high-fat diet (HFD) mice, fed either *ad libitum* (AL), during the light phase (LP) or during the dark phase (DP). The values are mean ± SEM. Interaction (I), diet (D) & time-restricted feeding (T) effects; two-way ANOVA with a Tukey's post hoc test $p < 0.05$, groups with the same letters are not significantly different from one another.

Table 3.3. The effect of time-restricted feeding on energy expenditure parameters.

Diet		SLD			HFD			Two-way ANOVA
Metabolic data		AL	LP	DP	AL	LP	DP	
Energy Expenditure (kJ/ lean mass)	24 hr	1.5±0.06	1.3±0.04	1.5±0.04	1.4±0.02	1.4±0.05	1.4±0.07	I $p > 0.05$ T $p > 0.05$ D $p > 0.05$
	LP	0.7±0.02	0.6±0.02	0.7±0.03	0.7±0.01	0.7±0.02	0.6±0.03	I $p > 0.05$ T $p > 0.05$ D $p > 0.05$
	DP	0.9±0.03	0.7±0.03	0.8±0.03	0.8±0.01	0.7±0.03	0.8±0.04	I $p > 0.05$ T $p < 0.01$ D $p > 0.05$
Movement (meters)	24 hr	139.3±3.8	171.9±9.7	153.8±9.8	123.9±4.8	165.4±5.2	146.8±18.6	I $p > 0.05$ T $p < 0.01$ D $p > 0.05$
	LP	33.5±2.4	44.0±3.7	27.2±3.8	30.6±2.2	42.2±1.6	31.0±3.8	I $p > 0.05$ T $p < 0.0001$ D $p > 0.05$
	DP	105.8±3.5	127.9±10.7	126.6±9.1	93.3±4.6	123.3±4.9	115.8±15.9	I $p > 0.05$ T $p < 0.05$ D $p > 0.05$
Respiratory Quotient (VCO_2/ VO_2)	24 hr	0.9±0.01	0.9±0.01	0.9±0.01	0.7±0.01	0.8±0.01	0.8±0.01	I $p > 0.05$ T $p > 0.05$ D $p < 0.0001$
	LP	0.8±0.01	0.9±0.02	0.8±0.02	0.7±0.01	0.8±0.01	0.7±0.01	I $p > 0.05$ T $p < 0.05$ D $p < 0.0001$
	DP	0.9±0.01	0.8±0.02	0.9±0.02	0.8±0.01	0.7±0.01	0.8±0.01	I $p > 0.05$ T $p < 0.01$ D $p < 0.0001$

Standard laboratory diet (SLD) and high-fat diet (HFD) mice, fed either *ad libitum* (AL), during the light phase (LP) or during the dark phase (DP). The values are mean ± SEM. interaction (I), diet (D) & time-restricted feeding (T) effects; two-way ANOVA.

Table 3.4. The effect of time-restricted feeding on parameters of circadian rhythmicity in blood glucose levels and relative mRNA levels of hepatic glucose transporter 2, insulin receptor and glycogen synthase.

Diet		**SLD**			**HFD**		
Food Availability		**AL**	**LP**	**DP**	**AL**	**LP**	**DP**
BGL	**JTK cycle *p*-value**	$p = 0.006$	$p = 0.008$	$p = 1$	$p = 0.01$	$p = 0.04$	$p = 0.05$
	Mean [95%CI]	9.9 [9.6, 10.4]	11.5 [11.0, 12.0]	11.3 [10.8, 11.8]	12 [11.6, 12.5]	11.7 [11.2, 12.2]	12.2 [11.7, 12.7]
	Mean diff [95%CI] ***p*-value**		1.5 [0.8, 2.2] $p < 0.0001$	1.3 [0.7, 1.9] $p = 0.0001$	2.1 [1.5, 2.6] $p < 0.0001$	1.7 [1.1, 2.4] $p < 0.0001$	2.2 [1.6, 2.9] $p < 0.0001$
	Amp [95%CI]	0.7 [0.1, 1.3]	1.8 [1.1, 2.6]	0.6 [-0.1, 1.3]	0.9 [0.3, 1.5]	1.7 [0.9, 2.4]	1.2 [0.4, 1.9]
	Amp diff [95%CI] ***p*-value**		1.1 [0.1, 2.1] $p = 0.03$	-0.09 [-1.0, 0.8] $p = 0.84$	0.2 [-0.7, 1.0] $p = 0.71$	0.9 [-0.03, 1.9] $p = 0.06$	0.4 [-0.5, 1.4] $p = 0.38$
	Peak [95%CI]	ZT 6.2 [3.4, 9.1]	ZT 15.5 [13.9, 17.1]	ZT 23.6 [18.8, 4.5]	ZT 8.7 [6.1, 11.2]	ZT 20.5 [18.8, 22.2]	ZT 8.9 [6.4, 11.4]
	Peak diff [95%CI] ***p*-value**		9.3 [6.1, 12.5] $p < 0.0001$	-6.6 [-12.2, -1.01] $p = 0.02$	2.4 [-1.4, 6.2] $p = 0.21$	14.3 [11.0, 17.6] $p < 0.0001$	2.6 [-1.1, 6.4] $p = 0.17$
Slc2a2	**JTK cycle *p*-value**	$p = 0.0003$	$p = 0.0002$	$p = 0.002$	$p < 0.0001$	$p < 0.0001$	$p = 0.01$
	Mean [95%CI]	-3.5 [-3.7, -3.2]	-2.7 [-3.1, -2.7]	-3.2 [-3.4, -2.9]	-3.5 [-3.7, -3.3]	-3.2 [-3.4, -3.0]	-3.1 [-3.3, -2.9]
	Mean diff [95%CI] ***p*-value**		0.6 [0.3, 0.9] $p < 0.0001$	0.3 [0.01, 0.6] $p = 0.04$	-0.08 [-0.4, 0.2] $p = 0.6$	0.2 [-0.1, 0.5] $p = 0.16$	0.4 [0.1, 0.7] $p = 0.01$
	Amp [95%CI]	0.5 [0.2, 0.8]	0.4 [0.1, 0.7]	0.3 [0.04, 0.6]	1.5 [1.2, 1.8]	0.4 [0.2, 0.7]	0.3 [0, 0.6]
	Amp diff [95%CI] ***p*-value**		-0.1 [-0.5, 0.3] $p = 0.62$	-0.2 [-0.6, 0.2] $p = 0.43$	0.9 [0.6, 1.4] $p < 0.0001$	-0.06 [-0.5, 0.3] $p = 0.77$	-0.2 [-0.6, 0.2] $p = 0.39$
	Peak [95%CI]	ZT 12.8 [10.4, 15.1]	ZT 23.8 [20.8, 26.7]	ZT 11.6 [8.0, 15.1]	ZT 4.5 [3.7, 5.2]	ZT 23.9 [21.2, 26.7]	ZT 7.9 [4.3, 11.5]
	Peak diff [95%CI] ***p*-value**		11 [7.2, 14.8] $p < 0.0001$	-1.2 [-5.5, 3.0] $p = 0.58$	-8.3 [-10.8, -5.8] $p < 0.0001$	11.1 [7.5, 14.8] $p < 0.0001$	-4.9 [-9.2, -0.5] $p = 0.03$
Irβ	**JTK cycle *p*-value**	$p = 0.06$	$p = 0.03$	$p = 0.0008$	$p = 0.001$	$p = 1$	$p < 0.0001$
	Mean [95%CI]	-4.5 [-4.6, -4.3]	-4.3 [-4.4, -4.1]	-3.9 [-4.1, -3.8]	-3.5 [-3.7, -3.3]	-4.2 [-4.4, -4.0]	-4.0 [-4.2, -3.9]
	Mean diff [95%CI] ***p*-value**		0.2 [-0.05, 0.4] $p = 0.12$	0.5 [0.3, 0.7] $p < 0.0001$	0.9 [0.7, 1.2] $p < 0.0001$	0.3 [0.1, 0.5] $p = 0.02$	0.4 [0.2, 0.7] $p = 0.0003$
	Amp [95%CI]	0.4 [0.1, 0.6]	0.3 [0, 0.5]	0.5 [0.3, 0.8]	0.6 [0.4, 0.9]	0.1 [-0.1, 0.3]	0.4 [0.2, 0.7]
	Amp diff [95%CI] ***p*-value**		-0.1 [-0.5, 0.2] $p = 0.45$	0.1 [-0.2, 0.5] $p = 0.39$	0.3 [-0.1, 0.6] $p = 0.15$	-0.3 [-0.6, 0.1] $p = 0.1$	0.04 [-0.3, 0.4] $p = 0.83$
	Peak [95%CI]	ZT 14.4 [12, 16.9]	ZT 18.1 [14.7, 21.6]	ZT 10.6 [8.9, 12.4]	ZT 8.0 [6.6, 9.5]	ZT 22.3 [13.0, 31.6]	ZT 3.1 [0.9, 5.3]
	Peak diff [95%CI] ***p*-value**		3.7 [-0.5, 7.9] $p = 0.08$	-3.8 [-6.8, -0.8] $p = 0.01$	-6.4 [-9.2, -3.6] $p < 0.0001$	7.9 [-1.7, 17.5] $p = 0.11$	-11.3 [-14.5, -8.0] $p < 0.0001$
Gys2	**JTK cycle *p*-value**	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.002$	$p < 0.0001$	$p = 0.002$
	Mean [95%CI]	-4.2 [-4.4, -4.0]	-3.9 [-4.1, -3.8]	-3.7 [-3.8, -3.5]	-4.0 [-4.2, -3.9]	-4.0 [-4.2, -3.9]	-3.6 [-3.8, -3.5]
	Mean diff [95%CI] ***p*-value**		0.3 [0.1, 0.5] $p = 0.007$	0.6 [0.4, 0.8] $p < 0.0001$	0.2 [0.01, 0.4] $p = 0.03$	0.2 [-0.1, 0.4] $p = 0.15$	0.6 [0.4, 0.8] $p < 0.0001$

	Amp [95%CI]	0.9 [0.7, 1.1]	0.8 [0.6, 0.9]	1.0 [0.8, 1.2]	0.7 [0.5, 0.9]	0.7 [0.5, 0.9]	0.4 [0.3, 0.6]
	Amp diff [95%CI] ***p*-value**		-0.1 [-0.4, 0.2] $p = 0.46$	0.2 [-0.1, 0.5] $p = 0.26$	-0.2 [-0.4, 0.1] $p = 0.31$	-0.2 [-0.5, 0.1] $p = 0.19$	-0.4 [-0.7, -0.1] $p = 0.003$
	Peak [95%CI]	ZT 13.9 [13, 14.8]	ZT 1.2 [0.2, 2.2]	ZT 13.7 [12.9, 14.4]	ZT 8.1 [6.9, 9.2]	ZT 2.0 [0.9, 3.3]	ZT 13.3 [11.5, 15.2]
	Peak diff [95%CI] ***p*-value**		11.3 [9.9, 12.7] $p < 0.0001$	-0.2 [-1.4, 0.9] $p = 0.74$	-5.8 [-7.2, -4.4] $p < 0.0001$	-11.8 [-13.3, -10.3] $p < 0.0001$	-0.5 [-2.6, 1.5] $p = 0.61$

Blood glucose levels (BGL; mmol/ L), hepatic glucose transporter 2 (Slc2a2), insulin receptor (Irβ) and glycogen synthase (Gys2) relative mRNA levels across 24 hr in standard laboratory diet (SLD) and high-fat diet (HFD) mice, fed either *ad libitum* (AL), during the light phase (LP) or during the dark phase (DP). Significant circadian variability determined by JTK cycle p-value < 0.05. Average 24 hr means, amplitudes and peak ZT followed by 95 % confidence intervals [95%CI]. Significant mean diff., amplitude diff. and peak diff. [95%CI] compared to SLD-AL mice determined by $p < 0.05$. Expression was relative to the housekeeping genes Gapdh and Ppia.

