Modulators of UCP1-dependent thermogenesis

Glucocorticoids, diet and novel research models

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Abstract

The activation and recruitment of brown adipose tissue (BAT) thermogenesis has been put forward as a promising strategy to reduce the disease burden of obesity and obesity-related diseases. Heat production by BAT can be attributed to the tissue-specific mitochondrial uncoupling protein 1 (UCP1). Upon activation, UCP1 uncouples substrate oxidation from ATP production, thereby dissipating energy solely as heat and thus facilitating the ‘wasting’ of energy. To date, cold exposure is the strongest known BAT activator. However, to harness the energy wasting potential of BAT as a weight-reducing agent, the search for alternative factors that alter the activation or recruitment state of BAT is ongoing. The goal of this thesis is to obtain a better understanding of compounds and processes that modulate UCP1-dependent thermogenesis.

We investigate glucocorticoids for their potential to alter the UCP1-dependent thermogenic capacity of mice. We provide the novel insight that glucocorticoid supplementation reduces total BAT UCP1 protein levels, but only in mice housed at thermoneutrality. This reduction occurs at the transcriptional level by direct binding of the liganded glucocorticoid receptor to Ucp1 regulatory regions. We also demonstrate that the glucocorticoid-induced reduction in BAT thermogenesis does not contribute to the development of glucocorticoid-induced obesity.

Further, we show that high-fat diet- and cafeteria diet-feeding induces the activation and recruitment of BAT UCP1 protein in the obesity-resistant 129S mouse strain. We demonstrate the importance of this diet-induced modulation of BAT thermogenic capacity by reporting an increased metabolic efficiency in UCP1-ablated mice compared to wild-type mice.

We finally present two novel models that can be used for the identification of novel modulators of BAT thermogenesis, namely a brown adipocyte clonal cell line derived from adult human BAT, and a UCP1-luciferase reporter mouse which facilitates real-time tracking of endogenous Ucp1 expression. Using these models, we identify the genes Mtus1 and Kcnk3, and the compound WWL113, as novel modulators of UCP1-dependent thermogenesis.

Keywords: brown adipose tissue: UCP1: glucocorticoids: diet-induced thermogenesis: obesity: physiology.
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Cover image by Ineke Luijten. Microscopy images of Phoenix cells transfected with a MSCV-GFP vector, differentiated WT1 cells stained with Oil Red O, and brown adipose tissues isolated from wild-type or DAdKO mice stained with hematoxylin & eosin.

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To my family
The great revelation had never come.  
The great revelation perhaps never did come.  
Instead there were little daily miracles,  
Illuminations,  
Matches struck unexpectedly in the dark.  

- Virginia Woolf, To The Lighthouse
This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Glucocorticoid-induced obesity develops independently of UCP1.**
   *Under review for Cell Rep.*

II. **Ucp1 transcription is regulated by glucocorticoid receptor binding at Ucp1 regulatory regions.**
    *Preliminary manuscript*

III. **Genetic and functional characterization of clonally derived adult human brown adipocytes.**
    *author contributed equally
    *Nat. Med. 2015. 21(4), 389-394*

IV. **Thermomouse: an in vivo model to identify modulators of UCP1 expression in brown adipose tissue.**
    *Cell Rep. 2014. 9(5), 1584-1593*

V. **In the absence of UCP1-mediated diet-induced thermogenesis, obesity is augmented even in the obesity-resistant 129S mouse strain.**
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ABBREVIATIONS

(gene name)-KO  (gene name) - knock-out
18FDG-PET  18F-fluoro-2-deoxy-D-glucose positron emission tomography
ACTH  Adrenocorticotropic hormone
ATF-2  Activating transcription factor-2
ATP  Adenosine triphosphate
ATGL  Adipose triglyceride lipase
(β3)AR  (beta-3) Adrenergic receptor
(I)BAT  (Interscapular) Brown adipose tissue
BMI  Body-mass index
BMP  Bone morphogenetic protein
BSA  Bovine serum albumin
C/EBP  CCAAT-enhancer-binding protein
cAMP  Cyclic adenosine monophosphate
CREB  cAMP-response element binding-protein
CRH  Corticotropin-releasing hormone
DEX  Dexamethasone
DNP  2,4-Dinitrophenol
FFA  Free fatty acid
FGF  Fibroblast growth factor
GDP  Guanosine diphosphate
GR  Glucocorticoid receptor
GRE  Glucocorticoid response element
HPA-axis  Hypothalamus-pituitary-adrenal-axis
HSD1/2  11β-hydroxysteroid dehydrogenase 1 and 2
HSL  Hormone-sensitive lipase
MR  Mineralocorticoid receptor
NE  Norepinephrine
OCR  Oxygen consumption rate
P38 MAPK  p38 Mitogen-activated protein kinase
PGC-1α  PPARγ coactivator-1α
PKA  Protein kinase A
POMC  Pro-opiomelanocortin
PPAR(γ)  Peroxisome proliferator-activated receptor (γ)
UCP1  Uncoupling protein 1
(Ing)(Is)WAT  (Inguinal)(Interscapular) White adipose tissue
INTRODUCTION TO UCP1-DEPENDENT THERMOGENESIS

1.1 THE BROWN

The ability to maintain a body temperature that is optimal for metabolic processes to occur is vital for eutherian mammals. Throughout the course of evolution, mammals have developed several strategies to defend their body temperature while being exposed to low environmental temperatures. One line of defense is shivering, i.e. the production of heat by involuntary muscle activity. Since shivering is not only uncomfortable but also limits a mammal’s ability to perform other tasks, brown adipose tissue (BAT) may have evolved as the main site for non-shivering thermogenesis to provide a more comfortable way of producing heat upon prolonged cold exposure.

BAT is considered to be a part of the adipose organ together with white adipose tissue (WAT) and brite/beige adipose tissue (WAT that can be induced to express brown-like characteristics) (Cinti, 2012). BAT is located at distinct sites throughout the body. In humans, the main BAT depots can be found in the supraclavicular, cervical, mediastinal and perirenal regions (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Nedergaard et al., 2007). In rodents, the main BAT depots can be found in the interscapular, axillary and cervical regions (Frontini and Cinti, 2010)(Fig. 1). Generally, the inguinal WAT depot is considered to be the most prone to ‘britening/beiging’.

Although BAT can and does store large amounts of energy in the form of triglycerides, BAT’s primary function is not energy storage, in contrast to WAT. Instead, BAT is specialized in producing heat upon cold exposure by the oxidation of substrates. BAT’s heat-producing capacity can be fully attributed to its high content of uncoupling protein-1 (UCP1), a member of the mitochondrial carrier protein family (Nedergaard et al., 2001). Located in the inner mitochondrial membrane, UCP1 creates a leak for protons (generated by the electron transport chain) to move from the mitochondrial intermembrane space into the mitochondrial matrix. By moving through UCP1, the protons circumvent the ATP synthase, and the proton gradient across the mitochondrial inner membrane that is usually used to create ATP is thus dissipated solely as heat.

BAT’s capacity for non-shivering thermogenesis is adaptive, meaning that brown adipocyte proliferation, mitochondrial biogenesis and UCP1 synthesis and activation are enhanced upon prolonged cold exposure or exposure to progressively decreasing
environmental temperatures. In humans and rodents, environmental temperature is sensed by a group of temperature sensitive ion-channels of the TRP (transient receptor potential channel) superfamily present on peripheral nerve endings (Zhang, 2015). Temperature can be sensed through these receptors both at the skin and the spinal cord. The signals from these receptors are integrated in distinct brain areas and ultimately signal to the hypothalamus where a perceived cold environment will lead to excitation of sympathetic nerves signaling to BAT (Morrison, 2016). In sympathetic peripheral neurons, catecholamines (among which is norepinephrine, NE) are produced from L-tyrosine, the rate-limiting step being catalyzed by tyrosine hydroxylase. BAT is richly innervated by fibers of the sympathetic nervous system, and brown adipocytes express α1, α2, β1, β2 and β3 adrenergic receptors (AR’s), of which the β3-adrenergic receptor (β3AR) is the most significant for thermogenesis in rodents (Bartness et al., 2010). Thus, when NE is released from sympathetic nerves upon cold exposure, it binds to β3ARs on the surface of brown adipocytes and sets off an intracellular signaling cascade (Cannon and Nedergaard, 2004)(overview in Fig. 1).

β3ARs belong to the G protein-coupled receptor class of membrane proteins, which means that they undergo a conformational change upon ligand binding which ultimately leads to dissociation of a G-protein subunit (in BAT of the Gαs subtype). The Gs protein successively activates adenylyl cyclase, an enzyme that catalyzes the conversion of ATP into cAMP and PPI, leading to an intracellular accumulation of cAMP (Paper II). cAMP functions as a crucial second messenger in brown adipocytes. Intracellular cAMP concentrations are determined by the rate of synthesis by adenylyl cyclase on the one hand and the rate of degradation by phosphodiesterases on the other hand (Coudray et al., 1999). cAMP activates protein kinase A (PKA) by binding to its regulatory subunits, releasing and thus activating the catalytic subunits. PKA phosphorylates a series of target proteins, among which are cAMP-response element binding-protein (CREB), p38 mitogen-activated protein kinase (p38 MAPK), hormone-sensitive lipase (HSL) and perilipin (Cao et al., 2004; Thonberg et al., 2002). Both PKA and perilipin have been shown to also successively regulate adipose triglyceride lipase (ATGL) activity (Pagnon et al., 2012; Wang et al., 2011). P38 MAPK successively continues the signaling cascade by phosphorylating activating transcription factor-2 (ATF-2) and peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) (Cao et al., 2004). The collective effects of activation of these downstream targets are activation and recruitment of BAT, through a release of free fatty acids (FFAs) from lipid droplets (ATGL, HSL and perilipin), an increase in mitochondrial biogenesis (PGC-1α) and activation of Ucp1 transcription at the proximal promoter and distal enhancer regions in the 5’ non-coding region of the Ucp1 gene (CREB, ATF-2) (Villarroya et al., 2017)(overview in Fig. 1).

Complementary to the adrenergic signaling system, BAT thermogenesis can also be activated by natriuretic peptides secreted by the heart (Shi and Collins, 2017). De
\textit{novo} recruitment of UCP1 is also facilitated by transcription factors such as C/EBPs (CCAAT-enhancer-binding protein) and PPARs, which regulate \textit{Ucp1} gene transcription (Sears et al., 1996; Yubero et al., 1994).

The intracellular release of FFAs upon adrenergic stimulation of BAT is generally accepted to activate heat production. In the inactivated, ‘resting’, state, purine nucleotides (e.g. GDP, ATP) are bound to UCP1 and fully prevent protons from leaking through the inner mitochondrial membrane (Shabalina et al., 2010). Through a yet unknown mechanism, FFAs (probably) activate UCP1 and thereby induce the uncoupling of substrate oxidation from ATP synthesis, leading to heat production (Bouillaud et al., 2016). Thus, adrenergic signaling in BAT both activates the UCP1 that is already present in the mitochondria through increasing the release of FFAs from brown adipocyte lipid droplets, and initiates additional transcription of \textit{Ucp1} through activation of transcription (co-) factors CREB, ATF-2 and PGC-1\(\alpha\). Additionally, FFAs serve another vital purpose in BAT thermogenesis, namely being used as substrates for oxidation, continuously ensuring the generation of sufficient protons for maintenance of the proton gradient across the inner mitochondrial membrane, thus continuously fueling thermogenesis.

\textbf{1.2 The BRITE}

As mentioned above, BAT’s heat production is fully dependent on the presence of UCP1. In contrast to brown adipocytes, ‘classical’ white adipocytes do not possess UCP1, meaning that they have practically no capacity for uncoupled respiration and thermogenesis. However, it has long been known that a subset of white adipocytes develops brown-like characteristics in mice exposed to cold (Young et al., 1984). The formation of these ‘brite’ (brown-like in white) adipocytes can also be induced by treating mice with an adrenergic agonist (Paper IV). The presence of white adipocytes that can acquire brown-like characteristics upon cold exposure or adrenergic stimulation has also been reported in humans (Chechi et al., 2017; Finlin et al., 2018; Sidossis et al., 2015).

When properly stimulated, the histological appearance of brite adipocytes switches from white-like to more brown-like. As mentioned above, BAT is richly innervated by fibers of the sympathetic nervous system. In addition, BAT is highly vascularized to provide the tissue with plenty of oxygen and nutrients, and to transport any produced heat to the rest of the body. Individual brown adipocytes contain many, large, UCP1-containing mitochondria in their cytoplasm and small, multilocular lipid droplets (Paper I; Cinti, 2012) (Fig. 1). In contrast, white adipocytes, being the main site for energy storage, contain very little cytoplasm, few, small mitochondria and usually have one large, unilocular lipid droplet (Paper I). Brite adipocytes appear as an intermediate form between brown and white adipocytes, containing several smaller lipid
droplets and several medium-sized mitochondria (Cinti, 2012; Keipert and Jastroch, 2014)(Fig. 1). The mitochondria in brite adipocytes contain UCP1, and this UCP1 is functionally thermogenic (Shabalina et al., 2013). How exactly white adipocytes ‘turn into’ brite adipocytes upon stimulation has not been deciphered yet. Upon cold exposure, brite adipocytes may be derived either from existing mature adipocytes in a process named transdifferentiation (Barbatelli et al., 2010; Lee et al., 2015; Rosenwald et al., 2013), or from de novo differentiation of preadipocytes into brite adipocytes (Wang et al., 2013). Alternatively, brite adipocytes may always be characteristically brite, but are masked by their white appearance in the absence of an adrenergic stimulus.

Another difference between brown and brite adipocytes may be their molecular origin. It has been postulated that at least some brite adipocytes are derived from different embryonic precursors than brown adipocytes, which are thought to be derived mainly from a Myf5-positive lineage; the same lineage from which skeletal muscle develops (Seale et al., 2008; Timmons et al., 2007). However, more recent research suggests that brown, white and brite adipocytes can all develop from several lineages and thus have multiple developmental origins in themselves (Long et al., 2014; Sanchez-Gurmaches and Guertin, 2014).

1.3 THE PHYSIOLOGICAL SIGNIFICANCE OF UCP1-DEPENDENT THERMOGENESIS

The identification of BAT as a major thermogenic organ in rodents dates back several decades. In humans, the role of BAT in neonatal thermogenesis has long been established (Lean, 1989). Human newborns lose a lot of heat due to a high surface-to-volume ratio, and thus have a high need for heat production to maintain their body temperature and protect them from hypothermia. As humans age, their surface-to-volume ratio decreases, and they can to a certain extent remove themselves from cold environments. For these reasons, it was long believed that after infancy, humans rapidly lose any relevant amounts of BAT. The majority of early studies into BAT functioning were therefore primarily aimed at unraveling the tissue’s function in thermoregulation in e.g. hibernating mammals, with little interest for the role of BAT in adult human physiology.

In 2007, interest in human BAT re-ignited when researchers speculated that BAT may be present in adult humans after all. Several groups re-analyzed combined data from $^{18}$FDG ($^{18}$F-fluoro-2-deoxy-D-glucose)–PET scans and computer tomography (CT) that were used to trace tumor metastasis in human adults (Nedergaard et al., 2007). They found symmetrical areas of high glucose uptake in the upper part of the body that were proposed to be BAT. Successive studies have shown that these areas indeed represent BAT, and that upon cold exposure or molecular analysis of neck fat, the tissue
can be detected in a high percentage of the adult population (Lee et al., 2011; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). The re-discovery of BAT in adult humans initiated a renaissance in BAT research.

In earlier studies, the burning of substrate in BAT in the absence of ATP production has been shown to facilitate metabolic inefficiency in mice, meaning that less of the total amount of ingested energy is stored in the body. In small adult mammals, cold-activated BAT can receive up to 34% of cardiac output, and may contribute for 40% to metabolic rate (Foster, 1984; Rothwell and Stock, 1979). We show that merely housing mice in mild cold (21 °C) doubles their resting energy expenditure over that measured at thermoneutrality (30 °C) (Paper I) (Fig. 2). Cold-activated BAT also significantly increases energy expenditure in humans. The increase may vary from 0-14% of resting metabolic rate during prolonged cold exposure to up to 30% of resting metabolic rate in situations of acute cold exposure (van Marken Lichtenbelt and Schrauwen, 2011). BAT thermogenesis may also be activated by the consumption of specific (high-fat) diets, thereby mediating facultative diet-induced thermogenesis (von Essen et al., 2017; Feldmann et al., 2009; Rowland et al., 2016; Paper V) (Fig. 1). Thus, diet-activated BAT may burn off excess ingested calories and prevent mice or humans from gaining as much weight as they otherwise would. Indeed, inverse relationships between the activity of BAT and BMI, body fat content and visceral fat accumulation in humans have been shown (Saito, 2013; but see Paper V). In addition, cold-activated BAT contributes to the clearance of glucose and FFAs from the blood (Bartelt et al., 2011; Labbé et al., 2015). Thus, cold- or diet-activated BAT is currently widely believed to contribute to a healthier phenotype in both mice and men.

Upon cold exposure, the browning of WAT has also been proposed to contribute to energy expenditure and weight loss in mice additionally to the activation of BAT (Guerra et al., 1998). However, the precise contribution of recruited brite fat to energy expenditure is still under debate (Chapter 2).

The significant energy-burning thermogenic capacity of BAT has been put forward by many as a novel target for pharmacological interventions aimed at reducing the prevalence of obesity (BMI ≥ 30) and obesity-related diseases such as diabetes (fasting blood glucose ≥ 7 mmol/l). In 2016, 13% of the adult population worldwide was obese and in 2014, 8.5% of the adult population worldwide suffered from diabetes (WHO, 2018). As these percentages are still increasing, the need for novel therapeutic interventions grows larger every day. The pharmacological activation of UCP1-dependent thermogenesis in BAT may be (part of) the answer to the growing obesity epidemic. In addition, the activation of brite adipose tissue may possibly fulfill the same purpose, but due to brite fat’s questionable contribution to energy expenditure, the importance and necessity of studying the tissue as a potential therapeutic target to lower obesity also remains unclear (Chapter 2).
Nevertheless, if we want to explore the possibility of reducing obesity by means of UCP1 activation and recruitment, it is vital that the research community identifies compounds that modulate UCP1-dependent thermogenesis in BAT and that thereby modulate energy expenditure. The aim of this thesis is therefore to provide further insights into modulators of UCP1-dependent thermogenesis.

In the next chapter, I first discuss ways in which the modulation of UCP1-dependent thermogenesis can be adequately measured in mice as a model for humans (Chapter 2). In Chapter 2, I also discuss the consumption of specific diets as a modulator of BAT thermogenesis, and novel research models that may find future use as tools to identify novel modulators of UCP1-dependent thermogenesis. Successively, I discuss glucocorticoids as potential modulators of UCP1-dependent thermogenesis (Chapter 3). Finally, I provide an overview of the main conclusions of this thesis and of areas regarding the modulation of UCP1-dependent thermogenesis that require further research.

