REVIEW

Adipose and skeletal muscle thermogenesis: studies from large animals

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Abstract

The balance between energy intake and energy expenditure establishes and preserves a 'set-point' body weight. The latter is comprised of three major components including metabolic rate, physical activity and thermogenesis. Thermogenesis is defined as the cellular dissipation of energy via heat production. This process has been extensively characterised in brown adipose tissue (BAT), wherein uncoupling protein 1 (UCP1) creates a proton leak across the inner mitochondrial membrane, diverting protons away from ATP synthesis and resulting in heat dissipation. In beige adipocytes and skeletal muscle, thermogenesis can occur independent of UCP1. Beige adipocytes have been shown to produce heat via UCP1 as well as via both futile creatine and calcium cycling pathways. On the other hand, the UCP1 homologue UCP3 is abundant in skeletal muscle and post-prandial thermogenesis has been associated with UCP3 and the futile calcium cycling. This review will focus on the differential contributions of adipose tissue and skeletal muscle in determining total thermogenic output and energy expenditure in large mammals. Sheep and pigs do not have a circumscribed brown fat depot but rather possess white fat depots that contain brown and beige adipocytes interspersed amongst white adipose tissue. This is representative of humans, where brown, beige and white adipocytes have been identified in the neck and supraclavicular regions. This review will describe the mechanisms of thermogenesis in pigs and sheep and the relative roles of skeletal muscle and adipose tissue thermogenesis in controlling body weight in larger mammals.

Key Words

- thermogenesis
- skeletal muscle
- adipose tissue
- weight loss
- obesity
- sheep
- pigs

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Introduction

The worldwide incidence of obesity has rapidly escalated and shows little sign of diminution; in 2017 the World Obesity Federation (WOF) estimated that over 600 million individuals were classified as obese (http://www.obesityday.worldobesity.org/). High rates of obesity are associated with greater economic burden (Tremmel *et al.* 2017) due to increased risk of type 2 diabetes (Bhupathiraju & Hu 2016), cardiovascular disease (Fuster *et al.* 2016, Ortega *et al.* 2016), cancer (LeRoith *et al.* 2008, Bhaskaran *et al.* 2014, Garg *et al.* 2014), Alzheimer's disease (Alford *et al.* 2017) and mental illness (Hillman *et al.* 2010). The WOF estimates that by 2025, the estimated cost of obesity, if left untreated, will almost reach $1.2 trillion as global obesity numbers are set to rise to approximately 850 million (http://www.obesityday.worldobesity.org/). In effort to curb this alarming rise, it is imperative to understand the pathophysiology of obesity and the mechanisms that confound long-term weight loss.

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It is widely recognised that body conformation is highly heritable and over 600 genes contribute to weight control (Perusse *et al*. 2005). It has been estimated that 40–70% of the innate variation in body mass index is determined by an individual's genetic background (Locke *et al*. 2015). Mutations in genes encoding various neuroendocrine factors including leptin, the leptin receptor, pro-opiomelanocortin (*POMC*), POMC post-translational processing enzymes and the melanocortin 4 receptor (*MC4R*) cause obesity in both humans and rodents (Jackson *et al*. 1997, Montague *et al*. 1997, Clement *et al*. 1998, Krude *et al*. 1998, Farooqi *et al*. 2003). Despite this, monogenetic mutations are extremely rare (Farooqi 2008). To add further complexity, pre-conception and early life environments impact on body mass via epigenetic modifications (Lillycrop & Burdge 2010) such as those to the tripartite motif-containing 28 (*TRIM28*) gene, which correlates with increased adiposity in humans (Dalgaard *et al*. 2016). Dogma now stipulates that various genetic and epigenetic factors lead to inherent susceptibility to become obese, which is typically unmasked by environmental pressure. Irrespective of this, it is well recognised that, once obese, it is extremely difficult to lose weight and maintain weight loss, due to homeostatic defence mechanisms that reset hunger and energy expenditure.

Early weight loss trials suggested that only 2% of the population could maintain long-term weight loss, at the two-year time point, in response to diet and exercise interventions (Stunkard & McLaren-Hume 1959). Successful weight loss is defined as >10% reduction in body weight, which is maintained for 1 year (Wing & Hill 2001, Wing & Phelan 2005). Numerous endocrine and neuroendocrine adaptations occur in response to weight loss, driving increased hunger and reduced energy expenditure (Lewis *et al*. 1993, Bi *et al*. 2003, Yu *et al*. 2009, Sumithran *et al*. 2011). In response to weight loss, circulating levels of the gut-derived appetite-stimulating hormone ghrelin are increased, whereas satiety factors such as leptin, amylin, cholecystokinin and glucagon-like peptide 1 are decreased (Sumithran *et al*. 2011). Importantly, this maladaptation in the secretion of numerous endocrine factors persists for up to 2 years post-weight loss (Sumithran *et al*. 2011). The aforementioned endocrine factors exert reciprocal effects on food intake and energy expenditure, where hormones that increase food intake typically reduce energy expenditure and vice versa (Kirchner *et al*. 2012, Park & Ahima 2015, Bauer *et al*. 2016). Thus, weight-loss-induced changes in circulating factors not only act to increase hunger but exert a dual effect to reduce energy expenditure, which predisposes individuals to regain lost weight (Leibel *et al*. 1995, Ravussin *et al*. 1988, Bosy-Westphal *et al*. 2013). A key component of the decrease in energy expenditure is reduced adaptive thermogenesis (Rosenbaum *et al*. 2008, Camps *et al*. 2015, Henry *et al*. 2017), which occurs in both skeletal muscle and brown adipose tissue (BAT). To investigate the specific metabolic adaptations that underlie these changes in thermogenesis at the tissue and molecular level, it is pertinent to use animal models. Rodents including mice, rats and hamsters have provided invaluable information to this field with widespread use of transgenic, optogenetic and knockout models (Lutz & Woods 2012, Barrett *et al*. 2016). The current review, however, will focus on metabolic flexibility of genetically heterogeneous or outbred populations of large animals, specifically sheep (*Ovis aries*) and pigs (*Sus*) and the role of thermogenesis in innate predisposition to obesity or inherent resistance to diet-induced weight loss.