Table 3.5. The effect of time-restricted feeding on parameters of circadian rhythmicity in plasma triglyceride levels and relative mRNA levels of hepatic acetyl-CoA carboxylase.

Diet		**SLD**			**HFD**		
Food Availability		**AL**	**LP**	**DP**	**AL**	**LP**	**DP**
PTG	**JTK cycle *p*-value**	$p = 0.74$	$p = 0.14$	$p = 0.17$	$p = 0.16$	$p < 0.0001$	$p = 0.005$
	Mean [95%CI]	1.3 [1.2, 1.4]	0.9 [0.8, 1.1]	0.9 [0.8, 1.1]	1.5 [1.4, 1.6]	0.8 [0.7, 1.0]	0.9 [0.7, 1.0]
	Mean diff [95%CI] ***p*-value**		-0.4 [-0.6, -0.2] $p = 0.0001$	-0.3 [-0.5, -0.1] $p = 0.0008$	0.2 [0.02, 0.3] $p = 0.03$	-0.5 [-0.6, -0.3] $p < 0.0001$	-0.4 [-0.6, -0.2] $p < 0.0001$
	Amp [95%CI]	0.3 [0.1, 0.5]	0.2 [-0.04, 0.4]	0.1 [-0.06, 0.3]	0.2 [0.03, 0.3]	0.3 [0.09, 0.5]	0.2 [-0.04, 0.3]
	Amp diff [95%CI] ***p*-value**		-0.1 [-0.4, 0.1] $p = 0.31$	-0.1 [-0.4, 0.1] $p = 0.25$	-0.1 [-0.4, 0.1] $p = 0.39$	-0.1 [-0.4, 0.1] $p = 0.85$	-0.1 [-0.4, 0.1] $p = 0.31$
	Peak [95%CI]	ZT 18.7 [16.7, 20.7]	ZT 11.4 [5.8, 16.9]	ZT 22.8 [16.2, 5.4]	ZT 20.4 [16.9, 23.8]	ZT 7.1 [4.7, 9.5]	ZT 23.2 [17.8, 4.5]
	Peak diff [95%CI] ***p*-value**		-7.3 [-13.2, -1.4] $p = 0.02$	4.1 [-2.8, 11.0] $p = 0.24$	1.7 [-2.3, 5.7] $p = 0.41$	-11.6 [-14.8, -8.5] $p < 0.0001$	4.5 [-1.3, 10.2] $p = 0.13$
Acc1α	**JTK cycle *p*-value**	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.03$	$p = 0.04$	$p = 0.09$
	Mean [95%CI]	-5.9 [-6.1, -5.7]	-6.0 [-6.2, -5.8]	-5.8 [-5.9, -5.6]	-5.6 [-5.8, -5.4]	-6.4 [-6.6, -6.2]	-6.5 [-6.7, -6.3]
	Mean diff [95%CI] ***p*-value**		-0.2 [-0.5, 0.1] $p = 0.25$	0.08 [-0.2, 0.4] $p = 0.59$	0.3 [0.02, 0.6] $p = 0.04$	-0.6 [-0.9, -0.3] $p < 0.0001$	-0.6 [-0.9, -0.4] $p < 0.0001$
	Amp [95%CI]	0.8 [0.486, 1.07]	0.5 [0.2, 0.8]	0.5 [0.3, 0.8]	0.8 [0.5, 1.1]	0.5 [0.2, 0.7]	0.2 [-0.06, 0.5]
	Amp diff [95%CI] ***p*-value**		-0.3 [-0.7, 0.1] $p = 0.16$	-0.2 [-0.6, 0.2] $p = 0.24$	0.03 [-0.4, 0.4] $p = 0.89$	-0.3 [-0.7, 0.1] $p = 0.12$	-0.5 [-0.9, -0.1] $p = 0.01$
	Peak [95%CI]	ZT 13.6 [12.2, 15.0]	ZT 5.8 [3.7, 7.9]	ZT 11.1 [8.9, 13.1]	ZT 5.6 [4.3, 6.9]	ZT 8.9 [6.6, 11.3]	ZT 5.5 [1.3, 9.7]
	Peak diff [95%CI] ***p*-value**		-7.8 [-10.3, -5.2] $p < 0.0001$	-2.5 [-5.1, -0.02] $p = 0.05$	-8 [-9.9, -6.1] $p < 0.0001$	-4.7 [-7.5, -1.9] $p = 0.0008$	-8.1 [-12.6, -3.7] $p = 0.0004$

Plasma triglyceride levels (PTG; mmol/ L) and relative hepatic acetyl-CoA carboxylase (Acc1α) mRNA levels across 24 hr in standard laboratory diet (SLD) and high-fat diet (HFD) mice, fed either *ad libitum* (AL), during the light phase (LP) or during the dark phase (DP). Significant circadian variability determined by JTK cycle p-value <0.05. Average 24 hr means, amplitudes and peak ZT followed by 95 % confidence intervals [95%CI]. Significant mean diff., amplitude diff. and peak diff. [95%CI] compared to SLD-AL mice determined by $p < 0.05$. Expression was relative to the housekeeping genes Gapdh and Ppia.

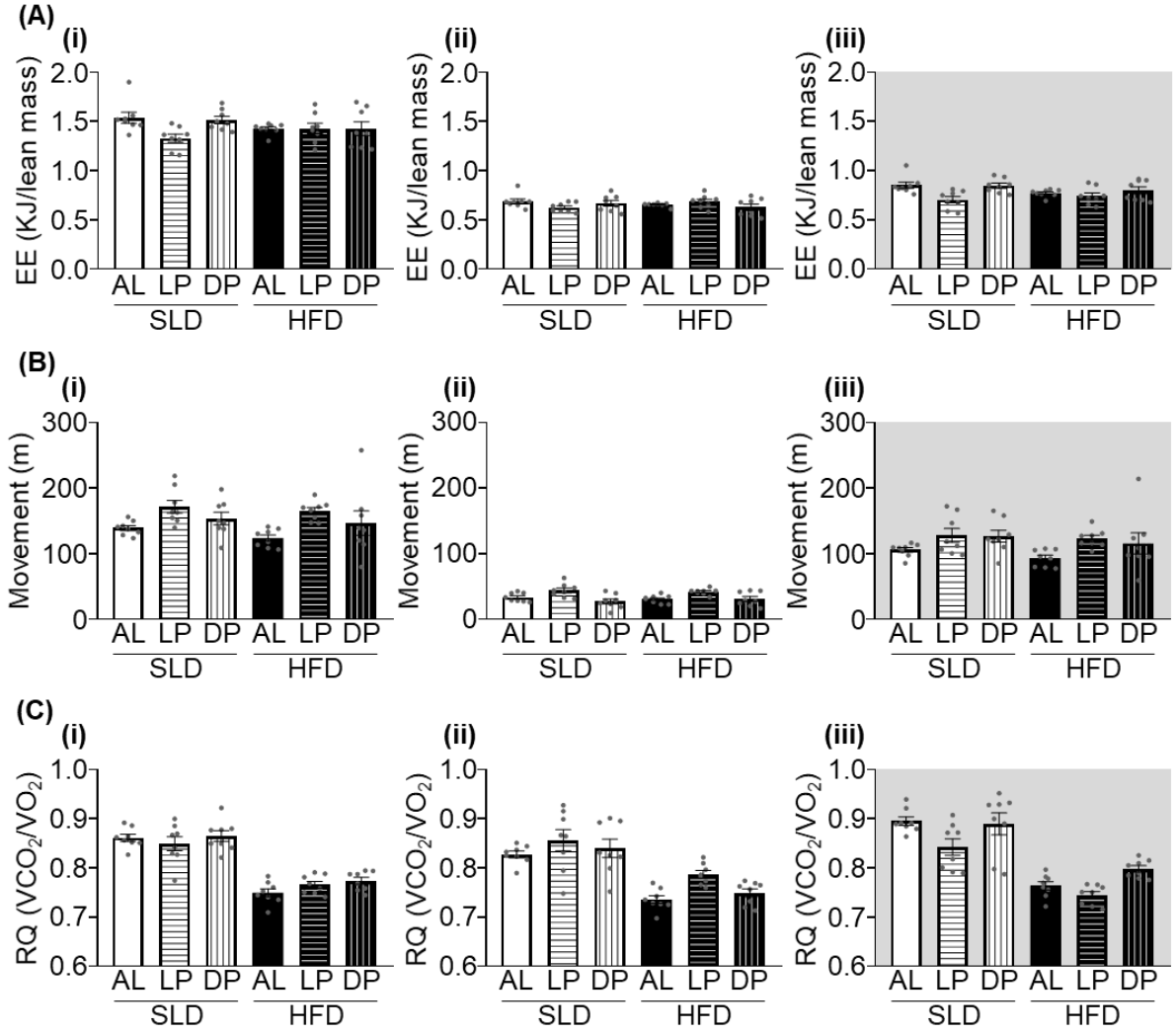

Figure 3.1. Energy expenditure parameters of standard laboratory diet (SLD) and high-fat diet (HFD) mice, fed either *ad libitum* (AL), during the light phase (LP) or during the dark phase (DP). (A) Energy expenditure normalised by lean mass (EE; kJ/ lean mass), (B) total activity (movement; m), and (C) respiratory quotients (RQ; VCO2/ VO2), (i) across 24 hr, (ii) during the LP and (iii) during the DP. The values are mean ± SEM, with the dots the individual data points. The statistics for this data is provided in Table 3.3.

