**Regulatory networks determining substrate utilization in brown adipocytes**

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**Abstract**

Brown adipose tissue (BAT) is often considered as sink for nutrients to generate heat. However, when the complex hormonal and nervous inputs and intracellular signaling networks regulating substrate utilization are considered, BAT appears much more as a tightly controlled rheostat, regulating body temperature and balancing circulating nutrient levels. Here we provide an overview of key regulatory circuits, including the diurnal rhythm, determining glucose, fatty acid and amino acid utilization and the inter dependency of these nutrients in thermogenesis. Moreover, we discuss additional factors mediating sympathetic BAT activation beyond β-adrenergic signaling and the limitations of glucose based BAT activity measurements, to foster a better understanding and interpretation of BAT activity data.

[Main text](file:///\\scidom.de\nas\IDO\Daten\IDO_Adipocytes_And_Metabolism\Internal\Manuscripts%20in%20preparation\Review%20TEM\Review%20structural%20draft.xlsx)

Introduction

The major role of brown adipose tissue (BAT) is to generate heat maintaining body temperature in endotherms. Activity of BAT in response to cold stimulation can vary dramatically dependent on the housing temperature from barely detectable (at 33°C) to up to 60% of total energy expenditure in mice housed at 4°C [1] and is predominantly regulated by the activation of sympathetic nerves innervating BAT. To generate heat through mitochondrial uncoupling (**see Glossary**), brown adipocytes consume a large amount of nutrients, such as glucose, fatty acids, and amino acids taken up from circulation or mobilized from intracellular stores. BAT activity therefore also acts to dispose of excess energy in rodents and humans [2]. Thus, an increase in BAT activity, especially diet-induced thermogenesis is pursued as a pharmacological approach to treat dyslipidemia, hyperglycemia and obesity [3].The energy substrates taken up in brown adipocytes are catabolized by distinct pathways feeding into the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, resulting in heat production through uncoupling protein (UCP) 1. In addition, recent studies have revealed UCP1-independent thermogenic mechanisms driven by futile cycles where reciprocal pathways concomitantly consume ATP [4-6].

Metabolic pathways fueling thermogenesis have been mainly described in context of β3-adrenergic receptor (ADRB3) signaling, whose ligand noradrenaline is released from active efferent nerve terminals as well as the adrenal glands. Thus, noradrenaline or ADRB3 agonist treatments are often synonymously used to mimic activation of brown adipocytes by the sympathetic nervous system. However, sympathetic nerves secrete various other factors [7,8] and BAT receives complex endocrine inputs regulating and fine-tuning thermogenic function [9] (**Figure 1**). This raises the question of how brown adipocytes processes these diverse external inputs to regulate catabolic fluxes merging into the TCA cycle, which impacts on systemic nutrient levels and metabolism. For example, the crosstalk of insulin and noradrenaline, which are both essential regulators of adipocytes, balances the utilization of glucose and lipids in brown adipocytes (**Box 1**). This review addresses the enigma of energy utilization decision making in BAT thermogenesis and aims to integrate the changes in metabolic fluxes upon metabolic adaptation to extracellular cues. A better understanding of these processes and their dependency on the metabolic state of the organism is key to design therapeutic strategies fully utilizing brown adipose tissue as rheostat for blood nutrient levels.

Energy Substrates for Thermogenesis

Brown adipocytes utilize three major types of nutrients: glucose, fatty acids, and amino acids. There have been several reviews discussing individual energy substrates for brown thermogenesis. Thus, we briefly summarize key facts here and refer to detailed metabolic pathways in a recent review [10].

**Glucose/Glycogen**

The gold standard method to visualize and quantify active BAT in humans and animal models is imaging of radioisotope-labeled glucose (18F-FDG) uptake by positron emission tomography-computed tomography (PET-CT) [11]. Unlike white adipocytes that rely on insulin-dependent GLUT4 mediated glucose uptake, murine brown adipocytes take up glucose mainly through GLUT1, which is independent of insulin but under the partial control of adrenergic signaling [12]. However, there is also GLUT4 mediated glucose uptake, linking glucose utilization in brown adipocytes to insulin action [13]. In contrast, human brown adipocytes appear to rely more on GLUT4 as GLUT1 expression declines with differentiation [14]. This, at first minor appearing, difference could point to fundamental differences in the hormonal versus neuronal control of substrate utilization and activity of BAT between mice and humans, as GLUT1 facilitates continuous glucose uptake with different transport kinetics than GLUT4 and is not under the control of the insulin-coupled glucose monitoring system of the organism.