Fig. 1. (page 13) Schematic overview of UCP1-dependent thermogenesis. (A) Brown adipocytes (mainly located in the interscapular region) and brite adipocytes (mainly located in the inguinal region) are activated upon cold exposure or the consumption of a high-fat diet. (B) Brite adipocytes (left) contain large lipid droplets and few mitochondria, brown adipocytes (right) contain multilocular small lipid droplets and many mitochondria. (C) UCP1 is recruited and activated through the adrenergic signaling pathway: norepinephrine (NE) binds to the β3-adrenergic receptor (β3AR). The Gs protein dissociates and activates adenylyl cyclase (AC): an enzyme that converts ATP into cAMP (+PPi). cAMP may converted into AMP by phosphodiesterases (PDE), or may activate protein kinase A (PKA). PKA upregulates the transcription of Uncoupling protein-1 (UCP1) through phosphorylation of several transcription factors (see Chapter 1.1 for details). PKA also phosphorylates lipases such as hormone-sensitive lipase (HSL). Through the action of lipases, free fatty acids (FFA) are released from lipid droplets and activate the UCP1 present in the inner mitochondrial membrane. UCP1 creates a leak for protons to circumvent the ATP synthase, thereby producing heat. (D) When activated, brown and brite adipose tissue contribute to energy expenditure (EE) and to the lowering of plasma FFA and glucose levels.
MEASURING THE MODULATION OF UCP1-DEPENDENT THERMOGENESIS

Research into BAT thermogenesis has experienced a profound revival ever since it has been hypothesized that BAT’s large energy burning capacity may be harnessed to combat obesity in humans. Currently, many research groups work to identify novel modulators of UCP1-dependent thermogenesis. Due to this overwhelming interest in BAT, the research field has also experienced the invention of new techniques that can be used to examine BAT physiology and to discover novel modulators of BAT thermogenesis. Although these techniques have provided us with many new insights and several potential novel modulators, there are pitfalls in the study of the modulation of UCP1-dependent thermogenesis that must be avoided in order to obtain meaningful results. Here, I discuss the main pitfalls and highlight two novel research models in the BAT field.

2.1 STUDYING THE MODULATION OF UCP1-DEPENDENT THERMOGENESIS AT TEMPERATURES BELOW THERMONEUTRALITY

One of the main concerns when investigating the modulation of thermal physiology in mice or humans is the environmental temperature at which the investigations are executed, as this temperature determines the animal’s need for thermogenesis. The temperature range where an animal’s basal metabolism is sufficient to maintain its predetermined body temperature is termed the thermoneutral zone (Cannon and Nedergaard, 2011). Thus, when living within this temperature range, animals do not have to expend any ‘extra’ energy to defend their body temperature. For mice, the thermoneutral zone is around 30-33 °C, as shown in Fig. 2. The costs of living below one’s thermoneutral zone can be quite significant (Fig. 2). Below the lowest temperature of the thermoneutral zone (the ‘lower critical temperature’), animals need first to activate non-energy costly mechanisms such as vasoconstriction and piloerection and thereafter energy costly mechanisms such as shivering and UCP1-mediated non-
shivering thermogenesis to defend their body temperature. The energy mice have to expend to produce a sufficient amount of heat to compensate for heat loss to the environment increases linearly with decreasing temperatures (Fig. 2). The amount of energy that has to be expended extra per extra degree of difference between environmental and internal temperature is determined by a mouse’s insulation (tail vein vasoconstriction, erection of fur etc.). Thus, the slope of the curve in Fig. 2 changes depending on insulation, e.g. when an animal loses fur, it loses more heat to the environment and will have to expend more energy to counteract any progressive decrease in environmental temperature. Accordingly, this will be visible in the curve as a steeper slope.

For naked humans, the thermoneutral zone is around 25-30 °C. However, as humans generally walk around clothed, we consider a thermoneutral ‘room temperature’ to be around 21 °C. Humans can to a certain extent alter the temperature of the environment they are in, and accordingly they will set indoor thermostats to 21 °C, so they can spend most of their lives comfortably within the thermoneutral zone. This means that in most animal facilities around the world, experiments on mice are conducted at 21 °C. As can be concluded from what is mentioned above, this is about 10 °C below the thermoneutral zone of mice. Thus, the majority of rodent studies into metabolism, physiology and also UCP1-dependent thermogenesis, have been executed on mice that expend double the amount of energy above their basal metabolic rate (Fig. 2, Paper I) to defend their body temperature, and therefore also eat significantly more than they would if they were housed at thermoneutrality (Paper I). As shown in Paper I of this thesis, housing mice below their thermoneutral zone can have significant impacts on research outcomes. The effects of modulators (such as glucocorticoids) of UCP1-dependent thermogenesis in mice may be masked when experiments are conducted at 21 °C, as they are overridden by the need for heat production when animals have to defend their body temperature. In addition, modulators or genetic alterations
that alter a mouse’s insulation (e.g. fur) may be falsely concluded to affect BAT thermogenesis, while the change in BAT thermogenesis is merely a secondary effect needed to compensate for the change in insulation (e.g. Westerberg et al., 2004). Thus, in order to find any real, and for humans relevant, modulators of UCP1-dependent thermogenesis, it is vital to conduct studies aiming to identify these modulators at thermoneutral temperatures.

Another example highlighting the influence that environmental temperature can have on experimental outcomes in the study of UCP1-dependent thermogenesis, is the initially erroneous conclusions that were drawn concerning the effects of BAT UCP1 ablation on mouse physiology. Initially, it was believed that UCP1 ablation in itself would induce obesity because it would block any possibility for cold- or diet-induced thermogenic energy expenditure (Fig. 1). However, this was shown not to be the case in animals housed at 23 °C (Enerbäck et al., 1997). As researchers later noted, this unexpected result was due to the fact that the energy expenditure needed to defend body temperature is not solely derived from BAT-derived non-shivering thermogenesis, as animals can also shiver to defend their body temperature. When animals are exposed to cold for prolonged periods of time, the increase in their metabolic rate remains constant, but the proportion of this increase that is mediated by non-shivering thermogenesis, rather than shivering thermogenesis, gradually increases (Sellers et al., 1954). Thus, UCP1-ablated animals can still constantly shiver to maintain their body temperature and thereby expend the energy that prevents them from becoming obese (Golozoubova et al., 2001).

However, when UCP1 knock-out (UCP1-KO) animals are housed at thermoneutrality, the ‘real’ effects of UCP1 ablation become visible. For example, when obesity-prone C57Bl/6 mice are housed at 30 °C and are fed a chow diet, UCP1 ablation in itself induces weight gain (Feldmann et al., 2009). When these animals receive a high-fat diet (more comparable to a standard, Western, human diet), it becomes evident that the consumption of this diet modulates BAT activity, as C57Bl/6J UCP1-KO mice become more obese on this diet than wild-type mice (Fig. 3). Thus, high-fat diet consumption increases BAT UCP1-dependent thermogenic capacity and thereby prevents C57Bl/6J mice from becoming quite as obese as would be expected based on their energy intake (Fig. 3). This phenomenon is termed UCP1-mediated facultative diet-induced thermogenesis.

The mediation of UCP1-dependent thermogenesis by high-fat diet-feeding is even visible in the obesity-resistant 129S mouse strain (Paper V). Indeed, wild-type mice from this strain show an increase in total BAT UCP1 protein levels when they are fed a high-fat diet (Fig. 3). In the absence of UCP1, 129S mice fed a high-fat diet store more of what they eat, visible as an increase in metabolic efficiency, WAT mass and a tendency to an increased body weight gain (Paper V) (Fig. 3). These effects of UCP1
ablation all indicate the contribution of the modulation of UCP1 levels by high-fat diet feeding to diet-induced thermogenesis in 129S mice (Fig. 3). Thus, UCP1-mediated facultative diet-induced thermogenesis also prevents obesity-resistant mice from getting as fat as they ‘should’ based on caloric intake, which shows the potential of the modulation of UCP1-dependent thermogenesis as a means of counteracting the development of obesity.

![Fig. 3](image_url)

**Fig. 3. Overview of UCP1-mediated facultative diet-induced thermogenesis.** (A) In wild-type (WT) mice housed at thermoneutrality, total UCP1 protein amount (green rectangles) in BAT increases upon high-fat diet feeding. (B) UCP1-ablated (UCP1-KO) mice housed at thermoneutrality and fed a high-fat diet gain more weight and accumulate more lipid in BAT than WT mice. Thus, UCP1 activation prevents WT mice from gaining weight upon consumption of a Western style diet.
2.2 STUDYING THE MODULATION OF UCP1-DEPENDENT THERMOGENESIS IN BRITE ADIPOSE TISSUE

In rodents, the modulation of UCP1-dependent thermogenesis, which may result in a successive modulation of energy expenditure, may not solely occur in BAT, but possibly also in UCP1-expressing brite fat. Thus, next to activation and recruitment of ‘classical’ brown fat, activation and recruitment of brite fat may also be a contributor to weight loss. For this reason, the identification of compounds that modulate the ‘britening’ of WAT (i.e. the UCP1-dependent thermogenic capacity of the tissue) in mice has been deemed increasingly important. However, the human relevance of research into murine brite fat for weight-loss purposes remains questionable, for reasons discussed below.

First, the comparability of brown fat and brite fat between mice and humans is an unresolved issue. As mentioned in Chapter 1.2, murine BAT and brite fat may originate from distinct developmental lineages. From this observation, the question arose whether it is relevant to compare human BAT to ‘classical’ murine BAT, since the human tissue might be more comparable to murine brite fat. Indeed, in isolated human neck fat, researchers have found cells that look like classical murine brown fat, but also an abundance of ‘paucilocular’ cells (cells with several medium-sized lipid droplets) that contain UCP1, thus phenotypically resembling murine brite fat rather than murine BAT (Zingaretti et al., 2009) (Fig. 4). This similarity between murine brite fat and human BAT would mean it might actually be more meaningful for human purposes to study the modulation of UCP1-dependent thermogenesis in murine brite fat rather than murine BAT.

Several efforts have been made to resolve this issue, with conflicting results (Jespersen et al., 2013; Sharp et al., 2012; Wu et al., 2012). One difficulty that arose with these previous analyses, has been the large cellular heterogeneity within BAT and WAT depots (mature adipocytes, preadipocytes, endothelial cells, immune cells). We have tried to tackle this issue by comparing clonally-derived murine and human adipocyte cell lines with each other (Paper III). Since murine brown and brite adipocytes have distinct gene expression patterns, it is possible to determine which one of these cell types has a transcriptome that is more comparable to the human BAT transcriptome. For this purpose, we have isolated the stromal vascular fraction of human neck fat (see Jespersen et al., 2013), immortalized it using SV40-T protein, and derived three adipogenic clones from it (Paper III, but see Chapter 2.3). Upon comparison of these human BAT-derived clonal cell line with murine BAT and WAT-derived clonal cell lines through unbiased genome-wide expression analyses, we provide evidence that clonally derived differentiated human adipocytes have a molecular signature that resembles differentiated murine brite adipocytes rather than differentiated murine brown adipocytes (Paper III).
Although the above-mentioned conclusion seems to provide clear evidence that for human purposes, it would be more fruitful to focus our efforts on studying the modulation of UCP1-dependent thermogenesis in murine brite fat rather than in BAT, several side notes have to be made to the research shown in Paper III. First, a clear downside of comparing clonally derived cells to one another is that in the end we are really comparing the transcriptomes of only three human brown adipocytes with the transcriptomes of only three mouse beige adipocytes and three mouse brown adipocytes. As mentioned, adipose tissue depots are very heterogenous. The nature of human brown fat has been shown to be dependent on the physical depth of the depot in the body, with deeper depots probably more closely resembling classical BAT (Cypess et al., 2013; Lidell et al., 2013; Nedergaard and Cannon, 2013a). Thus, the comparison of the few human and murine adipocytes presented in Paper III may be too limited to draw far reaching conclusion about the nature of human BAT.

Second, we again run into the issue of environmental temperature. The initial observations that human BAT seemed phenotypically to resemble murine brite fat rather than classical BAT were based on comparisons between tissues taken from mice housed at 21 °C (mild cold exposure), but from humans that spend most of their lives at thermoneutrality (Fig. 4). This is problematic, as it means that the adipose depots compared are in a very different ‘recruitment’ and ‘activation’ stage. We show in unpublished research coordinated by Natasa Petrovic that BAT from ‘humanized mice’ (housed at 30 °C and fed a high-fat diet for 7-8 months) is phenotypically remarkably similar to human BAT (de Jong et al.). Thus, when analyzed under comparable environmental conditions, human BAT and mouse BAT physiology may be directly compared. In our published study (Paper III), adipocyte precursors were isolated from mice housed at mild cold exposure and humans living at thermoneutrality (Jespersen et al., 2013; Wu et al., 2012). Even though the cells were differentiated in vitro, we show that differences in the transcriptomes between human brown and white adipocytes may already occur at the preadipocyte level (Paper III). Thus, even the comparison of precursor cells isolated from animals housed at different environmental temperatures may be false in the sense that these precursor cells already resemble an adipose tissue that is either thermogenically recruited or not. It may therefore be more interesting to compare the transcriptome of BAT/WAT from ‘humanized’ thermoneutral mice with that of BAT/WAT from thermoneutral humans (Fig. 4). Indeed, although BAT of humanized mice phenotypically more closely resembles WAT, Petrovic’s research shows that BAT of humanized mice does retain the molecular signature associated with BAT of mice housed at 21 °C, and can thus still be characterized as ‘real BAT’ (Cheng et al., 2018; de Jong et al.; Perdikari et al., 2018). Furthermore, comparative transcriptomics show a remarkable similarity between the transcriptomes of humanized mouse BAT and human BAT (de Jong et al.). These findings mean that human BAT is phenotypically and molecularly comparable to murine BAT studied under humanized
conditions. Thus, as long as the modulation of UCP1-dependent thermogenesis in murine classical BAT is studied in the correct way, it is more useful as a model for the modulation of UCP1-dependent thermogenesis in human BAT than the study of murine brite fat (Fig. 4).

Apart from the question as to whether human BAT is more comparable to murine BAT or brite fat, it may be so that the modulation of UCP1-dependent thermogenesis in murine brite fat is in any case irrelevant for human weight loss purposes, as the contribution of activated brite fat to total energy expenditure is questionable (Keipert and Jastroch, 2014). This questionability arises from the observation that even though the expression of *Ucp1* mRNA is induced ~100-fold in the inguinal WAT (ingWAT) and only ~3-fold in the interscapular BAT (IBAT) of mice moved from 30 °C to 4 °C, the absolute *Ucp1* mRNA levels at 4 °C are still 5- to 10-fold higher in IBAT compared to ingWAT (de Jong et al., 2015; Kalinovich et al., 2017; Nedergaard and Cannon, 2013b). This indicates that in the end, total – physiologically relevant – UCP1 levels in brite adipose tissue may be much lower than total UCP1 levels in BAT (Fig. 4). Indeed, as shown in **Paper I** of this thesis, we were unable to detect any relevant UCP1 protein

![Fig. 4. The comparison between murine brite adipose tissue and human BAT. (A) In mice housed at 21 °C, brite adipose tissue contains multilocular lipid droplets and low amounts of UCP1 protein, and is thus phenotypically comparable to BAT of humans housed at thermoneutrality (C). (B) In mice housed at thermoneutrality, lipid droplets in brite adipose tissue increase in size and the tissue does not contain detectable amounts of UCP1 protein. Thus, when studied under similar conditions, human BAT and murine brite fat are not comparable.](image-url)
levels in the ingWAT of C57Bl/6J mice housed at 30 °C (thermoneutrality) or 21 °C (mild cold exposure). These findings indicate that only quite severe cold exposure induces a more robust browning of WAT, and that this browning makes up only a small proportion of total thermogenic capacity. In summary, the contribution of brite fat to total UCP1-dependent thermogenic capacity appears to be minimal when mice are studied under circumstances that are physiologically relevant for humans.

Thus, if the ultimate goal is to aim at UCP1-dependent thermogenesis as a target for weight-loss in humans, the research community may be best off focusing their efforts on the discovery of modulators of UCP1-dependent thermogenesis in classical murine BAT.

### 2.3 Novel Research Models for Studying the Modulation of UCP1-Dependent Thermogenesis

As mentioned above, the discovery of novel modulators of UCP1-dependent thermogenesis in BAT is crucial if we want to use thermogenesis in the battle against obesity. This also signifies the importance of the availability of suitable research models that can be used to identify these novel modulators of UCP1-dependent thermogenesis. In this chapter, I will briefly discuss two novel research models that we have developed with the aim to contribute to the research field.

First, as mentioned in Chapter 2.2, we have developed novel clonal cell lines derived from the isolated stromal vascular fractions of human neck fat (Jespersen et al., 2013; Paper III). Although we aimed to immortalize the human BAT stromal vascular fractions by retroviral transduction with the SV40-T protein, the adipogenic clonal cell lines unfortunately stopped growing after multiple passages and thus appear to be not fully immortalized. We hypothesize that this may be due to reported difficulties with SV40 T-mediated immortalization of human cells (Bryan and Reddel, 1994; Sack and Obie, 1981; Shay and Wright, 1989). Nevertheless, early passages of the clonal cell lines can be differentiated in vitro upon treatment with an adipogenic cocktail (Paper III). When differentiated, the clones express Ucp1 mRNA and protein in an adrenergically-inducible way, accumulate lipids and have an increased mitochondria-derived oxygen consumption rate (OCR) upon stimulation with cAMP (Paper III). It has to be noted that the mitochondria-derived OCR as reported in Paper III was determined by subtracting the non-mitochondrial respiration (after rotenone / antimycin-A addition) from the total respiration (FCCP) and is therefore only a measurement of the number of mitochondria in the cell and not of UCP1 activity. As is shown in Paper I and research
published previously by our lab (Fischer et al., 2017), UCP1-mediated OCR can be measured in intact adipocytes only after addition of an adrenergic stimulus, or in isolated mitochondria after addition of GDP.

The potential of using early passages of these clonal cell lines to unravel the functioning of human BAT is underlined by the identification of Mtus1 (mitochondrial tumor suppressor 1) and Kcnk3 (potassium two pore domain channel subfamily K member 3) in these cells as potential novel human BAT marker genes (Paper III). As a side note, although the search for marker genes specific to murine/human BAT/brite fat has intensified over the years, only very few proposed markers actually seem helpful in reliably determining adipose tissue identity under a range of conditions (Zic1 being the most promising one) (de Jong et al., 2015). Although we show that the expression of both Mtus1 and Kcnk3 is increased in human BAT isolated from the neck region compared to human WAT isolated from the same individuals, and the expression of these genes is induced by cold exposure in human BAT, it is evident that these phenomena do not happen unanimously in all subjects (Paper III). Thus, the usefulness of these genes as human BAT-specific markers remains unclear.

Nevertheless, we show that Ucp1 mRNA levels, as well as UCP1 protein levels per µg protein, are decreased in cultured adipocytes lacking either Mtus1 or Kcnk3, thus providing an indication that these two proteins may be novel modulators of UCP1-dependent thermogenesis (Paper III).

In addition, we developed a novel mouse model in which luciferase is expressed under the control of the Ucp1 promoter region (Paper IV). This mouse model allows for the visualization of Ucp1 transcription in vivo upon injection of luciferase’s substrate luciferin. We show that the luciferase signal in vivo follows increases in BAT and ingWAT Ucp1 mRNA levels seen after cold exposure or adrenergic stimulation of the mice. This activity of Ucp1-luciferase was confirmed in an additionally developed Ucp1-luciferase mouse model (Wang et al., 2018). In addition, isolated Ucp1-luciferase brown adipocytes can be used as a tool to screen compounds for their capacity to modulate Ucp1 transcription in vitro, comparable to the analysis of Ucp1 mRNA levels by means of qPCR (Paper IV). The main advantage of this model is that Ucp1 transcription can be measured in one mouse on several subsequent occasions, thus making it possible for every mouse to be compared to its earlier self. This allows for the study of BAT Ucp1 transcription in models of e.g. aging, as is done in a comparable Ucp1-luciferase model later developed by a separate lab (Mao et al., 2017). These researchers show that luciferase activity (and thus Ucp1 transcription) declines steadily in mice when they age from 3 to 18 weeks (Mao et al., 2017).

However, there are several downsides to measuring Ucp1 transcription in vivo using the luciferase model, as pointed out by Fromme and colleagues (Birnbacher et al., 2018). These mainly concern factors that may interfere with the stable detection of the
luciferase signal, such as light absorption by fur and the varying thickness of the WAT tissue overlying the BAT tissue e.g. when mice age or become obese (Birnbacher et al., 2018). In addition, changes in body composition may alter the tissue distribution pattern of the injected luciferin substrate. Thus, using the Thermomouse to screen compounds for their capacity to modulate UCP-dependent thermogenesis may only be meaningful if proper controls for these factors are in place.
GLUCOCORTICOIDS AS MODULATORS OF UCP1-DEPENDENT THERMOGENESIS

3.1 WHY GLUCOCORTICOIDS?

As mentioned above, activating and recruiting brown fat has the potential to be a novel strategy to counteract the development of obesity. It is thus vital that the research community identifies compounds that modulate UCP1-dependent thermogenesis in BAT and that thereby modulate energy expenditure.