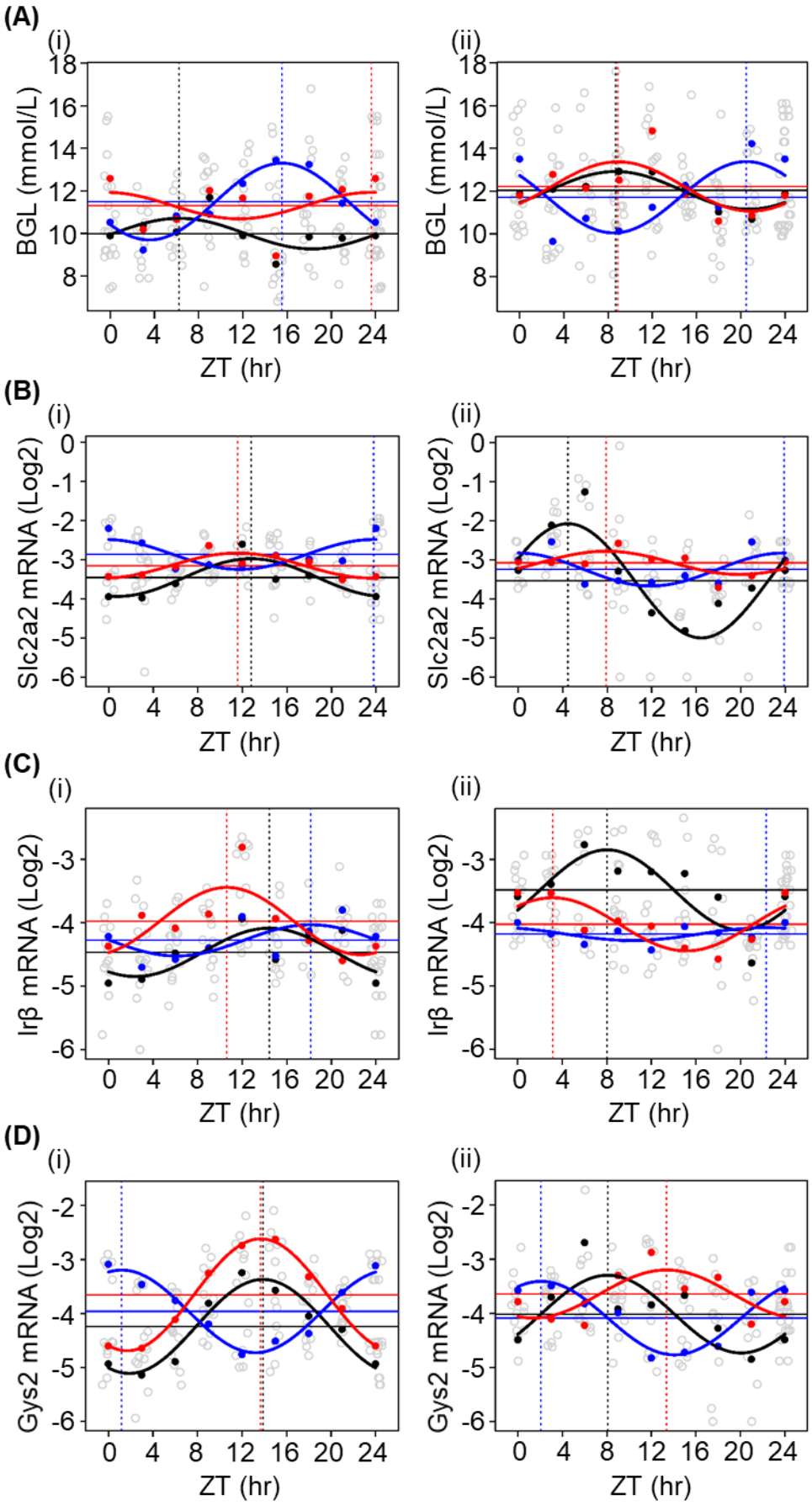
(A)
(i)
(ii)
BGL (mmol/L)
ZT (hr)
(B)
Slc2a2 mRNA (Log2)
(C)
Irβ mRNA (Log2)
(D)
Gys2 mRNA (Log2)

Figure 3.2. Circadian variation of (A) blood glucose levels (BGL; mmol/ L), (B) hepatic glucose transporter (Slc2a2), (C) hepatic insulin receptor (Irβ) and (D) hepatic glycogen synthase (Gys2) relative mRNA levels in (i) standard laboratory diet (SLD) mice and (ii) high-fat diet (HFD) mice, fed either *ad libitum* (AL; black line), during the light phase (LP; blue line) or during the dark phase (DP; red line). Grey circles are raw data. Vertical lines indicate estimated curve peak locations. Horizontal lines indicate estimated means. Expression was relative to the housekeeping genes Gapdh and Ppia.

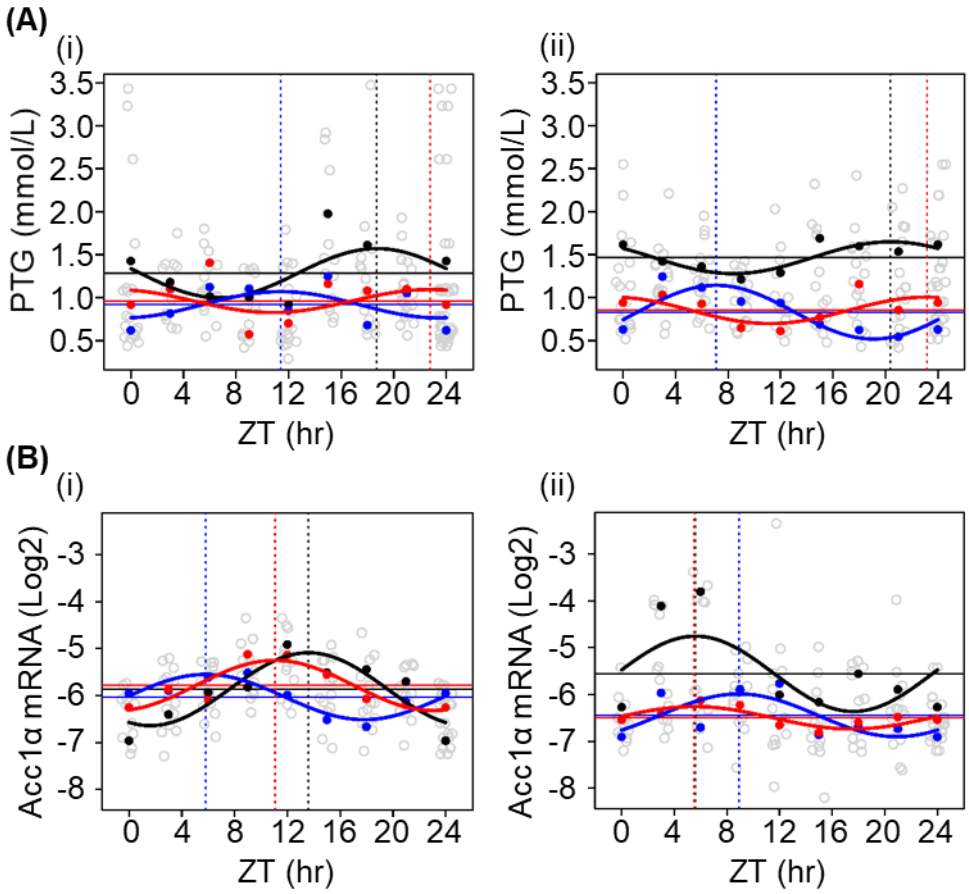

Figure 3.3. Circadian variation of (A) plasma triglyceride levels (PTG; mmol/ L), and (B) acetyl-CoA carboxylase (Acc1α) relative mRNA levels in (i) standard laboratory diet (SLD) mice and (ii) high-fat diet (HFD) mice, fed either *ad libitum* (AL; black line), during the light phase (LP; blue line) or during the dark phase (DP; red line). Grey circles are raw data. Vertical lines indicate estimated curve peak locations. Horizontal lines indicate estimated means. Expression was relative to the housekeeping genes Gapdh and Ppia.

CHAPTER 4. Pharmaceutical Intervention; Monotreme GLP-1 Analogues

Statement of Authorship

Title of Paper	The effect of monotreme GLP-1 analogues on glucose tolerance in lean and diet-induced obese mice
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Principal Author

Name of Principal Author (Candidate)	Rebecca O'Rielly		
Contribution to the Paper	Husbandary of animals, performed the experiments, collected and analysed the data and drafted the manuscript		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	16-06-2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised progression of work, assisted in data interpretation, contributed to the design of the project and manuscript revisions.		
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Contribution to the Paper	Supervised progression of work, assisted in data interpretation, contributed to the design of the project and manuscript revisions.		
Signature		Date	8/07/2020

The Effect of Monotreme GLP-1 Analogues on Glucose Tolerance in Lean and Diet-Induced Obese Mice

4.1 Abstract

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that plays a role in glycaemic regulation. The monotreme GLP-1 peptides are potent GLP-1 receptor agonists, resistant to degradation by dipeptidyl peptidase 4 in humans and mice. This study aimed to determine if treatment with a monotreme GLP-1 analogue improves glucose tolerance in lean and diet induced-obese mice. Male C57BL/6 mice (N = 112, 7 weeks old) were fed *ad libitum* with either a standard-laboratory diet (SLD) or high-fat diet (HFD) for 14 weeks (N = 56/diet). On week 13, mice were divided into 7 groups (N = 8/group/diet) and injected (intraperitoneal (IP)) twice daily (0700 hr & 1900 hr) with either 1) 0.2 M phosphate buffer (PB); 2) exendin-4 (Ex-4); 3) echidna GLP-1 (eGLP-1); 4) eGLP-1 derived F8S; 5) platypus GLP-1 (pGLP-1); 6) pGLP-1 derived N14S; or 7) pGLP-1 derived S26K for 7 days. On week 14, mice underwent an IP glucose tolerance test (IPGTT). HFD-mice gained more weight, had elevated fasting blood glucose levels and an elevated blood glucose AUC in response to the IPGTT, compared to SLD-mice. N14S reduced fasting blood glucose levels in SLD-mice compared to PB treated controls. Further, all treatment groups reduced the glucose AUC compared to PB treated controls. In HFD-mice, treatment with Ex-4, F8S and N14S reduced fasting blood glucose levels compared to PB treated controls. Further, treatment with Ex-4, pGLP-1, N14S and S26K reduced the glucose AUC compared to PB treated controls. Similar to treatment with Ex-4, treatment with eGLP-1, pGLP-1 and the monotreme GLP-1 analogues improved glucose tolerance compared to PB treated controls. Therefore, monotreme GLP-1 may be an effective intervention for type 2 diabetes.

LIST OF ABBREVIATIONS

In alphabetical order

AUC	Area under the curve
DPP4	Dipeptidyl peptidase 4
eGLP-1	Echidna GLP-1
Ex-4	Exendin-4
GLP-1	Glucagon-like peptide-1
HFD	High-fat diet
IP	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
PB	Phosphate buffer
pGLP-1	Platypus GLP-1
SLD	Standard laboratory diet

4.2 Introduction

Type 2 diabetes is an increasingly prevalent chronic metabolic disease, characterised by the progressive development of insulin resistance and ensuing hyperglycaemia (509). In healthy humans, daily blood glucose levels fluctuate in respect to circadian variation; patterns of activity and rest, feeding and fasting (295). Glycaemic control is achieved through a complex interplay between hormones and tissues. In response to the detection of nutrients such as glucose in the digestive tract, the incretin hormone glucagon-like peptide-1 (GLP-1) is released from enteroendocrine cells into the circulation (80). This leads to activation of GLP-1 receptors expressed in pancreatic islet cells, potentiating glucose-dependent insulin secretion and inhibiting glucagon release (168). Circulating insulin then promotes glucose uptake into tissues through enhanced glucose transporter action (254). GLP-1 receptors are also expressed in the vagal nerves and hypothalamus, where activation of the GLP-1 receptor inhibits appetite and food intake (80). In humans with type 2 diabetes, continuous subcutaneous infusion of GLP-1 for 6 weeks resulted in weight loss, reduced fasting blood glucose levels and improved postprandial blood glucose levels following a meal (169).

In the circulation, endogenous GLP-1 is rapidly inactivated, cleaved and degraded, mainly by the enzyme dipeptidyl peptidase 4 (DPP4) (168). A promising treatment strategy, several GLP-1 receptor agonists have been investigated in an attempt to identify a superior therapeutic compound that mimics the blood-glucose-lowering effect of endogenous GLP-1 but is resistant to DPP4 degradation (168). For example, Exendin-4 (Ex-4), originally isolated from the poisonous salivary secretions of the Gila monster (*Heloderma suspectum*) (460), is considered a highly potent GLP-1 receptor agonist (168,458). *In vitro* studies have demonstrated Ex-4 binds to the N-terminal fragment of the human GLP-1 receptor with ~400 times greater affinity, compared to human GLP-1 (462,463). However, the overall affinity of Ex-4 for the intact receptor is close to that of human GLP-1 (462,463). Further, the amino acid peptide sequence of Ex-4 is only ~50 % similar to human GLP-1, differences that provide resistance to cleavage by DPP4 (458). In diabetic animal models (439) and clinical studies (441,454,465), treatment with Ex-4 led to weight loss and a dose-dependent reduction in fasting and postprandial blood glucose levels. However, nausea is noted as a significant side effect (441,454,465), hindering treatment compliance (168).