Glycogen, as a major store of carbohydrates, is abundantly present in brown adipocytes, especially when *de novo* lipogenesis is limited [15]. Glycogen also accumulates following cold exposure [16]. However, the physiological role of glycogen in BAT thermogenesis is still under investigation. In principle, glucose metabolism branches into several pathways. The roles of glucose oxidation for thermogenesis are discussed in later sections but glucose also provides substrates and energy intermediates for lipogenesis [17]. In fact, glucose is primarily converted to glycerol 3-phosphate following short-term ADRB3 stimulation rather than acetyl-CoA [18].

**Fatty acids**

One characteristic morphological feature of brown adipocytes is that lipids are stored in multilocular small droplets whose enlarged surface area allows fast access of lipolytic enzymes enabling a quick response to lipolytic stimulation mediated by cAMP/PKA signaling [19]. During the initial few hours of cold exposure, fatty acids released from triglyceride stores in intracellular lipid droplets activate UCP1 and are oxidized into acetyl-CoA, followed by conversion into chemical energy in mitochondria [20]. Thereafter, circulating fatty acids provided by white adipose tissue (WAT) and diet are essential energy sources for euthermia [15,21]. In contrast, a feature of metabolic adaptation to prolonged cold exposure is the utilization of intracellular lipids produced by *de novo* lipogenesis, which becomes a large part of the energy resource during chronic cold exposure [17] (**Box 2**).

**Amino acids**

Overall, there is still an incomplete understanding on the role of amino acids in thermogenesis. Intracellular levels of tyrosine, alanine, threonine, and tryptophan as well as branched-chain amino acids (BCAA) are elevated in BAT upon cold challenge in mice and BCAA serve as fuel for thermogenesis in brown adipocytes [22]. In addition, serine, cysteine, and glycine uptake promotes UCP1-dependent thermogenesis in human brown/beige adipocytes [23]. The specific amino acid transporters are still being identified and in part controversially discussed. Serine, cysteine and glycine were suggested to be transported by the alanine/cysteine transporter 1, although this transporter was previously shown to be predominantly expressed in white and not brown adipocytes [24,25]. In contrast, brown adipocytes express the proton-coupled amino acid transporter 2 (PAT2) transporting alanine, glycine, and/or proline, on the plasma membrane [24] and PAT2 inhibition can suppress amino acid uptake fueling the protein futile cycle upregulated in brown adipocytes with defective mitochondrial respiration [6]. Alanine, serine, and glycine can also be metabolized to pyruvate and serine and glycine are being used for the synthesis of glutathione whose redox state regulates UCP1-dependent respiration via manipulating mitochondrial ROS [26]. Thus, amino acids can play various roles in the regulation of brown adipocyte thermogenesis. However, the exact physiological states, where they play the most important roles in BAT function remain to be determined.

Context-Dependent Energy Utilization in BAT

**Is glucose uptake a good predictor for BAT activity?**

To visualize and analyze BAT activity by PET-CT, radiolabeled glucose, fatty acids, and acetate are commonly used in clinical studies (see in review [11]). However, glucose uptake remains the most commonly used indicator of active BAT and is positively correlated with supraclavicular skin temperature [14] and fatty acid oxidation [27].

However, there have also been studies demonstrating that glucose uptake capacity dissociates from fatty acid oxidation or activity in human BAT. Scandinavians who have the custom to swim in winter possess BAT with higher responsiveness to cold environments but their BAT incorporates glucose comparable with control subjects under cold environments [28]. Another study revealed that cold exposure promotes fatty acid oxidation without affecting carbohydrate oxidation [29]. Moreover, BAT of type 2 diabetic subjects shows selective impairments in cold-induced glucose uptake with intact fatty acid uptake and subsequent oxidation [30]. Conversely, BAT of obese subjects showed impairments in cold-induced fatty acid uptake [31]. Similar effects have been observed in mouse models of brown adipocyte specific deletion of lipolytic enzymes, where glucose uptake compensates for fatty acid unavailability (see in the review [32]). Thus, BAT activity measurements relying only on the uptake of one energy source, most likely inadequately estimate brown fat activity. Furthermore, a retrospective cohort study using 18FDG-PET/CT revealed that BAT activity in patients taking statins is lower compered to patients without statin use [33]. Therefore, as well-reviewed by Ben T *et al*., [11], substrate utilization in BAT is complex and depends very much on long- and short-term environmental inputs, which needs to be considered. Moreover, in human studies, medication needs to be considered when studying substrate utilization in BAT.

