Turun yliopisto
University of Turku

Effect of melanocortin-1 receptor in the regulation of beta3-adrenoceptor agonist-induced BAT thermogenesis

Master's Thesis
Rapson Khadka, B.Pharmacy
MSc in Drug Discovery and Development
University of Turku

Supervisor
Eriika Savontaus, MD, PhD
Institute of Biomedicine, Research Center for
Integrative Physiology and Pharmacology, University of Turku

ABSTRACT

Adipose tissue is comprised of brown and white adipose tissues. In particular, brown adipose tissues (BAT) or brown fat contain rich amount of beta3-adrenoceptors (β3AR) and they are regulated by sympathetic nervous system (SNS). Indeed, BAT contains unique protein called uncoupling protein 1 (UCP1) that causes non-shivering thermogenesis via mitochondrial uncoupling of oxidative phosphorylation respiratory chain. Central melanocortin system (CMS) that involves melanocortin-3 and melanocortin-4 receptors regulates energy intake and energy expenditure (EE). CMS-mediated EE occurs through the involvement of BAT. It is known that defective CMS signaling produces obese phenotype because of altered CMS action in EE. Preliminary work from my research group has found the similar kind of EE alteration and obese characteristics in melanocortin-1 receptor deficient ($MC1R^{e/e}$) mice. The aim of the present experiment centers on finding if this defective EE in $MC1R^{e/e}$ mice is occurring via BAT thermogenesis. Using mutant yellow $MC1R^{e/e}$ mice and wild type C57BL/6 mice, β3AR agonist CL-316243 was administered intraperitoneally to induce BAT thermogenesis. Infrared thermography (IRT) technique was used to measure the temperatures of interscapular area overlying BAT and lower body (LB) area that is devoid of BAT. Results showed no significant difference between WT and $MC1R^{e/e}$ mice in terms of induced BAT temperature increment suggesting that defective MC1R responsible for reduced EE and increased adiposity is not associated with BAT thermogenic activity. However, small notable variance in temperature difference of BAT and LB between the genotypes implicated the possible role of MC1R in BAT-originated heat transmission across the body via impaired vasodilatation.

Keywords- Brown adipose tissue; BAT; BAT thermogenesis; UCP1; Melanocortin-1 receptor

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Adipose tissues

Adipose tissues are present in multiple areas of the mammalian body in the form of subcutaneous and visceral depots, collectively known as adipose organ. These fat depots can be brown, called as brown adipose tissue (brown fat/ BAT) or white, called as white adipose tissue (white fat/ WAT). Both of these tissues are vastly innervated and vascularize but BAT contains higher density of nerves and blood vessels (Cinti, 2005). WAT mainly helps to store surplus amount of energy as triglycerides (TG) whereas BAT helps to burn fats and dissipate energy in the form of heat. Brown-like adipocytes are present in white fat depots that mimic white adipocytes in basal metabolism and brown adipocytes during thermogenic stimulation. More importantly, thermogenic response of brown-like adipocytes is identical to that of response shown by brown adipocytes. These special adipocytes are termed as beige or brite cells (Park et al., 2014). All adipocytes express a specific type of adrenergic receptor, beta-3 adrenergic receptor (β3AR) (Mund and Frishman, 2013). Cellularly, BAT is composed of brown adipocytes which are multilocular that encompasses sufficient amount of larger and distinctive mitochondria. These mitochondria contain special type of protein, known as uncoupling protein 1 (UCP1). UCP1 is accountable for non-shivering thermogenesis, achieved via uncoupling of oxidative phosphorylation (Ricquier, 2011). BAT ability to burn fat has made it an interesting target for the treatment and prevention of obesity and obesity-related metabolic diseases (Mund and Frishman, 2013).

1.1.1 Anatomical locations of adipose tissues

Depots of adipose tissue are found in various locations (Figure 1). WAT is scattered throughout the body and are of two types- visceral WAT (vWAT), distributed around internal organs and subcutaneous WAT (sWAT), situated under the skin to provide insulation from heat/ cold. vWAT, based on location is sub-classified into mesenteric, perigonadal, retroperitoneal and omental adipose tissue. sWAT is located differently to that of vWAT i.e. found below the skin, inside the abdominal cavity and in the intramuscular fat intermixed

within the skeletal muscles. Inguinal WAT is a prime example of sWAT that is located on anterior region of the upper part of the hind limbs and below the skin. In humans, sWAT is normally dispersed around the hips, thighs and buttocks. Latest discovery of beige/brite adipocytes which are present in WAT has modified our perception in identifying and classifying adipose tissues. To differentiate between brown and beige adipocytes, brown adipocytes are occasionally referred as “classical brown adipocytes”. Brown adipocytes are predominantly distributed around the interscapular region in brown adipose tissues (iBAT) and in axillary, paravertebral and perirenal areas. iBAT, spread underneath the skin between the shoulders can be easily detached but perirenal BAT is difficult to remove without taking out the whole kidney. In humans, traces of iBAT are present in the thorax region (supraclavicular), chest and abdomen. It was widely accepted that BAT was restricted only in neonatal and early childhood phases and decreases as age advances. But, positron emission tomography (PET) scan to investigate BAT has clearly shown the presence of active and functional BAT in adult humans at distinct anatomical sites, specifically in the upper body part like cervical, supraclavicular, paravertebral and pericardial areas, and negligibly in mediastinal and mesenteric areas (Virtanen et al., 2009). Brown-like adipocytes have been discovered recently that share similar gene expression pattern with those of brown and white adipocytes. These unique kind of adipocytes which are distributed within WAT (mostly inguinal WAT) are known as beige/ brite adipocytes or inducible brown adipocytes since these adipocytes can be induced inside WAT by external stress like cold and diet (Wu et al., 2012). Human superficial neck fat has similar genetic expression pattern with mouse sWAT and that of deep neck fat with mouse iBAT (Cypess et al., 2013). These discoveries have led researchers to redefine and classify brown and beige adipocytes in more standard way.

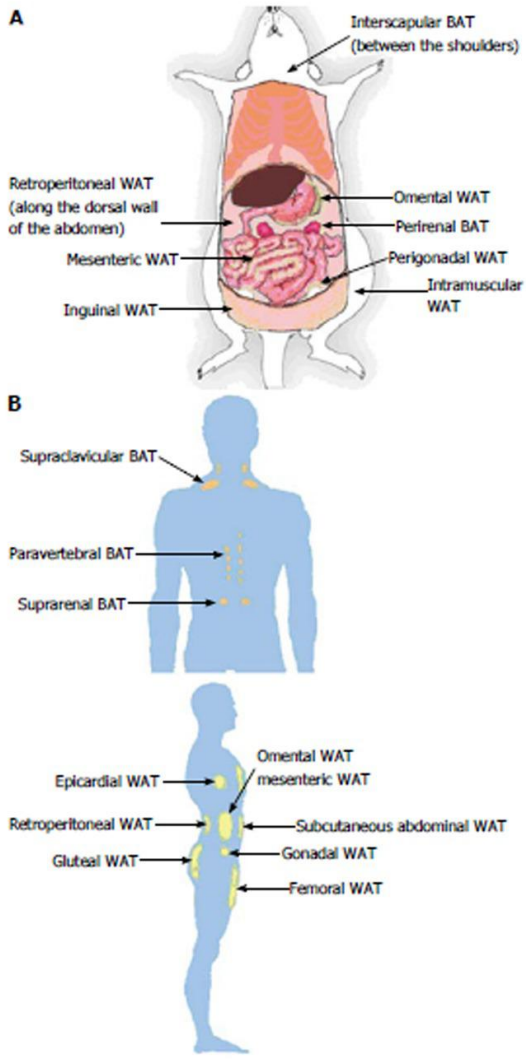


Figure 1: Locations of adipose tissue depots in a mouse (A) and an adult human (B). A: In a mouse model, subcutaneous (inguinal and intramuscular), visceral (mesenteric, omental, perigonadal and retroperitoneal) and brown (interscapular and perirenal) adipose tissue depots are shown. B: In a human model, subcutaneous (abdominal, femoral and gluteal), visceral (epicardial, gonadal, mesenteric, omental and retroperitoneal) and brown (paravertebral, supraclavicular and suprarenal) adipose tissue depots are shown. WAT- White adipose tissue; BAT- Brown adipose tissue. (Extracted from Park et al., 2014).

1.1.2 Characteristics and functions of adipocytes

Based on conventional classification, adipose tissues can be divided into two types- WAT and BAT with their own distinct colors, structure, metabolic and

biochemical functions, and gene expression patterns. WAT is the primary organ to store energy as lipids and BAT is the organ that helps in the regulation of body temperature by producing heat through the consumption of stored energy. Development of WAT mainly occurs after birth when dedicated fat storage cells are required to deliver fuel during fasting periods. UCP1 is almost absent in WAT but UCP2, an isoform of UCP1 along with some other genes like adiponectin, resistin, LPL and G3PDH are expressed in WAT (Park et. al., 2014). Brown adipocytes contain high number of UCP1-riched mitochondria. The red-brownish color of brown adipocytes is because of high mitochondrial content. When lipolysis occurs in the course of cold exposure, initiation of sympathetic nervous system (SNS) signaling takes place that dispels free fatty acids via UCP1 and finally, heat is generated. Thus, BAT primary function is to cause non-shivering/adaptive thermogenesis that disintegrates energy as heat when exposed to thermogenic stimuli (Nedergaard et al., 2001). BAT's mitochondrial thermogenin protein i.e. UCP1, upon activation by sympathetic provocations induces a proton leak across the inner membrane leading to heat generation through respiratory uncoupling reaction. UCP1- being the main player in BAT thermogenesis is regarded as primary marker of brown adipocytes (Golozoubova et al., 2006). Other BAT-localised markers are Ebf3, Eva1, Hspb7 and Pdk4 (Park et al., 2014). Since BAT contains sufficient amount of blood vessels, thermogenic inducement is rapid. Such generated heat is distributed all over the body via blood vessels.

BAT contains rich number of sympathetic nerves which on activation secrete norepinephrine (NE) (Figure 2). In brown adipocytes, NE binds to G-protein coupled cell membrane-localised β3 adrenergic receptors (β3AR). Binding of NE to β3AR stimulates the release of adenyl cyclase that mediates the conversion of AMP into cAMP which in turn causes the production of protein kinase A (PKA). PKA promotes mitochondrial UCP1 expression in mainly two ways. One way it does so is by facilitating the hydrolytic conversion of glycerides (mono-, di- and tri-glycerides) into free fatty acids (FFAs) with the help of intracellular lipases like MGL, pHSL and ATGL. Another pathway that PKA follows to enhance mitochondrial UCP1 content is by the help of biochemical processes such as gene transcription and translation and, cell

growth and differentiation. These biochemical setups are mediated by MAPK and ERK1/2, the kinase signaling pathways. FFAs enter mitochondria via carnitine palmitoyltransferase 1a (CPT1a) and undergoes β-oxidation and citric acid cycle producing NADH and FADH. These nucleotide coenzymes are oxidized by electron transport chain (ETC) which causes proton to pump out of mitochondrial matrix. This results in the formation of protonic force which pulls proton back into mitochondrial matrix through UCP1. The stored energy in protonic force is released generating heat and the overall process is known as BAT thermogenesis.

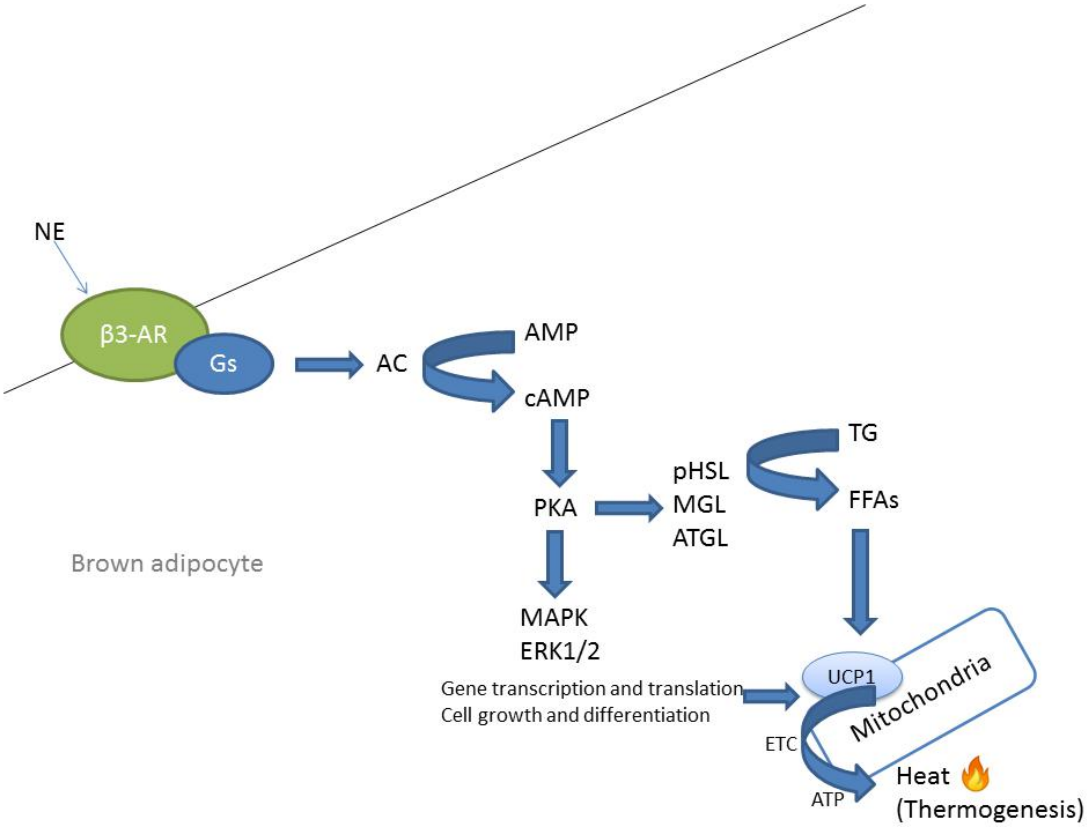


Figure 2: Brown adipose tissue uncoupling and thermogenesis. NE- Norepinephrine; AMP- Adenosine monophosphate; AC- Adenyl cyclase; cAMP- cyclic adenosine monophosphate; UCP1- Uncoupling protein 1; TGs- Triglycerides; MGL- Monoacylglycerol lipase; pHSL- active hormone-sensitive lipase; ATGL- Adipose triglyceride lipase; MAPK- Mitogen-activated protein kinase; ERK1/2- Extracellular signal-regulated kinase 1/2 pathway; NADH- Nicotinamide adenine dinucleotide + hydrogen; FADH- Flavin adenine dinucleotide + hydrogen. (Modified from Contreras et al., 2015).

The process of transdifferentiation into brown-like adipocyte (beige/brite cells) within the WAT is called browning or britening of WAT. Differential expressions of several genes have also been studied to distinguish between beige/ brite adipocytes and brown adipocytes. Tbx15, CD40, CD137 and Slc27a1 are examples of some proteins encoded by these genes (Walden et al., 2012; Sharp et al., 2012; Wu et al., 2012). Beige adipocytes exhibit both white and brown adipocytes features. During basal states, they contain unilocular, large lipid and similar gene expression pattern to those of white adipocytes but when exposed to cold environment, beige adipocytes change into intermediary cell structure with multilocular lipid droplets surrounding initial lipid droplets. This causes the increased expression of UCP1 which is one of the main characteristics of brown adipocytes. The induced browning process of WAT is reversible. It has been reported that approximately six weeks after the habituation to warmer condition (room temperature), the formed beige adipocytes were retransformed into white adipocytes with reduced level of mitochondrial UCP1 expression (Rosenwald et al., 2013). It is controversial that whether beige cells form by the transdifferentiation of pre-existing white adipocytes or by new adipogenesis from a subpopulation of precursor cells (Cinti, 2009; Wang et al., 2013). Differences between BAT, WAT and beige/brite adipocytes are summarized in the table below (Table 1).

Table 1: Distinguishing features between three types of adipocytes

	Brown	**White**	**Beige/ Brite**
Important location	Interscapular (iBAT), perirenal, axillary, paravertebral	Inguinal (sWAT), mesenteric, retroperitoneal, perigonadal, omental (vWAT)	Within inguinal WAT, in other inducible sites/ sWAT*
Morphology	Multilocular and smaller lipid droplets	Unilocular and large lipid droplets	Multiple small or singular large lipid droplets
Primary function	Heat generation	Energy storage in the form of	Adaptive thermogenesis

		TGs	
Level of mitochondria	Abundant	Scarce	Significant (on activation)
Iron content	Plenty	Few	Sufficient (on activation)
UCP1	+++	Insignificant	++ (on activation)
Blood vessels	Abundant	Few	Angiogenesis (on stimulation) in sWAT area
Sympathetic innervation	Rich in β3-adrenoceptors	Detectable presence of β3- and α2-adrenoceptors	β3 and/or α2*
Biomarkers	UCP1, Eva1, Pdk4, Ebf3, Hspb7	Ang, Resistin, LPL, G3PDH	Tmem26, Tbx1, Cited1, Shox2
Activators	Cold, thyroid hormone, thiazolidinediones, FGF21, Bmp7, Bmp8b, natriuretic peptide	High fat diet	Cold, thiazolidinediones, natriuretic peptides, FGF21, irisin, catecholamines, β-adrenoceptor agonists
Effect on obesity	Negative	Positive	Negative
Effect on insulin resistance	Negative	Positive	Negative

* indicates unclear data

1.1.3 Differentiation of adipocytes

The development and differentiation of BAT occurs prior to birth as it protects neonates from cold surrounding by causing non-shivering thermogenesis. Multipotent stem cells i.e. mesenchymal stem cells (MSCs) develop into adipoblasts and then into preadipocytes. Preadipocytes under the influence of thermogenic agent(s) change into mature adipocytes which is the last stage of differentiation (Rosen and MacDougald, 2006). Several sequences of mitosis occur until preadipocytes reach G1 phase of cell cycle. The re-entering, mitotic clonal expansion and exiting from the cell cycle continues till they gain metabolic characteristics of mature adipocytes, morphology change and collect cytoplasmic triglycerides. Finally, the division of mature adipocytes stops at the end of terminal differentiation. Mixture of inducing agents consisting of fetal bovine serum, insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) is required to bring differentiation in isolated cells of adipose tissue depots (Tang and Lane, 2012). It is important to understand that although both types of adipocytes have same origin (from MSCs), they are produced from different precursor cells. Myf5 acts as a primary myogenic regulatory factor. MSCs either differentiate into Myf5-negative cells (adipogenic) or Myf5-positive cells (myogenic) (Timmons et al., 2007). Adipogenic lineage produces white adipocytes whereas myogenic lineage produces brown adipocytes. Even though white adipocytes and brown adipocytes differ in precursor lineage, they have similar transcriptional sequences of adipogenic differentiation. The differentiation process also involves some common factors like key mediator of fat cell development, peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding proteins (C/EBPs) (Rosen and MacDougald, 2006; Tang and Lane, 2012).

Latest experiments have disapproved the assumptions of same precursor cell being the source of white and brown adipocytes (Park et al., 2014). Indeed, brown adipocytes as similar to skeletal muscles develop from Myf5-positive precursor cell that are formed from mesenchymal stem cell (Figure 3). Mature brown adipocytes develop from these Myf5-positive progenitor cells via intermediary pre-adipocytes mediated by several factors like BMP7, PRDM16,

PPAR-γ, C/EBP-β and PGC-1α. Beige/ brite adipocytes are functionally similar to brown adipocytes but are induced from white pre-adipocytes by cold exposure or from white mature adipocytes by endogenous or exogenous mediators like chronic cold exposure, β3AR agonists, PPAR-γ agonists' treatment, FGF21, irisin and natriuretic peptides.

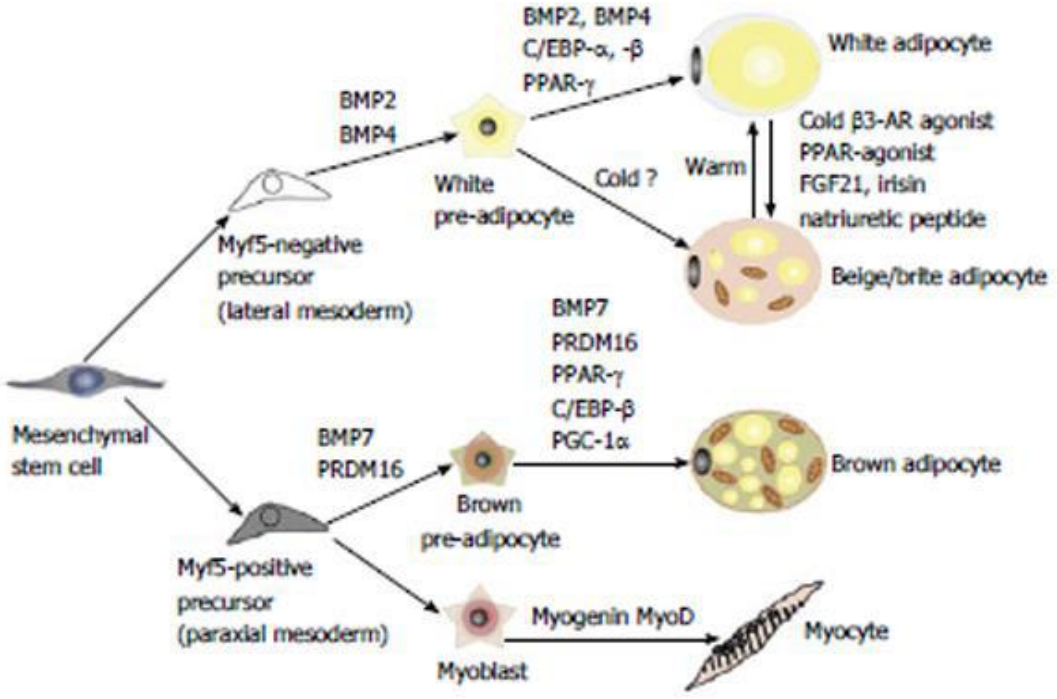


Figure 3: Differentiation of mesenchymal stem cell into white, beige/brite or brown adipocytes. BMP7- Bone morphogenetic protein 7; PRDM16- PR domain containing 16; PPAR-γ- Peroxisome proliferator activated receptor gamma; C/EBP-β- CCAAT/enhancer binding proteins; PGC-1α- Peroxisome proliferator activated receptor gamma coactivator 1 alpha; β3AR- Beta 3 adrenergic receptor; FGF21- Fibroblast growth factor. (Extracted from Park et al., 2014).