The most straightforward way of making use of BAT thermogenesis in the battle against obesity is to activate the UCP1 that is already present in BAT and/or to enhance the total whole-body amount of UCP1 (i.e. recruitment). To date, the best-known activators and recruiters of UCP1 are β-adrenergic agonists (cold, NE, CL-316 243), cAMP enhancers (forskolin), and the thiazolidinediones (rosiglitazone) (Paper I; Paper II). Growth factors such as the BMP’s and FGF’s, serine hydrolase inhibitors such as WWL113, and food ingredients such as capsinoids are also being investigated for their potential to modulate BAT functioning (Cypess and Kahn, 2010; Paper III, Paper IV). BAT has also been hypothesized to be activated and recruited by the consumption of specific diets, possibly through enhancing sympathetic output to the tissue. Indeed, we show that an increase in UCP1-dependent thermogenesis caused by the ingestion of diets high in fat lowers weight gain even in the obesity-resistant 129 mouse strain housed at thermoneutrality (Paper V) (Fig. 3). Additionally, the compound 2,4-dinitrophenol (DNP), a non-selective chemical uncoupler, has already been made commercially available in diet pills (Tainter et al., 1933). Although DNP greatly increases metabolic rate and reduces obesity, it has a too narrow therapeutic window and induces life-threatening side-effects – among them hyperthermia – and is therefore discontinued as a drug.

Next to the identification of modulators that induce UCP1-dependent thermogenesis, it is key to identify modulators that suppress BAT activity. If these negative BAT-modulators are exogenous to humans (e.g. a warm environment), awareness of them would make it easier for us to avoid them. Conversely, identifying suppressive factors of BAT thermogenesis that may be endogenous to humans makes it possible for us to suppress them, thereby activating BAT. In this respect, several
transcription factors have been found to suppress BAT thermogenesis, among them the retinoblastoma proteins (Harms and Seale, 2013).

Other proposed endogenous negative modulators of UCP1-dependent thermogenesis are the glucocorticoids. Glucocorticoids belong to the group of steroid hormones and are a vital part of human and animal physiology as modulators of inflammation and glucose homeostasis, primarily during the stress response (see Chapter 3.2). However, it is becoming more and more clear that glucocorticoids also play an important role in energy homeostasis and adipose tissue physiology. This can be inferred from reports describing rearrangements of fat tissues and a concomitant development of symptoms of the metabolic syndrome in patients suffering from diseases involving either elevated circulating levels of glucocorticoids (i.e. hypercortisolism) or reduced circulating levels of glucocorticoids (i.e. hypocortisolism) (Pasieka and Rafacho, 2016; Rockall et al., 2003).

Hypercortisolism can be due to an endogenous overproduction of glucocorticoids or exogenous administration of glucocorticoids as anti-inflammatory medication (Sharma et al., 2015). In patients with hypercortisolism (i.e. Cushing’s Syndrome), a profound accumulation of fat in central regions and wasting of fat in peripheral regions occurs. This puts patients at risk for developing insulin resistance and other symptoms of the metabolic syndrome (Pasieka and Rafacho, 2016). Cushing’s patients may also develop a ‘buffalo hump’: an accumulation of fat in the neck and the upper part of the back. On the other hand, in the case of endogenous hypocortisolism (i.e. Addison’s disease), patients experience weight loss and thereby fat wasting.

More recent experimental research has reported direct effects of glucocorticoids on WAT physiology on a cellular and molecular level. Glucocorticoid signaling in WAT promotes adipogenesis and adipose tissue expansion in both mice and humans (Infante et al., 2017; John et al., 2016; Kargi and Iacobellis, 2014; Lee et al., 2014; Peckett et al., 2011). Conversely, blocking glucocorticoid signaling in mice generally reverses these effects. Thus, from these and the above-mentioned observations, it can be concluded that glucocorticoids are important regulators of adipose tissue homeostasis.

The observed importance of glucocorticoid signaling for WAT functioning has led researchers to propose that glucocorticoids may also modulate BAT functioning. Indeed, BAT has been shown to be a glucocorticoid target organ (Feldman, 1978). Similar to the effects of glucocorticoids on WAT, glucocorticoids may induce lipid accumulation in BAT, which may or may not reflect an impaired UCP1-dependent thermogenic capacity. If glucocorticoids indeed suppress BAT thermogenesis, this may decrease cold- or diet-induced thermogenic energy expenditure and has therefore been proposed to contribute to the development of glucocorticoid-induced obesity in mice and men (Mousovich-Neto et al., 2019). Thus, unraveling the effects of glucocorticoids on BAT may take us one step closer to determining the cause of glucocorticoid-induced obesity,
which may possibly help to alleviate symptoms of Cushing’s Syndrome and to ultimately counteract obesity by inducing UCP1-dependent thermogenesis.

For the above-mentioned reasons, the modulation of BAT functioning by glucocorticoids has been investigated by many during the course of the past decades. After introducing glucocorticoids in Chapter 3.2, I will discuss the research on the modulation of UCP1-dependent thermogenesis by glucocorticoids in mice and men reported to date in Chapters 3.3-3.8.

3.2 INTRODUCTION TO GLUCOCORTICOIDS

The scientific and pharmaceutical history of glucocorticoids starts in the early 1900’s when Philip Hench, Edward Kendall and Tadeus Reichstein successfully extracted cortisone from the adrenal cortex and used the compound to treat rheumatoid arthritis. The trio’s work on hormones of the adrenal cortex would be rewarded with the Nobel prize in Physiology and Medicine in 1950. Later, the steroid hormones that are produced by the adrenal cortex and play a vital role in glucose metabolism would be named ‘glucocorticoids’, a contraction of the words glucose, cortex and steroid.

The family of steroid hormones is classified into five groups: the androgens, estrogens, progestogens, mineralocorticoids and glucocorticoids. The steroid hormones are divided among these groups based on which receptor(s) they bind to in peripheral tissues: the androgen receptor, the estrogen receptor, the progesterone receptor, the mineralocorticoid receptor and/or the glucocorticoid receptor. Glucocorticoids comprise several bioactive molecules that undergo enzymatic conversion between one another, including 11-deoxycortisol, cortisol (hydrocortisone), cortisone, 11-deoxycorticosterone, corticosterone, and 11-dehydrocorticosterone (Fig. 5). Of these glucocorticoids, cortisol is the most potent in humans, while corticosterone is the most potent in rodents (Chapter 3.4). The 11-dehydro variants cortisone and 11-dehydrocorticosterone are less potent than cortisol and corticosterone and are therefore considered to be their inactive forms. The 11-deoxy forms are mainly considered to be precursor molecules for cortisol and aldosterone synthesis, and are not major secretory hormones. Additionally, several synthetic glucocorticoids have been developed for pharmacological use, including the long-acting dexamethasone (DEX, t<sub>1/2</sub> = 36-54h, 27x more potent than cortisol), and the intermediate-acting methylprednisolone, prednisolone and prednisone (t<sub>1/2</sub> = 18-36h, 4-5x more potent than cortisol) (Fig. 5).

The production and release of glucocorticoids is conserved between mice and humans, and is under the control of the hypothalamus-pituitary-adrenal (HPA)-axis in both species. Upon perception of either a physical or cognitive stressor, or during specific times of the day, the hypothalamus is activated and secretes corticotropin-releasing hormone (CRH) (Chapter 3.3). CRH stimulates the pituitary to synthesize
adrenocorticotropic hormone (ACTH), which is released into the bloodstream (Chapter 3.3). ACTH travels to the adrenal cortex, where it stimulates the production and release of glucocorticoids in a process termed steroidogenesis (Chapter 3.4). Glucocorticoids released from the adrenal gland travel through the bloodstream bound to corticosteroid-binding globulin (also known as transcortin) to target organs (Chapter 3.5). When free glucocorticoids reach target organs, they generally move through cell membranes without the aid of a transporter, due to their lipophilic nature. Inside target cells, glucocorticoids may be converted from their active forms (cortisol / corticosterone) into their inactive forms (cortisone / 11-dehydrocorticosterone) or vice versa by the 11β-hydroxysteroid dehydrogenases (Chapter 3.6). Once converted into their active forms, glucocorticoids generally exert effects by binding to the cytoplasmic glucocorticoid receptor (GR) (Chapter 3.7). Upon ligand-binding, the GR translocates to the nucleus where it functions as a transcription factor, thus regulating the expression of target genes and thereby finally affecting mouse and human physiology (Chapter 3.7).

Almost every event in glucocorticoid signaling as described here has been investigated for its potential to modulate UCP1-dependent thermogenesis, and will be discussed further below in the chapters indicated above.
Fig. 5. Schematic overview of glucocorticoid synthesis. Enzyme abbreviations: P450scc: cholesterol side-chain cleavage enzyme, CYP17A1: 17α-hydroxylase, 3βHSD: 3β-hydroxysteroid dehydrogenase, CYP21a2: 21α-hydroxylase, CYP11b1: 11β-hydroxylase, 11βHSD1/2: 11β-hydroxysteroid dehydrogenase type 1/2.
3.3 THE REGULATION OF GLUCOCORTICOID RELEASE: THE HYPOTHALAMUS AND THE PITUITARY

In stressful situations, the release of energy from the body’s glycogen and fat stores is vital for survival. As mentioned above, glucocorticoids play an important role in regulating the supply of energy both during stress as well as post-stress, and are therefore often referred to as the ‘stress hormones’. Due to the vital nature of the role of glucocorticoids as stress hormones, it follows that circulating levels of glucocorticoids have to be tightly regulated. The regulation of glucocorticoid production and secretion is under central control of the HPA-axis, and will be further discussed here.

The hypothalamus is the main regulator of the production and release of glucocorticoids by the adrenal gland. In its paraventricular nucleus, sensory information concerning the internal state of the body or information concerning peripheral stimuli (sound, visual) is integrated. When the central nervous system signals the presence of a cognitive (e.g. a perceived threatening situation) or physical (e.g. thirst, hunger, pain) stressor to the hypothalamus, the hypothalamus secretes CRH at an area known as the median eminence (Fig. 6).

CRH is not only released by the hypothalamus as a response to stressors, but also in a circadian manner during specific times of the day (Fig. 6). The hypothalamus harbors an area named the suprachiasmatic nucleus, where clock cells reside that are under regulation of nerve cells, that in their turn are stimulated by daylight. Via this mechanism, the environmental day-and-night-cycle affects the expression of CLOCK genes in the clock cells of the suprachiasmatic nucleus. The circadian information from the suprachiasmatic nucleus is also integrated in the paraventricular nucleus of the hypothalamus, and may thereby lead to the release of CRH. The circadian manner in which CRH – and thereby glucocorticoids – is released is well-defined in humans and mice. In humans, the lowest circulating glucocorticoid levels (i.e. the nadir) can be measured around 11 PM, after which glucocorticoid levels gradually rise during sleep until they peak at the beginning of the light phase, around 8 AM. During the rest of the day, glucocorticoid levels slowly decrease until the nadir is reached again around midnight. As mice are nocturnal, their circadian pattern of circulating glucocorticoid levels is precisely inversed, i.e. the nadir is around 10 AM, when the mice go to sleep, and the peak is around 8 PM, when the mice wake up (Fig. 6). Because the GR is expressed in virtually every cell in the body (Chapter 3.7), and the expression of several CLOCK genes in peripheral tissues is regulated by the GR, glucocorticoids function as important second messengers that connect the central circadian clock with the peripheral circadian clock, thus aiding the synchronization of whole-body daily rhythms (Balsalobre et al., 2000; So et al., 2009).

As a side note, the fact that glucocorticoids play a central role in circadian homeostasis causes difficulties in optimizing glucocorticoid medication. Current ways of exogenous glucocorticoid administration, e.g. in the form of pills, do not simulate the
circadian rhythm of endogenous glucocorticoid release (Venneri et al., 2018). Consequently, patients receiving this type of medication to combat hypocortisolism often still suffer from poor quality of life. Additionally, mimicking the circadian pattern of endogenous glucocorticoid release has also proven to be an obstacle in rodent studies aiming to unravel the effects of glucocorticoids. Indeed, the continuous administration of glucocorticoids via injections, micro-osmotic pumps or implantation of slow release pellets does not succeed in maintaining the endogenous circadian pattern of glucocorticoid secretion seen in mice (Herrmann et al., 2009). The administration of glucocorticoids through the drinking water has been proposed because it is inexpensive, non-invasive and may more closely mimic endogenous circadian glucocorticoid release patterns, because mice will have high circulating glucocorticoid levels during the dark period, when they drink (Gasparini et al., 2016). However, as we show in Paper I, glucocorticoid levels are still profoundly elevated at the beginning of the light period (7:30AM, when the mice go to sleep) in rodents receiving glucocorticoids via the drinking water, which is not compatible with endogenous circadian glucocorticoid release patterns. It thus cannot be excluded that the metabolic effects we report in mice treated with glucocorticoids via the drinking water are not solely due to the glucocorticoid treatment, but also to a disturbed circadian rhythm of the hormone (Paper I).

As mentioned above, the paraventricular nucleus of the hypothalamus releases CRH at the median eminence in response to stressors or the time of the day (Fig. 6). CRH travels via the hypothalamo-hypophyseal portal system to the anterior lobe of the pituitary. The main function of CRH in the pituitary is to stimulate the production and release of ACTH by corticotropes (Fig. 6). ACTH is synthesized from pre-pro-opiomelanocortin (pre-POMC) through translational and post-translational modifications, and successively released into the vascular system, through which it is carried to the adrenal gland, where it stimulates glucocorticoid synthesis (Chapter 3.4) (Fig. 6).

Because glucocorticoid signaling partly regulates the provision of energy during the fight-or-flight response (together with catecholamines), the stimulation of glucocorticoid production and release by the HPA-axis occurs within a relatively rapid timeframe. Upon the perception of a stressor, the secretion of CRH by the hypothalamus, the successive secretion of ACTH from the pituitary, and finally the synthesis and release of glucocorticoids from the adrenal gland, happens within 20 min, when maximal circulating glucocorticoid levels can be measured.

While short-term hypercortisolism can be essential for survival, long-term hypercortisolism may be damaging (Pasieka and Rafacho, 2016). Following the induction of glucocorticoid production, it is thus also of importance that post-stress, the production of glucocorticoids is rapidly inhibited. The suppression of glucocorticoid production is therefore also tightly regulated, by means of negative feedback loops (Fig.
6). This means that circulating glucocorticoids in themselves rapidly (within minutes) inhibit the release of more glucocorticoids by binding to the GR in the paraventricular nucleus (but not the suprachiasmatic nucleus) of the hypothalamus. Hereby, the release of CRH from the hypothalamus is inhibited, which in its turn lowers the release of ACTH from the pituitary (Fig. 6). In the pituitary, glucocorticoids also suppress the transcription of POMC (Tremblay et al., 1988), thus regulating the availability of the ACTH precursor. The negative feedback mechanisms in the hypothalamus and pituitary may be mediated through both genomic and non-genomic GR signaling (Chapter 3.7). Finally, the peripheral clearance of glucocorticoids also contributes to making sure circulating glucocorticoid levels return to baseline post-stress.

3.3.1 ACTH and brown adipose tissue

Naturally, the activity of the HPA-axis ultimately affects the production and secretion of glucocorticoids. Glucocorticoids in their turn may or may not modulate UCP1-dependent thermogenesis in BAT (discussed in Paper I, Paper II, Chapters 3.4-3.8). However, first the possibility has to be discussed as to whether signaling by the HPA-axis in itself may also directly modulate BAT thermogenesis, and may thereby affect experimental outcomes regarding the effects of glucocorticoids on BAT.

As mentioned above, the HPA-axis is activated in response to cognitive or physical stressors (Fig. 6). In addition to e.g. hunger and pain, the physical stressors also include cold exposure, as this environmental condition puts an animal at risk of developing hypothermia. Indeed, plasma ACTH levels, fecal corticosterone levels, and the expression levels of steroidogenic enzymes in the adrenal gland are all increased in mice that are acutely exposed to 4 °C (van den Beukel et al., 2014). These data indicate an activation of the HPA-axis. In accordance with our data (Paper II), high concentrations of glucocorticoids (as they occur in stressful situations) may lower the transcription of Ucp1 (and possibly total UCP1 protein levels) in adipocytes differentiated in vitro (van den Beukel et al., 2014). Interestingly, high levels of ACTH in themselves also seem to acutely affect Ucp1 transcription in vitro, but in a manner completely opposite to corticosterone, namely an upregulation (van den Beukel et al., 2014; Schnabl et al., 2019). The acute upregulation of Ucp1 transcription and UCP1 protein per µg protein by ACTH treatment in cultured adipocytes has been reported by several groups (van den Beukel et al., 2014; Biswas, 2017; Iwen et al., 2008; Schnabl et al., 2019). In mice, an acute ACTH treatment increases GDP-binding to BAT, as well as NE-induced oxygen consumption, perhaps indicating an increased ‘unmasking’ of UCP1 (Rothwell and Stock, 1985; York and Al-Baker, 1984). The ACTH-induced upregulation of UCP1 levels is most likely mediated through an activation of the Gs-cAMP-PKA signaling pathway upon binding of ACTH to the melanocortin 2 receptor (Schnabl et al., 2019) (Chapter 3.4)
The proposed upregulation of UCP1-dependent thermogenesis in response to ACTH treatment may indicate that in physiological situations, ACTH mediates the acute effects of stress on BAT. Upon exposure to a stressor, circulating glucocorticoid levels rise after 20 min, while circulating ACTH levels rise much more rapidly. Thus, ACTH may initially enhance BAT activity, after which this enhancement is suppressed by the rise in glucocorticoids. Indeed, several reports indicate that corticosterone suppresses the ACTH-induced increase in UCP1 mRNA and protein levels in vivo and in vitro (van den Beukel et al., 2014; York and Al-Baker, 1984), although this remains controversial (Schnabl et al., 2019). However, it has to be kept in mind that the reported experimental concentrations of supplemented ACTH are profoundly supra-physiological. In mice, circulating ACTH levels are ~3 pg/ml at 8AM (e.g. Füchsl et al., 2013). Upon exposure to a stressor, these levels can rise to 500 pg/ml, or 1 ng/ml in extreme cases (van den Beukel et al., 2014; Füchsl et al., 2013). In the above-mentioned experiments, ACTH is supplemented at concentrations ranging from ~5 ng/ml to ~50 ng/ml (van den Beukel et al., 2014; Biswas, 2017; Iwen et al., 2008; Rothwell and Stock, 1985; Schnabl et al., 2019), which is thus at least 5x above maximal physiological concentrations. As the effects of ACTH on BAT are dose-dependent (van den Beukel et al., 2014; Schnabl et al., 2019) and only occur at these supraphysiological doses, it is unlikely that the proposed activation of UCP1-dependent thermogenesis by ACTH is physiologically relevant (Fig. 6).