**Is ADRB3 stimulation the main trigger of maximal BAT activity?**

In addition to glucose being the main substrate considered for quantifying BAT activity, activation through adrenergic receptors is often considered equal to sympathetic activation of BAT. Indeed the rs4994 polymorphism in the human *ADRB3* gene associates with lower resting energy expenditure [34]. However, the correlation of the polymorphism with body fat content in childhood and adolescence varies between ethnic groups. This polymorphism is associated with increased obesity risk in the East Asian population, but not in subjects from West Asia, Europe and Latin America [35]. Moreover, the ADRB3 agonist mirabegron has limited efficacy on inducing fatty acid oxidation in South Asians compared to Europeans despite the fact that South Asians equivalently respond to cold exposure [29]. Thus, the hypothesis that mirabegron increases energy expenditure through ADRB3 activation in BAT of humans was recently questioned. It was shown that the thermogenic effect of mirabegron at a maximal tolerated dose was less associated with BAT activity than with heart rate and fatty acid re-esterification in WAT through nonselective agonism on ADRB1/2 [36]. Moreover, cultured human brown adipocytes preferentially expressed ADRB2, rather than ADRB3 [36]. However, none of the ADRB2 agonists are associated with BAT activity [37,38]. Thus, further studies need to address the roles of adrenergic receptors in different ethnic groups and the details underlying their ability to induce brown adipocyte thermogenesis. Moreover, it is important to keep in mind, especially when discussing the complex regulation of substrate utilization in BAT that β-adrenergic receptor activation is only one element of sympathetic regulation of BAT activity.

The direct comparison between ADRB activation and sympathetic activation by cold reveals several differences. Cold exposure leads to the release of several lipid species into the circulation, whereas mirabegron only induces fatty acid release in humans [29]. Moreover, CL316,243 (ADRB3 agonist) treatment resulted in a lower triglyceride uptake than upon cold exposure [39]. Thus, intracellular energy sources differ between cold exposure and selective ADRB3 stimulation, suggesting that additional extracellular inputs besides ADRB3 regulate energy uptake and potentially utilization in brown adipocytes, allowing adaptation to the available nutrients in the circulation.

Diurnal Rhythm of Nutrient Utilization and BAT Activity

However, before other external inputs can be integrated into the model of substrate utilization, the intrinsic circadian rhythm of brown adipocytes needs to be considered [40]. The circadian rhythm induces oscillation in gene expression and protein abundance of metabolic enzymes and carriers. This results in an autonomous oscillation in nutrient uptake and utilization, directly affecting the thermogenic capacity of BAT. The cell-autonomous rhythm is synchronized to environmental cues ‘zeitgebers’ (time-givers), such as light exposure and food intake [40]. Thus, BAT activity follows a diurnal rhythm with a peak at the onset of awaking that is aligned with the biological rhythm (**Figure 2**). However, the cell-autonomous regulation interacts with diverse hormonal and nervous inputs sensing the nutritional and physiological status of the organism, resulting in a context dependent diurnal rhythm [40]. Thus, the following section discusses difference in energy utilization with respect to the diurnal rhythm of BAT activity.

**Diurnal glucose uptake by BAT**

Cultured human brown adipocytes, synchronized by serum shock, rhythmically express *GLUT4* mRNA and take up glucose upon translocation of GLUT4 to the plasma membrane in an approximately 30-hour cycle [14]. This rhythmic uptake is amplified by the presence of insulin [14]. The peak of glucose uptake by BAT in mice is reached around ZT 9 (zeitgeber time), where ZT 0 denotes the time at the onset of the light phase, while lowest glucose uptake is predicted around ZT 21 [41]. Importantly, the brown adipocyte molecular clock appears to act independently of corticosterone and sympathetic outflow [42], whereas the diurnal glucose uptake is under the control of both [43].

**Diurnal lipid uptake by BAT**

In humans, BAT lipid uptake is maximal at the onset of the wakeful phase (**Figure 2**). In the morning subjects with high BAT activity, defined by FDG uptake, expend more postprandial energy through fatty acid oxidation than those with low BAT activity that show a preference for glucose oxidation [27]. Remarkably, the differences in cold-induced thermogenesis fueled by fatty acid oxidation between individuals with high and low BAT activity are only distinguishable in the morning and not in the evening [27].

Murine studies revealed that lipid uptake by BAT oscillates at a higher amplitude than that of other metabolic organs, peaking at the onset of the dark phase [43]. The diurnal lipid uptake pattern is under the regulation of LPL activity that oscillates according to the intrinsic circadian gene expression of *Lpl* and *Angptl4* in brown adipocytes, rather than the sympathetic nervous system since the diurnal lipid uptake rhythm is maintained under thermoneutral (30°C) conditions, which per se blunts sympathetic outflow into BAT [43]. Further regulatory mechanisms of diurnal lipid utilization are summarized in **Box 2**.