There are several factors that result in the WAT browning process i.e. the formation of beige/ brite adipocytes in WAT. One of the main factors is cold exposure. In a prolonged cold environment (cold exposure for about 6 – 7 days), changes in metabolism and gene expression pattern in some cells of WAT are seen (Rosenwald et al., 2013). Also, stimulation of precursor differentiation into beige/ brite adipocytes occurs. Temperature of 27 – 33 ^{o}C

in vitro can directly influence the thermogenecity in sWAT but not in iBAT. It is well-known fact that BAT thermogenesis is controlled by β-adrenoceptor signaling pathway through sympathetic nervous system. Norepinephrine stimulates G-protein coupled β3AR which increases intracellular cAMP level causing fatty acid mobilization and increasing mitochondrial UCP1 expression in beige adipocytes, finally leading to non-shivering thermogenesis. Several old literatures have reported that catecholamines, a family of NE as well as β3AR agonists like CL-316243 can exhibit UCP1-derived thermogenecity (Himms-Hagen, 1990; Cousin et al., 1992; Lafontan and Berlan, 1993). WAT browning is also achieved by thiazolidinediones, a PPAR-γ activator via PRDM16 protein stabilization (Ohno et al., 2012). Other agents and molecules like FGF21, cardiac natriuretic peptides, glucagon-like peptide, Tbx15 and PRDM16 enhance both BAT mass and WAT browning; bone morphogenic protein 7, myostatin and twist 1 increases BAT mass only; irisin, VEGF-A, SirT1, Dbc1, brain-derived neurotropic factor (BDNF) and cyclooxygenase-2 helps in the browning of WAT and bone morphogenetic protein 8B can intensify BAT functions (Lee et al., 2013). More studies are required to determine how exactly these endogenous molecules would help in BAT thermogenic process.

1.1.4 Sympathetic and sensory innervation of BAT

BAT is richly innervated by SNS and stimulation of these sympathetic nerves is an important prerequisite for BAT-mediated thermogenesis (Morrison and Madden, 2014). Thermogenic stimuli like chronic cold, feeding (Labbe et al., 2015), stress (Razzoli et al., 2016), exercise, environmental enrichment and some drugs like β3AR (Kim and Plutzky, 2016) elicit the release of norepinephrine from its sympathetic nerve terminals and activation of BAT-localised β3AR takes place finally leading to thermogenesis via UCP1. Studies have found parasympathetic innervation in two minor BAT depots-mediastinal and pericardial BAT but not in iBAT (Bartness et al., 2010). It is also understood that BAT is supplied with sensory nerves which come into action in response to fatty acid accumulation or aiding feedback mechanism to normalize thermogenic-induced temperature increase to maintain thermal homeostasis. Retrograde pseudorabies virus transneuronal tract tracing

method has unveiled that CNS sympathetic innervation eventually ends in BAT (Bamshad et al., 1999; Cao et al., 2004). In consistent to this discovery, researchers have also found the greater extent of colocalization of melanocortin-4 receptor (MC4R) in sympathetic nerves leading to BAT (Song et al, 2008). Efforts are going on to pinpoint the precise SNS-BAT pathway and concerned endogenous or exogenous stimuli involved in various kind of induced thermogenesis. Pharmacological administration of MII, a MC3R/ MC4R agonist via fourth ventricular injection on decerebrated (removal of cerebrum) rodents stimulates iBAT UCP1 mRNA expression (Brito et al., 2007). This study implicates the potentiality of melanocortin system in stimulating BAT thermogenesis through BAT-SNS neural set-up. Thus, better understanding of nervous outflow of SNS to BAT and their neural communication with central melanocortin system would benefit us to develop novel pharmacotherapy for obesity by modulating energy homeostasis via thermogenesis.

Sympathetic innervation of WAT and their regulation by sympathetic nervous system (SNS) signals in the presence of various mediators provoke browning in WAT, particularly sWAT (Park et al., 2014). Such “browning mediators” can be irisin (secreted from skeletal muscle during physical challenge), FGF21 (secreted from liver), norepinephrine (stimulated by SNS in response to exogenous thermogenic stimuli like cold) or natriuretic peptides (secreted from heart). These proteins bind to their respective receptors and activate thermogenesis directly or indirectly via P38 MAPK initiated by AR-mediated cAMP-dependent PKA (Figure 4). PKA also releases Dio2 (that catalyzes the T4 into T3 transformation) and lipase (that catalyzes TAG conversion into FA). T3 and FA also contribute to thermogenic output.

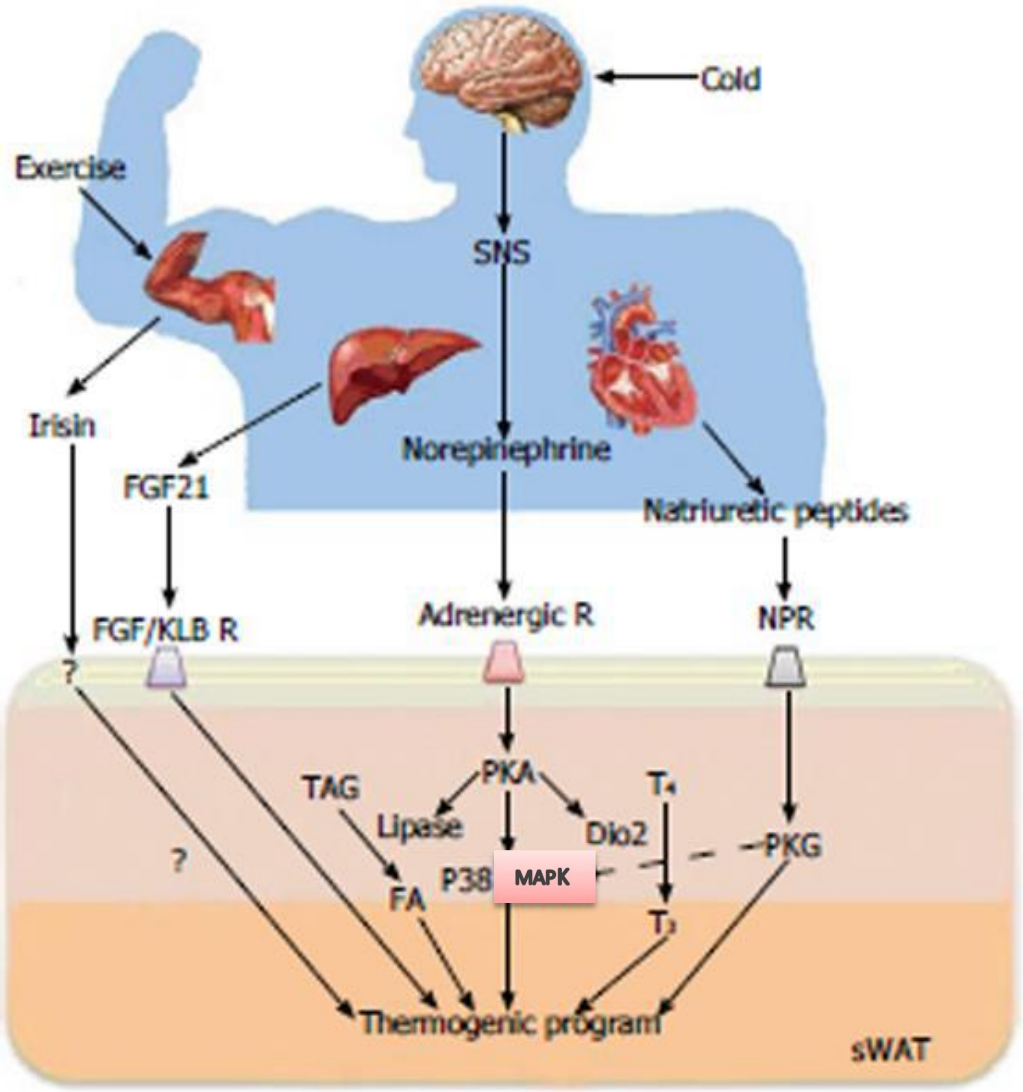


Figure 4: Key regulators of the browning process and their action. WAT- White adipose tissue; sWAT- subcutaneous WAT; FGF21- Fibroblast growth factor 21; P38 MAPK- P38 MAP kinase; AR- Adrenergic receptors; PKA- Protein kinase A; Dio2- 5' deiodinase 2; TAG- Triacylglycerol; FA- Fatty acid. (Extracted from Park et al., 2014).

1.1.5 Importance of BAT in regard to environmental temperature and condition

BAT-mediated thermogenesis can be stimulated by environmental factors like cold (cold-induced thermogenesis or CIT) and diet (diet-induced thermogenesis or DIT) or by agents or molecules mentioned above in the paper. CIT indicates BAT thermogenesis is totally dependent on temperature

of the surrounding i.e. cooler the periphery more stimulation of BAT activity and vice-versa. One study regarding surrounding enrichment has provided support for an association between outer environment and body weight (Cao et al., 2011). The test subjects were placed in comparatively cooler temperature, 22^{o}C in an enriched environment accompanied by toys and social interactions. It resulted in inducible browning of WAT and caused significant loss of fat mass independent of diet or physical activity. Finally, this study summarized that enriched environment in a hypothermic habitable temperature stimulates BAT, enhances energy expenditure (EE) and also helps in WAT browning via hypothalamic BDNF. BAT role in modulating metabolism in colder condition highlights the capability of BAT in improving body weight and metabolic health. We can relate socially enriched surrounding of mice experiment with the current human society but unlike the result of the study being mice metabolically healthy and losing weight, humans have been confronting obesity as one of the most challenging problem in today's sophisticated world. Only 10% of human subjects in an experiment conducted by Lee and his colleagues to check the prevalence of BAT in adult human had measurable functional BAT (Lee et al, 2010). Individuals lacking BAT tend to be obese, have weak metabolic history and are highly susceptible to suffer from type 2 diabetes mellitus. It has been discussed that extra WAT accumulation in obese individuals insulates body heat and the need of BAT thermogenesis is not felt. Another factor for smaller BAT volume and activity is due to progressing age (Cypess et al., 2009). The relationship between environmental temperature and condition, BAT activity and metabolic health has made us to realize the importance of each component and how the metabolic disorder like obesity can be manipulated partially by altering surrounding temperature. Interestingly, in the present world of social enrichment and luxurious life, BAT can play significant role in the development of anti-obesity medication.

1.1.6 Importance of BAT in energy metabolism

BAT plays a vital role in EE and thus helps in energy homeostasis. The main components of EE are physical activity, basal EE and BAT thermogenesis (Lee et al, 2013). These EE elements vary among individuals (van Marken et

al., 2002). Null or poor physical activity leads to obesity. It has been studied that among the obese, not only DIT is poorly stimulated but also the CIT, therefore producing a promising relationship between BAT thermogenesis and obesity vulnerability (Jonee and Bray, 1997; Argyropoulos and Harper, 2002; Eikelis and Esler, 2005; Wijers et al., 2010). Animal studies involving rodents showed BAT as a primary mediator organ for DIT and CIT. One of the previous experiments clarified energy-producing ability of BAT by measuring oxygen extraction across iBAT (Foster and Frydman, 1979). Also, mice fed with both chow and high-fat diet gained lesser weight when they were made to adapt in cold environment (Morrison, 1981). It showed an important role of BAT in energy homeostasis via CIT. One of the mouse studies also suggested BAT as a DIT regulator organ when high carbohydrate diet intake increased EE by 20%, measured by blood flow to BAT (Glick et al, 1984). In one experiment conducted at thermoneutrality, high-fat fed UCP1-KO group gained 50% more of its initial weight as compared to wild-type mice (Feldmann et al, 2009). At thermoneutrality, CIT is not required and this analysis regarding diet proved the idea of BAT as a DIT mediator organ. Therefore, DIT and CIT are important elements of total EE and directly influence energy homeostasis and weight increase (Wiljers et al, 2009).

Rothwell and Stock in one of their paper studied the role of BAT in DIT. When they observed rats fed with cafeteria diet (high fat and high sugar content) did not gain weight as expected, they assumed diet-induced BAT thermogenesis consumed extra calories (Rothwell and Stock, 1979). Knehans and Romsos investigated the BAT activity in obese leptin deficient (ob/ob) mice. Along with lean control, they were exposed to different temperatures (thermoneutral 33^{o}C, 25^{o}C and 14^{o}C) for three weeks. SNS-stimulated BAT thermogenesis as measured by norepinephrine (NE) turnover was higher in both ob/ob and control group at 14^{o}C. At 25^{o}C, NE turnover rate in BAT was significantly higher in ob/ob mice than control mice and at thermoneutrality, only ob/ob mice showed positive NE turnover (Knehans and Romsos, 1982). This data revealed the action of BAT in different temperatures and its metabolic-related association between lean and obese phenotype. New experiment performed the transplantation of BAT from lean wild-type mice into dorsal subcutaneous

region of equivalent age and sex leptin-deficient ob/ob mice (Liu et al., 2015). This resulted in increased oxygen consumption and decreased total body fat mass along with the improvement of the condition like insulin resistance. Most importantly, the significant reduction of body weight in BAT transplanted mice emphasizes ability of BAT in fighting obesity. Another experiment indicated the utmost developmental differences in BAT and WAT lipogenesis of genetically leptin receptor-deficient diabetic (db/db) mice and compared with normally functioning mice (Trayhurn and Wusteman, 1990). The study suggested the premature and enhanced development of insulin resistance in the BAT of leptin receptor-deficient db/db mice. Marette et al. examined the insulin and NE effect in isolated BAT adipocytes from obese (fa/fa) and lean Zucker rats. The results demonstrated the reduced sensitivity of obese brown adipocytes to NE-triggered thermogenic effects however their reaction to dibutyryl cyclic AMP was normal. These feedbacks were aided by lower lipolytic response and marked insulin resistance (Marette et al., 1990). Genetic ablation of BAT by using diphtheria toxin A in UCP1 expressed cells produced obese mice with decreased energy expenditure. The obesity was further heightened with the supply of high fat diet causing other metabolic disorders like insulin resistance and type 2 diabetes mellitus (Hamann et al., 1996). Using UCP1-KO mice, Feldmann et al. demonstrated deficiency of UCP1 causes lack of DIT and markedly enhances diet-induced obesity via increased metabolic efficiency (Feldmann et al., 2009). This experiment also intensified the fact that UCP1 is the sole mediator of DIT and it is important to consider ambient temperature in BAT-related study because it influences the result of metabolic activities. Another experiment utilizing UCP1 KO mice showed BAT UCP1 is not required for fibroblast growth factor 21 (FGF21) secretions (Keipert et al., 2015). Keipert and his team recently reported neither UCP1 nor voluntary/ involuntary increment of FGF21 is essential for maintaining energy metabolism and body weight in UCP1-KO mice (Keipert et al., 2017).

BAT weighs 60 – 100 g in human body which is a small proportion in terms of whole body weight and whether this small organ plays such a big role in human total body energy expenditure has been studied time and again. As

basal EE is an important component of total EE, studies have been conducted to explore the contribution of BAT in influencing basal EE. Cold-induced PET scanning study carried out in BAT-positive and BAT-negative individuals in thermoneutral temperature (i.e. 27^{o}C) revealed no significant differences (Yoneshiro et al., 2011). Another human-based experiment studied in cool environment (16^{0}C) for 2 hours before metabolic evaluation showed almost all individuals (23/24) to be BAT positive (van Marken Lichtenbelt et al., 2009). In short, these studies established the relationship of BAT activity with basal and cold-induced EE. Basal EE increases correspondingly when the surrounding temperature drops. Even the small changes in basal EE result in long-lasting body weight changes (Cunningham, 1982; Esparza et al., 2000). Thus, BAT ability to adjust basal EE could be utilized to compensate poorer physical activity to help in counteracting weight gain. On cold contact, [^{18}F] Fludeoxyglucose (^{18}FDG; a glucose tracer) uptake by BAT is multiplied by 10 to 12 times (Virtanen et al, 2009). If we assume glucose signifies 10% of total BAT fuel, BAT metabolism capacity to control basal EE approximates around 50% - 70% (Lee et al, 2013). In activated BAT individuals, lesser fasting glucose was reported suggesting metabolic role of BAT in depressing blood glucose (Cypess et al., 2009). Using [^{18}F] Fluoro-thiaheptadecanoic acid (^{18}FTHA; a free fatty acid tracer), it was reported that BAT metabolism of free fatty acid gives total energy consumption of approximately 6 kcal/day which was considerably less when ^{18}FDG was used (~ 100 kcal/day) (Lee et al., 2013). The differences in energy consumption value between two different tracers were experienced because glucose in comparison of fatty acid is a minor substrate of brown fat (Ma and Foster, 1986). Additionally, the utilization of glucose (GLUT1 and GLUT4) and lipid (ATGL, HSL and MGL) by BAT thermogenic process are shown by various rodent studies (Townsend et al., 2014). PET scanning cannot measure intracellular lipid, a source of most of the fatty acid fuel of brown adipocytes. However, scientists were able to mark down BAT utilization of intracellular lipid with the help of volumetric analytical method. One of the experiments found that one-third lipid of BAT (~ 28 g) was used to generate ~ 250 kcal of energy during chronic cold exposure of rodents (Lee et al., 2014). These studies have delivered solid support for thermogenic nature of BAT and its ability to burn body fat despite its insignificant size.

BAT thermogenic property makes it one of the important components of total EE. One experiment used two transgenic groups of mice, one with permanent BAT deficiency and other with temporary (Lowell et al., 1993). After two weeks, both groups gained sufficient weight to be classified as obese. One group with temporary BAT deficiency recovered from obese condition after BAT regeneration but the other group of permanent BAT deficiency continued gaining weight. This breakthrough examination showed BAT effectiveness in metabolism and body weight control. A prospective large sized cross-sectional human experiment which included 162 fit individuals, aged 20 - 73 years observed BAT-positive subjects had lesser body mass index (BMI), total body fat and abdominal fat than BAT-negative subjects. It concluded by stating that suppressed BAT activity might be the reason for body fat accumulation which keeps adding up with advancing age (Yoneshiro et al., 2011). The correlation of BMI and brown fat activity was also studied in further trials. In one experiment involving 35 test subjects, BAT-positive subjects had a mean BMI of around 4 kg/m^2 lower than BAT-negative subjects (Zingaretti et al., 2009) and another experiment containing 17 subjects showed inverse association between high supraclavicular fat UCP1 content and BMI suggesting UCP1 responsibility in significant BMI alterations (Lee et al., 2011). These human studies can be used as an evidence to show inverse correlation of BAT activity and body fat and how BAT could effectively influence body weight or vice versa. It is still not clear if BAT activity is a cause or effect of a lean body physique. Rich accumulation of fat in body makes BAT inactive as fat itself works as a heat insulator and requirement of BAT-mediated thermogenesis is not realized which subsequently shrink BAT size and activity. One experiment measuring BAT activity of 15 morbidly obese patients one year before and after bariatric surgery demonstrated the increment of BAT-positive subjects from 2 to 5 following the surgery (Vijgen et al., 2012). Also after the weight loss, individuals having active BAT show higher nonshivering thermogenesis than BAT-negative individuals. This study showed that non-obese healthy individuals have significant volume of functional BAT and explored the possibility of BAT recruitment in humans.

1.1.7 Drug-induced thermogenesis

Himms-Hagen et al. (1994) found that chronic treatment with β3AR agonists (CL-316243/ CL) increases body temperature and overall energy expenditure, primarily via increment of resting metabolic rate. Her group also discovered enlargement of iBAT size and significant multiplication of uncoupling protein (UCP) and cytochrome oxidase content following the treatment with CL. The nature of drug-induced iBAT hypertrophy and UCP content was different from that of CIT. In CL-treated rats, the drug even produced the loss of WAT weight and size. It also induced multilocular adipocytes in WAT depots for example, in inguinal, retroperitoneal, epididymal and mesenteric WAT. This experiment concluded that CL causes BAT mitochondrial proliferation stimulating thermogenesis. Thermogenesis directly affects overall energy expenditure and promotes leanness. Another follow-up experiment by Ghorbani et al. (1997) studied the characteristics of multilocular adipocytes that appeared in WAT depots. They documented the substantial increment (40 – 45%) of metabolic rate caused by CL transpires in brown adipocytes of BAT and WAT area. It was hypothesized that the multilocular adipocytes induced in WAT depots are the matured version of small brown preadipocytes present already in WAT and they contain few mitochondria with or without UCP. These so-called brown preadipocytes express β3 adrenergic receptors and become larger in size (hypertrophy) when they react with β3AR agonists. Chronic daily subcutaneous injection of CL (0.1 mg/kg) to 2.5 months old obese rats caused a remarkable loss in body weight and fat pad mass without any alteration in food intake (Umekawa et al., 1997). UCP mRNA level in BAT was increased significantly 2 – 3 folds with the stimulation of thermogenic protein in WAT area. Variants of UCP like UCP1, UCP2 and UCP3 display an uncoupling action when ectopically expressed. But, studies have proved that UCP1 is the only protein among these “uncouplers” that regulates adaptive adrenergic-stimulated BAT thermogenesis (Nedergaard et al., 2001). CL-treated obese group has also showed the improvement in metabolic dysfunction - hyperglycemia and hyperinsulinemia. Lack of distinct BAT morphology and activity in rodents’ obese condition can be treated by CL administration which functions *in vivo* by reducing white fat, stimulating WAT browning and activating BAT thermogenesis hence modifying energy homeostasis. CL was

also found to stimulate GLUT4 (Umekawa et al., 1997) and increase *in vivo* BAT glucose uptake (Olsen et al., 2014).