Mechanisms of thermogenesis

Brown adipose tissue

Body weight is determined by the balance between energy intake and energy expenditure, with the latter comprised of basal metabolic rate, physical activity and adaptive thermogenesis. Adaptive thermogenesis is defined as specialised heat production and occurs in BAT and skeletal muscle. Cold- and meal-associated stimuli are perceived by the brain, leading to activation of the sympathetic nervous system (SNS) and the induction of thermogenesis (Lowell & Spiegelman 2000, Cannon & Nedergaard 2004). Noradrenaline is released within BAT and activates uncoupling protein 1 (UCP1), which creates a proton leak across the inner mitochondrial membrane. This proton leak redirects protons away from ATP synthase and the production of ATP; cellular energy is dissipated in the form of heat (Fig. 1) (Cannon & Nedergaard 2004).

Across the lifespan, mice and rats retain a defined and circumscribed brown fat depot located in the interscapular region (Sbarbati *et al*. 1991, Morroni *et al*. 1995). This contrasts with larger adult mammals, including humans and sheep, where brown adipocytes are interspersed amongst white adipose tissue (WAT) (Cypess *et al*. 2013, Henry *et al*. 2017). Indeed, for many years, dogma stipulated that in humans BAT was only abundant during early life,

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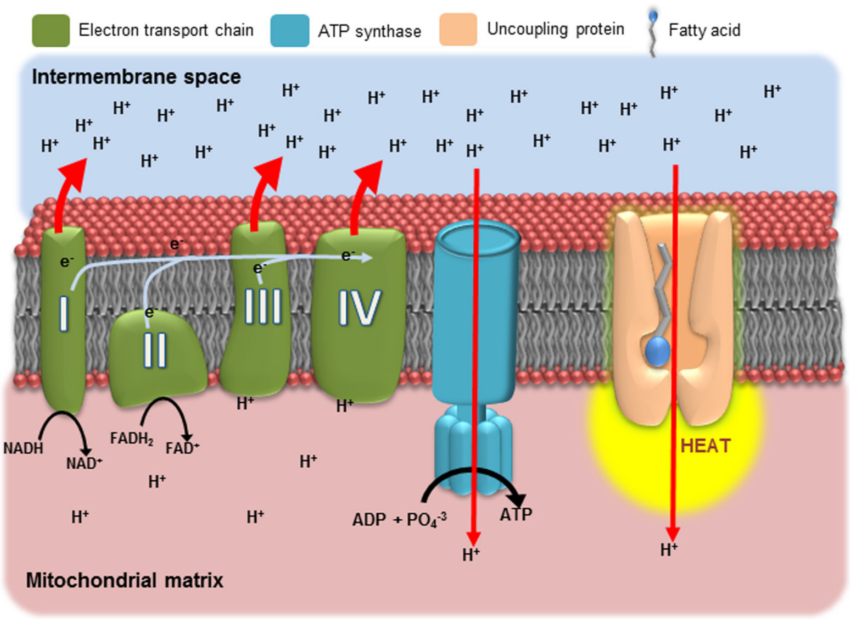

Figure 1
Schematic of mitochondrial uncoupling and the cellular process of thermogenesis. Metabolic processes such as glycolysis, β-oxidation and the citric acid cycle contribute electrons through the nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD^+) carriers to the electron transport chain. The action of the electron transport chain (complexes I–IV) results in the pumping of protons across the inner mitochondrial membrane from the matrix into the intermembrane space and the establishment of the electrochemical gradient. Normally, this proton motive force is harnessed by ATP synthase to produce ATP from ADP. UCPs provide an alternative means through which protons can cross the inner membrane. Fatty acids activate UCPs by binding to a hydrophobic pocket within the protein that increases proton conductance. The leak of protons across the inner mitochondrial membrane results in the dissipation of energy through heat production.

being fundamental to maintaining core body temperature in neonates (Aherne & Hull 1966, Heaton 1972, Cannon & Nedergaard 2004). Soon after birth, muscle acquires the ability to provide heat through shivering and thus BAT levels rapidly decline and were thought to be negligible in adult humans (Cunningham *et al*. 1985, Astrup 1986). A series of landmark papers have since identified pockets or islands of BAT particularly within the neck and supraclavicular region of adults (Nedergaard *et al*. 2007, Cypess *et al*. 2009, van Marken Lichtenbelt *et al*. 2009). These depots not only contain brown and white adipocytes, but also the morphologically distinct beige adipocytes (discussed in detail in the following section) (Sharp *et al*. 2012, Cypess *et al*. 2013, Jespersen *et al*. 2013, Nedergaard & Cannon 2013, Lee *et al*. 2014*a*). Functional BAT has since been observed in epicardial, paravertebral and perirenal adipose tissues (Cypess *et al*. 2015, Gaborit *et al*. 2015), although the physiological relevance of these depots to energy homeostasis remains to be elucidated.

Skeletal muscle

In addition to BAT, skeletal muscle is thermogenic, which occurs independent of UCP1. Myocytes express the *UCP1* homologue, *UCP3*, which is capable of uncoupling oxidative phosphorylation in mitochondria isolated from yeast cells (Gong *et al*. 1997). In addition to mitochondrial uncoupling, skeletal muscle produces heat via futile calcium cycling (Simonides *et al*. 2001, de Meis *et al*. 2005, Arruda *et al*. 2007, Clarke *et al*. 2012, Blondin *et al*. 2017) where calcium exits the sarcoendoplasmic reticulum (SR) via the ryanodine receptor (RyR). To maintain cytosolic calcium levels, activation of the sarcoendoplasmic reticulum ATPases (SERCA) propel calcium back into the SR; this effect is driven by the hydrolysis of ATP and results in heat production (Arruda *et al*. 2007, Bal *et al*. 2012) (Fig. 2). In rodents, sarcolipin is an endogenous activator of SERCA, which uncouples calcium transport from the hydrolysis of ATP, leading to an increase in the futile cycling of calcium and heat production (Fig. 2). In the absence of BAT (surgical removal) or UCP1 (genetic deletion), sarcolipin increases muscle thermogenesis and is essential for cold adaptation (Bal *et al*. 2012, 2016). Over-expression of sarcolipin in skeletal muscle increases oxygen consumption and fatty acid oxidation, which is associated with resistance to weight gain in mice fed a high fat diet (Maurya *et al*. 2015). The role of sarcolipin in thermogenesis in larger mammals, however, is relatively unexplored and requires closer investigation. Given that skeletal muscle accounts for approximately 40% of total body mass, it is hypothesised that, at least in large mammals, even small differences in muscle thermogenesis may contribute substantially to thermogenic capacity and total energy expenditure. Indeed, earlier work in adult humans suggested that skeletal muscle was the primary means of thermogenesis in response to sympathomimetic treatment (Astrup *et al*. 1985, Astrup 1986); ephedrine-induced thermogenesis is 10 fold higher in skeletal muscle than adipose tissue. It is important to emphasise, however, that this earlier work did not study adipose tissue in the neck and clavicular regions, where