Cross-regulatory mechanisms of metabolic flux switching

In nutrient-rich conditions, BAT with high thermogenic capacity burns excess energy substrates. However, the preference for either fatty acids or glucose might be related to diurnal activity [27] or insulin sensitivity [44], or both. However, the cold-induced thermogenic capability of BAT does not always reflect postprandial thermogenesis [45]. In line with this, habitual cold exposure does not alter the responsiveness to postprandial thermogenesis [46]. Thus, diet- and cold-induced thermogenesis appear to be under the regulation of various stimuli, creating a complex context dependent regulatory network. A better understanding of this network is important, when BAT activity should be pharmacologically increased to reduce selective nutrients such as glucose or lipids. However, there is a rapidly increasing number of hormonal and metabolite regulators of BAT activity and substrate utilization. Thus, the following sections discuss selected examples of stimuli regulating BAT thermogenesis.

**Neurotransmitters**

The role of noradrenaline and chemical mimetics thereof on brown fat thermogenesis have been extensively discussed here and in the literature [10,11]. However, it is often forgotten that in addition to noradrenaline, several other neural inputs regulate BAT thermogenesis. This is illustrated by studies using triple ADRB1-3 knockout mice that generate heat in response to sympathetic nervous activation [7], demonstrating that additional factors are released from the nerve terminal regulating brown adipocyte activity. The following sections discuss the role of adenosine and ATP, as examples, in terms of energy utilization. The detailed molecular mechanisms underlying the mechanism of action of the purinergic receptor-signaling pathways in adipocytes are discussed elsewhere [47].

***ATP/ Adenosine***

Extracellular ATP promotes mitochondrial uncoupling (**Box 3**) and stable ATP analogues induce *Ucp1* gene expression in cultured brown adipocytes [7]. Albeit, activation of ADRB3 triggers the release of ATP through pannexin 1 [48] ATP is also released together with noradrenaline from secretory granules of neurons [7]. However, relatively little is known about the exact receptor(s) within the P2Xs and P2Ys families that mediate the effects of extracellular ATP. Several purinergic calcium channels (P2X), as well as purinergic G-protein coupled receptors (P2Y) are expressed on brown adipocytes. Among them, the ATP activated calcium channel P2RX5 shows highest expression in brown adipocytes compared to various other tissues [24]. Interestingly, *P2rx5* mRNA expression positively correlates with *Ucp1* mRNA expression in brown adipocyte subpopulations [49]. Thus, P2RX5 could be the main ATP activated calcium channel regulating brown adipocyte activity. However, most humans have a single nucleotide polymorphism (SNP) in the *P2RX5* gene, resulting in a loss of function variant. Thus, the contribution of P2RX5 mediated ATP effects in human BAT remains unclear.

In addition to ATP adenosine is also released from excited efferent sympathetic nerve terminals inducing lipolysis and energy expenditure through binding to adenosine A2A receptor (A2AR), a member of Gs-protein coupled receptor, on brown adipocytes with an additive effect on noradrenaline/ADRB function [8]. Moreover, adenosine increases vasodilation and perfusion within BAT further promoting substrate delivery and metabolic activity of brown adipocytes. Interestingly, adenosine triggered energy expenditure associates with fatty acid oxidation rather than carbohydrate utilization in young male subjects [50].

**Hormones/growth factor**

Fasting promotes the secretion of glucagon from pancreatic α-cell and FGF21 from the liver to maintain basal blood glucose levels. Glucagon indirectly induces BAT thermogenesis through FGF21 [51], which activates hypothalamic FGF receptor/β-Klotho signaling followed by excitation of sympathetic neurons allowing higher energy expenditure in response to elevation of fatty acids as shown by high-fat diet feeding [52]. Chronotropic administration of FGF21 in diet-induced obese mice increased energy expenditure even at thermoneutrality, suggesting that FGF21 also directly acts on BAT [53]. Moreover, FGF21 acts as an insulin sensitizer to accelerate glucose disposal by BAT but the molecular mechanism is unclear [54].

In contrast, ingestion of dietary carbohydrates promotes secretion of insulin. The role of insulin in brown adipocyte glucose uptake is well characterized (**Box 1**). Moreover, it has been shown that in humans with high BAT activity and whole-body insulin sensitivity, postprandial BAT activity depends on glucose rather than fatty acids that are also taken up from the circulation but are used for lipid synthesis rather than oxidation [44]. Acute cold exposure induced lipid uptake mediated by LPL and CD36 appears insulin dependent [39], albeit the effects of acute cold exposure on insulin secretion remain controversial [44,55,56].

Secretion of secretin, an intestinal hormone, also increases postprandially to mediate satiety. Recently, it was shown that postprandial secretin levels in blood positively correlate with fatty acid and glucose uptake in BAT of human subjects [57]. Moreover, secretin directly acts on BAT to increase thermogenesis through a secretin receptor/cAMP/PKA pathway. This leads to an activation of HSL/ATGL resulting in lipolysis, independent of efferent sympathetic outflow into brown adipocytes [57]. Interestingly, as outlined above, postprandial BAT thermogenesis relies primarily on carbohydrate oxidation, probably due to effects of postprandial insulin secretion suppressing lipolysis and promoting anabolism of fatty acids [44]. In line with this, secretin administration increased FDG uptake that associates with increased energy expenditure in humans [58]. Thus, the role of secretin induced lipolysis to postprandial BAT activity remain to be studied in more detail.

