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THE ROLE OF THE SUCCINATE RECEPTOR IN BROWN ADIPOSE TISSUE FUNCTION

Ben Thomas McNeill

Thesis for the Degree of Doctor of Philosophy (PhD)

UNIVERSITY OF EDINBURGH
DECLARATION

I declare that this thesis has been composed entirely by the candidate, Ben Thomas McNeill. This work has not previously been submitted for a Doctor of Philosophy, a degree or any professional qualification. I have done all the work and data analysis, apart from the Western blots which were performed by Dr Alex Kelman.

Ben Thomas McNeill
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“Despair, or folly?’ ... It is not despair, for despair is only for those who see the end beyond all doubt. We do not.”

— J.R.R. Tolkien, The Fellowship of the Ring
ABSTRACT

Obesity increases the risk developing diseases such as type 2 diabetes mellitus, hypertension, ischaemic heart disease, and some cancers. The prevalence of obesity has increased substantially globally over the past 30 years despite current treatments and public health initiatives. Brown adipose tissue (BAT) is a thermogenic tissue that generates heat to maintain body temperature during cold exposure, as part of the process termed cold-induced thermogenesis (CIT). BAT activation is a potential therapeutic target for treating obesity-related cardiometabolic disease but the pathways regulating human BAT are not fully understood. Prior to this thesis our lab undertook RNA sequencing of human primary differentiated brown and white adipocytes. This revealed that SUCNR1, encoding the succinate receptor, was expressed ~20-fold greater in brown adipocytes. Previous work has demonstrated that succinate increased BAT thermogenesis, while Sucnr1−/− mice have impaired metabolic health when fed a high fat diet (HFD). We tested the hypothesis that SUCNR1 activation increases BAT thermogenesis in i) human adipocytes, ii) in mice with deletion of Sucnr1 and iii) in murine adipocytes.

i) To test the role of SUCNR1 in humans, primary brown and white differentiated pre-adipocytes were incubated with succinate or the selective SUCNR1 agonist cis-epoxysuccinic acid (C-ESA). Succinate increased both basal and noradrenaline-stimulated respiration in brown adipocytes, while C-ESA increased noradrenaline stimulated respiration. In white adipocytes, succinate increased both basal and nor-adrenaline stimulated respiration. Neither succinate nor C-ESA alter brown or white adipocyte mRNA expression of the thermogenic protein uncoupling protein 1 (UCP1). C-ESA reduced glycerol release, a marker of lipolysis, in human white adipocytes during noradrenaline stimulation. However, succinate or C-ESA did not alter lipolysis in brown adipocytes.

ii) To test the role thermogenic role of SUCNR1 in vivo in mice, we fed mice with selective disruption of Sucnr1 either a high fat (HFD) or control diet for 12 weeks and
measured energy expenditure when housed at both standard room temperature (21°C) and during more severe cold exposure (4°C). Male (but not female) Sucnr1<sup>-/-</sup> mice exhibited impaired glucose tolerance versus Sucnr1<sup>+/+</sup> littermates after just 2 weeks of HFD, and demonstrated increased fat mass following more prolonged feeding. However, energy expenditure, locomotor activity and tail vein temperature were similar between male Sucnr1<sup>-/-</sup> and Sucnr1<sup>+/+</sup> mice housed at 21°C or 4°C. In addition, expression of Ucp1 and Pgc1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) in BAT and inguinal WAT were similar between genotypes. Sucnr1 deletion did not alter fat mass or glucose tolerance in mice fed a control diet.

iii) To test whether SUCNR1 altered murine brown adipocyte function, pre-adipocytes were isolated, cultured and differentiated from the interscapular BAT (brown) and inguinal WAT (beige) from ~7-day old Sucnr1<sup>-/-</sup> and Sucnr1<sup>+/+</sup> mice prior to incubation with succinate or C-ESA. Basal and noradrenaline-stimulated respiration was similar between genotypes in both brown and beige adipocytes. Neither succinate nor C-ESA stimulated respiration in murine adipocytes. Basal and noradrenaline-stimulated Ucp1 mRNA levels were also similar in Sucnr1<sup>-/-</sup> and Sucnr1<sup>+/+</sup> adipocytes.

These data reveal that SUCNR1 activation enhances brown adipocyte respiration in human but not murine brown adipocytes, highlighting a novel pathway regulating BAT function in addition to differences between species. These data also reveal novel sex-specific differences in the metabolic effects of SUCNR1, although the reasons through which female mice protected from the adverse metabolic effects of Sucnr1 deficiency are unclear. SUCNR1 activation may be a novel therapeutic target to enhance human BAT thermogenesis, but further research is required to determine the mechanisms through which SUCNR1 activates BAT.
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ABBREVIATIONS LIST

\(^{18}\text{F-FDG} – \text{^{18}F-Fluoro-Deoxyglucose}

\(^{18}\text{FTHA} – \text{^{18}fluoro-6-Thia-Heptadecanoic~Acid}

\text{AA} – \text{Amino~Acid}

\text{AC} – \text{Adenyl~Cyclase}

\text{ACC1} – \text{Acetyl-Coa Carboxylase~1}

\text{acetyl-Coa} – \text{Acetyl-Coenzyme~A}

\text{ADIPOQ} - \text{Adiponectin}

\text{ADP} - \text{Adenosine~Diphosphate}

\text{AEE} – \text{Active~Energy~Expenditure}

\text{AMPK} – \text{AMP-Activated~Protein~Kinase}

\text{ANCOVA} – \text{Analysis~of~Covariance}

\text{ANOVA} – \text{Analysis~of~Variance}

\text{ATGL} – \text{Adipocyte~Triglyceride~Lipase}

\text{ATP} – \text{Adenosine~Triphosphate}

\text{AU} – \text{Arbitrary~Units}

\text{AUC} – \text{Area~Under~the~Curve}

\text{BAT} – \text{Brown~Adipose~Tissue}

\text{BMI} – \text{Body~Mass~Index}

\text{BMR} – \text{Basal~Metabolic~Rate}

\text{BSA} – \text{Bovine~Serum~Albumin}

\text{BW} – \text{Bodyweight}

\text{cAMP} – \text{Cyclic~Adenosine~Monophosphate}

\text{CD} – \text{Control~Diet}

\text{cDNA} – \text{Complimentary~DNA}

\text{C-ESA} – \text{Cis-Epoxysuccinic~Acid}

\text{CIT} – \text{Cold~Induced~Thermogenesis}

\text{CO}_2 – \text{Carbon~Dioxide}

\text{DBPS+P/S} – \text{Dulbecco’s~Phosphate~Buffered~Saline~+~Penicillin/Streptomycin}

\text{DGAT} – \text{Diacylglycerol~Acyltransferase}
DIT – Diet Induced Thermogenesis
DMEM – Dulbecco’s Modified Eagle Medium
DNA – Deoxyribonucleic Acid
EDTA – Ethylenediaminetetraacetic Acid
EE – Energy Expenditure
EI – Energy Intake
ELISA – Enzyme-Linked Immunosorbent Assay
ERK1/2 – Extracellular Signal-Regulated Kinases-1/2
Etoh – Ethanol
eWAT – Epididymal Adipose Tissue
FABP – Fatty Acid Binding Protein
FAS – Fatty Acid Synthase
FATP – Fatty Acid Transport Protein
FBS – Foetal Bovine Serum
FCCP – Carbonyl Cyanide-P-Trifluoromethoxyphenylhydrazone
FFA – Free Fatty Acid
FFM – Fat Free Mass
GCGR – Glucagon Receptors
GIP – Glucose-Dependent Insulinotropic Polypeptide
GLP-1 – Glucagon-Like Peptide-1
GLUT – Glucose Transporter Facilitators
GPCR G – Protein-Coupled Receptor
gWAT – Gonadal Adipose Tissue
HFD – High Fat Diet
HSL – Hormone Sensitive Lipase
IAT – Inguinal Adipose Tissue
IMM – Inner Mitochondrial Membrane
Kcal – Kilocalories
LEP – Leptin
LEPR – Leptin Receptor
LPL – Lipoprotein Lipase
MAPK - Mitogen-Activated Protein Kinase
MCT1 - Monocarboxylate Transporter 1
MGL - Monoacylglycerol Lipase
mRNA – Messenger RNA
mWAT – Mesenteric Adipose Tissue
Myf5 – Myogenic Factor 5
n – Sample Size
NADR – Noradrenaline
O₂ – Oxygen
OCR – Oxygen Consumption Rate
OGTT – Oral Glucose Tolerance Test
P/S – Penicillin/Streptomycin
PCR – Polymerase Chain Reaction
PDE3B – Phosphodiesterase 3
PET/CT Positron Emission Tomography Coupled With Computed Tomography
PKA – Protein Kinase A
PLIN1 – Perilipin 1
Pparg – Peroxisome Proliferator-Activated Receptor Gamma
PPARGC1alpha – Peroxisome Proliferator-Activated Receptor Gamma Co-activator 1-Alpha
pWAT – Peri-Renal Adipose Tissue
qPCR – Real-Time Quantitative Polymerase Chain Reaction
R/A – Rotenone/Antimycin
RAS – Renin-Angiotensin System
REE – Resting Energy Expenditure
RER – Respiratory Exchange Ratio
RET – Reverse Electron Transport
RNA – Ribonucleic Acid
RT-PCR – Reverse Transcription Polymerase Chain Reaction
SDH – Succinate Dehydrogenase
SDS – Sodium Dodecyl Sulphate
SEM – Standard Error Of The Mean
Slc13a3 – Solute Carrier Family 13 Member 3
Slc25a10 – Solute Carrier Family 25 Member 10
SNS –Sympathetic Nervous System
SUCNR1 – Succinate Receptor 1
T2DM – Type 2 Diabetes Mellitus
T3 – Triiodothyronine
TAE – Tris-Actetic Acid- Ethylenediaminetetraacetic Acid
TBE – Tris-Borate-EDTA
TBS – Tris-Buffered Saline
TBS/T – Tris-Buffered Saline/Tween-20
TDEE – Total Daily Energy Expenditure
TRIG - Triglyceride
UCP1 – Uncoupling Protein 1
uWAT – Uterine Adipose Tissue
V/V – Volume/Volume
VCO₂ – Volume Carbon Dioxide
VDACS – Voltage-Dependent Anion Channels
VLDLS – Very Low Density Lipoproteins
VO₂ – Volume Oxygen
W/V – Weight/Volume
WAT – White Adipose Tissue
β1/2/3-AR – β1/2/3 Adrenergic Receptors
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Chapter 1

Introduction
Chapter 1

1.1 ENERGY BALANCE AND OBESITY

1.1.1 Energy intake and expenditure

Metabolic processes require energy to function. In humans and animals, food is required to fuel metabolic demands. Fundamentally, energy balance is the relationship between energy intake and energy expenditure and how the differences between these two factors lead to body weight gain or loss (1). Energy intake is the net absorption of the dietary macronutrients following food consumption. This intake can be affected by several factors and varies among individuals and is dependent on the food types, how they are prepared, and intestinal factors affecting nutrient uptake (1). Energy expenditure is composed of resting energy expenditure (REE) or basal metabolic rate (BMR), physical activity, dietary induced thermogenesis, and cold induced thermogenesis (CIT) (2). Total daily energy expenditure can vary greatly between individuals but is typically between 1800-2200 Kcal in females and 2300-2700 Kcal in males. Typically, REE contributes the greatest amount to overall energy expenditure but varies on factors such as lean mass, gender, and age (3). Physical activity can also causes large changes in total daily energy expenditure but varies with exercise time and intensity (1).

It is only until very recently that food was as readily available as it is today (4, 5). During the evolutionary development of humans, food gathering was an energy intensive process as it required extensive foraging and hunting, or later manual labour in agriculture. Food scarcity was an issue during these times, therefore when food was plentiful there needed to be an effective method for storing excess energy from food intake for subsequent use later (6). As such, energy balance homeostasis was more precarious in these times and was critical for survival. While there are still many people around the world that this still applies to, in many countries modern agricultural practises have substantially reduced food scarcity. This has led to instances of energy imbalance due to increased food intake and the development of an obesogenic environment. Balance of energy expenditure and energy intake is a key component in the development of obesity, a disease state with significant health consequences.
1.1.2 Dysregulation of Energy intake and expenditure

The relationship between energy expenditure and intake is multifaceted and there is debate to what extent each side of the scale contributes to obesity (Figure 1.1) (7). Obese individuals have greater resting energy expenditure (REE) than non-obese individuals. However, when normalising REE to fat free mass (FFM), which includes skeletal muscle and other metabolically active organs, such as the heart and liver, the differences between obese and normal individuals is ablated. During periods of increased energy expenditure, such as exercise, the amount of energy required is proportionally the same between normal and obese individuals when normalised to FFM (7). Therefore, differences in energy expenditure may not greatly contribute to development of obesity and may predominantly be through differences in energy intake.

However, energy intake is difficult to measure as studies tracking caloric intake have identified that obese individuals typically underestimate their total daily caloric intake (8, 9). Additionally, being in a negative energy balance, either through reduced caloric intake or increased energy expenditure, leads to hormonal changes that alter appetite and stimulate neuronal circuitry of the hypothalamus to stimulate appetite. These hormonal changes include alterations to ghrelin and glucagon-like peptide-1 (GLP1) which stimulate hunger and potentially increase food intake balancing out the energy intake/expenditure axis. In obesity insulin- and leptin-resistance in the brain reduces the ability to suppress appetite, further driving increased caloric intake (10). These compensatory mechanisms make it difficult to maintain weight loss.
Figure 1.1 Energy balance in humans. Energy balance is controlled by the relationship between energy input (calories consumed) and energy output (energy expended). Obesity results from a chronic imbalance of increased energy intake above that of energy expenditure. White adipose tissue, with the use of intracellular triglycerides, is able to store this excess energy intake. Increased physical activity, cold induced thermogenesis, diet induced thermogenesis, and the body’s natural BMR are all energy intensive processes which can utilise these adipose stores in the post-state. Increased adipose tissue mass is associated with increased risk of Type-2 Diabetes Mellitus and cardiovascular diseases.

1.1.3 Obesity, a metabolic disease

1.1.3.1 Obesity prevalence
Obesity is a global health burden affecting an increasing number of people worldwide (11). As of 2022, approximately 650 million adult people and approximately 340 million children and adolescents (5-19 years) suffer from obesity worldwide (12). The World Health Organisation categorises obesity as a body mass index (BMI), an equation factoring of weight (Kg) per height\(^2\) (m\(^2\)), as being greater than or equal to 30 (12). Individuals with obesity have excess fat mass leading to increased incidence
metabolic diseases and risk of cardiovascular diseases and some cancers which severely impact life expectancy and quality. (11, 13) (14)

1.1.3.2 Current obesity treatments
Public health interventions to treat and reduce the increasing prevalence of obesity have been largely ineffectual. Lifestyle changes such as dietary caloric restriction and exercise, while effective over the short term, suffer from poor adherence and many individuals regain weight after intervention, often within 12 months post-intervention (13). There are several pharmacotherapies available globally which reduce caloric intake either through appetite suppression or reduced nutrient absorption. However, these pharmacotherapies often have uncomfortable side effects, including cardiovascular, mental health, and gastrointestinal issues and nausea, this has led to withdrawal of some drugs from world markets (15). There has been progress in recent years in improved pharmacotherapy for treating obesity, with effective treatments now available in many global markets such as the glucagon-like peptide-1 (GLP-1) receptor agonist semaglutide which significantly reduces appetite leading to weight loss of up to 15% body mass (16). There also additional drugs undergoing testing in clinical trials showing significant weight reductions in obese patients. These drugs are multi target and activate both GLP-1 and glucose-dependent insulintropic polypeptide (GIP), or target the aforementioned receptors and glucagon receptors (GCGR) (17, 18). Bariatric surgery can successfully treat obesity (19), although these procedures are invasive and can cause significant complications and are not suitable for many patients. Therefore, investigation into new treatments for obesity and T2DM are needed. The balance between energy intake and output is crucial in the development of obesity. However, to fully understand the impact of obesity on health and metabolic disease the biology of the adipocytes and other cell populations comprising adipose tissue must be considered.

1.1.3.3 Obesity health risks and associated conditions
Obesity is positively correlated with increased insulin resistance, hypertension, dyslipidaemia, together these factors significantly increase the risk of development
of T2DM (20, 21). Obesity induced insulin resistance in the liver and skeletal muscle increases the need for insulin production by pancreatic beta cells to attenuate the increased circulating glucose levels associated with obesity. Over a sustained period, this leads to reduced beta cell function and further insulin resistance which eventually leads to T2DM. There has been ample evidence to show that increased adiposity and obesity associated inflammation increase the risk of insulin resistance and T2DM (22-24). T2DM can be managed with appropriate treatment, however, if not well controlled it significantly increases the risks of CVD and macro and microvascular complications. The risks associated with T2DM include increased circulating levels of glucose and insulin, dyslipidaemia, and hypertension which increase CVD risk (25). Chronic inflammation and increased cell adipocyte apoptosis is frequently observed in adipose tissue during obesity and is associated with increased risk of atherosclerosis, insulin resistance, T2DM, and other metabolic diseases (26)(27). Adipose tissue morphology is affected by the sustained inflammatory state with increased levels of cytokines and chemokines, as well as infiltration by immune cells with macrophages forming crown-like structures around adipocytes (10). These crown-like structures occur to a greater extent in the visceral adipose depot (of both humans and mice) and are indicative of an adipocyte undergoing apoptosis. Taken together these obesity associated co-morbidities highlight the importance of managing and treating obesity in order to improve patient health and outcomes.
1.2 ADIPOSE TISSUE

In adult humans, white adipose tissue (WAT) provides storage capacity for excess caloric intake. WAT comprises a significant portion of human body mass, ranging from 8-19% in males (aged 20-40) and 21-32% in females (aged 20-40) in normal weight individuals, although these values increase with age and obesity (28, 29). Adipose tissue has an important role in metabolic health and endocrine function, acting as an energy storage organ for times of caloric deficit (30). There are numerous adipose tissue depots throughout the body and the adipocytes and cell types comprising them vary in both form and function (Figure 1. 2). Human and mouse adipose tissue share many similarities in both function and morphology, however, adipose depot distribution and location have some distinct differences (31). In humans WAT but is categorised into two broad types; subcutaneous WAT (scWAT) and visceral WAT (vWAT). Although these depots are present in mice their anatomical locations differ. In humans the scWAT are predominantly located in abdominal and gluteal/femoral, however in mice the scWAT (inguinal) depot is located in dorsolumbar region to the gluteal region. The vWAT locations in humans and mice can be found in the central abdominal region, however, in mice the gonadal depot comprises a proportionally greater mass of total vWAT (31).
Figure 1.2 Murine and human adipose tissue depots. In mice the largest brown adipose tissue (BAT) resides in the interscapular region. The inguinal (iWAT) depots in mice are comprised of beige adipocytes, whilst the gonadal, mesenteric, and peri-renal adipose depots are made up of white adipocytes. In adult humans BAT is located primarily in the cervical, supraclavicular, axillary, paravertebral and peri-renal regions. White adipose tissue depots in humans can be found abdominally in the visceral adipose tissue depot, subcutaneously, and in the gluteal-femoral regions.

1.2.1 White adipose tissue depots

1.2.1.1 Subcutaneous white adipose tissue

In humans, the scWAT depots are located beneath the dermal layer and, although widely distributed, the abdominal, gluteal, and femoral depots account for approximately 80% of total adipose tissue mass and has an important role in energy homeostasis through endocrine regulation of both leptin and adiponectin (32). Therefore, scWAT acts as the primary adipose depot for storage of excess energy intake in the form of lipids (33, 34). scWAT distribution throughout the body is highly
heritable (35). In mice, the subcutaneous region is found primarily in the inguinal and anterior depots, and function similarly to humans in regards to energy storage of caloric intake. However, the inguinal adipose tissue has a beige phenotype and can become active during cold exposure as some of the adipocytes resident in this depot contain Ucp1 (36).

1.2.1.2 Visceral white adipose tissue
The vWAT depot surrounds internal organs in the abdominal cavity, and can be subdivided into omental, mesenteric, and retroperitoneal depots. In lean healthy adults the visceral adipose tissue constitutes a relatively small proportion of total adipose tissue volume, however, vWAT mass increases substantially with obesity and is associated with poor metabolic and cardiovascular health outcomes (37). VWAT also has endocrine function and releases similar secretory factors to scWAT (38).

1.2.1.3 Bone marrow adipose tissue
Bone marrow adipose tissue (BMAT) is a dynamic tissue which comprises adipocytes resident in the bone marrow of the skeleton, but its function and regulation remain relatively unclear, although evidence suggest it supports haematopoiesis and bone function (39). BMAT can account for up to 70% of bone marrow content and approximately 10% of total adipose tissue mass (40). BMAT consists of two subgroups: constitutive and regulated, constitutive bone marrow adipocytes appear as a contiguous group at distal sites within the bone, whilst regulated bone marrow adipocytes are interspersed throughout the bone marrow (41). BMAT is also known to have endocrine function and secretes both leptin and adiponectin (39). BMAT mass increases with obesity, aging, and T2DM similar to WAT. However, BMAT has uncharacteristic properties compared to classical adipose tissue, during periods of caloric restriction BMAT mass increases, the mechanism driving this is currently unknown (42). BMAT also has a role in glucose homeostasis and during basal conditions has proportionally greater glucose uptake than WAT and the axial skeleton, whilst also being resistant to insulin stimulated glucose uptake (43). Whilst
the function and mechanisms regulating BMAT are still being elucidated, the data indicate it is an adipose tissue with metabolic importance and highlights the diversity of different adipose tissue sub-groups.

1.2.2 Obesity and adipose tissue

Obesity occurs when energy intake chronically outweighs energy expenditure, where the excess energy is then stored as lipids in adipose tissue. Although basic in principal, there are many other factors that contribute to this energy balance equation including lifestyle, hormonal, genetic and epigenetic factors (44). As obesity develops adipocytes can increase in size (hypertrophy) as a result of storing extra lipid but recruitment of new adipocytes (hyperplasia) from pre-adipocyte progenitors also occurs in scWAT and vWAT depots (44-46). Although both types of classical WAT share similarities they have distinct metabolic health effects dependant on their distribution and size. Increased scWAT and vWAT mass is positively associated with cardiovascular disease (CVD), hypertension, and other metabolic disorders such as dyslipidaemia and type 2 diabetes mellitus (T2DM) (47) (20, 21). Whereas, adipose tissue stored in the lower body, such as the gluteo-femoral depot, is associated with a protective effect of these metabolic risks (48). WAT distribution is affected by biological sex, generally females have greater adiposity than men. In females this adipose tissue is mainly stored in the lower body fat depots, whilst males will more often store fat in the abdominal scWAT and vWAT depots (49). This partially explains the increased risk of CVD and metabolic disorders in men. Whilst complete understanding of the mechanisms that drive this sexual dimorphism is not fully known sex hormones are believed to play an important role, as evidenced by fat deposition characteristics during puberty and in post-menopausal women where adiposity distribution changes. These data suggest the importance of oestrogen in fat distribution (49).

1.2.3 White adipocyte physiology and regulation

1.2.3.1 White adipocyte function

Adipose tissue stores excess energy in the form of triglycerides in lipid droplets. In the white adipocyte this lipid droplet is unilocular and comprises the majority of the
adipocyte, the lipid droplet will grow and shrink depending on metabolic requirements (Figure 1.3) (50). Lipogenesis refers to FA synthesis from acetyl CoA or the generation of triglycerides from FAs and glycerol, both of which are key stages of adipocyte lipid storage. Lipogenesis occurs following feeding where free fatty acids (FFAs) are sequestered into the fat cell following lipoprotein lipase (LPL) activity on circulating lipoproteins. The lipoproteins are generated from dietary lipids forming chylomicrons produced from the small intestine and very low density lipoproteins (VLDLs) synthesized by the liver (51). LPL is secreted from adipocytes, and skeletal and cardiac myocytes (52). LPL hydrolyses lipoproteins in the lumen of neighbouring capillaries to generate FFAs which are then transported into the cells via fatty acid transport protein (FATP), fatty acid binding proteins (FABP), and cluster of differentiation 36 (CD36) (53-55). When in the adipocyte following intracellular sequestration, FAs are re-esterified through the action of diacylglycerol acyltransferase (DGAT) to form intracellular triglycerides (56). In order for this process to occur, glucose must also be metabolised by the adipocyte to synthesize the glycerol backbone for the triglyceride (57). These processes are fuelled by dietary intake of carbohydrates and lipids, however, lipogenesis can also occur through de novo lipogenesis. When glucose levels are increased, for example following a high carbohydrate meal, there are increased acetyl-coenzyme A (acetyl-CoA) levels due to increased glucose oxidation (58). The FA palmitate can then be synthesized from acetyl-CoA via acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS), which can then be converted to other FAs (59). These newly generated FAs and the glycerol generated from glucose metabolism lead to the production of triglycerides completing de novo lipogenesis. De novo lipogenesis occurs both in adipose tissue and in the liver (60).

Conversely to lipogenesis, lipolysis is the breakdown of intracellular triglycerides into their constituent glycerol and FAs for use as metabolic substrates when energy requirements are increased due to increased energy expenditure during exercise, cold exposure, or due to lack of availability of substrates following the post-absorptive state (61). When a triglyceride is hydrolysed through the action of
adipocyte triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoacylglycerol lipase (MGL) a total of three FAs and a glycerol are generated as the end products (62). These metabolites can then be used for metabolic processes in different tissues other than adipose such as FAs used by skeletal muscle for oxidative phosphorylation (61), or glycerol being used for gluconeogenesis in the liver (63). Lipolysis is controlled by a signalling cascade under control of the sympathetic nervous system, this is initiated when nor-adrenaline (NADR) is released from innervating neurons which activate the β-adrenergic receptors (64). In white adipocytes β-3 adrenergic receptor (β3-AR) activation is primarily responsible for inducing lipolysis although β1-AR and β2-AR also contribute (65).

**Figure 1. 3 Lipogenesis and lipolysis in adipocytes.** Lipoprotein lipase (LPL) secreted from the adipocyte converts circulating lipoproteins (LP) into fatty acids (FAs) which are then sequestered by the adipocyte through transport through a combination of fatty acid transport protein (FATP), fatty acid binding proteins (FABP), and cluster of differentiation 36 (CD36). Circulating glucose is also transported into the adipocyte via both GLUT1 and GLUT4, from here glucose is oxidised through the glycolytic pathway.
pathway to form pyruvate which can be converted to acetyl-CoA. Acetyl-CoA can be converted into the FA palmitate through the action of acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS). Glucose can also be metabolised to produce glycerol. The FA pool generated in conjunction with the glycerol is converted into triglycerides (TRIG) via diacylglycerol acyltransferase (DGAT), the TRIGs are then stored in a lipid droplet in the cell. During lipolysis, noradrenaline (NADR) activates the beta 1/2/3 adrenergic receptors (β1/2/3-AR) which stimulate adenylyl cyclase (AC), converting adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The increased cAMP activates protein kinase A (PKA). PKA will then induce lipolysis of internal TRIG stored both directly and indirectly through activation of triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoacylglycerol lipase (MGL). The resulting products of this reaction are FAs and glycerol which are then transported out of the cell and used for metabolic processes.

Activation of β3-AR then activates adenylyl cyclase (AC), converting adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The increased cAMP activates protein kinase A (PKA) which stimulates lipolysis through ATGL and HSL which act upon the intracellular triglycerides (61). PKA activates HSL by direct phosphorylation, however, it indirectly activates ATGL via phosphorylation of perilipin 1 (PLIN1) which in turn releases CGI-58 and subsequently activating ATGL (66, 67). Additional methods of inducing lipolysis distinct from β-adrenergic receptors have been described, including natriuretic peptides, growth hormone, glucocorticoids, and tumour necrosis factor alpha (68). The mechanisms controlling these non-adrenergic pathways are not completely defined but could possibly be distinct from AC/cAMP/PKA signalling as they have been observed to have a synergistic increase in lipolysis during adrenergic stimulation (68). Insulin has an important role in regulating both lipolysis and lipogenesis. During the fed state circulating insulin levels are increased in response to food intake. Circulating insulin induces lipogenesis by activating LPL on the capillary endothelium adjacent to adipocytes stimulating the uptake of FAs into adipocytes. Additionally, insulin lowers cAMP production through increased phosphodiesterase 3 (PDE3B) activity, this
lowering of cAMP activity significantly blunts whole body lipolysis (68, 69). Insulin also increases the TORC1-Egr1 signalling pathway which inhibits ATGL, thereby suppressing lipolysis (70). During fasted periods both circulating glucose and insulin will significantly decrease, this is followed by an increase in NADR levels and whole-body lipolysis (71). These processes control the availability of FAs for metabolic processes and are vital in the regulation of energy expenditure during exercise or cold induced thermogenesis (CIT).

1.2.3.2 Adipocytes and endocrine function
Adipocytes also function as an endocrine organ producing hormones (often referred to as adipokines when originating from adipose tissue), as well as other signalling molecules such as cytokines, microRNAs, and other small signalling molecules (38, 72). Although there are numerous factors released from adipose tissue that have both local and systemic effects on metabolic control or immune function, there are two adipokines that are particularly well studied in the context of obesity and metabolic health, leptin (LEP) and adiponectin (ADIPOQ).

Leptin was first identified in mice colonies housed at Jackson laboratories, some mice would become spontaneously obese and were characterised as ob/ob mice. Further experimentation of these mice discovered the adipokine leptin as being responsible for this weight gain (73). Leptin is released from adipocytes where it then acts upon its cognate leptin receptor (LEPR), to regulate homeostatic control of adipose tissue mass through modulation of food intake and energy expenditure (74). Leptin deficiency induced hyperphagia, decreased basal metabolic rate, increased insulin resistance, and reduced body temperature (75, 76). Adiponectin is another key adipokine which acts as an insulin sensitising agent as well as regulating glucose homeostasis and lipid catabolism (77). Adiponectin binds to one of two adiponectin receptors on the cell surface (ADIPOR1 and ADIPOR2), this activates a signalling cascade involving AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor-α (PPARα) and a number of other regulatory proteins which
decrease liver gluconeogenesis, whilst increasing both skeletal muscle and WAT glucose uptake and simultaneously increasing FA oxidation in skeletal muscle (77, 78). These data highlight the importance of adipose tissue in the regulation of energy homeostasis and how WAT function extends beyond that of energy storage.
1.3 BROWN ADIPOSE TISSUE

1.3.1 Brown adipose tissue

Brown adipose tissue (BAT) is another type of adipose tissue distinct from WAT, the primary function of which is to generate heat during cold induced thermogenesis (CIT) (79). During this process BAT increases energy expenditure (80) and sequesters metabolites from the circulation, such as glucose and FAs, as well as hydrolysing intracellular triglyceride stores (81). These properties have caused significant research into BAT activation as a potential treatment for obesity and associated metabolic disease. Individuals with higher levels of BAT are protected from impaired cardiometabolic health, an effect seen in both lean and obese individuals (82). However, the mechanisms controlling BAT function have not been fully elucidated and further insights into its regulation are needed.

1.3.2 Brown adipocyte physiology

Brown adipocytes are distinct from white adipocytes and are characterised by their high number of mitochondria giving them a brown appearance (from which they derive their name) as well as many multilocular lipid droplets within the adipocytes (Figure 1. 4). Brown adipocytes are stimulated during cold exposure, which induces sympathetic activation of β3-adrenergic receptors (β3-AR, and possibly β1-AR and β2-AR (83, 84)). This induces intracellular lipolysis of intracellular triglyceride stores in an AC/ cAMP/ PKA dependant manner. FAs released from intracellular lipolysis then activate the inner mitochondrial membrane (IMM) transporter uncoupling protein 1 (UCP1) (85, 86). UCP1 transports protons generated from the electron transport chain across the IMM in a process uncoupled from ATP synthesis, generating heat for non-shivering thermogenesis (Figure 1. 5) (87). In rodents, two distinct types of thermogenic adipose tissue have been identified, classical BAT and beige. Classical BAT is found mainly in the interscapular region and is derived from myogenic factor 5 (myf5)-positive precursors (88) whilst beige fat is found primarily in the inguinal depot and is derived from different progenitor cells (reviewed in (89)). Beige adipocytes are recruited in response to cold or β-adrenergic stimulation (90), express UCP1 (although levels are substantially lower than in BAT) (90) and
contribute to thermogenesis (91, 92). Activation of the β3-AR induces BAT thermogenesis and administration of β3-agonists induces weight loss and improves hyperglycaemia in rodents (93).

Until recently BAT was thought to be found only in human infants. However, BAT has been identified in the cervical, supraclavicular, axillary, paraspinal, and perirenal regions of adults (Figure 1.2) (94-96) (97). Human BAT is also thermogenic in nature whilst having comparable UCP1 function to rodent BAT (98). Interestingly, the molecular signature of human BAT shares similarities with both rodent classical BAT and beige adipose tissue (99).

**Figure 1.4 Schematic diagram of differences between brown, beige, and white adipocytes.** Brown adipocytes are characterised by their high number of mitochondria and multilocular lipid droplets, these adipocytes have high expression of UCP1 and produce heat during thermogenesis. Conversely white adipocytes are unilocular and have a low number of mitochondria. Beige adipocytes have a moderate number of mitochondria (which express UCP1) and can produce heat.
during thermogenesis. Beige adipocytes contain multilocular lipid droplets but are larger than those found in brown adipocytes.

**Figure 1. 5 Brown adipocyte thermogenic pathway.** Sympathetic neurons release noradrenaline (NADR) from the synapse. NADR then binds to β-adrenergic receptors (β-AR) on the cell surface activating adenylyl cyclase (AC), converting ATP to cyclic adenosine monophosphate (cAMP). cAMP stimulates protein kinase A (PKA) activity triggering lipolysis of triglyceride stores and release of fatty acids (FA). FAs bind and activate UCP1 in the mitochondria. UCP1 generates heat via transport of protons (H+) across the inner mitochondrial member using the electron transport chain to facilitate this. This process uncouples respiration from ATP synthase. Uptake of circulating free fatty acids (FFA) and glucose contribute to the regeneration of intracellular triglyceride stores. FFAs are transported into the cell by fatty acid transport protein (FATP, fatty acid binding protein (FABP), and cluster of differentiation 36 (CD36). Glucose is transported into the cell via the glucose transporters GLUT1 and 4. Glucose also contribute to brown adipocyte function by being used in glycolysis and feeding into the TCA cycle to produce ATP. C1–4, complex 1–4; CoQ, co-enzyme Q; Cyto C, cytochrome C; e−, electron. Adapted from McNeill et al., 2021 (80).
1.3.3 Brown adipose tissue function and identification in humans

The primary function of BAT is to generate heat to maintain body temperature through non-shivering thermogenesis (NST) during cold exposure as a part of the wider CIT response (Figure 1. 6) (100). $^{18}$F-fluoro-deoxyglucose ($^{18}$F-FDG) positron emission tomography coupled with computed tomography (PET/CT) is a diagnostic tool used to identify malignancies, however, this technology led to the (re)-discovery of BAT in adult humans (101, 102). $^{18}$F-FDG is a radio-labelled analogue of glucose, where following injection into a subject PET/CT scans of the body and can be used to assess glucose uptake and glycolysis, and therefore detects metabolically active tissue (103). Identification of adult human BAT with the use of $^{18}$F-FDG has substantially increased interest in activating BAT to treat obesity (104). BAT activation in rodents substantially increases energy expenditure and improves other metabolic health parameters including insulin sensitivity (105) and dyslipidaemia (106), both of which are commonly associated of obesity and diabetes. Following the identification of BAT in adults, healthy volunteer studies confirmed that cold exposure substantially increased $^{18}$F-FDG uptake by BAT (96, 97, 107) and $^{18}$F-FDG-PET/CT has become the most commonly used technique to quantify BAT mass and activity (108). BAT remains metabolically active during warm conditions, with increased glucose and fatty acid uptake compared to WAT (109, 110). Review of historically performed clinical $^{18}$F-FDG-PET/CT scans performed at room temperature (for purposes distinct from identifying BAT) demonstrated that ~5% of individuals had detectable $^{18}$F-FDG uptake by BAT (111). In studies using cold exposure to activate BAT, the prevalence is as high as 95% in young healthy men (96). BAT mass is substantially lower than WAT mass (~16-22 Kg) even in normal weight adults (112, 113). The quantity of detectable BAT in humans ranges from ~10-300 grams (97, 114-116). Another technique which can be used to quantify BAT activity is $^{11}$C-acetate PET. Although similar in principal to $^{18}$F-FDG PET/CT, $^{11}$C-acetate PET measures oxidation as acetate is quickly metabolised in the body and converted into acetyl-CoA where the labelled carbon is detected with PET (117). Using $^{11}$C-acetate PET to measure BAT oxidative activity, has not revealed substantial BAT depots without
substantial glucose uptake (118) indicating that $^{18}$F-FDG-PET/CT estimates of BAT mass may be accurate.