The monotreme GLP-1 peptides, recently isolated from the venom and digestive tract of platypus and echidna (468), are also potent GLP-1 receptor agonists. The amino acid sequence

of the platypus (pGLP-1) and echidna (eGLP-1) GLP-1s are similar, 63.3 % and 43.3 % respectively, to that of human GLP-1 (468). While pGLP-1 and eGLP-1 are degraded in monotremes, the amino acid sequence differences correspond to changes in the DPP4 cleavage site, resulting in resistance to degradation in humans and mice (468). In fact, following incubation of human and monotreme GLP-1 in human serum containing DPP4, pGLP-1 and eGLP-1 remained almost entirely intact after 4 hr, whereas human GLP-1 had degraded by ~50 % after 1 hr and ~80 % after 4 hr (468). However, the pGLP-1 and eGLP-1 appeared to bind the human GLP-1 receptor with ~50 fold lower affinity and are less potent in their stimulation of intracellular signalling, demonstrated by reduced accumulation of cyclic adenosine monophosphate, compared to human GLP-1 in cultured rat insulinoma INS-1(832/13) cells (468). To improve affinity for the GLP-1 receptor and signalling, Grutzner *et al.* developed analogues of the monotreme GLP-1 peptides, where a single amino acid has been altered in either pGLP-1 or eGLP-1. This includes a phenylalanine to serine change at position 8 (F8S) of eGLP-1, an asparagine to serine change at position 14 (N14S) of pGLP-1 and a serine to lysine change at position 26 (S26K) of pGLP-1 (Ong et al. unpublished). The alteration of amino acid peptide sequences of these new analogues induced signalling bias, enhancing the strength of intracellular signalling (Ong et al. unpublished).

The current study aimed to investigate the effects of eGLP-1, pGLP-1, and the monotreme GLP-1 analogues (F8S, N14S and S26K) on glucose tolerance in lean and diet-induced obese mice. In particular, the aim of this study was to investigate the effects of one-week monotreme GLP-1 analogue treatment, compared with Ex-4 treatment, on fasting blood glucose and postprandial blood glucose levels in response to an intraperitoneal (IP) glucose tolerance tests (IPGTT).

4.3 Materials and Methods

Ethics Approval

This study was approved by the South Australian Health and Medical Research Institute Animal Ethics Committee. All experimental protocols were performed in alignment with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Study Design

Seven-week-old male C57BL/6 mice (N = 112) were housed in groups of four in a 12:12 hr light-dark cycle and temperature (24 ± 1^{o}C) controlled facility. Mice had *ad libitum* access to

either a standard laboratory diet (N = 56; SLD; 12 %, 23 % and 65 % of energy from fat, protein and carbohydrate respectively; Specialty Feeds, WA, Australia) or a high-fat diet (N = 56; HFD; 60 %, 20 % and 20 % of energy from fat, protein and carbohydrate respectively; adapted from Research Diets Inc., New Brunswick, USA) for 14 weeks. At the beginning of week 13, mice were randomly allocated into 7 groups per diet (N = 8/group/diet) and treated with either: 1) 0.2M phosphate buffer (PB); 2) Ex-4; 3) eGLP-1; 4) F8S; 5) pGLP-1; 6) N14S; or 7) S26K for 7 days.

Each day, mice underwent twice daily (0700 hr and 1900 hr) IP injections of the allocated compound at a dose of 1 mg/ kg of body weight for Ex-4 groups and 10 mg/ kg of body weight for the PB, eGLP-1, F8S, pGLP-1, N14S and S26K groups. Ex-4 administration twice daily via IP injection or at this dose is consistent with several human (440,441,454,465) and animal studies (439,510-512). All other compounds were administered at a 10 times greater dose than Ex-4 to compensate for reduced GLP-1 receptor affinity and intracellular signalling potency as previously described (468). The pGLP-1, eGLP-1, N14S, F8S and S26K are synthesised peptides (ChinaPeptides Co., Ltd., SH, China) with structural changes identified by comparative genetic analysis based on published (468) and unpublished genomic information of platypus and echidnas (Monotreme Resource Centre, The University of Adelaide). At no stage in this study were adult monotreme animals utilised to protect the conservation of the Australian monotreme populations

Body weight was measured weekly, during the 12 week diet period, and daily during the treatment period. However, due to intervention on the seventh day of treatment, weight loss was not measured. On week 14, mice underwent an IPGTT. Final body weight, gonadal fat pads, liver, heart and kidneys were then collected and weighed.

Intraperitoneal Glucose Tolerance Test

On the 7th day of monotreme GLP-1 treatment, mice were fasted for 6 hr (1300 hr -1900 hr). Following application of a topical anaesthetic cream (Emla, AstraZeneca) fasting blood glucose levels from a tail prick were determined with an Accu-Chek Performa monitor glucometer (Accu-chek, NSW, Australia). The mice were then given the final IP injection of the designated compound at 1900 hr. Thirty minutes later, the second reading of blood glucose levels was taken (baseline IPGTT; 0 min). Immediately after this, the mice received an IP

injection of 20 % D-glucose (2 g/ kg of body weight). Blood glucose levels were measured at 15, 30, 45, 60 and 120 min post glucose administration.

Statistical Tests

Values are expressed as mean ± SEM. Body weight from week 0-12 was analysed for any diet effects, time effects or diet by time interactions, using a two-way ANOVA followed by a Sidak's *Post hoc* test for multiple comparisons, using Graph Pad Prism v7.02 software. Body weight from weeks 13-14, body composition parameters and blood glucose levels were analysed for any diet effects, GLP-1 analogue treatment effects or diet by treatment interactions, using a two-way ANOVA followed by a Tukey's *Post hoc* test for multiple comparisons, using Graph Pad Prism v7.02 software. Blood glucose area under the curve (AUC) was analysed using IBM SPSS Statistics v26 software. $P < 0.05$ was considered statistically significant.

4.4 Results

Body weight gain prior to monotreme GLP-1 treatment

The effects of SLD and HFD feeding on body weight prior to GLP-1 analogue treatment are illustrated in Figure 1. Over the first 12 weeks, HFD-mice gained more weight than SLD-mice ($p < 0.0001$, F (1,1430) = 1023, diet effect; $p < 0.0001$, F (12,1430) = 374.6, time effect; $p < 0.0001$, F (12,1430) = 34.46, interaction; Figure 4.1).

At the end of week 12, prior to GLP-1 analogue treatment, HFD-mice had gained significantly more weight than SLD-mice ($p < 0.001$, F (1,96) = 125.0, diet effect; Table 4.1). There was also a significant diet by treatment interaction ($p < 0.05$, F (6,96) = 2.584, interaction; Table 4.1), despite there being no difference in weight gain between treatment groups.

Weight loss during the treatment period

The effects of PB, Ex-4 and monotreme GLP-1 analogue treatment on body weight during the treatment period are reported in Table 2. In all treatment groups of SLD- and HFD-mice, there was an initial large drop in body weight after the first day of treatment ($p < 0.0001$, F (6,82) = 33.25, treatment effect; $p < 0.05$, F (6,82) = 3.010, interaction; Table 4.2). This was followed by an incremental loss of body weight up to day 6 ($p < 0.0001$, F (1,90) = 28.77, diet effect; $p < 0.0001$, F (6,90) = 33.89, treatment effect; $p < 0.001$, F (6,90) = 5.085, interaction; Table

4.2). In SLD- and HFD-mice, after the 6th day of treatment, weight loss was greatest in mice treated with Ex4 compared to all other groups (All $p < 0.001$, Tukey's post hoc test; Table 4.2). Further in HFD-mice treated with eGLP-1, weight loss was lower compared to mice treated with F8S ($p < 0.05$), N14S ($p < 0.01$) and S26K ($p < 0.05$, Tukey's post hoc test; Table 4.2).

Final body weight, adiposity and body composition

Final body weight, gonadal fat pad mass and body composition parameters are displayed in Table 1. At the end of 14 weeks, HFD-mice had a higher body weight ($p < 0.0001$, F (1,98) = 86.54, diet effect; Table 4.1) and gonadal fat pad mass ($p < 0.0001$, F (1,97) = 167.7, diet effect; Table 4.1) compared to SLD-mice. There was no difference in final body weight and gonadal fat pad mass between treatment groups in SLD- and HFD-mice (Table 4.1).

There was no diet or treatment effect observed on liver mass, heart mass and average kidney mass in SLD- and HFD-mice (Table 4.1).

Fasting blood glucose levels

Fasting blood glucose levels are displayed in Table 4.3. On the final day of the treatment period, prior to the final treatment injection, HFD-mice had elevated fasting blood glucose levels compared to SLD-mice ($p < 0.001$, F (1,97) = 14.35, diet effect; Table 4.3). There was also a significant treatment effect observed on fasting blood glucose levels ($p < 0.0001$, F (6,97) = 9.779, treatment effect; Table 4.3). In SLD-mice, there was no difference in fasting blood glucose levels between mice treated with Ex-4 and PB. However, treatment with N14S significantly lowered fasting blood glucose levels compared to SLD-mice treated with pGLP-1 ($p < 0.05$) and PB ($p < 0.01$, Tukey's post hoc test; Table 4.3). In HFD-mice, treatment with Ex-4 ($p < 0.05$), F8S ($p < 0.05$) and N14S ($p < 0.01$) significantly lowered fasting blood glucose levels compared to treatment with eGLP-1 and PB (Tukey's post hoc test; Table 3). Further, treatment with N14S lowered fasting blood glucose levels further than treatment with pGLP-1 ($p < 0.05$, Tukey's post hoc test; Table 4.3).

Blood glucose levels following acute administration of GLP-1 analogues

On the final day of the treatment period, in response to acute administration of PB, blood glucose levels were elevated in both SLD- and HFD-mice compared to fasting blood glucose levels ($p < 0.0001$, F (6,97) = 14.35, treatment effect; $p < 0.01$, Tukey's post hoc test; Table

4.3). In SLD-mice, acute administration of eGLP-1, pGLP-1, F8S, N14S and S26k, reduced blood glucose levels compared to fasting blood glucose levels ($p < 0.05$, Tukey's post hoc test; Table 4.3). Acute administration of Ex-4, elevated blood glucose levels but to a lower extent than PB ($p < 0.05$, Tukey's post hoc test; Table 4.3). In HFD-mice, acute administration of Ex-4, eGLP-1, pGLP-1, F8S and S26k, reduced blood glucose compared to fasting blood glucose levels ($p < 0.05$, Tukey's post hoc test; Table 4.3). Acute administration of N14S elevated blood glucose levels but to a lower extent than PB ($p < 0.05$, Tukey's post hoc test; Table 4.3).