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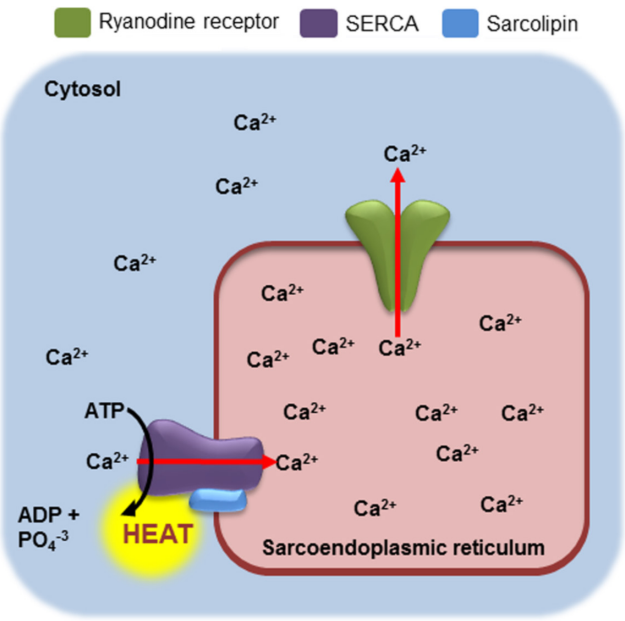

Figure 2
Schematic representation of futile calcium-cycling-mediated thermogenesis. At rest, the passive leak of calcium from the sarcoendoplasmic reticulum (SR) occurs via the ryanodine receptor. To maintain intracellular calcium homeostasis, calcium is propelled back into the SR via the sarcoendoplasmic reticulum ATPase (SERCA) pump. The movement of calcium against the concentration gradient requires the hydrolysis of ATP, which is thermogenic and produces heat. Sarcolipin is a key regulator of SERCA and activates thermogenesis via the futile calcium pathway.

brown/beige adipocytes are most abundant. Nonetheless, PET-CT studies in lean humans show that chronic low dose ephedrine treatment actually reduces BAT activity (Carey *et al*. 2015), which strongly suggests that tissues such as a skeletal muscle are important in whole body thermogenesis in humans.

On the other hand, the contribution of skeletal muscle to cold-induced adaptive thermogenesis in humans remains unclear. In obese and lean subjects, BAT is activated by cold exposure (Saito *et al*. 2009, van Marken Lichtenbelt *et al*. 2009, Wijers *et al*. 2010). In BAT, cold exposure increases *UCP1* levels with a concomitant increase in BAT activity, whereas in skeletal muscle there is no effect of cold on the expression of *UCP3*. Despite this, in lean men, cold exposure increases uncoupled respiration in permeabilised muscle fibres, which correlates with total energy expenditure (Wijers *et al*. 2008). Furthermore, in humans, skeletal muscle accounts for the vast majority of increased glucose uptake and utilisation during cold exposure compared to BAT (Blondin *et al*. 2015). A recent study, however, suggests that after 4 weeks of cold exposure, muscle-derived adaptive non-shivering thermogenesis is inhibited in favour of BAT thermogenesis (Blondin *et al*. 2017). This increase in BAT thermogenic capacity obviates the need for increased adaptive thermogenesis in skeletal muscle. Irrespective of the role of skeletal muscle in long-term cold adaptation, current evidence supports the notion that skeletal muscle is an important determinant of thermogenic capacity and contributes to total energy expenditure in adult humans.

Beige adipocytes

As alluded to above, adipose tissue is heterogeneous, containing numerous cell types. White adipocytes are unilocular, containing one large lipid droplet and few mitochondria, whereas brown adipocytes are multilocular and have numerous mitochondria (Cousin *et al*. 1992, Cinti 2001, Barbatelli *et al*. 2010). More recently, a third adipocyte, the beige cell has been identified (Himms-Hagen *et al*. 2000, Barbatelli *et al*. 2010). Beige adipocytes exhibit an intermediary phenotype and are referred to as paucilocular, as these cells contain more than one lipid droplet and multiple mitochondria (Himms-Hagen *et al*. 2000, Barbatelli *et al*. 2010). Beige adipocytes also express *UCP1* and have been termed 'recruitable' as these cells can be detected in small clusters within WAT in response to certain stimuli, such as cold (Shabalina *et al*. 2013, Lee *et al*. 2014*a*, Jankovic *et al*. 2015). Brown and beige adipocytes display distinct genetic fingerprints and importantly unlike brown adipocytes that show high basal expression of thermogenic genes such as *UCP1*, beige adipocytes only exhibit these genes in response to activating stimuli including cold and β-adrenoceptor agonists (Walden *et al*. 2012, Wu *et al*. 2012, Rosenwald *et al*. 2013). Furthermore, unlike brown adipocytes, which are derived from *Myf5* +ve precursor cells, beige adipocytes are derived from bipotent preadipocytes (Seale *et al*. 2008, Sanchez-Gurmaches *et al*. 2012, Harms & Seale 2013, Wang *et al*. 2013). The beige cell represents a novel adipocyte that contributes to thermogenesis in both rodents and humans (Cohen & Spiegelman 2015).

In addition to UCP1-dependent thermogenesis, beige adipocytes produce heat through futile creatine (Fig. 3) (Kazak *et al*. 2015, Bertholet *et al*. 2017) and futile calcium cycling pathways (Ikeda *et al*. 2017) (Fig. 2). In mice, proteomic analyses revealed a beige adipocyte-specific arginine-creatine metabolic pathway (Kazak *et al*. 2015), which is upregulated in response to cold exposure. Futile creatine cycling is important in

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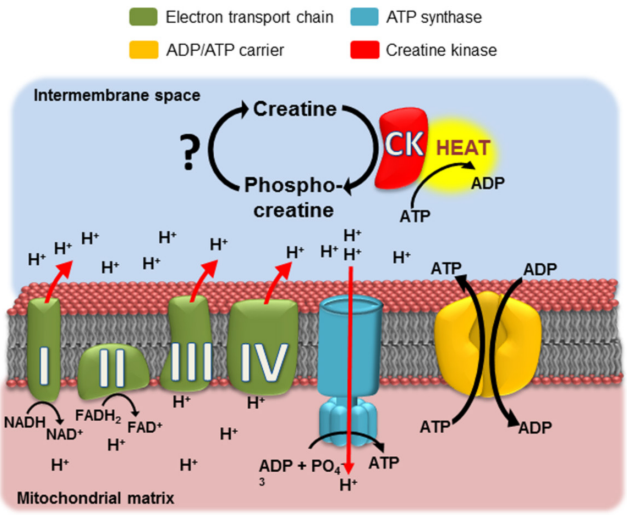