1.1.8 Detection and assessment of BAT activity

The techniques for detecting and assessing BAT function vary from simply weighing BAT to advanced methods involving sophisticated instruments like positron emission tomography combined with computer tomography (PET/CT). The investigation of BAT function depending only on BAT weight would be incomplete but changes in BAT weight give a basic idea that something has affected BAT. Obese mice have less active BAT and subsequently BAT weighs less than that found in lean mice. Acute cold condition irreversibly decreases BAT lipids and finally its weight without affecting BAT functionality. Fasting reduces both BAT weight and activity. Measuring BAT weight is simple and can be used as an indicator for potential BAT modifications demanding further analysis (Virtue and Vidal-Puig, 2013). Pair feeding method i.e. feeding same amount of food to the hyperphagic and the hypophagic group provide the suggestion for variations in metabolic rate. If the pair feeding does not fix body weight differences between the groups, it means there is a modification in metabolic rate. The important parameter to remember while pair feeding is the exact measurement of food intake. Although pair feeding gives the knowledge of modified energy expenditure, added experiments should be done to confirm if the changes are due to BAT. Remarkable weight loss to overnight deprivation of food (fasting response) suggests hypermetabolism. Sometimes performed under pair feeding experiment, the groups are not fed at all. The resulting significant weight loss corresponds to hypermetabolism but this experiment cannot confirm if it is BAT related because there might be involvement of other organs for such hypermetabolic phenotype. There are studies done to link BAT-related temperature changes and changes in core body temperature. There is no decrease in core body temperature or only a minor increase of temperature (0.25 – 1 ^{o}C) over the first hour of exposing rodents to a colder temperature (Lomax et al., 1964). It is assumed that the initial slight increase of core body temperature is due to elevated thermogenesis and peripheral vasoconstriction reducing peripheral heat loss. The reaction of rodents regarding core body

temperature following 3-4 hours of acute cold exposure depends on their thermogenic capability. One experiment showed the incompetence of mice kept at thermoneutrality to maintain core body temperature irrespective of thermogenic capacity during acute cold subjection (Golozoubova et al., 2001). The decrease in core body temperature does not always indicate the defective thermogenic capacity, it also occurs to conserve body energy as the metabolic rate decreases by 50%. However, this process is reversible and the animal shows recovery from the energy preserving state (Meyer et al., 2010). Sarcolipin gene imparts its role in shivering in muscle which also influences core body temperature (Bal et al., 2012). Hence, these animal studies have provided evidence for measuring core body temperature to indicate thermogenesis but cannot assure BAT-mediated thermogenesis. When mice are subjected to cold environment, there is rapid BAT delipidation. Endogenous lipids invade brown adipocytes to consume or synthesize lipids (Christoffolete et al., 2004). In one hour of cold exposure, expression of gene lipoprotein lipase (LPL) is induced that increases lipid uptake, the first physiological sign of BAT cold exposure (Mitchell et al., 1992). In 24 hours, increase in glycolytic genes and enzyme acetyl-CoA carboxylase causes de novo lipogenesis with a marked improvement in de novo lipogenic capacity (Christoffolete et al., 2004). In one week, BAT is largely refilled with lipids and the size of lipid droplets grows similar to that of mice kept at warmer temperature. Stearoyl-CoA desaturase 1 (SCD1) and fatty acid synthase (FAS) are hyperinduced in 3 weeks of cold exposure. It has been shown that not all changes in BAT lipid content are due to altered BAT metabolic rate only. Using lipocalin-prostaglandin D synthase KO mice (Virtue et al., 2012) and elongation of very long chain fatty acids protein 3 (Elovl3) KO mice (Westerberg et al., 2006), it was demonstrated that these genes cause changes in BAT lipid content at certain temperature. In a practical setting, BAT lipid content can be measured by histological morphometry or biochemical means (Virtue and Vidal-Puig, 2013). Staining agent haematoxylin and eosin stains lipid and non-lipid section of BAT giving white appearance to lipid droplets. Following phase analysis, total white area of a BAT section estimates % lipid content per brown adipocytes. Biochemical methods such as Folch extraction which involves weighing of BAT, extraction

of all lipid content by suitable solvents (chloroform: methanol) and finally weighing the lipid after solvent removal gives the measurement of total BAT lipid content. These various rodents' studies have shown clear influence of environmental temperature in BAT lipid accumulation and, also the magnitude and duration of housing temperature should be carefully considered when result is analyzed based on total BAT lipid content. Another important consideration is that due to the interaction of different physiological processes in affecting BAT lipid metabolism, the assessment of lipid content in isolated BAT in artificial medium does not give the exact measurement of BAT thermogenesis. Measurement of various mRNA markers accountable for BAT activation and WAT browning gives the idea of potential BAT thermogenic capacity rather than actual measure of BAT activity. Quantification of primary BAT marker i.e. UCP1 and other thermogenic related markers like Elovl3, peroxisome proliferator-activated receptor gamma coactivator 1α and 1β (PGC1α and PGC1β) and deiodinase 2 present the information regarding degree of thermogenic stimulation in rodents (Virtue and Vidal-Puig, 2013). UCP1 mRNA expression rises maximum after 4h of cold treatment and falls after 1-2 days. UCP1 mRNA level is normally expressed as a concentration per μg of RNA and is measured in terms of total UCP1 protein per specific BAT depot (Nedergaard and Cannon, 2013). This suggests UCP1 mRNA level as a marker of UCP1 protein content. Elovl3 (or Cig30) is stimulated to 200 fold in BAT after a 3 days treatment in 4^{o}C environment transferred from 30^{o}C (Tvrdik et al., 1997). PGC1α is an important transcriptional co-activator for the expression of BAT thermogenic and differentiation process and PGC1β helps in cold adaptive behavior of BAT. It has been shown that PGC1α is induced 5 fold and PGC1β is induced 3 fold in BAT when mice housed at 4^{o}C and 30^{o}C are compared (Lelliott et al., 2006). Mice lacking both PGC1α and PGC1β result in defective mitochondria and significant loss in oxidative phosphorylation capacity (Lai et al., 2008). Deiodinase 2 enzyme transforms thyroid hormone T4 into the active form T3. Thyroid hormone helps in BAT initiation and development. Deiodinase 2 is induced by 10 fold upon 6 days assimilation to cold treatment (Madsen et al., 2010). Lack of deiodinase 2 causes defective BAT thermogenesis.

PET/CT is currently preferred method to study the morphology and function of BAT. But, it has its own limitations. Standard uptake value (SUV) of the radiolabeled glucose substrate gives precise data only when BAT is actively functioning. In fact, magnitude of FDG uptake directly correlates with degree of BAT activation. Measurement of glucose substrate uptake by FDG-PET tends to undervalue BAT function in the influence of other biological condition like slight cold. BAT has a preference for endogenously produced fatty acids and TGs over exogenously provided glucose analogue. This is why there is variation in energy consumption calculation when two different (glucose and free fatty acid) substrates are used. Glucose uptake during PET/CT scan is also affected by other factors like insulin. If PET scanning is done after the meal or in insulin-defective person, BAT takes up more glucose producing false result. This is because of the insulin effect in stimulating glucose uptake. The extent of glucose uptake is not related with the increase in thermogenesis or local tissue blood flow or mitochondrial UCP1 activation. It is worth understanding that glucose not only helps in oxidation but also facilitates anaerobic glycolysis and lipogenesis. Although using F^{18}-FDG as a radiolabelled substrate in PET/CT scan gives high sensitivity (i.e. able to show the occurrence of metabolically active BAT), it is not highly specific imaging biomarker because glucose takes part in other physiological process apart of BAT thermogenesis. PET SUV result is affected by number of changing factors like dosage of substrate administered, inter-subject variations, effects of other tissues and organs in absorbing the substrate, radiotracer specific activity, diet and substrate uptake kinetics of BAT. These entities should be cautiously selected for the experiment as it makes differences in SUV and activated tissue volume estimation. It is recommended to implement quantitative dynamic PET/CT scanning merged with graphical interpretation. PET/CT is one of the expensive methods because of its high instrumentation cost. The subject cannot undergo repetitive exposure to PET/CT because already on the first exposure the subject is overexposed to safety risk. So, it is not safe to use in large population study where individuals are required to subject for imaging more than once in short break. F-FDG PET/CT gave us confirmation of prevalence of BAT in human but it cannot be regarded as

perfect choice for studying BAT morphology, activation and thermogenesis (Cypess et al., 2014).

Magnetic resonance imaging (MRI) can be used as a substitute for PET/CT. Work of MRI in identifying BAT is based on morphological difference of BAT from WAT. There is difference in saturated and di-unsaturated fatty acids and water content among the two types of adipose tissues. These cellular level variations bring changes in blood and show modifications in BAT metabolic activity via BAT oxygenation requirement (Khanna et al., 2012). Chen and his colleagues in their study mentioned that by using MRI they were able to differentiate BAT from WAT based on the fact that BAT has higher water-to-fat ratio and mitochondria-rich BAT is highly perfused making it highly susceptible to magnetic field associated with MRI. BAT activity by MRI was intuited after measuring hemodynamic changes accompanied by oxygenation demand (Chen et al., 2012). Thus achieved data were found consistent when compared to weighing dissected tissue *ex vivo*. Also, fMRI was capable to track drug-induced BAT thermogenic hemodynamic changes. Another study used MRI and CT to show the presence of big bilateral supraclavicular BAT depots in a 3-month old human child, confirmed later by tissue histology (Hu et al., 2012). These types of morphological, functional and metabolic recognitions of BAT by using MRI can give scientist an improved and alternative option to classical PET/CT BAT scanning technology.

Thermal imaging technique is comparatively newer method to diagnose BAT activity in rodents and humans. A group of researchers showed by using thermography technique that β3-induced dorsal surface temperature-change relates to actual estimation of UCP1-involved BAT thermogenesis (Crane et al., 2014). In the experiment, UCP1-null mice were used as negative control because of lack of increment in heat production. BAT thermogenesis was induced by β3AR agonist drug. The study validated the use of infrared thermography in promptly evaluating BAT-mediated thermogenesis. Skin which covers BAT in rodents is warmer than other parts of body because BAT on stimulation produces heat that can be measured using thermal camera. Heat, being an eventual function of BAT might not be correctly measured by PET/CT or MRI. Thus, detection of BAT activity via measurement of

produced-heat can be effortlessly done by using infrared thermography (IRT) method. Highly sensitive IRT can detect temperature variation between 0.1^{o}C. One experiment established the fact that changes in BAT temperature as measured by *in vivo* IRT increased proportionately with the increase in 18F-FDG accumulation (Carter et al., 2011). The difference between mean skin surface temperature overlying BAT and that of not overlying BAT increased from 0.82^{o}C to 1.26^{o}C after noradrenaline injection (Jackson et al., 2001). This study suggested IRT was able to differentiate between skin surfaces overlying and not overlying BAT. Another study concluded that change in temperature was significantly and consistently higher in BAT-positive individuals, assessed by IRT and PET/CT. The temperature change after two hours of cool contact, as detected by IRT indicates it as a promising non-invasive favorable technique for studying supraclavicular (SCV) BAT function (Jang et al., 2014). In another study involving healthy individuals of normal BMI with age range of 3 - 58 divided into three age groups after a standard cool challenge (20^{o}C) showed highly confined temperature increase in the SCV region along with a remarkable age-associated decline in both resting and stimulated conditions (Symonds et al., 2012). These experiments have revealed the validity of IRT and its convenience in detecting humans' and rodents' BAT-mediated thermogenesis.

1.2 Melanocortin system

The melanocortin system is composed of melanocortin peptides [α-, β-, γ-melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH)] derived from proopiomelanocortin gene; five G protein-coupled melanocortin receptors (MC1-5R); two endogenous antagonists [(agouti and agouti-related protein (AGRP)] and two ancillary proteins (mahogany and syndecan-3) which take part in different kind of biological functions for example, pigmentation, energy balance, pain modulation, inflammation, immunomodulation, temperature regulation, cardiovascular function, neuromuscular reinforcement, sexual function, steroidogenesis and exocrine discharge. These variant physiological functions of melanocortin system can be used in the therapeutic treatment of diseases and ailments like skin cancer and topical disorders, obesity, pain, inflammatory and immunologic diseases,

cardiovascular diseases, nerve injury, erectile dysfunction and cachexia (Gantz and Fong, 2003).

Proopiomelanocortin (POMC) preprohormone gene on post translation produces melanocortin peptides. The cleavage of POMC by prohormone convertases, PC1 and PC2 gives rise to number of peptides belonging to opioid and melanocortin families. The POMC-derived melanocortin peptides (α-, β-, γ-MSH and ACTH) contain same distinctive core structure of amino acid: His-Phe-Arg-Trp, a property attributable for binding to a member of melanocortin receptor family. These peptides resemble melanotropic and/or adrenocorticotropic activity, hence called 'melanocortin'. Along with the production of melanocortin peptides, POMC cleavage also generates opioid peptide called β-endorphin and corticotrophin-like intermediate lobe peptide. In the rodent, POMC is primarily situated in the intermediate lobe of pituitary gland, in the arcuate nucleus (ARC) of hypothalamus and in the nucleus of solitary tract (NTS) of brainstem (Joseph et al., 1983). However, POMC is also found in other areas like skin, pancreas, gut and placenta (Smith and Funder et al., 1988). It is well-known that melanocortin system is distinctively controlled by two internally produced antagonists which are agouti and agouti-related protein (AGRP) (Ollmann et al., 1997). Expression of agouti in hair follicular epithelial cells and its secretion controls adjacent follicular melanocytes' melanin appearances. AGRP is manifested in neuropeptide Y neurons (NPY) of ARC and in the adrenal cortex (Minor et al., 2009).

1.2.1 Melanocortin receptors

All five recognized melanocortin receptors, MC1R, MC2R, MC3R, MC4R and MC5R are named according to the order of cloning and are members of G protein-coupled receptor family. Expressed in number of locations, these receptors have significant affinities for particular melanocortin ligands. Stimulation of melanocortin receptors by ligands and antagonists cause specific downstream signaling cascades and manifest various physiological and homeostatic functions mediated through melanocortin system.

MC1R is primarily expressed in melanocytes of hair and skin and participates in the stimulation of eumelanin (black/brown) and pheomelanin (red/yellow)

pigmentation in many organisms (Rees, 2003). Binding of α-MSH to MC1R increases intracellular cAMP production and subsequently increases eumelanin: pheomelanin ratio giving rise to darker type of pigmentation (Cone et al., 1996). If agouti (an endogenous antagonist) binds to MC1R, it hinders the α-MSH activity at this receptor thus enhancing pheomelanin pigmentation and making red/ yellow a dominant pigmentation. Resulting manifestation of unusual MC1R action can be studied through agouti yellow mice (A^y) which have overly expressed agouti peptide, the reason why they are yellow ectopically (Wolff et al., 1999). These yellow mice are also physiologically obese because highly expressed agouti antagonist also act on MC4R that signals via various biological process causing obesity. MC1R also arbitrates anti-inflammatory and immunomodulatory properties due to its expression in leukocyte cells. MC1R is also present in vascular endothelial cells showing vasodilative effect (Rinne et al., 2015). Expression of MC1R is seen in macrophages which mediates anti-inflammation via α-MSH (Li and Taylor, 2008).

MC2R controls the action of ACTH receptor which is located on the adrenal cortex and helps in the regulation of corticosteroid and adrenal gland secretion in hypothalamic-pituitary-adrenal axis. MC2R is also found in adipose tissue of rodents and humans. It shows lypolytic action in mice (Wikberg, 1999).

MC3R is primarily expressed in hypothalamus and in lower level in limbic areas of central nervous system (CNS). It plays crucial role in physiological processes like energy balance, cardiovascular regulation and natriuresis (Roselli-Rehfuss et al., 1993). Preferential ligands for MC3R, α- and γ-MSH control natriuretic effects. MC3R is responsible for the regulation of energy homeostasis. This was proved by one of the experiment when scientists observed morbid obesity in MC3R deficient mice (Chen et al., 2000). The $MC3R^{-/-}$ mice, after 4 – 6 months of care unveiled increased adiposity, decreased lean mass and greater feed efficiency than control mice. The feeding behavior and metabolic rates were consistent for all mice during whole process. MC3R is expressed in POMC neurons in hypothalamus ARC. On peripheral parenteral administration of a potent and selective MC3R

agonist (D-Trp^{8}-γ-MSH), food intake in mice was stimulated which implicates inhibitory autoreceptor action in POMC neurons (Marks et al, 2006). These studies suggest the multifaceted and complex role of MC3R in the process of energy homeostasis as inhibitory anorexigenic effect of MC3R in POMC neurons is overruled by combined anorexigenic consequence when MC3R deficient mice lacking MC3R gene showed phenotypic obesity. Apart from MC3R expression in the CNS, it has also been found in minor quantity in other surrounding organs such as in adipose tissue, skeletal muscle, heart, kidney, stomach, duodenum, pancreas and placenta (Gantz et al., 1993; Ellacott et al., 2006). MC3R is also found responsible in salt-sensitive hypertension (Ni et al., 2003).

MC4R expression in CNS and its greater prevalence in the other organs and tissues than that of MC3R signify the diverse and valuable physiological functions of MC4R in maintaining energy homeostasis along with autonomic and neuroendocrine functions. MC4R is expressed in the multiple sites of rodent brain i.e. in amygdala, brainstem, cerebellum, cortex, hippocampus, hypothalamus, midbrain, thalamus and striatum (Mountjoy et al., 1994; Kishi et al., 2003). The primary ligand for MC4R is α-MSH and experimental evidence has revealed that MC4R is present wherever α-MSH shows its binding tendency (Tatro et al., 1990). In a study conducted by Huszar and co, deactivation of MC4R gene in MC4R deficient mutant mouse (MC4R$^{-/-}$) resulted in age-related obesity disorder accompanied by increased linear growth and symptoms like hyperphagia, hyperglycemia and hyperinsulinemia (Huszar et al., 1997). These phenotypic manifestations were found similar to that of agouti yellow mice deprived of MC1R, a gene responsible of hair and skin pigmentation suggesting agouti obesity syndrome is due to central melanocortin signaling antagonism mediated through MC4R interference. Also due to widespread expression of MC4R in organs and tissues other than CNS, it is involved in other physiological roles like cardiovascular regulation, pain processing, sexual behavior and penile erections which are controlled by CNS (Tao, 2010).

MC5R expression is found mainly in exocrine tissues such as sebaceous, adrenal and lacrimal glands. It helps in production of exocrine gland

secretions. Targeted deletion of MC5R gene in mice causes intense disruption of water repulsion and thermoregulation because of lower production of sebaceous lipids. MC5R is also essential for the production of harderian gland porphyrins and ligand-controlled lacrimal gland secretion of protein (Chen et al., 1997). Defective pheromone secretion in MC5R null mice causes aggression and protective behavior modifications (Morgan et al., 2004). Due to exocrine role of MC5R in synthesizing and secreting glandular secretion, it can be used as potential gene-level target for therapeutic treatment of topical disorders like dermatitis, acne and skin rashness (Gantz and Fong, 2003).

1.2.2 Role of central melanocortin system in regulating energy homeostasis

The central melanocortin system comprises neurons expressing POMC and its cleaved derivatives, neurons containing AGRP and CNS neurons which express MC3R and MC4R. These components undergo various processes and help in the regulation of energy homeostasis. Peripherally expressed MC3R and MC4R also possess probable energy homeostatic property. The two strains of agouti mouse, lethal yellow (A^y) and viable yellow (A^{vy}) are produced by spontaneous mutation. Phenotypically, A^{vy} mouse has black spots making it to appear as typical black/ yellow agouti color as compared to bright yellow coat color of A^y mouse (Yen et al., 1994). Dominant yellow color mutations at the mouse agouti point leads to recognizable effects including obesity, hyperinsulinemia, somatic growth activation, tumorigenesis and external coat coloration (Perry et al., 1994). As mentioned earlier, obesity in agouti mouse occurs due to antagonistic effect of agouti peptide at MC1R and MC4R. In fact after the cloning and hypothalamic localization of MC4R, it was supposed that agouti peptide cause an antagonistic effect in MC4R (Lu et al., 1994). Later, it was confirmed after the experiment on MC4R null mouse (Huszar et al., 1997). Use of MC3R/ MC4R agonist, Melanotan II (MTII) in wild-type littermates, agouti and leptin deficient ob/ob mice resulted in reduced food intake whereas MC3R/ MC4R antagonist- SHU9119 increased night time and fast-induced food intake. This implicates inhibitory effect of melanocortin system stimulated by altered food intake acts via centrally expressed MC3R and MC4R (Fan et al., 1997).

Adipocytes-produced leptin mixes into circulation, binds to centrally expressed leptin receptors and exerts its effect on energy homeostasis. Central melanocortin system imparts necessary role in intermediating the effects of leptin. Leptin receptors are highly localized in ARC's POMC neurons. SHU9119, a central melanocortin antagonist moderately reverses the anorexigenic effect of externally administered leptin molecule. This finding shows the central melanocortin system is the downstream result of leptin signaling cascade and is important in exerting leptin's effect (Seeley et al., 1997). Leptin regulates POMC and AGRP mRNA levels and the consistency of these proteins during exceptional energy homeostatic state like fasting and lactation (Chen et al, 1999; Mizuno et al., 1999). In *ex vivo* sample maintained at electrophysiological state, leptin controls the firing rate of POMC and AGRP/ NPY neurons in ARC (Cowley et al., 2001; Takahashi et al., 2005). Proteins taking part in leptin receptor signaling cascade like SOCS-3 and pSTAT-3 in ARC POMC neurons are also controlled by leptin hormone (Munzberg et al., 2003). Although leptin effects are mediated by POMC neurons, Boston and his coworkers demonstrated that the effects of central melanocortin facilitated disruptive POMC signaling and lack of leptin on weight increase in double-mutant yellow leptin deficient ($A^{y/a}$ lepob/lepob) mice were independent and synergistic. Also, they found that leptin resistance is due to inactive leptin signaling (Boston et al., 1997). Exogenously administered leptin can lead to energy homeostasis aided by MC4R by modifying nutrient intake (Trevaskis and Butler, 2005). Action of leptin does not depend only on MC4R-transduced central melanocortin pathway. It is important to note that leptin resistance is a resultant of various factors which arise from obesity. Anorectic neuromodulators like NPY and-, corticotrophin releasing factor (CRF) act autonomously or subsequently to MC4R signaling disregard of leptin resistance (Marsh et al., 1999).