**Signaling Molecules Regulating Substrate Utilization**

**The mechanistic target of rapamycin complex 1 and 2 (mTORC1/2)**

Extracellular signal integration occurs only in few instances at the receptor level, but is most often a result of interacting signaling pathways. A perfect example for this signal integration is the activation of the mechanistic target of rapamycin complex 1 (mTORC1). mTORC1 is a central intracellular energy sensor that integrates various external stimuli to adapt cellular metabolism to energy availability. In addition, mTORC2 regulates key cellular processes such as proliferation and survival and is important for Akt and mTORC1 activation. Asparagine was shown to promote glucose utilization by regulation of mTORC1 dependent protein synthesis of glycolytic enzymes [59]. Additionally, mTORC1 and 2 mediate insulin-induced glucose uptake [13,60]. Upon sympathetic activation, mTORC1 regulates lipolysis, mitochondrial biogenesis and oxidative metabolism [13,61]. In contrast, the activation of mTORC2 by adrenergic stimulation triggers glycolysis without affecting β-oxidation and mitochondrial uncoupling [62]. The role of mTORC1 and 2 in regulating catabolism in presence of both insulin and noradrenaline remains to be determined (**Box 1**).

Importantly, mTORC1 and 2 activity are differently regulated in BAT of mice exposed to cold. Adrenergic stimulation activates mTORC1 and mTORC2 with similar kinetics in cultured brown adipocytes. However, in mice, cold persistently activates mTORC1 whereas mTORC2 activity declines by prolonged cold stimulation [63]. The decline in mTORC2 activity promotes transcription of genes for lipid catabolism and UCP1 and suppression of Akt2/ChREBP-mediated *de novo* lipogenesis [60,64,65].

Intracellular Interactions of Substrate Utilization

The last section discusses metabolic flexibility in brown adipocytes depending on nutrient availability and external stimulation. At baseline, cultured brown adipocytes flexibly utilize available energy substrates such as glucose, fatty acids, and glutamine. Thus, each substrate has a similar capacity to compensate for the lack of others in cultured brown adipocytes [66]. However, changes in the nutritional state of the organism, such as fasting, impact on substrate availability and the preferential substrate utilization in brown adipocytes *in vivo* through a complex regulatory network. Therefore, *in vitro* studies easily provide an over simplified view on substrate utilization that poorly reflects the *in vivo* situation. This should not indicate that *in vitro* studies do not provide important insights into cellular function. Instead, great care should be taken when translating these findings to an *in vivo* situation. For example, fasting leads to *Pdk4* transcription by the transcription factor KLF15 [67]. PDK4 then inactivates pyruvate dehydrogenase (PDH) restricting pyruvate entry to the TCA cycle. This results in a preferential oxidation of fatty acid over glucose [67].

Conversely, adrenergic stimulation leads to PDH activation allowing pyruvate to enter the TCA cycle promoting uncoupled respiration [18] with a higher capacity of fatty acid oxidation [66] (**Figure 3A**). Interestingly, aerobic glycolysis following a large amount of glucose uptake is indispensable for fatty acid utilization [66] and thermogenesis [12,68], rather than lipogenesis (**Figure 3B**). When pyruvate supply to mitochondria is limited, such as by pharmacological inhibition [5], increased ATP-coupled respiration accounts for a majority of total oxygen consumption to replenish ATP consumed in the futile lipid cycle between esterification and acyl-glycerol breakdown (**Figure 3C**). This futile cycle probably produces heat by ATP hydrolysis contributing to thermogenesis, rather than uncoupled respiration. Instead of pyruvate, glutamine/glutamate can feed into the TCA cycle as α-ketoglutarate serving as carbon-body through the malate-aspartate shuttle [5]. However, glutamine cannot compensate for pyruvate deprivation upon adrenergic stimulation [18,66]. Instead, glutamine derived citrate and acetyl-CoA are used for the futile cycle via *de novo* lipogenesis [5]. This could be an explanation for the switch from mitochondrial uncoupling to futile cycles. In other words, fatty acid utilization is not necessarily linked to mitochondrial uncoupling as fatty acid utilization is also observed in *Ucp1-*knockout cells where respiratory capacity should be restricted [69].

Thus, activated brown adipocytes oxidize fatty acids to acetyl-CoA for mitochondrial thermogenesis, using glucose as the carbon-body in the TCA cycle. In glucose (or pyruvate) deprived conditions, however, thermogenesis switches to futile cycles as described above. Moreover, the protein futile cycle consumes ATP in the process of protein synthesis and degradation, also generating heat when mitochondrial respiration is restricted (**Figure 3C**). However, the physiological significance of these pathways remains unclear [6]. Interestingly, however, the protein futile cycle itself increases glucose uptake, most likely to compensate for ATP consumption. The versatility of glucose besides a role of carbon-donor in activated brown adipocytes is discussed in **Box 4**.