Figure 3
Schematic diagram depicting the futile creatine cycle. Within mitochondria, thermogenesis can occur in the ADP-depleted state via creatine cycling. Creatine is phosphorylated by creatine kinase (CK) and then dephosphorylated. The responsible phosphatase is currently unidentified. The process of creatine dephosphorylation is thermogenic via the hydrolysis of ATP.

beige adipocyte thermogenesis in ADP-depleted states, wherein this pathway drives the hydrolysis of ATP and thus increases oxygen consumption (Kazak *et al*. 2015). Cold exposure for 1 week increased the expression of both creatine kinase U-type, mitochondrial (*Ckmt1*) and creatine kinase S-type, mitochondrial (*Ckmt2*) in inguinal beige adipocytes, indicative of an upregulation in creatine cycling. Furthermore, treatment with the β3-adrenoceptor agonist, CL316 234, induced *Ucp1*-expressing beige adipocytes in the inguinal fat depot, as well as *Ucp1*-negative, *Ckmt2*-positive beige adipocytes in epididymal fat (Bertholet *et al*. 2017). Murine beige adipocytes also produce heat via futile calcium cycling and the activation of SERCA2b (Ikeda *et al*. 2017). Inhibition or downregulation of SERCA2b in inguinal adipose tissue attenuates the noradrenaline-induced increase in oxygen consumption (Ikeda *et al*. 2017), supporting the notion that futile calcium cycling is important in sympathetic-induced thermogenesis in beige adipocytes. To date, the vast majority of studies have utilised UCP1 protein or mRNA expression as a marker for beige adipocytes, and thus the abundance and importance of these cells have likely been underestimated. It is now apparent that multiple pathways contribute to thermogenesis in these unique beige cells. The role of beige adipocytes in determining thermogenesis and energy expenditure in larger mammals including sheep and pigs, however, requires further interrogation.

Neuroendocrine control of energy balance

Control of adaptive thermogenesis is mediated by the hypothalamus, and while only explained briefly here, has been well detailed by Morrison (Morrison 2016). In rodents, when exposed to cold, thermosensory inputs act via the median preoptic area to stimulate the dorsomedial hypothalamus (DMH) to increase sympathetic nerve activity to BAT through the rostral raphe pallidus (rRPa) (Hermann *et al*. 1997, Yoshida *et al*. 2009, Yu *et al*. 2016). Earlier work identified a subset of cold-inhibited and warm-activated GABA-containing neurons within the preoptic area that mediate cold-induced thermogenesis (Nakamura & Morrison 2007). Development of Designer Receptor Exclusivity Activated by Designer Drugs (DREADD) technology has allowed for further characterisation of these temperature-sensitive neurons. Use of DREADDS suggests that activation of GABA neurons in the preoptic area has little effect on body temperature or energy expenditure (Yu *et al*. 2016). Indeed, these studies show that leptin-receptor-expressing neurons in the preoptic area are integral to ambient temperature-induced metabolic (food intake and energy expenditure) adaptations (Yu *et al*. 2016). Within the preoptic area, there is clearly topographical neuronal organisation as cold exposure increases c-Fos levels in GABA neurons within the ventral part of the lateral preoptic area (Zhao *et al*. 2017) and optogenetic manipulation of these neurons influences body temperature. Optogenetic inhibition of this subset of GABA neurons causes hyperthermia, whereas activation of the same reduces body temperature (Zhao *et al*. 2017). Thus, within the preoptic area, there is an integrated network of neurons, including both GABA-ergic and leptin responsive cells, capable of sensing changes in skin temperature and modifying thermogenic output.

In addition to the aforementioned temperature-sensitive pathway, metabolic factors such as leptin, insulin and ghrelin modulate thermogenic activity via hypothalamic appetite-regulating peptides. Blood-borne factors can diffuse across the blood brain barrier via fenestrated capillaries and act directly on neurons in the arcuate nucleus (Banks 2009). The diffusion of metabolic hormones is controlled by local tanycytes (Balland & Cowley 2017). Importantly, two distinct sets of neurons are found in the arcuate nucleus, being either orexigenic or those that elicit satiety. The POMC neurons are activated by leptin (Elias *et al*. 1999, Cowley *et al*. 2001) and insulin (Claret *et al*. 2007, Williams *et al*. 2010), leading to release of α-melanocyte-stimulating

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hormone (aMSH), which elicits satiety via the MC4R in the paraventricular nucleus (PVN) (Cowley *et al*. 1999, Balthasar *et al*. 2005). A second population of neurons contain neuropeptide Y (NPY) and agouti-related protein (AgRP), which stimulate food intake in response to direct stimulation by ghrelin (Kamegai *et al*. 2001). The primary role of NPY/AgRP neurons is to protect against starvation; genetic deletion of *AgRP* reduces food intake and causes wasting, eventually leading to starvation and death (Luquet *et al*. 2005). NPY exerts an immediate effect to stimulate food intake, primarily via action at Y1 receptors in the PVN (Kask *et al*. 1998). On the other hand, AgRP acts as an inverse agonist at the MC4R to stimulate food intake (Nijenhuis *et al*. 2001). Hypothalamic appetite-regulating peptides exert reciprocal control to modulate food intake and energy expenditure, in particular BAT and muscle thermogenesis (Verty *et al*. 2010, Gavini *et al*. 2016). Indeed, pseudorabies-tracing studies show that appetite-regulating neurons of the hypothalamus ultimately project to neural networks controlling sympathetic outflow to peripheral tissues including BAT (Bamshad *et al*. 1999, Oldfield *et al*. 2002, Song *et al*. 2009, Ryu *et al*. 2015).