Moreover, even if the finding that ACTH activates BAT would be physiologically relevant, it seems unlikely to affect experimental outcomes when glucocorticoids are administrated exogenously. This is because exogenous glucocorticoid administration immediately suppresses HPA-axis activity, and thereby ACTH secretion from the pituitary, through the same negative feedback loop whereby endogenously released glucocorticoids suppress HPA-axis activity (Fig. 6). Thus, there is no rise in circulating ACTH levels upon exogenous glucocorticoid administration. Therefore, it is unlikely that any reported modulations of BAT by exogenous glucocorticoids as discussed below (and see Paper I) are altered by acute rises in ACTH levels. This does not take away the fact that the effects of exogenously administrated glucocorticoids on BAT may be different from what would be seen in physiological stress-situations, when there is a measurable peak in circulating ACTH levels.
Fig. 6. The effects of ACTH on BAT. Upon the perception of a stressor or in response to the circadian clock, the hypothalamus secretes corticotropin-releasing hormone (CRH) at the median eminence. CRH stimulates the pituitary to release adrenocorticotropic hormone (ACTH) into the bloodstream. At physiological concentrations, ACTH stimulates the production and secretion of glucocorticoids (GC) by the adrenal cortex, but does not in itself affect BAT functioning. Through a negative feedback mechanism, GC prevent the release of CRH by the hypothalamus, thereby lowering circulating levels of ACTH. When exogenously supplemented at supraphysiological concentrations (> 1 ng/ml), ACTH activates and recruits UCP1 in BAT.
3.4 GLUCOCORTICOID SYNTHESIS:  
THE ADRENAL GLAND

After being released into the vascular system by the pituitary, ACTH travels through the blood to the adrenal cortex (Fig. 6). The half-life of ACTH in the blood is around 10 min, thus ensuring a rapid ACTH turnover and thereby usually preventing the occurrence of prolonged hypercortisolism. Upon arrival at the adrenal gland, ACTH binds to surface ACTH receptors, i.e. the melanocortin 2 receptor, on cells of the adrenal cortex. The melanocortin 2 receptor is a G protein-coupled receptor that changes conformation upon ligand binding, after which the α-subunit of the G-protein dissociates and stimulates the production of cAMP by adenylate cyclase. Through a PKA signaling pathway, ACTH regulates multiple steps in the synthesis of steroid hormones, also named steroidogenesis.

Steroidogenesis is primarily located to the cortex of the adrenal glands. The adrenal cortex consists of three functionally distinct layers; the outer zona glomerulosa, the middle zona fasciculata and the inner zona reticularis (Fig. 7). Each cortical layer expresses a specific set of enzymes, thus regulating specific steps in steroidogenesis. This results in the production of mineralocorticoids in the zona glomerulosa (through specific expression of aldosterone synthase), glucocorticoids in the zona fasciculata and androgens (e.g. DHEA) in the zona reticularis (Fig. 7). Although differences occur between mice and men in zona reticularis development and presence (mice adrenals rather contain a transient X-zone) (Holmes and Dickson, 1971), the glucocorticoid-producing zona fasciculata is comparable between the two species.

Fig. 7. Adrenal cortex zonation in mice. ZG: zona glomerulosa (mineralocorticoid synthesis), ZF, zona fasciculata, ZR, zona reticularis (androgen synthesis), M: medulla (catecholamine synthesis). ACTH binds to melanocortin 2 receptors on cells of the ZF, thereby stimulating the synthesis and release of glucocorticoids (GC) into the bloodstream.
In addition to the circadian clock in the hypothalamus that ensures a circadian production and secretion of ACTH, the adrenal gland also contains a circadian clock. CLOCK genes in the adrenal gland are rhythmically expressed under control of the central nervous system, and control the transcription of genes that are important for steroidogenesis (Oster et al., 2006). Through this mechanism, the circadian clock in the adrenal gland determines the times of the day during which the zona fasciculata is most responsive to the induction of steroidogenesis by ACTH.

The production of all steroid hormones, including glucocorticoids, starts with cholesterol (Fig. 5). ACTH increases the uptake of cholesterol into the adrenal cortex by increasing the number of low-density lipoprotein receptors (Margioris and Tsatsanis, 2000). The first step in steroidogenesis is the cleavage of cholesterol’s side chain at the bond between carbon 20 and 22, thereby creating pregnenolone (Fig. 5). This cleavage is catalyzed by cholesterol side-chain cleavage enzyme (P450scc, encoded by the Cyp11a1 gene), an enzyme located in the inner mitochondrial membrane. ACTH upregulates the transcription of P450scc, thereby facilitating steroidogenesis (Hanukoglus et al., 1990) (Fig. 5). Although the conversion of cholesterol into pregnenolone by P450scc is sometimes considered to be the rate-limiting step in steroidogenesis, this is only the case in abnormal situations where there is not an abundance of cholesterol available in the adrenal cortex. In ‘normal’, physiological situations, the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane is considered to be the rate-limiting step in steroidogenesis. This cholesterol transport is mainly regulated by steroidogenic acute regulatory protein (StAR), which is also an ACTH-regulated enzyme. Once cholesterol is converted into pregnenolone in the mitochondria, pregnenolone moves to the endoplasmic reticulum where it is converted into 17α-hydroxy pregnenolone by 17α-hydroxylase (also a cytochrome P450 family member, CYP17a1) (Fig. 5). Subsequently, 17α-hydroxy pregnenolone is further converted into cortisol through several steps catalyzed by dehydrogenases and hydroxylases (Fig. 5). The expression of one of these hydroxylases (11β-hydroxylase/CYP11b1, a mitochondrial protein) is also under the control of ACTH signaling. The conversion of pregnenolone into 17α-hydroxy pregnenolone by 17α-hydroxylase/CYP17a1 is a crucial step for cortisol synthesis in humans. Interestingly, rodents do not express 17α-hydroxylase in their adrenal cortex, and consequently cannot produce cortisol (Fig. 5). Instead, they produce corticosterone from pregnenolone through steps catalyzed by the same dehydrogenases and hydroxylases that are present in humans (Fig. 5). In addition to upregulating the expression of steroidogenic enzymes, ACTH also induces growth and vascularization of the adrenal gland e.g. by regulating the transcription of VEGF. Finally, when the process of steroidogenesis is complete, glucocorticoids are released by the adrenal cortex into the vascular system.
In Chapters 3.4.1 and 3.4.2, I discuss the information we have gained concerning the modulation of UCP1-dependent thermogenesis by glucocorticoids from studies concerning the adrenal gland. First, I discuss how removal of the adrenal glands affects BAT functioning (Chapter 3.4.1), and second, I discuss how overproduction of glucocorticoids due to adrenal tumors (adrenal Cushing’s) affects BAT functioning (Chapter 3.4.2).

### 3.4.1 Underproduction of glucocorticoids: adrenalectomy

The most straightforward method to determine whether glucocorticoids modulate UCP1-dependent thermogenesis in mice seems to be the determination of BAT functioning after complete removal of the adrenal glands (i.e. adrenalectomy), and thus the complete removal of glucocorticoid production and secretion (Fig. 8). Indeed, the very first clues for a possible link between glucocorticoids and BAT functioning were derived from studies on adrenalectomized rodents. As early as 1949, researchers observed that adrenalectomy results in a depletion of lipid stores in BAT in lean rats and mice (Fawcett and Jones, 1949). Lipid store depletion is reversed in adrenalectomized rodents upon successive injection of glucocorticoids, indicating that the effect is glucocorticoid-dependent (Lachance and Page, 1953).

Additional indications of the possible regulation of UCP1-dependent thermogenesis by glucocorticoids became clear during the characterization of both genetically obese, leptin-deficient ob/ob mice and leptin receptor-deficient obese fa/fa Zucker rats. These rodent models first started to be used for research into the effects of glucocorticoids on BAT when it was realized that they exhibit abnormalities in HPA-axis activity. First, it was shown that ob/ob mice display adrenal cortical hypertrophy, mainly in the zona fasciculata (Naeser, 1975). Later it was reported that basal (measured 8-10 AM) serum corticosterone levels are significantly higher in ob/ob mice compared to lean littermates already before weaning (Dubuc, 1977). As these mice age, corticosterone levels follow a normal circadian rhythm, but at an elevated level (Saito and Bray, 1983). The finding that genetically obese ob/ob mice, as well as fa/fa rats, exhibit hypercortisolism has been reproduced many times in the following decades (Garthwaite et al., 1980; Martin et al., 1978; Okada et al., 1993).

In the 70’s, it was shown that adrenalectomy prevents the development of obesity in both ob/ob mice and fa/fa rats (Solomon et al., 1977; Yukimura and Bray, 1978). Although this is partly due to a reduction in food intake following adrenalectomy (Yukimura et al., 1978), it was hypothesized that adrenalectomy also prevents a glucocorticoid-induced suppression of BAT thermogenesis, thereby increasing energy expenditure and attenuating the development of obesity. Indeed, researchers have observed a reduction in GDP-binding to BAT mitochondria in fa/fa rats and ob/ob mice,
which can be restored by adrenalectomy, thus indicating that the reduction may be mediated by glucocorticoids (Himms-Hagen and Desautels, 1978; Holt and York, 1984, 1982; Holt et al., 1983; Marchington et al., 1983). This hypothesis was later confirmed by the fact that successive administration of corticosterone to adrenalectomized mice reduces GDP-binding to BAT mitochondria, as well as BAT Ucp1 mRNA levels, anew (Arvaniti et al., 1998; Holt et al., 1983; Shargill et al., 1989; Tokuyama and Himms-Hagen, 1987). It has to be noted that more recent findings indicate that there is no decrease in total BAT UCPI protein levels in ob/ob mice (Fischer et al., 2016). It therefore remains to be determined whether adrenalectomy induces a ‘true’ increase in total BAT UCPI protein levels in these mice, or whether this effect disappears when the correct measurements and calculations are applied (Chapter 2). Nevertheless, adrenalectomy does reverse morphological abnormalities seen in adipocytes of both ob/ob mice and fa/fa rats, i.e. larger lipid droplets and an enlargement of the mitochondria that also contain fewer cristae (Hogan and Himms-Hagen, 1980; Holt et al., 1983).

The above-mentioned changes in BAT physiology upon adrenalectomy of ob/ob mice and fa/fa rats appear to indicate an enhanced thermogenic capacity of the tissue. Thus, in the presence of glucocorticoids, BAT function may be repressed. Upon further study of intact and adrenalectomized rodents, it was proposed that this possible suppression of BAT thermogenesis by glucocorticoids is a secondary effect of glucocorticoid-induced effects on adrenergic output to BAT. It has been reported that NE turnover rate, measured by the half-time of disappearance of [3H]NE, is significantly lower in BAT of ob/ob mice (experiencing hypercortisolism) compared to lean mice (Knehans and Romsos, 1982). This reduction in NE turnover in BAT is most likely due to a decreased signalling from the sympathetic nervous system to BAT, as the firing rate of efferent nerve fibres to BAT is significantly reduced in fa/fa rats compared to lean controls (Holt and York, 1989). Seven days after adrenalectomy, the firing rate of these nerves is partly corrected towards levels seen in lean rats (Holt and York, 1989). Consequently, adrenalectomy also normalizes NE turnover in BAT to levels comparable to or higher than those in lean controls (Vander Tuig et al., 1984). These findings indicate a glucocorticoid-mediated suppression of sympathetic firing to BAT, perhaps resulting in the accumulation of lipids in the tissue and a reduction in UCP1 protein amount. Although the above-mentioned conclusion is drawn based on mouse studies performed below thermoneutrality, adrenalectomy also increases BAT-specific NE turnover in ob/ob mice housed at thermoneutrality (35°C), a temperature at which there is no need for thermoregulatory thermogenesis (Kim and Romsos, 1990) (Chapter 2).

Taken together, these data indicate that in mice, the complete removal of the production of glucocorticoids by adrenalectomy has profound effects on BAT physiology, i.e. a
reduction in lipid droplet size, an increase in GDP-binding to BAT mitochondria and a possible increase in BAT UCP1 protein per µg protein (Fig. 8). These effects thus indicate that when glucocorticoids are present, they may suppress UCP1-mediated thermogenesis. Moreover, the discussed studies provide indications that the effects of glucocorticoids on BAT functioning may be mediated indirectly through alterations in the sympathetic output to the tissue (Fig. 8).

Although variations in lipid droplet size in BAT are often used as indicators of an altered thermogenic capacity of the tissue, this may not necessarily be the case. For example, we show in Paper I that mice treated with glucocorticoids at 21 °C experience a profound lipid accumulation in their BAT compared to veh-treated mice, in the absence of changes in total BAT UCP1 protein levels or UCP1-dependent thermogenic capacity between the groups. In addition, lipid accumulation in BAT is similar in mice housed at 30 °C, while total BAT UCP1 levels in these mice are a ~100-fold lower than in mice housed at 21 °C. Thus, the decrease in lipid content in BAT upon adrenalectomy does not necessarily indicate an increase in thermogenic capacity of the tissue (Fig. 8).

From the above-mentioned studies on adrenalectomized rodents, it is also questionable to draw the conclusion that glucocorticoids lower BAT UCP1 levels (Fig. 8). Most of these studies are performed on animals housed below thermoneutrality, and report only BAT Ucp1 mRNA levels or UCP1 protein / µg BAT protein. Because these experimental conditions and calculations can provide a skewed image of the results (Chapter 2, Paper I), it is difficult to draw a definite conclusion from them.

Finally, there is the hypothesis that glucocorticoids may lower sympathetic output to BAT and thereby suppress UCP1-dependent thermogenesis. As we show that the capacity of BAT to respond to adrenergic stimulation is unaltered by glucocorticoid treatment (Paper I), this may mean that glucocorticoids somehow reduce the firing of sympathetic nerves or reduce the production or release of NE from sympathetic nerve endings. A reduction in sympathetic outflow to BAT may explain the effects of glucocorticoids on lipid accumulation in the tissue, as we report an increased lipid accumulation in BAT in vivo in mice but not in vitro in differentiated adipocytes upon glucocorticoid treatment (Paper I, Paper II). However, given the fact that we find that Ucp1 expression is downregulated in vitro by glucocorticoids even in cases when the intracellular adrenergic signalling pathway is upregulated, the effects of glucocorticoids on Ucp1 expression seem to be regulated transcriptionally in a cell-autonomous manner rather than through alterations in adrenergic output to BAT (Paper II) (Fig. 8).
3.4.2 Overproduction of glucocorticoids: adrenal Cushing’s

In addition to information obtained from mouse experiments that involve the complete removal of the adrenal glands and thereby the complete removal of glucocorticoid production, further information on the effects of glucocorticoids on BAT can be obtained from models in which the adrenal gland overproduces glucocorticoids. In humans, hypercortisolism due to excessive glucocorticoid production occurs in patients suffering from a glucocorticoid-producing tumour of the adrenal gland, a medical condition that constitutes roughly 25% of all cases of Cushing’s syndrome. Patients suffering from adrenal Cushing’s have continuously elevated plasma cortisol levels, which cannot be suppressed by high-dose exogenous administration of DEX. The most effective treatment for adrenal Cushing’s is the removal of the glucocorticoid-producing tumour. If surgery is not possible, glucocorticoid secretion can be suppressed with

Fig. 8. The effects of alterations in adrenal gland functioning on BAT. (A) In adrenalectomized (ADX) mice housed at ~21 °C, lipid droplets in BAT are decreased in size and BAT UCP1 protein per µg protein is possibly increased. The output of the sympathetic nervous system (SNS) to BAT may be increased. (B) Prkar1a-flox x Cyp11b2-Cre mice (DAdKO) housed at 21 °C develop adrenal, glucocorticoid (GC)-producing tumors and consequently become obese. Lipid droplets in BAT are increased in size and BAT Ucp1 mRNA levels are decreased.
Mammals other than humans can also develop adrenal Cushing’s, most notably dogs and hamsters. However, adrenal Cushing’s is not reported for wild-type mice, and thus the possibilities of using the mouse as a model organism to study the effects of adrenal Cushing’s on UCP1-dependent thermogenesis seem limited. Luckily, novel gene editing technologies have resulted in the development of genetically modified mice that develop glucocorticoid-producing adrenal tumours. For example, knocking out a regulatory subunit of PKA specifically in the adrenal cortex by crossing Prkar1a-lox (protein kinase cAMP-dependent type I regulatory subunit alpha) with Akr1b7-Cre (aldo-keto reductase family 1, member B7) mice, leads to mice developing Cushing’s due to the development of glucocorticoid-producing adrenal tumours (Sahut-Barnola et al., 2010). Prkar1a-lox x Akr1b7-cre mice develop frank Cushing’s syndrome between 5-10 months of age, including symptoms such as a doubling of circulating plasma corticosterone levels and fat accumulation in the interscapular region (Sahut-Barnola et al., 2010). Whether the interscapular fat represents ‘whitened’ BAT remains to be determined, as BAT function has so far not been investigated in this mouse model.

A more novel mouse model for Cushing’s Syndrome has been developed by crossing Prkar1a-lox with Cyp11b2-cre (cytochrome P450 family 11 subfamily B member 2, aldosterone synthase) mice (here termed DAdKO mice) (Dumontet et al., 2018) (Fig. 8). In these mice, adrenal tumours develop only in the definitive, adult, adrenal cortex, not in the fetal adrenal cortex (Dumontet et al., 2018 versus Sahut-Barnola et al., 2010). Analysis of the DAdKO mice in collaboration with the lab of Antoine Martinez (GReD, Université Clermont Auvergne, CNRS, INSERM, Clermont-Ferrand, France), reveals that their plasma corticosterone concentrations are doubled, probably due to an upregulation of steroidogenic gene expression in the adrenal cortex, and their body weight is significantly increased compared to wild-type mice (Figs. 8, 9; Dumontet et al., 2018). The increased body weight in DAdKO mice is due to an increase in total adipose tissue weight, as interscapular BAT and WAT, and inguinal and gonadal WAT are all increased in size, and there is a simultaneous decrease in lean mass (Fig. 9; Dumontet et al., 2018). Similar to our observations in mice with exogenous hypercortisolism (Paper 1), individual adipocyte size is increased in all adipose tissues in DAdKO mice with endogenous hypercortisolism (Fig. 10). Ucp1 mRNA levels are significantly decreased in the IBAT of DAdKO mice (Figs. 8, 11). Small amounts of Ucp1 transcripts are found in isWAT (interscapular WAT) and ingWAT, but these remain unaffected by hypercortisolism (Fig. 11). The expression of the adipogenic Fabp4 and Pparg genes is upregulated in all adipose tissues of DAdKO mice, except IBAT (Fig. 11).
**Fig. 9.** Endogenous hypercortisolism induces adipose tissue growth. Characteristics of 7-month old female wild-type (WT: As+/+ x Prkar1afl/fl; As+/Cre x Prkar1a+/+; As+/Cre x Prkar1a+/fl) and DAdKO mice (As+/Cre x Prkar1afl/fl). IBAT = interscapular BAT, isWAT = interscapular WAT, ingWAT = inguinal WAT, gWAT = gonadal WAT. Data are represented as mean ± SEM, WT n = 13, DAdKO n = 3. ** P < 0.01, *** P < 0.001, Student’s t-test.

**Fig. 10.** Endogenous hypercortisolism leads to adipocyte hypertrophy. Representative H&E stainings of IBAT, ingWAT and gWAT depots of wild-type (WT) and DAdKO mice (genotypes and abbreviations as in Fig. 9). Frequency distribution of mean adipocyte area as analyzed by Adiposoft. Data are represented as mean ± SEM, WT n = 6, DAdKO n = 3 (4-5 pictures per mouse). * P < 0.05, ** P < 0.01, *** P < 0.001 two-way ANOVA with Bonferroni post-test, significance within each bin size between genotypes.
From these data, we conclude that glucocorticoids may reduce BAT Ucp1 levels in mice experiencing endogenous hypercortisolism (Fig. 8). Although this conclusion seems to agree with the one drawn in Paper I, it does remain to be determined to what extent Cushing’s syndrome develops and affects BAT when DAdKO mice are housed at thermoneutrality. Furthermore, whole-body thermogenic capacity has not yet been determined in these mice. Finally, even if it will be found that UCP1-dependent thermogenesis is reduced in mice suffering from ‘Cushing’s’, the human implication of this might be minimal. As we show in Paper I, glucocorticoid-induced obesity develops independently of UCP1 levels in mice (and possibly in humans), and thus, reversing any reduction in UCP1-dependent thermogenesis may have no effects in patients suffering from obesity caused Cushing’s Syndrome.