Figure 1. 6 Organs involved in cold induced thermogenesis. Cold exposure stimulates lipolysis in WAT (2), releasing FAs into the circulation for utilisation by both BAT (1) and skeletal muscle (3) (grey arrows). BAT sequesters circulating FAs and glucose during thermogenesis. FAs released from internal triglyceride stores in BAT are used as the fuel for non-shivering thermogenesis (orange arrow). Skeletal muscle shivering accounts for the largest proportion of whole-body heat production, glucose and FA uptake during cold-induced thermogenesis (CIT) (pink arrows). Muscles that contribute substantially to shivering thermogenesis include the longus colli, sternocleidomastoid, pectoralis major, and the rectus femoris included in the diagram Adapted from McNeill et al., 2021 (80)
1.3.4 Brown adipose tissue activation and activators

During CIT, sympathetic neurons innervating BAT are stimulated and release NADR that activates β-adrenergic receptors (84, 86, 119, 120), which triggers a signalling cascade that results in the hydrolysis of local triglycerides, releasing fatty acids which then activate UCP1 (Figure 1. 5). In order for BAT to be a therapeutic target for treating obesity, understanding the mechanisms that regulate activation are key.

1.3.4.1 Cold induced BAT activation

Cold exposure is a common method utilised to activate BAT, in humans this is achieved either by reducing ambient room temperature (often to ~16-19°C) (84, 86, 119, 120) or using a wearable cold water infused cover (118, 121). Both methods elicit similar levels of BAT activation at least as measured by glucose uptake, however, it should be noted that the use of the water-cooled suits typically increase total energy expenditure to a far greater extent compared to ambient cooling (80, 109, 118, 122, 123). Repeated chronic cold exposure across a 6-week period increased BAT mass, activity, and energy expenditure (during cold exposure), whilst decreasing total fat mass (114, 124, 125). Using cold exposure techniques are time consuming (and possibly uncomfortable for patients), these studies reveal that short term cold exposure improves cardiometabolic markers and decreases fat mass in humans without the need for pharmacotherapy. Therefore, increasing BAT activation as part of CIT has therapeutic potential.

1.3.4.2 Pharmacological adrenergic BAT activation

Pharmacological activation of BAT is an attractive option as this would be a more comfortable and easier method compared to cold exposure protocols. Due to the low mass of BAT found in many adult humans, the optimal pharmacological activator of BAT would both expand total BAT mass while simultaneously increasing energy expenditure. Further, a subject must have sufficient BAT mass to effectively respond to activation. This is particularly critical in target patient groups (such as obese
subjects with type 2 diabetes who may be older) who typically have very little BAT (82, 126). As BAT expansion and activation are both under sympathetic regulation (127, 128), substantial research in this area has focused on the effect of sympathetic agonists. However, whilst the use of sympathetic agonists (such as mirabegron) increase EE and BAT activity there are several side effects (tachycardia and hypertension) that make their prolonged use dangerous (129), especially in the target group for obesity and metabolic disease treatment. Therefore, it is essential to determine the key pathways regulating BAT activation to assess the potential as a safe therapeutic tool for treating obesity and associated metabolic diseases.

Activation of the β3-AR induces BAT thermogenesis and administration of β3-agonists induces weight loss and improves hyperglycaemia in rodents (93). In humans, β3-agonist administration for 4-8 weeks improved insulin and lipid sensitivity indicating improvements in metabolic health, although body weight was unchanged (130, 131). A single dose of the β3-AR agonist mirabegron administered to healthy men, between ages 18 and 65 years with BMI between 18 and 40, increased $^{18}$F-FDG uptake in BAT and increased energy expenditure by ~200kcal/24 hours (during warm conditions) (119). Daily mirabegron use (for 4 weeks) in healthy women increased BAT mass/volume, and energy expenditure (129). However, there was no effect on body weight, but mirabegron did improved insulin sensitivity and increased HDL-C in these subjects (129). These data highlight that β3-AR agonism effectively activates BAT and can improve the metabolic profile of subject following chronic treatment. However, as mentioned above long term use would not be feasible, as such more study is needed to determine the benefit/cost ratio of using these drugs for BAT activation therapeutically or if more optimal treatment can be developed.

1.3.4.3 Other brown adipose tissue activators

In addition to sympathetic activation of BAT (either through cold exposure or β3 agonists) there are other drugs and compounds that increase BAT thermogenesis. In
rodents, bile acids increase BAT thermogenesis, and induce browning of beige adipose tissue, through the G-protein-coupled bile acid receptor (TGR5) and the cyclic-AMP-dependent thyroid hormone activating enzyme type 2 iodothyronine deiodinase (132-134). In humans, administration of the bile acid chenodeoxycholic acid for 2 days increased BAT activation and whole body EE in vivo and also increased mitochondrial uncoupling in human brown adipocytes through TRG5 activation (135).

Capsaicin and capsinoids are molecules naturally present in chilli peppers and their food derivatives, these molecules are agonists of the transient receptor potential vanilloid type 1 receptor (136). In humans, acute ingestion of capsinoids increased whole body energy expenditure in subjects with previously detectable BAT, an effect not seen in subjects lacking BAT (137). Chronic capsinoid supplementation increased thermogenesis in cold exposed healthy subjects (125) and increased resting energy expenditure in overweight subjects (138). Similarly, in rodents, capsinoids stimulate sympathetic activation of BAT and increase UCP1 expression in both BAT and WAT (136, 139).

In rodents, acute and chronic glucocorticoid excess decreases UCP1 expression and reduces BAT thermogenesis. However, in humans, acute glucocorticoid excess increases UCP1 and oxygen consumption in vitro and increases BAT glucose uptake, heat production and CIT in vivo (140-142). Although, following one week of glucocorticoid treatment, there were no significant differences in CIT energy expenditure compared to controls (in healthy male subjects) (143). Additionally, chronic glucocorticoid excess reduces BAT function (141, 144). However these data indicate, in the acute setting, that glucocorticoids have species specific differences in the regulation of human and murine BAT but are not appropriate for human use.
Overall, these data indicate that BAT can be activated through pathways distinct from sympathetic activation. Currently these methods are not suitable to chronically activate BAT, therefore, further study into mechanisms controlling BAT activation is needed if it is to be a therapeutic target for obesity.

1.3.5 Brown adipose tissue substrate utilisation

BAT is a metabolically active organ and during its activation (either via cold exposure or adrenergic stimulation) a variety of substrates are utilised to fuel this process, primarily intracellular triglycerides, glucose, and circulating fatty acids are used (Figure 1. 7). However, there are many other metabolites used including BCAAs (145), lactate, glutamate (109), and succinate (146).

1.3.5.1 Use of intracellular triglycerides

Data in both rodents and humans suggest that intracellular triglycerides are the primary fuel source for NST and BAT activity. Inhibition of lipolysis both in vivo and in vitro substantially inhibits rodent BAT activity (147, 148). Both global (149) and adipose specific ATGL knockout mouse models had impaired cold adaptation and thermogenesis, additionally, BAT took on a more WAT-like phenotype in this knockout. These data indicate that lipolysis of intracellular triglyceride stores are crucial for BAT function. Similar effects are also observed in humans whereby inhibition of lipolysis reduced BAT thermogenesis (150). Furthermore, quantification of BAT lipolysis was undertaken by measuring glycerol release from BAT using microdialysis in healthy lean men, this was substantially increased during cold exposure compared to WAT (109). Approximately 65 nmol/g/min of FA was estimated to release during BAT activation, potentially accounting for triglyceride usage in BAT thermogenesis (109). Due to glycerol recycling in BAT, lipolysis measurements may be inaccurate and underestimate total lipolysis. Glycerol kinase is highly expressed in rodent (147) and human BAT (109, 151), this enzyme converts glycerol released during lipolysis of triglyceride stores to glycerol-3-phosphate, subsequently this can be used as a part lipogenesis to replenish triglyceride stores.
During thermogenesis intracellular triglyceride stores are depleted, as demonstrated by both the reduced fat fraction when using MRI (152, 153) and increased radio-density of BAT observed when using CT scanning during cold exposure (118, 122), these data indicate a reduced lipid content in the cells following activation.

Fat fraction in BAT is also associated with metabolic health, individuals with higher BAT fat fraction have increased insulin resistance, these data suggest a role for BAT activity in glucose homeostasis and metabolic diseases such as T2DM (154). Additionally, age causes higher fat fraction in BAT compared with younger matched controls during both warm and cold conditions (121). Together these data show that triglyceride stores are depleted during BAT activation, although this effect is reduced in metabolically unhealthy individuals possibly due to reduced BAT function.

Figure 1. 7 Substrate utilisation and pathways in brown adipocytes. Glucose enters the brown adipocyte via GLUT4 and GLUT1 where it undergoes glycolysis to form...
pyruvate. Pyruvate is converted to lactate and exported from the cell by the monocarboxylate (MCT) transporters, this pathway accounts for the majority of glucose uptake by human BAT during both warm and cold exposure. Exported lactate may activate GPR81, inhibiting lipolysis. Pyruvate can also enter the mitochondria where it is converted to acetyl-CoA and incorporated into the TCA cycle. The TCA metabolite citrate (when exported out of the mitochondria) may also be converted to acetyl-CoA in the cytosol where it then is used as the first step of de novo lipogenesis (DNL). A proportion of glucose may be converted to glycerol-3-phosphate (Glycerol-3-P), generated from glyceraldehyde-3-phosphate during glycolysis. Glycerol-3-P then forms the backbone needed for replenishment of intracellular triglyceride stores (TRIGs). Fatty acids (FAs) hydrolysed from local TRIGs are the primary energy substrate used for uncoupled respiration during thermogenesis, which is mediated by mitochondrial UCP1. Glycerol released by this process can either be exported or recycled through conversion to Glycerol-3-P by glycerol kinase (GK) for subsequent TRIG synthesis. In addition to DNL, uptake of circulating FFAs either directly or following lipoprotein lipase (LPL)-mediated hydrolysis of triglyceride rich lipoproteins (TRLs) occurs via the fatty acid transporters or potentially by passive diffusion. BAT utilizes other circulating substrates during thermogenesis such as BCAAs, succinate and glutamate which are all likely incorporated into the TCA cycle. Arrows in red represent enzymatic conversion. Arrows in black indicate substrate transport/movement. Wording in blue indicate pathways confirmed in rodent BAT. Wording in black indicate pathway confirmed both in human and rodent BAT. Some reactions have been simplified/omitted for brevity. ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; AGPAT2, 1-acyl-sn-glycerol-3-phosphate acyltransferase beta; AQP7, aquaporin-7; ATGL, adipose triglyceride lipase; CD36, fatty acid translocase; CoQ, coenzyme Q; DGAT2, diacylglycerol acyltransferase 2; FABP, fatty acid binding protein; FASN, fatty acid synthase; FATP, fatty acid transport protein; GPAT3, glycerol-3-phosphate acyltransferase 3; GPR81, G-protein coupled receptor 81; HSL, hormone sensitive lipase; LDH, lactate dehydrogenase; MPC1/2, mitochondrial pyruvate carrier 1 and 2; PDH, pyruvate dehydrogenase; SLC13A3, solute carrier family 13 member 3; SLC25A10, solute carrier family 25 member 10; SLC25A44, solute carrier family 25 member 44. Adapted from McNeill et al., 2020 (81)
1.3.5.2 The role of glucose uptake by BAT

Glucose uptake increases during cold exposure in healthy subjects, suggesting an important role of glucose uptake in normal BAT function (97, 109, 121, 122). During cold exposure, glucose uptake by BAT is greater than skeletal muscle per gram of tissue but due to the low mass in adults BAT accounts for <1% of total body glucose uptake during thermogenesis, compared with ~50% by skeletal muscle (150). Repeated cold exposure can further increase glucose uptake by BAT and increase BAT oxidative metabolism and cold-induced thermogenesis (114, 122). This may be due to increased BAT mass or activation of previously dormant BAT (155, 156). However, this is not observed in all studies and comparisons are often confounded by substantial differences in protocol design in these studies. Some cooling protocols will drastically reduce body temperature necessitating skeletal muscle shivering which accounts for the majority of cold-induced thermogenesis (157, 158).

However, evidence suggests that glucose uptake, mediated by glucose transporters GLUT1 and GLUT4, into brown adipocytes is key for optimal function, where the sequestered glucose is used for glycolysis. In mice, both shRNA knockdown of GLUT1 in interscapular BAT and inhibition of glycolysis via the injection of 2-deoxy-D-glucose inhibited in vivo optogenetic stimulation of sympathetic nerve activation of non-shivering thermogenesis (159), highlighting the importance of glucose uptake in rodent BAT function. In mice, during cold exposure, glucose was shown to be broken down as part of glycolysis and enriched both glycolytic intermediates (lactate, pyruvate) and TCA cycle intermediates (succinate, fumarate, citrate, malate, α-ketoglutarate) (160). This was achieved using U\textsuperscript{13}C-glucose. Additional, U\textsuperscript{13}C-glucose tracer experiments in mice revealed that glucose is broken down into pyruvate and TCA cycle intermediates and knockout of the mitochondrial pyruvate transporter, MPC1, led to significantly reduced labelling of TCA cycle metabolites and impaired adaptation to cold tolerance (161). These data highlight the importance of glucose as substrate for efficient BAT function and that providing pyruvate for subsequent use in the TCA cycle is also key. In humans, glucocorticoid treatment increased \textsuperscript{18}FDG uptake by BAT in vivo, whilst increasing GLUT4 mRNA expression in differentiated
human brown adipocytes (141). Further, inhibiting lipolysis in BAT substantially decreases glucose uptake (150). Glucose may also contribute to the replenishment of intracellular triglyceride stores via conversion of glyceraldehyde-3-phosphate to glycerol-3-phosphate which subsequently forms the glycerol backbone during triglyceride synthesis. As discussed in the previous section intracellular triglyceride stores are the primary fuel source for BAT function, therefore, glycerol recycling paired with glucose uptake likely contribute to optimal BAT function. Supporting this, $^{13}$C-glucose tracing experiments in immortalised murine brown adipocytes (stimulated by a £3-AR agonist) revealed that a proportion of glycerol in triglycerides is derived from glucose sequestered from the cell culture media (162).

1.3.5.3 Fatty acid uptake by BAT

BAT lipid content acutely decreases following cold activation, although during prolonged cold exposure there is no further decrease, likely due replenishment of triglyceride stores (118, 163). As previously discussed, both glycerol recycling and glucose uptake contribute to this process, however, uptake of FAs from the circulation likely contribute to this process. Use of the FA PET tracer $^{18}$fluoro-6-thia-heptadecanoic acid ($^{18}$FTHA) confirmed uptake of FFAs by BAT during cold exposure (118), which coincides with increased whole body lipolysis, and therefore increased circulating fatty acid substrates (109, 164). However, due to the limitations of $^{18}$FTHA, the fate of these FAs following uptake by BAT is currently unclear, therefore it is unknown if FAs taken up by BAT are oxidised or incorporated into triglyceride stores.

As discussed in section 1.2.3.1, uptake of FAs from circulating TRLs occurs through the action of LPL (165). In rodents, LPL-mediated TRL uptake by BAT accounts for approximately 50% of systemic TRL clearance during cold exposure (106, 166, 167). In humans, cold exposure increases LPL expression BAT, indicating a potential role in systemic triglyceride clearance (164). When orally ingested as part of a lipid meal during cold exposure, there was detectable uptake of $^{18}$FTHA in BAT showing that BAT sequesters dietary FAs in humans (168). However, this post prandial uptake of
18FTHA was not increased following chronic cold exposure despite substantial increase in BAT activity. Differences in FA clearance between species may in part be due to lower proportional BAT mass in humans. Although, the exact fate of sequestered FAs remains unclear and further work is warranted to understand these processes in BAT. However, it remains that chronic activation of BAT in humans may induce a more favourable lipid profile which may reduce cardiovascular risk.

1.3.6 Brown adipose tissue as therapy for obesity in humans
Caloric expenditure, as part of CIT induced by ambient air cooling (to 17°C), can increase by ~250-300 kcal/24 hours and CIT is higher in subjects with greater BAT mass in some (125, 169), but not all studies (158). This increase in energy expenditure indicates that BAT activation and CIT are a possible means to treat obesity. Although BAT only contributes a small sum of the total energy expenditure increase during CIT, this may still be sufficient to improve weight loss outcomes in patients. To put this into context increasing energy expenditure by 50-60 kcal/ day for one year would result in weight loss of ~2. 5kg (170). However, the use of 15O2-PET (to measure oxygen consumption) during cold exposure, indicated that BAT accounts for a very small proportion of total energy expenditure even in those with large BAT mass, <20 kcal/24 hours (158, 171). These data suggest significant obstacles will need to be overcome for BAT to be an effective therapeutic target for treating obesity. However, activation of BAT through repeated cold exposure for several hours per day for up to 6 weeks increased BAT mass/activity (as measured using 18F-FDG), CIT and decreased fat mass (114, 124, 125). There is substantial data that BAT mass can expand or regress in response to different stimuli. For example, in colder climates greater BAT mass is found in individuals who work outdoors compared with indoor workers (172). Additionally, repeated cold exposure for approximately 7-10 days increased BAT glucose uptake, mass, and oxidative metabolism as well as increasing NST and wider CIT (114, 122). Cold acclimation has been observed in humans (tested during summer months) with repeated cold exposure, during this acclimation shivering steadily reduces from baseline levels (173). When subjects were tested during winter months there was already indications of reduced reliance on shivering thermogenesis.
Initially the mechanisms behind this acclimation and reduced reliance on shivering thermogenesis (generated by skeletal muscle) were unknown. However, as BAT has been shown to increase in mass and activity in both lean and obese individuals following repeated cold exposure (114, 122, 174), it is possible that BAT recruitment drives a reduction in the need for shivering thermogenesis by increasing the body’s capacity for NST, although BAT energy expenditure is still relatively small. However, it should be noted that NST does not solely rely on BAT function and both skeletal muscle and WAT are utilised with WAT providing a steady release of FAs to be used as fuels in this energy intensive process (157, 158).

Together, these data indicate that while energy expenditure from BAT is typically low in individuals, repeated activation leads to increased mass and energy expenditure. Therefore, expansion of BAT and subsequent activation could lead to sustained increased energy expenditure that could potentially treat obesity, although greater understanding of mechanisms controlling BAT function is required (104). As discussed in 1.1.3.2 there has been substantial progress recently with effective weight loss treatments particularly with regards to GLP-1 and GIP receptor agonists. However, weight re-gain following secession of treatment is a common occurrence in trial subjects. It is possible that co-treatment with BAT induced energy expenditure increase may prove to be an effective long-term treatment option for obesity and T2DM.

1.3.7 Murine models for investigating BAT regulation
1.3.7.1 Murine models for studying BAT physiology and function
Whilst substantial progress has been made in understanding the pathways that control and regulate BAT function there is still much unclear about BAT regulation. Although there are numerous in vivo studies that examine human BAT, these are limited in scope as to what can be tested due the difficulties involved in using invasive techniques on human subjects. To this end the mouse has been a common model for studying BAT. Human and mouse BAT share some similarities, including similar gene
expression profiles and physiology supporting the use of mice as models for studying BAT (98) (86, 175). Human and rodent BAT are both stimulated by sympathomimetics and cold exposure, and express high levels of UCP1 both at the protein and gene level (85, 98, 176). Additionally, in rodents repeated cold exposure leads to differentiation of new thermogenic beige adipocytes and is associated with improved metabolic health (46). As such studying browning of adipose tissue in rodents may elucidate novel mechanisms which could be applicable to human health.

Dysfunctional BAT has distinct effects on murine metabolic health, mice with genetically ablated BAT developed obesity (177). Whilst this represents the extreme phenotype of impaired BAT function there are multiple other models that are commonly used. Due to the ability to create both global and tissue specific knockout mice this allows for insight into detailed aspects of BAT function (178). As discussed in the previous section involving BAT substrate utilisation there have been numerous models used to elucidate the effects of specific genes of interest in BAT function, such as Atgl knockout mice which determined the importance of intracellular lipolysis. Therefore, mouse knockout models are a useful tool to investigate BAT function and its implications in metabolic health.

1.3.7.2 Considerations when comparing human and mouse models of BAT

However, it should be noted that there are some distinct differences in mouse and human BAT regulation. Mouse BAT makes up a proportionally larger component of body mass than in humans and during cold exposure contributes approximately 60% of total energy expenditure (179). The thermal neutral point of mice being between ~29°C in light phase and ~33°C in dark phase (180). In C57Bl/6 mouse models energy expenditure increases by 8% for every degree Celsius below thermoneutrality (181), although due to the range of thermoneutrality this value will vary. The thermoneutral temperature for humans is approximately 27°C when naked (182) but is approximately 22°C when lightly clothed (183). This means that when housing mice at room temperature during experimental conditions mice have comparatively
increased energy expenditure compared to humans. As such when making comparisons between human and mouse models or when interpreting energy expenditure data of mice housed at 21°C, it is important to take into consideration that these differences in energy expenditure could confound results.
Chapter 1

Introduction

1.4 SUCCINATE IN BROWN ADIPOSE TISSUE FUNCTION

1.4.1 Succinate

1.4.1.1 Succinate in the TCA cycle

Succinate is a dicarboxylic acid intermediate metabolite produced during the tricarboxylic acid (TCA) cycle (Figure 1. 8). The TCA cycle is responsible for the generation of ATP, a fundamental component of many cellular energetic processes (reviewed in (184)). ATP is hydrolysed into adenosine di-phosphate (ADP), during which energy is released fuelling multiple cellular processes. ATP is predominantly generated in TCA cycle, although it can be produced by other biochemical pathways such as glycolysis or through oxidation of ketone bodies by the mitochondria (184). The TCA cycle takes place in the mitochondria, where pyruvate (generated primarily through glycolysis but also other catabolic processes) is converted into Acetyl-CoA through the action of pyruvate dehydrogenase. Acetyl-CoA is then incorporated into the TCA cycle after the transfer of two acetyl groups to oxaloacetate to form citrate. Citrate, and the subsequent products of the downstream reactions in the TCA cycle generate ATP. Succinate is a key stage in this cycle and is generated from succinyl-CoA, before being oxidised by succinate dehydrogenase (SDH) to form fumarate. SDH forms complex II of the electron transport chain (184). Both succinate and SDH are key for cellular function but are involved in multiple cellular process outside of the TCA cycle (185).
Figure 1.8 The TCA cycle overview. Schematic diagram of the TCA cycle with key intermediates in blue boxes with key enzymes (green text 1-8) facilitating redox reactions to produce NADH for use in the electron transport chain. Succinate and succinate dehydrogenase are key steps in this metabolic process. Adapted from Creative Proteomics (186).
1.4.1.2 Succinate mitochondrial transport in cells

The mechanisms regulating succinate production during the TCA cycle are well characterised, however, transport of succinate throughout the cell and mitochondria remain unclear (Figure 1.9). Following synthesis, succinate can be exported from the inner mitochondrial membrane by the mitochondrial dicarboxylate carrier (DIC) transporter which is one of the solute carrier family 25 (SLC25) transporters (187) and is encoded by the gene SLC25A10. This family of transporters is one of the largest solute transporter families in humans with 53 members, and contain antiporter, uniporter, and symporter proteins (188). SLC25A10 has been shown to both import and export succinate indicating bi-directional transport across the mitochondrial membrane (189, 190). DIC is highly expressed in murine brown adipocytes (146). Succinate is then transported into the cytosol via voltage-dependent anion channels (VDACs) (187). Direct transport of succinate into the mitochondria is less clearly defined, however, succinate can accumulate in the inner mitochondrial space through import of other molecules, such as glutamine and γ-aminobutyric acid (GABA), leading to production of succinate. During glutamine-dependant anaplerosis, cytosolic glutamine is transported through the inner mitochondrial membrane via the SLC1A5 transporter (191). Amidohydrolase enzymes catalyze the conversion of glutamine into glutamate, which is subsequently converted into alpha-ketoglutarate (α-KG) by a number of enzymes including glutamate dehydrogenase 1, glutamic-pyruvic transaminase 2 and glutamic-oxaloacetic transaminase 2 (192). This increased α-KG pool is converted into succinate (by the action of α-KG dehydrogenase and succinyl CoA synthetase) as the next step of the TCA cycle. GABA transaminates with α-KG to form both glutamate and succinate semialdehyde through mitochondrial GABA transaminase. Succinate semialdehyde dehydrogenase can then convert succinate semialdehyde to succinate (193).
Figure 1. 9 Succinate transport across the plasma and mitochondrial membranes. Circulating succinate is sequestered into the cell via SLC13A3 and MCT1, the primary transporter has not been identified as inhibition of either transporter does not substantially impact total transport. These transporters are also able to release succinate from the cytosol into the interstitial fluid and circulation. Additionally, succinate produced in the mitochondria via the TCA cycle and succinate in the cytosol can be transported from the mitochondrial matrix (MM) across the mitochondrial membranes and into the cytosol. Intracellular succinate can be transported from the cytosol into the mitochondria and incorporated into the TCA cycle. However, many of the mechanisms regulating and facilitating this process are poorly understood.
1.4.1.3 Succinate plasma membrane transport in cells

Once in the cytosol, succinate can be transported out of the cell through monocarboxylate transporter 1 (MCT1) which is expressed in multiple tissues including BAT (194, 195). In skeletal muscle, succinate export occurred during acidification of the cytosol, induced by exercise in mice and humans. This cellular acidification caused protonation of succinate which facilitated its transport via MCT1 (195). During ischaemia of the heart, myocardial succinate concentrations increased ~2.5 fold (196, 197). During reperfusion, excess succinate is oxidised which drives substantial ROS production due to reverse electron transport (RET) in complex I of the electron transport chain, which leads to reperfusion injury (198). During these pathophysiological conditions myocardial acidification promotes efflux of succinate from the cytosol into the circulation via MCT1 to limit tissue damage (199). However, in BAT MCT1 has been shown to import succinate when in the monocarboxylic form (200). The suggested mechanism for this uptake is that adrenergic activation of the brown adipocyte causes cytosolic alkalinisation (relative to the interstitial fluid), and therefore transportation of monocarboxylate succinate is preferential and facilitated by MCT1. However, knockout of MCT1 in mice did not inhibit succinate uptake by BAT in vivo, suggesting there are compensatory mechanisms supporting brown adipocyte succinate transport, possibly through MCT4 or SLC13A3 (200). Various members of the SLC13 family of proteins are able to transport di- and tri-carboxylates (including succinate) into the cell, notably SLC13A2 and SLC13A3 (201). Despite being expressed in brown adipocytes SLC13A3 inhibition did not alter succinate uptake, however, as with MCT1, possible compensatory mechanisms likely facilitate continued succinate transport (200). MCT1 and 4 are also associated with regulating lactate levels in cells as well as succinate, and knockdown of MCT1 in brown adipocytes reduced adrenergic stimulated respiration, highlighting the importance of regulating intracellular concentrations of these metabolites (202).

1.4.2 Succinate in brown adipose tissue

During CIT, succinate concentrations increase in both mouse BAT and IAT, and to a lesser extent in eWAT. This increase is due to sequestration of circulating succinate,
which is then oxidised in the mitochondria, this was determined by injecting mice
with radiolabelled succinate and measuring concentrations of radiolabelled products
produced in the mitochondria of BAT (146). BAT succinate uptake was greater than
uptake of the other structurally similar mitochondrial dicarboxylic acids fumarate and
α-ketoglutarate, in keeping with selective uptake of succinate. The succinate
sequestered by BAT was shown to be released from shivering muscle, as treatment
with the nicotinic acetylcholine receptor inhibitor curare blunted cold dependent
accumulation of succinate in BAT (146). Muscle releases succinate during exercise
and cold exposure/shivering could lead to a similar process (195, 203). Supraphysiological succinate concentrations increase OCR in mature primary murine
brown adipocytes, immortalized mouse brown adipocytes, and immortalized human
brown adipocytes. This increased oxygen consumption is associated with increased
energy expenditure and ROS levels in brown adipocytes, and was attributable to
increased proton leak which is dependent on UCP1 and mitochondrial ROS (204). The
thermogenic effect of succinate was observed in isolated BAT mitochondria, where
the succinate transporter Slc25a10 was highly expressed and pharmacological
inhibition of this transporter blunted the succinate induced increase in oxygen
consumption. Inhibition of mitochondrial ROS production using either MitoQ (205),
and inhibition ROS dependent cysteine oxidation using N acetylcysteine both
significantly blunted succinate induced OCR increase (206). Additionally,
pharmacological inhibition of Na\(^+\)/K\(^+\) Adenosine-triphosphatase (catalyses
conversion of ATP to ADP) inhibited succinate stimulated respiration in brown
adipocytes indicating these transporters are necessary for succinate transport from
the circulation/interstitial fluid into the cell (146). The authors further showed that
superoxide production through succinate and SDH interactions are what led to this
increased ROS production. These data indicate that succinate drives thermogenesis
through transport of circulating succinate (likely derived from production in shivering
muscle) into the brown adipocyte and to the mitochondria where ROS drives
thermogenesis (146). However, succinate can also interact with its cognate receptor,
the succinate receptor (SUCNR1) (184). Succinate induced OCR increase was
preserved in brown adipocytes lacking SUCNR1. Neither the lack of SUCNR1 ligation,
elevation of cAMP levels, activation of PKA contributed to the succinate induced
increase in OCR. However, details of the model and method used to generate this knockout of SUCNR1 were unclear and the methods section did not specify whether this was in brown adipocytes taken from a Suncr1 knockout in vivo model or knockdown performed in an in vitro model. Additionally, the succinate doses used ranged from 1-10mM, significantly above the estimated EC$_{50}$ of SUCNR1 (27μM), and therefore, effects of SUCNR1 activation at more physiological concentrations could have been missed. In contrast to brown adipocytes, 1mM succinate reduces the OCR of human primary white adipocytes compared to basal conditions, although the mechanisms driving this are unclear but could indicate that brown and white adipocytes are under different regulation with regards to succinate treatment (146).

These data indicate an important role of succinate uptake and incorporation into the mitochondrial matrix during thermogenesis. However, despite substantial cold induced uptake of succinate into BAT, interestingly there is an overall net efflux of succinate from BAT during CIT in mice which was uncommon compared to other tested metabolites (207). Additionally succinate supplemented into mouse diet altered several genes associated with mitochondrial function and structure, and when these mice were given exogenous succinate in combination with liraglutide weight loss was increased compared to liraglutide alone (208). Succinate production is increased during hypoxic conditions and has been shown to act as a regulator of inflammation and redox stress (in conjunction with SDH) (209), it is therefore possible that intra and extracellular succinate concentrations are dynamic and act to both increase thermogenesis through increased ROS production but can be exported to prevent metabolic stress in the cell. The role that SUCNR1 plays in this is currently unclear.