**Concluding remarks and future perspectives**

Here we provide an overview of the complex regulatory elements determining utilization of glucose, fatty acids, and amino acids, also questioning the current ADRB3 centric view of brown adipocyte thermogenesis. Glucose and lipid utilization in brown adipocytes are integrated into whole-body energy homeostasis. This follows a diurnal rhythm, in which brown adipocyte activity is synchronized with awaking under the control of external inputs such as noradrenaline, other neurotransmitters and a variety of hormones and signaling metabolites. In this context, it appears reasonable to assume that sympathetic activity in response to environmental stresses rapidly directs energy preference, followed by slower modulation by hormonal inputs dependent on nutrient availability in brown adipocytes.

However, important differences between rodents and humans exist with regards to cellular composition and activity, as well as the physiological impact of BAT [70,71].

Active BAT exhibits the highest glucose and lipid disposal capacity among organs when normalized to a mass. However, total BAT activity only accounts for a few percent of whole-body energy disposal in humans. Thus, acute cold exposure does not decrease blood glucose, free fatty acids and triglycerides in humans [37,44]. In contrast, activation of BAT in mice can be responsible for disposal of excess blood glucose [12] and lipids [72] and account or up to 60% of energy expenditure in mice housed at 4°C [1]. Nevertheless, presence of detectable cold-activated BAT reduced metabolic disease in obese young adults [73]. In addition supraclavicular BAT in adult humans is composed of adipocytes showing features of murine beige, brown and white adipocytes, indicating that human BAT mass and function could be under an even more complex neural and hormonal regulation than murine BAT.

All these factors could contribute to the observed species differences in nutrient utilization. Intracellular lipolysis accounts for a larger proportion in acute cold-induced thermogenesis in humans than mice, which relies more on the supply from the circulation [15,21,56]. In addition, glucose uptake in humans appears under greater control of insulin, through its dependency on GLUT4, compared to GLUT1 mediated glucose uptake in mice.

Many external inputs trigger lipid catabolism through the cAMP/PKA pathway in the brown adipocytes. However, surprisingly little is known about the role of other signaling pathways in the regulation of substrate utilization. In addition, metabolic enzyme activity is also allosterically regulated by substrate abundancy. Thus, nutrient uptake itself can regulate substrate preference. In addition, the augmentation of β-adrenergic induced oxygen consumption by α-adrenergic stimulation is one example of integrating intracellular calcium dynamics with different extracellular cues to modulate energy utilization. Mitochondrial enzyme activity is regulated by mitochondrial calcium levels. Thus, it will be interest to see how intracellular calcium mobilization in response to external inputs modulates brown adipocyte metabolism. Additionally, although not detailed here, post-translational modifications such as acylation and phosphorylation of mitochondrial enzymes, regulated by sirtuins or kinase/phosphatase, respectively, also greatly affect metabolic enzyme activities [60,74,75]. Thus, a better understanding of the intracellular signaling networks integrating various extracellular cues will further deepen our knowledge on the molecular mechanism underlying maximal activation of brown adipocytes. Furthermore, many activators of brown adipocytes have been characterized but very little is known about inhibitors that are required for rhythmic BAT activity. (**Outstanding questions Box**).

Active brown adipocytes rely on fatty acid oxidation, using the TCA cycle with carbon-bodies provided by glucose for uncoupled respiration. Moreover, increased glucose uptake, and increased glycolytic flux could be required to satisfy the cellular demand for ATP created by a switch to uncoupled respiration. Impaired uncoupled respiration or limitation of TCA intermediates will induce a switch to futile cycle-oriented thermogenesis in brown adipocytes, which itself will further drive glucose uptake and glycolysis. Thus, increased glucose uptake does not necessarily indicate uncoupled respiration. UCP1-independent futile cycles consuming glucose and fatty acids through ATP production could also provide interesting therapeutic strategies to dissipate excessive energy. However, these mechanisms appear insufficient to maintain euthermia upon cold exposure and even worsen insulin sensitivity [68].

Taken together, this review emphasizes that the key role of brown adipocytes in systemic metabolism could be to maintain whole-body nutrient homeostasis through different mechanisms of energy expenditure. Therefore, further research is required to fully understand the cross-regulatory mechanisms of substrate utilization in context of orchestrating systemic nutrient balance. (**Outstanding questions Box**).

**Glossary**

**Mitochondrial uncoupling:** dissipating a proton electrochemical gradient across the inner mitochondrial membrane through uncoupling protein (UCP1) to generate heat instead of ATP synthase (coupling).