Fig. 11. Gene expression in IBAT, isWAT, ingWAT and gWAT of WT and DAdKO mice. Abbreviations and genotypes as in Fig 9. 18S (reference gene), uncoupling protein 1 (Ucp1), cell death-inducing DFFA-like effector A (Cidea), fatty acid-binding protein 4 (Fabp4), peroxisome proliferator activated receptor gamma (Pparg), pparg co-activator 1 alpha (Ppargc1a), iodothyronine deiodinase 2 (Dio2), adrenergic receptor beta 3 (Adrb3), glucocorticoid receptor (Nr3c1). Data are represented as mean ± SEM, WT n = 8, DAdKO n = 3. * P < 0.05, ** P < 0.01, *** P < 0.001, Student’s t-test.
3.5 **GLUCOCORTICOID TRANSPORT:**

**TRANSCORTIN**

After glucocorticoids are produced and released by the adrenal cortex, they travel through the blood stream to target organs. Due to the low water solubility of glucocorticoids, they cannot travel through the vascular system by themselves, but have to be bound to a transport protein in order to remain in solution. The main transport protein that glucocorticoids are bound to in the vascular system is termed corticosteroid-binding globulin, also known as transcortin.

Transcortin is an alpha-globulin produced and secreted by the liver, and has a high affinity for binding glucocorticoids. Consequently, when the adrenal secretion of glucocorticoids is low, up to 95-99% of circulating glucocorticoids can be bound to transcortin. Because glucocorticoids can only diffuse into target tissues when they are un-bound (Mendel, 1989), it is vital that under stressful conditions, there are mechanisms in place that ensure that a sufficient amount of circulating free glucocorticoids is available to exert effects on target tissues. For this reason, there are several ways in which the amount of free glucocorticoids in the circulation can be increased in response to a stressor. First, circulating transcortin can become saturated with glucocorticoids. This means that, when circulating glucocorticoid levels are high, more free glucocorticoids will be available for diffusion into target tissues. Second, glucocorticoids themselves inhibit the synthesis of transcortin in the liver (Verhoog et al., 2014). Thus, when circulating glucocorticoid levels are high, circulating transcortin levels are low, and vice versa. Third, the binding affinity of transcortin for glucocorticoids is variable. More specifically, the dissociation constant (Kd) with which transcortin binds glucocorticoids increases in response to an increased body temperature or a lower glycosylation state of transcortin (Chan et al., 2013). This means that in these situations, glucocorticoids are bound less tightly to transcortin and thus, more glucocorticoids will be available for diffusion into target tissues. Through these processes, the bioavailability of glucocorticoids is adequately regulated, so that glucocorticoids can only exert effects on target tissues when needed.

In addition to being bound to transcortin, a small percentage of circulating glucocorticoids is bound to albumin. Albumin has a higher capacity but lower affinity for binding glucocorticoids than transcortin and consequently, glucocorticoids bound to albumin become more rapidly available to target tissues (Breuner and Orchinik, 2002).

In general, around 80-90% of circulating glucocorticoids are bound to transcortin, 5-10% are bound to albumin, and 3-10% are free (Perogamvros et al., 2012). Because these numbers may vary and because only free glucocorticoids are bioactive, it has been hypothesized that measuring free circulating glucocorticoid levels may provide more information about the health status of a human or animal than measuring total circulating glucocorticoids levels (as in *Paper I*). Whether this is true remains to be determined.
The regulation of the transport and bioavailability of glucocorticoids by transcortin is increasingly recognized as an important requirement for proper glucocorticoid signaling. Indeed, alterations in the functioning of transcortin may affect the metabolic outcomes of glucocorticoid signaling. Through genetic studies, a link between mutations in the transcortin gene and adiposity or susceptibility to diabetes has been found (Moisan, 2010). In addition, experimental studies report that disturbances in circulating transcortin levels may directly affect adipose tissue physiology. One of these disturbances may be the complete absence of transcortin, as is the case in the transcortin-KO mouse (Petersen et al., 2006). Although free plasma corticosterone levels are increased 10-fold in transcortin-KO mice, the physiological consequences of this increase on adipose tissue physiology are not necessarily comparable to the effects seen in mice experiencing endogenous or exogenous hypercortisolism (Petersen et al., 2006). For example, transcortin-KO mice do not gain extra weight when fed a high-fat diet, and accumulate less subcutaneous WAT than their wild-type counterparts (Gulfo et al., 2016) (contrary to Paper I). Although researchers have proposed that this might be due to a desensitization of the mice to high levels of free corticosterone (Gulfo et al., 2016), this has not been experimentally confirmed.

Because research into the link between transcortin and adipose tissue physiology is rather recent and still emerging, firm conclusions on the topic have not yet been reached. Moreover, no results have been reported concerning the relevance of transcortin for BAT functioning. Because the reported effects of the ablation of transcortin on WAT appear inconsistent and do not necessarily corresponded to previously reported effects of hypercortisolism on WAT, the effects of transcortin on BAT may also be surprising and worthy of further investigation. Thus, the link between transcortin and BAT may be an exciting new area of research and may provide us with further information on the modulation of UCP1-dependent thermogenesis by glucocorticoids.

### 3.6 Glucocorticoid Activation:

**The 11β-Hydroxysteroid Dehydrogenases**

After glucocorticoids have been produced by the adrenal gland and have been transported bound to transcortin by the vascular system, they reach their targets, which may be virtually every cell in the body. When un-bound glucocorticoids reach a target cell, they generally diffuse through the cell membrane without the aid of a transporter (but see Chen and Farese, 1999). Although it would be expected that glucocorticoids then exert their effects on cells by binding to the GR (Chapter 3.7), it has been discovered that glucocorticoid-target cells possess enzymatic machinery to convert the
bioactive forms of glucocorticoids into their inactive forms and vice versa. The enzymes catalyzing these reactions are termed the 11β-hydroxysteroid dehydrogenases.

There are two main 11β-hydroxysteroid dehydrogenases, coded for by the Hsd11b1 and Hsd11b2 genes. 11β-hydroxysteroid dehydrogenase type 1 (HSD1) is an NADPH-dependent reductase that is responsible for the conversion of inactive cortisone (11-dehydrocorticosterone in mice) to active cortisol (corticosterone in mice) (Fig. 5). 11 β-hydroxysteroid dehydrogenase type 2 (HSD2) is an NAD-dependent dehydrogenase that inactivates cortisol (/corticosterone) in the reverse reaction (Fig. 5). The synthetic glucocorticoid DEX is not converted by the HSD enzymes, but the synthetic prednisolone is converted into prednisone and vice versa (Fig. 5).

The HSD1 and HSD2 enzymes are differentially expressed across tissues. Whereas HSD1 is widely expressed in key metabolic tissues such as the liver, muscle and adipose tissues, HSD2 is expressed predominantly in the kidneys, colon and salivary gland, i.e. tissues that rely heavily on mineralocorticoid receptor action. This specific tissue distribution of the HSDs may have evolved because of the fact that glucocorticoids can also bind to the mineralocorticoid receptor (MR). The MR can bind both aldosterone and glucocorticoids with high affinity (Gomez-Sanchez and Gomez-Sanchez, 2014). Because circulating concentrations of glucocorticoids are ~100 x higher than circulating concentrations of aldosterone, the MR, when present, is virtually always occupied by glucocorticoids (Funder, 2005; Gomez-Sanchez and Gomez-Sanchez, 2014). To ensure that epithelial tissues are only regulated by mineralocorticoids and not glucocorticoids, HSD2 inactivates around 90 % of intracellular glucocorticoids (Funder, 2005). Thus, HSD2 is able to protect the mineralocorticoid receptor in sodium-transporting epithelia from glucocorticoid-binding, and thereby confers aldosterone specificity.

HSD1 is expressed in BAT of mice housed both at 21 °C and 30 °C, as we show in tissues isolated from 14-week-old mice (Fig. 12; average Ct value ~23). Very low HSD2 mRNA levels have been found in subcutaneous WAT of rodents, but so far, these have not been shown to be physiologically relevant (Milagro et al., 2007). Low levels of HSD2 mRNA have also been found in WAT and BAT of humans, but again it remains unclear whether these levels are sufficient to affect BAT glucocorticoid concentrations in vivo (Ramage et al., 2016).

The presence of the HSD enzymes means that the dose of active glucocorticoids available to exert effects on target tissues is not solely determined by the amount of glucocorticoids in the circulation, but also by the conversion of these glucocorticoids into their bioactive forms in a local tissue- (or even cell-) specific manner.
Indeed, intracellular HSD1 levels have been shown to be important regulators of tissue-specific glucocorticoid effects (Morgan et al., 2014). For example, corticosterone levels are doubled in mice overexpressing HSD1 specifically in adipose tissues compared to wild-type mice, in the absence of a change in circulating glucocorticoid levels (Masuzaki et al., 2001). The physiological significance of this becomes evident from the development of visceral obesity and insulin resistance in these mice. In contrast, mice fully lacking HSD1 lack the ability to convert inactive glucocorticoids into active glucocorticoids, and show a reduced WAT mass and improved glucose tolerance upon glucocorticoid administration, again while having circulating glucocorticoid levels similar to wild-type mice (Morgan et al., 2014). Also mice overexpressing HSD2 in adipose tissue and thereby locally inactivating glucocorticoids are protected against diet-induced obesity (Kershaw et al., 2005).

As a sidenote, because a reduction in tissue glucocorticoid levels has been shown to reverse symptoms of the metabolic syndrome, selective HSD1 inhibitors have been developed and tested in both mice and humans. Treatment with these inhibitors modestly reverses high-fat diet-induced insulin resistance and obesity (Feig et al., 2011; Peng et al., 2016).

The significance of the local conversion of inactive glucocorticoids into active glucocorticoids for the correct functioning of WAT raises the question whether the HSD enzymes play a similarly important role in BAT physiology. Furthermore, by studying the effects of increasing or decreasing the levels of active glucocorticoids in BAT on the thermogenic capacity of the tissue, we may increase our understanding about the extent

Fig. 12. The expression of Hsd11b1 in murine adipose tissues. Hsd11b1 gene expression levels in interscapular BAT (IBAT), inguinal WAT (ingWAT) and epidydimal WAT (eWAT) of C57Bl/6J mice housed at 21 °C or 30 °C and treated with vehicle (0.25 % EtOH) or corticosterone (50 µg/ml) in the drinking water for two weeks. Forward primer: CTCTCTGTGTCCTTGGCCTC, reverse primer: AGTACACCTCGCTTTTGCGT. Data are represented as mean ± SEM, n = 7. ** P < 0.01, *** P < 0.001, Student’s t-test.
to which glucocorticoids modulate UCP1-dependent thermogenesis. For this purpose, I discuss how downregulation of the HSD enzymes affects BAT functioning in Chapter 3.6.1, and I discuss how upregulation of the HSD enzymes affects BAT functioning in Chapter 3.6.2.

### 3.6.1 Downregulation of HSD1

Unsurprisingly, the actions of glucocorticoids on BAT are heavily dependent on the amount of active glucocorticoid available in the tissue. One way to investigate the effects of low active glucocorticoid levels on BAT functioning is by pharmacological inhibition of HSD1 in rodents. By applying this technique, it was found that in mice supplemented orally with the selective HSD1 inhibitor BVT.2733 for 4 weeks, BAT Ucp1 gene expression and the expression of lipolytic genes (e.g. carnitine palmitoyltransferase 1A and perilipin) in BAT is increased (Liu et al., 2013) (Fig. 15A). This presumably leads to an increase in lipolysis and a decrease in lipid droplet size in BAT (Fig. 15A). The inhibition of the HSD1 enzyme has also been shown to affect BAT in rats. Rats supplemented with the HSD1 inhibitor 4-heteroarylbicyclo [2.2.2] octyltriazole in their diet for 3 weeks show a decrease in BAT weight and a doubling in BAT Ucp1 gene expression (Berthiaume et al., 2007) (Fig. 15A). Although the uptake of circulating triglycerides by BAT is increased in these rats, there is a decrease in the total triglyceride content in BAT, indicating a high lipid turnover. Unfortunately, non-shivering thermogenic capacity was not measured in these rodent studies, and it is thus not possible to conclude whether the increase in Ucp1 gene expression in the absence of active glucocorticoids in BAT leads to an increase in UCP1-dependent thermogenic capacity.

Additional evidence that shows that a low conversion rate of inactive glucocorticoids into active glucocorticoid affects BAT functioning has been shown by analysis of the HSD1 knock-out mouse (HSD1-KO). This mouse model allows for the study of the physiological effects of HSD1 depletion without the need for exogenous supplementation with HSD1 antagonists. The deletion of HSD1 is achieved by replacement of exons 3 and 4 of the Hsd11b1 gene with a neomycin-resistance cassette (Kotelevtsev et al., 1997). Indeed, HSD1-KO mice are unable to convert supplemented inactive 11-dehydrocorticosterone into active corticosterone, as is evident from low plasma corticosterone concentrations after supplementation (Kotelevtsev et al., 1997). Although HSD1 enzyme activity has been shown to be decreased in the BAT of the HSD1-KO mice, no direct measurements of the absolute amounts of 11-dehydrocorticosterone and corticosterone have been made (Doig et al., 2017; Zeng et al., 2016). Thus, the levels of active glucocorticoid in the BAT of these mice are unknown. Interestingly, BAT histology is unaffected by the absence of HSD1, but
HSD1-KO mice do display an increase in core body temperature (Morton et al., 2004; Zeng et al., 2016) (Fig. 15B). The nature of this increase remains to be determined.

More pronounced effects of the loss of HSD1 on BAT functioning are visible when HSD1-KO mice are challenged with an excess of circulating glucocorticoids. In wild-type mice housed both at 21 °C and 30 °C and supplemented with 50 µg/ml corticosterone in their drinking water, we show an increase in BAT weight, lipid accumulation in BAT, and BAT Hsd11b1 gene expression (Paper I; Figs. 12, 15C). In addition, mice with endogenously high circulating levels of glucocorticoids (DAdKO mice) also show an increase in Hsd11b1 gene expression across several adipose tissues (Fig. 13). In a model of glucocorticoid excess similar to our studies, HSD1-KO mice are housed at 21 °C and supplemented with 100 µg/ml corticosterone in their drinking water (Doig et al., 2017). The effects of glucocorticoid excess on BAT in wild-type mice and DAdKO mice housed at 21 °C that we report are all attenuated in these HSD1-KO mice (Doig et al., 2017) (Fig. 15D). In addition, the decrease in BAT UCP1 levels per µg protein that we report in response to glucocorticoid treatment (Paper I) is also reversed in the HSD1-KO mice (Doig et al., 2017) (Fig. 15D). Unfortunately, Doig and colleagues provide no data on total UCP1 protein levels in the BAT of HSD1-KO mice following corticosterone treatment, nor do they test nonshivering thermogenic capacity. Since we show that total BAT UCP1 protein levels, as well as whole-body thermogenic capacity, in mice housed at 21 °C are not affected by hypercortisolism (Paper I), it remains unclear whether the absence of HSD1 affects BAT thermogenic functioning, or whether it merely affects the accumulation of lipids in BAT in response to glucocorticoid treatment.

Additional information about the effects of glucocorticoids on BAT can be obtained from the study of aged HSD1-KO mice. In wild-type mice aged 100 weeks, BAT HSD1
protein levels are increased 4-fold compared to young mice (15 weeks old), which correlates with reported decreases in BAT UCP1 protein levels (per µg protein) in aging mice (Graja and Schulz, 2015). The reported decrease in UCP1 protein in aging mice may partly be due to high exposure to glucocorticoids. This hypothesis is supported by the observation that aged HSD1-KO mice show a 3-fold increase in BAT UCP1 protein levels (per µg protein) compared to aged wild-type mice, even in the absence of any exogenous glucocorticoid treatment (Doig et al., 2017). Although it again remains to be determined whether this is a ‘true’ increase in total BAT UCP1 protein levels and to what extent this translates into alterations in whole body UCP1-dependent thermogenic capacity, it provides a tentative indication that a glucocorticoid-mediated suppression of UCP1 may contribute to the development of obesity associated with age.

It may be so that the reported changes in BAT functioning due to lowering whole-body HSD1 levels in vivo either pharmacologically or through genetic modifications are secondary to effects that the absence of HSD1 has on other tissues. Indeed, the downregulation of HSD1 (and thus the lowering of active glucocorticoid levels) only in visceral WAT and liver using i.p. injected antisense oligonucleotides mildly influences gene expression in BAT, as it results in an upregulation of the transcription of genes such as Dio2, Dgat1 and Cox8b (Li et al., 2012). Although it is currently not possible to test in vivo whether the effects of the ablation of HSD1 on BAT are direct or secondary to other physiological effects, as BAT-specific HSD1-KO mouse models do not exist, it may be possible to some draw conclusions on this matter from the inhibition of HSD1 in vitro in cell cultures of brown adipocytes.

First, we show that treatment of cultured primary brown adipocytes with 1 µM DEX during differentiation increases Hsd11b1 gene expression 5-fold, and reduces Ucp1 gene expression 2-fold (Paper II, Fig. 14). In primary brown adipocytes isolated from HSD1-KO mice and differentiated in the presence of 1 µM DEX, Ucp1 mRNA levels are significantly increased compared to the levels seen in brown adipocytes isolated from wild-type mice (Doig et al., 2017). Finally, both the pharmacological inhibition of HSD1 by BVT.2733 as well as the downregulation of HSD1 by siRNA significantly increases Ucp1 gene expression in cultured primary brown adipocytes differentiated in the presence of 1 mM cortisone (Liu et al., 2013). In these brown adipocytes, also lipid droplet size is significantly decreased in the absence of HSD1, which may be due to an upregulation of the levels of proteins associated with lipid droplets and fatty acid β-oxidation (Liu et al., 2013).

Taken together, these results indicate that in vitro, lower levels of HSD1, and thus of active glucocorticoids, are associated with an increase in Ucp1 transcription in brown adipocytes. This means that the effects of the ablation of HSD1 on brown adipocytes are at least partly cell autonomous, and not secondary to effects of HSD1 ablation on other tissues.
The above-mentioned data indicate that in situations of prolonged hypercortisolism, the conversion of inactive glucocorticoids into their bioactive form is a crucial part in the mediation of the cell-autonomous effects of glucocorticoids on BAT. In the absence of HSD1, Ucp1 transcription is increased both in vivo in the BAT of mice, as well as in vitro in brown adipocytes grown in culture.

As mentioned, the physiological significance of the reported increase in Ucp1 transcription and BAT UCP1 levels per µg protein in the absence of HSD1 remains to be determined. Because we show that hypercortisolism does not affect UCP1-dependent thermogenic capacity in mice housed at 21 °C (Paper I), the modulation of intracellular active glucocorticoid levels by HSD1 in the BAT of animals housed at this temperature may be irrelevant. Since glucocorticoids do downregulate total BAT UCP1 protein levels in animals housed at thermoneutrality, the effects of HSD1 downregulation on UCP1-dependent thermogenic capacity may be worth investigating in animals housed at this temperature.

It does seem likely that lipid accumulation in brown adipocytes in situations of prolonged hypercortisolism is decreased in the absence of HSD1 (Fig. 15). However, as we show in Paper I and discuss in Chapter 3.4, lipid content in BAT is not necessarily a direct reflection of the thermogenic capacity of the tissue. Nevertheless, it is clear that without the possibility to activate intracellular glucocorticoids – even though circulating glucocorticoid levels may be chronically increased – lipid droplet size in BAT decreases. Because the uptake of triglycerides into BAT is simultaneously increased in the absence of HSD1 (Berthiaume et al., 2007), as well as the expression of lipolytic enzymes (Liu et al., 2013), there may be an increased lipolytic capacity of the tissue (Fig. 15). For these reasons, it may be so that the glucocorticoid-induced expansion of adipocytes and hypertrophy of BAT that we see in mice housed both at 21 °C and 30 °C can be reversed through the suppression of HSD1 (Paper I). It has to be noted that it seems to be the case that the effects of the ablation of HSD1 on Ucp1 transcription and lipid accumulation in BAT may be only relevant in situations of prolonged...
hypercortisolism. Indeed, the downregulation of Ucp1 mRNA levels in response to a short-term treatment of wild-type adipocytes with 1 µM corticosterone (in accordance with our own data), is not significantly reversed in the absence of HSD1 (Doig et al., 2017).