### 1.4.3 Identification of SUCNR1 as a gene of interest in human brown adipocytes

Our group performed RNA sequencing of human paired primary brown and white adipocytes (210) to identify novel pathways regulating BAT activation and function. These data identified a possible important role of the succinate and its cell surface receptor SUCNR1, SUCNR1 expression was >12-fold higher in human primary brown adipocytes ($P < 5 \times 10^{-9}$, Figure 1. 10) which were confirmed by qPCR (Figure 1. 10).
**Figure 1.** Comparative RNA sequencing of human brown and white adipocytes identifies differential SUCNR1 expression. (A) Transcriptional profiling of paired human white and brown adipocytes identified SUCNR1 as being more highly expressed in brown adipocytes compared to white (n=4 for both brown and white adipocytes). Adapted from Suchacki et al., 2023 (210). (B) Differences in SUCNR1 expression were confirmed with qPCR analysis of differentiated human brown and white adipocytes (performed by Stimson lab, n=6 for brown and white adipocytes). Data are presented as means +/- SEM and were analysed by Paired t-test. **=p<0.01.
1.5 SUCNR1

1.5.1.1 G-protein coupled receptors

The primary physiological purpose of succinate is as a metabolite in the TCA cycle, however, it is also a ligand for the G protein-protein coupled receptor (GPCR) SUCNR1 (211). GPCRs are a large and diverse group of 7 domain transmembrane protein receptors responsible for signal transduction across plasma membranes, many of these receptors show high affinity for specific molecules, due to these traits GPCRs are targets for approximately 60% of marketed drugs for humans (212, 213). At a basic level a GPCR consists of a receptor, a G protein, and an effector. In vertebrates there are five families of GPCRs based on sequence and structure, these families are rhodopsin-like, secretin, glutamate, adhesion, and Frizzled/Taste2 (212). Rhodopsin-like GPCR’s can be further divided in α, β, and γ sub-units (Figure 1. 11). These subunits form a heterotrimeric complex consisting of the sub-unit (which binds and hydrolyses GTP), and the β/γ sub-units which are paired in a dissociable bond. When a GPCR is activated by its ligand a conformational change occurs in the receptor, causing the bound α and β/γ sub-units of the G protein to dissociate (212). This is facilitated by the exchange of GDP for GTP in the α sub-unit, following receptor activation. Both α and β/γ sub-units once released, can interact with their respective downstream effectors. Following activation of the α sub-unit effector, the GTP bound to the unit is hydrolysed to GDP, allowing for the re-association of the α and β/γ sub-units. Subsequently the heterotrimeric G protein can bind again to its associated receptor and a signalling cycle is renewed (212). The downstream effects of the dissociated α and β/γ sub-units vary depending on the sub-class of α sub-unit. There are four subclasses consisting of Gs, Gq/11, Gq, and G12/13. Typically, receptors are able to activate more than one of the different α unit sub-types with this being tissue specific causing tissue specific differences in receptor activation (212). Gs and Gs12/13 are involved in migration, growth and cell division (215).
Figure 1. 11 Overview of GCPR activation and downstream effects. G-protein coupled receptors are cell surface receptors that have α, β, and γ sub-units bound in a heterotrimeric complex consisting of the sub-unit (1). When the receptor is activated by a ligand, a conformational change occurs in the receptor. This is facilitated by the exchange of GDP for GTP in the α sub-unit (2). Following conformational change, the α sub-unit and a β/γ dimer are released from the receptor (3). Both the α sub-unit and β/γ dimer can interact with downstream targets (such as modulation of gene expression, or AC/cAMP/PKA signalling pathways). The effects of the α sub-unit are dependent on its class which include the Gs, Gi/o, Gq, and G12/13. Following dissociation from the receptor and initiation of downstream signalling, the GTP bound to the α sub-unit is hydrolysed to GDP, allowing for the re-association of the α and β/γ sub-units to the cell surface receptor (4).
1.5.1.2 SUCNR1

*SUCNR1*, originally called GPR91, was identified as an orphan GPCR on chromosome 3q24-25, with an amino acid (AA) length of 330AA. *SUCNR1* belongs to the largest GPCR subclass, the rhodopsin-like family (216). *SUCNR1* is expressed in a number of tissues in both humans and mice with the receptor having an approximate 68% homology between the species, although the murine protein sequence is 12AA shorter than in human at the C-terminal, at approximately 318AA (217). The crystal structure of human *SUCNR1* has not yet been confirmed (218). When succinate was first identified as the endogenous ligand for *SUNCR1* the EC\textsubscript{50} was estimated to be 28μM or 56μM, using the aequorin assay or FLIPR assay respectively (211). In this study 200 different carboxylic acids were tested, only maleate and methylmalonate also activated the *SUCNR1* receptor but with 5-10-fold less potency. However, since then a number of other TCA metabolites have been shown to activate *SUCNR1*, albeit to a much lower extent than succinate (EC\textsubscript{50}= 17μM); these metabolites include oxaloacetate (EC\textsubscript{50}= 171μM), L-malate (EC\textsubscript{50}= 207μM), and α-ketoglutarate (EC\textsubscript{50}= 7.3mM), although these concentrations are above typical circulating levels (219). Initially, *SUCNR1* activation was believed to induce both G\textsubscript{i} and G\textsubscript{q} pathways when *SUCNR1* was overexpressed in HEK293 cells, as succinate inhibited forskolin stimulated cAMP production which was abolished by pertussis toxin (a G\textsubscript{i} protein inhibitor) (211). In addition, succinate treatment induced inositol phosphate and intracellular Ca\textsuperscript{2+} increase which were associated with G\textsubscript{q} signalling (211). The role of G\textsubscript{q} signalling as the cause of the observed Ca\textsuperscript{2+} increase has been disputed, and may be attributed to PLC-β activation by the β/γ sub- unit (220, 221). However, recent work has identified that activation of *SUCNR1* with either succinate or the specific *SUCNR1* agonist cis-epoxysuccinic acid (C-ESA) induce both G\textsubscript{q} and G\textsubscript{i} signalling in macrophages, these effects were inhibited with the use of the *SUCNR1* antagonist NF-56-EJ40 supporting the importance of *SUCNR1* in these responses (222).

1.5.1.3 SUCNR1 tissue distribution

*SUCNR1* is widely expressed throughout the body and has a diverse set of functions (Figure 1. 12). *SUCNR1* is expressed in kidney, liver, spleen, and white adipose tissue...
Additionally, *Sucnr1* is expressed in immune cell populations including myeloid cells (224) and dendritic cells (225). *Sucnr1* is also expressed in cardiac muscle tissue (226), *SUCNR1* is lowly expressed in skeletal muscle myocytes but is expressed to a much greater extent in muscle resident M2 macrophages (195, 222). RNA seq data in several tissues comparing human and mouse *SUCNR1* expression highlighted that both humans and mice have high levels of expression in WAT and the liver, but *SUCNR1* is more highly expressed in human than murine macrophages (222).

### 1.5.1.4 SUCNR1 and brown adipose tissue

The interplay between succinate and BAT function also has systemic effects on metabolic health and liver function. In *Ucp1* KO mice there is substantial depletion of most mitochondrial catabolic enzymes in brown and beige adipocytes (227, 228). When fed an obesogenic diet in standard housing conditions (21°C), *Ucp1* KO mice had comparable body weight, fat mass, energy expenditure, and caloric intake to wild-type controls, an effect commonly reported and associated with compensatory mechanisms for energy homeostasis (229, 230). The proteome of *Ucp1* KO mice was analysed and compared to wild-type controls following 14 weeks of a western diet (a high fat diet with approximately 45% of calories from fat which also contains high levels of cholesterol and sucrose (231, 232). In the *Ucp1* knockout mice fed a HFD there was substantial upregulation of liver inflammation and proteins associated with hepatic stellate cell (HSC) activation, which are the major cell type that drive tissue fibrosis and inflammation in the liver (228). BAT succinate concentrations in the *Ucp1* knockout mice were significantly depleted compared to wild type controls whilst liver extracellular succinate concentrations were increased. During western diet feeding a common side effect is local liver tissue hypoxia (233), as previously discussed succinate concentrations are increased following hypoxic cellular conditions which could have contributed to this increased succinate liver concentration seen in the *Ucp1* knockout model. The authors assessed how *Ucp1* and *Sucnr1* knockout contributed to the observed liver phenotype with the use of dual *Ucp1/Sucnr1* knockout model. Comparing the proteome of wild-type, *Ucp1* knockout, and *Ucp1/Sucnr1* knockout mice fed a western diet, it was shown that
removal of Sucnr1 from these mice improved the inflammatory phenotype in the liver (228). When western diet fed Sucnr1 knockout mice were house at thermoneutrality they had reduced liver inflammation and pathology compared to wild type controls, this effect was not seen during standard 21°C housing, further implicating thermogenic adipose tissue activity as having a role with SUCNR1 induced liver inflammation. These data highlight an intricate balance between BAT mediated clearance of systemic circulating succinate and SUCNR1 activated pro-inflammatory response in the liver.

However, the role of SUCNR1 in BAT function remains relatively unclear. While succinate increased BAT thermogenic activity (146) and Sucnr1 disruption in mice increased adiposity and impaired metabolic health during high fat feeding (234), little research has been performed to adequately explore the role of this receptor during cold exposure in mice. Global knockout of the receptor does not change Ucp1 expression in BAT (234), however, there are other aspects of BAT function that could be affected by Sucnr1 such as cold tolerance or energy expenditure. Our lab’s data indicate that, in humans, Sucnr1 is highly expressed in brown differentiated adipocytes (see Figure 1.10) and could therefore have a role in brown adipocyte function. Additionally, when succinate has been used to activate thermogenesis in vitro, supra-physiological concentrations have been used which have activated thermogenesis via intracellular pathways which may elicit differing responses to SUCNR1 activation in brown adipocytes. While the metabolically favourable phenotype seen in control diet fed Sucnr1 KO mice may be due to increased WAT lipolysis and reduced leptin production, the cause of the metabolic dysfunction in high fat fed Sucnr1 KO mice is unclear. Due to the importance of succinate and succinate transport in BAT thermogenesis it is plausible that SUCNR1 plays an important role in regulating BAT function and knockout of the receptor may contribute to the metabolic dysfunction observed in this model. This thesis aims to explore the role of SUCNR1 in both human and murine brown adipose tissue function.
Figure 1. 12 Effects of SUCNR1 and succinate in different tissues. Succinate is produced in the TCA cycle and by succinate producing bacteria in the intestines as well as being found in dietary sources. Microbiome succinate production is increased in obesity and T2DM. Circulating succinate has a wide variety of systemic effects in the body. In brown adipose tissue succinate stimulated thermogenesis through enhanced mitochondrial reactive oxygen species production, an effect shown to be independent of SUCNR1. In WAT activation of SUCNR1 inhibits lipolysis. Sucnr1 global knockout increases adiposity when male mice are fed a high fat diet. In adipose tissue specific knockout mice have reduced leptin levels as well as increased adiposity on high fat diet. Additionally, both knockout models show mice have impaired glucose handling. Activation of the SUCNR1 increases stimulates renin release and subsequently increases blood pressure. In the liver activation of SUCNR1 leads to a pro-inflammatory phenotype, which is exacerbated in Ucp1 knockout mouse, an effect associated with the ability of BAT to sequester large volumes of circulating succinate. Skeletal muscle can release succinate during exercise conditions which leads to activation of SUCNR1 in surrounding cells which induces a switch from fast
to slow twitch muscle fibres and increasing muscular strength. SUCNR1 in cardiomyocytes leads to cardiac hypertrophy and increased Ca²⁺ concentrations, increased intracellular succinate concentrations in cardiomyocytes has been associated with increased reperfusion injury during ischaemia.

1.5.2 SUCNR1 cell specific functions

1.5.2.1 SUCNR1 in blood pressure regulation

One of the first identified roles of SUCNR1 was in the regulation of the renin-angiotensin system (RAS) in the kidneys (211). SUCNR1 is expressed in the proximal tubules and in the luminal membrane of the macula densa of the juxtaglomerular apparatus in close proximity to renin-producing granular cells, the cortical thick ascending limb, and cortical and inner medullary collecting duct cells (211, 235). Succinate was found to increase blood pressure in a dose dependant manner in rats by increasing renin cells (211, 235). This effect was attenuated by either bilateral nephrectomy or through the angiotensin-converting enzyme inhibitor captopril, confirming that the site of action was the kidney and that renin production was a key component. Sucnr1 knockout mice were also protected from succinate induced hypertension, but there were no differences in basal blood pressure. Additionally, in vitro assays showed that the SUCNR1 agonist cis-epoxysuccinic acid (C-ESA) decreased cAMP levels with an approximate 10-fold increase in potency (EC₅₀ 2.7μM) compared to succinate, whilst also stimulating an increase in rat blood pressure in vivo (236). Circulating succinate levels are increased in hypertensive rats compared to controls, however, this was not observed in humans (237). Further, there is evidence that succinate-SUCNR1 signalling plays an important role in the regulation of blood pressure in T2DM-induced renal disease. During T2DM increased circulating glucose induced renin release, SUCNR1 was believed to be part of this signalling cascade as local hypoxic conditions led to renal succinate accumulation and subsequent activation of SUCNR1 leading to activation of the RAS pathway (238). Global Sucnr1 knockout mice had reduced renin release and hypertension in a streptozotocin induced model of type 1 diabetes (238). However, the authors did not measure local succinate concentrations in the juxtaglomerular apparatus so the
extent of succinate-SUCNR1 signalling in diabetic kidney disease requires further study.

1.5.2.2 Succinate and SUCNR1 in the gut microbiota

The gut-microbiome has recently been shown to have a significant role in regulating both gut and whole body health particularly with regards to obesity and T2DM (239). Additionally, succinate is produced in large quantities by many different species of bacteria found in the gut microbiome (240, 241). Increased dietary succinate has also been shown to improve metabolic health in animals. Mice fed sodium-succinate in drinking water reduced weight gain during high fat feeding and reduced the impact of diet induced glucose intolerance in a dose-dependent manner, an effect attributed to increased BAT activation and therefore energy expenditure (146).

Another study identified that dietary fibre (previously shown to improve metabolic and cardiovascular health with increased consumption) increased numbers of succinate producing bacteria and caecal succinate levels (242). Succinate in the digestive tract, either through dietary consumption or bacterial production, also activates SUCNR1 expressed on intestinal tuft cells. Dietary succinate in the drinking water of mice increased tuft and goblet cell hyperplasia in wild type but not Sucnr1−/− mice (243). These data show that the microbiome has a significant role in regulating succinate levels and that dietary succinate can improve metabolic health.

Circulating succinate concentrations are increased in humans from approximately 20μM in lean adults to 75μM and >100μM in obese and diabetic individuals respectively, revealing dysregulation of succinate levels in metabolic disease and a correlation between increased circulating succinate levels and CVD risk (241). These differences in circulating succinate levels were associated with differing compositions of the patients’ gut microbiome. Increased succinate levels were also positively associated with blood glucose, insulin, and triglycerides, and negatively
associated with scWAT ATGL and HSL expression (241). Another study showed that young adults individuals with higher succinate levels had higher levels of circulating triglycerides, C-reactive protein, and pro-inflammatory omega-6 oxylipins than individuals with lower succinate levels (244). Additionally, higher succinate levels were associated with increased vWAT and diastolic blood pressure, and were also higher in metabolically unhealthy individuals than in healthy overweight/obese subjects (244). However, circulating succinate was not associated with BAT volume (as measured by $^{18}$FFDG) or activity and did not affect faecal microbiota composition in this study. Patients with obesity and T2DM were also shown to have reduced circulating succinate following gastric by-pass surgery (245). Additionally, following a mixed meal challenge, patient circulating succinate levels were increased similar to healthy controls. These data show, following weight loss intervention, that dysregulation in circulating succinate levels can be recovered (245). It should also be noted that in murine models that circulating concentrations of succinate are increased in obesity and T2DM (237, 238). Together these data highlight an important role of both dietary and microbiome produced succinate on systemic metabolic health and that circulating succinate is dysregulated in metabolic diseases.

1.5.2.3 SUCNR1 in skeletal muscle

During exercise succinate is released from the skeletal muscle, as mentioned previously this is facilitated by intracellular acidification during anaerobic conditions during exercise leading to the protonation of succinate and subsequent export (195). This occurs in both mouse and humans through MCT1 but not MCT2 or MCT4, while SLC13A3 is not expressed in skeletal muscle (246). In mouse femoral muscle extracellular succinate levels were approximately 20μM at baseline, and increased substantially to 60-200μM post exercise, meaning that SUCNR1 should be activated in these conditions (195). However, SUCNR1 was not expressed in myotubes derived from whole muscle but were found in non-myofibrillar resident cell types, including stromal, endothelial, and satellite cell populations (195). Following exercise, RNA sequencing transcriptomics were performed on these cell populations from both wild-type and global SUCNR1 knockout mice. Satellite and stromal cells had
decreases in transcripts involved in neuronal projections and axon guidance in Sucnr1 knockout mice (195). These changes were due to being downstream of PKA and mitogen-activated protein kinase (MAPK) pathways activated by SUCNR1 agonism (217, 247). Further, Sucn1 knockout mice were less responsive to physiological benefits of exercise and showed substantially reduced strength gain and fast twitch muscle fibres following resistance training compared to wild-type mice (203). Additionally, the Sucnr1 knockout mice had impaired insulin sensitivity post exercise which the authors attributed to the skeletal muscle phenotype (203). In addition, dietary succinate supplementation increased both muscle grip strength, running endurance, and hang time in mice in a dose dependent manner whilst increasing the ratio of slow to fast twitch muscle fibres in the gastrocnemius muscle (203). This change in muscle fibre type was due to upregulation of MyHC I, MyHC IIa, PGC-1α, myoglobin, and TnnT1, all associated with slow twitch muscle fibre development, both MyHC IIb and TnnT3 (associated with fast twitch muscle fibre development) were downregulated. In conjunction with these remodelling changes succinate increased oxidative capacity in the muscle due to increased mitochondrial biogenesis. In this study exercise increased Sucnr1 mRNA expression in both soleus and gastrocnemius muscles. Sucnr1 deletion, either globally or in the gastrocnemius, prevented the physiological improvements and genetic changes of dietary succinate (203). The data from these studies highlight the importance of SUCNR1 signalling in regulating skeletal muscle response to exercise and how increased dietary succinate can improve metabolic health. However, both succinate and SUNC1 affect other types of muscle, most notably cardiomyocytes.

1.5.2.4 SUCNR1 in cardiac muscle
As previously discussed, myocardial intracellular succinate concentrations increase during cardiac ischaemia and is released from cardiomyocytes during reperfusion (196, 197). Administration of intravenous succinate to rats for 5 days upregulated hypertrophic markers including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (MYH7) and α-skeletal actin (α-SkA). Cardiomyocyte size was also increased in these rats and was associated with
pathological cardiac hypertrophy (248). These effects were not observed in knockout mice so are mediated by SUCNR1. The gene expression changes were also validated in neonatal rat cardiomyocyte primary cell cultures. These data indicate that SUCNR1 is required for succinate induced cardiac hypertrophy. The extracellular signal-regulated kinases-1/2 (ERK1/2) hypertrophic signalling pathway and HDAC5 pathway were activated following SUCNR1 activation leading to increased intracellular Ca\(^{2+}\) concentrations in cardiomyocytes (248). Additional studies have shown that succinate signalling through SUCNR1 increases intracellular Ca\(^{2+}\) concentrations in rat cardiomyocytes by increasing phosphorylation of the ryanodine receptor and phospholamban through PKA-dependant pathways (226). The authors additionally showed that succinate treatment decreased cardiomyocyte viability due to SUCNR1 induced activation of the caspase-3 activation pathway. The activation of ERK1/2 in cardiomyocytes (through succinate/SUCNR1 signalling) induced mitochondrial fission and dysfunction which caused cardiomyocyte apoptosis (249). Together these data implicate an important role of succinate-SUCNR1 signalling and cardiomyocyte viability.

### 1.5.2.5 SUCN1 in macrophages

In addition to being a key regulator of metabolism, succinate and by extension SDH are involved in inflammatory processes which can be both pro- and anti-inflammatory (250). Succinate accumulation can occur if SDH activity is inhibited (251), or when α-KG anaplerosis increases glutamine metabolism (252). Succinate accumulation in macrophages increases HIF-1α expression through inhibition of prolyl hydroxylases (PHDs) causing downstream activation of multiple targets which induce pro-inflammatory processes such as interleukin-1β production (187, 252, 253). Succinate and SDH are also involved in reactive oxygen species (ROS) production (254). Increased succinate levels can reverse electron flow through complex I of the electron transport chain as part of RET (255), resulting in a high level of hydrogen peroxide generation (256). SDH produces ROS with electrons supplied from succinate when ubiquinone re-oxidation through complex I and complex III is prevented and succinate concentrations are low (257). ROS are known to exacerbate
and cause inflammation injury (258). Succinate is released by activated pro-
inflammatory macrophages (259), which can activate SUCNR1 on macrophage plasma membranes producing anti-inflammatory effects and is part of an apparent inflammation resolving mechanism (219, 224). Sucnr1 knockout in macrophages found in the scWAT caused a pro-inflammatory phenotype which was not observed in wild type controls. Conversely, macrophages found in the vWAT inSucnr1 knockout mice display an anti-inflammatory phenotype, with reduced markers of inflammation compared to wild-type controls (224). Similar effects of succinate/SUCNR1 signalling to reduce inflammation have been observed with succinate released from macrophages activating SUCNR1 on neural stem cells which causes prostaglandin E2 secretion, while scavenging of extracellular succinate by increased expression of SLC13A3, which initiates resolution of inflammation during pathophysiological conditions (260). These data demonstrate the complex role of succinate in inflammation, due to succinate signalling with HIF1-α and succinate/SDH mediated generation of ROS contributing to inflammatory processes whilst SUCNR1 acts to resolve inflammatory processes.

1.5.2.6 SUCNR1 in WAT

Our group has shown that SUCNR1 is expressed in human brown and white adipocytes, although to a much lesser extent in white (see Figure 1. 10). However, tissue transcriptional profiling of GPCRs identified Lucnr1 as being highly expressed in mouse WAT, activation of the receptor (using succinate) inhibited isoproterenol stimulated lipolysis in WAT explants in a dose dependant manner (223). In a separate study, succinate decreased isoproterenol-stimulated lipolysis in gonadal explants from wild type but not Sucnr1 knockout mice (234). This was mirrored in vivo, where male chow-fed Sucnr1 KO mice were leaner and had improved glucose tolerance compared to wild-type mice, additionally Sucnr1 knockout mice had reduced circulating leptin and increased food intake during dark periods. In addition, energy expenditure was increased in Sucnr1 knockout mice compared to wild type controls. However, when fed an obesogenic HFD, male KO mice had increased adiposity despite smaller overall bodyweight in addition to impaired glucose and insulin
tolerance and increased hepatic steatosis (234). There were no differences in energy expenditure, RER, locomotor activity, or food intake between the genotypes when fed a HFD. The authors observed decreased adiponectin and increased aspartate transaminase and alanine aminotransferase in the blood, however, circulating NEFAs and triglycerides were unchanged between genotypes. Although the exact mechanisms driving this phenotype were not determined in the high fat fed mice it is likely that perturbed adipokine signalling may contribute (261). In mice, local WAT succinate concentrations increased 2.3 fold during high fat feeding (262).

Although the mice in the previous study were from a global Sucnr1 knockout mouse, similar results were recapitulated in an adipose-specific Sucnr1 knockout model that was published recently (263). Control diet fed Sucnr1 knockout mice had reduced iWAT and gWAT depot size and improved glucose and insulin tolerance despite greater food intake at room temperature. These mice also had increased Ucp1 expression in iWAT depots, indicating that SUCNR1 signalling in adipocytes may suppress beiging (263). However, this study did not investigate cold tolerance or thermogenic capacity of brown or beige adipose tissue so the effect of adipose tissue specific Sucnr1 knockout in in vivo was not determined. When fed a HFD the knockout mice had greater adiposity and glucose and insulin intolerance despite reduced food intake (263). When fed a control diet, adipose tissue specific Sucnr1 knockout mice had significantly reduced Lep expression in gWAT and circulating leptin and had a severely blunted response to feeding induced increases in circulating leptin. Additionally, oral administration of sodium succinate increased circulating leptin levels and Lep expression in iWAT and eWAT in wild-type but not knockout mice. These data were recapitulated in vitro in 3T3-L1 adipocytes where both succinate and C-ESA treatment increased Lep expression. Additionally, leptin release was increased in human adipocytes treated with succinate and C-ESA. These changes in leptin expression were mediated through an AMPK/ JNK/ C/EBPα-dependent manner. Leptin administration for 4 weeks increased adipocyte size and impaired glucose tolerance in adipose tissue specific Sucnr1 knockout mice, partially ablating the metabolically favourable phenotype observed in control diet fed mice. The authors
attributed the WAT browning and low leptin levels to the reduced adiposity seen in these mice. Furthermore, in humans increased LEP and SUCNR1 expression in scWAT and vWAT depots positively correlated with both obesity and circulating succinate. SUCNR1 expression was also independently associated with increased LEP expression, indicating that obesity-associated hyperleptinemia could be linked to succinate/SUCNR1 signalling in adipose tissue (263). Increased circulating succinate has been observed in obese humans (241, 244), therefore, succinate-SUCNR1 signalling could play a role in the pathology or development of obesity, however, further insight to the mechanisms involved in these processes are needed.

Table 1. 1 Summary of Sucnr1 knockout mouse models. Description of the phenotypes of the various global and tissue-specific knockout mouse models.

<table>
<thead>
<tr>
<th>Model publication</th>
<th>Knockout details</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Diepen et al. (264)</td>
<td>Global Sucnr1 knockout</td>
<td>Sucnr1 knockout mice had significantly reduced numbers of macrophages and crown-like structures in adipose tissue and improved glucose tolerance compared to wildtype mice fed an HFD.</td>
</tr>
<tr>
<td>Mills et al. (228)</td>
<td>Global Sucnr1 knockout</td>
<td>Liver immune cell infiltration and pathology is regulated by systemic circulating succinate and Sucnr1 signalling. Infiltration of immune cells was significantly reduced in the knockout model</td>
</tr>
<tr>
<td>McCreath et al. (234)</td>
<td>Global Sucnr1 knockout</td>
<td>When fed a standard chow diet global knockout mice had a</td>
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...
metabolically favourable phenotype with reduced adipose tissue mass. However, when fed a HFD diet mice had increased adiposity and impaired glucose tolerance. *Sucnr1* knockout white adipocytes have increased lipolysis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Experimental Model</th>
<th>Outcome</th>
<th>Details</th>
</tr>
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<tbody>
<tr>
<td>Villaneuva-Carmona <em>et al.</em> (263)</td>
<td>Adipocyte specific <em>Sucnr1</em> knockout</td>
<td>Adipocyte specific knockout</td>
<td>Mice had increased adiposity and reduced food intake when fed a HFD, in addition to perturbed circadian clock gene expression and leptin signalling.</td>
</tr>
<tr>
<td>Keiran <em>et al.</em> (224)</td>
<td>Myeloid specific <em>Sucnr1</em> knockout</td>
<td>Myeloid-specific <em>Sucnr1</em> deficiency</td>
<td>Promoted a local pro-inflammatory phenotype, disrupted glucose homeostasis in mice fed a normal chow diet, exacerbated the metabolic consequences of diet-induced obesity and impaired adipose-tissue browning in response to cold exposure.</td>
</tr>
<tr>
<td>Wang <em>et al.</em> (203)</td>
<td>Global <em>Sucnr1</em> knockout</td>
<td>Dietary succinate</td>
<td>Increased endurance exercise ability, myosin heavy chain I expression, aerobic enzyme activity, oxygen consumption, and mitochondrial biogenesis in mouse skeletal muscle,</td>
</tr>
<tr>
<td>Reddy et al. (195)</td>
<td>Global Sucnr1 knockout</td>
<td>Succinate-SUCNR1 signalling is required for paracrine regulation of muscle innervation, muscle matrix remodelling, and muscle strength in response to exercise training.</td>
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<tr>
<td>Lei et al. (243)</td>
<td>Global Sucnr1 knockout</td>
<td>Sucnr1 knockout mice showed diminished immune responses to treatment with polyethylene glycol and streptomycin, which are known to enhance microbiota-derived succinate.</td>
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</table>
1.6 HYPOTHESES AND AIMS

The overarching hypothesis of this thesis is that the succinate receptor regulates both human and murine BAT function and that it has protective role in the development of obesity and diabetes during an obesogenic challenge. This hypothesis can be sub-divided as follows:

1. Activation of SUCNR1 in murine and human brown adipocytes increases thermogenesis.
2. Disruption of the succinate receptor impairs BAT function and reduces energy expenditure during cold exposure and high fat feeding, increasing metabolic dysfunction during high fat feeding.
3. Brown adipocytes lacking SUCNR1 will have reduced thermogenic capacity.

To test these hypotheses, this thesis aimed to address these by using both in vitro and in vivo models. In Chapter 3, I investigated the effects of succinate and C-ESA on oxygen consumption, gene expression and lipolysis in differentiated human brown and white pre-adipocytes. In Chapter 4, I used a global Sucnr1 knockout model to investigate how disruption of the receptor alters metabolic health in male and female mice, housed both at room temperature and during cold exposure, fed a control or high fat diet. In Chapter 5, I investigated the effect of succinate and C-ESA on differentiated brown and inguinal (beige) pre-adipocytes from both wild-type and Sucnr1 knockout mice, measuring the same parameters as in chapter 3 to allow comparison with the human adipocytes.
Chapter 2

Materials and Methods:
2.1 MATERIALS
All chemicals were purchased from Sigma Aldrich (Dorset, UK), unless otherwise stated. All microcentrifuge tubes were purchased from Sarstedt (Nümbrecht, Germany). All cell culture plates were purchased from Corning (New York, USA), with the exception of plates for respirometry assays purchased from Agilent Technologies (California, USA). Sources other than these are indicated in parentheses. Room temperature was 18°C-21°C.

2.2 COMMONLY USED SOLUTIONS

2.2.1.1 Common lab bench solutions
- Krebs-Henseleit buffer – 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.25 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3, 11 mM glucose
- Dulbecco’s Phosphate Buffered Saline Solution – DPBS (Gibco, UK)
- 70% EtOH – Ethanol and water (70:30, v:v)
### Materials and Methods

#### 2.2.1.2 Cell culture compositions

**Table 2.1 Cell culture media composition.** Table describing composition of cell culture media used for in vitro culture and experimental assays.

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
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<tbody>
<tr>
<td>Proliferation medium</td>
<td>High glucose DMEM media (Lonza, BE12-614Q, or Fisher Scientific, 13476146; dependant on stock), 10% Hyclone foetal bovine serum (FBS, Gibco, ThermoFisher Scientific) volume/volume (v/v) , with 1% Pen/Strep v/v (penicillin and streptomycin, 15070063, ThermoFisher Scientific) and 1% v/v L-glutamine (GlutaMAX™ Supplement, 35050061, ThermoFisher Scientific)</td>
</tr>
<tr>
<td>Differentiation medium</td>
<td>Proliferation media with the addition of 1nM tri-iodothyronine (T3) (T6397), 20nM insulin, 1µM rosiglitazone (R2408), 500µM IBMX (I5879), 500nM dexamethasone (D4902)</td>
</tr>
<tr>
<td>Post-differentiation (A) medium</td>
<td>Proliferation media with the addition of 1nM T3, 20nM insulin, 1µM rosiglitazone</td>
</tr>
<tr>
<td>Post-differentiation (B) medium</td>
<td>Proliferation media, 1nM T3, 20nM insulin</td>
</tr>
<tr>
<td>Serum free media</td>
<td>High glucose DMEM media, 1nM T3, 20nM insulin</td>
</tr>
<tr>
<td>2% BSA media</td>
<td>Serum free media, 2% BSA (A3294) weight/volume (w/v)</td>
</tr>
<tr>
<td>Lipolysis media</td>
<td>Phenol red free high glucose DMEM media (Lonza, BE12-917F), 2% fatty acid free BSA (Merck, 10775835001) w/v</td>
</tr>
<tr>
<td>Full XF media</td>
<td>Agilent seahorse base XF media (Agilent 102353-100) with the addition of 17.5mM glucose and 1mM sodium-pyruvate</td>
</tr>
</tbody>
</table>
2.3 INTRODUCTION
The majority of the in vivo and laboratory techniques and assays used in this thesis were routinely in use in this laboratory. Consequently, I learned and adapted the techniques as required. Some techniques had not been performed in this group previously or required substantial optimisation for the purposes of this thesis, these techniques included:

- Murine primary brown and inguinal cell culture
- RNA extraction from murine cell culture lysates using isopropanol and ethanol precipitation techniques

Consequently, I undertook development of these techniques and optimised these methods accordingly. The murine primary cell culture protocol was developed to maximise proliferation and differentiation of adipocytes from brown and inguinal depots. For human primary cell culture, the technique used was designed to more closely mimic the murine technique to allow direct comparison.

2.4 CELL CULTURE TECHNIQUES
2.4.1 Stromal vascular fraction pre-adipocyte isolation
Murine and human pre-adipocytes isolated from the stromal vascular fraction (SVF) of their respective depots (detailed below) were cultured and differentiated for use as in vitro models. Both murine and human brown, and white (inguinal for murine) adipocytes have previously been cultured by this lab using a similar technique utilised in this thesis to assess adipocytes function in response to drug treatment (265). Cell culture techniques were modified to include rosiglitazone during the differentiation process. Rosiglitazone enhances adipose tissue browning by binding PPARγ and sensitising to insulin (266, 267). During optimisation of murine adipocyte cell culture techniques, rosiglitazone enhanced the percentage of differentiated adipocytes and increased Ucp1 expression and was therefore included in both murine and human cell culture models. Further optimisation for murine adipocyte culture was performed, identifying that pooling of adipose tissue depots from 3 separate mice between 6-10 days old provided a sufficient mass of adipose tissue precursors to
ensure adequate proliferation and subsequent differentiation percentage. Pre-adipocytes from older mice would not proliferate or differentiate sufficiently. The protocols for culture techniques for human and murine adipocytes are detailed below.

2.4.1.1 Murine BAT and IAT dissection for cell culture

Mouse pups for in vitro tissue culture were generated by homozygous crossing of male/female $Sucnr1^{+/+}$ or $Sucnr1^{-/-}$ mice. Homozygous crosses were used due to genotyping restrictions on mice under 14 days old, as pups were culled between days 6-10 of age. Only male pups were used for cell culture to minimise confounders, males were chosen as female $Sucnr1^{-/-}$ mice developed no metabolic phenotype (see chapter 4). Murine pre-adipocytes were isolated from the SVF of iBAT and IAT depots. Pups were culled with schedule 1 cervical dislocation and confirmation was performed with brachial artery severing. Adipose tissue depots were pooled from 3 mice for the SVF isolation protocol.

2.4.1.2 Human tissue dissection for cell culture

Human BAT and WAT tissues were sourced from patients undergoing elective neck surgery at the Royal Infirmary Edinburgh. Male and female euthyroid subjects, aged 18-80 years, were recruited who were due to undergo elective thyroid or parathyroid surgery in the Royal Infirmary of Edinburgh (under research ethics committee approval number 20/ES/0061). All patients gave informed consent to have tissue taken for research purposes. During their operation, a small amount of BAT was obtained from the central compartment of the neck, superior to the clavicle and deep to the lateral thyroid lobe either adjacent to the longus colli muscle or to the oesophagus. WAT was isolated from the subcutaneous neck tissue. All samples were taken by the same surgeon and placed in 37°C Krebs-Henseleit buffer before being transported to CVS, QMRI for isolation of the SVF.
2.4.1.3 Murine pre-adipocyte isolation

Krebs-Henseleit buffer, Dulbecco’s phosphate buffered saline with pen/strep (DBPS+P/S) and proliferation media DMEM were heated to 37°C prior to use. Dissected IAT and BAT were added to 5ml Krebs-Henseleit in separate 15ml Falcon tubes (352196, Fisher Scientific, UK). Samples were stirred with a spatula to remove excess blood. Each tissue sample was transferred into a separate 15ml Falcon tube containing 5ml DPBS + P/S to sterilise and wash, this process was repeated in a fresh falcon tube. Samples were transferred into a 7ml bijou containing sterile Krebs-Henseleit with collagenase type-I (LS005275, Worthington-biochem, New Jersey, USA), to digest collagen. Tissue was macerated by chopping with scissors whilst swirling and transferred to 15mL Falcon tubes. Falcons were incubated at 37°C in a shaking water bath, for 45 minutes. After digestion, 5ml Krebs-Henseleit was added to each 15ml falcon to dilute the collagenase I, following this the mixture was subject to centrifugation at 900rcf for 10 minutes at room temperature. Following centrifugation, the supernatant was removed and the pellet re-suspended in 5ml Krebs-Henseleit then transferred to a new 50ml Falcon tube through a 100µm strainer (352360, SLS, UK). A 5ml volume Krebs-Henseleit buffer was added to the used 15ml falcon, mixed and pipetted over the cell strainer to ensure transfer of any remaining cells. Samples were then subject to centrifugation at 200rcf for 5 minutes at room temperature, then the supernatant was removed. Cells were re-suspended in 4ml of proliferation media per depot, and suspension plated into 2 collagen coated wells of a 6 well plate (2ml in each well). Cells were washed the following day twice with 0.5ml DPBS+P/S and 2mL proliferation media was replaced. This was termed P0 (passage 0).

2.4.1.4 Human pre-adipocyte isolation

The SVF isolation was performed as detailed in 2.4.1.3 with the following changes. Firstly, samples were washed in 7ml bijous containing DPBS, rather than 15ml Falcon tubes. Cells were transferred into Nalgene pots (Nalgene, UK) following maceration rather than 50ml Falcon tubes. Finally, the stromal vascular fraction was plated into
cell culture plates that were not collagen coated. The protocol was otherwise identical.