Blood glucose levels in response to an intraperitoneal glucose tolerance test

Blood glucose levels in response to an IPGTT and glucose AUC are illustrated in Figure 4.2. HFD feeding elevated the glucose AUC compared to SLD-mice ($p < 0.0001$, F (1,97) = 55.52, diet effect; Figure 4.2 Aiii & Biii). There was also a significant treatment effect observed ($p < 0.0001$, F (6,97) = 13.53, treatment effect; Figure 4.2 Aiii & Biii). In SLD-mice, treatment with Ex-4 ($p < 0.01$), eGLP-1 ($p < 0.05$), F8S ($p < 0.01$), pGLP-1 ($p < 0.0001$), N14S ($p < 0.0001$) and S26K ($p < 0.0001$) significantly reduced the glucose AUC compared to PB treated controls (Tukey's post hoc test; Figure 4.2 Aiii). There was no difference between glucose AUC in SLD-mice treated with Ex-4, eGLP-1, F8S, pGLP-1, N14S and S26K (Figure 4.2 Aiii). In HFD-mice, treatment with Ex-4 ($p < 0.01$), pGLP-1 ($p < 0.001$), N14S ($p < 0.001$) and S26K ($p < 0.0001$) significantly reduced the glucose AUC compared PB treated controls (Tukey's post hoc test; Figure 4.2 Biii). There was no difference between glucose AUC in HFD-mice treated Ex-4, eGLP-1, F8S, pGLP-1, N14S and S26K (Figure 4.2 Biii).

4.5 Discussion

The monotreme GLP-1 peptides are naturally occurring long-acting GLP-1 receptor agonists (468). The monotreme GLP-1 peptides, like Ex-4 discovered in a reptile, have evolved resistance to degradation (468) and therefore have potential for development as an anti-diabetes treatment (168). In the current study, we compared the monotreme GLP-1 peptides and the monotreme derived GLP-1 analogues, F8S, N14S and S26K with Ex-4 in lean and diet-induced obese mice to investigate the effects of 1 week treatment on body composition and glucose tolerance.

In this study, consistent with previous studies (198,380), HFD-mice gained significantly more weight and had greater fat pad mass compared to SLD-mice. During the GLP-1 analogue treatment period, significant weight loss was observed in all treatment groups, in both SLD-

and HFD-mice. However, the total weight loss observed in mice treated with eGLP-1, pGLP-1, F8S, N14S and S26K was similar to the weight loss observed in the PB treated controls. This suggests the weight loss may be attributed to a confounding factor, rather than a direct result of the treatment. For example, acute and chronic stress has been demonstrated to induce weight loss in mice through reduced food intake (513). Therefore, in this study, the weight loss may be due to the stress generated during restraint and IP injection of the treatment compounds. Further, in this study, in both SLD- and HFD-mice, treatment with Ex-4 promoted significantly more weight loss than observed in any other group, including the PB control group. This effect of Ex-4 is consistent with previous rodent (439) and human studies (454). For example, in Zucker Diabetic Fatty rats, 6 weeks of Ex-4 treatment induced a dose-dependent reduction in food intake and body weight compared to saline-injected rats (439). Similarly, in human volunteers with metformin-controlled type 2 diabetes, 30 weeks of twice-daily Ex-4 (Exenatide) injections, significantly reduced body weight compared to placebo controls (454). However, in this and other human studies (441,454,465), Ex-4 treatment resulted in mild to moderate side effects, including nausea with higher incidence during the initial treatment period. This suggests early weight loss may have been attributed to nausea-induced suppression of appetite. Future studies should include an investigation of the acute and chronic effects of eGLP-1, pGLP-1 and the monotreme derived GLP-1 analogues on food intake, appetite, nausea and body weight regulation. In addition, regular handing of the mice throughout the study and acclimatisation to the IP injections prior to the actual treatment period might minimise the impact of stress on body weight.

In uncontrolled type 2 diabetes, chronic hyperglycaemia damages nerves, vascular and nephron tissues, and vision (301). In the current study, consistent with previous studies (198), HFD feeding elevated fasting blood glucose levels compared to SLD-mice. In HFD-mice, treatment with Ex-4, F8S and N14S, significantly reduced fasting blood glucose levels compared to PB treated controls, although there was no difference in the effects of these different treatment groups. Similarly, in SLD-mice, treatment with N14S, but not Ex-4, reduced fasting blood glucose levels compared to PB treated controls. However, it should be noted that the concentration of GLP-1 analogues was ten times higher than Ex-4, and therefore, the differences in effect may be due to the difference in concentration. In addition, there are differences in the amino acid peptide sequence, between not only Ex-4 and the monotreme GLP-1s, but also between the different monotreme GLP-1 analogues, which affect how they bind to the GLP-1 receptor in mice (468). These differences in chemical structure likely

influence analogue functionality, including their effectiveness to reduce blood glucose levels in vivo. The current study is the first to investigate the blood glucose lowering effect of monotreme GLP-1 in vivo and further studies are required to clarify this effect. For example, dose-response curves for all monotreme GLP-1 analogues are required to establish potential differences in potency. Nonetheless, the results suggest that the monotreme analogues, F8S and N14S, similar to Ex-4, are effective in lowering fasting blood glucose levels and may be considered good candidates for further pre-clinical and clinical development, particularly N14S which seemed more effective in reducing fasting glucose levels in lean mice.

Following administration of the PB control compound, there was an acute elevation in blood glucose levels. This may be attributed to a stress-induced elevation in corticosterone release (513,514) which, in animal studies, is demonstrated to induce hyperglycaemia (515). Further, in SLD-mice, treatment with eGLP-1, pGLP-1, F8S, N14S and S26K, but not Ex-4, reduced blood glucose levels following administration. Similarly, in HFD-mice, treatment with Ex-4, eGLP-1, pGLP-1, F8S and S26K, reduced blood glucose levels following administration. However, there was no significant difference between the blood glucose lowering effects of eGLP-1, pGLP-1, F8S and S26K compared to Ex-4 treatment. This suggests the acute blood glucose lowering effect of these GLP-1 analogues, particularly pGLP-1 in SLD-mice, was sufficient to overcome an acute stress-induced elevation in blood glucose levels. It is well known that endogenous GLP-1 plays a role in glycaemic control (80). Acting upon receptors in the pancreas, GLP-1 induces secretion of insulin which in turn lowers blood glucose levels by promoting glucose uptake into tissues (168). In volunteers with type 2 diabetes, continuous infusion of endogenous GLP-1, for 6 weeks, significantly reduced fasting plasma glucose levels, attributed to increased insulin secretion, measured by elevated plasma C-peptide levels (169). It is likely that the GLP-1 analogues tested also enhance insulin secretion as a mechanism to reduce fasting blood glucose levels, however, this requires further investigation.

Consistent with previous studies, HFD feeding in the current study impaired glucose tolerance during the IPGTT compared to lean mice (472). In HFD-mice, treatment with Ex-4, pGLP-1, N14S and S26K, significantly reduced the blood glucose AUC compared to PB treated controls, although there was no difference in effects between treatment groups. Similarly, in SLD-mice, treatment with Ex-4, eGLP-1, pGLP-1, F8S, N14S and S26K significantly reduced the blood glucose AUC compared to PB treated controls with no difference in the effects between different treatment groups. Previous studies (441,454,465) have demonstrated Ex-4

treatment improved glucose tolerance. In volunteers with medication-controlled type 2 diabetes, 30 weeks of Ex-4 (Exenatide) administration significantly reduced postprandial blood glucose levels in response to a meal (441,454). Chronic Ex-4 treatment also reduced haemoglobin A1c levels, an indicator of long-term glycaemic control (441,454,465). In the current study, eGLP-1, pGLP1, and the monotreme derived GLP-1 analogues had a similar effect to Ex-4, suggesting an effective role of these compounds in improving glucose tolerance. Further, during the IPGTT, mice treated with pGLP-1, N14S and S26K, there was a trend for a greater reduction in blood glucose compared to Ex-4 treated mice. Although this trend was not significant it is worthy of further investigation of the dose-response relationships.

In addition to the aforementioned insulinotropic properties of endogenous GLP-1, the administration of GLP-1 has been demonstrated to slow gastric emptying in humans (169). Delaying the rate at which nutrients empty from the stomach into the intestines, slows the rate of glucose absorption in the intestine (80), which, in turn, influences postprandial blood glucose levels (225). Short-acting GLP-1 receptor agonists, such as Ex-4, are generally considered to delay gastric emptying (452). However, in rodent studies, central and peripheral administration of Ex-4 (Exendin) has been shown to have no significant effect on the rate of gastric emptying (516). Further, Ex-4 in combination with exogenous GLP-1 administration was shown to abolish the inhibitory effect of GLP-1 alone, on gastric emptying (516). A limitation of the current study is that the effect of eGLP-1, pGLP-1 and the monotreme derived GLP-1 analogues on gastric emptying was not determined. This requires further investigation.

4.6 Conclusion

In conclusion, treatment with Ex-4, eGLP-1, pGLP-1, and the monotreme derived GLP-1 analogues, F8S, N14S and S26K, reduced fasting blood glucose levels and lowered blood glucose levels in response to an IPGTT. This suggests monotreme GLP-1 analogues may be an effective treatment for type 2 diabetes.

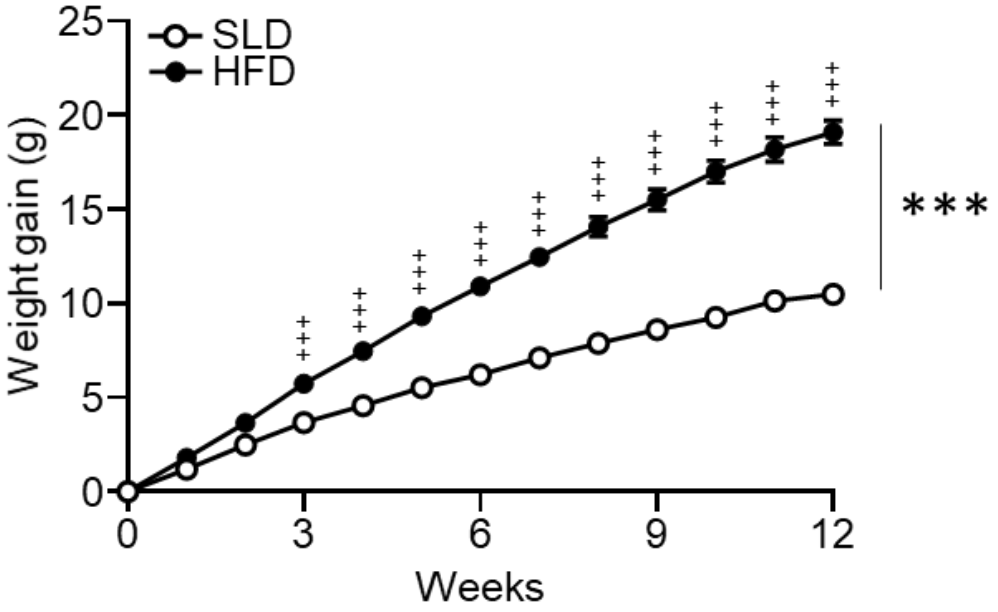

Figure 4.1. Body weight gain during weeks 0-12 in standard laboratory diet (SLD)-mice (N = 56) and high-fat diet (HFD)-mice (N = 56). Values are mean ± S.E.M. *** $p < 0.001$, diet effect, two-way ANOVA. +++ $p < 0.001$ vs SLD, Sidak's post hoc test.