Finally, it is important to note that lowering HSD1 levels, and thus the amounts of active glucocorticoids in tissues, in vivo in mice, lowers food intake (Li et al., 2012). This agrees with our data that show that exogenous corticosterone supplementation increases food intake in mice (Paper I). Although part of the effects of lower tissue levels of active glucocorticoids may be secondary to the effect of HSD1 ablation on food intake, both we and other groups have shown that the glucocorticoid-induced changes in body weight, fat mass and UCP1 protein levels happen at least partly independently of changes in food intake (Li et al., 2012; Paper I). Thus, it is likely that the presence of HSD1 affects BAT physiology at least partly independently of effects on food intake in a cell-autonomous manner.

Fig. 15. (page 52) The effects of the absence of HSD1 on BAT. (A) In wild-type (WT) mice housed at 21 °C and treated with an HSD1 inhibitor, the expression of the lipolytic enzymes perilipin and carnitine palmitoyltransferase IA (CPT1a) is upregulated, but also triglyceride (TG) uptake into BAT is increased. The expression of Ucp1 is upregulated; whether this alters total BAT UCP1 protein levels remains to be determined. (B) In mice lacking HSD1 (HSD1-KO) housed at 21 °C, no differences are visible in BAT histology compared to WT mice. HSD1-KO mice do show an increased core body temperature (BT). (C) WT mice housed at 21 °C and treated with glucocorticoids become obese, and show an increase in lipid accumulation and a decrease in UCP1 protein per µg protein in BAT. (D) HSD1-KO mice housed at 21 °C and treated with glucocorticoids do not become obese, and do not show an increase in lipid accumulation nor a decrease in UCP1 protein per µg protein in BAT.
3.6.2 Overexpression of HSD1

The effects of glucocorticoids on BAT lipid metabolism and possibly thermogenesis are further highlighted in studies showing the effects of overexpression rather than downregulation of HSD1.

In transgenic mice overexpressing HSD1 under control of the Fabp4 promoter-enhancer region, HSD1 enzyme activity, and thereby the amount of active glucocorticoid in the tissue, is increased in all adipose tissue depots, including BAT (Masuzaki et al., 2001). BAT Ucp1 mRNA levels are significantly downregulated in these mice (Masuzaki et al., 2001). Unfortunately, no measurements have been made of UCP1 protein levels or in vivo thermogenic capacity of HSD1-transgenic mice.

A mouse model in which the overexpression of HSD1 is established in a more indirect manner has also been developed. The expression of Hsd11b1 is endogenously controlled by the lysine-specific demethylase 1 (LSD1), a corepressor that binds to the promoter region of the Hsd11b1 gene. To reverse the inhibition of Hsd11b1 gene expression by LSD1 and thus induce an upregulation of HSD1 protein levels, mice have been created that lack LSD1 in all adipose tissues (Zeng et al., 2016). In these mice, BAT Hsd11b1 mRNA levels are upregulated and BAT HSD1 enzyme activity is increased, thus most likely leading to a higher content of active glucocorticoids in BAT (although this has not been measured). The increased activity of BAT HSD1 in these mice goes hand in hand with an increase in BAT mass and lipid content, a 2-fold decrease in BAT Ucp1 mRNA levels, and a decrease in NE-induced brown adipocyte respiration rate (Zeng et al., 2016). These effects are reversed in animals in which HSD1 is simultaneously knocked-out together with LSD1, indicating that they are the result of the upregulation of HSD1 through the absence of LSD1.

Finally, the effects of overexpression of HSD1 on brown adipocytes have been investigated in vitro. A 4-fold increase in HSD1 protein levels is achieved in cultured primary brown adipocytes by lentiviral infection (Liu et al., 2013). The overexpression of HSD1 decreases brown adipocyte Ucp1 transcription, and increases lipid droplet size in brown adipocytes. These effects can all be reversed by successive treatment of the cells with the HSD1 inhibitor BVT.2733, again indicating that they are due to the increased HSD1 levels (Liu et al., 2013).

In summary, an increase in the levels of active glucocorticoids in BAT as a result of HSD1 overexpression leads to an increase in BAT mass and lipid accumulation in BAT, and reduces brown adipocyte Ucp1 transcription. These results agree with the data discussed in chapter 3.6.1 and our own reported data (Paper I, Paper II).

The finding that NE-induced brown adipocyte respiration is decreased when HSD1 activity is increased indicates that glucocorticoids may affect UCP1-dependent thermogenic capacity in a physiologically relevant manner. This is in accordance with our findings in mice housed at thermoneutrality (Paper I). However, to draw this
conclusion, measurements are needed of total thermogenic capacity in animals in which HSD1 levels are increased and that are housed at thermoneutrality.

However, independent of the effects of glucocorticoids on UCP1, it does seem clear that in the presence of high tissue-specific levels of glucocorticoids, lipid accumulation in BAT is increased.

Fig. 16. The effects of the overexpression of HSD1 on BAT. In mice overexpressing HSD1 (HSD1-tg) housed at 21 °C, lipid accumulation in BAT is increased and the expression of Ucp1 is downregulated, whether this alters total BAT UCP1 protein levels remains to be determined. Brown adipocytes isolated from these mice show a decreased oxygen consumption rate (OCR).
3.7 GLUCOCORTICOID SIGNALING: THE GLUCOCORTICOID RECEPTOR

After the diffusion of un-bound circulating glucocorticoids into target cells, and after the successive intracellular conversion of inactive forms of glucocorticoids into their active forms by the HSD1 enzyme, active glucocorticoids can exert their intracellular effects. Glucocorticoids mainly affect cellular physiology through binding to the intracellular GR. In this chapter, I will discuss in what way the signaling of glucocorticoids through the GR affects UCP1-dependent thermogenesis. First, I discuss the different isoforms and the tissue distribution of the GR (Chapter 3.7.1). Next, I discuss the function of the GR as a ligand-dependent transcription factor, i.e. the ways in which the liganded GR can affect gene expression (Chapter 3.7.2). The effects of pharmacological inhibition of the GR or genetic modification of the GR on BAT functioning in rodents is discussed respectively in Chapters 3.7.3 and 3.7.4. Finally, I discuss whether glucocorticoids may affect BAT through nongenomic signaling pathways (Chapter 3.7.5).

3.7.1 Isoforms and tissue distribution

The GR is encoded by the *Nr3c1* gene, located on chromosome 5 in humans and on chromosome 18 in mice. The *Nr3c1* gene contains 9 exons in both species. Exon 1 forms the 5′-untranslated region, while the subsequent exons code for an N-terminal transactivation domain (exon 2), a central DNA-binding domain (exon 3,4), a hinge-region (exon 5) and a C-terminal ligand-binding domain (exon 6-9). Two isoforms of the GR, GRα and GRβ, are generated by alternative splicing of exon 9 in humans, creating transcripts that differ in their C-termini (Oakley et al., 1996). In mice, GRβ is formed by alternative splicing of intron 8 (Hinds et al., 2010). Human and mouse GRβ are 87% identical and are both unable to bind glucocorticoids due to alterations in their ligand-binding domains (Hinds et al., 2010). In addition to the GRα and GRβ isoforms, 8 isoforms of the human GRα are formed by alternative initiation of translation at the N-terminus in exon 2 (GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, GRα-D3) (Kadmiel and Cidlowski, 2013). These translational isoforms of GRα are also present in mice, and GRβ is predicted to also exist in these translational isoforms (Lu and Cidlowski, 2005). Finally, the translated GR protein can undergo several post-translational modifications, including phosphorylation, acetylation, ubiquitination and SUMOylation (Anbalagan et al., 2012).

The GR is ubiquitously expressed throughout the human and rodent body. However, the expression of the different splice variants, as well as the variants formed by alternative initiation of translation, is highly variable between and within tissues.
Different isoforms of the GR have been shown to function in distinct ways. For example, GRβ always resides in the nucleus, cannot bind glucocorticoids and does not directly regulate gene expression, but inhibits the effects of GRα on gene expression (Kino et al., 2009). Therefore, its level of expression within a certain tissue is believed to determine the ability of that tissue to perceive glucocorticoid resistance. All 8 isoforms of GRα have been detected in most tissues of mice (Lu and Cidlowski, 2005). Although all isoforms of the GRα are capable of binding both ligand and DNA to a similar extent, their subcellular localization and ability to recruit coregulators to target gene promoters differs (Oakley and Cidlowski, 2011). These isoform-selective transcriptional effects are believed to fine-tune glucocorticoid signaling and lead to tissue-specific effects of glucocorticoids (Oakley and Cidlowski, 2011).

Forty years ago, it was discovered that BAT is also a glucocorticoid target organ (Feldman, 1978). The GR is expressed in both pre- and mature brown adipocytes (Fig. 17), as well as in other cell types present in BAT, e.g. endothelial cells. The main GR isoforms expressed in BAT seem to be GRα-A and GRα-B, but not GRα-C and GRα-D (Lu and Cidlowski, 2005). No data have been published concerning the expression of GRβ in BAT.

![Fig. 17. The expression of \( Nr3c1 \) and \( Pparg \) in differentiating WT1 cells. GR (\( Nr3c1 \)) and \( Pparg \) gene expression levels in WT1 cells during day 2, 4, 6, 8 or 10 of differentiation. Primers as in Paper II. Data are represented as mean ± SEM, \( n = 6 \). Experiment performed by Yun Gao.](image)

### 3.7.2 Genomic effects of glucocorticoids

The GR is a part of the nuclear receptor class of proteins, meaning that it functions as a ligand-dependent transcription factor. Unliganded, GR α resides mainly in the cytoplasm, bound to several chaperone proteins as a multiprotein complex (Pratt et al., 2006). These chaperone proteins include heat-shock protein 90 (Hsp90), heat-shock
protein 70 (Hsp70), Hsp70-Hsp90 organizing protein (Hop), heat-shock protein 40 (Hsp40) and P23. The chaperone proteins facilitate the formation of GR-Hsp90 heterocomplexes, which is essential for GR’s ability to bind steroid. Hsp90, together with the chaperone proteins, opens up GR’s steroid-binding cleft in an ATP-dependent manner. The steroid-binding cleft remains open as long as Hsp90 is bound. Upon binding of a glucocorticoid in the steroid-binding cleft of the GR, conformational changes expose the GR’s nuclear localization sequences, which direct the transport of liganded GR towards and into the nucleus. This process involves binding of the chaperone proteins to dynein proteins that move along microtubules. The transport of the liganded GR into the nucleus is dependent on importin proteins (Freedman and Yamamoto, 2004). Inside the nucleus, the liganded GR may homodimerize, and successively uses its DNA-binding domain containing two zinc-finger motifs to bind to specific DNA sequences, named glucocorticoid response elements (GREs). Recent research has shown that the binding of the liganded GR to a GRE and its transcriptional activity when bound is also dependent on the GR chaperone proteins (Conway-Campbell et al., 2011).

The binding of the GR to GREs in the promoter or enhancer regions of target genes can elicit multiple effects. GRs can bind to GREs that either activate or repress the transcription of target genes, in processes named transactivation and transrepression (Ratman et al., 2013). The ‘simple’ GREs increase the transcription of target genes through direct binding of the liganded GR and its association with coactivators (e.g. histone acetyl transferases, C/EBPβ) (Lonard and O’Malley, 2007). Alternatively, transactivation can occur through composite GREs or tethering GREs. The former consist of binding sites for GR and associated binding sites for other transcription factors. Gene transcription in this case is regulated synergistically upon binding of both the GR and the additional transcription factor. Tethering GREs do not bind the liganded GR, but rather attract other transcription factors that in their turn recruit the GR, thereby indirectly activating gene transcription (e.g. NF-κB, AP-1) (Kassel and Herrlich, 2007). Transrepression can be mediated through composite or tethering GREs in a similar manner to transactivation. In addition, the liganded GR can bind to a response element where another transcription factor may usually bind, and thereby in a competitive manner block that transcription factor from binding, thus repressing transcription (Ratman et al., 2013). It is usually assumed that transrepression does not occur through the ‘simple’ direct binding of a liganded GR to a target gene promoter region. However, more recent research has shown that, in several cases, the liganded GR and corepressors do bind to negative GREs and thereby directly suppress gene transcription (Surjit et al., 2011).

The effects of glucocorticoids on adipose tissue physiology are also mediated through the binding of the GR to GREs in regulatory regions of target genes and thereby
affecting gene expression. The GR has binding sites in the regulatory regions of many genes in WAT, as several researchers have determined by ChIP-seq analysis. In 3T3-L1 adipocytes treated with DEX, 619 genes have been shown to be regulated by glucocorticoids (421 activated, 198 repressed) and of these, 337 contain a GRE in or around their regulatory regions (Yu et al., 2010). Most of these genes code for proteins involved in triglyceride synthesis as well as in lipolysis. The regulation of gene expression by the GR has also been shown in human white adipocytes isolated from the abdominal region and treated with DEX (Singh et al., 2015). In these cells, 123 GR target genes have been identified, associated with 136 GREs (4 genes had multiple GREs). For BAT, GR binding sites have previously only been determined in SV40 T-immortalized preadipocytes isolated from murine BAT 0 and 4 hours after the induction of differentiation (in the presence of DEX) (Park and Ge, 2017). 247 genes are upregulated in a GR-dependent manner during this time-frame, among them many genes that promote adipogenesis, such as the C/EBPs. In this thesis, we show in BAT isolated from C57Bl/6 mice that also the Ucp1 gene has GR binding sites in its promoter and enhancer regions (Paper II) (Fig. 18).

The mere presence of a GR binding site in the regulatory regions of e.g. the Ucp1 gene does not confer any information on whether the binding of the GR will result in an up- or down-regulation of the expression of that gene. To determine this, information has to be obtained on the type of GRE that is present in the regulatory region.

The consensus GRE is a 6 bp inverted repeat separated by 3 nonspecific bases: 5’-AGAACAnnnTGTTCT-3’ (Strähle et al., 1987). However, the GRE may be present in several variations of this consensus sequence and may thereby differ in its affinity for binding the liganded GR. For example, a tethering GRE does not contain a binding-site for GR, but binding sites for other transcription factors. Correspondingly, one study found that in human lung epithelial carcinoma cells, only 62 % of the DEX-induced DNA-binding of GR occurs at sites containing at least one GRE (Reddy et al., 2009). Thus, the binding of the GR on Ucp1 regulatory regions may or may not indicate the presence of a GRE and may or may not indicate an upregulation of gene transcription.

Researchers have so far been unable to predict whether the binding of the liganded GR to a GRE will activate or suppress the transcription of the target gene. One indication for the future effects of the GR on transcription may however be the distance of the specific GRE the GR binds to from the transcription start site of the target gene. GREs are known to be present not only in the promoter region or the 5´-untranslated region of a target gene, but also very much upstream of the target gene. Target genes the expression of which is upregulated by the GR are much more likely to have a GRE close to the transcription start site compared to target genes of which the expression is downregulated by GR (Reddy et al., 2009). The nearest GR binding occurs on an average distance of 6 kb from the transcription start site in upregulated genes and 119
kb from the transcription start site in downregulated genes (Reddy et al., 2009). These very distant GREs may still affect gene expression upon GR-binding due to the looping nature of chromatin.

The binding of GR to GREs in *Ucp1* regulatory regions that we report is found both 2-5 kb upstream of the transcription start site of *Ucp1*, and also around 12 to 13 kb upstream of the *Ucp1* transcription start site (Paper II) (Fig. 18). Based on the information provided above, it may be expected that glucocorticoids upregulate the expression of *Ucp1*, due to the proximity of these GR binding sites to the *Ucp1* transcription start site. However, in Paper II we report on the basis of luciferase reporter assays that upon a 24 h DEX treatment, the liganded GR actually downregulates *Ucp1* gene expression through binding at a regulatory region 0-4 kb upstream of the *Ucp1* transcription start site (Fig. 18). Although initially surprising, this may indicate that there is a direct negative GRE present at this site. On the other hand, it is possible that the binding of the GR at this site interferes or works synergistically with the binding of factors that are present in the cell culture serum, and thereby downregulates gene expression. Based on our reported experiments, further conclusions on this matter cannot be drawn. From our research, it can also not be concluded whether the reported GR binding sites in *Ucp1* regulatory regions are the only ones that may influence *Ucp1* gene expression. There may be additional distal GR binding sites regulating *Ucp1* transcription, but we have not performed any luciferase reporter assays for GREs further upstream than 13 kb from the *Ucp1* transcription start site. Furthermore, *Ucp1* gene expression is interestingly upregulated in this system after a 48 h DEX treatment (Paper II). What this indicates remains to be determined.

As a side note, where the liganded GR binds in the regulatory regions of target genes may not solely be determined by the presence of GREs. Recent research suggests that the genome-wide binding patterns of the GR also seem to be determined to a large extent by pre-existing chromatin availability. For example, the in vitro DNA-binding of the GR upon DEX treatment shows a high correspondence with patterns of high chromatin availability determined in the cells pre-DEX treatment (John et al., 2011). The availability of chromatin for the GR to bind may depend on the methylation status of the chromatin, as it has been shown that transcription factor binding sites are often hypomethylated (Choy et al., 2010). This may also be relevant for GR-binding in BAT. For example, in SV40 T-immortalized preadipocytes isolated from murine BAT 4 hours after induction of differentiation (in the presence of DEX), binding of the GR colocalizes with H3K4me1 and H3K27ac, an enhancer mark and an active enhancer mark, respectively (Park and Ge, 2017). Also, we show in Paper II that in BAT isolated from C57Bl/6 mice, the DNA-binding sites of the GR around the *Ucp1* gene show a high correspondence to H3K27ac. Thus, in BAT, histone acetylation may also be a
determinant of where the GR will bind to the DNA, although we cannot conclude to which extent these chromatin acetylations were already in place before GR binding.

**Fig. 18. The binding of the liganded GR to Ucp1 regulatory regions.** A 24 h dexamethasone treatment induces glucocorticoid receptor (GR) translocation into the nucleus. In the nucleus, the liganded GR binds to regulatory regions 2, 3, 4, 5, 12 and 13 kb upstream of the Ucp1 transcription start site (TSS). The Ucp1 promoter region contains binding sites for CREB and C/EBPs (among others), the Ucp1 distal enhancer region ~2.5 kb upstream of the TSS contains binding sites for PPARs and ATF2 (among others). Binding of the GR to a region 0-4 kb upstream of the Ucp1 TSS (blue box) inhibits Ucp1 gene expression.

### 3.7.3 Pharmacological inhibition of the GR

As shown in Paper I, Paper II and Chapters 3.3-3.6, glucocorticoids affect BAT physiology in vivo and brown adipocyte functioning in vitro. We show that glucocorticoids rapidly downregulate the expression of Ucp1, and that this most likely happens through binding of the liganded GR at a Ucp1 regulatory region 0-4 kb upstream of the Ucp1 transcription start site (Paper II) (Fig. 18). The significance of
this finding for the physiological functioning of UCP1-dependent thermogenesis may be determined in models in which GR functioning is altered, for example through pharmacological inhibition. Indeed, several groups have investigated to what extent pharmacological inhibition of the GR in vivo and in vitro affects BAT functioning. Their findings will be discussed here.