2.4.2 Proliferation and differentiation of isolated murine pre-adipocytes

Proliferation media was changed every 2-3 days. Cells were passaged once 90-95% confluence was reached. Details of the passage method for murine cells is described in 2.4.4. 1 well of a 6 well plate at P0 would be split into 12 wells of a 24 well-plate, or where applicable 1 well at P0 would be used to plate into 20 wells of a 24 well V24 Cell Culture Microplate (Agilent, 100777-004). Cells were designated Day 0 (D0) once fully confluent. At D2 the differentiation protocol began with the addition of differentiation media to cells for 3 days (from D2-D5). On D5, cells were switched to post-differentiation media (A) until D6 at which point cells were switched to post-differentiation media (B). Experimental assays were performed between D7 and D9.

2.4.3 Proliferation and differentiation of isolated human pre-adipocytes

Once human primary cells were isolated and plated, proliferation media was changed every 2-3 days, and cells were passaged once 85-95% confluence was reached. Human pre-adipocytes were initially plated into 1 well of 6 well-plate and would be split in 1/4, this would be performed for 2 passages to give a total of 16 wells of a 6 well-plate. Once at P2, the cells were passaged a final time once >90% confluent and plated in a 1/1 ratio into wells of a 12 well-plate, or where applicable 2 wells at P2 were plated into 20 wells of a 24 well V7 Cell Culture Microplate (Agilent, 100777-004). Once the cells were plated at P3 in either a 12 well plate or V7 microplate, cells were monitored until fully confluent which was designated Day 0 (D0). At D2 the differentiation protocol began with cells switched from proliferation media to differentiation media for 7 days (D2-D9). At D9, cells were switched to post-differentiation media (B) until D14 at which point cells were ready to use for experimentation. All experiments were performed between D14-17.
2.4.4 Dissociation and passaging of cells

Once ready for passaging, cell culture media was removed and cells were washed twice with 2mL of DPBS. To each well 750μL of 0.05% trypsin-EDTA solution (Gibco, 25300054) and incubated at 37°C for 3-5 minutes until cells were dissociated from the well. A volume of 2mL proliferation media was added to each well to inactivate the 0.05% trypsin-EDTA solution. Suspended cells and media were collected in a 15mL or 50mL Falcon (depending on cell media volume) and subject to centrifugation for 5 minutes at 200rcf at room temperature. Proliferation media was removed, the pelleted cells were re-suspended in appropriate volume for the number of wells required for next passage, volumes listed below:

- Human cells P0 -> P1, 8mL
- Human cells P1 -> P2, 32mL
- Human cells P2 -> P3, 16mL
- Murine cells P0 -> P1, 6ml
- Seahorse plates, 2mL (initially 50μL in each well, with the addition of 450μL approximately 2 hours later once cells had adhered to the well)

2.4.5 Respirometry assays

2.4.5.1 Human respirometry assays

Cellular respiration was measured using a Seahorse XFe24 analyser (Agilent, USA). The day prior to the start of the assay, the Seahorse cartridge was prepared by adding 1ml Seahorse calibrant to each well of the calibration plate and attaching the hydration booster and sensor cartridge. The cartridge was placed in a sealed square bag in an oven at 37°C overnight. Both serum free media and full XF assay media were prepared for the following day, sterilised by passing through a 40μM mesh filter following heating to 37°C. Stock solutions (water based) of 500mM and 50mM succinate, 50mM, 5mM, and 0.5mM C-ESA were added to aliquots of serum-free and Full XF media. Final media concentrations were 100µM or 1000µM succinate, or 1µM, 10µM or 100µM C-ESA, as well as vehicle (sterile distilled water). Media aliquots had pH adjusted to 7.4 using 1M sodium hydroxide or 1M hydrochloric acid. All conditions were replicated in triplicate, with the exception of vehicle and 100µM C-ESA which were in quadruplicate. The seahorse plate was washed twice with 500µL
DPBS per well and switched to serum-free media containing vehicle/succinate/C-ESA. Cells were incubated at 37 °C for 1 hour in 5% CO₂. Following incubation cells were washed twice with 500μL Full XF media and switched to drug containing Full XF media. Cells were placed in an oven heated to 37°C with atmospheric CO₂ levels for a further hour. Following incubation, cells were analysed in the Seahorse XFe 24 instrument, each measurement cycle consisted of 2 minutes mixing, 4 minutes waiting, and 2 minutes measurement of oxygen consumption rate. These conditions were chosen following optimisation that demonstrated these were ideal times to allow for equilibration between measurement cycles. Measurements occurred during basal conditions and following injections of full XF media containing 15.34µM NADR (final concentration 2µM), 17.34µg/ml oligomycin (final concentration 2µg/mL), 19.34µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (final concentration 2µM), and 2.13µM/26.666µM rotenone/antimycin A (R/A) (final concentration 0.2µM /2.5µM). Each condition was measured for 3 cycles, with the exception of following addition of NADR which was measured for 6 cycles (Figure 2.1). NADR was added to activate uncoupled respiration of the adipocyte. Oligomycin was added to measure the contribution of ATP linked and uncoupled respiration, oligomycin inhibits complex V (ATP-synthase) of the electron transport chain. FCCP was added to measure maximal cellular respiration by allowing free transport of protons across the mitochondrial membrane. Rotenone and antimycin A were injected simultaneously to assess non-mitochondrial respiration. Rotenone and antimycin A, which inhibit complexes I and III in the electron transport chain respectively, lead to arrest of the electron transport chain (Figure 2.2). Four of the wells in each plate were left as ‘blanks’ having no cells plated in the well. During the assay these wells had media and drug conditions added as normal to serve as a baseline measurement. OCR data was presented by subtracting non-mitochondrial oxygen consumption (OCR when treated with rotenone/antimycin A).

2.4.5.2 Murine respirometry assays
Respirometry for murine adipocytes was performed as in 2.4.5.1, with minor alterations to the protocol. Final concentration of injected NADR was reduced to
500nM as higher concentrations inhibited oligomycin from reducing OCR. Oxygen consumption was also measured for three cycles rather than six during NADR stimulation.

**Figure 2. 1. Representative diagram of seahorse respirometry assay.** Schematic diagram of the human adipocyte respirometry assay with drug injections indicated at the respective cycles. Final OCR values were calculated by subtracting non-mitochondrial oxygen consumption values.
Figure 2. Representative diagram of the electron transport chain and the effects of rotenone, antimycin A, and oligomycin. Protons are transported across the inner mitochondrial membrane through complex 1, 3, and 4 (C1, C2, C4) due to excitation of electron transport across C1, complex 2 (C2), coenzyme Q (CoQ), C3, cytochrome C (Cyto C), and finally C4. This transport of protons and electrons generate and electrochemical proton gradient which is utilised by ATP synthase to transport protons across the inner mitochondrial membrane to generate ATP (adenosine triphosphate) from ADP (adenosine di-phosphate). Rotenone and Antimycin A inhibit C1 and C3 function, respectively, leading to electron transport chain arrest. Oligomycin inhibits ATP synthase activity preventing ATP production. Adapted from McNeill et al., 2021 (80).
2.4.6 Effects of succinate and cis-epoxysuccinic acid on mRNA expression

2.4.6.1 Human gene expression assay protocol

At D14, differentiated brown and white adipocytes were washed twice with 1mL of DPBS warmed to 37°C. Cells were then incubated for 8 hours with serum free media containing either vehicle (sterile water), succinate, C-ESA, in the presence or absence of NADR. All conditions were performed in duplicate. After 8 hours of incubation, media was removed and cells were lysed as described in 2.6.2.

2.4.6.2 Murine gene expression assay protocol

Murine adipocytes followed the same protocol as in 2.4.6.1 with minor alterations as detailed below:

- Experiment began at D9 rather than D14.
- Cells were washed with 500μL of DPBS.
- Cells were incubated for 8 hours in 2% BSA media with drug treatment.

2.4.7 Lipolysis assays

2.4.7.1 Human differentiated adipocyte lipolysis assays

At D14, differentiated brown and white adipocytes were washed twice with 500μL of DPBS heated to 37°C. Cells were pre-incubated for 1 hour in 500μL of lipolysis media containing either vehicle (sterile distilled water) or 1μM NADR. Cells were washed twice with 500μL of DPBS. Following the pre-incubation and wash, lipolysis media containing either vehicle, succinate, in the presence or absence of NADR was added to wells. All conditions were performed in duplicate. Aliquots of media (60μL) were obtained at 8 and 24 hours and placed on dry ice prior to storage at -80°C until analysis. Cells were then washed and the remaining media removed. Cells were then lysed for protein as described in section 2.7.1.

2.4.7.2 Murine differentiated adipocyte lipolysis assays

Murine adipocytes lipolysis assay was performed as in 2.4.7.1, with the following changes to protocol:
• Experiment started at D9.
• Aliquots were taken at 8 hours only.

2.5 MURINE IN VIVO TECHNIQUES

2.5.1 Animal welfare and husbandry
All animal experiments were approved by the Home Office and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals. Animals were kept under traditional housing conditions with a 12 hour light/dark cycle with 07:00-19:00 light and 19:00-07:00 dark. Cages were housed at room temperature. Animals were housed at either The Centre for Regenerative Medicine or Little France animal units based at the Bioquarter, University of Edinburgh.

2.5.2 DNA extraction and genotyping
Genotyping of Sucnr1 mice was performed both in-house and by Transnetyx®. Ear clips were collected during weaning (approximately 21 days old) and DNA was extracted using Direct PCR ear reagent (402-E, Viaten Biotech, USA). To each ear clip, 100μl of reagent containing 20μg/ml proteinase K inhibitor (539470, Merck, Germany) was added and incubated at 55°C for 1 hour, the sample was agitated during this time. Following incubation, samples were heated to 85°C for 45 minutes to deactivate the proteinase K inhibitor, and extracted DNA was stored at -20°C until use. Extracted DNA was subject to direct PCR using primers and PCR conditions as provided by Charles River lab Genetic testing services (see Table 2.2 for details). Mastermix containing 5μL GoTaq buffer, 1μL of each primer Sucnr1 Forward, Sucnr1 Reverse, and NeoF2, 0.5μL dNTPs, 0.25μl GoTaq enzyme, and 16.25μL water were added to each DNA sample for amplification. Samples were run on a 2% agarose Tris-borate-EDTA (TBE) gel made containing gel red (5μL/100μL of agarose gel). Bands were identified as per Charles River labs guidance, these bands were the same size (750bp for the wild-type band, and 330bp for the mutant band).
as previously observed in this mouse model (264). Hyper ladder 1 (BIO-33053, Meridian Bioscience, USA) was used as a reference for DNA length. Additionally, Transnetyx genotyping services were also used to genotype mice when there were delays in reagent availability and during genetic monitoring of the mouse model (see Table 2.2 for Transnetyx primer details). The in-house (Charles Rivers genotyping assay) primers used targeted the full length of the Sucnr1 transcript as well as the neomycin cassette (named NeoF2 by Charles River) insertion in the Sucnr1+/− mice, whilst the Transnetyx assay targeted the LacZ transcript.

Table 2.2 Genotyping primers for Sucnr1+/− mice. Primer sequences for both Charles River Labs genotyping protocol (same as in-house primers) and Transnetyx primer sequences for genotyping.

<table>
<thead>
<tr>
<th>Primer:</th>
<th>Charles River</th>
<th>Transnetyx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucnr1 Forward</td>
<td>GCTGCTGGGCTTTAGTGAC</td>
<td>TCCCTACATCCTTCAGGACATGA</td>
</tr>
<tr>
<td>Sucnr1 Reverse</td>
<td>GCTGCTTCTGTAGTTTATCAAGT</td>
<td>ACTGCACAGCAGTTTATCAAGT</td>
</tr>
<tr>
<td>NeoF2</td>
<td>ATCGCCTTCTTGAGGACAGGC</td>
<td>N/A</td>
</tr>
<tr>
<td>LacZ Forward</td>
<td>N/A</td>
<td>CGATCGTAAATCAGCGGAGGT</td>
</tr>
<tr>
<td>LacZ reverse</td>
<td>N/A</td>
<td>CGTTGGCCTGACTCATTCC</td>
</tr>
</tbody>
</table>

2.5.3 Body weight assessment
Mice were placed in a weighing boat on a calibrated balance (Ohaus, PR Series, Switzerland). Body measurements were recorded 3 times over the course of 15 seconds to account for variation to bodyweight causes by movement of mouse on balance. Measurements were performed at the same time each day to reduce confounders from diurnal variation of body weight and feeding routine (268).
2.5.4 Time-domain nuclear magnetic resonance assessment of body composition

Body composition was assessed using time-domain nuclear magnetic resonance (TD-NMR) using the Bruker™ minispec LF50 system (7.5MHz, 0.175T) (269) and operated using the Minispec Plus software (Bruker, Germany). Mice were loaded into a plastic tube and securely held in place before entering the instrument and scanned for approximately two minutes. Mice were measured every 14 days during the experiment at the same time of day at each reading to reduce the confounding effect of circadian rhythm on food intake which could alter body composition values (268).

2.5.5 Oral glucose tolerance tests

Mice were switched to clean cages free of food remnants, and fasted for 5 hours (mice began fasting between 07:00 and 07:30) before being administered a 25% (w/v) glucose/water bolus via oral gavage. The dose was normalised to 2mg/g of lean body mass, obtained from TD-NMR analysis of body composition as in 2.5.4. Venesection of the tail vein was performed at T=0 minutes (before glucose bolus) to allow for blood glucose monitoring and blood sample collection. Blood samples were obtained at T0, T+ 15, 30, 60, and 120 minutes after glucose administration. Blood glucose was measured with an Accu-Chek Nano glucose meter (Roche Diagnostics Ltd, UK) from fresh blood directly at the sight of venesection. Blood was collected from tail vein venesection in ethylenediaminetetraacetic acid (EDTA)-coated capillary tubes (Sarsted, 16.444.100) and stored on wet ice until sample processing, approximately 20-50μl of blood was taken at each time point. Blood samples were then subject to centrifugation at 2000rcf for 10 minutes at 4°C to separate the plasma fraction of the blood. Plasma samples were transferred to clean microcentrifuge tubes. Samples were stored at -80°C until insulin quantification. Insulin was measured by ultra-sensitive ELISA (protocol described in 2.9) (ChrystalChem, Chicago, IL, USA) to obtain glucose-stimulated insulin secretion (GSIS) measurements.
2.5.6 Indirect calorimetry and metabolic phenotyping

Energy expenditure, food intake, respiratory exchange ratio, and physical activity were all measured through the Phenomaster system (TSE systems, Germany). Seven days prior to housing in Phenomaster metabolic cages, mice were single housed and trained to use Phenomaster specific water bottles. Water consumption and mouse bodyweight were measured twice daily to monitor training efficacy, at 7 days mice were deemed ready to enter metabolic cages provided they had regularly consumed water and bodyweight had dropped by no more than 10% of starting weight. Mice housed in the Phenomaster were subject to the same 12-hour light/dark cycle and their usual *ad libitum* experimental diets. Water and food were accessible from suspended hoppers to allow for quantification of consumption by weight change. Caloric intake was then calculated as grams of food eaten multiplied by the calories/gram of each respective diet. Mice were housed in the Phenomaster for a total of 144 hours, 72 hours at room temperature (21°C) and 72 hours of cold exposure (4°C cabinet temperature). The initial 24 hours of each 72-hour phase was used as an acclimation period and data has been omitted from metabolic phenotyping, with the exception of the change in energy expenditure following the initial switch to cold exposure. Metabolic phenotyping measurements were sampled continuously for 3 minutes in two cages simultaneously, the 8 metabolic cages and a control chamber (containing ambient air) were all sampled every 15 minutes for the duration. Animal welfare checks were performed twice daily during a 15-minute window, these ‘check windows’ were removed from data analysis to reduce confounders from the data. To calculate changes in energy expenditure and respiratory exchange ratio (RER), the oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were measured in each cage (compared to a supplied gas exchange control cage). The formula to calculate RER was as follows (270):

\[
RER = \frac{VCO_2}{VO_2}
\]

If the value of the RER is closer to 0.7 fat is the predominant macronutrient utilised for EE, if the RER is closer to 1.0 carbohydrates are the predominant macronutrient.
utilised (270). To calculate energy expenditure the Phenomaster software utilises a modified Weir’s equation (271):

\[ EE(\text{Watts}) = \left( (3.941 \times \text{VO2}) + (1.106 \times \text{VCO2}) \right) \]

Technical outliers, such as during animal welfare checks when cages were opened, were removed from the Phenomaster data set. EE data was presented as a repeated measures time plot not adjusted for body weight or lean mass. However, an analysis of covariance (ANCOVA, a general linear model combining ANOVA and regression) was performed using the lean mass of mice as the co-variable to determine whether this variable altered EE. ANCOVA analysis is considered a statistically sound approach to analysing mouse energy expenditure to account for factors of bodyweight and composition (272).

Locomotor activity was calculated as the number of beam breaks in each measured period using a scan rate of 100Hz, this was measured throughout the 15 minute window and given as a total sum of those 15 minutes. The Phenomaster software measures both frequency of movement and distance travelled.

### 2.5.7 Infrared thermography

Using a FLIR infrared thermography camera (T650sc, Teledyne FLIR LLC, USA), mouse tail surface temperature was measured at both 21°C and 4°C. The emissivity (effectiveness of a surface/material emitting energy in the form of thermal radiation) was set to a ratio of 0.95 (this ratio is calculated as the thermal radiation from a surface to the radiation from an ideal black surface at the same temperature as given by the Stefan–Boltzmann law) (273). Although emissivity can be affected by different factors such as mammal pelage a range of 0.95-1.00 is often used as standard for mammalian studies. Mice were removed from metabolic cages following 72 hours at 21°C and placed in an open container. Mice were photographed 2-4 times capturing the whole mouse in the image (camera approximately 30-50cm away from mouse), mice were then placed into metabolic cages for cold exposure. Following 72 hours of cold exposure, prior to culling, mice were removed from the Phenomaster and placed in a container on ice (to maintain relative cold ambient temperature) and
photographed as described. Images were downloaded from the camera and analysed using FLIR Tools software. ROIs were drawn at the proximal tail (approximately 1-1.5 cm away from tail base) and distal regions (1-1.5 cm away from tail end) avoiding areas of tail which had been touched during handling to reduce variation from heat transference. Mean temperature of the ROI was calculated and presented.

2.5.8 Core body temperature
Following infrared thermography imaging during cold exposure (2.5.7), core body temperature was immediately measured in mice. A rectal probe thermometer (BAT-12, Physitemp, USA) was inserted into mice and measurements were taken after 5-10 seconds to ensure probe was adequately positioned. Mice were secured by holding the tail to reduce warming from handling.

2.5.9 Dissection and tissue collection
Mice were culled at the end of experiment (17-18 weeks old) using the cervical dislocation schedule 1 method, confirmation of schedule 1 was performed with decapitation. Immediately following decapitation blood was collected in EDTA coated capillary tubes (Sarsted, 16.444.100) and processed as in 2.5.5. Tissues collected during dissection included interscapular BAT (BAT), inguinal WAT (iWAT), gonadal WAT (gWAT), mesenteric WAT (mWAT), peri-renal WAT (pWAT), kidney, liver, and gastrocnemius and soleus muscles. Once dissected, tissues were weighed with a fine balance (Ohaus, Switzerland). The tissues were placed on dry ice prior to storage at -80°C until subsequent RNA or protein analysis. Tissue dissection and culls were performed at the same time of day ranging from 09:00-12:00 to preventing confounders from circadian rhythm. Additionally, at time of culling, mice were subject to ad libitum feeding.
2.6 RNA QUANTIFICATION

2.6.1 Whole tissue homogenisation for RNA
Frozen tissue samples were cut, on dry ice, into sample sizes of ~30mg for kidney, 40-50mg for iBAT, and ~70mg for iWAT and gWAT. Differing masses of tissue were used based on expected RNA yields from RNA extraction protocols used in the lab. A 1mL volume of Qiazol lysis reagent (79306, Qiagen, Germany) was added to each sample and a 7mm stainless steel bead (autoclaved to remove RNases and DNases) (69989, Qiagen, Germany) was added in 2mL microcentrifuge tubes and placed on ice. Samples were then homogenised using the Tissue Lyser II (Qiagen, Germany), using a 30Hz frequency for 2 minutes. If the sample was not fully homogenised following the initial 2-minute period, a further 2-minute homogenisation was performed. The tissue homogenate was then frozen on dry ice and stored at -80°C until RNA extraction.

2.6.2 In vitro differentiated adipocyte lysis for RNA
For both human and murine differentiated adipocytes, cell culture media was removed from wells and washed with DPBS prior to addition of 500μL of Qiazol lysis buffer. Wells were scraped with a pipette tip before repeated aspiration to ensure lysis of cells. Samples were then frozen on dry ice and stored at -80°C until RNA extraction.

2.6.3 RNA extraction of human in vitro cell culture lysates and murine whole tissue lysates
RNA was extracted from murine whole tissue lysates using the RNeasy lipid Mini Column Kit (Qiagen, USA) and performed following manufacturer’s instructions using a modified protocol as follows:

Tubes containing the homogenate were placed on the benchtop at room temperature for 5 min. A 200 μL volume of chloroform was added to each sample. Samples were vortexed for 15 seconds. Samples were incubated on the benchtop at room temperature for 3 min. Samples were subject to centrifugation at 13000rcf for
15 min at 4°C. The upper aqueous phase (approximately 520μL) was transferred to a new 1.5mL microcentrifuge tube. A 520μL volume of 70% ethanol was added to the upper aqueous phase, and mixed thoroughly by vortexing for 15 seconds. A 700μL volume of the sample was transferred to an RNeasy Mini spin column placed in a 2mL collection tube (supplied in kit). The sample was subject to centrifugation at 13000rcf for 30 seconds at room temperature and the flow-through discarded. This step was repeated with the remaining aqueous phase and ethanol mix. A 700μL volume Buffer RW1 (supplied in kit) was added to the RNeasy Mini spin column. The sample was subject to centrifugation at 13000rcf for 30 seconds at room temperature to wash the membrane and the flow through discarded. A 500μL volume Buffer RPE (supplied with kit) was added to the RNeasy Mini spin column. The sample was subject centrifugation at 13000rcf for 30 seconds at room temperature to wash the membrane. Flow through was discarded. This step was repeated with sample subject to centrifugation at 13000rcf for 2 minutes at room temperature. The RNeasy Mini spin column containing RNA sample was placed in a new 2mL collection tube (supplied in kit). The sample was subject to centrifugation at 13000rcf for 2 minutes at room temperature to eliminate any possible carryover of Buffer RPE or if residual flow through remained on the outside of the RNeasy Mini spin column after wash steps. The RNeasy Mini spin column was placed in a new 1.5mL collection tube (supplied in kit). A 30μl volume of RNase-free water was added directly to the spin column membrane. Samples were incubated at room temperature for 1 minute to allow the membrane to soak in RNase-free water. Samples were subject to centrifugation at 13000rcf for 2 minutes at room temperature to elute RNA from the membrane. Samples were placed on wet ice. The concentration of sample RNA was quantified using the Nanodrop and Nanodrop 1000 software (Marshall Scientific, USA). Nucleotide free water was used as a blank control. The concentration was measured using a 2μL volume of the sample. Following quantitation samples were placed on dry ice and then stored at -80°C until cDNA generation.
2.6.4 RNA extraction of murine in vitro cell culture lysates

RNA was extracted from murine cell culture lysates using an isopropanol and ethanol precipitation protocol as collagen coated plates used for murine cell culture reduced RNA yield with mini column kits. The protocol was performed as follows:

Tubes containing the homogenate were placed on the benchtop at room temperature for 5 min. A 200 µL volume of chloroform was added to each sample containing 500µL of Qiazol lysate. Samples were vortexed for 15 seconds. Samples were incubated on the benchtop at room temperature for 3 minutes. Samples were subject to centrifugation at 13000rcf for 15 minutes at 4°C. The upper aqueous phase (approximately 250µL) was transferred to a new tube 1.5mL microcentrifuge tube. A 250µL volume of 100% isopropanol was added to the upper aqueous phase, and mixed thoroughly by vortexing for 15 seconds. The sample was incubated at room temperature for 10 minutes and then subject to centrifugation at 13000rcf for 10 minutes at 4°C. The supernatant was removed and discarded, leaving behind the RNA pellet. A 1mL volume of 75% EtOH was added to sample tube, then vortexed for 5 second intervals to wash the pellet, the process was performed carefully to ensure RNA pellet integrity. The sample was subject to centrifugation at 13000rcf for 5 minutes at 4°C and the supernatant discarded. The EtOH wash step and centrifugation was repeated. Using a pipette the maximal volume of supernatant was removed from the sample tube, ensuring the RNA pellet was intact and minimal EtOH remained. Samples were air dried for 5-10 minutes. A 30µl volume of RNase-free water was added to the sample. Samples were incubated on wet ice for 5 minutes to allow the RNA pellet to soak in RNase-free water and dissolve. Samples were aspirated multiple times with a pipette to ensure mixing of sample. The concentration of sample RNA was quantified using the Nanodrop and Nanodrop 1000 software (Marshall Scientific, USA). Nucleotide free water was used as a blank control. The concentration was measured using a 2µL volume of the sample. Following quantitation samples were placed on dry ice and then stored at -80°C until cDNA generation.
2.6.5 Reverse transcription of mRNA for cDNA synthesis
The QuantiTect Reverse Transcription Kit was used to generate cDNA. RNA was thawed on wet ice. A mass of 500ng RNA was added to 0.2mL microcentrifuge tubes, the sample volume was calculated from the concentration obtained in 2.6.3 and 2.6.4. The sample volume was made up to 12μL with RNase-free water. A 2μL volume of gDNA wipe-out buffer (supplied in kit) was added to each sample. Samples were incubated at 42°C for 4 minutes, then placed immediately on ice. A 6μL mastermix (containing 4μL Quantiscript RT buffer, 1μL RT primer mix, and 1μL Quantiscript Reverse Transcriptase, per sample) was added to each sample. Samples were incubated at 42°C for 20 minutes, before heating to 95°C for 3 minutes. Samples were then cooled to 4°C. Upon generation of cDNA an equal volume from each individual sample were pooled in order to generate a standard curve using serial dilutions, which were as follows: 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256. The remaining volume of individual cDNA samples were then diluted 1/20 using nucleotide free water. Diluted samples and the standard curve were stored at -20°C.

2.6.6 Real time quantitative PCR of cDNA using SYBR green
Quantification of cDNA was performed by real-time quantitative polymerase chain reaction (RT-qPCR) using the LightCycler 480 system (Roche Diagnostics, Switzerland) and SYBR green I reagent. SYBR green I is a nucleic acid stain and can therefore be used to quantify cDNA (274). All standards, samples, and controls were ran in triplicate for each gene measured. Reaction mixtures were made up to 10μL comprising of the following constituents:

- 5μL of SYBR Green I mastermix (Roche diagnostics, 04887352001)
- 0.1-0.3μL of Forward primer & 0.1-0.3μL Reverse primer (Primer concentrations were optimised for standard curve efficiency, see Table 2. 3 for details)
- 2μL of 1/20 cDNA samples, standards, or negative controls
- 2.4-2.8μL of nucleotide free water (volume dependant on combined primer volume)
Components of reactions were added to a white 384 well-plate (AB1384W, ThermoFisher, UK) whilst on wet ice. Once samples were added a sealing film (AB0558, ThermoFisher, UK) was carefully placed over the wells and secured. The plate was subject to centrifugation at 800rcf for 2 minutes at room temperature. The plate was then immediately used for RT-qPCR or wrapped in foil to avoid light exposure and stored at 4°C overnight. The LightCycler programme was set as per manufacturer’s instructions as detailed in Figure 2.3. Upon completion of the run data were analysed and cleaned manually using the LightCycler 480 software (Roche diagnostics) to remove anomalous triplicate values. Outliers were removed when standard deviations (stdev) of each triplicates Cp were >0.5. The software then calculated a standard curve and the relative concentrations of samples were derived from the curve. The standard curve of each individual gene was optimised for primer concentration to give a curve with efficiency ranging from 1.9-2.2. Samples were then normalised to the mean relative expression of two housekeeping genes, Tbp and RNA18S5 were used for murine samples and RNA18S5 and PPIA were used for human samples.

![Table](image)

**Figure 2.3** Lightcyler programme for qPCR of genes quantified using SYBR green reagent. Hydrolysis programme for qPCR reactions quantified using Roche SYBR
Green I reagent. Programme consisted of a pre-incubation cycle, 45 amplification cycles, before a final melt curve and subsequent analysis.

2.6.7 Real time quantitative PCR of cDNA using Taqman assays
If primer sequences could not be optimised for use with the SYBR green protocol, Taqman probe assays (ThermoFisher, 4331182) were substituted (see Table 2. 4 for details), the LightCycler 480 system (Roche Diagnostics) was also used to run these samples. The reaction mixture composition differed from SYBR green and was as follows:

- 5µL of PerfeCTa qPCR FastMix II (QuantaBio, 97065)
- 2µL of 1/20 cDNA samples, standards, or negative controls
- 2.5µL of nucleotide free water
- 0.5µL of Taqman probe containing primers (ThermoFisher, 4331182)

Results of the run were analysed and cleaned as in 2.6.6, however, the standard curve was not validated as Taqman kits are optimised prior to use.
Table 2. 3 Primer concentrations used for SYBR Green qPCR reactions. Table of SYBR green primer sequences for human and murine genes of interest (including housekeeper genes). Concentrations are for each forward and reverse primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Individual primer concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA18S5</td>
<td>Human</td>
<td>cttccacaggagcctacac</td>
<td>cgcaaaatatgctggaactt</td>
<td>400</td>
</tr>
<tr>
<td>PPIA</td>
<td>Human</td>
<td>gactgagtggtagtgagcc</td>
<td>tcaatggagtgtagctgcaag</td>
<td>400</td>
</tr>
<tr>
<td>UCP1</td>
<td>Human</td>
<td>gtgtgcccaactgtgcaatg</td>
<td>acgtccaggatccagtcga</td>
<td>400</td>
</tr>
<tr>
<td>Rna18s5</td>
<td>Mouse</td>
<td>aaaaggtgctacasatccagaa</td>
<td>cttccatgctgtagctgtaa</td>
<td>400</td>
</tr>
<tr>
<td>Tbp</td>
<td>Mouse</td>
<td>gggagaatctggaggacagaa</td>
<td>gatggaattccaggagtcag</td>
<td>400</td>
</tr>
<tr>
<td>Ucp1</td>
<td>Mouse</td>
<td>gccaagtccccttcagat</td>
<td>tgatttgccttgagatgcc</td>
<td>400</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>Mouse</td>
<td>gaaaggcccaacagagaga</td>
<td>gtaaatcacagggcgtcctt</td>
<td>400</td>
</tr>
<tr>
<td>Sucnr1</td>
<td>Mouse</td>
<td>cccatttctagtttagctttcc</td>
<td>ggacccagctgtgtacctcag</td>
<td>400</td>
</tr>
<tr>
<td>Slc13a3</td>
<td>Mouse</td>
<td>tgtgctccttcctctttg</td>
<td>ctgcgtagctgtagtt</td>
<td>600</td>
</tr>
<tr>
<td>Slc25a10</td>
<td>Mouse</td>
<td>tgggttaactggagcctcg</td>
<td>tccagggcatgagtagttg</td>
<td>400</td>
</tr>
</tbody>
</table>

Table 2. 4 Taqman kits used for qPCR reactions. Table of Taqman kits used for human and murine genes of interest that were unable to be validated for SYBR green.

<table>
<thead>
<tr>
<th>PPARGC1A</th>
<th>Human</th>
<th>Hs00173304_m1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUCNR1</td>
<td>Human</td>
<td>Hs00908230_m1</td>
</tr>
<tr>
<td>Pparg</td>
<td>Mouse</td>
<td>Mm00440940_m1</td>
</tr>
</tbody>
</table>
2.7 PROTEIN QUANTIFICATION

2.7.1 Crude protein extraction in differentiated adipocytes
Media was removed from wells containing differentiated adipocyte cultures and were washed twice with DPBS and placed on wet ice. A 200μL volume of ice cold (approximately 4°C) 0.5% Sodium dodecyl sulphate (SDS) solution was added to each well. Wells were scraped with a P1000 pipette tip to detach cells from the well base, and lysate was aspirated repeatedly to ensure cell lysis. Samples were transferred to a 1.5mL microcentrifuge tube and vortexed for 15 seconds to homogenise the sample. Samples were then subject to centrifugation at 13,000rcf for 15 minutes at 4°C, the supernatant was removed and transferred to a fresh 0.5mL microcentrifuge tube and stored at -80°C.

2.7.2 Whole tissue protein extraction for Western blot
Whole tissue iBAT and iWAT were cut into ~30mg and ~60mg pieces respectively and placed in separate 2mL microcentrifuge tubes each containing one 2mm bead, 400μL RIPA buffer (Thermo Scientific, 89900) and 4μL Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, 1861281), samples were then kept on wet ice. Samples were then homogenised using the Tissue Lyser II set at 30 Hz for 20 seconds and immediately placed on wet ice, these steps were repeated until samples were fully homogenised. The homogenate was transferred to a new 1.5mL microcentrifuge tube and subject to centrifugation at 13000rcf for 15 minutes at 4°C, the supernatant was stored in aliquots at -80°C.

2.7.3 Total protein quantification
Protein concentration and mass were calculated using values obtained using the DC protein assay kit (Bio-rad, 5000112). The protocol was carried out as per manufacturer’s instructions as follows:

A 20μl volume of reagent S was added to each ml of reagent A needed for all samples, this mixture was termed reagent A’. Standard curve dilutions of a BSA solution were prepared containing 0.2 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, and 1.5 mg/ml protein. A 5 μL volume of standards and samples were transferred into a clean, clear
Chapter 2

Materials and Methods

96 well plate. Standards were ran in duplicate and samples in singlicate. A 25µL volume of reagent A' was added to each well. A 200µL volume of reagent B (supplied in kit) was added to each well. The plate was gently agitated to mix the reagents. After 15 minutes, absorbance was read at 750 nm using a plate reader (Molecular devices, USA).

Samples were diluted 1/5 for cell lysates, 1/10 for iWAT and 1/20 for iBAT. All samples were diluted in their respective lysis buffer. Protein concentrations were calculated using sample optical density (OD) values and read off the standard curve, the values were adjusted for their respective dilution factor.