Central melanocortin system is not only actively involved in the regulation of energy homeostasis via factors like leptin but also shows its action in conciliating hunger and satiety cues. POMC and MC4R mRNA are also situated in the dorsal vagal complex (DVC) of the brainstem (Mountjoy et al., 1994). DVC as we know is an end-point for vagal afferent nerve fibers and

vital in instant transmission of satiety related information between stomach and brain. MTII, a MC3R/ MC4R agonist decreases a meal size whereas antagonist SHU9119 increases a meal size, both regardless of meal frequency (Sutton et al., 2005). This data acts as an evidence for satiety inducing characteristics of central melanocortin system. Cholecystokinin (CCK), a popular satiety factor and meal-stimulated satiety activates NTS POMC neurons (Fan et al., 2004). Upsurge of NTS pERK (phosphorylation of extracellular-signal regulated kinase) by CCK or MTII can be retreated with the prior administration of antagonist SHU9119 which advocate that tonic pERK increment is reliant on pristine MC3R and MC4 signaling, supporting the proof for CCK interaction with central melanocortin system (Sutton et al., 2005). Imparting important role in CCK-induced satiety cues, central melanocortin system is also responsible for mediating hunger cues or meal initiation. Ghrelin, an endogenous hunger-inducing hormone triggers nutrient demand needed for the body. Before the meal, the systemic level of ghrelin is increased which lowers after food intake. This indicates importance of ghrelin in meal commencement. Also, lowering of after-meal ghrelin level is directly correlated with ingested nutrient caloric value (Callahan et al., 2004). Expression and release of ghrelin in hypothalamic region of CNS stimulates the synthesis of orexigenic peptides and an inhibition of POMC firing (Cowley et al., 2003). This region of CNS also contains NPY/AGRP neurons. ARC NPY neurons activation via growth hormone secretagogue receptor (GHSR) increases the appetite (Willesen et al., 1999). Prolonged central administration of ghrelin peptide in rat initiates meal intake and subsequent increment of body weight without affecting plasma insulin, glucose, leptin and GH level. There is also rise of both NPY and AGRP mRNA levels in ARC (Kamegai et al., 2001). These studies suggest the interactive connection between hunger stimulant ghrelin, satiety stimulant leptin and central melanocortin system mainly involving MC3R and MC4R and their important role in mediating energy homeostasis via appetite and surfeit cues.

Genetic deletion of melanocortin 4 receptor function in mice produces MC4R null mice with melanocortin obesity syndrome that have phenotypic resemblance with agouti yellow mice. The cause of obesity in these strains of

mice is due to hyperphagia and hypometabolism. However, it has been showed that hyperinsulinemia occurs prior to these symptoms (Fan et al., 2000). Melanocortin system can also modulate pancreatic sympathetic signaling cascade. MC4R null mice do not have natural homeostatic approaches to diet fat modifications (Butler et al., 2001). They become oversensitive to obesity-causing effects of high fat diet. These results are not seen in leptin deficient and MC3R null mice. It is also discovered that MC4R null mice are not able to increase energy expenditure despite being hyperphagic. However, this incapability is not associated with environmental temperature as MC4R null mice adapt to lower temperature by BAT mediated thermogenesis. But, again this cold environment adaptation is not seen in ob/ob mice and leptin administered MC4R null mice (Marie et al., 2000; Butler et al., 2001).

Role of central melanocortin receptors in metabolism and obesity can be further studied in terms of whether it has any association or contribution in cardiovascular disorders. Metabolic syndrome, defined as a cluster of biochemical as well as physiological defects with the symptoms like abdominal obesity, hyperlipidemia, hyperglycemia, hypertension and low HDL (high-density lipoprotein) cholesterol level elevates the risk for coronary heart disease and other health problems like diabetes. Regarding the connection between metabolism and cardiovascular diseases, there is several hypothesis and studies on the effect of central melanocortin system in the physiological and pathological role of cardiovascular system. In one study, it was demonstrated that mean arterial pressure (MAP) was elevated by chronic stimulation of hypothalamic MC3R/ MC4R with no change in food intake. However, MAP remained unchanged following hypothalamic MC3R/ MC4R inhibition but in this case, there was significant weight increment. This experiment was in consistent with the hypothesis that intact MC3R/ MC4R are essential in elevating arterial blood pressure in obese individuals (Kuo et al., 2003). Another experiment involving homozygous and heterozygous MC4R deficient mice confirmed this hypothesis by showing homozygous MC4R null mice were neither hypertensive nor salt sensitive even after possessing many features of metabolic syndrome like obesity, hyperglycemia, hyperinsulinemia

and hyperleptinemia (Tallam et al., 2005). Hence, these data supports the competence of central melanocortin system in obesity and obesity-associated cardiovascular disorders like hypertension and tachycardia.

1.2.3 Role of central melanocortin system in adiposity

MC4R signaling helps in the regulation of metabolism. To investigate this, pair-feeding studies had been done that have shown MC4R null mice had increased fat pad accumulation in the absence of hyperphagia (Marie et al., 2000). This experiment also exposed significant weight differences between female test and female control groups which were not seen in male group that suggest the possible role of sex steroid hormones in metabolism. More buildup of fat pad in male test mice than its control without significant weight differences indicates the importance of central melanocortin pathway in maintaining adiposity. This study has also stressed defective MC4R signaling enhances caloric efficiency, the result which can be seen in agouti mice and also in MC3R null mice. Another study done few years later tried to focus on hyperphagia rather than hypometabolism for the cause of early-onset obesity (Weide et al., 2003). MC4R also affects lipid storage in liver and increase in lipogenic liver enzyme might be associated with adiposity (Albarado et al., 2004). MC3R that is expressed on adipocytes can modulate fat mass content as displayed by MC3R null mice, which have greater adiposity and feed efficiency value but reduced lean mass as compared to wild types (Chen et al., 2000). $POMC^{-/-}$ mice are found to be hypometabolic, hyperphagic to high-fat diet and obese but show regular reaction to anorexigenic peptide YY or PYY_{3-36} (Challis et al., 2004).

Central melanocortin system plays certain part in BAT-mediated thermogenesis by modulating sympathetic nerve activity that innervates brown fat. Agonist of MC3R/ MC4R (e.g., α-MSH) stimulates sympathetic nerve activity and elevates BAT temperature whereas antagonist (e.g., AGRP) lowers BAT temperature by inhibiting activity of BAT-innervated sympathetic nerve (Yasuda et al., 2004). Ventricular administration of MTII (MC3R/ MC4R agonist) in hindbrain and forebrain region increases UCP1 mRNA expression in BAT, mediated by sympathetic outflow to BAT (Williams et al., 2003). These

experiments indicate that central melanocortin system can regulate sympathetic nerve activity of BAT resulting in thermogenesis and subsequently helps in energy expenditure. Leptin, an adipocyte-derived satiety-inducing hormonal factor contributes to EE by BAT mediated thermogenesis via hypothalamus (Pandit et al., 2017). Among other signaling cascades, regulation of BAT thermogenesis is done by preoptic area - rostral raphe pallidus (POA-rRPa) pathway and dorsomedial hypothalamus/ dorsal hypothalamic area (DMH/ DHA) pathway. It has been suggested that neurons expressing leptin in DMH raise sympathetic outflow to BAT and the other way round. Central administration of leptin in ob/ob mice brought about normal body temperature which directs that leptin regulates EE by initiating BAT activation and thermogenesis via DMH/ DHA neurons (Jo and Buettner, 2014). By injecting pseudorabies virus (which acts as a polysynaptic retrograde tracing agent) into iBAT, sympathetic outputs from brain to interscapular region have been discovered (Zhang and Bi, 2015). It was reported that iBAT-injected pseudorabies virus were significantly found in various hypothalamic areas like paraventricular hypothalamus (PVN), lateral hypothalamus (LH), DMH, ARC and POA whereas in ventromedial hypothalamus (VMH), few or no virus was found. Upon viral injection into WAT, viruses were found in PVN, DMH and POA area. Discovery of these active sites have acknowledged that the downstream signaling from various hypothalamic regions regulate sympathetic nerve activity of BAT and WAT which initiates BAT thermogenesis along with WAT browning. However, Butler et al. found no involvement of BAT or leptin in DIT but suggested the connection of MC4R in mediating thermogenesis caused by dietary changes (Butler et al., 2001). These revolutionary findings have propounded that BAT thermogenesis is centrally supervised by hypothalamic melanocortin signals which leads to energy homeostasis and the possibility of targeting central melanocortin receptors during the metabolic disorders to tempt enduring energy balance state.

Cold-induced BAT thermogenesis is regulated by POA-DMH-rRPa pathway (Zhang and Bi, 2015). POA of hypothalamus contains sensory neurons that sense the external hypothermic (cold) condition (Figure 5). rRPa contains

sympathetic premotor neurons that react to thermoregulatory information received from POA and DMH by generating heat if necessary. This DMH-rRPa pathway also centrally governs stress-induced BAT thermogenesis and hyperthermia. Stress mediates through DMH to activate rRPa neurons and hence cause thermogenesis resulting from sympathetic outflow. Upstream neural network from DMH caused by stress is not properly understood. Expression of anorexigenic (appetite-suppressing) POMC neurons and orexigenic (appetite-enhancing) NPY/AGRP neurons in the ARC also helps in central maintenance of thermoregulation. Similarly, presence of NPY neurons in the DMH and, orexin and MCH neurons in the LH also helps in temperature equilibrium. Excessive high fat (HF) diet modifies the function of these neurons to eventually cause energy homeostatic state by increasing energy expenditure via BAT thermogenesis. However, central neuronal pathway in this regard (i.e. diet-induced thermogenesis) is not properly defined. Endogenous factors like BMP8 (BAT), estrogens (ovarian) and GLP-1 (intestine) mediates through VMH. These organ-specific stimulations are considered peripheral signals which also cause BAT activation or beiging and involvement of VMH demonstrates the secondary contribution of hypothalamus in BAT thermogenesis via neuroendocrine circuit. Above figure shows that central as well as peripheral pathway acts on classical BAT (iBAT) and beige/ brite cells in white adipose tissue (WAT). These both cells are sympathetically controlled by hypothalamic neural populations.

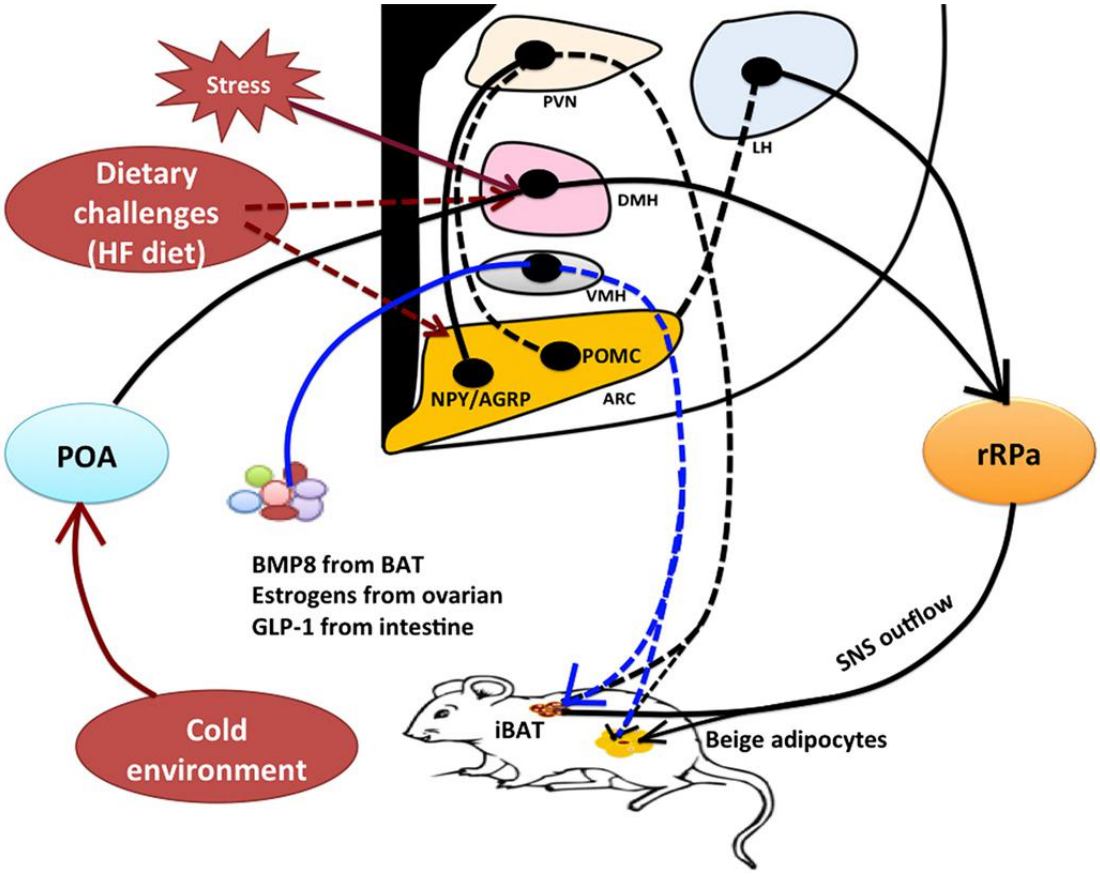


Figure 5: Model of hypothalamic neural regulation of BAT thermogenesis. (Solid lines/arrows indicate established pathways and dash lines/arrows indicate unascertained pathways]. BAT- Brown adipose tissue; iBAT- interscapular BAT; POA- Preoptic area; DMH- Dorsomedial hypothalamus; rRPa- rostral Raphe pallidus; POMC- Proopiomelanocortin; NPY- Neuropeptide Y; AGRP- Agouti-related peptide; ARC- Arcuate nucleus; MCH- Melanin concentrating hormone; LH- Lateral hypothalamus; BMP8- Bone morphogenetic protein 8; GLP-1- Glucagon-like peptide 1; VMH- Ventromedial hypothalamus; PVN- Paraventricular hypothalamus; SNS- Sympathetic nervous system. (Extracted from Zhang and Bi, 2015).

1.2.4 Other functions of central melanocortin system

It is well-known that MC4R mediates the linear growth. Genetic or natural disruption of MC4R function causes enhanced linear extension in rodents and humans (Huszar et al., 1997; Farooqi et al., 2003). Even though the systemic GH level is reduced in obese children, effects such as hyperinsulinemia, hyperprolactinemia, increased insulin-like growth factor 1 (IGF-1) and

increased insulin-like growth factor binding protein 3 (IGFBP-3) levels are experienced (Attia et al., 1998; Park et al., 1999). MC4R suppression in rodents result in obesity similar to human but along with weight gain in rodents, increase in linear growth and muscle mass are also seen. In fact, there is overall increase in lean mass in MC4R null mice. In agouti-derived yellow obese mice, increased level of serum IGF-1 during the early stage (2^{nd} – 4^{th} week) of life-cycle is assumed for enhanced longitudinal growth despite reduced systemic GH level (Wolff et al., 1999). One experiment summarized MC4R blockade by chronic infusion of central melanocortin antagonist, SHU9119 produced an obesity phenotype without any notable side-effects on the reproductive and somatotropic axes [GH, IGF-1, growth hormone releasing hormone (GHRH)]. In this investigation, hypothalamic neuropeptide Y (NPY) gene expression was specifically inhibited to suppress NPY-induced hyperphagia. Another experiment found that central melanocortin pathway can alter the GH secretion pattern but not the overall GH secretion level (Watanobe et al., 2003). Established anatomical data supports the idea of central melanocortin system in mediating GH secretion by displaying the close association between hypothalamic somatostatin and POMC neurons (Fodor et al., 1998). Arcuate nucleus contains elements of melanocortin and somatotropic system like POMC, AGRP, GHRH and somatostatin receptors like ss1 and ss2. Indeed, some POMC neurons express active binding sites for somatostatin. α-MSH interacts with somatostatin in PVN. Limited data implicate the necessity of more researches to specifically understand the effect of central melanocortin system in linear extension.

In addition to affecting linear growth, central melanocortin system also affects bone metabolism subsequently contributing to increased lean mass as seen in MC4 null mice. It has been found that there is higher expression of Rankl and Cart factor in the bones and hypothalamus of MC4R null mice (Elefteriou et al., 2005). Normal bone formation and reduced bone resorption occurs in half-year old MC4R null mice. MC4R deficient individuals have decreased serum Dpd and crosslaps CTX. One research group has reported that all five melanocortin receptors, MC1-5R are expressed in different concentration in osteoblast-like and osteoclasts cells (Zhong et al., 2005). Additionally, the

authors advised direct and indirect effects of POMC-derived hormones on bone cell subspecies and the involvement of steroidal hormone secretion. α-MSH, a melanocortin peptide directly stimulates bone cell proliferation but *in vivo* it causes reducing effect in trabecular bone which is mediated by α-MSH action that decreases adiposity and pancreatic anabolic hormones (Cornish et al., 2003). ACTH helps in the generation of chondrocyte phenotype from mesenchymal progenitor cell populations and also enhances production of matrix and differentiation of responsible chondrocytes (Evans et al., 2004). One study induced cachexia in MC3R null mice and wild-type mice by tumorigenesis (Marks et al., 2003). Cachexia is defined as a chronic metabolic disease because of decreased appetite (or food intake) and increased metabolism of lean as well as fat mass. In the experiment, researchers observed the remarkable loss of lean mass in MC3R null mice than wild-type controls. This experiment tried to explain the crucial role of MC3R in maintaining lean mass in a physiological abnormal state. Also, due to absence of functional MC3R in the diseased MC3R null mice, energy homeostasis was severely disrupted along with unregulated lean mass. The anatomical, pharmacological, physiological and pathological evidences to show the expression of central melanocortin system components during the somatic growth, osteoregulation and pathological studies illustrate the importance of overall melanocortin signaling in the regulation of lean mass in rodents and humans.

2 AIM OF THE EXPERIMENT

The present experiment has used genetic mutant $MC1R^{e/e}$ yellow mice having C57BL/6 background. $MC1R^{e/e}$ mice are produced by frameshift mutation between exon 4 and 5 that results in non-functional MC1R (Robbins et al., 1993). They have yellow fur color due to MC1R mutation that causes higher pheomelanin expression in the melanocytes of hair. In one of the unpublished experiment performed by our research group, it was found that $MC1R^{e/e}$ mice fed on standard chow diet had remarkable higher adipose tissue mass and decreased whole body energy expenditure than their WT counterparts.

Interestingly, these phenotypic changes seen in $MC1R^{e/e}$ mice coincide with the characteristics of MC3R and MC4R deficiency. On the other hand, it is already known that central melanocortin system (MC3R and MC4R) regulate body temperature via BAT thermogenesis. We wanted to know if the increased adiposity and decreased energy expenditure as seen in $MC1R^{e/e}$ mice is related with BAT thermogenesis thereby checking the importance of MC1R in mediating BAT thermogenesis.

3 MATERIALS AND METHODS

3.1 Animals, care and ethics

The experiment was carried out on 3 to 4 months old adult male mice. The mice were housed in the Central Animal Laboratory, University of Turku. Temperature was maintained at 21 ± 2 ^{o}C with relative humidity of 36 ± 2%. Twelve hours light/dark cycle was maintained throughout the experiment without any restriction on water and diet. Diet provided was standard chow diet. The 3R principle of replacement, reduction and refinement was followed. Euthanasia was done after the completion of experiment meeting standard guidelines. The experiment was performed in accordance to national and E.U. directives regarding animal care during experiment and approved by national animal care and use committee.

3.2 Animal models and drug used

Two different varieties of male adult mice were used- wild-type C57BL/6 (black) mice and mutant $MC1R^{e/e}$ (yellow) mice on a C57BL/6 background. $MC1R^{e/e}$ yellow mice are homozygous for the $MC1R^{e}$ allele and they have defective MC1R function because of single base deletion mutation. All the mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in Central Animal Laboratory, University of Turku.

CL-316243 (1.00 mg/kg, CL-316243 disodium salt, Tocris Bioscience, UK), a highly selective β3AR agonist was administered i.p. to induce BAT thermogenesis. Food and water were provided ad-lib in an indoor environment

of set room temperature (21 ± 2 °C). Saline (0.9%, Natriumklorid Braun 9 mg/ml, B. Braun Melsungen AG, Germany) was used as control. The handling was avoided as much as possible because stress caused by handling also influences body temperature and drug effect can be accentuated.

3.3 Experimental setup

Prior to main experiment, pilot study was conducted for few weeks taking only wild-type (WT) mice and injecting them with saline and CL (i.p). The primary reason behind this pilot project was to determine the experimental setup. We drew some important conclusions that would be applied in the main experiment. Sedative agent (medetomidine) needs to be used to calm the mice because in awake mice, the stress of handling and injection increases the BAT temperature and hence the CL-effect could not be differentiated. Medetomidine-induced hypothermia was managed by using heating mat and recovery of induced sedation was made possible by atipamezole i.p. injected at the end of experiment. We also noticed that shaving of ROI (interscapular and lower body region) was necessary as infrared camera can precisely detect and measure the temperature in the shaved area. The procedure was mainly based on the published paper where infrared camera was used to specifically measure BAT thermogenesis *in vivo* (Crane et al., 2014). Comparison of skin temperature between interscapular region (overlying BAT) and lower body region was necessary to compare the CL-induced BAT temperature increment with normal body-temperature rise (Jackson et al., 2001).