The most widely used pharmacological GR antagonist is the synthetic steroid RU-38486, also known as mifepristone (here referred to as RU486). RU486 has a high affinity for the GR, 2-3 times higher than that of DEX, and a very low dissociation rate from its complex with the GR (t1/2 >> 24 h, DEX t1/2 = 12 h) (Gagne et al., 1985). Consequently, RU486 fully inhibits the cellular effects of DEX. RU486 does not exhibit any glucocorticoid agonist activity at the GR below concentrations of 10 µM in cell culture (liver cells) and 50 mg/kg in rats (Gagne et al., 1985), and can therefore be considered to solely exhibit antagonistic properties in most studies.

Being a non-selective inhibitor, RU486 not only blocks GR signaling, but also blocks signaling through the progesterone receptor. Although the progesterone receptor is predominantly expressed in reproductive organs, the bladder and smooth muscle cells, the presence of progesterone receptor mRNA has also been reported in BAT (although the Ct values for detection are not mentioned) and progesterone treatment may influence BAT gene expression in vitro (Rodriguez-Cuenca et al., 2007; Rodriguez et al., 2002). Thus, it cannot be fully ruled out that some of the reported effects of RU486 on brown adipocyte functioning are in part a result of a reduction in progesterone receptor signaling.

The antagonizing of GR signaling by RU486 has been shown to significantly affect brown adipocyte functioning in vitro. Specifically, RU486 treatment reverses the above-mentioned effects of glucocorticoids on brown adipocytes (Chapters 3.3-3.7.2). For example, in the murine T37i and HIB-1B cell lines, RU486 treatment reverses the downregulation of NE-induced Ucp1 mRNA levels by glucocorticoids (van den Beukel et al., 2014; Soumano et al., 2000). Also in SV40-T immortalized preadipocytes isolated from murine BAT and differentiated in vitro, the downregulation of NE-induced Ucp1 mRNA levels by corticosterone is reversed upon simultaneous RU486 treatment, but only partially (Kroon et al., 2018). These findings confirm the notion that the downregulation of Ucp1 expression by glucocorticoids is at least partly mediated by the GR.

In vivo, the chronic administration of RU486 blocks the actions of endogenously circulating glucocorticoids. That this affects BAT function becomes clear from a study in which rats receive an 8-day treatment with RU486 (Hardwick et al., 1989). In these rats, GDP-binding to BAT mitochondria (a measure of UCP1 protein) increases by over 50 % (Hardwick et al., 1989). This could be physiologically significant, as metabolic efficiency decreases in these rats (Hardwick et al., 1989). The physiological effects of
the inhibition of glucocorticoid signaling through the GR are also visible in mice (Fig. 19). In C57Bl/6 mice receiving RU486 in their food for 4 weeks, BAT Ucp1 mRNA levels show a 50 % increase, BAT Pgc1a, Lpl and Cd36 mRNA levels also increase, and BAT lipid content decreases (van den Heuvel et al., 2016) (Fig. 19). These results indicate that even in the absence of hypercortisolism, physiological concentrations of glucocorticoids may suppress Ucp1 transcription and may induce lipid accumulation in BAT. These effects are reversed upon the inhibition of the GR (Fig. 19).

Interestingly, the effects of RU486 administration on BAT are visible already after a single injection. In rats, one peripheral injection of RU486 leads to a rapid (30-60 min) increase in O2 consumption (Hardwick et al., 1989). This increase is mediated through the adrenergic signaling pathway, as it is prevented by prior treatment with the β-adrenergic antagonist propranolol, and may be UCP1-dependent, as GDP-binding to BAT mitochondria of these rats is increased (Hardwick et al., 1989). These results have been reproduced in ob/ob mice, where a single RU486 injection increases GDP-binding to BAT mitochondria after 120 min (Chen and Romsos, 1994). Additionally, in adrenalectomized ob/ob mice (lacking endogenous glucocorticoid production), an acute RU486 treatment reverses the decrease in BAT mitochondrial GDP-binding that results from an acute DEX injection (Chen and Romsos, 1994). These rapid effects of RU486 may indicate that in normal situations, UCP1 is ‘masked’ by liganded GR, and RU486 induces an ‘unmasking’. How this manifests itself remains to be determined.

From these data, it can be concluded that glucocorticoids inhibit the expression of Ucp1 in brown adipocytes perhaps even at normal physiological circulating levels through binding to the GR (Fig. 19). These effects most likely occur in a cell-autonomous manner, as they occur both in vivo and in vitro. The increase in BAT lipid content seen upon an in vivo glucocorticoid treatment (Paper I) may also be a result of glucocorticoid signaling through the GR. Furthermore, the reversal of the glucocorticoid-induced downregulation of Ucp1 expression by RU486 may be physiologically significant, as RU486 treatment decreases metabolic efficiency. However, no measurements have been made of total BAT Ucp1 mRNA or protein levels nor of whole-body UCP1-dependent thermogenic capacity in response to RU486 treatment. Thus, whether the increase in Ucp1 expression is real, and whether it successively affects energy expenditure in animals housed at thermoneutrality, remains to be determined (Fig. 19).

As a side note: RU486 may exert effects on UCP1-dependent thermogenesis in itself (i.e. independent of its GR-antagonism) when administrated in high concentrations. In primary brown pre-adipocyte cultures differentiated in vitro and treated with 10 µM RU486 for 24 h in serum-free medium, NE-induced Ucp1 mRNA levels are upregulated (Rodriguez and Palou, 2004). In these cultures, basal and NE-induced UCP1 protein levels are upregulated at an RU486 concentration >100 µM (Rodriguez and Palou, 2004). Since there cannot be any glucocorticoids present in the
culture medium in serum-free conditions, it can be excluded that these effects are due to GR antagonistic properties. Thus, they have to be due to intrinsic agonist properties of RU486 itself. However, for most studies, this finding may be irrelevant, as RU486 is usually supplemented at concentrations below 10 µM, and will therefore solely exhibit receptor antagonist properties.

As mentioned above, RU486 is an antagonist for the GR, as well as for the progesterone receptor. This lack of selectivity is an issue when RU486 is used for pharmacological purposes, as e.g. Cushing’s patients treated with RU486 can suffer from endometrial thickening or irregular vaginal bleeding as a result of RU486’s progesterone antagonism (Hunt et al., 2017). In addition, also as mentioned above, RU486 exhibits partial agonist effects when administrated in high concentrations.

Because of these disadvantages of RU486, researchers have been developing novel, more selective GR antagonists that do not exhibit partial agonist properties. One of these novel GR antagonists is CORT125281. This compound inhibits cellular corticosterone signaling through the GR at an IC\textsubscript{50} of ~500 nM (IC\textsubscript{50} of RU486 ~50 nM, HEK293T cells), and does not affect progesterone signaling through the progesterone receptor (Hunt et al., 2017; Kroon et al., 2018). In vivo, CORT125281

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**Fig. 19. The effects of the pharmacological inhibition of the GR on BAT.** In wild-type (WT) mice housed at 21 °C and treated with the GR antagonist RU486, the expression of the lipolytic enzyme lipoprotein lipase (Lpl), and of an enzyme involved in lipid uptake, cluster of differentiation 36 (Cd36), are upregulated. The expression of Ucp1 in BAT is upregulated, whether this alters total BAT UCP1 protein levels remains to be determined. The inhibition of GR signaling may reduce metabolic efficiency (ME).
administration lowers plasma TG and FFA levels in mice fed a high-fat diet, and increases whole-body fatty acid oxidation (Kroon et al., 2018). Whether this GR antagonist affects BAT functioning has also been investigated. Similarly to the effects of RU486, treatment with CORT125281 partially reverses the downregulation of NE-induced Ucp1 mRNA levels by corticosterone (Kroon et al., 2018). CORT125281 also increases FFA uptake in BAT, but simultaneously reduces lipid content (Kroon et al., 2018). Similarly to results reported in mice supplemented with an HSD1 inhibitor, this may indicate an enhanced lipid turnover in the absence of glucocorticoid signaling (Berthiaume et al., 2007; Kroon et al., 2018; see Chapter 3.6). Although these results again indicate that UCP1-dependent thermogenesis may be increased, and lipid accumulation may be decreased, in the absence of glucocorticoid signaling through the GR, again no measurements have been made of the thermogenic capacity of these mice. It therefore remains unclear whether these effects lead to physiologically relevant changes in UCP1-dependent thermogenic capacity, and also whether the increased lipid turnover may be a result of an increase in BAT UCP1 recruitment or activation.

Another novel GR antagonist that has been investigated for its effects on BAT physiology is C108297 (van den Heuvel et al., 2016). Interestingly, in mice fed a high-fed diet, BAT lipid accumulation and gene expression remain unchanged compared to control mice upon treatment with C108297 (van den Heuvel et al., 2016). These results are in contrast to the reported effects of RU486 and CORT125281 on BAT, and thus indicate that the effects of GR antagonism may be antagonist-specific. Nevertheless, it is interesting to note that mice treated with CORT125281 and C108297 both show a decrease in body weight and fat mass (van den Heuvel et al., 2016; Kroon et al., 2018). Thus, it can be concluded that these effects happen independently of the presence or absence of glucocorticoid signaling through the GR in BAT. From this, it can be extrapolated that these data are in agreement with our data that show that BAT UCP1 is dispensable for the development of glucocorticoid-induced obesity (Paper I).

### 3.7.4 In vivo knock-outs of the GR

The significance of the glucocorticoid-mediated suppression of Ucp1 expression for the physiological functioning of UCP1-dependent thermogenesis has been further investigated in mice that have been genetically modified to not express the GR (i.e: GR-KO). The first GR-KO mice were developed by the group of Günther Schultz in Heidelberg, by the placement of a neomycin resistance cassette in exon 2 of Nr3cl, the gene coding for the GR (Cole et al., 1995). Although these mice express a truncated version of the GR that contains the ligand-binding domain and that can therefore bind DEX, they are profoundly resistant to physiological actions of glucocorticoids (Cole et al., 2001). Consequently, around 90% of GR-KO mice die at birth from respiratory
distress and an inability to activate perinatal gluconeogenesis in the liver (Cole et al., 1995). The remaining 10% of mice survive to adulthood but show a reduction in catecholamine (mainly epinephrine) production in the adrenal gland (Cole et al., 1995).

A second mouse model for a whole-body knock-out of the GR was created by crossing actin-Cre mice with mice homozygous for a loxP-flanked Nr3c1 gene (Bauerle et al., 2018). In these mice, the embryonic development of white fat pads has been tracked, and has been reported to develop normally (Bauerle et al., 2018). This may indicate that GR signaling is not required for in vivo white adipogenesis.

The relevance of the whole-body presence of the GR for BAT functioning has not been investigated in either of these two GR-KO models, most likely due to the low number of mice that survive to adulthood. Because of the difficulties that studying the GR-KO mouse presents, several labs have developed mouse models that allow for the study of the significance of the presence of the GR only in adipose tissues. These are mice in which the GR is knocked-out in an adipose tissue-specific manner (here denoted AGR-KO).

One way of making an adipose-specific GR-KO mouse, is by crossing adiponectin-Cre mice with GR$^{fl/fl}$ (exon 2 or 3 of the Nr3c1 gene) mice (Bose et al., 2016; Desarzens and Faresse, 2016; de Kloet et al., 2015; Mueller et al., 2017; Shen et al., 2017). In these mice, the GR is functionally knocked-out in mature brown and white adipocytes, but is still present in the adipose tissue stromal-vascular fractions containing preadipocytes. Knocking-out the GR in this adipose-tissue specific manner might attenuate the development of obesity as well as the accumulation of WAT mass in response to high-fat diet feeding and aging, but these results are still controversial (Bose et al., 2016; Desarzens and Faresse, 2016; de Kloet et al., 2015; Mueller et al., 2017; Shen et al., 2017). Concerning the functioning of BAT, there seems to be little difference between AGR-KO and wild-type mice. It has been reported that AGR-KO mice show a significantly decreased core body temperature compared to wild-type mice upon acute cold exposure, but this may be an indication of a reduced shivering capacity rather than a reduced UCP1-dependent thermogenic capacity, as no differences in BAT UCP1 or respiratory complex protein levels have been reported in AGR-KO mice (Mueller et al., 2017; Shen et al., 2017). Furthermore, in AGR-KO animals fed a high-fat diet for 14 weeks, no histological differences are observed in BAT tissues compared to wild-type mice (Shen et al., 2017). Although these results seem to indicate that the presence of the GR is completely dispensable for BAT functioning, a relevant function for the GR in BAT physiology nevertheless becomes visible in situations of hypercortisolism (Fig. 20). For example, when AGR-KO mice are injected with DEX, the upregulation of the expression of genes involved in lipolysis (Hsl, Atgl) and lipogenesis (Gpat4, Dgat2) normally seen in BAT after DEX injections is severely blunted (Bose et al., 2016) (Fig. 20). Moreover, in contrast to wild-type mice, no lipid accumulation is seen in the BAT of AGR-KO mice after an 8-week DEX treatment (Shen et al., 2017). These changes in
BAT lipid metabolism in the absence of the GR happen despite the fact that UCP1 protein levels remain unchanged (Shen et al., 2017) (Fig. 20). This thus indicates that in situations of hypercortisolism, the glucocorticoid-induced lipid accumulation in BAT requires signaling through the GR. However, this lipid accumulation but may be a result of altered expression patterns of lipogenic and lipolytic enzymes rather than of a reduction in BAT thermogenic capacity, in agreement with Paper I of this thesis.

A second mouse model that can be used to test the necessity for glucocorticoid signaling through the GR for BAT functioning, is a mouse model in which the GR is deleted specifically in brown mature- and pre-adipocytes (and skeletal muscle and interscapular WAT). This is achieved by crossing Myf5-Cre with GRfl/fl mice (here denoted BAGR-KO) (Park and Ge, 2017). In agreement with the analysis of AGR-KO mice, analysis of BAGR-KO mice also shows that the GR most likely has a minimal

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**Fig. 20. The effects of the genetic ablation of the GR in adipose tissues (AGR-KO) on BAT functioning.** (A) In wild-type (WT) mice housed at 21 °C and treated with glucocorticoids, the expression of the lipolytic enzymes hormone-sensitive lipase (Hsl) and adipose triglyceride lipase (Atgl), and of the lipogenic enzymes glycerol-3-phosphate acyltransferase 4 (Gpat4) and diacylglycerol O-acyltransferase 2 (Dgat2) are upregulated in BAT. (B) In AGR-KO mice housed at 21 °C and treated with glucocorticoids, the expression of lipolytic and lipogenic enzymes in BAT remains unaffected, as well as the expression of Ucp1.
role in BAT functioning when circulating glucocorticoid levels are in the physiological range; BAGR-KO mice do not show a change in cold tolerance or cold-induced Ucp1 gene expression compared to wild-type mice (Park and Ge, 2017). The GR is also not essential for the development of BAT, as both 18.5-day-old embryos and 6-week-old BAGR-KO mice do not show any changed in the expression of genes used as markers for adipogenesis or thermogenesis compared to wild-type mice.

Thus, in situations of hypercortisolism, the GR may play an important role in regulating the accumulation of lipids in BAT. However, and surprisingly, the transcription of BAT Ucp1 is not affected by the absence of the GR. Furthermore, under normal physiological situations, the GR is most likely dispensable for BAT functioning.

3.7.5 Non-genomic effects of glucocorticoids

The ‘classical’ model for glucocorticoid signaling through the GR as discussed above dictates that the liganded GR affects the expression of target genes. On average, the signaling cascade from binding of the glucocorticoid to the GR, translocation of the liganded GR into the nucleus, binding of the GR to the DNA, and activation or repression of gene transcription takes about 30-60 min (Cato et al., 2002). Thus, any measurable outcomes of glucocorticoid treatment are expected to arise after this time interval. It therefore seems surprising that some effects of glucocorticoid treatment are measurable already within seconds to a few minutes. For example, at high concentrations (0.1 mM), DEX inhibits glucose uptake into fat cells within 1 min after addition (Livingston and Lockwood, 1975). Furthermore, in an experiment using more physiologically relevant glucocorticoid concentrations, corticosterone (0.02 µg/ml) suppresses glucose-induced insulin secretion from pancreatic islets between 1-8 min after administration (Billaudel and Sutter, 1979). These intracellular actions of glucocorticoids occur so rapidly that they cannot involve gene transcription through the ‘classical’ GR signaling pathway. To describe these effects more precisely, researchers have termed them ‘non-genomic’ effects of glucocorticoid signaling.

Additional later research has shown that rapid, non-genomic effects of glucocorticoids (and other steroid hormones) can occur in several distinct ways, and the effects have therefore been classified into three categories: (I) non-receptor-mediated effects that occur at the plasma membrane (II) membrane receptor-mediated effects that occur at the plasma membrane (III) cytosolic receptor-mediated effects that occur at the plasma membrane (Cato et al., 2002). I will discuss here each of the categories in more detail, and will finally discuss to what extent the non-genomic effects of glucocorticoids may be relevant for BAT functioning.
Non-genomic actions of glucocorticoids in category I involve glucocorticoids altering the biophysical properties of the plasma membrane (Fig. 21A). For example, in vivo glucocorticoid administration decreases the fluidity of the plasma membrane in several cell types (Gerritsen et al., 1991; Neu et al., 1986). This decrease in membrane fluidity is associated with an increase in the cholesterol/phospholipid ratio of membrane lipids (Gerritsen et al., 1991). Additionally, glucocorticoids may affect the function of ion channels or receptor proteins that are embedded in the membrane (Fig. 21A). For example, the rapid suppression of glucose-induced insulin secretion by glucocorticoids is believed to be due to a glucocorticoid-mediated inhibition of $\text{Ca}^{2+}$-channels and thereby an inhibition of calcium influx into the cell (Billaudel et al., 1984). The actions of glucocorticoids on the plasma membrane are so far only measurable at high doses of glucocorticoid administration, and it is therefore questionable whether they are physiologically relevant. However, because endogenous glucocorticoid levels increase many-fold in response to stress, the direct effects of glucocorticoids on the plasma membrane may be relevant in these situations.

Non-genomic actions of glucocorticoids in category II involve glucocorticoid signaling through a plasma-membrane receptor that is distinct from the classical cytosolic GR (Fig. 21B). The presence of this glucocorticoid signaling pathway has been proposed after observations of rapid, non-genomic glucocorticoid-induced effects that are only observed when cells are treated with membrane-impermeable forms of glucocorticoids (glucocorticoids linked to bovine serum albumin (BSA) e.g. DEX-BSA, corticosterone-BSA) (Liu et al., 2007). In addition, these rapid effects cannot be inhibited by simultaneous treatment with a GR antagonist and do not occur when glucocorticoids are delivered intracellularly (Liu et al., 2007). Thus, these actions are proposed to be mediated by a glucocorticoid-binding receptor that is distinct from the classical GR. The nature of this additional glucocorticoid-binding receptor has not been discovered yet, but the most likely candidates have been proposed to be G protein-coupled receptors (Groeneweg et al., 2012) (Fig. 21B). Indeed, glucocorticoid-induced endocannabinoid synthesis depends on a Gs-cAMP-PKA signaling pathway (Di et al., 2003). However, glucocorticoid-binding sites on the membrane vary in affinity and selectivity for glucocorticoids, and therefore possibly consist of several non-classical glucocorticoid-binding receptors that remain to be discovered (Groeneweg et al., 2012).