2.7.4 Western blot

Protein aliquots from 2.7.2 were thawed on wet ice. In a 1.5ml microcentrifuge tube 5µL of loading buffer, 2µL of Dithiothreitol (DTT) buffer, 3-12µL of water, and 2mg/µL of sample were added for a total reaction volume of 20µL. If the protein concentration was sufficiently high to require less than 2µL either a 1/10 or 1/20 dilution was performed (using RIPA buffer with halt protease) prior to addition to the microcentrifuge tube, this was done to reduce the likelihood of introducing a pipetting error by using small volumes. Samples were then denatured by incubation at 100°C for 5 minutes in a microcentrifuge tube heat block before immediately being placed on wet ice. The following steps were all performed at room temperature. A gradient mini-gel (Bio-Rad, 4568084) was prepared in a mini gel tank as per manufacturer’s instructions (Bio-RAD, 1704270). In brief, the SDS-PAGE gel was secured in place and running buffer was added to the tank until the wells of the gel were covered. Using a syringe, gel wells were washed to ensure removal of acrylamide. Samples were then loaded into the gel with one lane used for the Chameleon Duo Pre-stained Protein Ladder (Li-Cor, 928-60000). Gels were ran for approximately 75 minutes at 110V. Thereafter, gels were washed in transfer buffer prior to transfer of protein to a nitrocellulose blot using the Trans-Blot Turbo system (Bio-RAD, 1704150). Transfer stacks were comprised of upper and lower ion reservoir stacks (3 upper & 4 lower pads soaked in transfer buffer) with the nitrocellulose membrane and the gel placed atop the membrane between the stacks. The protein
was transferred at 110 volts for 3 minutes for one gel transfer or 7 minutes if transferring two concurrent gels. Following transfer, membranes were washed in Tris-buffered saline with 0.1% Tween-20 (TBS-T) with gentle agitation. Membranes were then immersed in Ponceau S Staining Solution (A40000279, ThermoFisher, UK) for 5 minutes and then washed with ultrapure water until the desired Ponceau stain intensity (approximate visually assessed intensity, not quantified) was achieved to determine successful protein transfer, the remaining stain was removed with further ultrapure water washes. The membrane was then blocked using 5% low fat milk powder (Tesco, UK) in TBS-T for 1 hour at room temperature with agitation, the membrane was then washed three times each for 5 minutes in TBS-T. Following washes the membrane was incubated in primary antibody solution containing both anti-UCP1 and anti-β-actin (5% BSA in TBS-T with a rabbit anti-Ucp1 primary antibody 1:1000, U6382 Merck, Germany; or rabbit anti-β-actin 1:500, ab8227 abcam) overnight at 4°C with agitation. Following overnight incubation, the membrane was washed 3 times each for 10 minutes with TBS-T to remove primary antibody solution. The membrane was then incubated in secondary antibody solution (5% BSA in TBS-T with, for 45 minutes at room temperature before a final three 10 minute TBS-T wash. The membrane was imaged using the Odyssey CLX instrument (Li-Cor, USA) and analysed using Odyssey software (Li-Cor, USA), reading at 700nM for UCP1 and 650nM for β-actin.

Protein expression was calculated by normalising the UCP1 protein band fluorescence to a control protein (β-actin) and then comparing mean fluorescence intensity between groups.

2.8 GLYCEROL ASSAY

Cell culture media samples taken during the lipolysis assay (2.4.7) were thawed on wet ice. Glycerol standard solution (Merck, G7793-5ML) was used to create a serial dilution standard curve encompassing 0.625, 1.25, 2.5, 5, 10, 20, 40 nmoles (per 20μL of standard), with Phenol red free DMEM media used as a blank control and the standard curve diluent. Into a clear 96 well-plate, 20μL of either sample or standards
were added in duplicate. Glycerol reagent (Merck, F6428-40ML) was reconstituted with 40ml of milli-Q water and 160μL of reagent was added to each well. The plate was then incubated at 37°C for 5 minutes before being read at 540nm on an Optimax tuneable microplate reader (Molecular devices, USA). The equation of the standard curve was calculated and used to determine the glycerol concentration from sample OD values, which was then used to calculated glycerol release at each time point by multiplying the concentration by the well media volume remaining at that time-point.

2.9 INSULIN ELISA
Plasma samples taken during the OGTT (2.5.5) were thawed on wet ice. Plasma insulin levels were quantified using the Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, 90080), according to the manufacturer’s instructions for wide range measures. The protocol was performed as follows:

Samples from mice in HFD fed groups were diluted 1/2 in sample diluent (supplied with kit), CD fed mice were analysed undiluted. The Ultra-sensitive insulin ELISA kit was left to equilibrate at room temperature for 30 minutes before use. The stock insulin powder was reconstituted using 100μL of distilled water, to a concentration of 25.6 ng/ml. A serial dilution standard curve, ranging from 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 ng/ml, was derived from the insulin stock solution using the sample diluent. A 5μL volume of standard or sample was added to ELISA kit assay wells (supplied in kit), standards were run in duplicate and samples in singlicate. A 95μL volume of sample diluent was then added to each well prior to incubation at 4°C for 2 hours. Samples were removed from wells using inversion and dried by repeated tapping onto an absorbent surface. Wells were washed 7 times with 200μL of wash buffer (supplied in kit), between each wash the plate was inverted to remove wash buffer and dried. A 100μL volume of conjugate solution was added to each well and incubated for 30 minutes at room temperature. Wash steps were repeated as previously performed. A 100μL volume of substrate solution (supplied in kit) was added to each well and incubated for 40 minutes at room temperature in the dark. A 100μL volume of stop solution (supplied in kit) was added to each well. The plate
was read at 450/630nm, the concentration of samples was derived from the equation of the line of the standard curve.

2.10 STATISTICAL ANALYSIS

2.10.1 Statistical Analysis software
Statistical analyses were performed with GraphPad Prism (version 8.4.30), with Excel software (Microsoft Office Professional Plus 2016) used for formatting of raw data files and calculation of mean values for inputting into GraphPad Prism. Data were presented throughout as a scatter plot with means and, where appropriate, as mean +/- standard error of the mean (SEM). Each dataset was assessed for normal distribution using Shapiro-Wilk, D'Agostino & Pearson test, and Sidak’s multiple comparison. All data sets analysed were normally distributed, therefore, parametric tests were used to perform statistical comparisons. A p-value of 0.05 was the threshold applied for significant statistical effects. Details on sample size, statistical comparisons and significance are described in each figure.

2.10.2 Power calculations
Energy expenditure calculations were powered using Suchacki et al. (43) as a reference for the primary outcome measure of change in energy expenditure in mice. G*Power software was used for power calculations. We determined that a sample size of 8 mice per group would provide power to detect a 20% change in energy expenditure with 90% confidence across sex, diet, and genotype (see Table 2.5 for data). Each cohort of mice in experiment held 10-16 mice to ensure that sufficient n per group were able to be housed in the Phenomaster following 12 weeks of dietary intervention. In total 9 cohorts of mice were needed to fill the study. Cell culture experiments were powered using data taken from Ramage et al (141).
**Table 2.5 Energy expenditure data for power calculations.** Data taken from Suchaki et al. (43) was used to calculate statistical power and necessary numbers per group.

<table>
<thead>
<tr>
<th></th>
<th>Energy expenditure (W/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.93967</td>
</tr>
<tr>
<td>2</td>
<td>21.01431</td>
</tr>
<tr>
<td>3</td>
<td>23.55834</td>
</tr>
<tr>
<td>4</td>
<td>18.56702</td>
</tr>
<tr>
<td>6</td>
<td>20.98374</td>
</tr>
<tr>
<td>8</td>
<td>--</td>
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<tr>
<td></td>
<td>Mean EE</td>
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<tr>
<td>1</td>
<td>21.61664</td>
</tr>
<tr>
<td>2</td>
<td>35.51597375</td>
</tr>
<tr>
<td>3</td>
<td>37.37907875</td>
</tr>
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<td>4</td>
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</tr>
<tr>
<td>6</td>
<td>40.92518</td>
</tr>
<tr>
<td>8</td>
<td>43.39766</td>
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<tr>
<td></td>
<td>st.dev</td>
</tr>
<tr>
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<td>1.914709453</td>
</tr>
<tr>
<td>2</td>
<td>8.43374146</td>
</tr>
<tr>
<td>3</td>
<td>5.046846817</td>
</tr>
</tbody>
</table>
Chapter 3

The Role of the Succinate Receptor in Human Brown and White differentiaded Adipocytes
3.1 INTRODUCTION

SUCNR1 was identified as a G\textsubscript{i} protein coupled receptor with succinate as its endogenous ligand and is expressed both in human brown and white adipocytes (211, 236). As discussed in Chapter 1, SUCNR1 activation regulates multiple cellular processes and has tissue specific functions (275, 276). One of the first identified roles of the succinate receptor was the regulation of blood pressure through interactions with the renin-angiotensin system (211, 237, 238). Since then, SUCNR1 has been shown to regulate skeletal muscle remodelling during exercise inducing a fast to slow switch muscle fibre change (195). In addition, succinate-SUCNR1 signalling has been shown to induce inflammatory responses both in the liver (228) and in macrophages (259). However, myeloid cell specific knockout of Sucnr1 leads to a pro-inflammatory response and activation of SUCNR1 is key in a macrophage inflammation resolution, indicating that SUCNR1 can have both pro- and anti-inflammatory effects (224). These effects are driven by G\textsubscript{i} signalling, however, in cardiomyocytes SUCNR1 activation increases cAMP production and activates the protein-kinase A (PKA) pathway and suggests action through non-G\textsubscript{i} protein coupled activation (248). Together these data highlight that SUCNR1 activation and its signalling pathways remain unclear and that differing responses can occur depending on cell type.

As discussed in chapter 1, SUCNR1 has role in energy homeostasis inhibiting lipolysis in WAT. The knockout of Sucnr1 leads to a moderately lean phenotype in male mice control diet fed mice, but Sucnr1 knockout on a HFD increased adiposity and induced glucose tolerance (234). This contradictory effect is likely due to dysregulation in circadian clock genes during HFD, leading to reduced leptin expression in WAT. This reduced leptin expression resulted in dysregulation of energy homeostasis and hyperphagia leading to increased adiposity and glucose intolerance (263). Whilst these data indicate an important role of SUCNR1 in adipose tissue biology and whole-body energy expenditure, the role of SUCNR1 in BAT is poorly understood. In differentiated murine brown adipocytes, supra-physiological doses of succinate increased brown adipocyte basal oxygen consumption, indicating activation of thermogenesis (146). This increased OCR was through uptake of succinate into the
Chapter 3

The Role of the Succinate Receptor in Human Brown and White differentated Adipocytes

Brown adipocyte leading to increased reactive oxygen species (ROS) generation via oxidation of SDH, a key enzyme in the TCA cycle, and was mediated through an UCP1-dependant manner in mice. However, the 1-10mM doses of succinate used exceed typical circulating concentrations and as such do not accurately reflect in vivo physiological conditions which show circulating succinate at concentrations of ~25μM in healthy individuals, but range from 50-100μM in obese or individuals with T2DM (241, 244). Additionally, myeloid specific knockout of Sucnr1 in mice impaired white adipocyte browning in response to cold exposure, highlighting the role of SUCNR1 signalling in adipose tissue resident macrophages on WAT thermogenesis (224). These data indicate that both SUCNR1 and succinate have a role in modulating thermogenesis, however, the role of SUCNR1 activation in BAT is currently unknown. As discussed in section 1.4.3 RNA-sequencing analysis of human differentiated brown and white adipocytes identified ~20-fold greater expression of SUCNR1 in brown adipocytes, these data suggest that the succinate receptor may have a role in regulating human brown adipocyte function.

During brown adipocyte thermogenesis there is significant uptake of metabolites to fuel thermogenesis including succinate (146, 207). However, succinate overall is net released from BAT during both cold exposure and β3-AR activation (207) although untested it is possible that this succinate release could increase interstitial concentrations and activate SUCNR1 during thermogenesis. As discussed in section 1.4.1.2 succinate transport in the cell is not fully understood. Succinate is produced in the mitochondria but is exported out of the mitochondria and the cell via SLC25A10 (an inner mitochondrial membrane transporter found in both brown and white adipocytes) (146, 277). Additionally succinate can be imported into the cell from interstitial fluid via SLC13A3, a surface dicarboxylic acid transporter (278, 279). MCT1 was also shown to facilitate succinate transport into the brown adipocyte, however, knockout of the transporter did not significantly alter total succinate transport (200). As such, succinate transport in brown adipocytes remains unclear.
Circulating succinate is increased in overweight/obese individuals and in patients with T2DM (241), it is also associated with increased VAT mass, serum triglycerides, and serum C-reactive proteins levels (241, 244). The concentration of circulating succinate seen in obesity/T2DM (50-100μM) would be sufficient to activate SUCNR1 (224, 228, 234). As both succinate and SUCNR1 are linked with metabolic health it is important to understand the mechanisms they have in regulating BAT function and if this has an impact on energy expenditure and thermogenesis. In order to determine the role of SUCNR1 and succinate in brown adipocyte thermogenic processes an appropriate experimental model is needed. Using a human in vivo model to study the effect of succinate on BAT function would be difficult. Administering succinate intravenously would likely induce off target effects in other tissues, and whilst a more targeted approach such as the microdialysis technique to infuse succinate into the interstitial fluid surrounding BAT is possible, measuring and quantifying the effects on BAT function and activity would difficult. However, using differentiated in vitro cultures of brown and white adipocytes allow for direct measurement of the cellular responses to SUCNR1 agonism.

The Stimson lab has extensive experience of culturing human primary brown and white adipocytes as an in vitro model to study regulation of human BAT (109, 141). Pre-adipocytes are isolated from the SVF of human subjects undergoing elective neck surgery and are then differentiated in culture. These differentiated brown adipocytes demonstrate increased expression of many of the genes associated with thermogenesis such as UCP1, PPARGC1A, and PRDM16 compared to paired white adipocytes. Additionally, these cultured cells respond to adrenergic stimulation increasing respiration and UCP1 expression, further validating the in vivo relevance of this model. Therefore, this is a suitable approach to determine the role of SUCNR1 in human brown adipocytes, through incubation with both succinate and the specific SUCNR1 agonist C-ESA (211, 236). During thermogenesis, brown adipocyte oxygen consumption is increased with the metabolic demand of increased energy expenditure (280, 281). As such, quantification of cellular respiration is a marker of thermogenesis. The respirometry assays in this chapter were designed to detect...
differences in the role of succinate and SUCNR1 activation in the adipocytes with the use of the agonist C-ESA. C-ESA does not cross the plasma membrane (211), and is a potent agonist of SUCNR1 and has previously been shown to elicit activation in human cell lines (236). Therefore, using both succinate and C-ESA would identify how both SUCNR1 agonism and intracellular succinate uptake altered adipocyte respiration. Thermogenic gene expression was measured in both brown and white adipocytes to indicate if SUCNR1 activation induced a browning phenotype. Additionally, proliferation of cells \textit{in vitro} allowed for multiple comparisons to be made within an individual patient. Lipolysis was also measured in this \textit{in vitro} model to determine whether SUCNR1 activation inhibited lipolysis in BAT in addition to WAT (234) but succinate treatment increasing respiration in BAT (228) appear contradictory with regards to \textit{SUCNR1} activation increasing BAT thermogenesis.

\textbf{3.2 HYPOTHESIS}

Activation of the succinate receptor increases human brown adipocyte respiration and \textit{UCP1} expression.

\textbf{3.3 AIMS}

1. To determine the effect of succinate and SUCNR1-agonist treatment on basal and noradrenaline-stimulated respiration in brown and white differentiated pre-adipocytes;

2. To determine the effect of succinate and SUCNR1-agonist treatment on basal and noradrenaline-stimulated thermogenic gene expression in brown and white differentiated pre-adipocytes;

To determine the effect of succinate and SUCNR1-agonist treatment on basal and noradrenaline-stimulated lipolysis in human primary brown and white differentiated pre-adipocytes.
3.4 METHODS

3.4.1 Human pre-adipocytes isolation and cell culture
Paired samples of human BAT and WAT tissues were obtained from patients undergoing elective neck surgery at the Royal Infirmary Edinburgh and were isolated as described in Chapter 2.4.2.2. Cells were cultured and differentiated as described in 2.4.4.

3.4.2 Quantification of cell culture media succinate concentrations LC-MS/MS
Succinate concentrations were quantified in cell culture medium by LC-MS/MS, using a novel method developed locally. Succinate standard was sourced from Sigma Aldrich (398055) all other reagents were HPLC grade quality and sourced from VWR (Leicestershire, England). The standard curve used concentrations as follows: 2μM, 5μM, 10μM, 20μM, 50μM, 80μM, and 100μM. Standards were made up in HPLC grade water with 0.1% formic acid. This method utilised the Waters Acquity I-Class UPLC system, and an Intrada organic acid column (150 x 2 mm; 3 um) (TR09A, Imtakt, USA) for the LC component with a temperature of 37°C and flow rate of 0.2 mL/min. For the mass spectrometry component a Sciex Qtrap 6500+ mass spectrometer operated in ESI in negative ion MRM mode (500C, -4.5kV) was used. The mobile phases used were:

- Mobile phase A – Water/Acetonitrile (90:10) 0.1% formic acid
- Mobile phase B – 100 mM ammonium formate/acetonitrile (90:10 v/v)

These mobile phases were selected as per manufacturer’s instructions for the Intrada organic acid column. The total run time was 15 minutes with Mobile phase B being introduced as a gradient elution at 2 minutes and reaching 100% of flow on column at 7 minutes and continued for a further 3 minutes (10 minutes total) before Mobile Phase A was re-introduced to re-equilibrate column to 100% Mobile phase A. The mass to charge transitions (m/z) of succinate were 117->99.
3.4.3 Effect of succinate and C-ESA on human brown and white adipocyte respiration

Human differentiated brown and white pre-adipocytes were treated with vehicle control (sterile dH₂O), 100μM succinate, 1000μM succinate, 1μM C-ESA, 10μM C-ESA, and 100μM C-ESA (6 groups in total, see Figure 3.1 for plate map). Within each cell culture plate conditions were performed in triplicate with the exception of vehicle and 100μM C-ESA which were performed in quadruplicate. A total of 9 subjects were used for brown and white adipocyte culture.

<table>
<thead>
<tr>
<th>Blank well</th>
<th>Succinate 1000μM</th>
<th>Vehicle 2</th>
<th>C-ESA 1μM</th>
<th>Succinate 1000μM</th>
<th>Blank well</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Succinate 100μM 2</td>
<td>C-ESA 100μM 2</td>
<td>C-ESA 1μM 3</td>
<td>Vehicle 4</td>
</tr>
<tr>
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<td>C-ESA 10μM 1</td>
<td>Succinate 1000μM</td>
<td>Vehicle 3</td>
<td>C-ESA 10μM 3</td>
<td>C-ESA 100μM 4</td>
</tr>
<tr>
<td>Blank well</td>
<td>C-ESA 100μM 1</td>
<td>C-ESA 1μM 2</td>
<td>Succinate 100μM 3</td>
<td>C-ESA 100μM 3</td>
<td>Blank well</td>
</tr>
</tbody>
</table>

Figure 3.1 Plate map for human respirometry assays. Schematic diagram of 24 well plate and conditions for respirometry assays using Seahorse V7 plates for brown and white differentiated pre-adipocytes.

Respirometry assays were performed as described in 2.4.8.1. In brief, human pre-adipocytes were cultured in Agilent seahorse plates and differentiated as per protocol. Once fully differentiated, paired primary brown and white human adipocytes were incubated with drug treatments. Drug treatment media did not contain FBS or BSA. Doses of succinate used were greater than the EC50 of the receptor (27-50μM), as with C-ESA which is a more potent agonist (2.3μM) (282). Differentiated adipocytes were treated either vehicle (sterile distilled water), 100μM succinate, 1000μM succinate, 1μM C-ESA, 10μM C-ESA, or 100μM C-ESA (6 groups in total) for 2 hours prior to measurement of OCR. Following incubation cells were
analysed in a Seahorse XFe 24 instrument measuring oxygen consumption rate, with injections of NADR (2µM), oligomycin (2µg/ml), Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (2µM), and rotenone/antimycin A (R/A) 0.2µM/2.5µM. NADR was injected to measure adrenergic-stimulated respiration, followed by oligomycin to quantify uncoupled respiration, and FCCP to quantify maximal respiratory capacity. The injection of rotenone/antimycin A was done in order to subtract non-mitochondrial respiration (see Figure 2.1). Data from the respirometry assays are presented as the percentage of the vehicle group during basal conditions. Data were presented this way as variation in total differentiation percentage of adipocytes between subjects caused significant variation in absolute OCR values. Data were then presented as OCR during total time course of the experiment, the mean basal, NADR stimulated OCR, mean ATP linked respiration (calculated by subtracting mean oligomycin OCR from the final NADR OCR value), and mean maximal respiration (see Figure 2.1).

3.4.4 Effects of succinate and cis-epoxysuccinic acid on UCP1 mRNA expression in human brown and white adipocytes

3.4.4.1 Dose response to succinate and cis-epoxysuccinic acid on UCP1 expression

To determine the effect of SUCNR1 agonism on UCP1 mRNA levels, human differentiated brown and white pre-adipocytes were treated with vehicle control (sterile dH₂O), 100µM succinate, 1000µM succinate, 1µM C-ESA, 10µM C-ESA, and 100µM C-ESA in serum free media (6 groups in total, see Figure 3.2 for plate map). Pre-adipocytes were isolated from 10 subjects in this experiment. However, 2 of the subjects brown adipocytes demonstrated no UCP1 expression so were determined to in fact be white adipocytes and were excluded from the analyses. Following 8 hours incubation with drug containing media, cells were lysed with Qiazol reagent for RNA extraction and subsequent qPCR. UCP1 mRNA expression was quantified and normalised to the mean expression of housekeeping genes RNA18S5 and PPIA.
**Chapter 3**  
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<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Vehicle/Vehicle</th>
<th>C-ESA 1μM</th>
<th>C-ESA 1μM</th>
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<tr>
<td>Succinate 100μM</td>
<td>Succinate 100μM</td>
<td>C-ESA 10μM</td>
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<td>Succinate 1000μM</td>
<td>Succinate 1000μM</td>
<td>C-ESA 100μM</td>
<td>C-ESA 100μM</td>
</tr>
</tbody>
</table>

**Figure 3.2 Plate map for mRNA dose response assay.** Schematic diagram of 12 well plate and conditions for mRNA expression assays investigating dose response to succinate and C-ESA.

3.4.4.2 **Effects succinate and cis-epoxysuccinic acid on thermogenic gene expression in basal and noradrenaline stimulated adipocytes**

To determine the role of SUCNR1 on mRNA expression in differentiated brown and white adipocytes during both basal and NADR stimulated conditions, cells were treated with either vehicle, 1000μM succinate, or 10μM C-ESA at basal conditions or in the presence of 1μM NADR in serum free media (6 groups in total in a 2x3 design (see Figure 3. 3 for plate map). Pre-adipocytes were isolated from 8 subjects for use in this experiment. However, 2 of the subjects showed no UCP1 expression and were therefore excluded from the analysis. All conditions were run in duplicate. Following 8 hours incubation, cells were lysed with Qiazol reagent for RNA extraction and subsequent qPCR. *UCP1, PPARGC1A, and SUCNR1* mRNA expression were quantified and normalised to the mean expression of housekeeping genes *RNA18S5* and *PPIA*. 

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Figure 3. 3 Plate map for basal and NADR stimulated mRNA expression assays, and lipolysis assays. Schematic diagram of 12 well plate and conditions for mRNA expression assays investigating basal and NADR stimulated mRNA expression and lipolysis after drug treatment of succinate and C-ESA

3.4.4.3 qPCR quantification of mRNA expression in human differentiated adipocytes
RNA was isolated and processed as described in 2.6.2, 2.6.4, 2.6.5. The mRNA expression was quantified as described in 2.6.6.

3.4.5 Effect of succinate and C-ESA on brown and white adipocyte lipolysis
To determine whether succinate and C-ESA altered glycerol release (a marker of lipolysis), human brown and white differentiated pre-adipocytes were treated with either vehicle, 1000μM succinate, or 10μM C-ESA at basal conditions or in the presence of 1μM NADR in serum free media (6 groups in total in a 2x3 design. See Figure 3. 3 for plate map). Aliquots of media (60μL) were obtained at 8 and 24 hours and placed on dry ice and storage at -80°C until analysis. Pre-adipocytes were isolated from 8 subjects for use in this experiment. However, 2 of the brown adipocyte cultures failed to differentiate and as such were not used in the experiment due to no lipid accumulation or differentiation.

Glycerol was measured as described in 2.8. Initial testing of cell culture media samples identified 8 and 24 hour as the optimal time points, as glycerol concentrations were below the lowest point of the standard curve at earlier time
points. The glycerol concentration was normalised to total protein content per well, with cells lysed for protein following the 24 hour time point (2.7.1). Cellular protein content was measured as described in 2.7.3.

3.4.6 Statistical analysis
Statistical tests and analysis were performed as described in 2.10.1. Data were analysed with either RM two-way or RM one-way ANOVA and presented as mean +/- SEM. Data were tested for normality using Shapiro-Wilk and D'Agostino & Pearson test. Significance was determined when p<0.05.
3.5 Results

3.5.1 Quantification of cell culture media succinate concentration

In empty DMEM media (without the addition of FBS) there was no detectable succinate. However, the succinate concentration in proliferation media (DMEM with the addition of 10% v/v FBS) was measured at 32.5μM (Table 3.1). As such, subsequent experiments were performed in serum free media to reduce confounders of additional succinate.

Table 3.1. Succinate is present at sufficient concentrations in cell culture media to activate SUCNR1. LC-MS/MS analysis of proliferation media shows that succinate is present at 32.5μM. Standard curve $R^2 = 0.9979$.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Succinate (m/z 117 - &gt; 99) μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation media</td>
<td>32.5</td>
</tr>
</tbody>
</table>

3.5.2 Succinate and C-ESA increase human brown adipocyte respiration

Both succinate (100μM and 1000μM concentrations) and C-ESA (10μM) increased brown adipocyte oxygen consumption (Figure 3. 4 A). Both succinate concentrations increased basal OCR while only 1000μM succinate increased OCR following NADR stimulation (Figure 3. 4 B-C). 10μM C-ESA increased NADR-stimulated respiration compared to vehicle control (Figure 3. 4 C). Neither succinate nor C-ESA altered ATP-linked or maximal respiration in brown adipocytes (Figure 3. 4 D-E). In white adipocytes, 1000μM succinate increased OCR both during basal conditions and following NADR (Figure 3. 5 B-C), without altering ATP-linked or maximal respiration (Figure 3. 5 D-E). C-ESA did not alter OCR in white adipocytes.
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A. Brown adipocytes oxygen consumption rate

B. Mean basal oxygen consumption rate

C. NADR stimulated oxygen consumption

D. Mean ATP linked oxygen consumption rate

E. Maximal oxygen consumption rate
Figure 3.4 Oxygen consumption rate increases in human differentiated brown adipocytes in response to SUCNR1 agonism. Oxygen consumption rate in differentiated human brown (n=9/group) adipocytes treated with either vehicle (black columns), 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 2 hours and measured at basal conditions (1) and following NADR treatment (2), Oligomycin treatment (3) and FCCP treatment (4), (A). OCR during basal conditions (B), following NADR (C), mean ATP linked respiration (D), and maximal respiratory capacity (E). Data are presented as mean +/- SEM, data were analysed using (A) two-way RM ANOVA with Fisher’s post-hoc LSD testing; (B-E) one-way RM ANOVA, Fisher’s LSD test; # = p<0.05 for 100μM succinate vs vehicle; *=p<0.05, **=p<0.01, ***=p<0.001 for 1000μM succinate vs vehicle; @=p<0.05 for 10μM C-ESA.
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A. White adipocytes oxygen consumption rate

B. Mean basal oxygen consumption rate

C. NADR stimulated oxygen consumption

D. Mean ATP linked oxygen consumption rate

E. Maximal oxygen consumption rate

Drug treatment; p=0.0047

Drug treatment; p=0.01

Drug treatment; p=0.66

Drug treatment; p=0.3

Succinate/C-ESA treatment
p=0.091
Figure 3. 5 Succinate but not C-ESA increases respiration in human differentiated white adipocytes. Oxygen consumption rate in differentiated human white (n=9/group) adipocytes treated with either vehicle (black columns), 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 2 hours and measured at basal conditions (1) and following NADR treatment (2), Oligomycin treatment (3) and FCCP treatment (4), (A). OCR during basal conditions (B), following NADR (C), mean ATP linked respiration (D), and maximal respiratory capacity (E). Data are presented as mean +/- SEM, data were analysed using (A) two-way RM ANOVA with Fisher’s post-hoc LSD testing; (B-E) one-way RM ANOVA, Fisher’s LSD test; *=p<0.05, **=p<0.01 for 1000μM succinate vs vehicle.
3.5.3 Succinate and C-ESA do not alter *UCP1* expression in human brown or white adipocytes

Neither succinate nor C-ESA, at any dose, altered *UCP1* mRNA levels in human brown or white adipocytes in the basal state (Figure 3. 6 A-B). Therefore, the effect of succinate and C-ESA was determined in a separate experiment during noradrenaline stimulation. Similar to the initial experiment, *UCP1* expression was unaltered by succinate or C-ESA during vehicle or noradrenaline stimulation in brown and white adipocytes (Figure 3. 7 A-B). However, NADR significantly increased *UCP1* expression in brown adipocytes and tended to do so in white adipocytes (283). *PPARGC1A* expression was unchanged in either the basal or NADR stimulated states in either human brown (Figure 3. 7 C) or white adipocytes (Figure 3. 7 D). *SUCNR1* expression was also unaltered following succinate or C-ESA treatment in both brown and white adipocytes and unaltered by noradrenaline (Figure 3. 7 E-F).
**Figure 3.6 Succinate and C-ESA do not alter UCP1 expression.** UCP1 mRNA levels in differentiated human (A) brown (n=8/group) and (B) white (n=10/group) adipocytes treated with either vehicle (black columns), 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 8 hours. Data are mean ± SEM and were analysed by two-way repeated measures ANOVA.
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Figure 3.7 Succinate and C-ESA do not alter thermogenic gene expression of SUCNR1 at basal and NADR stimulated conditions. Basal and NADR stimulated mRNA expression of UCP1 (A-B), PPARG1A (C-D), SUCNR1 (E-F) in differentiated human brown (A, C, E) (n=6/group) and white (B, D, F) (n=8/group) adipocytes incubated with either vehicle (black), 1000μM succinate (orange), or 10μM C-ESA (blue). Data are represented as mean ± S.E.M and were analysed by RM two-way ANOVA.
3.5.4 SUCNR1 agonism reduces white but not brown adipocyte lipolysis

Glycerol release in human brown adipocytes was not altered by either succinate or C-ESA treatment in either the basal state or during NADR stimulation (Figure 3.8 A-B). In human white adipocytes, neither succinate nor C-ESA changed basal glycerol release (Figure 3.8 C), however, 10μM C-ESA reduced glycerol release during NADR stimulation (Figure 3.8 D).
Figure 3. 8 Succinate and C-ESA do not alter glycerol release in human brown adipocytes. Glycerol release in differentiated human brown (n=6 brown adipocytes) (A-B) and white (n=8 white adipocytes) (C-D) adipocytes in the basal state (A, C) and during NADR stimulation (B, D) following incubation with either vehicle (black), 1000μM succinate (orange), or 10μM C-ESA (blue). Data are represented as mean ± S.E.M, RM two-way ANOVA. *; p<0.05 for vehicle vs 10μM C-ESA.
3.6 Discussion

This lab previously identified that mRNA expression the succinate receptor (encoded by SUCNR1) was 20-fold higher in human differentiated brown adipocytes compared to white adipocytes (210). Succinate had also been shown to increase respiration in both human and murine brown adipocytes (146), while succinate was sequestered by brown adipocytes during CIT. Sucnr1−/− mice have increased adiposity and impaired glucose tolerance in both global (234) and adipose tissue specific (263) knockout models. However, the role of the succinate receptor in BAT function remains unclear.

In this chapter I aimed to determine the role of SUCNR1 in human brown and white adipocyte function and if activation of the receptor increased thermogenesis. To achieve this, we treated human differentiated adipocytes with both succinate and C-ESA and assessed alterations in brown adipocyte function. In brief, succinate and C-ESA treatment increased NADR-stimulated respiration in brown adipocytes, in the white adipocytes only succinate increased respiration. Gene expression was unaffected by either succinate or C-ESA treatment in brown and white adipocytes. C-ESA treatment reduced glycerol release in white adipocytes only, no effect was seen by succinate or C-ESA in brown adipocytes.

In the respirometry experiment, brown and white adipocytes were treated with either succinate or C-ESA for 2 hours as acute succinate treatment has previously been tested (146). Succinate increased both basal and NADR-stimulated respiration, while C-ESA only increased NADR-stimulated respiration. Therefore, these results are consistent with the hypothesis that succinate increases brown adipocyte respiration both intracellularly, through succinate uptake into the cell and mitochondria, and extracellularly, through activation of SUCNR1. The succinate results are in keeping with data demonstrating that succinate drives brown adipocyte thermogenesis through increased reactive oxygen species (ROS) generation which was unaffected by SUCNR1 knockout (146, 228). However, as C-ESA increased NADR-stimulated respiration, this shows that activation of the receptor contributes to thermogenesis in brown adipocytes. Only high dose succinate increased respiration in white adipocytes, which occurred basally and during NADR stimulation. C-ESA had no effect
on white adipocyte OCR which is consistent with the low SUCNR1 expression in these cells. These data indicate that high dose succinate increases OCR independently of SUCNR1 activation in both white and brown adipocytes. However, SUCNR1 activation in the brown adipocytes does increase NADR stimulated respiration, highlighting differing effects of SUCNR1 agonism between white and brown adipocytes. It should be noted that the observed increase in NADR respiration was using 1000μM succinate which is a supraphysiological dose so the in vivo relevance of this data is unclear.

As UCP1 is instrumental to brown adipocyte function it was necessary to assess how incubation with succinate and C-ESA altered UCP1 expression. However, neither succinate nor C-ESA altered UCP1 expression during either basal conditions or following NADR stimulation in brown or white adipocytes. These data suggest that during this 8-hour incubation period SUCNR1 agonism does not affect UCP1 mRNA expression and does not induce browning in white adipocytes despite succinate increasing respiration. These data may indicate that succinate and C-ESA increase respiration via a UCP1-independent mechanism. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) is a transcriptional co-activator which regulates BAT differentiation through increasing mitochondrial biogenesis (284). Ppargc1a deficient mice are cold intolerant and are unable to maintain core body temperature in excess of 6 hours at 4°C (285), highlighting its key role in thermogenic function. Following 8 hours incubation with either succinate or C-ESA there was no change observed in PPARGC1A mRNA expression, suggesting that neither succinate nor SUCNR1 agonism regulates thermogenic gene expression in brown or white adipocytes. However, only mRNA expression was quantified due to a lack of cell material which excluded quantification of protein expression. Therefore, protein expression of these genes should be quantified to corroborate these data. Post-translational modification of UCP1 has been shown to alter its expression and the mRNA analysis alone may not depict the functional levels of UCP1 (286, 287).
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However, another possibility for the lack of any change in UCP1 could be the prevailing succinate concentrations in the cell media. For this reason, we utilised a tandem mass spectrometry assay to measure succinate in the cell media to determine if it was a potential confounder. We found that succinate was abundant in the cell culture media (due to the FBS) with concentrations in the reported EC50 range of human SUCNR1. Therefore, it is likely that during both proliferation and differentiation of cell culture that the succinate receptor is being continuously activated, albeit, with lower doses (~30μM) than those used in our experiments. Chronic SUCNR1 activation may confound measurements by causing activation of the receptor prior to commencement of the experiment. Removing FBS from the media and culturing differentiated adipocytes without serum for extended periods is often detrimental to cellular function, our attempts to culture human differentiated adipocytes without serum caused significant cell death after 24-48 hours. As such, a prolonged washout period prior to experimental start was deemed unfeasible, it is possible that the lack of transcriptional changes observed in UCP1 and PPARGC1A mRNA levels could be attributed to this lack of succinate washout. Future experimental designs to accommodate this requirement to culture cells with succinate containing FBS would be to perform siRNA-induced knockdown SUCNR1 in differentiated brown adipocytes, this technique has been successfully performed previously with target genes of interest (288).