**Text Boxes**

**Box 1. Interplay of Insulin and Noradrenaline**

Glucose uptake in human brown adipocytes is highly sensitive to insulin compared to white adipose tissue, possibly due to high levels of GLUT4 expression in supraclavicular adipose tissue [55]. Insulin and β-adrenergic stimulation activate pyruvate dehydrogenase (PDH) a fuel switch in mitochondria and promote glucose uptake [18,76]. Noradrenaline-induced glucose uptake is partially mediated by AMPK activation through ADRB1/2, but not ADRB3 [77]. Co-treatment with noradrenaline blunts insulin-dependent glucose uptake, glycolysis and fatty acid synthesis while increasing oxygen consumption in rat brown adipocytes [78]. However, it remains unclear if this can be extrapolated to human physiology as mild cold exposure reduces blood insulin levels [55]. Moreover, human BAT takes up more glucose in the fasting state than in the postprandial state, which should raise insulin levels, whereas skeletal muscle takes up glucose mainly after eating [79].

**Box 2. Diurnal Lipid Utilization**

**Intrinsic regulation:** During acute cold exposure circulating lipids are the main energy source for BAT. However, intracellular lipid mobilization is the main source for energy production upon chronic cold exposure in BAT of humans [80] and mice [81]. The diurnal rhythm of gene expression related to fatty acid oxidation and *de novo* lipogenesis are under the regulation of SREBP1c and fluctuate synchronously with a peak at ZT 16 in BAT of chronic cold-exposed mice [81]. These diurnal rhythms of gene expressions are absent in BAT under thermoneutrality [81]. During the light phase (resting, ZT 0-12), when triglyceride content is lowest in BAT, food consumption remains high [81]. In other words, the energy obtained from the oxidation of fatty acids synthesized by *de novo* lipogenesis in BAT to maintain body temperature cannot be compensated by oxidation of circulating lipids and glucose [81].

**Hormonal regulation:** Glucocorticoid (corticosterone in rodents) is an oscillatory hormone with a peak at the middle of the wakeful phase affecting BAT activity. Interventions that reduce blood corticosterone levels abolish the peak of BAT lipid uptake at awaking [82]. However, the mechanisms remain unclear as adipocyte-specific glucocorticoid receptor deficient mice show intact diurnal lipid uptake [82].

The circadian rhythm of blood glucocorticoid level synchronizes with adrenocorticotropic hormone (ACTH) that promotes secretion of glucocorticoid, making a feedback loop to suppress ACTH secretion. ACTH levels are augmented by cold exposure [83] and supraphysiological ACTH stimulation activates Gs-coupled receptor/cAMP/PKA signaling leading to lipolysis and increased mitochondrial respiration in brown adipocytes [84]. In contrast, long term dexamethasone treatment, a mimetic of corticosterone, blunts cAMP production and mitochondrial respiration [84]. These suggest that the ACTH-glucocorticoid axis could regulate brown adipocyte activity whereby ACTH stimulates fatty acid utilization whereas glucocorticoid suppresses further activation by suppression of gene expression.

**Box 3. ATP Action in Brown Adipocytes**

Brown adipocytes express two types of purinergic receptor; P2X: ligand-gated cation channel provoking Ca2+ flux across the plasma membrane and P2Y: Gq-protein-coupled receptor leading Ca2+ release from stores [9]. ATP treatment raises cytosolic Ca2+ level to a similar extent as α-adrenergic receptor agonists. ATP treatment can mildly increase heat production in isolated rat brown adipocytes although not to the extent of noradrenaline [85]. The effect of noradrenaline on oxygen consumption is maximal by engaging α- and β-adrenergic signaling [86], suggesting that not only cAMP but also an increase in cytosolic Ca2+ regulates thermogenesis. However, no additive effect of ATP on noradrenalin induced heat production is measurable in brown adipocytes [85]. Thus, it remains unclear to what extend Ca2+ dynamics are linked to substrate utilization and oxygen consumption in brown adipocytes.

**Box 4. The Versatility of Glucose in Thermogenesis**

**Glycolysis serves as source for ATP upon mitochondrial uncoupling**

In a steady state a subset of mitochondria are localized in proximity to lipid droplets (lipid-droplet associated mitochondria) that produce ATP through coupled oxidative respiration, which in part is used for lipogenesis. However, in an activated state, mitochondrial fission results in a dissociation from lipid droplets and a switch to uncoupled respiration fueled by fatty acids provided by lipolysis [87]. Thus, glycolysis could compensate the drop in mitochondrial ATP production. Indeed, chemical uncoupling increases glucose uptake and lactate production, an indication of glycolytic activity [66]. Moreover, cold-induced lactic acid production is abolished in *Ucp1*-knockout mice [69]. An advantage of aerobic glycolysis is that lactic acid acts as a redox carrier in the metabolism of pyruvate by lactate dehydrogenase, providing NAD+ that is recycled later in glycolysis [88]. Moreover, excess lactic acid is excreted through monocarboxylate transporter(s) [12], suggesting that this efflux system enables sustainable intracellular redox cycling (**Figure 3A and C**).