As mentioned earlier, activation of the SNS and the release of catecholamines, in particular, noradrenaline are fundamental to BAT thermogenesis. Indeed, genetic deletion of all three β-adrenergic receptors (βAR) in brown adipocytes of mice causes profound obesity by negating thermogenesis (Bachman *et al*. 2002). Interestingly, in humans, isoprenaline (a non-specific βAR) treatment increases energy expenditure without an associated activation of BAT (Vosselman *et al*. 2012). Similarly, blockade of the βAR with propranolol had no effect on cold-induced BAT thermogenesis in humans (Wijers *et al*. 2011). This lack of effect, however, is likely due to receptor specificity as both isoprenaline and propranolol show preferential agonistic and antagonistic affinity to the β1AR and β2AR, respectively. Indeed, in healthy lean men, administration of the β3AR-specific agonist, mirabregon, activates BAT and causes a concurrent increase in resting metabolic rate (Cypess *et al*. 2015). Furthermore, a 64 Trp/Arg genetic polymorphism in the β3AR is linked to the decline in BAT function with ageing in men (Yoneshiro *et al*. 2012). Together, these studies highlight that in humans, the β3AR is essential to catecholamine-mediated BAT thermogenesis. It remains possible, however, that the effects of isoprenaline to increase energy expenditure via the β1/β2 AR (Vosselman *et al*. 2012) are mediated via skeletal muscle thermogenesis (Blaak *et al*. 1993).

In addition to catecholamines, thyroid hormones (TH) are notable endocrine regulators of BAT activity. Brown adipocytes contain the deiodinase type 2 (DIO2) enzyme, allowing for local conversion of thyroxine (T4) to triiodothyronine (T3) (Carvalho *et al*. 1991); T3 exhibits greater biological potency than T4. In rodents, TH act directly at nuclear thyroid hormone receptors located in brown adipocytes to transcriptionally upregulate *UCP1* expression (Weiner *et al*. 2017). Furthermore, clinical data demonstrate that BAT activity is higher in the subclinical hyperthyroid state than in the hypothyroid state (Broeders *et al*. 2016), supporting the notion that endogenous TH regulate BAT thermogenesis. Although the classical action of T3 is thought to be peripherally mediated, more recent studies have shown that TH can also act centrally within the hypothalamus to regulate BAT thermogenesis. Tanycytes in the mediobasal hypothalamus express DIO2 and thus convert T4 to T3 (Coppola *et al*. 2007). Furthermore, in mice, intracerebroventricular administration of T3 increases BAT thermogenesis via reduced hypothalamic levels of AMP kinase (AMPK) and subsequent activation of the SNS (Lopez *et al*. 2010). Indeed, sub-chronic (6 days) central administration of T3 leads to browning of WAT in mice (Alvarez-Crespo *et al*. 2016).

To date, much of the work defining the regulation of thermogenesis and its contribution to energy balance has been in rodents. This has provided invaluable information and understanding of the neuroendocrine mechanisms that control thermogenesis. More recently, a number of large animal models have been employed including pigs and sheep, which provide further insight into the role of thermogenesis in long-term regulation of body weight in mammalian species.

Regulation and significance of thermogenesis in pigs

It is well recognised that pigs lack a functional UCP1 protein (Hou *et al*. 2017), which previously led to the assumption that this species did not possess brown adipocytes. Pigs, specifically those belonging to the *Suidae* species, do not have exons 3–5 of the *UCP1* gene, rendering animals prone to hypothermia-induced death as neonates (Berg *et al*. 2006). With only exons 1 and 2, *UCP1* can still be transcribed; however, protein translation does not occur (Hou *et al*. 2017). Hence, previous histological studies failed to detect UCP1 protein immunoreactivity at baseline (Rowlatt *et al*. 1971) or in response to cold

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exposure (Trayhurn *et al*. 1989), and thus pigs were deemed to be lacking BAT. It has since been proposed that pigs do indeed possess functional BAT; however, adaptive thermogenesis occurs via UCP1-independent mechanisms (Ikeda *et al*. 2017, Lin *et al*. 2017). Indeed, recent work comparing cold-tolerant Tibetan pigs to cold-sensitive Bama pigs has provided direct evidence of adaptive thermogenesis in subcutaneous (sWAT) and perirenal WAT (Lin *et al*. 2017) (Fig. 4). Both of these pig breeds lack *UCP1* expression, yet when subjected to cold exposure (4°C for 4 h) PET-CT identified metabolically active subcutaneous and perirenal adipose tissue (Lin *et al*. 2017) in cold-tolerant animals, which contributed to the maintenance of core body temperature. Furthermore, morphological studies show that in response to cold exposure, subcutaneous adipose tissue displays evidence for beige cell recruitment with an increase in multilocular adipocytes, increased mitochondrial DNA copy number and increased expression of *PGC1a* and the beige cell marker *CD137* (Wu *et al*. 2012, Lin *et al*. 2017). Furthermore, in Tibetan pigs (cold tolerant), cold exposure increased the expression of the UCP3 gene and protein in isolated subcutaneous adipocytes and this is associated with increased uncoupled respiration, providing evidence to suggest an increase in UCP3-driven thermogenesis (Lin *et al*. 2017) (Fig. 4).

The contribution of UCP3 to brown fat thermogenesis has been contentious and appears to be dependent on the species studied. In mice, earlier work suggested that BAT thermogenesis was dependent on UCP1 (Matthias *et al*. 2000). Despite this, hamsters that lack functional UCP3 specifically in brown adipocytes have increased propensity to weight gain, which is indicative of a reduction in energy expenditure (Fromme *et al*. 2009). Although innate differences in *UCP3* expression in adipose tissue of pigs have been linked to cold tolerance, to date, there are no data on BAT-specific UCP3 function and the control of body weight in this species.

In addition to UCP3-associated uncoupling and thermogenesis, recent data suggest that SERCA-driven beige cell thermogenesis also occurs in pigs. Indeed, the work by Ikeda *et al*. (Ikeda *et al*. 2017) demonstrate strong conservation of the beige adipocyte SERCA2b pathway across species. Retroviral expression of PRDM-16 in subcutaneous porcine adipocytes increases the expression of beige-cell-specific markers including *CIDEA* and *TMEM26* (Ikeda *et al*. 2017). Furthermore, decreased *SERCA2b* expression reduced basal and noradrenaline-induced oxygen consumption and extracellular acidification rates in isolated pig adipocytes (Ikeda *et al*. 2017). Thus, it is now clear that adipose tissue thermogenesis and the associated energy expenditure are not solely mediated via UCP1 and mitochondrial uncoupling, but in fact, a number of cellular pathways, across both adipose tissue and skeletal muscle, act in concert to determine total thermogenic potential.