Induction of lipolysis is a key component of brown adipocyte thermogenesis triggered by the increase in cAMP following activation of adrenergic receptors (289). As FAs generated by intracellular lipolysis are utilised by UCP1 to facilitate the transport of protons across the mitochondrial membrane, modulation of FA availability can affect brown adipocyte function (87, 290). Succinate induced activation of SUCNR1 inhibits isoproterenol stimulated lipolysis in murine WAT explants cultured without the presence of insulin (223, 234). However, the role of SUCNR1 in lipolysis had not been investigated in human white or brown differentiated adipocytes. C-ESA reduced glycerol release in human differentiated white adipocytes only during NADR stimulation. These data show that both human
and murine white adipocyte lipolysis is regulated by SUCNR1. However, brown adipocyte lipolysis was unaffected SUCNR1 agonism in both the basal and NADR stimulated state, this is despite brown adipocytes having greater expression of SUCNR1. Therefore, it seems likely that the activation of SUCNR1 in brown adipocytes has a different regulatory function than white adipocytes, at least in humans. Succinate is sequestered from circulation by brown adipocytes and increases thermogenesis by generation of ROS (146, 228). This increased thermogenesis could in turn increase intracellular lipolysis mitigating the inhibitory effects of SUCNR1 activation. C-ESA was also unable to reduce lipolysis in the brown adipocytes which indicates that SUCNR1 activation does not regulate brown adipocyte lipolysis. Previous research on the efficacy of C-ESA has predominantly been in rodent models. However, using bioluminescence resonance energy transfer (BRET) assays monitoring G protein activation, C-ESA has been shown to effectively activate SUCNR1 more potently than succinate in human cell lines (291). These data indicate that C-ESA likely activates SUCNR1 to the same extent in the cell culture model used in this study and the lack of reduction in lipolysis is unlikely to be due to lack of efficacy of the agonist. Despite being below the statistical threshold of $p=0.05$, it should be noted that in the brown adipocytes the vehicle control group had large variation within the data, but mean values were approximately 35-40% higher in the vehicle group compared to both succinate and C-ESA, these differences and variation could indicate that this experiment was underpowered to statistically detect the observed effect. Due to glycerol recycling in BAT (109), it is likely this could have confounded measurements in the cell culture assay. However, with available data it appears SUCNR1 agonism does not affect brown adipocyte lipolysis but does decrease white adipocyte lipolysis, which could indicate differing G protein response to activation. Although previously reported as primarily a $G_i$-coupled GPCR (211), it has been speculated that SUCNR1 may also have $G_q$ coupled receptor functions (248, 291). Therefore, the cell specific differences in SUCNR1 activation between white and brown adipocytes could be mediated through differing signalling cascades as a result of altered G protein action. $G_i$ signalling decreases cAMP mediated through adenylyl cyclase (AC) modulation, however, $G_q$ signalling induces calcium ($Ca^{2+}$) release from intracellular reservoirs (215). As the succinate receptor has tissue-
specific functions and roles with apparent differences in G protein signalling (276), it is therefore possible that brown and white adipocytes are under differing regulatory control of SUCNR1. However, we did not selectively inhibit G\textsubscript{i} and G\textsubscript{q} signalling in cell culture assays for each cell type in this chapter. Inhibition of G\textsubscript{i} and G\textsubscript{q} signalling in these cells would be key to determine through which mechanisms activation of the receptor is driving the observed phenotypes and if they differ between white and brown adipocytes.

To further elucidate the role of SUCNR1 in human brown adipocytes either a knockdown or inhibition of the succinate receptor would be required. In murine adipocytes due to the availability of global or tissue-specific knockout mice it is easier to acquire cells lacking the receptor, however, in humans other methods are needed to generate such a cell culture model. The receptor could either be inhibited by an antagonist or through knockdown using siRNA protocols that would reduce/inhibit expression of the gene and therefore protein (288). Additionally, there is a commercially available SUCNR1 antagonist, named NF-56-EJ40 (MedChemExpress, USA), with an IC\textsubscript{50} of 25nM (218, 292). Use of antagonists can be an effective way to determine the role of an individual receptor but it can be challenging to determine if responses are due to the desired inhibitory effect or if there are off target effects from the antagonist being non-specific. Therefore, knockdown of this receptor would be a preferable approach for future research. Our group have recently undertaken siRNA knockdown of the serotonin receptors (HTR2A and HTR2B) in human differentiated brown adipocytes previously, this demonstrated ~85% knockdown showing proof of concept of the technique’s efficacy (210). Similar experiments to those detailed in this chapter could be repeated in such cells.

Since SUCNR1 does not alter UCP1 expression or modulate intracellular lipolysis (and by extension the available FA to be utilised by UCP1), the activation of the receptor may be driving thermogenesis through non-UCP1 mediated pathways. Creatine cycling has been identified as having a role in thermogenesis in both brown and beige
adipocytes in murine models. Creatine enhances the respiration of beige adipocytes and cold exposure increased creatine kinase activity (92). Creatine kinase can catalyse the reversible transfer of the γ-phosphate group of ATP to the guanidine group of creatine to form ADP and phosphocreatine, producing energy. Further, pharmacological reduction of creatine levels in mice, reduced β3-agonist-stimulated energy expenditure and BAT/beige adipose tissue metabolic activity (92). Importantly, heat production secondary to creatine cycling was observed in beige adipocytes with and without UCP1 (293), and reducing creatine levels in Ucp1 knockout mice lowered core body temperature (92). Futile Ca\(^{2+}\) cycling in beige adipocytes also contributes to thermogenesis via ATP-dependent Ca\(^{2+}\) cycling mechanisms mediated through SERCA2b and the Ca\(^{2+}\) release channel ryanodine receptor 2 (RyR2) (294). It is possible that SUCNR1 increases brown adipocyte thermogenesis through UCP1-independent pathways, but these were not investigated in this chapter and require further research to elucidate the possible mechanisms.

In conclusion, these data reveal that SUCNR1 agonist treatment increases noradrenaline-stimulated respiration in human differentiated brown adipocytes, without altering thermogenic gene expression or intracellular lipolysis. This indicates that there is a functional role of SUCNR1 in human brown adipocyte thermogenesis and energy expenditure but the mechanisms driving this are not clear. This research has also determined that SUCNR1 activation in white adipocytes does not induce a beiging phenotype, while high dose succinate increases white adipocyte respiration independent of SUCNR1. The subsequent chapters will address how Sucnr1 knockout in an in vivo murine model effects energy expenditure at the whole organism level specifically with regards to cold induced thermogenesis.
Chapter 4

Effects of Succinate Receptor Disruption on Murine BAT Function and Metabolic Health
4.1 INTRODUCTION

In Chapter 3, I determined that succinate and C-ESA treatment increased respiration in differentiated human brown adipocytes. Although succinate and C-ESA increased energy expenditure in brown adipocytes, the effect of succinate was more pronounced than C-ESA despite the increased potency of C-ESA as a SUCNR1 agonist (211). The data indicate succinate may activate brown adipocytes through distinct pathways from SUCNR1 activation. Human in vivo studies on SUCNR1 function in brown adipose tissue are not feasible due to the lack of licensed SUCNR1 agonists for human use. However, in mice due to the potential for transgenic manipulation the effect of SUCNR1 can be more easily investigated, indeed, there are previously characterised and commercially available Sucnr1−/− mice (224, 234, 264, 295). Mouse BAT is active at 21°C, the typical housing temperature for experimental mice (181). The typical thermoneutral range of an adult mouse ranges from 28-33°C and varies diurnally (180, 296). Housing mice at standard room temperature can increase the metabolic demands (caloric intake and energy expenditure) by approximately 50% than at thermoneutral conditions (297). Indeed, due to these alterations in metabolic requirements it is possible to interpret data as false positives when housed at room temperature if the genetic modification of the mouse effects thermoregulatory processes that do not directly involve BAT function (297). Mice with deletion of Ucp1 are unable to tolerate cold exposure (4°C) without appropriate conditioning, showing the importance of functional BAT during acute cold exposure (298). Therefore, assessing BAT function at more than one temperature (e.g., housing at 4°C and 21°C or 30°C) is preferable to determine if BAT function is altered in the Sucnr1−/− mouse. Although mice have different dietary and nutritional requirements to humans, they respond similarly to HFD with regards to development of obesity and T2DM (reviewed in (299)). Therefore, diet induced obesity mouse models are an ideal method in which to study obesity and T2DM (300). Sucnr1−/− male mice have increased adiposity when fed a HFD (234), and dietary succinate supplementation reduced body fat mass in male mice (146). Additionally, myeloid specific knockout of Sucnr1 reduced browning in mouse inguinal adipose tissue (224), which supports reported data showing succinate as a thermogenic activator of brown and beige adipocytes (146). As discussed in 1.5.2.6, recent phenotyping of an adipose tissue
specific *Sucnr1<sup>-/-</sup>* model found reduced leptin production and signalling during HFD, which led to increased food intake due to impaired leptin signalling changing energy homeostasis (263). *Ucp1* expression was increased in differentiated brown adipocytes (from control diet fed mice) in these adipose-specific *Sucnr1<sup>-/-</sup>* mice, however, the authors did not assess the effect of cold exposure and BAT thermogenesis. These data, in combination with our data in chapter 3, justify testing the effect of deletion of *Sucnr1* on murine energy expenditure and BAT function in vivo.

When using rodents as an experimental model a congenic strain reduces genetic variability and potential confounders. Several murine strains are frequently used as experimental models, each with their own benefit for use in different research areas. The C57BL/6 strain (and its various sub-strains) is commonly used for metabolic research due to the ease in which these mice develop obesity, glucose intolerance (301, 302). C57BL/6 mice have previously been used to study the metabolic effects of *Sucnr1* deletion (234). A *Sucnr1<sup>-/-</sup>* murine model generated by Amgen was previously reported to be on a non-specific C57BL/6 background (264).

Mice with non-functional BAT due to deletion of *Ucp1* are more pre-disposed to obesity than wild-type controls when housed at thermoneutrality (303). By comparing mice on control and high fat diets, we can determine how an obesogenic diet can exacerbate metabolic challenge compared to the normal physiological conditions of a control diet. Furthermore, as *Sucnr1* disruption in mice leads to a lean phenotype during CD but increased adiposity during an obesogenic challenge (234, 263), it is important to determine any interaction between diet, genotype and BAT function.

Male and females, in both humans and mice, have differing responses to HFD and resulting metabolic health outcomes, in part due to the protective metabolic effects
of oestrogen (304). As such, both male and female mice should be included in metabolic studies to assess the total population and identify any sex-specific differences, which can have important therapeutic implications. *Sucnr1* gene expression has not previously been shown to be different between sexes in humans or mice in the tissues discussed in sections 1.5.2, and at least in humans gender does not significantly alter circulating succinate levels in either young adults (244) or adults with obesity and/or T2DM (241). At present, there are no data investigating succinate concentrations in female mice and with studies only performed in males (237, 305). Therefore, very little is known about the role of SUCNR1 in female mice.

Reduced BAT function could potentially explain the previously reported phenotypes of increased adiposity and glucose intolerance in *Sucnr1*−/− mice on an obesogenic diet. To determine the role of SUCNR1 on BAT function, I phenotyped male and female *Sucnr1*−/− and *Sucnr1*+/+ mice either fed a control or high-fat diet that were housed at standard room temperature and at 4°C.

4.2 HYPOTHESES

1. *Sucnr1* disruption induces brown adipose tissue dysfunction, reduces energy expenditure and cold tolerance in mice fed a high fat diet, increasing adiposity and body weight.

4.3 AIMS

1. To determine the effect of *Sucnr1* disruption on weight gain, body composition and glucose tolerance in mice fed a control or HFD.

2. To determine whether *Sucnr1* disruption alters energy expenditure in mice housed at 21°C and 4°C.

3. To determine if there are sex-specific differences in the metabolic effects of *Sucnr1* deletion.
4.4 METHODS
4.4.1 Generation of Sucnr1^{−/−} mice
As C57BL/6NCrl mice are frequently used as model for studying obesity and metabolic phenotyping (301) and were readily available at the LFR animal units, it was decided this would be the most appropriate background to have the mouse line reconstituted on as it would allow for easier colony maintenance. As such, Sucnr1 global knockout mouse was obtained from Amgen, with a cryorecovery of heterozygote sperm that was carried out by Charles River Labs (Massachusetts, USA).

Cryorecovery was performed as described in 2.5.2, in brief heterozygote sperm from Sucnr1^{−/−} mice were injected into donor eggs of C57BL/6NCrl females prior to insertion in surrogate dams. This mouse line was previously generated by Amgen, through the insertion of an IRES/lacZ/neomycin cassette into exon 2 which caused a deletion of this exon by homologous recombination (211, 264). This deletion in exon 2 removes the binding domain for succinate making the receptor non-functional (211, 264). However, this deletion does not cause an early stop codon so protein translation still occurs, albeit, generating a non-functioning protein (Figure 4. 1). Initially, two male and two female mice were to be provided by Amgen, but due to Covid-19 restrictions on animal imports mice were held in quarantine for 6 months prior to delivery. Further breeding in USA led to the eventual transfer of 18 mice (7 males, 11 females) to our animal facility. However, following transfer it was clear the mice were not congenic. Typically, C57BL/6NCrl mice have a black coat colour but there were a mixture of black, white, and brown coated mice (Figure 4. 2 D). Amgen were unable to provide the genetic strain information on these mice.

Therefore, we used Transnetyx genetic monitoring services to identify SNPs associated with different murine strains to characterise the genetic backgrounds of these mice. The genetic monitoring panel assesses >10,000 SNPs against a library of mouse genomic profiles to determine the percentage each strain contributes to the mouse’s background. This revealed that the mouse model was an approximate 50:50
mix of the mouse strains C57BL/6N and BALB/c, with <1% SNPs associated with the 129 strain (Figure 4. 2 B). These strains have distinct metabolic responses to a HFD, for example BALB/c mice are resistant to obesity and T2DM compared to C57BL/6 mice (306) so this genetic variability could confound our measurements of the effect of Sucnr1 disruption. Generating a congenic strain of mice requires multigenerational breeding and can typically take in excess of 10 mating cycles (307). Due to the project timeline this was not possible, so we undertook a more targeted backcross using the mice with the greatest C57BL/6N and least BALB/c from the Transnetyx monitoring panel. Initially, a female with the greatest percentage of detectable C57BL/6NCrl SNPs was mated with a C57BL/6NCrl male mouse purchased from Charles River Labs, this was done to refresh the Y chromosome in the mutant line (308). SNP genetic monitoring was performed on the male progeny from this mating. The three male mice with the greatest percentage of C57BL/6NCrl were selected for the next breeding with 2 C57BL/6NCrl stock female mice each. This would allow for a refreshed X chromosome to enter the mutant line (308). This backcrossing step was performed for 2 further generations followed by a subsequent heterozygous cross of littermates.

![Figure 4. 1 Schematic map of the Sucnr1+/+ locus. Promoter (grey bar), untranslated regions (UTRs; white bars), intron (line) and coding sequence (CDS; black bars) are indicated. The IRES/lacZ/neo cassette was inserted to delete a large part of exon 2, by homologous recombination](image-url)
Figure 4. 2 Genetic monitoring and backcrossing of Sucnr1\(^{-/-}\) mice. Analysis of Genetic monitoring SNP panels (performed by Transnetyx) was performed on a C57BL/6NCrl mouse (A), a 1st generation Sucnr1\(^{-/-}\) mouse (B), and 3\(^{rd}\) generation Sucnr1\(^{+/-}\) mouse (C). Littermates from a 1\(^{st}\) generation litter pictured highlighting obvious strain contamination of the C57BL/6NCrl background (D), compared to a 4\(^{th}\) generation mouse (E).
4.4.2 Murine in vivo study design

Following breeding, C57BL/6NCrl Sucnr1\textsuperscript{-/-} and Sucnr1\textsuperscript{+/+} littermates were assigned to groups 1-8 following measurement of bodyweight at 28-35 days of age (Table 4.1). The groups were split in a 2x2x2 design, with factors being sex (M/F), genotype (+/+, -/-), and diet (CD/HFD). There were 8 mice per group for energy expenditure measurements, with the exception of male HFD Sucnr1\textsuperscript{-/-} mice which only had 7 per group due to sealing issues with the cage during measurement of respiratory gases. Groups were matched for bodyweight and age within each sex. Once assigned to designated dietary groups, mice were fed their respective experimental diet ad libitum for 12 weeks. The CD (Special Diet Services, 801151) comprised 7.5% Kcal from fat, and the high fat diet (Research Diets, D12331) comprised 58% Kcal from fat (see Table 4.2 for dietary information). The mice began their dietary intervention at 4-5 weeks of age, considered juvenile age for mice, this was done due to project time and budget constraints and the requirement to accelerate experimental timelines to meet these targets. The study was designed to characterise the phenotypic changes of Sucnr1\textsuperscript{-/-} and Sucnr1\textsuperscript{+/+} mice in response to CD and HFD (Figure 4.3). Bodyweight was recorded weekly (as described in 2.5.5) and body composition bi-weekly (2.5.6) throughout the protocol duration. Oral glucose tolerance tests (OGTTs) were performed after 2 weeks of dietary intervention and after 11 weeks of dietary intervention to assess glucose tolerance (described in 2.5.7) and insulin response to glucose challenge. After 12 weeks of diet, mice (aged approximately 16 weeks old) were placed in metabolic cages linked to the TSE Phenomaster system. Measurements of energy expenditure, RER, locomotor activity, and food intake were performed in mice housed initially at 21ºC for 3 days and then subsequently at 4ºC for a further 3 days. The parameters measured during this time are described in 2.5.8. Using infrared thermography, thermal images were taken of mice after each period in the metabolic cages at 21ºC and at 4ºC to assess heat dissipation from the proximal and distal regions of the tail (described in 2.5.9). Core body temperature was measured using a rectal probe thermometer after 72 hours of 4ºC cold exposure (described in 2.5.9 and 2.5.10). Following measurement of metabolic parameters mice were culled and tissues were dissected and weighed (described in 2.5.11) prior
to storage at -80°C for subsequent analysis of mRNA and protein expression. Tissues dissected included interscapular BAT (BAT), inguinal WAT (iWAT), gonadal WAT (gWAT), mesenteric WAT (mWAT), peri-renal WAT (pWAT), kidney, liver, and gastrocnemius and soleus muscles.

**Figure 4. 3 Schematic diagram of study design.** Diagram depicts weeks on diet and timings of experimental procedures during the study. Groups depicted in diagram show male groups 1-4, and were replicated in females numbered 5-8.
Table 4.1 Study groups. Groups 1-8 showing the sex, genotype, and diet of each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Genotype</th>
<th>Diet</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>Sucnr1⁺/⁺</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>Sucnr1⁺/⁺</td>
<td>High Fat</td>
</tr>
<tr>
<td>3</td>
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<td>Sucnr1⁻/⁻</td>
<td>Control</td>
</tr>
<tr>
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<td>Male</td>
<td>Sucnr1⁻/⁻</td>
<td>High Fat</td>
</tr>
<tr>
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<tr>
<td>8</td>
<td>Female</td>
<td>Sucnr1⁻/⁻</td>
<td>High Fat</td>
</tr>
</tbody>
</table>

Table 4.2 Macronutrient composition of diet. Table describing macronutrient composition as a percentage of total calories in the control and high fat diet.

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<th>Kcal %</th>
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<tr>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td>HFD</td>
<td>58</td>
</tr>
</tbody>
</table>

4.4.3 Glucose and insulin measurements

Whole blood glucose and plasma insulin were measured in all groups of mice during the OGTTs at 2 and 11 weeks of dietary intervention to investigate the effects of short term and more chronic high fat feeding compared to control diet. Mice within the same cohort were assessed within a 3-day period of each another, with 4-7 mice assessed per day depending on cohort size. The OGTT protocol was performed as described in described in 2.5.7. Glucose and insulin concentrations were quantified
as described in 2.5.7 and 2.9 respectively. HOMA-IR was also calculated with the following formula:

\[
\text{fasting insulin (microU/L) } \times \text{ fasting glucose (nmol/L)} / 22.5 = \text{HOMA-IR}
\]

### 4.4.4 qPCR of murine whole tissue following 4°C cold exposure

Real time qPCR was performed to determine how *Sucnr1*−/− altered expression of thermogenic genes and succinate transporters in brown, inguinal, and gonadal adipose tissue depots. Genes measured included *Ucp1, Ppargc1a, Slc25a10, Slc13a3*, and *Sucnr1* (see Table 2.3). Sample gene expression was normalised to the mean relative expression of two housekeeping genes, *Tbp* and *Rna18s5*.

### 4.4.5 Western blot analysis of murine whole tissue

UCP1 expression was quantified in BAT by Western blot as described in 2.7.4. Membranes were imaged using the Odyssey CLX instrument (Li-Cor, USA) and analysed using Odyssey software (Li-Cor, USA). Protein expression was calculated by normalising the UCP1 protein band fluorescence (wavelength 650nm) to a control protein (β-actin, wavelength 700nm), the mean fluorescence intensities were then compared between groups.

### 4.4.6 Statistical analysis

Statistical tests and analyses were performed as described in 2.10.1 with the addition of using ANCOVA analysis (described in 2.5.8) to use the lean mass of mice as the covariate for energy expenditure analysis, as this is considered the gold standard for energy expenditure analysis.
4.5 RESULTS

4.5.1 Generation of a Sucnr1\(^{-/-}\) mouse model on the C57BL/6NCrl

Following four generations of backcrossing, no SNPs from contaminating backgrounds (BALB/c or 129) could be identified in the panel and the percentage of SNPs associated with the C57BL/6N strain were comparable to the in-house C57BL/6N control mice (Figure 4. 2 A-E). Following the targeted backcrossing, Sucnr1\(^{-/-}\) mice were used to generate the experimental animals for the \textit{in vivo} study in addition to the \textit{in vitro} culture experiments reported in Chapter 5. In house genotyping of mice confirmed the presence or the deletion of exon 2 in the Sucnr1\(^{+/+}\) and Sucnr1\(^{-/-}\) mice (Figure 4. 4 C) which identified bands at 700bp in the Sucnr1\(^{+/+}\) and Sucnr1\(^{-/-}\) mice, and the mutant band at 300bp in the Sucnr1\(^{-/-}\) and Sucnr1\(^{-/-}\) mice. These data corroborated previously observed exon 2 deletion in this mouse model by Charles River Labs and as previously performed by other research groups using this model (264)( Figure 4. 4 A and B).
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A. Charles River Labs genotyping

<table>
<thead>
<tr>
<th>Observed Product Size:</th>
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<th>Allele Description</th>
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<td>WT</td>
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Display PCR products on LabChip Gx (Perkin Elmer)

B. Van Diepen 2017 genotyping

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</thead>
<tbody>
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</tr>
<tr>
<td>Exon 2 + 3’ UTR (c+d)</td>
<td>701</td>
</tr>
<tr>
<td>Neomycin + 3’ UTR (e+d)</td>
<td>333</td>
</tr>
</tbody>
</table>

C. In house genotyping

![Genotyping results](image)

Figure 4. 4 Genotyping results of murine model. Genotyping results from Charles River Labs (A), and van Diepen, 2017 (B), compared to genotyping results from in-house genotyping of the Sucnr1<sup>−/−</sup> line (C), with Sucnr1<sup>−/−</sup> (1), Sucn1<sup>−/−</sup> (2), and Sucnr1<sup>+/−</sup> (3) mice.

4.5.2 Sucnr1 disruption does not alter bodyweight

In male mice, a high fat diet increased bodyweight but genotype did not alter weight in either the CD or HFD fed groups (Figure 4. 5 A and B). Genotype did not alter bodyweight in female mice on either diet (Figure 4. 5 C). However, a HFD increased bodyweight in the female mice by week 11 of the experiment (Figure 4. 5 D).
Figure 4. 5 Sucnr1 disruption does not alter bodyweight. Bodyweight of male (A, B) and female (C, D) mice during the experiment (A,C) and following 11 weeks of each diet (B,D) Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1+/- mice fed a control (blue circles) or HFD (pink circles) (n=10-15 per group). Data were analysed by RM three-way ANOVA (A & C) and two-way ANOVA (B & D) with Tukey’s multiple comparison. **, p<0.01.
4.5.3 Male Sucnr1⁻/⁻ mice have increased adiposity on a HFD

In male mice, both HFD and genotype altered fat mass (with an interaction between both factors). Sucnr1 disruption increased fat mass only on a HFD (Figure 4.6 A & B). By week 10 of diet, fat mass was increased in Sucnr1⁻/⁻ mice compared to Sucnr1⁺/⁺ on a high fat diet, but not on control diet (Figure 4.6 C). In female mice, a HFD increased fat mass although there was no effect of genotype (Figure 4.6 D-F). A HFD reduced lean mass in both male and female mice (Figure 4.7 A-F). Genotype had no effect on lean mass in either sex.
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Figure 4.6 Sucnr1−/− in male mice increased adiposity during high fat diet. TD-NMR measurement of fat mass was performed in male (A-C) and female (D-F) mice to calculate body fat (A, D), percentage fat (B, E), and fat mass at week 10 (C, F). Data are mean ± SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=10-15 per group). Data were analysed by RM three-way ANOVA (A, B, D & E), and two-way ANOVA (C & F) with post-hoc testing using Tukey’s multiple comparison. *p<0.05; *** p<0.001; **** p<0.0001.
Figure 4.7 Lean mass of mice is unaffected by Sucnr1 knockout. TD-NMR measurement of lean mass was performed in male (A-C) and female (D-F) mice to calculate lean mass (A, D), percentage lean mass (B, E), and lean mass at week 10 (C, F). Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=10-15 per group). Data were analysed with RM three-way ANOVA (A, B, D & E), two-way ANOVA (C & F) with Tukey’s multiple comparison. *; p<0.05. **; p<0.01.
4.5.4 Sucnr1⁻/⁻ male mice develop glucose intolerance

4.5.4.1 Sucnr1⁻/⁻ male mice develop glucose intolerance after two weeks of HFD

After 2 weeks of dietary intervention in male mice, blood glucose was higher in Sucnr1⁻/⁻ compared to Sucnr1⁺/⁺ mice, and was increased by HFD (Figure 4. 8 A). The glucose area under the curve (AUC) was greater in Sucnr1 knockout and HFD fed male mice. HFD increased blood glucose in female mice (Figure 4. 8 D & E), but there was no effect of genotype. Fasting glucose was not affected by either diet or genotype in the male or female mice (Figure 4. 8 C & F).

The insulin response to glucose bolus was increased in high fat-fed mice of both sexes increasing both AUC and fasting insulin (Figure 4. 9 A - F). In the male mice there was an interaction between diet and genotype with Sucnr1⁻/⁻ mice developing higher insulin on a HFD but lower insulin on CD compared with Sucnr1⁺/⁺ mice (Figure 4. 9 A). Male Sucnr1⁻/⁻ HFD mice had greater insulin AUC than Sucnr1⁻/⁻ CD mice as well as higher fasting insulin (Figure 4. 9 B & C).
Figure 4.8 Glucose tolerance is impaired in male Sucnr1−/− mice after 2 weeks of a high fat diet. Blood glucose levels during the 120 minutes following oral glucose administration (A, D), with AUC (B, E), and fasting plasma glucose (C, F) for male (A-C) and female (D-F) mice. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=10-15 per group). Data were analysed with RM three-way ANOVA (A & D), two-way ANOVA (B, C, E, & F) with Tukey’s multiple comparison. *; p<0.05.
**Figure 4. 9 Sucnr1 deletion alters insulin levels in male mice after two weeks of diet.**

Plasma insulin in male mice during a 120 minute time course following oral glucose administration (A), with AUC (B), and fasting plasma insulin (C). The same data were presented in female mice (D-F). Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=9-10 per group). Data were analysed with RM three-way ANOVA (A & D), two-way ANOVA (B, C, E, & F) with Tukey’s multiple comparison. **; p<0.01. ***; p<0.001.
4.5.4.2 Sucnr1⁻/⁻ male mice develop fasting hyperglycaemia after chronic high fat feeding

After 11 weeks of dietary intervention, fasting blood glucose and following glucose bolus were higher in high fat-fed male mice (Figure 4. 10 A-B). Fasting glucose was increased in Sucnr1 knockout mice on both control and high fat diets (Figure 4. 9 C) and with a similar trend during the OGTT (p=0.075, Figure 4. 9 A). A HFD increased blood glucose (and AUC) during OGTT in the female mice but with no effect of genotype (Figure 4. 10 D-E). Fasting glucose was not affected by either diet or genotype in the female mice (Figure 4. 10 F).

Insulin concentrations (fasting and during the OGTT) were increased by HFD in both male and female mice but were unaltered by genotype (Figure 4. 11A-F). HOMA-IR was unchanged by Sucnr1 knockout but was increased when mice were fed a HFD, except for female mice at 2 weeks of dietary intervention (Figure 4. 12 A-D)
Figure 4. 10 Sucnr1 deletion induces fasting hyperglycaemia in male mice after 11 weeks of diet. Plasma glucose for male (A-C) and female (D-F) mice undergoing OGTT showing glucose levels over 120 minutes (A,D), area under curve (B,E) and fasting glucose (C,F). Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8-12 per group). Data were analysed with RM three-way ANOVA (A & D), two-way ANOVA (B, C, E, & F) with Tukey’s multiple comparison. *; p<0.05.
Figure 4. **Insulin levels are unchanged by Sucnr1 disruption after 11 weeks of dietary intervention.** Plasma insulin for male (A-C) and female (D-F) mice undergoing OGTT showing plasma insulin levels over 120 minutes (A,D), area under curve (B,E) and fasting insulin (C,F). Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=10-15 per group). Data were analysed with RM three-way ANOVA (A & D), two-way ANOVA (B, C, E, & F) with Tukey’s multiple comparison. *; p<0.05. **; p<0.01.
Figure 4. 12 HOMA-IR is unaffected by Sucnr1 knockout in male and female mice.

HOMA-IR was quantified for male (A-B) and female (C-D) mice at 2 and 11 weeks of either control diet or HFD. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8-10 per group). Data were analysed with two-way ANOVA.
4.5.5 Energy expenditure in mice housed at 21°C is unaffected by Sucnr1 disruption

In male mice, the energy expenditure was similar between genotypes over the 48-hour measurement period on either diet when housed at room temperature. High fat feeding increased energy expenditure and reduced the diurnal variation in energy expenditure (Figure 4.13 A). The mean energy expenditure for each time period (day and night) was also unaffected by genotype (Figure 4.13 C). However, in the female mice neither genotype nor diet altered energy expenditure either across the 48-hour measurement period or when comparing the day or night periods (Figure 4.13 B & D). In male mice, the ANCOVA analysis of energy expenditure (2.5.8), using lean mass as the covariate, similarly showed no effect of genotype on energy expenditure on either diet (Figure 4.14 A-B), but the effect of HFD remained (Figure 4.14 C). In the female mice, ANCOVA analysis also found no effect of either genotype or diet on energy expenditure (Figure 4.14 D-F). In both male and female mice, energy expenditure was greater during nocturnal periods, while energy expenditure was greater in males.
Figure 4.13 Energy expenditure at 21°C is unaffected by Sucnr1 disruption. Energy expenditure across 48 hours in male (A) and female (B) mice. Mean energy expenditure for day and night in male (C) and female (D) mice. Data are represented as mean only (A & B) or mean ±S.E.M (C & D), for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=8-10 per group). Data were analysed with RM three-way ANOVA (A & B), RM two-way ANOVA (C & D) with Tukey’s multiple comparison. *; p<0.05.
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A. Male CD energy expenditure

B. Male HFD energy expenditure

C. Male CD and HFD energy expenditure

D. Female CD energy expenditure

E. Female HFD energy expenditure

F. Female CD and HFD energy expenditure

p=0.13

p=0.26

p=0.025

p=0.65

p=0.96

p=0.90
Figure 4. 14 Sucnr1⁻/⁻ does not alter energy expenditure at 21°C in male mice. Simple linear regression analysis of covariance (ANCOVA) of energy expenditure at 21°C, with lean mass as the covariate, of male Sucnr1⁺/⁺ and Sucnr1⁻/⁻ mice on CD (A) and HFD (B), CD and HFD fed male mice (C), female Sucnr1⁺/⁺ and Sucnr1⁻/⁻ mice fed a CD (D) or HFD (E), and CD and HFD fed female mice (F) (n=8 per group in A-B and D-E, n=16 per group in C and F). Data are represented as line regression correlation. Data were analysed using MMPC (see 2.5.8).

4.5.6 Sucnr1 disruption does not affect RER, locomotor activity, or food intake at 21°C
Genotype did not alter RER in both male and female mice, but a HFD reduced RER in both sexes (Figure 4. 15). Locomotor activity was measured by determining the total distance across the cage travelled by each mouse over the 48-hour measurement which was split into day and night periods. As to be expected, activity was greater during the nocturnal period in both sexes, but neither diet nor genotype altered the total distance travelled (Figure 4. 16). Similarly, food intake over the 48-hour period was unaffected by either diet or genotype in both sexes (Figure 4. 17).
Figure 4. 15 Respiratory exchange ratio at 21°C is unaffected by Sucnr1 knockout. RER across 48 hours was measured in male (A) and female (B) mice. Mean RER for both day and night periods was calculated in male (C) and female (D) mice. Data are represented as mean only (A & B) or mean ±S.E.M (C & D) for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM three-way ANOVA (A & B), RM two-way ANOVA (C & D) with Tukey’s multiple comparison. ****; p<0.0001.
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Figure 4. 16 Locomotor activity is not altered by diet or Sucnr1 knockout in mice housed at 21°C. Total distance travelled in 48 hours was measured in male (A) and female (B) mice at 21°C. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM three-way ANOVA (A & B), with Tukey’s multiple comparison.

Figure 4. 17 Caloric intake is unaffected by Sucnr1 knockout at 21°C. Total caloric intake over 48 hours was measured in male (A) and female (B) mice at 21°C. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=8 per group).
Data were analysed with RM two-way ANOVA with post hoc testing by Tukey’s multiple comparison.

4.5.7 Cold acclimation and energy expenditure at 4°C are unaffected by Sucnr1 disruption
In both male and female mice there was an increase in energy expenditure when housed at 4°C compared to 21°C. However, neither diet nor genotype altered the response to cold exposure during the cold acclimation period (Figure 4. 18 A-B) or during the 48-hour measurement window (Figure 4. 19 A-D). Energy expenditure remained higher during the night in both male and female groups (Figure 4. 19 A-D). There was also no effect of sex on energy expenditure when comparing the male and female groups both during the day and night (Figure 4. 19). ANCOVA analysis accounting for lean mass also demonstrated no effect of genotype or diet on energy expenditure at 4°C in either the male or female mice (Figure 4. 20 A-D).
Figure 4. 18 *Sucnr1* knockout does not alter energy expenditure adaptation to 4°C.

Energy expenditure was measured in male (A) and female (B) mice during cooling of metabolic cages (C) (*N*=8 per group). Data are means only (A-B) or mean +/- SEM (C).