3.3.1 Main experiment

Total of 18 male mice (9 WT and 9 mutant ($MC1R^{e/e}$)) were divided into six groups with each group containing same genotype mice. The interscapular and lower body regions were shaved one day prior to imaging basal body temperature. Shaving was carried out under the influence of isoflurane anesthesia (4% induction, 1.5% maintenance; Attane vet, 1000 mg/g, Piramal Healthcare, UK) followed by trimming (Ordinary trimmer, Isis, Aesculap, Germany) and shaving (Veet hair removal cream, Reckitt Benckiser, UK). Then, dorsal body temperatures were imaged with the help of infrared camera

(FLIR T620bx, FLIR Systems AB, USA) in awake, freely moving mice after two hour habituation to individual housing in a new cage. On the following day, same groups of mice were injected i.p. with either saline or CL in a randomized crossover manner. Before saline/CL injection, mice were anesthetized with medetomidine (0.33 mg/kg, Cepetor vet 1mg/ml, ScanVet Oy, Finland) and then transferred to heating mat (776 X 274 mm, 230 V, 41.5 W, MAT Solutions BV, Netherlands) to counter the hypothermic effect of medetomidine. Infrared images were then shot (referred as 'images 00:00' or 'baseline images') by keeping mouse out of heating mat on open-top cage. After the saline/CL administration, infrared images were taken in various time points in 00:20, 00:40, 01:00 and lastly in 02:00 hours before the administration of atipamezole (2.00 mg/kg, Antisedan vet 5mg/ml, Orion Pharma, Finland) i.p. (Figure 6).

Figure 6: Time-based flowchart showing the procedure for the measurement of BAT thermogenesis using infrared thermography technology. HM – Heating Mat.

Below are the examples of infrared images for treated genotype which were produced using FLIR software (Figure 7). Rectangular and circular/oval box were drawn in the image to restrict the measurement within the interscapular

ROI (represented by Bx1) and lower body ROI (represented by El1) respectively. Interscapular ROI encompasses BAT and lower body ROI encompasses region of body devoid of BAT and works as reference to body temperature. The image precisely shows the magnitude of maximum, minimum and average temperature within ROIs. Colorful linear side bar is the index for extent of total body temperature that varies between the images due to the software automatic operation to make suitable contrasts however higher limit does not exceed 40^{o}C.

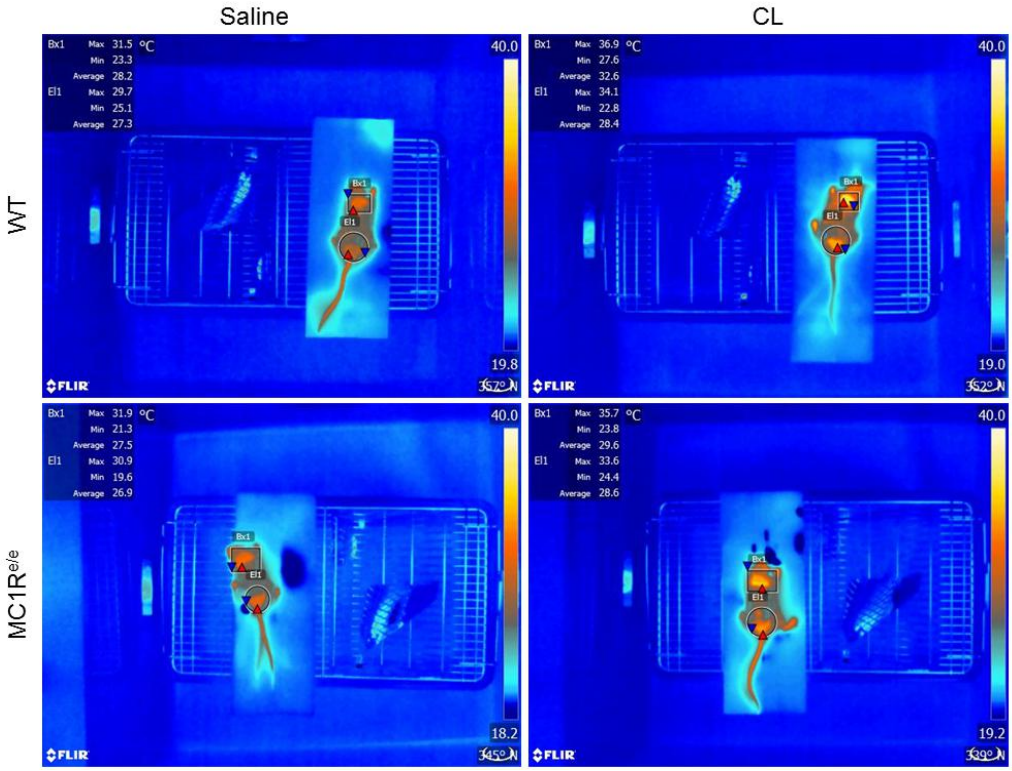


Figure 7: Representative infrared images of WT and MC1R$^{e/e}$ mice 20 minutes after saline/CL administration. WT- Wild type; MC1R$^{e/e}$- MC1R deficient; BAT- Brown adipose tissue; LB- Lower body.

3.4 Preparation of drug solution

Powder grade CL was mixed with saline to prepare 20 aliquots of stock solution (1 mg/ml) and stored in deep freezer (-70^{o}C). Dose of CL administered was 0.001 mg/g and suitable concentration was prepared depending on weight of mice and required volume.

3.5 Data production from thermal images

Thermal images shot by infrared camera were imported in the computer and data production was done by FLIR Tools (version 5.6.16078.1002). Multiple images were taken in each of the allocated time points but during data production, only the best five images for each time point were taken into account. All the images were consistent in terms of their picture properties-dimensions of 561 x 421, horizontal and vertical resolutions of 72 dpi and bit depth of 24. Interscapular ROI was marked within rectangular box and lower body ROI was marked within circular/ oval boundary. Only the highest temperature within these restricted areas was taken into consideration. Surface temperature of skin overlying BAT within interscapular region denotes the β3AR agonist induced BAT thermogenesis (Crane et al., 2014).

3.6 Statistical methods

All the statistical analyses were carried out using licensed GraphPad Prism software (version 6.01). Data were presented as mean ± SEM value. P-value of less than or equal to 0.05 (p-value ≤ 0.05) was regarded statistically significant. The temperature (^{o}C) vs. time point (min.) graphs were drawn for BAT, LB and ΔT (BAT-LB) temperatures and the comparisons were made using two way ANOVA test and Sidak's multiple comparison test. Same kind of comparison tests were performed for the basal temperature analysis. In addition, magnitude of CL effect in inducing BAT thermogenesis was studied with the help of area under the curve (AUC).

4 RESULTS

4.1 Body temperature in awake mice

BAT and LB temperatures were imaged with the help of infrared camera in mice allowed to move freely in an individual open-top cage in a normal room temperature without the influence of the drug or handling. Statistical test showed marked difference between BAT and LB temperature in the genotypes (Figure 8). BAT temperature was significantly higher than LB temperature in both genotypes (p-value < 0.0001 body region by two-way ANOVA). Importantly, statistically significant difference was not found in body

temperatures between WT and MC1R$^{e/e}$ mice (p-value = 0.07 genotype by two-way ANOVA, interaction of genotype and body region = not significant). This study shows the dominance of BAT temperature over LB temperature which is regardless of MC1R deficiency.

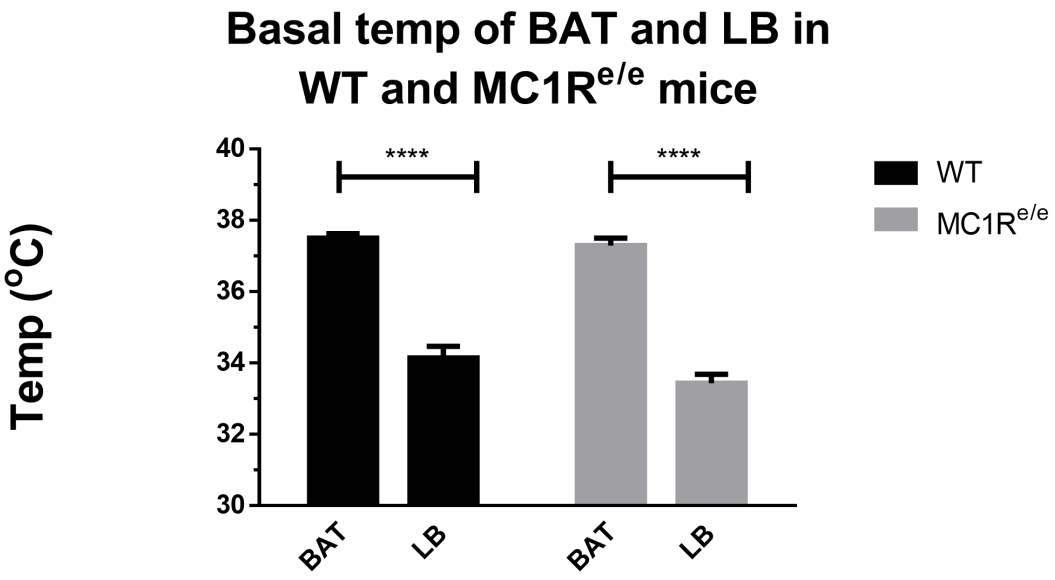
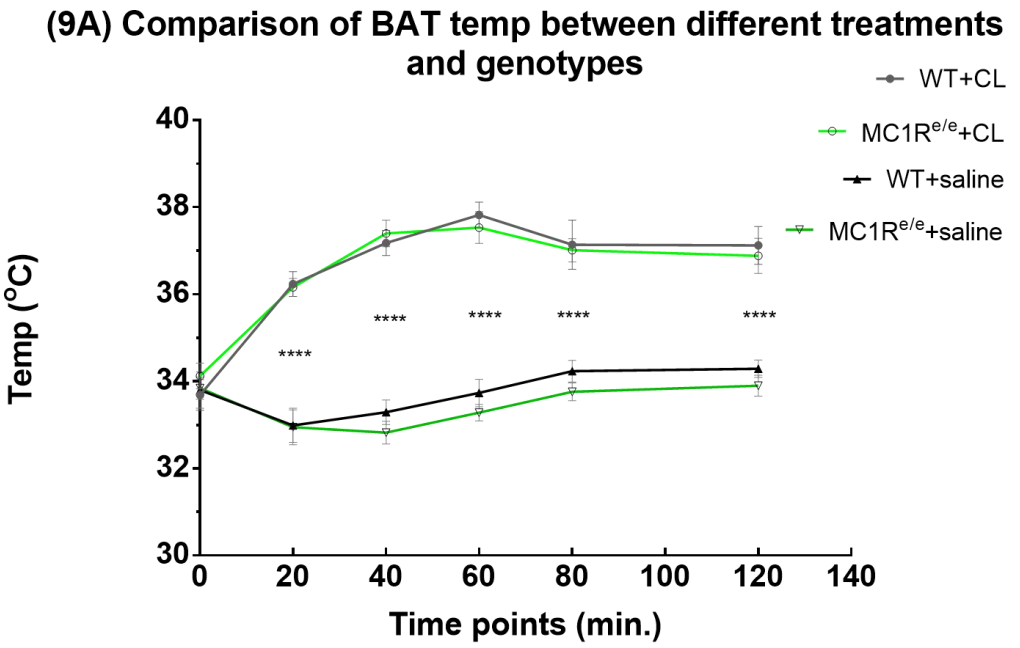


Figure 8: Comparison of basal temperature of BAT and LB in WT and MC1R$^{e/e}$ mice. Statistically significant difference exists between BAT and LB basal temperatures in WT and MC1R$^{e/e}$ mice (p-value < 0.0001). In both genotypes, temperature of BAT is markedly higher than that of LB area. More importantly, statistically significant difference was not found between the genotypes' BAT and LB temperatures (p-value = 0.07). Data are presented as mean ± SEM. ****, p-value < 0.0001. WT- Wild type; MC1R$^{e/e}$- MC1R deficient; BAT- Brown adipose tissue; LB- Lower body.

4.2 Lack of significant differences in BAT temperature of WT and MC1R$^{e/e}$ mice

CL caused an increase in BAT temperature of both, WT and MC1R$^{e/e}$ mice (Figure 9A). In fact, even treatment with saline induced BAT temperature increment in both of the genotypes except during the first time point (i.e. 00:20). However, such rise of BAT temperature caused by saline was significantly less when compared to CL. Statistical evaluation showed

increment of BAT temperature prominently in CL treatment group than saline group (p-value < 0.0001). One of the important things to notice is that, within CL group BAT temperature increment was not markedly different between WT and $MC1R^{e/e}$ mice (p-value > 0.05). Along with BAT temperatures, LB temperatures were also studied within the same set of mice. LB, deprived of brown or beige/brite adipocytes was also our ROI as LB suitably represents the temperature of body that lacks BAT. After the analysis, the nature of temperature vs time points graph of LB temperatures (Figure 9B) was found to be similar to that of BAT temperatures. However, temperatures peaked during LB temperature measurement were considerably lower as compared to BAT temperatures. Significant numerical differences but proportional relation between BAT and LB temperatures was observed.



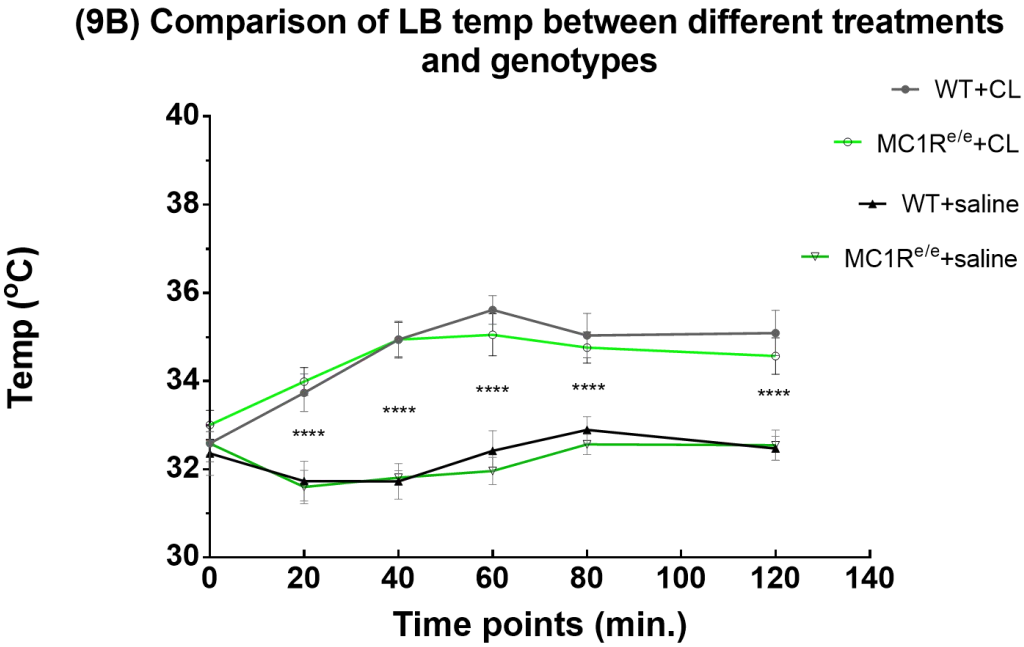


Figure 9: Temperature (°C) vs time point (min.) graph for (A) BAT and (B) LB temperature. Figure 9A shows statistically significant differences between the treatment groups (p-value < 0.0001) as evaluated by multiple comparison test which means significant temperature increment was seen in CL-injected group

than saline-injected group. However, Sidak's multiple comparison test within CL injected group showed lack of statistically significant differences between the genotypes that suggests the lack of role of MC1R in modifying BAT temperature. Similar kind of graph was obtained for LB temperatures (Figure 9B). The main difference between these two graphs- 9A and 9B was the peak temperature reached in individual time points. Data are presented as mean ± SEM. ****, p-value < 0.0001. WT- Wild type; $MC1R^{e/e}$- MC1R deficient; BAT- Brown adipose tissue; LB- Lower body.

Area under the curve (AUC) against time was drawn for BAT temperatures and analyzed by two-way ANOVA (Figure 10). AUC of BAT temperature over time was found to be significantly higher after CL injection than saline injection in both WT and $MC1R^{e/e}$ mice (p-value < 0.0001). CL-induced BAT temperature was insignificantly lower in $MC1R^{e/e}$ mice than WT mice (p-value = 0.1566 genotype).

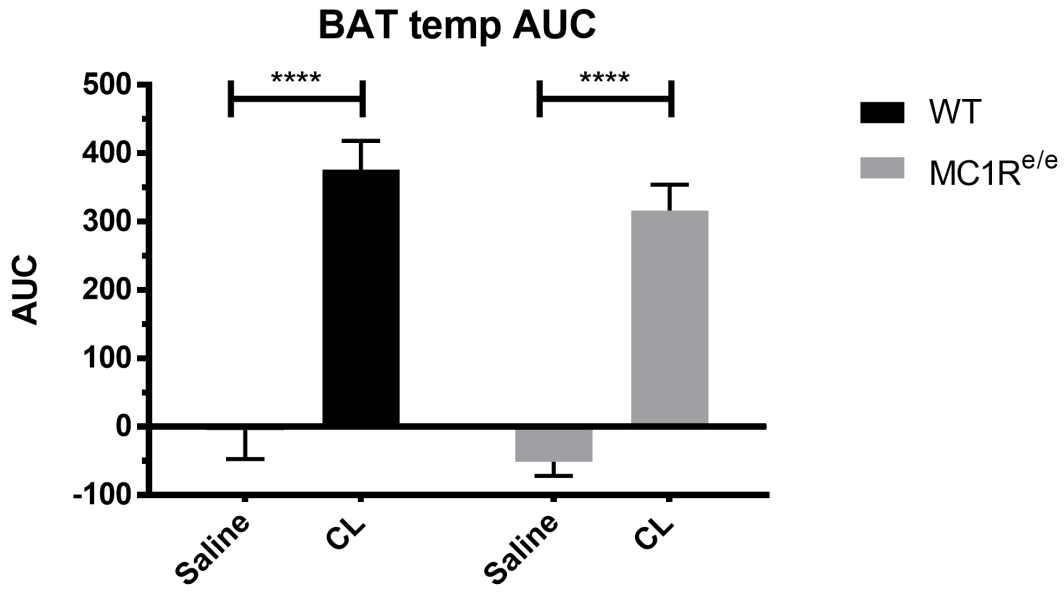


Figure 10: AUC of BAT temperature over time in the genotypes. BAT temperature is significantly higher following CL injection than saline injection

in both genotypes (p-value < 0.0001). Non-significant difference was observed in CL effect between the genotypes (p-value = 0.1566). Data are presented as mean ± SEM. ****, p-value < 0.0001. WT- Wild type; MC1R$^{e/e}$- MC1R deficient

4.3 Comparison of temperature difference in BAT-LB (BAT minus LB or Δt) between wild-type and MC1R$^{e/e}$ mice

When proportional relation between BAT and LB temperatures from above analysis (4.1) was realised, temperature difference between BAT and LB (Δt) vs time point was calculated (Figure 11). Statistically significant interaction between genotype and time (p-value < 0.05) was found in Δt of WT and MC1R$^{e/e}$ mice after CL injection. The result was different for saline-treated groups as no statistically significant difference (p-value > 0.05) was found between the genotypes. CL-induced Δt peaked earlier in WT mice but in MC1R$^{e/e}$ mice, Δt raised slowly and remained higher than WT mice that might suggest a difference in heat transmission in β3-agonist-induced thermogenesis possible via MC1R-mediated vasodilative action.

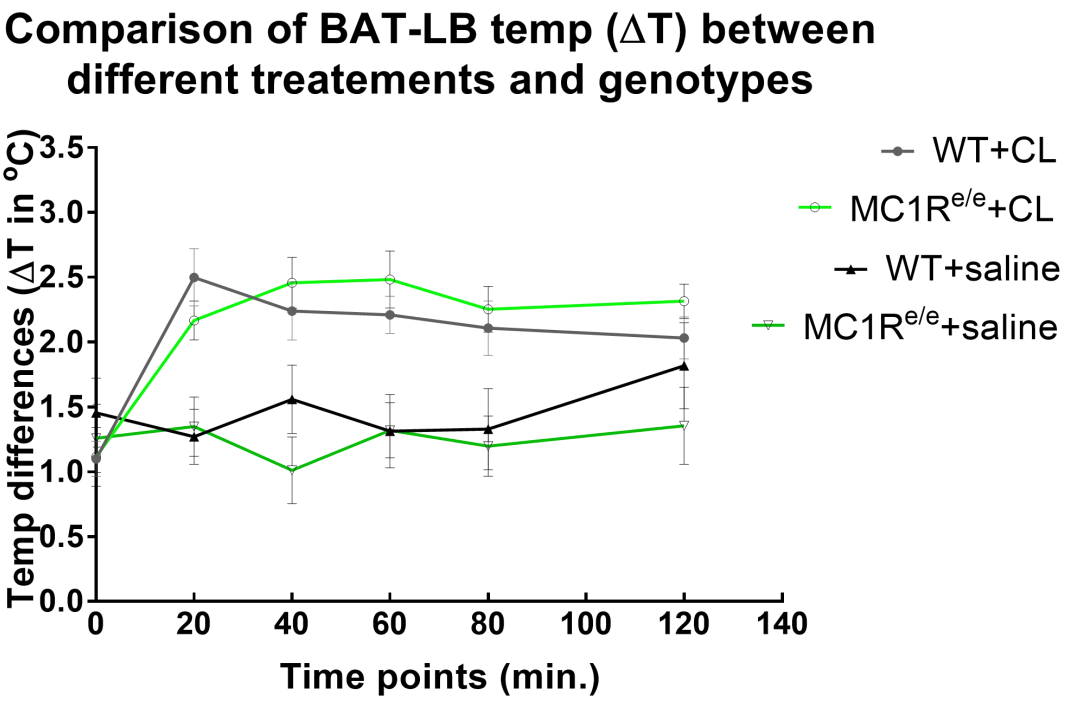


Figure 11: Temperature (°C) vs time point (min.) graph for Δt in WT and MC1R$^{e/e}$ mice following treatments. Significant interaction was observed between genotype and time in CL-induced Δt of WT and MC1R$^{e/e}$ mice (p-value < 0.05 for interaction in two-way ANOVA). In contrast when saline-treated groups were compared, significant difference was not found between WT and MC1R$^{e/e}$ mice. Data are presented as mean ± SEM. WT- Wild type; MC1R$^{e/e}$- MC1R deficient; BAT- Brown adipose tissue; LB- Lower body; Δt- BAT minus LB.