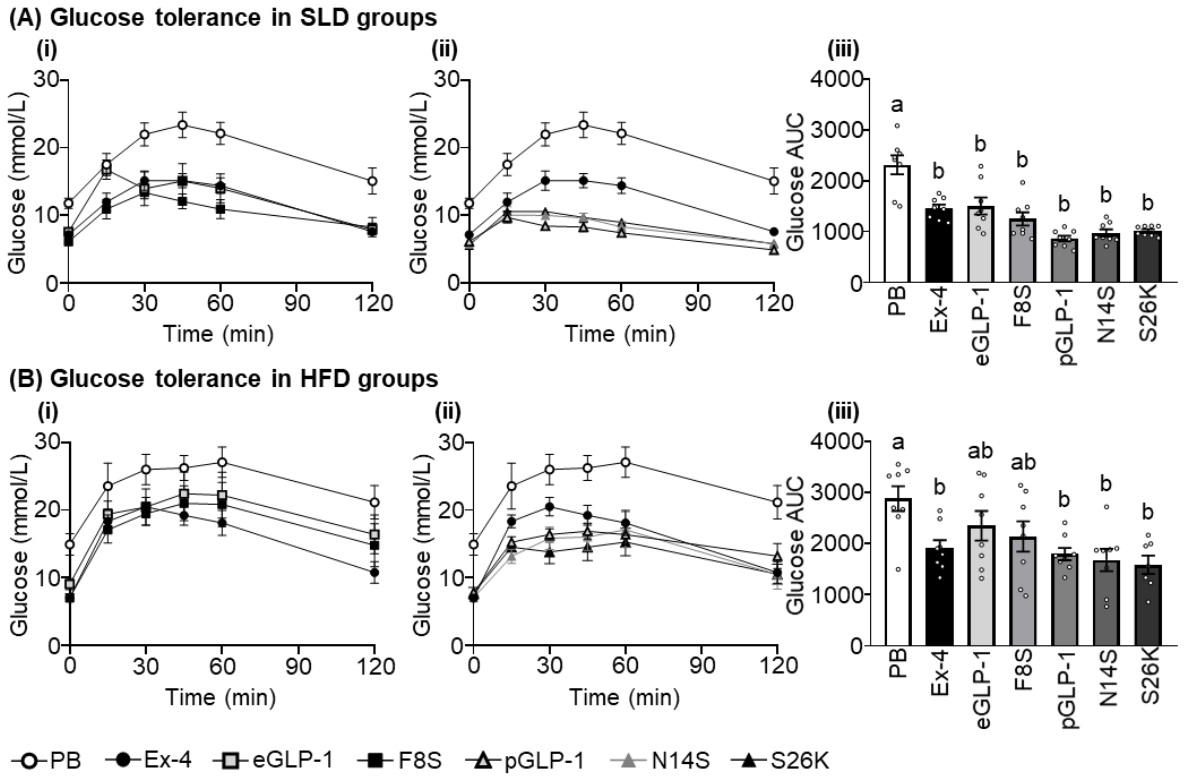

Figure 4.2. Blood glucose levels in response to a glucose tolerance test and glucose area under curve (AUC) in (A) standard laboratory diet (SLD)- and (B) high-fat diet (HFD)-mice treated with PB, Ex-4 compared to either (i) eGLP-1, F8S, or (ii) pGLP-1, N14S and S26K. Values are mean ± S.E.M. The grey open circles symbolise the individual values. Two-way ANOVA with Tukey's post hoc test for multiple comparisons between groups where $p < 0.05$ a vs b. (i.e. different letters indicate a significant difference).

Table 4.1. The effect of monotreme GLP-1 treatment on body weight and body composition parameters.

Monotreme GLP-1 Treatment Groups									
Diet Group		**PB**	**Ex-4**	**eGLP-1**	**F8S**	**pGLP-1**	**N14S**	**S26K**	**Two-way ANOVA**
Body weight at 12 weeks (g)	**SLD**	35.1±0.7	38.6±1.1	35.0±0.8	34.0±0.5	34.7±0.9	33.7±1.6	34.8±0.7	I $p < 0.05$ D $p < 0.0001$ T $p = 0.4186$
	HFD	45.2±1.8	40.5±1.9	44.4±1.6	42.9±2.2	42.5±1.8	43.0±2.1	44.5±2.5	
Final body weight (g)	**SLD**	32.8±0.6	32.5±0.8	33.0±0.5	31.5±0.4	32.5±0.9	31.4±1.2	32.5±0.7	I $p = 0.3221$ D $p < 0.0001$ T $p = 0.2499$
	HFD	41.2±1.5^{a}	34.8±1.6^{b}	39.0±1.2^{ab}	39.3±1.9^{ab}	40.0±1.5^{ab}	38.8±1.1^{ab}	40.4±2.1^{ab}	
Gonadal fat pads (g)	**SLD**	0.8±0.1	1.0±0.1	0.8±0.1	0.7±0.1	0.8±0.1	0.9±0.2	0.7±0.1	I $p = 0.0660$ D $p < 0.0001$ T $p = 0.4112$
	HFD	1.9±0.2^{ab}	1.5±0.2^{a}	2.1±0.1^{ab}	1.9±0.2^{ab}	2.3±0.1^{b}	1.7±0.1^{ab}	1.7±0.2^{ab}	
Liver (g)	**SLD**	1.5±0.1	1.4±0.1	1.5±0.1	1.5±0.1	1.5±0.1	1.6±0.1	1.3±0.1	I $p = 0.1906$ D $p = 0.9177$ T $p = 0.2378$
	HFD	1.7±0.1	1.3±0.1	1.4±0.1	1.3±0.2	1.5±0.2	1.4±0.1	1.6±0.1	
Heart (g)	**SLD**	0.18±0.02	0.18±0.03	0.19±0.03	0.15±0.02	1.18±0.02	0.18±0.02	0.15±0.02	I $p = 0.2641$ D $p = 0.7252$ T $p = 0.7404$
	HFD	0.16±0.02	0.19±0.03	0.16±0.02	0.16±0.02	0.15±0.03	0.14±0.02	0.21±0.01	
Kidneys Average (g)	**SLD**	0.18±0.02	0.19±0.01	0.19±0.02	0.17±0.02	0.18±0.02	0.15±0.01	0.19±0.02	I $p = 0.7730$ D $p = 0.3585$ T $p = 0.2016$
	HFD	0.18±0.02	0.20±0.02	0.16±0.02	0.18±0.02	0.15±0.02	0.14±0.01	0.18±0.02	

Standard laboratory diet (SLD)- and high-fat diet (HFD)-mice treated with phosphate buffer (PB), exendin-4 (Ex-4), echidna GLP-1 (eGLP-1), eGLP-1 derived F8S (F8S), platypus GLP-1 (pGLP-1), pGLP-1 derived N14S (N14S) and pGLP-1 derived S26K (S26K). Values are mean ± S.E.M. interaction (I), diet (D) & treatment (T) effects; two-way ANOVA with Tukey's post hoc test $p < 0.05$ a vs b.

Table 4.2. The cumulative weight loss effect of monotreme GLP-1 treatment.

Monotreme GLP-1 Treatment Groups										
Diet Group			**PB**	**Ex-4**	**eGLP-1**	**F8S**	**pGLP-1**	**N14S**	**S26K**	**Two-way ANOVA**
Cumulative weight loss (%)	**1 Day**	**SLD**	2.5±0.3^{b}	8.5±0.2^{a}	1.9±0.5^{b}	2.5±0.5^{b}	1.9±0.4^{b}	3.1±0.3^{b}	2.2±0.5^{b}	I $p < 0.05$ D $p = 0.4238$ T $p < 0.0001$
		HFD	2.5±0.7^{b}	6.3±0.5^{a}	2.1±0.5^{b}	3.2±0.4^{b}	2.8±0.2^{b}	2.0±0.4^{b}	2.5±0.5^{b}	
	2 Days	**SLD**	3.8±0.6^{b}	10.5±0.5^{a}	1.7±1.2^{b}	3.1±0.4^{b}	2.5±0.5^{b}	2.8±0.5^{b}	2.3±0.4^{b}	I $p < 0.01$ D $p < 0.05$ T $p < 0.0001$
		HFD	3.5±0.6^{b}	8.1±0.5^{a}	3.1±0.3^{b}	4.8±0.4^{b}	3.8±0.5^{b}	4.0±0.5^{b}	3.9±0.4^{b}	
	3 Days	**SLD**	5.3±0.5^{b}	11.2±0.3^{a}	4.9±0.7^{b}	3.7±0.6^{b}	3.1±0.6^{b}	3.4±0.7^{b}	3.8±0.8^{b}	I $p < 0.05$ D $p < 0.05$ T $p < 0.0001$
		HFD	4.9±0.9^{b}	10.11±0.6^{a}	4.0±0.2^{b}	5.6±0.5^{b}	4.9±06^{b}	5.3±0.4^{b}	5.1±0.4^{b}	
	4 Days	**SLD**	6.5±0.6^{b}	12.5±0.6^{a}	4.9±1.0^{b}	4.9±0.4^{b}	3.5±0.5^{b}	3.9±0.9^{b}	4.1±1.1^{b}	I $p < 0.05$ D $p < 0.01$ T $p < 0.0001$
		HFD	5.6±1.0^{b}	11.3±0.9^{a}	5.2±0.3^{b}	6.5±0.6^{b}	5.8±0.5^{b}	6.5±0.5^{b}	6.7±0.5^{b}	
	5 Days	**SLD**	6.8±0.6^{b}	13.9±0.7^{a}	6.3±0.8^{b}	5.3±0.6^{b}	4.5±0.6^{b}	4.9±0.9^{b}	4.8±1.0^{b}	I $p < 0.01$ D $p < 0.001$ T $p < 0.0001$
		HFD	7.4±0.7^{b}	12.3±1.1^{a}	5.6±0.4^{b}	8.0±0.5^{b}	7.3±0.9^{b}	8.0±0.5^{b}	7.9±0.6^{b}	
	6 Days	**SLD**	7.3±0.7^{b}	15.0±0.5^{a}	6.4±0.7^{b}	5.6±0.7^{b}	4.9±0.5^{b}	5.7±0.7^{b}	5.7±0.8^{b}	I $p < 0.001$ D $p < 0.0001$ T $p < 0.0001$
		HFD	7.4±0.7^{bc}	14.0±0.9^{a}	6.5±0.3^{c}	9.4±0.6^{b}	7.7±0.7^{bc}	9.8±0.6^{b}	9.3±0.6^{b}	

Cumulative weight loss during 6 days of monotreme GLP-1 treatment, as a percentage of week 12 body weight, in standard laboratory diet (SLD)- and high-fat diet (HFD)-mice treated with the phosphate buffer (PB), exendin-4 (Ex-4), echidna GLP-1 (eGLP-1), eGLP-1 derived F8S (F8S), platypus GLP-1 (pGLP-1), pGLP-1 derived N14S (N14S) and pGLP-1 derived S26K (S26K). Values are mean ± S.E.M. interaction (I), diet (D) & treatment (T) effects; two-way ANOVA with Tukey's post hoc test $p < 0.05$ a vs b vs c.

Table 4.3. The effect of monotreme GLP-1 treatment on fasting blood glucose levels and glucose tolerance.