Data were analysed by RM three-way ANOVA (A & B).
Figure 4. 19 Energy expenditure at 4°C is unaffected by Sucnr1 knockout. Energy expenditure across 48 hours was measured in male (A) and female (B) mice. Mean energy expenditure for both day and night periods was calculated in male (C) and female (D) mice. Data are represented as mean only (A & B) or mean ±S.E.M (C & D) for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM three-way ANOVA (A & B), RM two-way ANOVA (C & D) with Tukey’s multiple comparison testing.
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A. Male CD energy expenditure

B. Male HFD energy expenditure

C. Male CD and HFD energy expenditure

D. Female CD energy expenditure

E. Female HFD energy expenditure

F. Female CD and HFD energy expenditure

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p = 0.084

p = 0.58

p = 0.55

p = 0.32

p = 0.31

p = 0.57
Figure 4. Energy expenditure at 4°C is unaffected by Sucnr1 knockout when lean mass is factored in analysis. Simple linear regression analysis of covariance (ANCOVA) of energy expenditure at 4°C, with lean mass as the covariate, of male Sucnr1+/+ and Sucnr1−/− CD mice on CD (A) and HFD (B), CD and HFD fed male mice (C), female Sucnr1+/+ and Sucnr1−/− mice fed a CD (D) or HFD (E), and CD and HFD fed female mice (F) (n=8 per group in A-B and D-E, n=16 per group in C and F). Data are represented as linear regression correlation. Data were analysed using MMPC (see 2.5.8).
4.5.8 *Sucnr1* disruption does not alter RER or distance travelled at 4°C

RER when housed at 4°C was lower in all groups compared to 21°C, while RER remained lower in HFD-fed mice at 4°C. The loss of diurnal variation in RER of HFD-fed mice persisted at 4°C. There was no effect of genotype on RER in either male or female mice (Figure 4. 21 A-D). The total distance travelled during the night was the same at 21°C and 4°C, although distance travelled during the day was lower at 4°C in male mice (Figure 4. 22). Cold exposure did not alter the total distance travelled in female mice either during the day or night. Neither diet nor genotype altered the total distance travelled in either the day or night for male and female mice (Figure 4. 22). Food intake was increased when mice were housed at 4°C versus 21°C (Figure 4. 21 A-B), but there were no effects of diet or genotype on caloric consumption in the male mice (Figure 4. 23 A). However, in female mice food intake was increased in the *Sucnr1*−/− mice while HFD mice ate less than their control diet littermates (Figure 4. 23 B).
Figure 4.2: Respiratory exchange ratio at 4°C is unaffected by Sucnr1 knockout.

RER in male (A) and female (B) mice across the 48-hour period. Mean RER for both day and night periods in male (C) and female (D) mice. Data are represented as mean only (A & B) or mean ±S.E.M (C & D) for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM three-way ANOVA (A & B), RM two-way ANOVA (C & D) with Tukey’s multiple comparison. ****, p<0.0001.
Figure 4.22 Locomotor activity is not altered by Sucnr1 knockout during cold exposure. Total distance travelled in 48 hours was measured in male (A) and female (B) mice at 4°C. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM three-way ANOVA (A & B).

Figure 4.23 Caloric intake is increased in female Sucnr1-/- mice housed at 4°C. Total caloric intake 48 hours was measured in male (A) and female (B) mice at 4°C. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM three-way ANOVA (A & B).
4.5.9 Core temperature is increased in male Sucnr1−/− mice housed at 4°C

Thermal imaging of cold exposed mice was performed to determine thermoregulatory control of body heat in Sucnr1−/− and Sucnr1+/+ mice at both 21°C and 4°C. Cold exposure reduced tail temperature in both sexes but neither diet nor genotype altered proximal or distal tail temperatures (Figure 4. 24). Immediately following thermal imaging at 4°C, core body temperature was recorded. In males, core body temperature was increased in Sucnr1−/− mice on both control and HFD. Additionally, HFD lowered core temperature in male mice. In female mice there were no effect of genotype or diet on core temperature (Figure 4. 24).
Figure 4. 24 Sucnr1 knockout increases core temperature in male mice at 4°C. Core body temperature was measured in male (A) and female (B) mice at 4°C. Infra-red thermography, (C) with an example image, was performed on mice at 21°C (D) and 4°C (E). Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM two-way ANOVA (A & B), RM three-way ANOVA (D & E).
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4.5.10 Tissue weights are similar in Sucnr1+/- and Sucnr1-/- mice

BAT weight was not altered by either diet or genotype in male or female mice. HFD increased both IAT and pWAT mass in both sexes, there was no effect of genotype. Male epididymal WAT and female uterine WAT were increased in HFD diet groups, however, there was no effect of genotype in either sex (Figure 4. 25, Figure 4. 26). The mWAT of male and female mice was unaffected by either Sucnr1 knockout or HFD. Male liver weights were increased with HFD, however this was not observed in females. Male and female muscle mass in the gastrocnemius and soleus was unaffected by either genotype or diet. (Figure 4. 25, Figure 4. 26). Similar results were seen when tissue mass was normalised to total bodyweight although the effect of HFD increasing liver weight in male mice was lost and HFD significantly reduced mWAT mass in females (Figure 4. 27, Figure 4. 28).
Figure 4. 25 Sucnr1 deletion does not alter tissue mass in male mice after 4°C exposure. After 4°C cold exposure tissues were dissected from male mice including (A) BAT, (B) IAT, (C) gWAT, (D) mWAT, (E) pWAT, (F) Liver, (G) Gastrocnemius, and (H) Soleus. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD.
(orange circles) and Sucnr1/- mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM two-way ANOVA (A-H), with Tukey’s multiple comparison. *; p<0.05. **; p<0.01. ***; p<0.001. ****; p<0.0001.
Figure 4. 26 Sucnr1 deletion does not alter tissue mass in female mice after 4°C exposure. After 4°C cold exposure tissues were dissected from female mice including (A) BAT, (B) IAT, (C) gWAT, (D) mWAT, (E) pWAT, (F) Liver, (G) Gastrocnemius, and (H) Soleus.
Soleus. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM two-way ANOVA (A-H), with Tukey’s multiple comparison. *; p<0.05. **; p<0.01. ***; p<0.001. ****; p<0.0001.
Figure 4. 27 Sucnr1 deletion does not alter normalised tissue mass in male mice after 4°C exposure. After 4°C cold exposure tissues weight was normalised to mouse bodyweight measuring (A) BAT, (B) IAT, (C) gWAT, (D) mWAT, (E) pWAT, (F) Liver, (G) Gastrocnemius, and (H) Soleus. Data are mean +/- SEM for Sucnr1+/+ mice fed a
control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM two-way ANOVA (A-H), with Tukey’s multiple comparison. **; p<0.01. ****; p<0.0001.
Figure 4. Sucnr1 deletion does not alter normalised tissue mass in female mice after 4°C exposure. After 4°C cold exposure tissues weight was normalised to mouse
bodyweight measuring (A) BAT, (B) IAT, (C) gWAT, (D) mWAT, (E) pWAT, (F) Liver, (G) Gastrocnemius, and (H) Soleus. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=8 per group). Data were analysed with RM two-way ANOVA (A-H), with Tukey’s multiple comparison. *; p<0.05 **; p<0.01.

4.5.11 Gene and protein expression of Sucnr1<sup>-/-</sup> and Sucnr1<sup>+/+</sup> mice

Following ~72 hours of cold exposure at 4°C, neither diet nor genotype affected mRNA expression of any of the genes measured in BAT or gWAT in either sex (Figure 4. 29 A-H). In the IAT of male mice there was an effect of diet on Ucp1 mRNA expression, and an interaction between diet and genotype with Sucnr1<sup>-/-</sup> decreasing Ucp1 expression on CD but increasing Ucp1 on HFD (Figure 4. 29 A), this effect was not observed in females (Figure 4. 29 B). mRNA expression of the remaining genes was unaffected by Sucnr1 deletion or diet in IAT of male and female mice. The expression of Sucnr1 was analysed in the Sucnr1<sup>+/+</sup> mice, there was no effect of diet on Sucnr1 expression in any of the adipose depots (Figure 4. 29 I-J). Sucnr1 expression tended to be lower in the BAT of both sexes compared to gWAT and IAT, although this did not reach significance (p=0.054).

Neither diet nor genotype altered UCP1 protein expression in the BAT of either sex, both normalised to β-actin expression and for total BAT mass (Figure 4. 30 C).
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A. Male *Ucp1* expression
- Genotype; p=0.33
- Diet; p=0.83
- Tissue; p=0.0014

B. Female *Ucp1* expression
- Genotype; p=0.99
- Diet; p=0.51
- Tissue; p=0.0029

C. Male *Pgc1α* expression
- Genotype; p=0.83
- Diet; p=0.39
- Tissue; p<0.0001

D. Female *Pgc1α* expression
- Genotype; p=0.48
- Diet; p=0.94
- Tissue; p<0.0001

E. Male *Slc25a10* expression
- Genotype; p=0.28
- Diet; p=0.2
- Tissue; p=0.02

F. Female *Slc25a10* expression
- Genotype; p=0.57
- Diet; p=0.94
- Tissue; p=0.029

G. Male *Slc13a3* expression
- Genotype; p=0.98
- Diet; p=0.77
- Tissue; p=0.0037

H. Female *Slc13a3* expression
- Genotype; p=0.31
- Diet; p=0.54
- Tissue; p=0.21

I. Male *Sucnr1* expression
- Diet; p=0.99
- Tissue; p=0.032

J. Female *Sucnr1* expression
- Diet; p=0.7
- Tissue; p=0.23
Figure 4. **Sucnr1 knockout does not alter thermogenic gene expression in cold exposed mice.** After 4°C cold exposure tissues mRNA expression was measured in the BAT, IAT, and gWAT of male (A, C, E, G, I) and female (B, D, F, H, J) mice. Gene measured include Ucp1, Ppargc1a, Slc25a10, Slc13a3, and Sucnr1. Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1-/- mice fed a control (blue circles) or HFD (pink circles) (n=5-6 per group). Data were analysed with RM two-way ANOVA, with Tukey’s multiple comparison. *; p<0.05.
Figure 4. **Sucnr1 knockout does not alter UCP1 protein expression in cold exposed mice.** UCP1 protein expression was measured in mouse BAT and quantified by normalising to β-actin protein expression. Protein expression was measured in 4°C exposed Sucnr1+/+ and Sucnr1−/− males (A-B) and females (C-D). Data are mean +/- SEM for Sucnr1+/+ mice fed a control (black circles) or HFD (orange circles) and Sucnr1−/− mice fed a control (blue circles) or HFD (pink circles) (n=2-3 per group). Data were analysed with two-way ANOVA.
4.6 Discussion

In the present study, the role of SUCNR1 in BAT function was assessed in mice with Sucnr1 disruption fed either a control or high fat diet. The aim of this experiment was to identify whether knockout of the receptor would impair BAT function and reduce energy expenditure at room temperature and during a more severe cold stress. Male Sucnr1<sup>-/-</sup> mice developed increased adiposity (when fed a HFD) and impaired glucose tolerance, which was not observed in female mice. However, energy expenditure at 21°C and 4°C was unchanged by Sucnr1 disruption indicating the metabolic phenotype of these mice was not driven by dysfunctional BAT.

On a CD, Sucnr1<sup>-/-</sup> mice of both sexes had similar weights and body composition as Sucnr1<sup>+/+</sup> littermates. These results are different to what has previously been observed in Sucnr1<sup>-/-</sup> mouse models which have reported a leaner phenotype (despite no differences in bodyweight) when mice are fed a low fat or control diet (234, 263). Control diets used in these previously reported models were different than in this study and the dietary fat content was marginally higher (7.5% vs 10%), therefore, macronutrient differences could possibly explain the lack of leanness in this model. However, it is important to consider that these models were on a unspecified C57BL/6 background and not specifically the C57BL/6N which can affect the metabolic phenotype of the mouse, as the C57BL/6N gains weight faster on HFD compared to other sub-strains (301). Additionally, one of the models used was an adipose specific knockout (263) rather than the global knockout used in these studies, meaning there could be contributions from other metabolic tissues to our observed phenotype. Importantly, the previous studies only investigated male mice so these data are the first reports of the metabolic effect of Sucnr1 disruption in female mice.

When fed a HFD, male Sucnr1<sup>-/-</sup> mice had increased fat mass compared to Sucnr1<sup>+/+</sup> high fat-fed mice, despite no differences in bodyweight or lean mass. This difference in adiposity was only observed in male mice and not females, indicating sex
differences in the importance of SUCNR1 in metabolic health. Additionally, the increase in total adiposity observed in male mice was not attributed to any individual adipose depot as there were no effect of genotype on adipose tissue mass in the mice. These data show that although the total mass of adipose tissue was greater in Sucnr1−/− mice the distribution was similar to that of the Sucnr1+/+ mice. Although HFD increased adiposity in the female mice compared to CD, Sucnr1 disruption did not alter fat mass in female mice highlighting sex differences in this response. These data are consistent with other Sucnr1−/− murine models which have increased fat mass following a HFD as seen in global (234), adipose specific (263), and in myeloid specific knockouts (224).

Sucnr1 deletion has been reported to improve glucose tolerance of male mice when fed a low fat diet (3.1% Kcal from fat) (263), although this was not seen in this study. However, as with the lack of a lean phenotype in the mice, the difference in mouse model and age of mice in the study are potentially responsible for this observation. Male Sucnr1−/− mice had increased fasting glucose and displayed glucose intolerance after only two weeks of a HFD. Fasting glucose remained higher in the knockouts after 11 weeks of HFD, although glucose tolerance and insulin levels were similar between genotypes. These data corroborate previously identified metabolic dysfunction in HFD fed Sucnr1−/− mice (234), but with the novel identification of sex-specific differences. The impaired glucose tolerance in the HFD males was also to be expected from previous data (224, 234, 263), but was milder than previously reported, that could be due to a reduced time on diet in the present study (12 vs 16 weeks). The glucose intolerance phenotype seen in these mice could be driven by multiple factors in the global knockout mouse. Sucnr1−/− mice have increased rates of lipolysis in WAT depots (234) leading to dysregulation of circulating lipids when on a HFD, this may impair glucose tolerance due to possible impaired glucose transport or β-cell function (309). Indeed, the interaction between diet and genotype in the plasma insulin curve (after 2 weeks of high fat diet) possibly indicate impaired insulin signalling in the mice. However, in the present study this has not been interrogated in enough detail and use of hyperinsulinemic-euglycemic clamps would elucidate
whether Sucnr1 deletion causes insulin resistance (310). Additionally, global Sucnr1-/ mice have impaired liver function and NAFLD when fed a HFD (311); due to the liver’s role in systemic glucose homeostasis, hepatic dysfunction may contribute to this phenotype. However, analysis of collected liver tissues was unable to be performed in the project’s timeframe so it remains unclear whether the mice had fatty liver or defects in glucose signalling in this model. SUCNR1 activation in mice induces a muscle fibre switch from fast-twitch to slow-twitch fibres (203), a process which may be dysregulated in the model used in this study. As skeletal muscle is a large source of glucose uptake (203), dysfunction in this tissue could contribute to the observed glucose intolerance. However, it should be noted that the adipose specific knockout (263) had similar glucose intolerance to this model, indicating that Sucnr1 knockout in adipose tissue rather than muscle is the major cause of glucose intolerance. Another caveat to consider when comparing the metabolic outcomes of these mice is the age at which the mice started the dietary intervention. As these mice were juveniles when the study began their growth had not been completed and as such this may have added confounders to the data (312). Juvenile rats have been shown to have comparable energy intake when fed a HFD compared to a control chow diet, however, following 9 weeks of diet the HFD fed rats had increased adiposity compared to the control mice despite similar bodyweights, a phenotype the authors termed normal-weight obesity (313). However, in the present study the HFD fed mice had both increased bodyweight and adiposity (with further increased adiposity in the Sucnr1-/- HFD fed mice) indicating that although these mice were started on the diet as juveniles, the 12 week dietary intervention was sufficient to observe differences in the metabolic parameters measured, although the magnitude of change may have differed if these mice started their respective diets once adults (301).

Our data suggest that impaired BAT function is not the cause of the metabolic phenotype seen in this model, as despite increased adiposity in the male high fat fed Sucnr1-/- mice there was no detectable difference in energy expenditure when housed at either 21°C or 4°C when compared to Sucnr1+/- mice. There was also no
difference in energy expenditure between the CD male groups, or between any of the female groups. This is in contrast to previous data from global Sucnr1-/- male mice which have reported increased energy expenditure compared to wild-type controls on a low-fat diet when housed at 21°C (234). The only difference in energy expenditure observed in the present study was between male mice fed either a CD or HFD at 21°C which was observed with both raw energy expenditure output and with ANCOVA analysis. This increased energy expenditure on a HFD is likely due to the increased fat mass of these mice, as fat mass is known to have a significant contribution to energy expenditure in mice (314). However, there were no differences in energy expenditure between the high fat fed Sucnr1+/- mice and the Sucnr1-/- mice, despite the Sucnr1-/- mice have greater fat mass, although the differences in fat mass were smaller between these groups than compared to the control diets.

There was also no impairment of adaptation to cold and all mice had similar increases in energy expenditure following the transition to 4°C. Therefore, it is unlikely that BAT function is impaired by Sucnr1 knockout, as mice with dysfunctional BAT are less able to tolerate acute cold exposure unless appropriately acclimated (298). In addition to the energy expenditure data, mRNA and protein expression of UCP1 were also not altered in BAT by Sucnr1 knockout. Therefore, mechanisms other than BAT dysfunction are responsible for the difference in adiposity and glucose intolerance observed in the male HFD-fed Sucnr1-/- mice. Recently, an adipose specific Sucnr1-/- mouse model was characterised in a similar manner to the present study (263). In this model, when fed a high fat diet the knockout mice developed glucose intolerance with a trend for increased adiposity, whilst also having no differences in energy expenditure whilst at 21°C. The adipose specific knockout mouse had hypoleptinemia, this was due to dysregulation of core-clock genes governing circadian rhythm (263). The researchers showed that succinate and SUCNR1 signalling (through an AMPK-JNK dependant mechanism), controls BMAL1-C/EBPα-mediated regulation of leptin production, and that disturbances in this pathway led to increased food intake on control diet but decreased food intake on high fat diet,
Despite the increased adiposity of the knockout mice on HFD. It is therefore likely that changes in circulating succinate from dietary intake (315) and production in the intestine (305) act as signalling pathway to control energy homeostasis via SUCNR1 activation and leptin regulation. Leptin expression or signalling was not measured in the present study as the manuscript describing these mechanisms was not published until late into the project, as there had not been any previous literature implicating Leptin being under regulation of succinate-Sucnr1 signalling this was not considered for investigation. However, further research using the mouse model in this study should examine leptin signalling and the impact of Sucnr1 knockout on circadian clock gene regulation to compare to other published models.

However, in this study food intake was unaffected by either diet or genotype in male mice at either 21°C or 4°C, and at 21°C in females, although food intake was increased in female Sucnr1−/− mice at 4°C. No previous food intake in female Sucnr1 knockout mice has previously been reported, and what data has been reported in males would indicate that food intake should be increased at 21°C. However, a possible explanation for this increased food intake in the females could be related to the increased systemic lipolysis coupled with the increased energy requirements for thermogenesis led to an increase in caloric intake for energy homeostasis. Although this does not explain the lack of change in male food intake nor absence of differences in energy expenditure. It is possible that these potential differences in food take and energy expenditure were below detectable limits in the time measured.

These data coupled with the energy expenditure data are discordant with the increased weight gain seen in the HFD-fed mice and the increased adiposity observed in male HFD Sucnr1−/− mice. However, measurement periods within the Phenomaster were limited to 72 hours per temperature period (21°C and 4°C) with the initial 24 hours of each period removed as data are often inaccurate at these times due to the stress of moving to a new environment. A 48-hour measurement period represents
approximately 2% of the total 13 weeks from study start to end which is a narrow window into the total energy expenditure and intake throughout the study. Although no differences in body mass were observed in the male HFD-fed mice, the mean fat mass of the \textit{Sucnr1}\textsuperscript{-/-} mice on a HFD was 4.3 grams greater than the respective \textit{Sucnr1}\textsuperscript{+/+} HFD-fed group, this equates to a 38.7Kcal difference across the whole experiment, assuming 1 gram of fat is 9Kcal. Therefore, if the \textit{Sucnr1}\textsuperscript{-/-} mouse had an approximately 0.5Kcal/day difference in energy intake than the \textit{Sucnr1}\textsuperscript{+/+} this would account for the difference in adiposity over the duration of the study. Whilst these numbers are extrapolated from means gathered during the experiment and there is substantial variability at the individual level, these data highlight how subtle differences could be missed during measurement of energy expenditure or food intake during such a small measurement window. Other models of \textit{Sucnr1}\textsuperscript{-/-} have increased caloric intake when on a standard diet (234, 263), although when fed a HFD intake is similar to wild type mice (234) or even decreased in adipose specific knockout (263) seemingly paradoxical to the increased adiposity observed. These data show that the pathways governing \textit{Sucnr1} control of energy homeostasis are complex and vary between models used. However, in these previous models female mice were not assessed so a comparison cannot be made, and data collected in this study data could be indicative of additional sex differences in the role of \textit{Sucnr1} food intake requirements during 4\textdegree C cold exposure.

The RER measured during both 21\textdegree C and 4\textdegree C showed no effect of genotype on lipid or glucose utilisation in any group of the mice, indicating that SUCNR1 does not change fuel preferences during CIT or standard housing conditions. There was a loss of diurnal variation in RER in the HFD groups which is consistent with diet-induced obesity (316, 317). However, these data contradict previous reports demonstrating increased RER in both global (234) and adipose specific \textit{Sucnr1} knockout (263) models on a standard diet. Furthermore, in adipose specific knockout mice high fat feeding decreased RER, indicating increased lipid metabolism in the mice, which was not observed in this study. These data further highlight how the effects of SUCNR1 on the metabolic phenotype are tissue and background dependant.
In addition to the lack of effect *Sucnr1* knockout on BAT function, thermogenic gene expression of the IAT depot was also similar between genotypes. These data suggest that SUCNR1 does not drive a browning phenotype in IAT at 4°C. A lack of WAT browning was also observed in another model of global *Sucnr1*<sup>−/−</sup> (234). However, these data are in contrast to the adipose specific knockout, which observed increased WAT browning in differentiated adipocytes derived from *Sucnr1*<sup>−/−</sup> mice indicating that SUCNR1 inhibits browning in scWAT (263). However, it should be noted this was not tested *in vivo* or during cold exposure which may explain the differences seen. A myeloid specific *Sucnr1* knockout model showed a reduction in scWAT browning following 4°C cold exposure. These data indicate that SUCNR1 plays a tissue dependant role in WAT browning and that responses vary depending on the model used. Therefore, it is possible that compensatory mechanisms act to maintain normal BAT and WAT function during CIT.

Although there were no differences in energy expenditure or thermogenic gene expression in either BAT or IAT following cold exposure, male *Sucnr1*<sup>−/−</sup> mice had higher core temperatures than the *Sucnr1*<sup>+/+</sup> mice on both CD and HFD following cold exposure. As discussed already, energy expenditure was similar but there were also no significant differences in tail temperatures, suggesting that this is not due to altered heat dissipation, although we could not quantify dissipation through the fur. In skeletal muscle succinate is secreted in response to exercise induced pH changes in myocytes, where there is paracrine signalling through SUCNR1 to control muscle remodelling transcriptional programs (195). SUCNR1 is also known to control fast-twitch to slow-twitch muscle fibres changes (203). Skeletal muscle has an important role in CIT as well as BAT (318), and as SUCNR1 plays a role in skeletal muscle function it is possible that a skeletal muscle phenotype is driving the increase in core body temperature. Another possible explanation for increased core temperature in the *Sucnr1*<sup>−/−</sup> mouse could be mediated through liver function. In hepatocytes, SUCNR1 signalling mediates an inflammatory response in the liver when activated by circulating succinate (228). The group observed that in mice with *Ucp1*<sup>−/−</sup> in BAT that
systemic clearance of succinate from the circulation was ablated leading to increased activation of SUCNR1 in the liver and driving inflammation from modulating hepatic stellate cell and macrophage populations. Dual Ucp1/Sucnr1−/− mice were rescued from this phenotype. It is therefore possible that in the model used in this study there is a liver inflammatory phenotype that has not been characterised, and given the variation of metabolic profiles of different Sucnr1−/− lines it could contribute to increased core body temperature if there is systemic inflammation through liver dysfunction (319, 320). However, this is speculative and further investigation is needed to understand why core temperature is different in the Sucnr1−/− mouse and to what extent, if any, that skeletal muscle or the liver contribute to this.

The mRNA expression of the succinate transporters Slc25a10 and Slc13a3 was unchanged in the BAT of all groups, both male and female, suggesting that neither SUCNR1 nor HFD alter brown adipose tissue succinate transport during cold exposure. These data suggest that extracellular SUCNR1 signalling does not alter succinate transport across the mitochondrial or plasma membrane. Importantly though, we did not measure protein expression of these transporters. Furthermore, quantification of uptake of labelled succinate by BAT would have been required to determine this. Recent reports have also identified other transporters such as monocarboxylate transporter 1 (Mct1) as having a role in BAT succinate import into murine BAT, although ablation of this transporter did not affect succinate transport (200). This indicates that succinate transport can compensate for loss of individual transporter expression in BAT.

Sucnr1 mRNA levels were reduced in BAT compared to IAT and gWAT depots in male mice, while a similar pattern was observed in females this was not significant. Sucnr1 expression in male and female mice was comparable showing no sex differences in expression of Sucnr1 despite the differences in response to knockout. Female mice and humans are often resistant to metabolic disorders due to the protective effects of oestrogen (reviewed in (321)), however, this was not established as the
mechanism driving the observed sex differences and as such further investigation is needed to confirm this. To determine if it is the effects of oestrogen that are protecting the females from the metabolic consequences of Sucnr1−/−, an ovariectomy could be performed in juvenile female mice and studied alongside intact females and males to see how the phenotypes compare. Previous reports have shown ovariectomy impairs female mouse metabolic health and have a comparable phenotype to male mice (322-324).

It should be noted the expression pattern observed in mouse adipose tissue is contrary to that in human differentiated adipocytes seen in chapter 3, which had highest expression in brown adipocytes. These data are indicative of species-specific differences of the role of SUCNR1 in mouse and human BAT function and metabolic health. SUCNR1 activation in human differentiated brown adipocytes increased respiration indicating activation of thermogenesis, however, in this mouse model there was no evidence of SUCNR1 modulating brown or beige adipose tissue function. These data indicate that whilst succinate increases brown adipocyte thermogenesis in mouse (146) and human (Figure 3. 4) brown adipocytes, these effects are independent of Sucnr1 in the mouse. However, as C-ESA increased NADR stimulated respiration in the human adipocytes SUCNR1 activation may also contribute to brown adipocyte thermogenesis.

Overall, it appears that whilst SUCNR1 plays an important role in murine metabolic health, particularly with regards to adiposity and glucose tolerance, it is not through modulating BAT or beige adipose tissue activity. However, the full scope of BAT has not been fully investigated in the present study. During thermoneutrality BAT is inactive, in mice this is observed when mice are housed between 28°C-33°C. At these conditions, NADR can be injected into the mouse tail vein to induce BAT activation (325). Changes in energy expenditure measured during this time will be attributed to BAT function occurring separately from CIT, energy expenditure changes could be measured in metabolic cages to assess how BAT function differs between wild-types
and knockouts. As well as measuring energy expenditure metabolite handling of BAT could be measured using $^{18}$FDG-PET or $^{11}$C-acetate-PET (to measure glucose uptake and oxidative capacity). These techniques in conjunction with either cold exposure or NADR injection would be able to assess whether Sucnr1 disruption leads to impaired glucose metabolism or lipid oxidation in BAT, which was unable to be performed within the present study. Additionally, SUCNR1 may have affected brown adipose tissue function but compensatory mechanisms may have been able to alleviate this dysfunction. Therefore, using a BAT specific knockout would eliminate these possible confounders. Using a BAT specific knockout model would determine whether dysfunction in other tissues is contributing to the increased fat mass in male HFD Sucnr1$^{-/-}$ mice. Modifications to the experimental design performed in this chapter should be added to address current knowledge gaps. Increasing the period of energy expenditure measurement may reduce variability between individual days and more accurately represent daily energy expenditure. Food intake was only measured during housing in metabolic cages, however, as previously discussed the differences in energy balance needed to explain the difference in fat mass between male HFD fed Sucnr1$^{+/+}$ and Sucnr1$^{-/-}$ mice is small. Therefore, measuring food intake throughout the course of the study would allow for assessment of total caloric intake which may well identify such differences. This would also circumvent the possible confounder of stress altering the food intake of the mice when changing housing to the metabolic cages. Additionally, faecal analysis to assess nutrient absorption during digestion would also help to determine energy intake and if differences in fat mass are in part due to food intake/nutrient absorption differences.

These additional experiments would allow for a more complete characterisation of the role of SUCNR1 in BAT to elucidate to what extent BAT contributes to the observed metabolic phenotypes if at all. However, as these were not feasible in the scope of this thesis, the next chapter focuses on the culture of brown and inguinal adipocytes derived from both Sucnr1$^{+/+}$ and Sucnr1$^{-/-}$ mice and how treatment with both succinate and C-ESA alter cellular function. Data from this cell model will help elucidate how Sucnr1 knockout affects brown adipocyte function and allow direct
comparisons with the human data obtained in chapter 3 to identify any potential species-specific differences.
Chapter 5

The Effect of Succinate Receptor Disruption on Murine Brown and Inguinal Adipocyte Function
5.1 INTRODUCTION

In chapter 3, succinate increased respiration in human brown and white adipocytes, whilst the succinate receptor agonist C-ESA increased noradrenaline-stimulated respiration only in brown adipocytes. To explore this effect on BAT function in vivo, in chapter 4 we undertook metabolic phenotyping of a global Sucnr1−/− murine model during cold exposure. However, contrary to the hypothesis, BAT function was unaffected by deletion of Sucnr1, despite male Sucnr1−/− mice developing increased adiposity and glucose intolerance on a high fat diet, as previously reported (234, 263).

Additionally, mRNA expression of thermogenic genes and UCP1 protein levels were unchanged in Sucnr1−/− mice both on control and high fat diets. However, as this was a global knockout and CIT is a complex multi-organ process, compensatory mechanisms in other tissues could have obscured dysfunctional BAT. Compensatory mechanisms have been previously observed in other global knockout mouse models (326, 327), and in mice with dysfunctional BAT compensatory browning of WAT can occur (328), however, this was not observed in our model. The data from these previous chapters suggest possible species-specific differences in the role of the succinate receptor in human and murine BAT. Using an in vitro model with cells derived from Sucnr1+/+ and Sucnr1−/− mice allows us to compare how succinate and C-ESA treatment alter murine and human adipocyte function and determine whether succinate mediates any of its effects in murine brown adipocytes through SUCNR1.

There are several models of both human and mouse immortalised adipocyte cell lines, typically similar proliferation protocols are used, and will use common cell culture media compositions to drive differentiation (329). However, immortalised cell lines have several undesirable features as a cell culture model. In order to become immortalised, genes controlling cell cycle arrest must be altered to favour continuous proliferation (329). Indeed immortalised human brown adipocytes (175) show a different gene profile to human primary brown adipocytes (210). Additionally, repeated proliferation and culture of cell lines leads to genetic drift through the occurrence of random mutations, which can alter the phenotype of these cells and
the response to drug treatment (330). These issues mean that during prolonged in vitro experiments the genetic drift and changes to cellular process in these immortalised cell lines can cause confounders in the results contributing to variation. Using primary cell cultures, some of the limitations can be mitigated (329). Primary cell lines (both murine and human) proliferate at a similar rate to their respective immortalised cell lines, however, after repeated clonal expansion primary cells will reduce in viability to a much greater extent than immortalised lines (331). Whilst primary cells lines can’t be expanded or used for repeated experiments to the same extent as immortalised cell lines, due to the cells being isolated directly from in vivo adipose tissues they more closely resemble the genetic expression of the cell than immortalised cell lines (329, 331). As such, this makes primary brown adipocyte cultures a good model in which to study the functional role of SUCNR1. However, as primary cells are derived from individual animals there is greater genetic variation between samples. This variation has been shown to effect inguinal/beige adipocyte differentiation in primary cell culture models in two murine strains (332). However, as genetic monitoring and testing of the mouse model used in this study was performed, the cells derived from the Sucnr1+/+ and Sucnr1-/− should show minimal variability and are an appropriate in vitro model to use. A major benefit of using cells derived from these mice as the in vitro model is that Sucnr1 is already knocked out and circumvents the need for pharmacological inhibition or siRNA interruption of the receptor. Additionally, the isolation and culture techniques used in this in vitro model allow for direct comparison with the human adipocytes as it uses the same protocol.

We planned to replicate the human in vitro experiments undertaken in chapter 3, investigating the effect of succinate and C-ESA on cellular respiration, gene expression and lipolysis in in murine primary adipocytes. Sucnr1 disruption in mice, increased glycerol release in ex vivo gWAT explants, due to the lack of inhibitory effects on lipolysis following Sucnr1 activation (234). However, this was not investigated in inguinal or brown adipose tissue, so the role of SUCNR1 activation on lipolysis is unclear in murine thermogenic adipose tissue. This is particularly
important because FAs generated from intracellular lipolysis are key to BAT function (333). Succinate acutely increases murine brown adipocyte respiration independently of SUCNR1 (146), however, this was observed using supraphysiological doses of succinate (1-5mM) and without activation of SUCNR1 through the use of an agonist such as C-ESA. This effect was preserved in cells lacking Suncr1, although, it was unclear how this model was generated as the manuscript did not detail either mouse model or knockout/knockdown protocol. Further, chronic treatment with succinate and its effects on respiration was not assessed, therefore, the role of chronic SUCNR1 agonism in murine brown adipocyte respirometry remains unclear.

5.2 HYPOTHESIS

Activation of the succinate receptor in murine brown and beige adipocytes increases cellular respiration and increases expression of thermogenic genes.

5.3 AIMS

1. To determine the effect of succinate and SUCNR1-agonist treatment on basal and noradrenaline-stimulated respiration and determine whether these effects are abated in Sucnr1 knockout derived brown and inguinal adipocytes.

2. To determine the effect of succinate and SUCNR1-agonist treatment on basal and noradrenaline-stimulated thermogenic gene expression in murine differentiated brown and inguinal pre-adipocytes.

3. To determine the effect of succinate and SUCNR1-agonist treatment on basal and noradrenaline-stimulated lipolysis in murine primary brown and inguinal differentiated brown and inguinal pre-adipocytes.
5.4 METHODS

5.4.1 Murine pre-adipocyte isolation and cell culture
Murine pre-adipocytes were isolated, cultured and differentiated as described in 2.4.2. In brief, murine iBAT and IAT were dissected from 6-10 day old male mouse pups. Only male mouse pups were used to remove sex as a potential confounder, particularly as female mice did not show any phenotypic differences in the previous chapter. Tissues from 3 mice were pooled together, from the entire iBAT depot and the right inguinal depot respectively. Following collection, tissues were digested and SVF was isolated and plated into collagen-coated cell culture plates. Cells were proliferated and differentiated until ready for experimentation at D7-9.