AUC data showed distinctly increased Δt for CL-treated group than saline-treated groups in WT and MC1R$^{e/e}$ mice (Figure 12). Statistically significant difference was observed between saline-induced and CL-induced Δt in both genotypes (p-value < 0.0001). Nonsignificant difference was observed when CL-induced Δt was compared between genotypes (p-value = 0.5604).

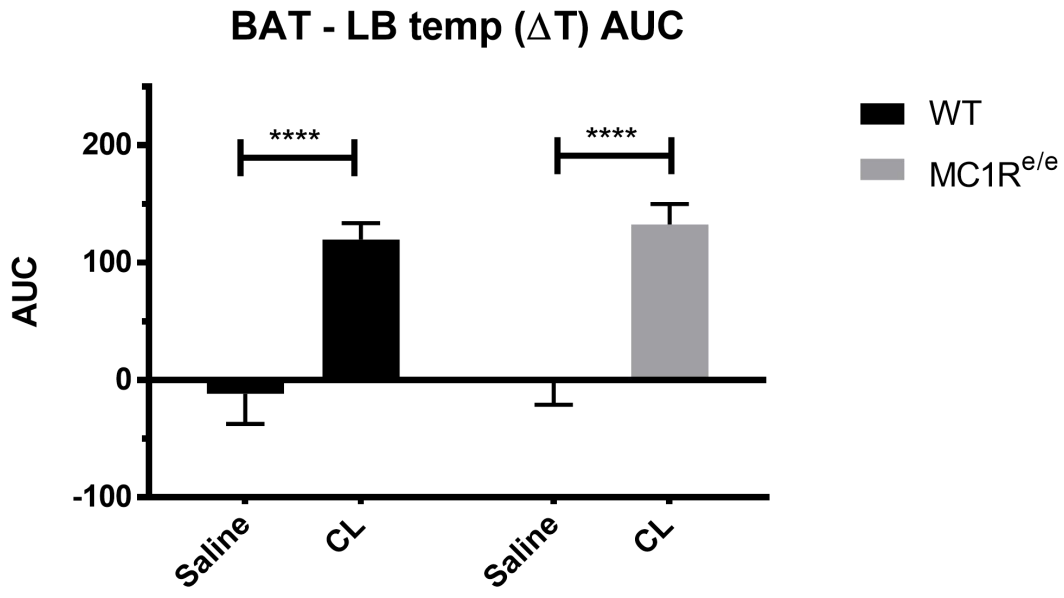


Figure 12: AUC of BAT-LB temperature (Δt) over time in the genotypes. Marked difference was seen between saline-treated and CL-treated group in both genotypes (p-value < 0.0001). CL-induced Δt was nonsignificantly different between the genotypes (p-value = 0.5604). Data are presented as mean ± SEM. ****, p-value < 0.0001. WT- Wild type; MC1R$^{e/e}$- MC1R deficient.

5 DISCUSSION

5.1 Methodological consideration

CL-316243, a highly selective β3AR agonist causes BAT mitochondrial proliferation and thermogenesis which is linked to total energy expenditure and leanness. In addition, CL has shown the effect in WAT hyperplasia by impeding its growth during initial period of diet-induced obesity and also causing WAT browning by inducing hypertrophy to small brown preadipocytes in WAT that express β3-adrenoceptors (Himms-Hagen et al., 1994; Ghorbani et al., 1997). In the present experiment, CL (1.00 mg/kg i.p.) was used to acutely stimulate BAT mediated thermogenesis in mice. Injection of drug and stress of handling of mice during the experiment increased BAT temperature which masked the effect of drug. To solve this problem, medetomidine was used (Cruz et al., 1998). The dose of medetomidine was kept lower (0.33 mg/kg) because the purpose was to bring mild sedation rather than anesthesia. The light sedation prevented the mice to move frequently. Mice were kept on the tissue paper individually over the top-grid of the cage and thermal images were photographed by fixed top-positioned infrared camera. Due to the mice inactive movement brought about by medetomidine, infrared photography was performed with convenience and consistence. One of the serious side effects of medetomidine in rodents includes hypothermia that decreases the body temperature and can be life-threatening (Cruz et al., 1998). To solve this issue, mice were kept on normal heating mat by which they were able to maintain their body temperature. Heating mat was used until the experiment lasted to consistently provide the warm surrounding to the mice and bring uniformity in the experimental procedure. The use of heating mat might influence the result as it increases the skin temperature of mice. However from ethical point of view, heating mat provides survival surrounding for sedated mice prone to intense hypothermia. In the end of experiment, mice were injected with atipamezole (2.00 mg/kg i.p.) that reverses the effects of medetomidine (Cruz et al., 1998). Atipamezole is an α2 agonist drug that treats α2-mediated effects like sedation, hypothermia and acute

cardiovascular and respiratory dysfunction (Sinclair et al., 2003). Six time points were chosen for infrared imaging including one time point (i.e. 00:00) before the administration of drug or saline- regarded as baseline and the other five time points after the drug/ saline administration. Those images were taken in every 20 minutes till 80th minute before the last imaging which was taken in 120th minute (00:20, 00:40, 01:00, 01:20 and 02:00). Decision for selecting particular time points, doses of drug and substances and route of administration were achieved from the data of pilot experiments performed prior to the main experiment. To reduce treatment biasness and enhance reproducibility, experimental mice were treated with either saline or drug in separate days in a randomized and cross over manner.

Thermogenesis mediated by UCP1 (i.e. BAT thermogenesis) *in vivo* can be specifically measured by infrared thermography (Crane et al., 2014). In the experiment following i.p. injection with CL to UCP1$^{-/-}$ mice and WT (UCP1$^{+/+}$) mice, significant increase in interscapular and whole body dorsal surface temperature was observed in UCP1$^{+/+}$ mice than UCP1$^{-/-}$ mice. There was also increased oxygen uptake in UCP1$^{+/+}$ mice. The result suggested that infrared thermography can accurately sense and measure UCP1-mediated thermogenic changes in β3 stimulated mice. The experiment was also performed in UCP1$^{+/+}$ mice which were bred and housed in two different temperatures (thermoneutral temperature of 30^{o}C and comparatively cooler temperature of 23^{o}C). On β3 stimulation by CL injection, mice maintained at 23^{o}C showed notable increase in oxygen consumption and interscapular and whole body dorsal surface temperature than mice maintained at thermoneutral temperature. These results have not only helped to establish a positive correlation between whole body energy metabolism and BAT thermogenesis but also provided evidence that changes in UCP1-mediated BAT temperature can be evaluated by infrared thermography. The infrared thermography (IRT) technique has been used in human subjects also to measure the surface temperature overlying BAT-containing supraclavicular (SCV) fossa and the lateral upper chest (Jang et al., 2014). The average left SCV temperature and temperature difference between left or right SCV and chest (Δtemp) were higher in BAT +ve subjects (subjects with significant amount of functional BAT)

than BAT –ve subjects before and during cold exposure (19 ^{o}C). The experiment showed IRT as a promising novel method to study BAT activity. Many other experiments have also used IRT for detecting and quantifying BAT-mediated adaptive thermogenesis in mice and human that highlights the convenience and accuracy of this infrared-based imaging technique (Lee et al., 2011; Symonds et al., 2012; David et al., 2013; Maurer et al., 2015; El Hadi et al., 2016; Robinson et al., 2016; Salem et al., 2016; Ang et al., 2017; Scotney et al., 2017).

5.2 Role of MC1R in BAT thermogenesis

MC1R$^{e/e}$ mice have defective MC1R function. MC1R, mainly expressed in melanocytes of hair and skin is responsible for pigmentation (Ellacott et al., 2006). MC1R has higher affinity towards α-MSH and ACTH. α-MSH, an endogenous agonist gives darker pigmentation while agouti, an endogenous antagonist gives lighter pigmentation. Effect of ligand interaction with MC1R can be realized in agouti mice. Agouti mice are yellow in color due to the antagonistic effect of agouti peptide in melanocyte MC1R. The agouti peptide also acts on centrally expressed MC4R that fluctuates energy homeostasis and produces obese phenotype. In the present experiment, MC1R$^{e/e}$ mice are used as genetic model for dysfunctional MC1R which have abnormal eumelanin:pheomelanin ratio that results in lighter pigmentation (or yellow color fur). Recent finding by Rinne et al. (2015) has shown the linkage of disruptive MC1R signaling with increased arterial rigidity and the consequence in endothelium-related vasodilation. Conclusively, the group suggested a functional role of MC1R in the management of arterial tone. However, the purpose of the present experiment for using MC1R$^{e/e}$ mice was concerned only with their obese phenotype. BAT thermogenesis is regulated by the CNS with the main involvement of CMS that consists of MC3R and MC4R (Girardet and Butler, 2014; Contreras et al, 2015). CMS helps in energy homeostasis by influencing whole body energy metabolism and adaptive thermogenesis via hormonal and central pathway. Because of the CMS crucial role in body weight maintenance, it is not surprising that rodents lacking CMS receptors produce obese phenotype. Treating APOE*3-Leiden.CETP transgenic mice with SHU9119, Kooijman et al. (2014) showed inhibition of central MC3/4R

pathway disrupts BAT function, along with decreasing EE and increasing adiposity. One of our previous experiment conducted by different researchers showed similar kind of reduced EE and higher adipose tissue mass in $MC1R^{e/e}$ mice. The present experiment tried to address the reason behind such observational changes in $MC1R^{e/e}$ mutant mice and if BAT activity is involved in the process. In the experiment, BAT activity was stimulated by exogenously administered β3-adrenoceptor agonist (CL-316243) as BAT contains rich amount of specific β3 nerves. The endpoint response of BAT activation is observed by the increment in BAT temperature (thermogenesis) mediated by UCP1, a specific mitochondrial protein expressed in brown adipocytes. Increased BAT temperature was assessed by infrared thermography (IRT) technique by measuring superficial temperature of interscapular ROI underlying BAT. To compare the BAT temperature with other areas of body, IRT was also used to measure lower body area temperature in the same mice simultaneously.

Results obtained from the current experiment can be discussed in two main points- one when thermogenesis was measured in basal state and another when thermogenesis was stimulated by using CL. On the second day of each experimental week, both genotypes from the respective study groups were measured for its BAT and LB temperature in an unstimulated normal room temperature (21^{o}C). Mice were first transferred to individual new cage, left there for two hours without any food or water then top grid of cage was removed and left for another half an hour. Thermography was done with top-mounted infrared camera. The study on basal temperature revealed that BAT temperature is always higher than temperature of body that do not overly BAT. When CL-induced rising BAT temperatures were compared between the genotypes, statistically significant difference was not observed that simply implicated the lack of the MC1R role in maintaining basal temperature. However, one can oppose the notion as there is a natural tendency for a decreasing body temperature. Conclusively, MC1R does not interfere in basal temperature and the temperature of region that overly BAT is consistently higher than that region which lacks BAT, as detected by IRT. Next, the present experiment analyzed β3-agonist-induced BAT thermogenesis in WT

and MC1R$^{e/e}$ mice. When data obtained from thermographed images were statistically evaluated, it did not yield significant difference between the genotypes. Two-way ANOVA tests of measured BAT temperature in different time points showed statistically significant difference between saline-treated and CL-treated group but not between genotypes. Since it is well established fact that CL stimulates BAT thermogenesis (Himms-Hagen et al., 1994), the positive rise of BAT temperature observed in CL-treated groups was not a novel finding but the lack of significant difference between CL-treated genotypes suggests for the first time that there is no active role of MC1R in regulating BAT thermogenesis. Our experimental setting easily demonstrated the thermogenesis effect of CL in mice. Furthermore when temperature difference between BAT-LB (Δt) was compared among genotypes, the differences were significantly different between the genotypes over time with slower increase and larger temperature difference in MC1R$^{e/e}$ mice. In-detail comparison of Δt between the groups showed prominent difference between saline-treated and CL-treated group in WT mice in the first 20 minutes time. However, in MC1R$^{e/e}$ mice such significant difference was observed in later time points (i.e. in all time points except the first 20 minutes). The result showed that difference between CL-induced Δt and saline-induced Δt was significantly larger in MC1R$^{e/e}$ mice than WT mice which is observed in later time points. The analysis entails MC1R is somehow responsible for inconsistent rate of heat distribution produced by β3 activation. In a living body, heat is distributed via circulatory mechanism. Heat generated in BAT by β3 stimulation is transmitted to peripheral organs through blood. Disproportionate temperature variation in BAT-LB in MC1R$^{e/e}$ mice might be associated with impaired vasodilatation. Although MC1R$^{e/e}$ mice have defective MC1R, they have functional MC3/4R- the main components of CMS which is sole responsible for hypothalamic control of brown fat thermogenesis. This is the reason why MC1R$^{e/e}$ mice were still able to produce thermogenic response to β3 drug alike their WT counterparts. This theory however fails to justify the reason behind decreased energy expenditure and increased adiposity observed in MC1R$^{e/e}$ mice. MC1R has also been detected in periaqueductal gray matter as suggested by anatomical studies (Xia et al., 1995) aided by its physiological role in nociception and anti-inflammation

(Caruso et al., 2014) but its direct role in energy homeostasis is not properly explained. The present experiment was able to demonstrate the lack of role of MC1R in brown fat thermogenesis but to describe the reason behind it in molecular level, further researches are required. These MC1R deficiency-related hypometabolism and weight gain could be linked with CMS-independent pathway that modulates energy homeostasis. Indeed, this assumption was further supported by observation when $MC1R^{e/e}$ mice showed similar effect as WT mice during the basal temperature measurement.

Feeding behavior and energy expenditure are two main factors that lead to obesity, a metabolic disorder characterized by increased fat with cardiovascular and hyperglycemic complications. Currently, it affects large population worldwide i.e. 13% of world's adult population (WHO Factsheets, 2014) and is responsible for comorbid conditions like hypertension, type 2 diabetes mellitus, dyslipidemia, other cardiovascular diseases, stroke, gallbladder disorder, gout and osteoarthritis (Khaodhiar et al., 1999). Clinical manifestation observed in obese is due to excessive fat mass accumulation and this abnormal weight gain is centrally controlled by CNS which involves key neuronal system called melanocortin system. Through various neural pathways, melanocortin system impacts diverse kind of physiological processes from pigmentation to energy homeostasis and from steroidogenesis to exocrinal secretion. Most important of all, central melanocortin system role in influencing energy intake, satiety and energy expenditure via centrally expressed MC3/4R and endogenous melanocortin peptides (α-MSH and AgRP) directly relates to body weight regulation (Hill and Faulkner, 2017). Hypothalamic control of brown fat thermogenesis works downstream to CMS leading to the dissipation of stored energy as heat from the body (Cannon and Nedergaard, 2004). Circulating plasma level of anorexigenic α-MSH was lower and that of orexigenic AgRP was higher in obese children highlighting the role of these melanocortin peptides in the regulation of appetite (Vehapoglu et al., 2016). α-MSH is an important endogenous ligand in melanocortin system as its binding to melanocortin receptors show significant functions like pigmentation (MC1R) and appetite suppression (MC3/4R). Conversely, these melanocortin receptors work via

same ligands to show diverse kind of physiological function. Although the data obtained from the current experiment were not sufficient to explain the weight gain in $MC1R^{e/e}$ mice on a genetic basis, one can postulate that the adiposity seen in these mutant mice might be due to presumed alternative feeding behavior accompanied by decreased energy expenditure but irrespective of BAT thermogenesis. In the experiment, the basal temperature was measured on the basis of thermogenic response and it did not show any significant differences between MC1R-deficient and WT mice strengthening the fact that feeding action is causing over-weight. Lack of MC1R in mice might affect hormonal and neuronal pathways leading to the change in feeding behavior (mealing frequency and meal size) causing alteration in body weight. Additionally, prior experiment has shown deficiency in MC1R causes reduction in energy expenditure. Combining the observed evidences, it can be encapsulated that MC1R does not influence BAT thermogenesis and basal temperature but it can affect the appetite-hunger balance causing increase in adipose tissue mass, accompanied by decreased energy expenditure. Apart from MC1R function in skin/ hair pigmentation, pain response modification and anti-inflammatory reaction, more research can establish MC1R as a novel target for treating obesity as scientific work have demonstrated its deficiency causes weight gain.

6 CONCLUSION

It is not a recent discovery that profound the importance of melanocortin system in the central control of energy homeostasis. With the help of antagonistically binding ligands and centrally expressed melanocortin receptors, this system is epitomized as overseer of energy balance regulation. Developments in neuroscience and molecular biology had helped scientific community to grab a better understanding in melanocortinergic neuronal and signaling pathways and their direct involvement in hunger, satiety, energy expenditure, glycemic equilibrium and sympathetic rush. On the other hand, recent confirmation of presence of BAT in adult humans (previously thought to be present only in neonates) had intensified scientific work on brown fat and

attempts are being carried out to activate or enlarge this tissue by external means to develop new pharmacotherapy for obesity and related disorders. The common role of melanocortin system and brown fat in energy expenditure and body weight regulation has led researchers to reckon the relationship between these two energy-regulating factors. Not surprisingly, it was found that central melanocortin system regulates energy expenditure via BAT and modulation of either of these can be proved as beneficial for obesity and associated complications. But, the side-effects they can cause by selectively targeting these receptors and ligands are in investigation phase. In such regard, the present experiment has studied if any relationship exists between particular member of melanocortin receptor family (i.e. MC1R) and the main thermogenic organ (BAT) in the induction of non-shivering thermogenesis. Despite our finding showing lack of any association between MC1R and BAT, the role of MC1R in modifying whole body energy expenditure and weight variation as revealed by previous experiment can still prove as effective in conducting further works to study MC1R and its potential to unearth new attractive target for anti-obesity medication.

7 ACKNOWLEDGEMENTS

First and foremost, I would like to express my genuine gratitude to my supervisor Eriika Savontaus for the persistent support of my master's thesis and for her time, motivation, patience, enthusiasm and boundless knowledge. Her guidance was proved so important in every step of the research and writing of my thesis report. I could not have completed my thesis without her support. I would also like to thank Jin-Lei Wang, Cristina Contreras Jiménez and Wei Zhang for giving me permission to use and modify figures from their scientific literature.

I would like to thank Satu Mäkelä who introduced me about the lab settings and the study design, staffs in the central animal laboratory and the whole farmis colleagues. My heartfelt thanks also go to Markku Koulu, Sanna Soini and Eeva Rainio for their continuous academic support during the master's programme. I would like to show my gratitude to my master's classmates for the journey of short but memorable two years.

Last but not the least, I would like to thank my parents, Dirgha Bahadur Khadka/ Mina Khadka and my sister Roshani Khadka for being with me every time I would ask for any kind of help and teaching me the value of life.

8 ABBREVIATIONS LIST

AGRP – Agouti-Related Protein

ARC – Arcuate nucleus

β3AR – Beta-3 Adrenergic Receptor

BAT – Brown Adipose Tissue

CIT – Cold-Induced Thermogenesis

CMS – Central Melanocortin System

CNS – Central Nervous System

DIT – Diet-Induced Thermogenesis

EE – Energy Expenditure

iBAT – Interscapular BAT

IRT – Infrared Thermography

MC1R – Melanocortin 1 Receptor

POMC – Proopiomelanocortin

UCP1 – Uncoupling Protein 1

WAT – White Adipose Tissue

9 REFERENCES

Albarado, D.C., McClaine, J., Stephens, J.M., Mynatt, R.L., Ye, J., Bannon, A.W., Richards, W.G. and Butler, A.A., 2004. Impaired coordination of nutrient intake and substrate oxidation in melanocortin-4 receptor knockout mice. *Endocrinology*, *145*(1), pp.243-252.

Ang, Q.Y., Goh, H.J., Cao, Y., Li, Y., Chan, S.P., Swain, J.L., Henry, C.J. and Leow, M.K.S., 2017. A new method of infrared thermography for quantification of brown adipose tissue activation in healthy adults (TACTICAL): a randomized trial. *The Journal of Physiological Sciences*, *67*(3), pp.395-406.

Argyropoulos, G. and Harper, M.E., 2002. Invited review: uncoupling proteins and thermoregulation. *Journal of Applied Physiology*, *92*(5), pp.2187-2198

Attia, N., Tamborlane, W.V., Heptulla, R., Maggs, D., Grozman, A., Sherwin, R.S. and Caprio, S., 1998. The metabolic syndrome and insulin-like growth factor I regulation in adolescent obesity. *The Journal of Clinical Endocrinology & Metabolism*, *83*(5), pp.1467-1471.

Bal, N.C., Maurya, S.K., Sopariwala, D.H., Sahoo, S.K., Gupta, S.C., Shaikh, S.A., Pant, M., Rowland, L.A., Bombardier, E., Goonasekera, S.A. and Tupling, A.R., 2012. Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nature medicine*, *18*(10), pp.1575-1579.

Bamshad, M., Song, C.K. and Bartness, T.J., 1999. CNS origins of the sympathetic nervous system outflow to brown adipose tissue. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *276*(6), pp.R1569-R1578.

Bartness, T.J., Vaughan, C.H. and Song, C.K., 2010. Sympathetic and sensory innervation of brown adipose tissue. *International journal of obesity (2005)*, *34*(0 1), p.S36.

Boston, B.A., Blaydon, K.M., Varnerin, J. and Cone, R.D., 1997. Independent and additive effects of central POMC and leptin pathways on murine obesity. *Science*, *278*(5343), pp.1641-1644

Brito, M.N., Brito, N.A., Baro, D.J., Song, C.K. and Bartness, T.J., 2007. Differential activation of the sympathetic innervation of adipose tissues by melanocortin receptor stimulation. *Endocrinology*, *148*(11), pp.5339-5347.