Monotreme GLP-1 Treatment Groups									
Diet Group		**PB**	**Ex-4**	**eGLP-1**	**F8S**	**pGLP-1**	**N14S**	**S26K**	**Two-way ANOVA**
Fasting blood glucose (mmol/ L)	**SLD**	8.9±0.5^{a}	6.9±0.4^{ab}	8.2±0.6^{ab}	7.19±0.3^{ab}	8.46±0.8^{a}	6.0±0.4^{b}	7.0±0.4^{ab}	I p = 0.6089 D p < 0.001 T p < 0.0001
	HFD	10.9±0.8^{a}	7.8±0.6^{bc}	10.3±0.6^{a}	7.6±0.6^{bc}	9.1±0.7^{ab}	6.6±0.3^{c}	8.5±0.8^{abc}	
Blood glucose after treatment (mmol/ L)	**SLD**	11.8±0.8^{a}	7.1±0.6^{b}	7.5±0.5^{b}	6.1±0.3^{b}	6.1±0.6^{b}	5.8±0.4^{b}	5.6±0.4^{b}	I p = 0.3751 D p < 0.0001 T p < 0.0001
	HFD	14.9±1.6^{a}	7.0±0.3^{b}	9.0±0.8^{b}	7.0±0.6^{b}	7.8±0.5^{b}	7.0±0.5^{b}	7.7±0.9^{b}	
Change in blood glucose (mmol/ L)	**SLD**	2.8±0.4^{a}	0.3±0.5^{b}	-0.6±0.7^{bc}	-1.1±0.3^{bc}	-2.3±0.5^{c}	-0.3±0.2^{bc}	-1.5±0.5^{bc}	I p = 0.1971 D p = 0.1973 T p < 0.0001
	HFD	3.9±0.9^{a}	-0.8±0.5^{b}	-1.3±0.3^{b}	-0.5±0.5^{b}	-1.3±0.4^{b}	0.4±0.4^{b}	-0.7±0.4^{b}	
Glucose AUC	**SLD**	2312±187.1^{a}	1454±75.9^{b}	1503±165.0^{b}	1248±130.8^{b}	865.4±57.7^{b}	973.1±70.5^{b}	1014±35.1^{b}	I p = 0.7823 D p < 0.0001 T p < 0.0001
	HFD	2882±241.8^{a}	1910±159.3^{b}	2349±290.6^{ab}	2140±294.3^{ab}	1795±121.8^{b}	1682±219.5^{b}	1588±179.6^{b}	

Fasting blood glucose, blood glucose after treatment, change in blood glucose levels and blood glucose area under curve (AUC), in standard laboratory diet (SLD) and high-fat diet (HFD) mice treated with the phosphate buffer (PB), exendin-4 (Ex-4), echidna GLP-1 (eGLP-1), eGLP-1 derived F8S (F8S), platypus GLP-1 (pGLP-1), pGLP-1 derived N14S (N14S) and pGLP-1 derived S26K (S26K). Values are mean ± S.E.M. interaction (I), diet (D) & treatment (T) effects; two-way ANOVA with Tukey's post hoc test p< 0.05 a vs b vs c.

CHAPTER 5. General Discussion and Conclusion

LIST OF ABBREVIATIONS

In alphabetical order

AL	*Ad libitum*
BCAA	Branched-chain amino acid
DP	Dark phase
DPP4	Dipeptidyl peptidase 4
Ex-4	Exendin-4
GLP-1	Glucagon-like peptide-1
HFD	High-fat diet
IPGTT	Intraperitoneal glucose tolerance test
LP	Light phase
OGTT	Oral glucose tolerance test
SLD	Standard laboratory diet
TRF	Time-restricted feeding

In this modern era, the global prevalence of metabolic diseases such as obesity and type 2 diabetes is rapidly rising (14,16). A wide variety of dietary, lifestyle, pharmacological and surgical strategies currently exist to treat these diseases, however, the efficacy and effectiveness of these treatment strategies are limited. Therefore, novel or improved interventions are required to combat these metabolic diseases. This thesis aimed to investigate the potential roles of three new dietary, lifestyle or pharmaceutical options for treating obesity and type 2 diabetes. The principal advantage and disadvantages of these strategies are displayed in Figure 5.1.

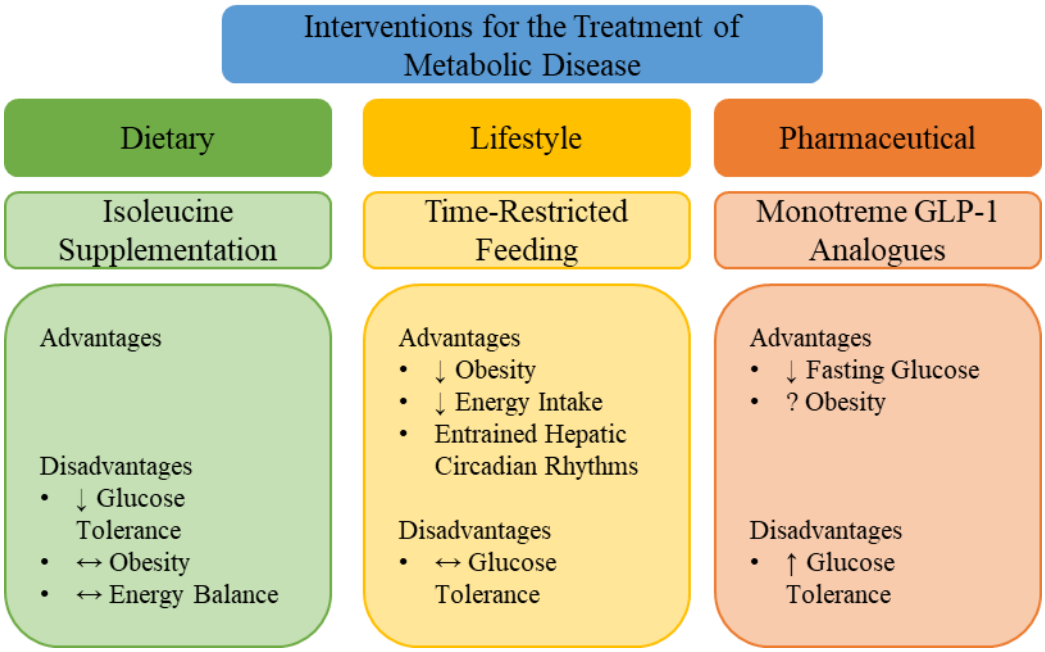

Figure 5.1 Principal advantages and disadvantages of three potential interventions for the treatment of metabolic disease, including isoleucine supplementation, time-restricted feeding and monotreme GLP-1 analogues. ↑ Designates an increase; ↓ designates a decrease; ↔ designates no effect; ? designates insufficient evidence to report an effect.

5.1 Dietary Intervention

Previous literature

Population studies demonstrate an association between a high dietary intake of the branched-chain amino acids (BCAAs), isoleucine, leucine and valine, and a low prevalence of overweight and obesity in adults (358,359). In addition, chronic isoleucine supplementation has been shown to protect mice from high-fat diet (HFD)-induced obesity without restricting

food intake (349). This evidence suggests isoleucine supplementation may have a protective effect against obesity. However, whether chronic isoleucine treatment ameliorated weight gain and adiposity in mice with established obesity remained to be established. Therefore, chapter 2 aimed to investigate the impact of chronic isoleucine supplementation on weight gain and adiposity in lean and obese mice.

Dietary BCAA supplementation has also been shown to improve glucose tolerance in glucose-intolerant rats (517). Specifically, several studies have reported that acute isoleucine administration reduces blood glucose levels in response to an oral glucose tolerance test (OGTT) in lean and obese rodents (350,351,427,428). In addition, chronic isoleucine supplementation has been shown to reduce fasting blood glucose levels in glucose-intolerant mice (351). Overall, this evidence suggests that isoleucine treatment may be beneficial for treating type 2 diabetes. However, the impact of chronic isoleucine supplementation on glucose tolerance, especially blood glucose levels in response to an OGTT, in lean and obese mice was unknown. Hence, chapter 2 aimed to investigate this.

Current findings

In chapter 2, chronic isoleucine supplementation did not affect weight gain, adiposity, energy intake or energy expenditure in standard laboratory diet (SLD)- or HFD-mice (a disadvantage depicted in Figure 5.1). In addition, acute and chronic isoleucine treatment had no beneficial effect on glucose tolerance in SLD- or HFD-mice, despite using a similar dose and duration (0.3 g/ kg and 1.5 % in drinking water for 6 weeks) to previous studies (350,351). In fact, chronic isoleucine treatment adversely elevated blood glucose levels in response to the OGTT in SLD-mice (a disadvantage depicted in Figure 5.1). In addition, gastric emptying rate, which has previously been shown to impact postprandial blood glucose levels in humans (225), was assessed in SLD-mice to determine if the observed effects are due to isoleucine induced changes in gastric emptying. Neither, acute or chronic isoleucine treatment affected the rate of gastric emptying and, therefore, it is unlikely that the elevated blood glucose levels in response to the OGTT, in chronic isoleucine treated SLD-mice, is due to a change in gastric emptying.

Limitations and future directions

In the current study, 6 weeks of chronic isoleucine did not affect body weight or energy balance in obese mice. This is inconsistent with a previous study that reported 4 weeks of chronic isoleucine supplementation prevented HFD-induced weight gain and adiposity in mice through

increased energy expenditure, specifically increased fatty acid uptake and oxidation in the liver and skeletal muscle (349). However, in that study, isoleucine supplementation was initiated after only two weeks of HFD feeding (349), which is arguably an insufficient time-course to establish obesity in mice (106). It is possible that a longer treatment duration may have been required for isoleucine to reverse obesity in the current study. Nevertheless, the current differences in between study outcomes suggest isoleucine may prevent HFD-induced obesity, but is ineffective in ameliorating weight gain in established obesity.

In previous studies, acute isoleucine administration in lean mice, at a dose of 0.1 g/ kg and 0.3 g/ kg, induced a dose-dependent reduction in blood glucose levels during an OGTT (350,351,428). Similarly, acute isoleucine administration reduced plasma glucose levels in leptin receptor-deficient (*db/db*) mice, a model of type 2 diabetes, but at a higher dose of 0.5 g/ kg (351). This evidence suggests that a larger dose of isoleucine may have been required to significantly lower blood glucose levels in response to the OGTT in the current study, especially in the HFD-induced obese mice. Therefore, future studies are needed to re-evaluate the dose-response effects of acute isoleucine on glucose tolerance in established obesity.

A limitation of the current study is that the mechanisms which attributed to the adverse elevation of blood glucose levels following chronic isoleucine supplementation in lean mice were not identified and, therefore, remain an area of focus for future studies. In particular, future studies should investigate the impact of chronic isoleucine on intracellular insulin signalling and glucose uptake in skeletal muscle since previous studies have identified a link between acute isoleucine and insulin-stimulated actions (413,424,426). In addition, recent new evidence has identified an association between a high dietary intake of BCAAs and an increased risk of insulin resistance (423). There is also evidence to suggest that high plasma BCAA levels are biomarkers for an increased risk of future type 2 diabetes (367,368). Therefore, it would be prudent to investigate the adverse effect of chronic isoleucine supplementation on glucose tolerance to provide further insight into the link between BCAAs and type 2 diabetes.

5.2 Lifestyle Intervention

Previous literature

The timing of food intake is an often overlooked determinant for metabolic diseases (518). In the modern era, food intake in humans generally spans more than half of the 24 hr solar light

cycle (199). This is particularly true of shift workers who frequently consume food during the rest phase and, despite having similar energy intake compared to individuals working 'normal' daylight hrs (200), are at an increased risk of metabolic diseases, including obesity (187,201) and type 2 diabetes (285,286). This increased risk of metabolic disease is, at least in part, due to dysregulation of the circadian system (172-174).