5.4.2 Assessment of pre-adipocyte total differentiation
To determine how Sucnr1 disruption altered differentiation of brown adipocytes cells underwent the differentiation protocol as described in 2.4.2. When fully differentiated at D9 cells were imaged using light microscopy (EVOS Cell Imaging Systems, ThermoFisher Scientific) at 20x magnification. Images were captured with the built in digital camera system. A 4x4 grid overlay was added to the field of view image and undifferentiated cells were counted and compared to total differentiated cells.

5.4.3 The effect of succinate and C-ESA on respiration in differentiated pre-adipocytes from Sucnr1+/+ and Sucnr1−/− mice
To determine whether succinate and C-ESA altered basal and NADR-stimulated respiration, differentiated pre-adipocytes from Sucnr1+/+ and Sucnr1−/− mice were treated either vehicle (sterile distilled water), 100μM succinate, 1000μM succinate, 1μM C-ESA, 10μM C-ESA, or 100μM C-ESA (6 groups in total) for 2 hours prior to measurement of OCR. All conditions were run in triplicate with the exception of vehicle and 100μM C-ESA that were ran in quadruplicate (see Figure 5. 1 for plate map). A total of 18 mice were used for each genotype. Cellular respiration was
performed in the murine differentiated adipocytes as described in 2.4.8.2 to measure OCR in these cells. Non-mitochondrial respiration (see Figure 2.1) was subtracted from the OCR values presented.

![Figure 5.1 Plate map for murine respirometry assays. Schematic diagram of 24 well plate and conditions for respirometry assays using Seahorse V28 plates for brown and beige adipocytes.](image)

### 5.4.4 Effects of succinate and cis-epoxysuccinic acid on mRNA expression in adipocytes from Sucnr1+/+ and Sucnr1−/− mice

To determine the role of SUCNR1 on mRNA expression in differentiated brown and inguinal adipocytes during both basal and NADR stimulated conditions, cells were treated in a 3x2 design (6 groups in total with 18 mice used in the Sucnr1+/+ adipocytes and 7 mice used in the Sucnr−/− adipocytes) and were incubated with either vehicle, 1000μM succinate, or 10μM C-ESA, in the presence or absence 1μM NADR (see Figure 5. 2). All conditions were run in duplicate. At D7-9 both brown and inguinal adipocytes were treated as described in 2.4.6.3. Following 8 hours incubation, cells were lysed with Qiagen reagent for RNA extraction and subsequent qPCR. *Ucp1, Ppargc1a, Pparg, Slc25a10, Slc13a3* and *Sucnr1* mRNA expression were
quantified and normalised to the mean expression of housekeeping genes Rna18s5 and Tbp.

<table>
<thead>
<tr>
<th>Vehicle/Vehicle</th>
<th>Vehicle/Succinate 1000μM</th>
<th>Vehicle/C-ESA 100μM</th>
<th>NADR/Vehicle</th>
<th>NADR/Succinate 1000μM</th>
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<tr>
<td>Vehicle/Vehicle</td>
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<td>NADR/Vehicle</td>
<td>NADR/Succinate 1000μM</td>
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**Figure 5. 2 Plate map for mRNA and lipolysis assays.** Schematic diagram of 24 well plate and conditions for mRNA expression assays and lipolysis assays

**5.4.5 Effect of succinate and C-ESA on murine adipocyte lipolysis**

To determine the role of SUCNR1 in differentiated brown and inguinal adipocytes on lipolysis during both basal and NADR stimulated conditions, cells were treated in a 3x2 design (6 groups total with 18 mice used in the Sucnr1+/+ adipocytes and 7 mice used in the Sucnr1-/- adipocytes) and were incubated with either vehicle, 1000μM succinate, or 10μM C-ESA, in the presence or absence 1μM NADR (see Figure 5. 2). All conditions were run in duplicate. Following differentiation (D7-9), brown and inguinal adipocytes were treated as described in 2.4.7.2. Glycerol was measured as described in 2.8. Initial testing of cell culture media samples identified the 24–hour time-point as the optimal sample for subsequent measurements. The glycerol concentration was normalised to total protein content per well, with cells lysed for protein following the 24-hour time point (2.7.1). Cellular protein content was measured as described in 2.7.3.
5.4.6 Statistical analysis

Statistical tests and analysis were performed as described in 2.10.1. Data were analysed with either RM two-way or RM one-way ANOVA and presented as mean +/- SEM. Data were post hoc tested using Shapiro-Wilk, D'Agostino & Pearson test, and Sidak’s multiple comparison. Significance was determined when p<0.05.
5.5  RESULTS

5.5.1  Brown and inguinal adipocyte oxygen consumption rate is unaffected by Sucnr1 disruption

Sucnr1 disruption did not affect respiration in brown adipocytes (Figure 5.3 A-B). In brown adipocytes, 1000μM succinate increased basal OCR, with a significant difference only seen by post-hoc testing in Sucnr1−/− cells (Figure 5.3 C). However, 1000μM succinate decreased NADR-stimulated respiration in both genotypes (Figure 5.3 D). Succinate treatment did not alter brown adipocyte ATP-linked respiration in either genotype (Figure 5.3 E). C-ESA did not alter respiration in brown adipocytes of either genotype (Figure 5.3 C-E). In inguinal adipocytes, respiration was unaffected by Sucnr1 disruption (Figure 5.4 A-B). Neither succinate nor C-ESA altered basal respiration (Figure 5.4 C), but like the brown adipocytes, there was an effect of drug treatment on NADR stimulated respiration. Treatment with 100μM C-ESA increased NADR-stimulated respiration in inguinal adipocytes from Sucnr1−/− mice (Figure 5.4 D). ATP-linked respiration was unaffected by either succinate or C-ESA treatment in both brown and inguinal adipocytes (Figure 5.3 E and Figure 5.4 E). Unexpectedly, FCCP injection did not increase OCR in either brown or inguinal adipocytes from either genotype, therefore, analysis of data during this phase of the respirometry assay was not undertaken.
Figure 5.3 Murine differentiated brown adipocyte OCR is unaffected by Sucnr1 disruption. Oxygen consumption rate of brown adipocytes treated with either vehicle (black columns), 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (pink columns) in Sucnr1+/+ (A) and Sucnr1−/− (B) derived adipocytes measured during
basal conditions (1) and following addition of NADR (2), oligomycin (3), and FCCP (4). Mean basal (C), mean NADR (D), and mean ATP linked respiration (E). Data are presented as mean ±S.E.M, A-F analysed with two-way RM ANOVA with Sidak’s multiple comparison post hoc test, n=6/group. (*; p<0.05).
Chapter 5

The Effect of Succinate Receptor Disruption on Murine Brown and Inguinal Adipocyte Function

Figure 5. 4 Murine differentiated inguinal adipocyte OCR is unaffected by Sucnr1 disruption. Oxygen consumption rate of inguinal adipocytes treated with either vehicle (black columns), 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (pink columns) in Sucnr1^+/+ (A) and Sucnr1^-/- (B) derived adipocytes measured at basal (1), NADR stimulation (2), oligomycin stimulation (3), and FCCP stimulation (4). Mean
basal (C), the initial response to NADR (D), and mean ATP linked respiration (E). Data presented as mean ±S.E.M, A-E analysed with two-way RM ANOVA with and Sidak’s multiple comparison (*; p<0.05) n=6/group.
5.5.2 The effects of Sucnr1 disruption on adipocyte mRNA expression

5.5.2.1 Succinate and C-ESA do not alter Ucp1 expression in murine adipocytes

NADR increased Ucp1 expression in both murine brown and inguinal adipocytes (Figure 5.5 A-D). In brown adipocytes, treatment with succinate or C-ESA did not alter basal or NADR-stimulated Ucp1 expression (Figure 5.5 A-B). Genotype did not statistically alter Ucp1 expression in brown adipocytes, although Sucnr1 disruption tended to reduce Ucp1 expression following NADR treatment (Figure 5.5 A-B). In inguinal adipocytes, both succinate and C-ESA reduced Ucp1 expression in the Sucnr1+/+ cells during both basal and NADR stimulated conditions. In Sucnr1−/− inguinal adipocytes, succinate reduced Ucp1 during basal conditions only. Genotype had no effect on inguinal adipocyte Ucp1 expression (Figure 5.5 C-D).
Figure 5. Brown adipocyte Ucp1 expression is unaffected by Sucnr1−/− or succinate/C-ESA treatment. Ucp1 mRNA levels in differentiated murine (A-B) brown (n=6/group) and (C-D) inguinal (n=6/group) adipocytes treated with either vehicle (black columns), 1000μM succinate (orange columns), 10μM C-ESA (blue columns) for 8 hours with or without 1μM NADR. Data are mean ± SEM for and were analysed by two-way repeated measures ANOVA.
5.5.2.2 *Sucnr1* disruption increases adipocyte *Ppargc1a* expression during NADR stimulation

*Ppargc1a* expression was increased in noradrenaline treated brown and inguinal adipocytes from *Sucnr1*−/− mice compared with cells from WT controls, and succinate and C-ESA treatment reduced *Ppargc1a* expression (Figure 5.6 B and D). In brown adipocytes, succinate or C-ESA did not alter *Ppargc1a* expression during basal conditions in either *Sucnr1*+/+ and *Sucnr1*−/− cells (Figure 5.6 A). In inguinal adipocytes, succinate and C-ESA treatment lowered *Ppargc1a* expression during basal conditions compared to vehicle in both genotypes (Figure 5.6). In both murine brown and inguinal adipocytes, there was no effect of NADR treatment on *Ppargc1a* expression (Figure 5.6 A-D).
Figure 5. 6 Sucnr1 deletion increases brown and inguinal adipocyte Ppargc1a expression during NADR stimulation. Ppargc1a mRNA levels in differentiated murine (A-B) brown (n=6/group) and (C-D) inguinal (n=6/group) adipocytes treated with either vehicle (black columns) 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 8 hours with or without 1μM NADR. Data are mean ± SEM for and were analysed by two-way repeated measures ANOVA, with Sidak’s multiple comparison test. (*; p<0.05)
5.5.2.3 Sucnr1 disruption does not alter succinate transporter expression in murine adipocytes

Sucnr1 disruption did not alter mRNA expression of Pparγ, Scl13a3, or Slc25a10 in either brown or inguinal adipocytes. Succinate, C-ESA and NADR also had no effect on expression of these genes or Sucnr1 (Figure 5. 7-10).
Figure 5. 7 Neither Succinate/C-ESA treatment nor Sucnr1 disruption alter Pparγ mRNA expression in brown or inguinal adipocytes. Pparγ mRNA levels in differentiated murine (A-B) brown (n=6/group) and (C-D) inguinal (n=6/group) adipocytes treated with either vehicle (black columns) 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 8 hours with or without 1μM NADR. Data are mean ± SEM for and were analysed by two-way repeated measures ANOVA.
Figure 5. Neither Succinate/C-ESA treatment nor Sucnr1 disruption alter Slc13a3 mRNA expression in brown or inguinal adipocytes. Slc13a3 mRNA levels in differentiated murine (A-B) brown (n=6/group) and (C-D) inguinal (n=6-7/group) adipocytes treated with either vehicle (black columns) 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 8 hours with or without 1μM NADR. Data are mean ± SEM for and were analysed by two-way repeated measures ANOVA.
Figure 5.9 Neither Succinate/C-ESA treatment nor Sucnr1 disruption alter Slc25a10 mRNA expression in brown or inguinal adipocytes. Slc25a10 mRNA levels in differentiated murine (A-B) brown (n=6/group) and (C-D) inguinal (n=6/group) adipocytes treated with either vehicle (black columns) 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 8 hours with or without 1μM NADR. Data are mean ± SEM for and were analysed by two-way repeated measures ANOVA.
Figure 5. Neither Succinate/C-ESA treatment alter Sucnr1 mRNA expression in brown or inguinal adipocytes. Sucnr1 mRNA levels in differentiated murine (A) brown (n=6/group) and (B) inguinal (n=6/group) adipocytes treated with either vehicle (black columns) 100μM succinate (orange columns), 1000μM succinate (blue columns), 1μM C-ESA (red columns), 10μM C-ESA (grey columns), or 100μM C-ESA (brown columns) for 8 hours with or without 1μM NADR. Data are mean ± SEM for and were analysed by two-way repeated measures ANOVA.
5.5.3 C-ESA increases NADR stimulated lipolysis in brown but not inguinal adipocytes

NADR treatment increased lipolysis in both brown and inguinal adipocytes from both genotypes (Figure 5.11 A-D). In murine brown adipocytes, glycerol release at basal conditions was unaltered by succinate, C-ESA or genotype (Figure 5.11 A). C-ESA increased NADR-stimulated glycerol release in Sucnr1+/+ (and tended to do the same in Sucnr1−/−) brown adipocytes (Figure 5.11 B). In murine inguinal adipocytes, neither succinate/C-ESA treatment nor Sucnr1 disruption affected basal glycerol release (Figure 5.11 C). However, Sucnr1 disruption increased glycerol release during NADR treatment (Figure 5.11 D).
**Figure 5.** Sucnr1 disruption does not alter brown adipocyte lipolysis. Glycerol release from murine Sucnr1\(^{+/+}\) and Sucnr1\(^{-/-}\) brown and inguinal adipocytes after an 8 hour incubation with vehicle, 1000μM succinate, or 10μM C-ESA during treatment with vehicle (basal) (A, brown, n=4-5/group) (C, inguinal, n=5-6/group) or 1μM NADR (B, n=5-6/group) (D, inguinal, n=5-6/group) state. Data are represented as mean ±S.E.M, data were analysed by RM two-way ANOVA with Tukey’s multiple comparison (*; p<0.05).
5.6 DISCUSSION

5.6.1 General discussion
In chapter 3, I showed that both succinate and C-ESA increased human brown adipocyte respiration. However, in chapter 4, both BAT function and CIT were unaffected by global disruption ofSucnr1in mice when fed either a control or HFD. These murine data seemingly contradicted what was observed in the human in vitro model, suggesting possible species-specific differences in succinate receptor regulation of BAT function. In this chapter I aimed to investigate whether SUCNR1 regulated brown adipocyte function by using adipocytes isolated from the SVF of both Sucnr1+/+ and Sucnr1−/− mice. I showed that murine brown adipocyte function was largely unaffected by Sucnr1 disruption, with similar respiration and lipolysis compared with adipocytes from wild type controls. Additionally, Ucp1 expression was unchanged in both brown and inguinal adipocytes from Sucnr1+/+ and Sucnr1−/− mice. These results were similar to the in vivo data presented in chapter 4 and indicate that Sucnr1 does not play a significant role in murine BAT function.

Succinate acutely increases murine brown adipocyte OCR both in primary and immortalised cells, through SDH activity inducing ROS production (146). However, the increased OCR occurred when succinate was acutely added to the cells during respirometry. In the respirometry assays performed in the current study, adipocytes were incubated with succinate and C-ESA for two hours prior to the start of the assay. This was done to investigate how more sustained activation of SUCNR1 would affect respiration. During basal conditions, high dose succinate increased respiration in Sucnr1−/− brown adipocytes, meaning this effect is independent of SUCNR1. In addition, C-ESA had no effect on OCR in both Sucnr1+/+ and Sucnr1−/− brown adipocyte basal respiration. The OCR values for both Sucnr1+/+ and the Sucnr1−/− brown adipocytes were similar to what was previously reported at basal conditions (146) and disruption of Sucnr1 did not alter basal respiration. Unexpectedly, in both brown and inguinal adipocytes of either genotype, FCCP did not increase respiration, possibly due to the NADR treatment increasing respiration to near maximal levels.
and preventing further increases in respiration. However, this does not explain the decrease in respiration following FCCP injection. ROS production can inhibit respiration in adipocytes and although these products can be scavenged by pyruvate in the cell culture media the substantial increase in respiration seen following NADR treatment could indicate that further respiration was inhibited (334). Further, excessive ROS production can even lead to apoptosis in adipocytes and the decreases in respiration seen during the assay could have been indicative of cell death (335). However, ROS production and post assay cell death were not quantified and as such the cause for the lack of increased OCR post-FCCP remains unclear.

During NADR stimulation, 100μM C-ESA increased respiration in the Sucnr1<sup>−/−</sup> cells, indicating that C-ESA may have off target effects and is increasing respiration through mechanisms distinct from SUCNR1 activation. These data call into question the respirometry data observed in the human differentiated adipocytes where C-ESA increased human brown adipocyte NADR stimulated respiration. If C-ESA can alter brown or white adipocyte function through mechanisms distinct from SUCNR1 activation this indicates the previously observed increase in human brown adipocyte respiration in chapter 3 may not stem from SUCNR1 activation. As discussed in chapter 3, this further highlights the need for additional research involving knockdown of the receptor in human adipocytes to ascertain the function of SUCNR1 in brown adipocyte respiration.

Additionally, during lipolysis assays C-ESA treatment increased NADR stimulated lipolysis in the Sucnr1<sup>+/−</sup> brown adipocytes and tended to do the same in the KO cells. These data indicate that C-ESA may increase brown adipocyte lipolysis through mechanisms distinct from SUCNR1 activation. However, there have not been previously reported instances of off target effects of C-ESA and has not elicited changes in cells lacking the succinate receptor (236). The succinate receptor can be activated by other TCA cycle metabolites which share a similar structure to succinate
including oxaloacetate, L-malate, and α-ketoglutarate, as well as some non-TCA cycle compounds (219). It is also possible that C-ESA can bind other GPCRs although no such effects have previously been described. Hydrocarboxylic acid receptor 1 (HCAR1), a GPCR that is activated by lactate, another carboxylic acid metabolite important in the TCA cycle, regulates glucose uptake into BAT during high fat feeding (336, 337) and also inhibits lipolysis in adipocytes (338). These data show that small carboxylic acids and their respective receptors can modulate BAT function and that carboxylic acid molecules can bind to similar receptors that are not their cognate receptor. Therefore, a key experiment would be to determine if these changes in human brown adipocyte respiration persist with C-ESA treatment following knockdown of the succinate receptor as discussed in chapter 3. Inguinal adipocytes from Sucnr1−/− mice had higher rates of lipolysis than Sucnr1+/+ adipocytes following NADR stimulation. gWAT explants taken from Sucnr1−/− mice demonstrated increased lipolysis during isoprenaline stimulation (234, 263). Although WAT explants typically have greater levels of glycerol release during lipolysis assays this work demonstrates that both WAT and inguinal adipocyte lipolysis is under the regulation of SUCNR1. However, the lack of response to succinate and C-ESA treatment in Sucnr1+/+ derived inguinal adipocytes may suggest that SUCNR1 activation is not altering lipolysis. However, succinate was demonstrated to be present in the FBS used for proliferation and differentiation of cells up until the media was changed for the lipolysis assay, and chronic SUCNR1 agonism may confound these results. The lipolysis media did not contain FBS, but it did contain fatty acid free BSA which should not contain succinate. It is possible that succinate release from the adipocytes could be sufficient to achieve concentrations high enough in the vehicle groups to activate SUCNR1 and confound results within the Sucnr1−/+ cells. Therefore, further work is required to determine the role of SUCNR1 activation in both brown and inguinal adipocyte lipolysis.

The results from the gene expression assays were similar to those in the human adipocytes. Ucp1 mRNA expression was unaffected by knockout or succinate/C-ESA treatment in Sucnr1+/+ or Sucnr1−/+ brown adipocytes. However, in inguinal adipocytes
succinate and C-ESA decreased Ucp1 expression in the Sucnr1+/+ cells (both at basal and NADR stimulated conditions) and succinate caused a lesser reduction in Ucp1 expression in Sucnr1−/− cells during basal respiration. Although genotype did not significantly alter Ucp1 expression inguinal adipocytes it should be noted that Sucnr1−/− derived adipocytes had approximately a two-fold higher expression compared to Sucnr1+/+ derived adipocytes, this could be indicative of under powering and warrants potential follow up with increased replicates. Sucnr1 disruption has been shown to impair adipose tissue browning of WAT in a myeloid specific knockout model (224) but increased Ucp1 expression in brown adipocytes (263). The data collected in the in vivo study in chapter 4 indicated that Sucnr1 knockout did not alter BAT or IAT Ucp1 expression. With the data gathered in this chapter it appears that whilst succinate/C-ESA treatment and SUCNR1 may have a role in inguinal adipocyte browning these effects are not observed in vivo (possibly due to compensatory mechanisms) and indicate the role of Sucnr1 in inguinal thermogenic function is minimal.

However, Ppargc1a expression was increased in Sucnr1−/− brown and inguinal adipocytes compared to Sucnr1+/+ adipocytes following NADR stimulation. Ppargc1a is a regulator of mitochondrial biogenesis and expression is increased with increased ROS production (339). As SUCNR1 has previously been implicated in ROS production signalling (146) these data could highlight an interplay between SUCNR1 signalling and Ppargc1a mediated mitochondrial biogenesis during cellular redox stress, in this case induced by NADR stimulation in the adipocytes (340). However, in vivo, Ppargc1a mRNA expression was unaltered by Sucnr1 disruption in either the BAT or IAT during cold exposure so the relationship between these genes remains unclear. Succinate reduced Ppargc1a mRNA expression even in Sucnr1−/− adipocytes when incubated with NADR, indicating this reduction is independent of SUCNR1 agonism. Interestingly C-ESA also reduced Ppargc1a expression in Sucnr1−/− adipocytes, which further supports the possibility of C-ESA affecting adipocyte function through mechanisms independent of SUCNR1. As Sucnr1 knockout increases Ppargc1a, which
is important in adipocyte browning (341) and metabolic health (342, 343), investigation of potential SUCNR1-mediated regulation of *Ppargc1a* expression is warranted. However, neither succinate nor C-ESA treatment altered *PPARGC1A* expression in the human adipocytes indicating this may be an effect only observed in murine adipocytes. As discussed in chapter 3, siRNA knockdown of *SUCNR1* would be an ideal way to further investigate the role of the succinate receptor in human adipocytes and could confirm the possible species-specific differences identified. *Pparγ* mRNA expression was unchanged by *Sucnr1* disruption or with succinate/C-ESA suggesting SUCNR1 does not play a major role in adipocyte differentiation, supporting the observed similarity in total differentiation of the adipocytes of both genotypes. However, this would need to be investigated by quantifying lipid accumulation as a marker of differentiation for example with the use of Oil Red O (344). Although no differences in proliferative or differentiation capacity were observed during cell culture this was not investigated experimentally.

Succinate transporter expression was unaffected in either brown or inguinal adipocytes. However, other transporters such as MCT1 (200) and the protein levels of these genes should be investigated in the future to support this as the exact mechanisms governing succinate transport have not been fully elucidated. As discussed in section 1.4.1.2 transport of succinate across the plasma membrane is poorly understood, and when either SLC13A3 or MCT1 are inhibited succinate transport is only slightly altered, further highlighting the need for this to be investigated. Additionally, in the *Sucnr1*+/+ brown and inguinal adipocytes, *Sucnr1* mRNA expression was unaffected by NADR treatment. These data indicate that SUCNR1 activation is not regulated by adrenergic stimulation and therefore is unlikely to be altered by cold exposure.

Overall, the data presented in this chapter suggest that the succinate receptor has minimal role in murine brown adipocyte function. These data are consistent with the
results from chapter 4, where disruption of the succinate receptor did not alter energy expenditure in mice either housed at 21°C or at 4°C. In this mouse model, *Sucnr1* expression is highest in white adipocytes and lowest in brown (with inguinal as an intermediate), as previously reported (234). This is in contrast to the human RNA seq and qPCR data in differentiated adipocytes. Species-specific differences in the control of brown and white adipose tissue have previously been reported (36). Acute glucocorticoid stimulation acutely increases human BAT activity, whereas it reduces it in mice (141). Additionally, the primary activating receptor of brown adipocytes in mice is the β3-adrenergic receptor, however, there is evidence that β1 and β2-adrenergic receptors are the primary drivers of human brown adipocyte activation (84, 120). Further, human and mouse brown adipose tissue have differing anatomical locations and activation temperatures due to the physiological differences in anatomy (345). These differences highlight substantial differences in regulation of BAT between humans and mice, as such there remains a possibility that C-ESA, through activation of SUCNR1, does increase human brown adipocyte respiration, although further work is required to confirm this mechanism.
Chapter 6

Final Discussion and Future Research
6.1 DISCUSSION

As discussed in chapter 1, the global burden of obesity is rising (346, 347) and with it the prevalence of diseases such as T2DM and cardiovascular disease (11, 13). Obesity occurs when energy intake exceeds energy expenditure leading to increased adiposity (348), however, the mechanisms controlling each side of this energy balance equation are complex. In evolutionary terms there is a strong drive to increase energy intake during times of food abundance as an adaptation to survive periods of scarcity (349). However, in recent decades, many areas of the world have increased food security and abundance year round, creating a situation where the availability and access of food is ever present (350). Additionally, there has been a reduction in overall daily energy expenditure in the last few decades (7), although the exact cause (increased sedentary lifestyle or dietary changes) has yet to be confirmed. These factors together are possibly contributing to the increased global levels of obesity. Research into the exact contribution to which side of the energy balance equation needs adjusting to reduce obesity is currently ongoing. Dietary and lifestyle changes to encourage increased physical activity, although effective treatments for obesity, are difficult to maintain consistently over an individual’s lifetime (13). Additionally, available pharmacotherapies for obesity treatment, such as the GLP-1 receptor agonist Semaglutide (351-353), are becoming an increasingly promising method of body weight control and treatment of obesity. However, the re-gaining of lost body weight and fat mass is a potential issue with such medications as side effects of pharmacotherapies limit adherence, while these medications have only been approved for a 2-year period and weight regain occurs following cessation of treatment (354). Further, processes regulating increased energy intake and expenditure are tightly regulated by the body, and homeostatic controls are in place to maintain energy balance by compensating for changes to energy expenditure or intake (355). Therefore, the need for additional research into obesity treatments is warranted.

BAT is one such potential target for an anti-obesity treatment. When BAT is activated during CIT, energy expenditure is increased to meet the metabolic demands of the
tissue. Although this increase in energy expenditure is small, a caloric deficit of 50-60 Kcal per day, if sustained for a period of 12 months, equates to a ~2.5Kg weight loss (170). This increased energy expenditure coupled with appetite suppression treatments could be an effective way to treat obesity over the long-term. Indeed, individuals with increased BAT mass are associated with fewer instances of cardiovascular disease, even in obese individuals (82). The plasticity of brown adipose tissue as an expandable organ during prolonged periods of cold exposure (122) or adrenergic receptor agonism (129), provide evidence that the depot can increase in mass and possibly increase energy expenditure during chronic activation.

However, BAT function in humans is not fully understood. This thesis aimed to address knowledge gaps in mechanisms controlling BAT function and activation. The underlying hypothesis of this thesis was that the succinate receptor, which had high differential expression in human brown adipocytes compared to white adipocytes, played a role in modulating BAT function when activated by the endogenous ligand succinate. Succinate has been implicated in activation of brown adipocytes via sequestering succinate from circulation which in turn leads to ROS production driving thermogenesis (146). Sucnr1 disruption in mice has been shown to have effects on metabolic health by inducing leanness in control diet fed mice but leading to excessive fat mass in HFD fed mice (234). Additionally, succinate receptor signalling has been implicated in skeletal muscle remodelling following exercise (195). These studies have highlighted the importance of both succinate and SUCNR1 in metabolic health, however, the interplay between SUCNR1 activation and BAT function have not been fully elucidated. We hypothesised that activation of SUCNR1 in human brown adipocytes activates thermogenesis and therefore increases energy expenditure.

To test this hypothesis, both in vitro and in vivo models were used to elucidate the role of the succinate receptor in human and murine BAT. Human differentiated brown and white adipocytes, isolated from the SVF of patients undergoing elective
neck surgery, were treated with both succinate and C-ESA to determine the effect on brown adipocyte function. C-ESA treatment reduced lipolysis in white adipocytes, consistent with observed results in mouse WAT explants (234). Additionally, treatment with succinate or C-ESA was able to increase respiration in human brown adipocytes during basal and NADR stimulated conditions. These data indicate that activation of SUCNR1 is able to increase energy expenditure in human brown adipocytes, although the exact mechanism driving this increase is unclear.

To explore how this SUCNR1 modulation of BAT energy expenditure translated to BAT function in a whole organism a global Sucnr1 knockout mouse model was used to test this in chapter 4. Sucnr1−/− male mice had increased adiposity and impaired glucose tolerance when fed a HFD, an effect not observed in CD males or females irrespective of diet. These sex differences observed have not previously been reported in other models investigating Sucnr1 in metabolic health, as female mice have been omitted from study (234, 263). This novel data indicates that females are protected from the deleterious effects of Sucnr1 knockout, although the mechanisms governing this protection were unable to be assessed in this thesis. Sucnr1 disruption did not impair adaptation to cold exposure or energy expenditure during 4°C in male or female mice. These data suggest that in mice, Sucnr1 does not play an important role in BAT function. This was supported with the in vitro models used in chapter 5 which highlighted Sucnr1 disruption had minimal effect on gene expression with the exception of increased Ppargc1a mRNA expression. Sucnr1 disruption did not significantly alter respiration of brown or inguinal adipocytes. However, in the brown adipocytes succinate did increase basal respiration in both Sucnr1+/+ and Sucnr1−/− adipocytes. These corroborate previously identified succinate driven increase in brown adipocytes OCR, and as Sucnr1 disruption did not alter respiration, the likely mechanism behind this increase is through ROS production in the cell.

Together, the data gathered throughout this thesis indicate possible species-specific differences in the role of the succinate receptor in human and murine BAT function.
While SUCNR1 activation increased respiration in human differentiated brown pre-adipocytes, Sucnr1 knockout did not alter murine adipocyte respiration. However, an important caveat is the potential off-target effects of C-ESA, highlighted by this drug increasing respiration and lipolysis in murine brown adipocytes not expressing SUCNR1.

Therefore, to further understand the role of SUCNR1 in human BAT function additional investigation is required. Recent work in the Stimson lab has demonstrated that siRNA knockdown of target genes is feasible and effective using a similar cell culture protocol as was performed in this thesis (210). Knockdown of SUCNR1 in human adipocytes would highlight whether the effects of C-ESA treatment were indeed through SUCNR1 and if there are species specific differences in the importance of SUCNR1 in BAT function. This disruption of SUCNR1 would allow for similar experiments to those detailed in this chapter 3 to be repeated, most importantly to determine whether the observed effects of C-ESA on brown adipocyte respiration were mediated through SUCNR1 activation or off-target effects. Alternatively, the receptor could be inhibited by an antagonist such as NF-56-EJ40. Use of antagonists can be an effective way to determine the role of an individual receptor but it is difficult to determine if responses are due to the desired inhibitory effect or if there are off target effects from the antagonist being non-specific. Therefore, the knockdown of SUCNR1 in cultured human adipocytes is likely the best option. Human in vivo work would also be vital to assess the importance of SUCNR1 in BAT function. A potential experimental design to achieve this would be to use microdialysis techniques to infuse BAT positive humans with either saline or a succinate containing solution into the left and right supraclavicular depot respectively. Following which glucose uptake and glycerol/FA release could be quantified to determine differences in metabolite flux and indicate activity level. This could further be developed with the inclusion of cold exposure and subsequent $^{18}$FDG or $^{11}$C-acetate PET/CT to quantify how succinate infusion affects BAT activation and oxidation during cold exposure. These experiments would ideally be performed in both sexes to establish whether possible sex differences in humans are present in
SUCNR1 regulation of BAT as indicated by the sex differences of SUCNR1 in murine metabolic health. Additionally, both normal weight and obese individuals to assess how obesogenic challenge impacts succinate induced activation of BAT.

In the global SUCNR1 knockout model used for this study no differences in energy expenditure at either 21°C or 4°C were observed. Additionally, Sucnr1 disruption did not alter the expression of Ucp1 or Ppargc1a mRNA and UCP1 protein levels in whole brown adipose tissue, highlighting that Sucnr1 does not play a role in BAT function. However, there remains untested facets of BAT function. For instance, glucose uptake in BAT was not assessed and with the effect of SUCNR1 regulating glucose tolerance this could indicate perturbed metabolite handling in BAT. Using 18FDG-PET/CT to monitor both cold induced and NADR stimulated glucose uptake in the mice would be the ideal technique to identify these differences. This technique would also allow for assessment of other metabolically active tissues such as liver or skeletal muscle, both of which SUCNR1 has been shown to impact their function (195, 228). These data may highlight the tissues associated with the glucose tolerance phenotype observed in the male Sucnr1−/− mice. Additionally, use of euglycaemic-hyperglycaemic clamp techniques would be able to further assess the glucose and insulin tolerance of these mice which was unable to be performed in this study. Further, these mice could be housed at thermoneutrality in metabolic cages and treated with NADR to stimulate BAT activation, this would potentially highlight smaller differences in energy expenditure during BAT activation that may not have been observed in the present study. If further study is to be done in this model closer attention to food intake would be imperative. Although, food intake was measured during housing in the metabolic cages. However, this accounted for only a small window of the study and due to stress associated with changing housing prior to measurement are possibly not representative of normal feeding behaviour. Adipose specific Sucnr1 knockout models have been shown to have increased food intake when on control diet and decreased food intake when on high fat diet (263). These data seemingly contradict the observed adiposity phenotype but was determined to be caused by perturbed Lep expression and whole body energy homeostasis. Both
Lep expression and food intake should be measured in this model during future study to determine if these effects are preserved in this model. As previously discussed in chapter 4 oestrogen is a likely candidate for this difference. Comparing the metabolic health of female mice with ovariectomies, sham control females, and male mice would be a logical next step (322-324). Finally, a currently untested in vivo model would be to utilise a BAT specific Sucnr1 knockout, possibly using an Ucp1-Cre model to knockout Sucnr1 in BAT only (178). Utilising this model would highlight whether the impaired metabolic health observed in the HFD fed male mice can be attributed to BAT function or were primarily through other tissues, such as WAT (263).

6.2 CONCLUSIONS

The overarching hypothesis of this thesis was that succinate receptor activation would drive thermogenesis in human and murine brown adipose tissue. The data gathered in human adipocytes suggest that the succinate receptor does have a positive role in brown adipocyte function but the exact mechanisms through which SUCNR1 increases energy expenditure remain unclear. Conversely, murine in vivo data show that Sucnr1 disruption impairs metabolic health in HFD-fed males without reducing energy expenditure or cold tolerance. Similarly murine cell cultures demonstrate no substantial effect of SUCNR1 on adipocyte thermogenesis, revealing species-specific differences in the importance of the succinate receptor in murine and human brown adipocytes. Further research is needed to elucidate the extent to which SUCNR1 activation modulates human BAT function and whether this is therapeutically relevant in the context of obesity and metabolic disease.
7.1 References

14. BHF.org.uk. Heart and circulatory disease deaths in under 75’s see first sustained rise in 50 years. 2019.


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298. Shabalina IG, Hoeks J, Kramarova TV, Schrauwen P, Cannon B, Nedergaard J. Cold tolerance of UCP1-ablated mice: a skeletal muscle mitochondria switch toward lipid oxidation with marked UCP3 up-regulation not associated with increased basal, fatty acid- or ROS-induced uncoupling or enhanced GDP effects. Biochim Biophys Acta. 2010;1797(6-7):968-80.


346. Collaboration NCDRF. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based