Butler, A.A. and Cone, R.D., 2002. The melanocortin receptors: lessons from knockout models. *Neuropeptides*, *36*(2), pp.77-84.

Butler, A.A., Marks, D.L., Fan, W., Kuhn, C.M., Bartolome, M. and Cone, R.D., 2001. Melanocortin-4 receptor is required for acute homeostatic responses to increased dietary fat. *Nature neuroscience*, *4*(6), p.605.

Callahan, H.S., Cummings, D.E., Pepe, M.S., Breen, P.A., Matthys, C.C. and Weigle, D.S., 2004. Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load

but does not predict intermeal interval in humans. *The Journal of Clinical Endocrinology & Metabolism*, *89*(3), pp.1319-1324.

Caruso, V., Lagerström, M.C., Olszewski, P.K., Fredriksson, R. and Schiöth, H.B., 2014. Synaptic changes induced by melanocortin signalling. *Nature reviews. Neuroscience*, *15*(2), p.98.

Cannon, B. and Nedergaard, J.A.N., 2004. Brown adipose tissue: function and physiological significance. *Physiological reviews*, *84*(1), pp.277-359

Cao, L., Choi, E.Y., Liu, X., Martin, A., Wang, C., Xu, X. and During, M.J., 2011. White to brown fat phenotypic switch induced by genetic and environmental activation of a hypothalamic-adipocyte axis. *Cell metabolism*, *14*(3), pp.324-338.

Cao, W.H., Fan, W. and Morrison, S.F., 2004. Medullary pathways mediating specific sympathetic responses to activation of dorsomedial hypothalamus. *Neuroscience*, *126*(1), pp.229-240.

Carter, E.A., Bonab, A.A., Paul, K., Yerxa, J., Tompkins, R.G. and Fischman, A.J., 2011. Association of heat production with 18F-FDG accumulation in murine brown adipose tissue after stress. *Journal of Nuclear Medicine*, *52*(10), pp.1616-1620.

Challis, B.G., Coll, A.P., Yeo, G.S.H., Pinnock, S.B., Dickson, S.L., Thresher, R.R., Dixon, J., Zahn, D., Rochford, J.J., White, A. and Oliver, R.L., 2004. Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY3-36. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(13), pp.4695-4700.

Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Xiao-Ming, G., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L. and Metzger, J.M., 2000. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nature genetics*, *26*(1), p.97.

Chen, P., Li, C., Haskell-Luevano, C., Cone, R.D. and Smith, M.S., 1999. Altered expression of agouti-related protein and its colocalization with neuropeptide Y in the arcuate nucleus of the hypothalamus during lactation. *Endocrinology*, *140*(6), pp.2645-2650.

Chen, W., Kelly, M.A., Opitz-Araya, X., Thomas, R.E., Low, M.J. and Cone, R.D., 1997. Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell*, *91*(6), pp.789-798.

Chen, Y., Cypess, A.M., Sass, C.A., Brownell, A.L., Jokivarsi, K.T., Kahn, C.R. and Kwong, K.K., 2012. Anatomical and functional assessment of brown adipose tissue by magnetic resonance imaging. *Obesity*, *20*(7), pp.1519-1526.

Christoffolete, M.A., Linardi, C.C., de Jesus, L., Ebina, K.N., Carvalho, S.D., Ribeiro, M.O., Rabelo, R., Curcio, C., Martins, L., Kimura, E.T. and Bianco, A.C., 2004. Mice with targeted disruption of the Dio2 gene have cold-induced overexpression of the uncoupling protein 1 gene but fail to increase brown adipose tissue lipogenesis and adaptive thermogenesis. *Diabetes*, *53*(3), pp.577-584.Cinti, S., 2007. The adipose organ. In *Adipose tissue and adipokines in health and disease* (pp. 3-19). Humana Press.

Cinti, S., 2009. Transdifferentiation properties of adipocytes in the adipose organ. *American Journal of Physiology-Endocrinology and Metabolism*, *297*(5), pp.E977-E986.

Cone, R.D., Lu, D., Koppula, S., Vage, D.I., Klungland, H., Boston, B., Chen, W., Orth, D.N., Pouton, C. and Kesterson, R.A., 1996. The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. *Recent progress in hormone research*, *51*, pp.287-317.

Contreras, C., Gonzalez, F., Fernø, J., Diéguez, C., Rahmouni, K., Nogueiras, R. and López, M., 2015. The brain and brown fat. *Annals of medicine*, *47*(2), pp.150-168.

Cornish, J., Callon, K.E., Mountjoy, K.G., Bava, U., Lin, J.M., Myers, D.E., Naot, D. and Reid, I.R., 2003. α-Melanocyte-stimulating hormone is a novel regulator of bone. *American Journal of Physiology-Endocrinology and Metabolism*, *284*(6), pp.E1181-E1190.

Cousin, B., Cinti, S., Morroni, M., Raimbault, S., Ricquier, D., Penicaud, L. and Casteilla, L., 1992. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *Journal of cell science*, *103*(4), pp.931-942.

.Cowley, M.A., Smart, J.L., Rubinstein, M. and Cerdán, M.G., 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature*, *411*(6836), p.480.

Cowley, M.A., Smith, R.G., Diano, S., Tschöp, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L. and Garcia-Segura, L.M., 2003. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron*, *37*(4), pp.649-661

Crane, J.D., Mottillo, E.P., Farncombe, T.H., Morrison, K.M. and Steinberg, G.R., 2014. A standardized infrared imaging technique that specifically detects UCP1-mediated thermogenesis in vivo. *Molecular metabolism*, *3*(4), pp.490-494.

Cruz, J.I., Loste, J.M. and Burzaco, O.H., 1998. Observations on the use of medetomidine/ketamine and its reversal with atipamezole for chemical restraint in the mouse. *Laboratory animals*, *32*(1), pp.18-22.

Cunningham, J.J., 1982. Body composition and resting metabolic rate: the myth of feminine metabolism. *The American journal of clinical nutrition*, *36*(4), pp.721-726.

Cypess, A.M., Haft, C.R., Laughlin, M.R. and Hu, H.H., 2014. Brown fat in humans: consensus points and experimental guidelines. *Cell metabolism*, *20*(3), pp.408-415.

Cypess, A.M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A.B., Kuo, F.C., Palmer, E.L., Tseng, Y.H., Doria, A. and Kolodny, G.M., 2009. Identification and importance of brown adipose tissue in adult humans. *New England Journal of Medicine*, *360*(15), pp.1509-1517.

Cypess, A.M., White, A.P., Vernochet, C., Schulz, T.J., Xue, R., Sass, C.A., Huang, T.L., Roberts-Toler, C., Weiner, L.S., Sze, C. and Chacko, A.T., 2013. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nature medicine*, *19*(5), pp.635-639.

David J., Knowles S., Taschereau R., Chatziioannou A. and Stout D., 2013. Murine brown fat metabolism measured by infrared thermography correlates with PET FDG uptake. *J Nucl Med*, 54(2), pp 1120.

Eikelis, N. and Esler, M., 2005. The neurobiology of human obesity. *Experimental physiology*, *90*(5), pp.673-682.

El Hadi, H., Frascati, A., Granzotto, M., Silvestrin, V., Ferlini, E., Vettor, R. and Rossato, M., 2016. Infrared thermography for indirect assessment of activation of brown adipose tissue in lean and obese male subjects. *Physiological measurement*, *37*(12), p.N118.

Elefteriou, F., Ahn, J.D., Takeda, S. and Starbuck, M., 2005. Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature*, *434*(7032), p.514.

Ellacott, K.L. and Cone, R.D., 2006. The role of the central melanocortin system in the regulation of food intake and energy homeostasis: lessons from mouse models. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *361*(1471), pp.1265-1274.

Esparza, J., Fox, C., Harper, I.T., Bennett, P.H., Schulz, L.O., Valencia, M.E. and Ravussin, E., 2000. Daily energy expenditure in Mexican and USA Pima Indians: low physical activity as a possible cause of obesity. *International journal of obesity*, *24*(1), p.55.

Evans, J.F., Niu, Q.T., Canas, J.A., Shen, C.L., Aloia, J.F. and Yeh, J.K., 2004. ACTH enhances chondrogenesis in multipotential progenitor cells and matrix production in chondrocytes. *Bone*, *35*(1), pp.96-107.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J. and Cone, R.D., 1997. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*, *385*(6612), p.165.

Fan, W., Dinulescu, D.M., Butler, A.A., Zhou, J., Marks, D.L. and Cone, R.D., 2000. The central melanocortin system can directly regulate serum insulin levels. *Endocrinology*, *141*(9), pp.3072-3079.

Fan, W., Ellacott, K.L., Halatchev, I.G., Takahashi, K., Yu, P. and Cone, R.D., 2004. Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nature neuroscience*, *7*(4), p.335.

Farooqi, I.S., Keogh, J.M., Yeo, G.S., Lank, E.J., Cheetham, T. and O'rahilly, S., 2003. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *New England Journal of Medicine*, *348*(12), pp.1085-1095.

Fedorenko, A., Lishko, P.V. and Kirichok, Y., 2012. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell*, *151*(2), pp.400-413.

Feldmann, H.M., Golozoubova, V., Cannon, B. and Nedergaard, J., 2009. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell metabolism*, *9*(2), pp.203-209.

Figure 1: Park et al., 2014. Locations of adipose tissue depots in a mouse (A) and an adult human (B). Available at: https://www.wjgnet.com/1948-0210/full/v6/i1/WJSC-6-33-g001.htm [accessed 28.08.2017]

Figure 2: Contreras et al., 2015. Brown adipose tissue uncoupling and thermogenesis. From: Contreras, C., Gonzalez, F., Fernø, J., Diéguez, C., Rahmouni, K., Nogueiras, R. and López, M., 2015. The brain and brown fat. *Annals of medicine*, *47*(2), pp.150-168.

Figure 3: Park et al., 2014. Differentiation into white, beige/brite or brown adipocytes. Available at: https://www.wjgnet.com/1948-0210/full/v6/i1/WJSC-6-33-g002.htm [accessed 28.08.2017]

Figure 4: Park et al., 2014. Key regulators of the browning process and their action mechanisms. Available at: https://www.wjgnet.com/1948-0210/full/v6/i1/WJSC-6-33-g003.htm [accessed 28.08.2017]

Figure 5: Zhang and Bi, 2015. Model of hypothalamic neural regulation of BAT thermogenesis. Available at: http://www.frontiersin.org/files/Articles/154504/fendo-06-00136-HTML/image_m/fendo-06-00136-g001.jpg [accessed 28.08.2017]

Fodor, M., Csaba, Z., Epelbaum, J., Vaudry, H. and Jegou, S., 1998. Interrelations between hypothalamic somatostatin and proopiomelanocortin neurons. *Journal of neuroendocrinology*, *10*(2), pp.75-78.

Foster, D.O. and Frydman, M.L., 1979. Tissue distribution of cold-induced thermogenesis in conscious warm-or cold-acclimated rats reevaluated from changes in tissue blood flow: the dominant role of brown adipose tissue in the replacement of shivering by nonshivering thermogenesis. *Canadian journal of physiology and pharmacology*, *57*(3), pp.257-270.

Gantz, I. and Fong, T.M., 2003. The melanocortin system. *American Journal of Physiology-Endocrinology And Metabolism*, *284*(3), pp.E468-E474.

Gantz, I., Konda, Y.T.T.S.Y.M.H.M.G.W.S.J.D.J.T., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S.J., DelValle, J. and Yamada, T., 1993. Molecular cloning of a novel melanocortin receptor. *Journal of Biological Chemistry*, *268*(11), pp.8246-8250.

Ghorbani, M., Claus, T.H. and Himms-Hagen, J., 1997. Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of diet-induced obesity in rats treated with a β3-adrenoceptor agonist. *Biochemical pharmacology*, *54*(1), pp.121-131.

Girardet, C. and Butler, A.A., 2014. Neural melanocortin receptors in obesity and related metabolic disorders. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1842*(3), pp.482-494.

Glick, Z., Wickler, S.J., Stern, J.S. and Horwitz, B.A.. 1984. Blood flow into brown fat of rats is greater after a high carbohydrate than after a high fat test meal. *The Journal of nutrition*, *114*(10), pp.1934-1939.

Golozoubova, V., Cannon, B. and Nedergaard, J., 2006. UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *American Journal of Physiology-Endocrinology And Metabolism*, *291*(2), pp.E350-E357.

Golozoubova, V., Hohtola, E.S.A., Matthias, A., Jacobsson, A., Cannon, B. and Nedergaard, J.A.N., 2001. Only UCP1 can mediate adaptive nonshivering thermogenesis in the cold. *The FASEB Journal*, *15*(11), pp.2048-2050.

Hamann, A.N.D.R.E.A.S., Flier, J.S. and Lowell, B.B., 1996. Decreased brown fat markedly enhances susceptibility to diet-induced obesity, diabetes, and hyperlipidemia. *Endocrinology*, *137*(1), pp.21-29.

Hill, J.W. and Faulkner, L.D., 2017. The role of the melanocortin system in metabolic disease: new developments and advances. *Neuroendocrinology*, *104*(4), pp.330-346.

Himms-Hagen, J.E.A.N., 1990. Brown adipose tissue thermogenesis: interdisciplinary studies. *The FASEB Journal*, *4*(11), pp.2890-2898.

Himms-Hagen, J., Cui, J.I.N.G.Y.I.N.G., Danforth, E., Taatjes, D.J., Lang, S.S., Waters, B.L. and Claus, T.H., 1994. Effect of CL-316,243, a thermogenic beta 3-agonist, on energy balance and brown and white adipose tissues in rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *266*(4), pp.R1371-R1382.

Hu, H.H., Tovar, J.P., Pavlova, Z., Smith, M.L. and Gilsanz, V., 2012. Unequivocal identification of brown adipose tissue in a human infant. *Journal of Magnetic Resonance Imaging*, *35*(4), pp.938-942.

Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D. and Smith, F.J., 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell*, *88*(1), pp.131-141.

Jackson, D.M., Hambly, C., Trayhurn, P. and Speakman, J.R., 2001. Can non-shivering thermogenesis in brown adipose tissue following NA injection be quantified by changes in overlying surface temperatures using infrared thermography?. *Journal of thermal biology*, *26*(2), pp.85-93.

Jang, C., Jalapu, S., Thuzar, M., Law, P.W., Jeavons, S., Barclay, J.L. and Ho, K.K., 2014. Infrared thermography in the detection of brown adipose tissue in humans. *Physiological reports*, *2*(11), p.e12167.

Jennissen, K., Siegel, F., Liebig-Gonglach, M., Hermann, M.R., Kipschull, S., van Dooren, S., Kunz, W.S., Fassler, R. and Pfeifer, A., 2012. A VASP-Rac-soluble guanylyl cyclase pathway controls cGMP production in adipocytes. *Sci Signal*, *5*, p.ra62.

Jo, Y.H. and Buettner, C., 2014. Why leptin keeps you warm. *Molecular metabolism*, *3*(8), p.779.

Jonsson, A.L., Granqvist, A., Elvin, J., Johansson, M.E., Haraldsson, B. and Nyström, J., 2014. Effects of melanocortin 1 receptor agonists in experimental nephropathies. *PloS one*, *9*(1), p.e87816.

Johnson, F., Mavrogianni, A., Ucci, M., Vidal-Puig, A. and Wardle, J., 2011. Could increased time spent in a thermal comfort zone contribute to population increases in obesity?. *Obesity Reviews*, *12*(7), pp.543-551.

Jonee, L. and Bray, G.A., 1997. The thermic effect of food and obesity: a critical review. *Obesity*, *5*(6), pp.622-631.

Joseph, S.A., Pilcher, W.H. and Bennett-Clarke, C., 1983. Immunocytochemical localization of ACTH perikarya in nucleus tractus solitarius: evidence for a second opiocortin neuronal system. *Neuroscience letters*, *38*(3), pp.221-225.

Kamegai, J., Tamura, H., Shimizu, T., Ishii, S., Sugihara, H. and Wakabayashi, I., 2001. Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. *Diabetes*, *50*(11), pp.2438-2443.

Keipert, S., Kutschke, M., Lamp, D., Brachthäuser, L., Neff, F., Meyer, C.W., Oelkrug, R., Kharitonenkov, A. and Jastroch, M., 2015. Genetic disruption of uncoupling protein 1 in mice renders brown adipose tissue a significant source of FGF21 secretion. *Molecular metabolism*, *4*(7), pp.537-542.

Keipert, S., M. Kutschke, M. Ost, T. Schwarzmayr, E. M. van Schothorst, D. Lamp, L. Brachthauser, I. Hamp, S. E. Mazibuko, S. Hartwig, S. Lehr, E. Graf, O. Plettenburg, F. Neff, M. H. Tschop, and M. Jastroch. 2017. Long-Term Cold Adaptation Does Not Require FGF21 or UCP1. Cell Metab 26: 437-446 e435.

Khanna, A. and Branca, R.T., 2012. Detecting brown adipose tissue activity with BOLD MRI in mice. *Magnetic resonance in medicine*, *68*(4), pp.1285-1290.

Khaodhiar, L., McCowen, K.C. and Blackburn, G.L., 1999. Obesity and its comorbid conditions. *Clinical cornerstone*, *2*(3), pp.17-31.

Kim, S.H. and Plutzky, J., 2016. Brown fat and browning for the treatment of obesity and related metabolic disorders. *Diabetes & metabolism journal*, *40*(1), pp.12-21

Kishi, T., Aschkenasi, C.J., Lee, C.E., Mountjoy, K.G., Saper, C.B. and Elmquist, J.K., 2003. Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *Journal of Comparative Neurology*, *457*(3), pp.213-235.

Knehans, A.W. and Romsos, D.R., 1982. Reduced norepinephrine turnover in brown adipose tissue of ob/ob mice. *American Journal of Physiology-Endocrinology and Metabolism*, *242*(4), pp.E253-E261.

Kooijman, S., Boon, M.R., Parlevliet, E.T., Geerling, J.J., van de Pol, V., Romijn, J.A., Havekes, L.M., Meurs, I. and Rensen, P.C., 2014. Inhibition of the central melanocortin system decreases brown adipose tissue activity. *Journal of lipid research*, *55*(10), pp.2022-2032.

Kuo, J.J., Silva, A.A. and Hall, J.E., 2003. Hypothalamic melanocortin receptors and chronic regulation of arterial pressure and renal function. *Hypertension*, *41*(3), pp.768-774.

Labbé, S.M., Caron, A., Lanfray, D., Monge-Rofarello, B., Bartness, T.J. and Richard, D., 2015. Hypothalamic control of brown adipose tissue thermogenesis. *Frontiers in systems neuroscience*, *9*.

Lafontan, M. and Berlan, M., 1993. Fat cell adrenergic receptors and the control of white and brown fat cell function. *Journal of lipid research*, *34*(7), pp.1057-1091.

Lai, L., Leone, T.C., Zechner, C., Schaeffer, P.J., Kelly, S.M., Flanagan, D.P., Medeiros, D.M., Kovacs, A. and Kelly, D.P., 2008. Transcriptional coactivators PGC-1α and PGC-lβ control overlapping programs required for perinatal maturation of the heart. *Genes & development*, *22*(14), pp.1948-1961.

Lee, P., Greenfield, J.R., Ho, K.K. and Fulham, M.J., 2010. A critical appraisal of prevalence and metabolic significance of brown adipose tissue in adult humans. *American Journal of Physiology-Endocrinology and Metabolism*.

Lee, P., Ho, K.K.Y. and Greenfield, J.R., 2011. Hot fat in a cool man: infrared thermography and brown adipose tissue. *Diabetes, Obesity and Metabolism*, *13*(1), pp.92-93.

Lee, P., Swarbrick, M.M. and Ho, K.K., 2013. Brown adipose tissue in adult humans: a metabolic renaissance. *Endocrine reviews*, *34*(3), pp.413-438.

Lee, P., Zhao, J.T., Swarbrick, M.M., Gracie, G., Bova, R., Greenfield, J.R., Freund, J. and Ho, K.K., 2011. High prevalence of brown adipose tissue in adult humans. *The Journal of Clinical Endocrinology & Metabolism*, *96*(8), pp.2450-2455.

Lelliott, C.J., Medina-Gomez, G., Petrovic, N., Kis, A., Feldmann, H.M., Bjursell, M., Parker, N., Curtis, K., Campbell, M., Hu, P. and Zhang, D., 2006. Ablation of PGC-1β results in defective mitochondrial activity, thermogenesis, hepatic function, and cardiac performance. *PLoS biology*, *4*(11), p.e369.

Li, D. and Taylor, A.W., 2008. Diminishment of α-MSH anti-inflammatory activity in MC1r siRNA-transfected RAW264. 7 macrophages. *Journal of leukocyte biology*, *84*(1), p.191.

Liu, X., Wang, S., You, Y., Meng, M., Zheng, Z., Dong, M., Lin, J., Zhao, Q., Zhang, C., Yuan, X. and Hu, T., 2015. Brown adipose tissue transplantation reverses obesity in Ob/Ob mice. *Endocrinology*, *156*(7), pp.2461-2469.

Lomax, P., Malveaux, E. and Smith, R.E., 1964. Brain temperatures in the rat during exposure to low environmental temperatures. *American Journal of Physiology--Legacy Content*, *207*(3), pp.736-739.

Lowell, B.B., Vedrana, S., Hamann, A., Charles, A., Lawitts, J.A., Himms-Hagen, J., Boyer, B.B., Kozak, L.P. and Flier, J.S., 1993. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature*, *366*(6457), pp.740-742.

Lu, D., Willard, D., Patel, I.R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R.P., Wilkison, W.O. and Cone, R.D., 1994. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature*, *371*(6500), pp.799-802.