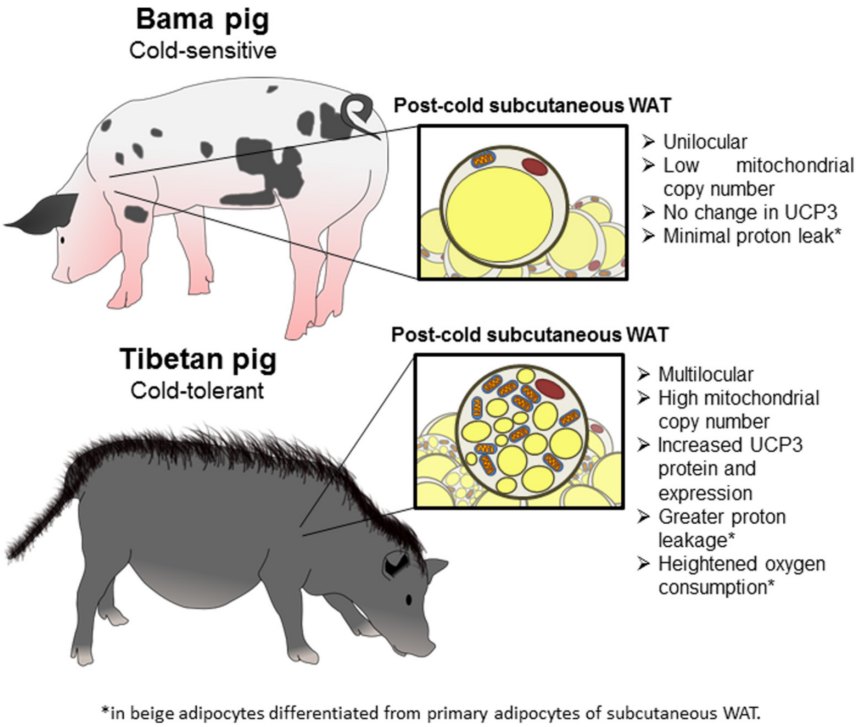

Figure 4
Role of thermogenesis in determining cold tolerance in pigs. Tibetan pigs are cold tolerant and this coincides with the recruitment of beige adipocytes in subcutaneous WAT in response to cold exposure. Although pigs do not that express functional uncoupling protein (UCP) 1, adipocytes exhibit UCP3 and this mediates mitochondrial uncoupling and adipose tissue thermogenesis. In contrast the Bama pig is unable to induce 'browning' in WAT of white adipose tissue and thus is characterized as cold sensitive. *Denotes findings from beige adipocytes differentiated from primary adipocytes of subcutaneous WAT.

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Role of thermogenesis in sheep

In lambs, the expression of *UCP1* is maximal in perirenal adipose tissue on the first postnatal day, rapidly declining with the expansion of WAT (Symonds 2013, Pope *et al.* 2014). Mapping of *UCP1* mRNA in lambs shows abundant expression in sternal and retroperitoneal adipose depots compared to omental fat, which is a predominantly WAT depot (Symonds *et al.* 2012). Indeed, adult sheep retain *UCP1* expression in both sternal and retroperitoneal fat and this coincides with post-prandial heat production, albeit this response is greater in the sternal fat depot (Henry *et al.* 2017). This coincides with the expression of UCP1 protein, where UCP1-positive brown-like adipocytes were only detectable in sternal adipose tissue of adult ewes (Henry *et al.* 2017). Data logger temperature probes have been employed to measure longitudinal heat production in multiple tissues to index thermogenic output in sheep. Sheep are a grazing species and therefore do not display typical meal-associated excursions such as changes in ghrelin secretion. Despite this, temporal food restriction in sheep entrains a pre-prandial rise in ghrelin (Sugino *et al.* 2002, Takahashi *et al.* 2008) and post-prandial increases in thermogenesis in skeletal muscle and adipose tissue (Henry *et al.* 2008, 2017), similar to what is seen in humans (Johnston *et al.* 2002, Stob *et al.* 2007, van Baak 2008). In adult sheep, post-prandial skeletal muscle thermogenesis is associated with increased expression of *UCP3* as well as an increase in protein and/or gene expression of markers of futile calcium cycling (Clarke *et al.* 2012). Furthermore, post-prandial thermogenesis in both skeletal muscle and retroperitoneal adipose depots is markedly enhanced by intracerebroventricular infusion of leptin (Henry *et al.* 2008). Thus, in spite of relatively low levels of UCP1 in adult sheep, skeletal muscle and specific adipose depots retain thermogenic capacity. Over recent years, we have utilised the sheep to dissect the differential roles of adipose tissue and skeletal muscle thermogenesis in the long-term control of body weight, which is discussed in detail in the following section.

Role of thermogenesis in controlling long-term changes of body weight in sheep

Similar to other species, ovine body weight can be readily manipulated through dietary management (Henry *et al.* 2000, 2017, Iqbal *et al.* 2001, 2003). Sheep are ruminants and thus body weight is increased through feeding a high-energy diet enriched in lupin grain and oats. Diet-induced obesity, however, is not associated with any change in heat production in adipose tissues or skeletal muscle of sheep (Henry *et al.* 2017). On the other hand, long-term food restriction and low body weight are associated with a homeostatic decrease in thermogenesis in sternal and retroperitoneal adipose tissue and skeletal muscle (Henry *et al.* 2017) (Fig. 5). Importantly, similar to humans, the reduction in thermogenesis caused by food restriction and low body weight is still evident at one year post-weight loss, which suggests that homeostatic changes in thermogenesis contribute to impaired weight loss and increased long-term weight regain (Henry *et al.* 2017).

The homeostatic reduction in thermogenesis is coordinated by the hypothalamus. Long-term weight loss in ovariectomised ewes increases the expression of the orexigenic neuropeptides *NPY* in the arcuate nucleus and melanin-concentrating hormone (*MCH*) in the lateral hypothalamus (LH) to increase hunger and reduce energy expenditure (Henry *et al.* 2000) (Fig. 5). Regarding the anorexigenic melanocortin pathway, the effect of low body weight on the expression of *POMC* is controversial with data showing a decrease (Backholer *et al.* 2010) or no effect (Henry *et al.* 2000). This is not surprising since POMC is the precursor to multiple neuropeptides, only one of which includes aMSH and the ultimate end product is dependent on post-translational processing (Mountjoy 2010). On the other hand, increased *Agrp* and *Npy*