Previously, evidence has demonstrated that time-restricted feeding (TRF) during the active phase (dark phase (DP) in rodents (325,326), light phase (LP) in humans (327)) is an effective lifestyle intervention for the correction of circadian dysregulation and the treatment of obesity and glucose intolerance. However, the beneficial effects of TRF during the rest phase were less clear and potentially important for individuals, such as shift workers, who need to eat during the rest phase. Therefore, chapter 3 investigated in lean and obese mice the impact of a 12 hr TRF regime for 8 weeks during the LP compared to TRF during the DP on body weight and glycaemic control.

Current findings

Chapter 3 demonstrates TRF during the DP or LP are both effective to reduce HFD-induced obesity but not HFD-induced hyperglycaemia (advantages and a disadvantage, respectively, depicted in Figure 5.1). Specifically, HFD-LP and HFD-DP mice had reduced weight gain and adiposity compared to HFD-mice fed *ad libitum* (AL), but there was no difference in blood glucose levels between groups. This effect on body weight was mainly due to reduced energy intake since the HFD-LP and HFD-DP mice had lower energy intake than HFD-AL mice. Comparatively, the SLD-LP and SLD-DP mice increased their meal size and meal number, compensating for the restricted window of food access (as previously reported (380)), resulting in similar body weights to SLD-AL mice.

Chapter 3 also demonstrates that TRF during the DP or LP aligns circadian rhythms of hepatic markers of glucose metabolism to the timing of food intake. In the current study, the HFD-AL mice exhibited circadian desynchrony of hepatic glucose metabolism markers with, in general, increased mean and phase-advanced peak expression levels compared to SLD-AL mice. This effect could be due to the change in food intake patterns in HFD-AL mice, with a 'grazing-like' behaviour and an increase in food intake during the LP compared to SLD mice. In HFD groups, TRF during the LP, in general, resulted in a phase-reversal of hepatic gene expression, compared to SLD-AL mice. Similarly, TRF during the DP, in general, resulted in a phase-advance of hepatic gene expression, compared to SLD-AL mice.

Limitations and future directions

Chapter 3 demonstrates TRF during either the LP or DP reduced HFD-induced weight gain and aligned circadian rhythms of hepatic markers of glucose metabolism to the timing of food intake. It is well established that the timing of food intake and TRF regimes entrain metabolic gene expression in peripheral tissues (reviewed in (176,183,322)). This is particularly true in the liver, which must respond immediately to food intake to metabolise nutrients and maintain proper glycaemic control (100,182). Therefore, it is not surprising that in chapter 3, TRF entrained the peak expression of genes involved in hepatic glucose metabolism.

Overall, in chapter 3, 8 weeks of TRF during the LP or DP reduced HFD-induced weight gain and adiposity in HFD-mice, suggesting that TRF during the rest phase may reduce the risk of obesity in individuals whose occupation induces circadian desynchrony, such as night-shift workers. However, TRF during the LP or DP had no effect on blood glucose levels in HFD-mice. In previous studies, TRF during the DP in HFD-mice ameliorated blood glucose levels by reducing the overexpression of genes involved in hepatic glucose production (109,295,519). This difference in findings is likely due to the nutritional status of the mice when the blood glucose levels were evaluated (i.e. under AL feeding conditions in the current study vs under fasting condition in previous studies (109,295)). Regardless, it is necessary for future studies to investigate the long-term health outcomes resulting from TRF, specifically the paradoxical combination of improved body weight but not blood glucose levels should first be investigated in a long-term animal study.

5.3 Pharmaceutical Intervention

Previous literature

In humans, the gut hormone glucagon-like peptide-1 (GLP-1) is involved in several physiological roles, including appetite suppression (80,520,521) and glycaemic control (47,168). In a clinical study, administration of GLP-1 reduced body weight and fasting blood glucose levels through suppression of appetite and food intake (169). This evidence suggests that exogenous GLP-1 administration may be beneficial for treating obesity and type 2 diabetes. However, human GLP-1 is rapidly degraded within the bloodstream by the enzyme dipeptidyl peptidase 4 (DPP4), and any potential anti-obesity and anti-diabetes effects of human GLP-1 administration would be short-lived (168,522). Therefore, research is focused on alternative more stable GLP-1 analogues for treating obesity and type 2 diabetes. For

example, exendin-4 (Ex-4) is a highly potent, naturally occurring GLP-1 receptor agonist (168,458) resistant to degradation by DPP4 (217,458). The commercial synthetic form of Ex-4 is exenatide which is used under the trade names Byetta and Bydureon among others (458). In diabetic animal models (439,464) and clinical studies (441,454,465), Ex-4 treatment promotes weight loss and lowers blood glucose levels in a dose-dependent manner. However, Ex-4 treatment in humans is also associated with several side effects, including mild to moderate nausea (433,441,454,465), vomiting, diarrhoea and mild hypoglycaemia (217,433).

Comparatively, GLP-1 peptides derived from echidna and platypus are also naturally occurring, long-acting GLP-1 receptor agonists. In a preliminary *in vitro* study, these monotreme GLP-1 analogues demonstrated resistance to degradation by DPP4 (468), similar to Ex-4. In addition, they were found to bind the human GLP-1 receptor with a lower affinity and were less potent in their stimulation of intracellular signalling compared to Ex-4 (468). This suggests that the monotreme GLP-1 analogues may elicit long-lasting physiological actions without the risk of side effects, such as hypoglycaemia, an improvement over Ex-4. However, the effectiveness of the monotreme GLP-1 analogues in treating type 2 diabetes had yet to be determined *in vivo*. For the first time, chapter 4 investigated the impact of monotreme GLP-1 analogue treatment on glucose tolerance in lean and obese mice.

Current findings

Chapter 4 demonstrated 1 week of monotreme GLP-1 analogue treatment-induced weight loss in all groups. However, on the 6th day of treatment, cumulative weight loss was highest in mice treated with Ex-4 compared to any other group. Further, monotreme GLP-1 analogue treatment was shown to be effective in reducing fasting and postprandial blood glucose levels, with a single dose of the monotreme GLP-1 analogues sufficient to lower fasting blood glucose levels (an advantage depicted in Figure 5.1). In comparison, administration of Ex-4 and the control compound resulted in an elevation in fasting blood glucose levels, likely an acute stress-induced hyperglycaemic response due to the intraperitoneal injection procedure (515). In addition, monotreme GLP-1 analogue treatment, similar to Ex-4, reduced blood glucose levels in response to an intraperitoneal glucose tolerance test (IPGTT) in lean and obese mice. This evidence demonstrates that the monotreme GLP-1 analogues are potentially effective for treating type 2 diabetes.

Limitations and future directions

In chapter 4, treatment with the monotreme GLP-1 analogues resulted in weight loss, suggesting that, like Ex-4, the monotreme GLP-1 analogues have potential benefit in treating obesity. However, most of this weight loss was observed in all groups on the first day of treatment, suggesting that the weight loss may have been caused by acute stress (523) due to the injection protocol. Future studies should include a period of placebo injections to acclimatise the mice to the procedure to minimise this effect. Regardless, 1 week treatment is not a sufficient time period to accurately investigate the impact of monotreme GLP-1 analogues on body weight, appetite or energy balance. Therefore, future studies should investigate the effect of chronic (e.g. $\geq$ 12 weeks) monotreme GLP-1 analogue treatment on body weight, adiposity and energy balance, perhaps via the use of metabolic monitoring cages, in lean and obese mice. In addition, previous studies demonstrate that Ex-4 therapy is associated with side effects such as nausea and gastrointestinal distress (433,441,454,465), factors that likely influence appetite and energy balance. Therefore, future studies should also monitor for nausea-like symptoms in mice, by, for example, observing kaolin clay ingestion (524). Another limitation of Ex-4 therapy, as well as other commercially available GLP-1 receptor agonists is its dependency on subcutaneous injection administration, which is considered unfavourable by patients (458). Future pre-clinical and clinical studies into the development of monotreme GLP-1 analogues for the treatment of obesity and type 2 diabetes should consider use of an alternate pathway, such as oral administration.

Chapter 4 also demonstrated that the monotreme GLP-1 analogues were effective blood glucose-lowering agents. Specifically, monotreme GLP-1 analogue treatment, at a dose of 10 mg/ kg of body weight, lowered fasting blood glucose levels and reduced blood glucose levels in response to an IPGTT in lean and obese mice. However, this blood glucose-lowering effect during the IPGTT was not more potent than that stimulated by Ex-4 at a lower dose of 1 mg/ kg of body weight. Therefore, future studies should utilise a dose-response testing method to investigate the maximal impact of acute monotreme GLP-1 analogue administration on fasting blood glucose levels and glucose tolerance. Following dose optimisation, future studies should then evaluate if these blood glucose-lowering effects persist following a chronic treatment regime in lean and obese mice, and in models of type 2 diabetes, such as leptin- and leptin receptor-deficient (*ob*/*ob* and *db*/*db*) mice.

5.4 Conclusion

This thesis investigated three new or improved dietary, lifestyle and pharmaceutical interventions for treating obesity and type 2 diabetes. Chapter 2 demonstrates isoleucine supplementation was ineffective at reducing weight gain or improving glucose tolerance in lean or obese mice, despite previous reports of obesity-protective (349) and blood glucose-lowering effects (350,351). Instead, chronic isoleucine supplementation in lean mice worsened glucose tolerance compared to controls, but not through altered gastric emptying. This study provides an avenue of evidence for future studies to investigate the mechanisms behind the link between high plasma BCAAs and insulin resistance (368). Next, chapter 3 demonstrates TRF during the DP or LP is similarly effective for treating obesity by reducing diet-induced weight gain and adiposity. However, TRF during the LP and DP was ineffective at reducing HFD-induced hyperglycaemia. The broader health implications of TRF-LP/DP-induced weight loss, despite persistent HFD-induced hyperglycaemia should be investigated in future studies. Finally, chapter 4 demonstrates monotreme GLP-1 analogue treatment is effective to improve glucose tolerance in lean and obese mice. Specifically, the monotreme GLP-1 analogues, similar to Ex-4, are potent blood glucose-lowering agents responding to an IPGTT. However, future studies are required to optimise dose and evaluate the safety and effectiveness of these monotreme GLP-1 analogues on glucose tolerance and body weight during chronic treatment. This study provides foundational evidence to optimise monotreme GLP-1 analogues as effective pharmaceutical agents for treating type 2 diabetes and obesity.

Overall, this thesis is a collection of health interventions, each with principal advantages and disadvantages for treating obesity and type 2 diabetes. Therefore, rather than a single approach, improved health outcomes might require a multifaceted approach. Throughout the literature, there is abundant evidence highlighting multifactorial health strategies are more effective than single interventions. To begin with, interventions with a combined focus on diet and exercise/lifestyle are shown to be ~20 % more effective than dieting alone in promoting and maintaining weight loss (318). Further, the usage of anti-obesity or anti-diabetes medications in combination with dietary/lifestyle interventions are shown to be more effective than drug monotherapy alone (525-527). For example, one clinical trial reported that in individuals with obesity and type 2 diabetes, the adoption of dietary/lifestyle changes in addition to their anti-diabetes medications resulted in significantly greater reductions in plasma glycated haemoglobin levels, fasting blood glucose levels, body weight, body mass index and waist

circumference compared to baseline (527). Therefore, to meaningfully improve the health outcomes of individuals with metabolic disease, future health interventions might incorporate a number of different strategies, including some of the novel interventions from this thesis, to improve the health and wellbeing of individuals with obesity and type 2 diabetes.

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