**Pentose Phosphate Pathway for Oxidative Stress Defense**

Mitochondria are the main source of reactive oxygen species (ROS) in cells. ROS generated through succinate oxidation is important for UCP1-dependent respiration [89]. Furthermore, mitochondrial ROS stabilizes HIF1α protein, inducing glycolytic gene transcription [90]. Inversely, excess ROS damages mitochondria. Thus, ROS levels need to be tightly controlled. An important reductant is NADPH, synthesized through the pentose phosphate pathway (PPP) branched off from glycolysis. Knockdown of the first glycolytic enzyme hexokinase 2 has a stronger impact on the reduction of oxidative respiration in response to β-adrenergic stimulation, compared to knockdown of the last glycolytic enzyme pyruvate kinase [66]. This suggests that glycolysis is important in active brown adipocytes, not only for ATP production but also for intermediates produced through the PPP. Indeed, cold exposure redirects glucose to the PPP, which is not observed in *Ucp1*-knockout mice [69]. Hence, PPP could protect against surplus oxidative stress in mitochondria [17,69].

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**Author contribution**

All authors studied and wrote the manuscript.

**Declaration of Interests**

The authors declare no competing interests

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**Figure Legends**

**Figure 1. External inputs regulating brown adipose tissue activity**

Brown adipose tissue (BAT) is regulated by the sympathetic nervous system, diet nutrients, and hormones. Sympathetic nerve excitation releases noradrenalin, adenosine, and ATP. Activation of BAT by the sympathetic nervous system induces uptake of circulating triglycerides and fatty acids liberated from white adipose tissue (WAT) by lipolysis in response to adrenergic stimulation. Ingestion of dietary nutrients leads to heat production by BAT (postprandial energy expenditure), which in part is regulated by secretin and insulin. During fasting, glucagon indirectly activates BAT through FGF21 and hypothalamic FGF receptor. FGF21 also directly activates BAT. Diurnally secreted hormones ACTH and glucocorticoids balance BAT activity. The encircled symbols of plus and minus indicate stimulation or suppression of energy catabolism in BAT, respectively. This figure was created using  BioRender (<https://biorender.com/>).

**Figure 2. Relationship between diurnal hormonal rhythm, energy utilization and BAT activity**

Left graphs show diurnal rhythm of each external input. Glucose uptake peaks about 3 hours earlier than lipid uptake that peaks at onset of awaking when BAT activity is maximized. The circled symbols of plus, minus and slash indicate stimulation, suppression or no-effect of energy catabolism in BAT, respectively. Controversial evidences are shown with plus and no-effect. No evidence is shown as blank. This figure was created using  BioRender (<https://biorender.com/>).

**Figure 3. Intracellular energy substrate utilization**

(**A**) Glucose (left) and lipid utilization (right) upon noradrenergic (Gs-coupled protein receptor: GsPCR) stimulation. Glucose is taken up through GLUT1/4 and catabolized into pyruvate (Glycolysis). Glycolysis branches to the pentose phosphate pathway (PPP). Pyruvate is converted to lactate by LDH that provides NAD+ for glycolysis. Pyruvate enters the mitochondrial matrix through MPC1/2 and is catabolized by PDH to acetyl-CoA fueling for the TCA cycle. On the other hand, lipid uptake from the circulation and lipolysis are induced by GsPCR/cAMP/PKA stimulation. Free fatty acids are oxidized into acetyl-CoA feeding into the TCA cycle. The TCA cycle provides electron carriers to the electron transport chain (ETC) creating a proton gradient across the inner-mitochondrial membrane. UCP1 dissipates the proton gradient generating heat. (**B**) Upon insulin stimulation, glucose supplies glycerol 3-phosphate (G3P) branched off from glycolysis for esterification of free fatty acids to store in intracellular lipid droplets. (**C**) UCP1-independent futile cycle thermogenesis is an alternative system when pyruvate is deprived (left) and the TCA cycle is limited (right). The lipid futile cycle (left) generates heat by hydrolysis of ATP in the re-esterification of fatty acids liberated by lipolysis. ATP is synthesized in ATP-coupled respiration in the mitochondria fueled by glutaminolysis. Protein futile cycle (right) generates heat by hydrolysis of ATP between protein synthesis and degradation. ATP is produced by glycolysis, which is accelerated by lactate production. The thickness of arrows indicates the relative flow rate. This figure was created using  BioRender (<https://biorender.com/>).