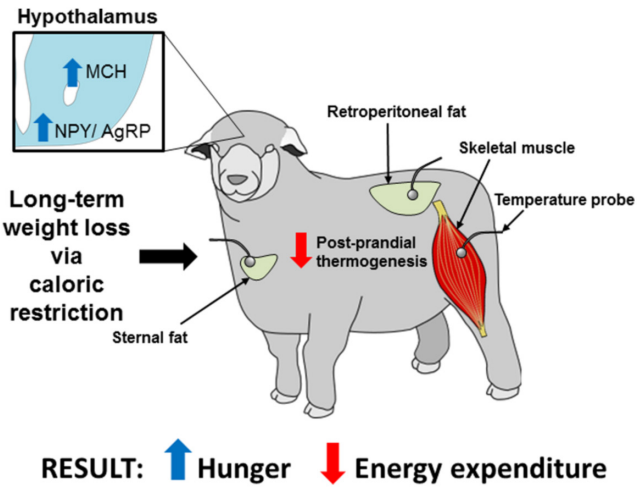

Figure 5
Effect of chronic food restriction and weight loss on adaptive thermogenesis in ewes. Tissue temperature recordings show that caloric restriction and low body weight cause a homeostatic decrease in night time thermogenesis in ovariectomised ewes. This metabolic adaptation occurs in both sternal adipose tissue (adipose tissue enriched in uncoupling protein 1) and skeletal muscle and to a lesser extent in retroperitoneal adipose tissue. The reduction in thermogenesis is associated with increased expression of neuropeptide Y (NPY) in the arcuate nucleus and melanin-concentrating hormone (MCH) in the lateral hypothalamus.

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expression and reduced *Pomc* mRNA have been observed in rodents (Bi *et al*. 2003, Rogers *et al*. 2016) and lambs (McShane *et al*. 1993). Thus, weight-loss-induced changes in hypothalamic gene expression are likely to reduce thermogenesis, whilst causing a concurrent increase in hunger drive. This represents a homeostatic mechanism to protect against weight loss and promote weight regain in calorie-restricted individuals.

Polygenic models of predisposition to obesity in sheep

Over thousands of years of domestication, the modern sheep has undergone much human-imposed selection to the point that there exists polygenic populations predisposed to either obesity or leanness (Afonso & Thompson 1996, Morris *et al*. 1997). Animals were originally selected for innate differences in adiposity by measuring back fat thickness and two lines were created via selective breeding strategies. A key feature of the genetically lean and obese sheep is an inherent difference in the growth hormone (GH) axis, where lean animals have increased mean GH concentration in plasma and an associated increase in pituitary gland weight (Francis *et al*. 1998). The increase in pituitary gland weight is primarily due to a greater number of cells in the lean animals (Francis *et al*. 2000). Furthermore, expression of *GH* and the GH secretagogue receptor (*GHSR*) is greater in genetically lean sheep, indicating differential responses to ghrelin, an agonist of the GHSR (French *et al*. 2006). This suggests that innate differences in the set-point of the GH axis may underpin differences in adiposity in the genetically lean and obese sheep; however, this is only one aspect that could contribute to this phenotype.

Interestingly, food intake is similar in genetically lean and obese sheep as is the expression of *POMC, Leptin Receptor* and *NPY* in the arcuate nucleus. On the other hand, lean animals have elevated post-prandial thermogenesis in retroperitoneal adipose tissue and this coincides with increased expression of *UCP1* in this tissue (Henry *et al*. 2015). The divergence in thermogenesis is specific to adipose tissue since post-prandial thermogenesis is similar in genetically lean and obese animals (Henry *et al*. 2015). Despite similar expression of appetite-regulating peptides in the arcuate nucleus of the hypothalamus, genetically lean sheep have increased expression of *MCH* and pre-pro-orexin (*ORX*) in the LH compared to obese animals (Anukulkitch *et al*. 2010). While both neuropeptides are considered orexigenic (Shimada *et al*. 1998, Hara *et al*. 2001, Ito *et al*. 2003, Segal-Lieberman *et al*. 2003), MCH and orexin have differing effects on energy expenditure (Teske *et al*. 2008). Deletion of *MCH* in mice results in hypophagia and a lean phenotype (Shimada *et al*. 1998), while deletion of *ORX* leads to obesity despite also causing hypophagia (Hara *et al*. 2001). Orexin is critical in the embryonic development of BAT in mice (Sellayah *et al*. 2011), and loss of orexin neurons impairs stress- (Zhang *et al*. 2010) and cold-induced thermogenesis (Mohammed *et al*. 2016). Thus, increased expression of *ORX* in the LH of lean sheep may be an important physiological determinant of increased thermogenesis in retroperitoneal fat and the associated changes in adiposity.

The stress axis, cortisol responsiveness and obesity in sheep

It is widely recognised that there is marked variation in the glucocorticoid response to stress or activation of the hypothalamo-pituitary adrenal (HPA) axis (Cockrem 2013, Walker *et al*. 2017). The activity of the HPA axis in response to stress is impacted on by age (Sapolsky *et al*. 1986*a*,*b*, Turner *et al*. 2010), pregnancy (Brunton & Russell 2011), lactation (Tilbrook & Clarke 2006), sex (Turner *et al*. 2010) and sex steroids (Turner *et al*. 2002, 2006). Nonetheless, in any given population individuals can be characterised as either high (HR) or low (LR) glucocorticoid responders (Epel *et al*. 2001, Newman *et al*. 2007, Knott *et al*. 2008, Touma *et al*. 2008, Lee *et al*. 2014*b*). It is important to note that female LR and HR sheep have similar basal plasma cortisol concentration and divergence in glucocorticoid secretion only occurs in response to ACTH or stress (Lee *et al*. 2014*c*, Hewagalamulage *et al*. 2016). Previous studies have suggested that obesity itself causes perturbation of the HPA axis with impaired glucocorticoid-negative feedback (Jessop *et al*. 2001) and hypersecretion of cortisol in response to corticotropin-releasing factor (CRF) or stress (Mårin *et al*. 1992, Pasquali *et al*. 1993, Rosmond *et al*. 1998). Furthermore, cortisol directly impacts on metabolic function; however, this will not be addressed in the current review. Initial studies in rams show that high cortisol response to adrenocorticotropin (ACTH) is associated with lower feed-conversion efficiency (Knott *et al*. 2008). Furthermore, in rams, adiposity is correlated to cortisol responses to ACTH (Knott *et al*. 2008). More recent work shows that identification of high (HR) and low (LR) cortisol responders in female sheep can predict altered propensity to gain weight when exposed to a high-energy diet, where HR gain more adipose tissue than LR (Lee *et al*. 2014*b*). Thus, at least in female sheep, data suggest that

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cortisol responses can be used as a physiological marker that predicts propensity to become obese.