Ma, S.W. and Foster, D.O., 1986. Uptake of glucose and release of fatty acids and glycerol by rat brown adipose tissue in vivo. *Canadian journal of physiology and pharmacology*, *64*(5), pp.609-614.

Madsen, L., Pedersen, L.M., Lillefosse, H.H., Fjære, E., Bronstad, I., Hao, Q., Petersen, R.K., Hallenborg, P., Ma, T., De Matteis, R. and Araujo, P., 2010. UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PloS one*, *5*(6), p.e11391.

Marette, A.N.D.R.E., Geloen, A.L.A.I.N., Collet, A.N.D.R.E. and Bukowiecki, L.J., 1990. Defective metabolic effects of norepinephrine and insulin in obese Zucker rat brown adipose tissue. *American Journal of Physiology-Endocrinology and Metabolism*, *258*(2), pp.E320-E328.

Marie, L.S., Miura, G.I., Marsh, D.J., Yagaloff, K. and Palmiter, R.D., 2000. A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors. *Proceedings of the National Academy of Sciences*, *97*(22), pp.12339-12344.

Marks, D.L., Butler, A.A., Turner, R., Brookhart, G. and Cone, R.D., 2003. Differential role of melanocortin receptor subtypes in cachexia. *Endocrinology*, *144*(4), pp.1513-1523.

Marks, D.L., Hruby, V., Brookhart, G. and Cone, R.D., 2006. The regulation of food intake by selective stimulation of the type 3 melanocortin receptor (MC3R). *Peptides*, *27*(2), pp.259-264.

Marsh, D.J., Hollopeter, G., Huszar, D., Laufer, R., Yagaloff, K.A., Fisher, S.L., Burn, P. and Palmiter, R.D., 1999. Response of melanocortin–4 receptor–deficient mice to anorectic and orexigenic peptides. *Nature genetics*, *21*(1), pp.119-122.

Maurer, S.F., Fromme, T., Grossman, L.I., Hüttemann, M. and Klingenspor, M., 2015. The brown and brite adipocyte marker Cox7a1 is not required for non-shivering thermogenesis in mice. *Scientific reports*, *5*.

Meyer, C.W., Willershäuser, M., Jastroch, M., Rourke, B.C., Fromme, T., Oelkrug, R., Heldmaier, G. and Klingenspor, M., 2010. Adaptive thermogenesis and thermal conductance in wild-type and UCP1-KO mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *299*(5), pp.R1396-R1406.

Minor, R.K., Chang, J.W. and De Cabo, R., 2009. Hungry for life: How the arcuate nucleus and neuropeptide Y may play a critical role in mediating the benefits of calorie restriction. *Molecular and cellular endocrinology*, *299*(1), pp.79-88.

Mitchell, J.R., Jacobsson, A.N.D.E.R.S., Kirchgessner, T.G., Schotz, M.C., Cannon, B.A.R.B.A.R.A. and Nedergaard, J., 1992. Regulation of expression of the lipoprotein lipase gene in brown adipose tissue. *American Journal of Physiology-Endocrinology And Metabolism*, *263*(3), pp.E500-E506.

Mizuno, T.M., Makimura, H., Silverstein, J., Roberts, J.L., Lopingco, T. and Mobbs, C.V., 1999. Fasting regulates hypothalamic neuropeptide Y, agouti-related peptide, and proopiomelanocortin in diabetic mice independent of changes in leptin or insulin. *Endocrinology*, *140*(10), pp.4551-4557.

Morgan, C., Thomas, R.E. and Cone, R.D., 2004. Melanocortin-5 receptor deficiency promotes defensive behavior in male mice. *Hormones and behavior*, *45*(1), pp.58-63.

Morrison, S.D., 1981. Cold-specific feeding response of rats to cold exposure and energy density of body weight change. *Journal of Applied Physiology*, *51*(2), pp.327-334.

Morrison, S.F. and Madden, C.J., 2014. Central nervous system regulation of brown adipose tissue. *Comprehensive Physiology*.

Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B. and Cone, R.D., 1994. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Molecular endocrinology*, *8*(10), pp.1298-1308.

Mund, R.A. and Frishman, W.H., 2013. Brown adipose tissue thermogenesis: β3-adrenoreceptors as a potential target for the treatment of obesity in humans. *Cardiology in review*, *21*(6), pp.265-269.

Münzberg, H., Huo, L., Nillni, E.A., Hollenberg, A.N. and Bjørbæk, C., 2003. Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin. *Endocrinology*, *144*(5), pp.2121-2131.

Nedergaard, J., Golozoubova, V., Matthias, A., Asadi, A., Jacobsson, A. and Cannon, B., 2001. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1504*(1), pp.82-106.

Nedergaard, J. and Cannon, B., 2013. UCP1 mRNA does not produce heat. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1831*(5), pp.943-949.

Ni, X.P., Pearce, D., Butler, A.A., Cone, R.D. and Humphreys, M.H., 2003. Genetic disruption of γ-melanocyte–stimulating hormone signaling leads to salt-sensitive hypertension in the mouse. *Journal of Clinical Investigation*, *111*(8), p.1251.

N.J. Rothwell, M.J. Stock. 1979. A role for brown adipose tissue in diet-induced thermogenesis. *Nature*. 281, pp. 31-35.

Ohno, H., Shinoda, K., Spiegelman, B.M. and Kajimura, S., 2012. PPARγ agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell metabolism*, *15*(3), pp.395-404.

Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I. and Barsh, G.S., 1997. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science*, *278*(5335), pp.135-138.

Olsen, J.M., Sato, M., Dallner, O.S., Sandström, A.L., Pisani, D.F., Chambard, J.C., Amri, E.Z., Hutchinson, D.S. and Bengtsson, T., 2014. Glucose uptake in brown fat cells is dependent on mTOR complex 2–promoted GLUT1 translocation. *J Cell Biol*, *207*(3), pp.365-374.

Pandit, R., Beerens, S. and Adan, R.A., 2017. Role of leptin in energy expenditure: the hypothalamic perspective. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *312*(6), pp.R938-R947.

Park, A., Kim, W.K. and Bae, K.H., 2014. Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. *World journal of stem cells*, *6*(1), p.33.

Park, M.J., Kim, H.S., Kang, J.H., Kim, D.H. and Chung, C.Y., 1999. Serum levels of insulin-like growth factor (IGF)-I, free IGF-I, IGF binding protein (IGFBP)-I, IGFBP-3 and insulin in obese children. *Journal of Pediatric Endocrinology and Metabolism*, *12*(2), pp.139-144.

Perry, W.L., Copeland, N.G. and Jenkins, N.A., 1994. The molecular basis for dominant yellow agouti coat color mutations. *Bioessays*, *16*(10), pp.705-707.

Qian, S.W., Tang, Y., Li, X., Liu, Y., Zhang, Y.Y., Huang, H.Y., Xue, R.D., Yu, H.Y., Guo, L., Gao, H.D. and Liu, Y., 2013. BMP4-mediated brown fat-like changes in white adipose tissue alter glucose and energy homeostasis. *Proceedings of the National Academy of Sciences*, *110*(9), pp.E798-E807.

Raposinho, Paula D., Einar Castillo, Violaine D'alleves, Pierre Broqua, François P. Pralong, and Michel L. Aubert. "Chronic Blockade of the Melanocortin 4 Receptor Subtype Leads to Obesity Independently of Neuropeptide Y Action, with No Adverse Effects on the Gonadotropic and Somatotropic Axes." *Endocrinology* 141, no. 12 (2000): 4419-4427.

Razzoli, M., Frontini, A., Gurney, A., Mondini, E., Cubuk, C., Katz, L.S., Cero, C., Bolan, P.J., Dopazo, J., Vidal-Puig, A. and Cinti, S., 2016. Stress-induced activation of brown adipose tissue prevents obesity in conditions of low adaptive thermogenesis. *Molecular metabolism*, *5*(1), pp.19-33.

Rees, J.L., 2003. Genetics of hair and skin color. *Annual review of genetics*, *37*(1), pp.67-90.

Ricquier, D., 2011. Uncoupling protein 1 of brown adipocytes, the only uncoupler: a historical perspective. *Frontiers in endocrinology*, *2*.

Rinne, P., Ahola-Olli, A., Nuutinen, S., Koskinen, E., Kaipio, K., Eerola, K., Juonala, M., Kähönen, M., Lehtimäki, T., Raitakari, O.T. and Savontaus, E., 2015. Deficiency in melanocortin 1 receptor signaling predisposes to vascular endothelial dysfunction and increased arterial stiffness in mice and humans. *Arteriosclerosis, thrombosis, and vascular biology*, pp.ATVBAHA-114.

Robbins, L.S., Nadeau, J.H., Johnson, K.R., Kelly, M.A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K.G. and Cone, R.D., 1993. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell*, *72*(6), pp.827-834.

Robinson, L.J., Law, J.M., Symonds, M.E. and Budge, H., 2016. Brown adipose tissue activation as measured by infrared thermography by mild anticipatory psychological stress in lean healthy females. *Experimental physiology*, *101*(4), pp.549-557.

Roselli-Rehfuss, L., Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Low, M.J., Tatro, J.B., Entwistle, M.L., Simerly, R.B. and Cone, R.D., 1993. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proceedings of the National Academy of Sciences*, *90*(19), pp.8856-8860.

Rosen, E.D. and MacDougald, O.A., 2006. Adipocyte differentiation from the inside out. *Nature reviews. Molecular cell biology*, *7*(12), p.885.

Rosenwald, M., Perdikari, A., Rülicke, T. and Wolfrum, C., 2013. Bi-directional interconversion of brite and white adipocytes. *Nature cell biology*, *15*(6), p.659.

Salem, V., Izzi-Engbeaya, C., Coello, C., Thomas, D.B., Chambers, E.S., Comninos, A.N., Buckley, A., Win, Z., Al-Nahhas, A., Rabiner, E.A. and Gunn, R.N., 2016. Glucagon increases energy expenditure independently of brown adipose tissue activation in humans. *Diabetes, Obesity and Metabolism*, *18*(1), pp.72-81.

Scotney H., Symonds M.E., Law J., Budge H., Sharkey D., Manolopoulos K.N.. 2017. Glucocorticoids modulate human brown adipose tissue thermogenesis in vivo. *Metabolism*. 70. pp 125-132.

Seeley, R.J., Yagaloff, K.A., Fisher, S.L., Burn, P., Thiele, T.E., van Dijk, G., Baskin, D.G. and Schwartz, M.W., 1997. Melanocortin receptors in leptin effects. *Nature*, *390*(6658), p.349.

Sharp, L.Z., Shinoda, K., Ohno, H., Scheel, D.W., Tomoda, E., Ruiz, L., Hu, H., Wang, L., Pavlova, Z., Gilsanz, V. and Kajimura, S., 2012. Human BAT possesses molecular signatures that resemble beige/brite cells. *PloS one*, *7*(11), p.e49452.

Sinclair, M.D., 2003. A review of the physiological effects of α2-agonists related to the clinical use of medetomidine in small animal practice. *The Canadian veterinary journal*, *44*(11), p.885.

Smith, A.I. and Funder, J.W., 1988. Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocrine reviews*, *9*(1), pp.159-179.

Song, C.K., Vaughan, C.H., Keen-Rhinehart, E., Harris, R.B., Richard, D. and Bartness, T.J., 2008. Melanocortin-4 receptor mRNA expressed in sympathetic outflow neurons to brown adipose tissue: neuroanatomical and functional evidence. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *295*(2), pp.R417-R428.

Sutton, G.M., Duos, B., Patterson, L.M. and Berthoud, H.R., 2005. Melanocortinergic modulation of cholecystokinin-induced suppression of feeding through extracellular signal-regulated kinase signaling in rat solitary nucleus. *Endocrinology*, *146*(9), pp.3739-3747.

Symonds, M.E., Henderson, K., Elvidge, L., Bosman, C., Sharkey, D., Perkins, A.C. and Budge, H., 2012. Thermal imaging to assess age-related changes of skin temperature within the supraclavicular region co-locating with brown adipose tissue in healthy children. *The Journal of pediatrics*, *161*(5), pp.892-898.

Tang, Q.Q. and Lane, M.D., 2012. Adipogenesis: from stem cell to adipocyte. *Annual review of biochemistry*, *81*, pp.715-736.

Takahashi, K.A. and Cone, R.D., 2005. Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. *Endocrinology*, *146*(3), pp.1043-1047.

Tallam, L.S., Stec, D.E., Willis, M.A., da Silva, A.A. and Hall, J.E., 2005. Melanocortin-4 receptor–deficient mice are not hypertensive or salt-sensitive despite obesity, hyperinsulinemia, and hyperleptinemia. *Hypertension*, *46*(2), pp.326-332.

Tao, Y.X., 2010. The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. *Endocrine reviews*, *31*(4), pp.506-543

Tatro, J.B., 1990. Melanotropin receptors in the brain are differentially distributed and recognize both corticotropin and α-melanocyte stimulating hormone. *Brain research*, *536*(1), pp.124-132.

Timmons, J.A., Wennmalm, K., Larsson, O., Walden, T.B., Lassmann, T., Petrovic, N., Hamilton, D.L., Gimeno, R.E., Wahlestedt, C., Baar, K. and Nedergaard, J., 2007. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proceedings of the National Academy of Sciences*, *104*(11), pp.4401-4406.

Trayhurn, P. and Wusteman, M.C., 1990. Lipogenesis in genetically diabetic (db/db) mice: developmental changes in brown adipose tissue, white adipose tissue and the liver. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *1047*(2), pp.168-174

Trevaskis, J.L. and Butler, A.A., 2005. Double leptin and melanocortin-4 receptor gene mutations have an additive effect on fat mass and are associated with reduced effects of leptin on weight loss and food intake. *Endocrinology*, *146*(10), pp.4257-4265.

Tvrdik, P., Asadi, A., Kozak, L.P., Nedergaard, J., Cannon, B. and Jacobsson, A., 1997. Cig30, a mouse member of a novel membrane protein gene family, is involved in the recruitment of brown adipose tissue. *Journal of Biological Chemistry*, *272*(50), pp.31738-31746.

Umekawa, T., Yoshida, T., Sakane, N., Saito, M., Kumamoto, K. and Kondo, M., 1997. Anti-obesity and anti-diabetic effects of CL316, 243, a highly specific β3-adrenoceptor agonist, in Otsuka Long-Evans Tokushima Fatty rats: induction of uncoupling protein and activation of glucose transporter 4 in white fat. *European journal of endocrinology*, *136*(4), pp.429-437.

Valverde, P., Healy, E., Jackson, I., Rees, J.L. and Thody, A.J., 1995. Variants of the melanocyte–stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nature genetics*, *11*(3), pp.328-330.

van Marken Lichtenbelt, W.D., Schrauwen, P., van de Kerckhove, S. and Westerterp-Plantenga, M.S., 2002. Individual variation in body temperature and energy expenditure in response to mild cold. *American Journal of Physiology-Endocrinology and Metabolism*, *282*(5), pp.E1077-E1083.

van Marken Lichtenbelt, W.D., Vanhommerig, J.W., Smulders, N.M., Drossaerts, J.M., Kemerink, G.J., Bouvy, N.D., Schrauwen, P. and Teule, G.J., 2009. Cold-activated brown adipose tissue in healthy men. *New England Journal of Medicine*, *360*(15), pp.1500-1508.

Vehapoğlu, A., Türkmen, S. and Terzioğlu, Ş., 2016. Alpha-melanocyte-stimulating hormone and agouti-related protein: do they play a role in appetite regulation in childhood obesity?. *Journal of clinical research in pediatric endocrinology*, *8*(1), p.40.

Vijgen, G.H.E.J., Bouvy, N.D., Teule, G.J.J., Brans, B., Hoeks, J., Schrauwen, P. and van Marken Lichtenbelt, W.D., 2012. Increase in brown adipose tissue activity after weight loss in morbidly obese subjects. *The Journal of Clinical Endocrinology & Metabolism*, *97*(7), pp.E1229-E1233.

Villarroya, F. and Vidal-Puig, A., 2013. Beyond the sympathetic tone: the new brown fat activators. *Cell metabolism*, *17*(5), pp.638-643.

Virtue, S., Feldmann, H., Christian, M., Tan, C.Y., Masoodi, M., Dale, M., Lelliott, C., Burling, K., Campbell, M., Eguchi, N. and Voshol, P., 2012. A new role for lipocalin prostaglandin d synthase in the regulation of brown adipose tissue substrate utilization. *Diabetes*, *61*(12), pp.3139-3147.

Virtue, S. and Vidal-Puig, A., 2013. Assessment of brown adipose tissue function. *Frontiers in Physiology*, *4*.

Virtanen, K.A., Lidell, M.E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N.J., Enerbäck, S. and Nuutila, P., 2009. Functional brown adipose tissue in healthy adults. *New England Journal of Medicine*, *360*(15), pp.1518-1525.

Waldén, T.B., Hansen, I.R., Timmons, J.A., Cannon, B. and Nedergaard, J., 2012. Recruited vs. nonrecruited molecular signatures of brown,“brite,” and white adipose tissues. *American Journal of Physiology-Endocrinology and Metabolism*, *302*(1), pp.E19-E31.

Wang, Q.A., Tao, C., Gupta, R.K. and Scherer, P.E., 2013. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. *Nature medicine*, *19*(10), pp.1338-1344.

Watanobe, H. and Yoneda, M., 2003. Evaluation of the role of melanocortin 3 and 4 receptors in leptin-stimulated and spontaneous growth hormone secretion in rats. *Neuroendocrinology*, *78*(6), pp.331-338.

Weide, K., Christ, N., Moar, K.M., Arens, J., Hinney, A., Mercer, J.G., Eiden, S. and Schmidt, I., 2003. Hyperphagia, not hypometabolism, causes early onset obesity in melanocortin-4 receptor knockout mice. *Physiological genomics*, *13*(1), pp.47-56.

Westerberg, R., Månsson, J.E., Golozoubova, V., Shabalina, I.G., Backlund, E.C., Tvrdik, P., Retterstøl, K., Capecchi, M.R. and Jacobsson, A., 2006. ELOVL3 is an important component for early onset of lipid recruitment in brown adipose tissue. *Journal of Biological Chemistry*, *281*(8), pp.4958-4968.

Wijers, S.L., Saris, W.H. and van Marken Lichtenbelt, W.D., 2009. Recent advances in adaptive thermogenesis: potential implications for the treatment of obesity. *obesity reviews*, *10*(2), pp.218-226.

Wijers, S.L., Saris, W.H. and Lichtenbelt, W.D., 2010. Cold-induced adaptive thermogenesis in lean and obese. *Obesity*, *18*(6), pp.1092-1099.

Wikberg, J.E., 1999. Melanocortin receptors: perspectives for novel drugs. *European journal of pharmacology*, *375*(1), pp.295-310.

Willesen, M.G., Kristensen, P. and Rømer, J., 1999. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology*, *70*(5), pp.306-316.

Williams, D.L., Bowers, R.R., Bartness, T.J., Kaplan, J.M. and Grill, H.J., 2003. Brainstem melanocortin 3/4 receptor stimulation increases uncoupling protein gene expression in brown fat. *Endocrinology*, *144*(11), pp.4692-4697.

WHO Factsheets. 2014. Obesity and overweight. Available at: http://www.who.int/mediacentre/factsheets/fs311/en/ . Accessed at: 7 October 2017.

Wolff, G.L., Roberts, D.W. and Mountjoy, K.G., 1999. Physiological consequences of ectopic agouti gene expression: the yellow obese mouse syndrome. *Physiological genomics*, *1*(3), pp.151-163.

Wu, J., Boström, P., Sparks, L.M., Ye, L., Choi, J.H., Giang, A.H., Khandekar, M., Virtanen, K.A., Nuutila, P., Schaart, G. and Huang, K., 2012. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell*, *150*(2), pp.366-376.

Xia, Y., Wikberg, J.E. and Chhajlani, V., 1995. Expression of melanocortin 1 receptor in periaqueductal gray matter. *Neuroreport*, *6*(16), pp.2193-2196.

Yang, X., Enerbäck, S. and Smith, U., 2003. Reduced expression of FOXC2 and brown adipogenic genes in human subjects with insulin resistance. *Obesity*, *11*(10), pp.1182-1191.

Yasuda, T., Masaki, T., Kakuma, T. and Yoshimatsu, H., 2004. Hypothalamic melanocortin system regulates sympathetic nerve activity in brown adipose tissue. *Experimental biology and medicine*, *229*(3), pp.235-239.

Yen, T.T., Gill, A.M., Frigeri, L.G., Barsh, G.S. and Wolff, G.L., 1994. Obesity, diabetes, and neoplasia in yellow A (vy)/-mice: ectopic expression of the agouti gene. *The FASEB Journal*, *8*(8), pp.479-488.

Yoneshiro, T., Aita, S., Matsushita, M., Kameya, T., Nakada, K., Kawai, Y. and Saito, M., 2011. Brown adipose tissue, whole-body energy expenditure, and thermogenesis in healthy adult men. *Obesity*, *19*(1), pp.13-16.

Yoneshiro, T., Aita, S., Matsushita, M., Okamatsu-Ogura, Y., Kameya, T., Kawai, Y., Miyagawa, M., Tsujisaki, M. and Saito, M., 2011. Age-related decrease in cold-activated brown adipose tissue and accumulation of body fat in healthy humans. *Obesity*, *19*(9), pp.1755-1760.

Zhang, W. and Bi, S., 2015. Hypothalamic regulation of brown adipose tissue thermogenesis and energy homeostasis. *Frontiers in endocrinology*, *6*

Zhong, Q., Sridhar, S., Ruan, L., Ding, K.H., Xie, D., Insogna, K., Kang, B., Xu, J., Bollag, R.J. and Isales, C.M., 2005. Multiple melanocortin receptors are expressed in bone cells. *Bone*, *36*(5), pp.820-831.

Zingaretti, M.C., Crosta, F., Vitali, A., Guerrieri, M., Frontini, A., Cannon, B., Nedergaard, J. and Cinti, S., 2009. The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. *The FASEB Journal*, *23*(9), pp.3113-3120