Non-genomic actions of glucocorticoids in category III involve glucocorticoid signaling through a membrane-bound ‘classical’ cytosolic GR (Fig. 21C). The presence of the classical GR in the plasma membrane was demonstrated in the 90’s by experiments showing the binding of radiolabeled DEX to membrane proteins, and successive immunoblotting with antibodies against the cytosolic GR (Gametchu et al., 1991, 1993). The membrane-bound GR (mGR) is currently hypothesized to be transcribed from the same gene as the cytosolic GR (Nr3c1), to undergo similar splicing patterns as the cytosolic GR, but to undergo differential posttranslational modifications.
that transform the cytosolic GR into a membrane protein (Strehl et al., 2011). However, so far, no transmembrane domains or modifications that facilitate insertion of the GR into the membrane have been found. The mGR is physiologically present only in low numbers and is therefore challenging to detect, but is nevertheless believed to have a significant physiological relevance. Scientists have used membrane-impermeable DEX-BSA and corticosterone-BSA to show that mGR signaling in the brain is essential for the rapid negative feedback of glucocorticoids on the HPA-axis (Chapter 3.3) and for the rapid changes in adaptive behavior and memory in stressful situations (Groeneweg et al., 2012). The rapid actions of glucocorticoids on the immune and cardiovascular system (inhibition of inflammation and vasoconstriction) are also mediated by the mGR (Groeneweg et al., 2012). Correspondingly, rapid, non-genomic effects of glucocorticoids through mGR signaling are believed to align with classical glucocorticoid effects through cytosolic GR signaling, and are therefore proposed to ‘prime’ the cell for these classical glucocorticoid effects (Vernocchi et al., 2013). How the mGR exerts its intracellular effects is not fully known, and may vary considerably across cells. The liganded mGR may transduce signals through p38MAP kinase, the PI3K-Akt pathway and by altering adenyl cyclase or ion-channel activity, but no generally accepted model has been put forward so far (Groeneweg et al., 2012; Strehl and Buttgereit, 2014) (Fig. 21C).

Fig. 21. Non-genomic glucocorticoid signaling. (A) Glucocorticoids may alter the biophysical properties of the plasma membrane or may alter the functioning of ion channels or receptor proteins that are embedded in the membrane. (B) Glucocorticoids may bind to a membrane receptor other than the classical, cytosolic glucocorticoids receptor (GR). This receptor is most likely a GPCR and functions through a Gs-cAMP-PKA signaling pathway. (C) Glucocorticoids may bind to a membrane-bound classical ‘cytosolic’ GR. Hereby, they may alter AC activity, alter ion-channel activity, or signal through P38MAPK or PI3K.
Concerning BAT, no rapid, nongenomic effects of glucocorticoids have been reported. It thus remains to be determined whether any of the effects of glucocorticoids on BAT function that we report (Paper I, Paper II) are mediated through nongenomic mechanisms. However, the data discussed above (Chapter 3.7) do provide some indications. For example, the phenotype of AGR-KO mice (Chapter 3.7.4) indicates that the effects of glucocorticoid administration on lipid accumulation in BAT require signaling through the classical GR and do not depend on nongenomic signaling mechanisms. On the other hand, the effects of glucocorticoids on Ucp1 transcription in brown adipocytes occur very rapidly. As shown in Paper II, DEX treatment inhibits NE-induced Ucp1 transcription in primary brown adipocytes and WT1 cells within 24 h after treatment. In unpublished data, we have shown that even when DEX is added simultaneously with NE 2 hours before harvest of the cells, Ucp1 transcription is inhibited in primary brown adipocytes (experiments by Ana Lukic). However, these effects still occur within the time-frame required for genomic signaling to occur. Indeed, when DEX is added only 1 hour before harvest, NE-induced Ucp1 transcription remains unaltered (Soumano et al., 2000). In addition, the inhibition of Ucp1 transcription by glucocorticoids can be reversed by RU486 (Chapter 3.7.3). Thus, the effects of glucocorticoids on BAT are most likely mediated through classical, cytosolic GR signaling, with little or no role for non-genomic GR signaling.

3.8 GLUCOCORTICOIDS AS MODULATORS OF BROWN FAT IN HUMANS

The rodent studies discussed throughout Chapter 3 seem to provide significant indications that glucocorticoids suppress BAT Ucp1 transcription and simultaneously induce the development of obesity. The simultaneous occurrence of these two events has led researchers to propose the theory that in rodents, the suppression of BAT thermogenesis (partially) induces the development of glucocorticoid-induced obesity (Mousovich-Neto et al., 2019) (but see Paper I). If this theory is correct, we may extrapolate that glucocorticoids also modulate BAT thermogenesis in humans, which may thereby also be the (partial) cause for the development of glucocorticoid-induced obesity in humans, as is seen in cases of Cushing’s Syndrome. However, this extrapolation has to be made with caution, as there are several factors that call into question the applicability of the rodent research discussed above to the human condition. I will discuss these factors in Chapter 3.8.1.

A more scientifically sound means of achieving the full picture of the effects of glucocorticoids on BAT in humans is by performing direct observational and experimental studies in humans experiencing hypercortisolism. These studies, and their similarities to and differences from rodent studies, are discussed in chapter 3.8.2.
3.8.1 Extrapolating rodent data to humans

The first factor that complicates direct extrapolation of rodent data to humans, is that the notion that glucocorticoids always suppress BAT thermogenesis in rodents is false. As we show in Paper I, in mice housed at 21 °C, the increased sympathetic output to BAT (needed to activate the production of heat) overrules any suppressive effects of glucocorticoids on BAT UCP1 protein levels. This effect only becomes visible upon calculation of total IBAT UCP1 protein levels. Thus, in order to draw any conclusions about physiologically relevant BAT UCP1 levels, it is crucial to include this calculation in the analysis of the data. Because none of the studies mentioned above include this calculation, it can be assumed that at least part of the conclusions drawn from these studies about the effects of glucocorticoids on BAT in rodents are erroneous. Thus, it may be speculated that also in humans, hypercortisolism does not affect total BAT UCP1 protein levels.

Second, it has to be considered that the vast majority of the studies mentioned above has been executed on rodents housed at 21 °C. As mentioned in Chapter 2, this temperature is ~10 °C below the thermoneutral zone of mice, and thus causes a continuous activation of thermoregulatory energy expenditure, followed by a continuous compensatory increase in food intake. Humans, on the other hand, spend the vast majority of their lives at thermoneutrality and thus do not expend extra energy or consequently increase their food intake to defend their body temperature. Thus, even if a ‘true’ glucocorticoid-induced decrease in total BAT UCP1 protein levels would have been reported in mice housed at 21 °C, the applicability of this finding to humans would be questionable. To circumvent this issue, we report the effects of glucocorticoids on BAT in mice housed at thermoneutrality (30 °C) (Paper I). These mice can thus be said to be thermally humanized. At this temperature, glucocorticoids do suppress the transcription of Ucp1 in BAT, ultimately leading to a functionally lower total BAT UCP1 protein content. This indicates that glucocorticoids may indeed also lower total BAT UCP1 protein content in thermoneutral humans. However, it still remains to be determined whether this affects the development of glucocorticoid-induced obesity, as we show in rodents that glucocorticoid-induced obesity develops independently of UCP1 (Paper I).

3.8.2 Human studies

Direct research on the effects of glucocorticoids on BAT in humans has been very limited, mainly due to the complex nature of sample collection. However, there are indications that glucocorticoids have a suppressive effect on BAT thermogenesis in humans. For example, the prevalence of BAT-positive individuals is much lower in
patient-groups suffering from cortisol-producing adenomas and secondary hypercortisolism (33.3 % and 16.7 % respectively) than in patient-groups suffering from aldosterone-producing adenomas, pheochromocytomas and non-functioning adenomas (46.9 %, 62.5 % and 100 % respectively) (Betz et al., 2013). In addition, in the same study, a trend was found for a negative correlation between urinary cortisol secretion and retroperitoneal UCP1 expression (P=0.094) (Betz et al., 2013). The prevalence of BAT-positive individuals is also decreased in patients receiving chronic glucocorticoid medication (1.7 %) compared to matched controls (6.7 %) (Ramage et al., 2016). These findings suggest that long-term hypercortisolism suppress BAT function (or at least 18FDG uptake) in humans.

In addition to these observational studies, an experimental study has been published in which healthy volunteers received long-term exogenous glucocorticoid supplementation. In this double-blind cross-over placebo-controlled trial, 18FDG uptake in BAT, as well as supraclavicular skin temperature, in response to cooling (19 °C) was found to be decreased in individuals pre-treated with oral prednisolone for 7 days (Thuzar et al., 2018) (Fig. 22A). Although both of these measurements do not necessarily provide information about BAT thermogenic capacity, they do provide an indication that BAT functioning in humans responds to long-term glucocorticoid treatment in a manner similar to that in rodents.

Interestingly, the effects of glucocorticoids on human BAT seem to be strongly time-dependent. Specifically, a more short-term infusion with hydrocortisone (14 h) leads to an increased basal and isoprenaline-induced supraclavicular skin temperature (Scotney et al., 2017). Short-term (36 h, 10 mg every 12 h) administration with prednisolone also increases 8FDG uptake in BAT activated by exposure to mild cold (16 – 17 °C) (Ramage et al., 2016) (Fig. 22B). This increase occurs despite a decrease in whole-body 18FDG uptake. Additionally, the same short-term prednisolone supplementation leads to an increased supraclavicular skin temperature upon cold exposure (Ramage et al., 2016) (Fig. 22B). These data indicate that, in contrast to results reported for long-term hypercortisolism, short-term spikes in glucocorticoid levels may increase BAT thermogenesis in humans.

The effects of glucocorticoids on human brown adipocytes have also been investigated in vitro. In primary cultures of supraclavicular human brown adipocytes differentiated for 9 days in the presence of 10 μM DEX, basal Ucp1 as well as Cidea and Ppargc1a mRNA levels are upregulated (Barclay et al., 2015). In primary cultures of deep supraclavicular human BAT, 24 h treatment with 100 nM cortisol also increases basal Ucp1 and Glut4 levels (Ramage et al., 2016). In contrast, a 5 h DEX treatment significantly suppresses the adrenergically-induced upregulation of Ucp1 expression and uncoupled VO2 rate in mature primary brown adipocytes (Barclay et al., 2015). Thus, basal Ucp1 expression in human adipocytes is affected by glucocorticoids in a manner distinct from in rodent adipocytes (i.e. increased), whereas adrenergically-
induced *Ucp1* expression is affected by glucocorticoids similarly in human and rodent adipocytes (i.e. decreased).

The length of the glucocorticoid treatment also seems to influence experimental outcomes *in vitro*. For example, whereas 24 h treatment with 100 nM cortisol increases basal *Ucp1* expression in human adipocytes, 48 h treatment with the same dose of cortisol does not (Ramage et al., 2016). Moreover, 24 h treatment with 1 µM cortisol also leaves *Ucp1* gene expression unaffected, and 48 h 1 µM treatment even decreases basal *Ucp1* mRNA levels (Ramage et al., 2016).

Thus, although most studies indicate that also in humans, glucocorticoids may suppress BAT function, this effect is probably both time- and dose-dependent. In addition, no human study has reported to what extent alterations in BAT activity affect the development of glucocorticoid-induced obesity.

![Figure 22](image)

**Fig. 22. The effects of glucocorticoid administration on human BAT.** (A) A 7-day glucocorticoid supplementation decreases supraclavicular skin temperature and 18FDG uptake in human BAT upon acute cold exposure. (B) A 36-h glucocorticoid supplementation increases supraclavicular skin temperature and 18FDG uptake in human BAT upon acute cold exposure.
CONCLUSIONS AND FUTURE PERSPECTIVES

Recruiting and activating UCP1-dependent thermogenesis is a key strategy to increase energy expenditure, and thereby possibly to lower body weight. Studies aiming to identify novel modulators of UCP1-dependent thermogenesis are vital if we want to harness UCP1-dependent thermogenic energy expenditure in the battle against obesity and obesity-related diseases. However, in order to obtain meaningful results, it is important that such studies are executed under optimal experimental conditions. When studying thermal physiology at temperatures below thermoneutrality, any changes in e.g. insulation, shivering capacity, etc. due to genetic mutations or compound supplementation have to be taken into account. Moreover, we find that the only relevant thermogenic measure for physiological purposes is that of total, whole-body, thermogenic capacity, thus including e.g. measurements of total BAT UCP1 protein.

In experiments executed in line with the guidelines above, we find that UCP1 mediates facultative diet-induced thermogenesis in obesity-resistant mice fed a Western style diet, and thus prevents these mice from gaining as much weight as they would in the absence of UCP1 (Paper V). It remains to be determined to what extent UCP1-dependent facultative diet-induced thermogenesis also prevents weight gain in humans.

We further present two novel research models that, when experimented with under the appropriate conditions, can be used to identify novel modulators of UCP1 (Papers II, III). Especially the Thermomouse can facilitate the screening of compounds for their effects on Ucp1 transcription, and can further be used in studies regarding UCP1 and aging.

We investigate the potential of glucocorticoids to modulate UCP1-dependent thermogenesis (Papers I, II). Although we find that glucocorticoids are important regulators of lipid metabolism in BAT, the proposed suppression of UCP1-dependent thermogenesis by glucocorticoids is questionable. In situations of physiological circulating glucocorticoid levels, BAT thermogenesis (but not lipid metabolism) remains unaffected by the absence of HSD1 or of the GR. Upon glucocorticoid administration at temperatures below thermoneutrality, UCP1 protein per µg BAT protein is decreased, but not total UCP1 protein or whole-body thermogenic capacity. In mice housed at thermoneutrality, glucocorticoids functionally decrease total UCP1 protein. This most likely happens independently of the adrenergic signaling pathway, but through direct binding of the liganded GR to Ucp1 regulatory regions. The exact mechanism behind this remains to be determined, but the physiological impact may be minimal.

The studies of murine ‘classical’ BAT (rather than murine brite fat) presented here imply that certain modulators of UCP1-dependent thermogenesis may affect body weight also in humans. However, we show that the amount of UCP1 protein does not affect the development of glucocorticoid-induced obesity in mice. Thus, even though the effects of glucocorticoids on human brown adipocytes remain to be further specified, the modulation of UCP1-dependent thermogenesis may not be the answer to reducing obesity induced by glucocorticoids.
Rekrytering och aktivering av brun fettvävnad för värmeproduktion ses som en ny möjlighet för att bekämpa övervikt, fetma och dess följdssjukdomar. Värmen som produceras av brun fettvävnad alstras av Uncoupling Protein-1 (UCP1), ett protein som endast finns i de bruna fettcellernas mitokondrier. Efter rekrytering och aktivering särskiljs UCP1 att oxidationen av näringsämnen är frikopplad från produktionen av ATP. Det betyder att all energi från näringsämnen omvandlas till värme istället för ATP och därigenom förbrukas. Om vi vill använda denna frikoppling av energi genom brun fettvävnad som ett sätt att gå ner i vikt, måste vi kunna rekrytera brun fettväv och aktivera värmeproduktionen på ett kontrollerat sätt. Hittills är köldexponering det huvudsakliga sättet att uppnå detta mål, men då kyla är obehagligt vore det önskvärt att hitta nya sätt för brunt fett att aktiveras och rekryteras. Syftet med denna avhandling är att ge en djupare inblick i flera nya faktorer och processer som modulerar UCP1-medierad värmeproduktion i brunt fett.

Vi undersöker i vilken utsträckning glukokortikoider påverkar musens förmåga att producera värme via UCP1 i brunt fett. När vi behandlar möss med glukokortikoider ser vi en tydlig minskning av mängden UCP1-protein i brunt fett, men endast när mössen lever i termoneutralitet. Detta är en ny insikt som här rapporteras för första gången. Vi visar att glukokortikoider minskar det genetiska uttrycket för UCP1 eftersom receptorn för glukokortikoider binder direkt till de genetiska sekvenser som reglerar UCP1: s uttryck. Vi visar också att den översvik som utvecklas till följd av glukokortikoidbehandling inte påverkas av mängden UCP1-protein i brunt fett.

Dessutom undersöker vi effekten av olika dieter vid rekrytering och aktivering av UCP1 i brunt fett. Vi visar att möss som normalt är resistent mot fetma får mer UCP1-protein i deras bruna fett när de äter en diet med hög fetthalt. Om vi genetiskt modifierar dessa möss så att de inte längre har UCP1-protein blir de tjockare än möss som har UCP1-protein. Detta visar att rekrytering och aktivering av UCP1 i brunt fett som ett resultat av konsumtionen av feta dieter är en viktig mekanism emot utveckling av fetma.

Slutligen presenterar vi två nya modeller som kan användas för att upptäcka nya faktorer som modulerar värmeproduktionen av UCP1 i brunt fett. Den första är en cellinje gjord av mänskliga bruna fettceller, den andra är en mus som uttrycker proteinet luciferas styrt från en UCP1-promotor. Med hjälp av dessa modeller har
vi upptäckt två nya gener som är viktiga vid reglering av UCP1-genuttryck, nämligen *Mtus1* och *Kcnk3*, och substansen WWL113, som modulerar värme produktionen av UCP1 i brunt fett.
Het rekruteren en activeren van warmteproductie door bruin vetweefsel wordt gezien als een nieuwe strategie om overgewicht en ziektes gerelateerd aan overgewicht tegen te gaan. De warmte die bruin vetweefsel produceert wordt gegenereerd door Uncoupling Protein-1 (UCP1), een eiwit dat gevonden wordt in de mitochondriën van uitsluitend bruine vetcellen. Na rekrutering en activatie zorgt UCP1 ervoor dat de oxidatie van nutriënten losgekoppeld wordt van de productie van ATP. Dit betekent dat alle energie uit nutriënten wordt omgezet in hitte in plaats van in ATP, en dus wordt verspild. Als we de verspilling van energie door bruin vetweefsel willen gebruiken als een manier om gewicht te verliezen, zullen we de productie van hitte door bruin vetweefsel moeten rekruteren en activeren. Tot nu toe is blootstelling aan kou de voornaamste manier die we kennen om dit doel te bewerkstelligen. Echter, vanwege het oncomfortabele karakter van blootstelling aan kou is het noodzakelijk om nieuwe manieren te ontdekken waarop bruin vet geactiveerd of gerekruteerd kan worden. Het doel van dit proefschrift is om een dieper inzicht te verschaffen in verschillende nieuwe factoren en processen die hitte productie door UCP1 in bruin vet moduleren.

We onderzoeken in hoeverre glucocorticoïden de capaciteit van muizen beïnvloeden om hitte te produceren via UCP1 in bruin vet. Als we muizen behandelen met glucocorticoïden, zien we een duidelijke reductie in de hoeveelheid UCP1 eiwit in bruin vet, maar alleen wanneer de muizen thermoneutraal zijn gehuisvest. Dit is een nieuw inzicht dat hier voor de eerste keer gerapporteerd wordt. We laten zien dat glucocorticoïden de genetische expressie van UCP1 verminderen doordat de receptor voor glucocorticoïden direct bindt aan genetische sequenties die de expressie van UCP1 reguleren. We laten ook zien dat het overgewicht dat zich ontwikkelt ten gevolge van de behandeling met glucocorticoïden niet beïnvloed wordt door de hoeveelheid UCP1 eiwit in bruin vet.

Hiernaast onderzoeken we ook het effect van verschillende diëten op de rekrutering en activatie van UCP1 in bruin vet. We laten zien dat muizen die normaal gesproken resistent zijn voor obesitas meer UCP1 eiwit in hun bruin vet krijgen als ze gevoed worden met een dieet met een hoog vetpercentage. Als we deze muizen genetisch modificeren zodat ze geen UCP1 eiwit meer bezitten, worden ze dikker dan muizen die wel UCP1 eiwit hebben. Dit laat zien dat de rekrutering en activatie van UCP1 in bruin vet ten gevolge van de consumptie van diëten met veel vet een belangrijk mechanisme is tegen de ontwikkeling van obesitas.
Tenslotte presenteren we twee nieuwe modellen die gebruikt kunnen worden om nieuwe factoren te ontdekken die hitte productie door UCP1 in bruin vet moduleren. De eerste is een cellijn gemaakt van humane bruine vetcellen, de tweede is een muis die het eiwit ‘luciferase’ tot expressie brengt onder de genetische promoter van UCP1. Met behulp van deze modellen hebben we twee nieuwe genen ontdekt die belangrijk zijn in de regulatie van UCP1 genexpressie, namelijk *Mtus1* en *Kcnk3*, en het stofje WWL113, dat hitte productie door UCP1 in bruin vet moduleert.
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