Previous studies in women suggest that HR eat more after a stressful episode than LR (Epel *et al*. 2001). Furthermore, HR individuals display preference for foods of high fat and sugar in response to psychological stress (Tomiyama *et al*. 2011). Similarly, in ewes, baseline food intake is similar in LR and HR, but HR eat more following either psychosocial (barking dog) or immune (lipopolysaccharide exposure) stressors (Lee *et al*. 2014*c*). In addition to altered food intake, HR ewes have reduced thermogenesis in skeletal muscle only; in response to meal feeding, post-prandial thermogenesis in skeletal muscle is greater in LR than in HR (Lee *et al*. 2014*b*). This again exemplifies divergence in the control of adipose tissue and skeletal muscle thermogenesis (Fig. 6).

Gene expression analyses reveal that LR and HR exhibit differences in 'set-point' in a number of hypothalamic systems. For example, at baseline in the non-stressed resting state, HR individuals show an overall upregulation of the HPA axis, with increased expression of *CRF* and arginine vasopressin, but reduced expression of oxytocin in the PVN (Hewagalamulage *et al*. 2016). In addition to altered expression of genes within the HPA axis, a key neuroendocrine feature of the LR and HR animals is altered expression of the *MC3R* and *MC4R* in the PVN (Fig. 6). Reduced *MC4R* expression coincides with the development of melanocortin resistance. Central infusion of leptin reduces food intake in both LR and HR animals, but intracerebroventricular infusion of aMSH reduces food intake in LR only. Thus, reduced *MC4R* expression appears to be central to the metabolic phenotype of HR that confers increased propensity to become obese in HR individuals (Fig. 6). Interestingly, gene expression of *NPY*, *AgRP* and *POMC* in the arcuate nucleus is equivalent in LR and HR (Hewagalamulage *et al*. 2015). Hence, differences in the control of food intake and thermogenesis are most likely manifest at the level of the melanocortin receptor. Indeed, previous work in sheep has shown the MC4R to be central in mediating the reduction in food intake caused by immune challenge (Sartin *et al*. 2008). Furthermore, in rodents, direct injection of the melanocortin agonist melanotan II into the ventromedial nucleus of the hypothalamus increases skeletal muscle thermogenesis (Gavini *et al*. 2016). We propose that reduced expression of the *MC4R* in HR animals underpins the metabolic phenotype wherein food intake is relatively increased in response to stress and reduced post-prandial thermogenesis in skeletal muscle is associated with propensity to become obese.

Conclusion

Historically, thermogenesis was considered to primarily occur in brown adipocytes and was solely driven by UCP1. It is now recognised that beige adipocytes and skeletal muscle also contribute to total thermogenic capacity and that thermogenesis is differentially regulated in these tissues. Indeed, in beige adipocytes, thermogenesis occurs via three distinct mechanisms, with these being UCP1-

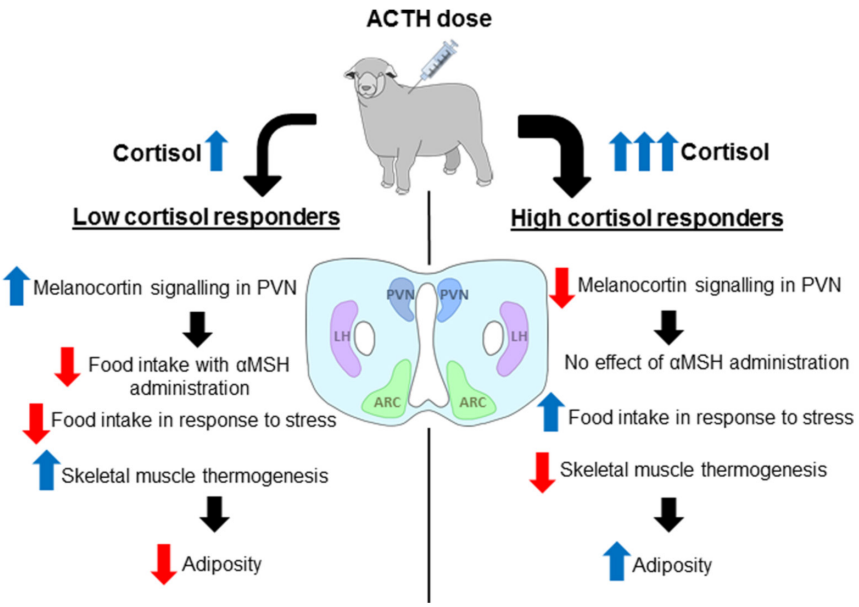

Figure 6
Schematic depiction of the altered metabolic phenotype in animals selected for either high or low cortisol responsiveness. Sheep are characterised as either high (HR) or low (LR) cortisol responders when given a standardised dose of adrenocorticotropic hormone. Animals characterized as HR have increased propensity to become obese, which is associated with perturbed control of food intake and reduced energy expenditure. Post-prandial thermogenesis in skeletal muscle is decreased in HR compared to LR ewes. Furthermore, food intake in response to stress is greater in HR than in LR and the former are resistant to the satiety effect of alpha-melanocyte stimulating hormone (aMSH). High-cortisol-responding animals have reduced expression of the melanocortin 4 receptor (MC4R) in the paraventricular nucleus of the hypothalamus (PVN). We propose that the decreased levels of MC4R underpin the altered metabolic phenotype and increased propensity to become obese when compared to LR.

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driven mitochondrial uncoupling, futile creatine cycling and futile calcium cycling. On the other hand, in skeletal muscle, thermogenesis is associated with UCP3 and futile calcium cycling. Unlike rodents, large mammals including sheep and pigs do not contain a defined or circumscribed brown fat depot but have dispersed brown adipocytes within traditionally white fat depots. Large animals have provided invaluable insight into alternative mechanisms of thermogenesis. The sheep has been particularly useful in delineating the differential role of adipose tissue and skeletal muscle in the control of body weight. Furthermore, sheep models have allowed characterisation of the neuroendocrine pathways that may contribute to altered thermogenesis. We have shown that in sheep, both skeletal muscle and BAT differentially contribute to thermogenesis and therefore total energy expenditure. Changes in thermogenesis, however, do not exclusively associate with altered gene expression at the level of the arcuate nucleus. Indeed, decreased *MC4R* expression in HR animals and reduced orexin expression in the genetically obese animals coincide with altered thermogenic output. This review highlights the importance of the use of large animal models to ascertain the contribution and control of thermogenesis in multiple tissues and the relative role in the regulation of body weight.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

Both authors contributed equally to the writing and preparation of this review.

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