IDENTIFICATION OF FKBP51 AS A NOVEL SUSCEPTIBILITY GENE FOR METABOLIC DYSFUNCTION

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Table of Contents

Table of Contents i
Abstract iv
Abbreviations v
Publications ix
Chapter 2.1 ix
Chapter 2.2 ix
Chapter 2.3 ix
Chapter 2.4 ix
Chapter 2.5 ix
Additional Contributions ix
Declaration of Contributions xi
Chapter 2.1 xi
Chapter 2.2 xi
Chapter 2.3 xi
Chapter 2.4 xii
Chapter 2.5 xii
1. General Introduction 1
1.1. Obesity 1
1.2. Pathophysiologies Associated with Obesity 1
1.2.1. Type 2 Diabetes (T2D) 2
1.2.2. Depression 2
1.3. Energy Balance 3
1.3.1. Energy Intake 4
1.3.2. Energy Expenditure 6
1.4. Tissue-Specific Regulation of Glucose Homeostasis 7
1.5. Genetics of Obesity 9

1.6. Signaling Molecules of Energy Homeostasis....10

1.6.1. Leptin....11
1.6.2. Insulin....12
1.6.3. Nutrients....14
1.6.4. Glucocorticoids....22

1.7. Therapeutic Targets/Pathways to Treat Obesity and Obesity-Related Disorders....25
1.8. Novel Molecular Targets in the Regulation of Whole Body Energy Metabolism....26

1.8.1. FK506 binding protein 51 (FKBP51) Characterization....26
1.8.2. Established Regulatory Functions of FKBP51....27
1.8.3. FKBP51 in Gene x Early Life Environment Interactions....29
1.8.4. FKBP51 Knockout Studies....29
1.8.5. FKBP51 as a Potential Therapeutic Target in the Treatment of Metabolic-Related Disorders....30

1.9. Rationale and Thesis Objectives....31

2. Research Articles....32

2.1. The Interplay between Diet-Induced Obesity and Chronic Stress in Mice: Potential Role of FKBP51....32
2.2. Prenatal Exposure to Maternal Obesity Alters Anxiety and Stress-Coping Behaviors in Aged Mice....45
2.3. Loss or Inhibition of FKBP51 Protects Against Diet-Induced Metabolic Disorders by Shaping Insulin Signaling....61
2.4. FKBP51 Enhances AMPK Inhibition of mTOR: A Preliminary Report....85
2.5. FKBP51 Acts in the Hypothalamus to Regulate Body Weight....100

3. General Discussion....118

3.1. Summary....118

3.1.1. *Fkbp5* Expression is Modulated by the Dietary Context....118
3.1.2. FKBP51 Impacts Body Weight, Glucose Homeostasis, and Metabolic Signaling Cascades: Possible Therapeutic Implications....119
3.1.3. Hypothalamic FKBP51 Overexpression Paradoxically Improves the Metabolic Outcome in Mice....122

3.2. Significance to the Research Field....123

3.2.1. FKBP51: A Genetic Risk Factor in Metabolic Diseases 123
3.2.2. FKBP51: Novel and Established Molecular Mechanisms Governing Energy and Glucose Metabolism 125
3.2.3. FKBP51: Implications for the Treatment of Obesity and Type 2 Diabetes.....129
3.3. Limitations and Future Directions..130
3.3.1. Tissue-Specificity of FKBP51 130
3.3.2. Responsiveness of FKBP51 to Environmental Cues 132
3.4. Closing remarks..132
4. Bibliography 133
Curriculum Vitae 163
Acknowledgements..164
Assertion/ Eidesstattliche Erklärung 166

Abstract

FKBP51 is an immunophilin protein best known as a regulator of the stress response system. Recently, a genome-wide association study demonstrated that a number of genetic variants within the gene encoding FKBP51, *FKBP5*, associate with traits related to type 2 diabetes (T2D). In this thesis, we characterized the function of FKBP51 in energy and glucose homeostasis. Initially we addressed whether FKBP51 gene expression is responsive to the dietary environment. Interestingly, FKBP51 gene expression is affected by both in utero and adult high fat diet exposure when examined in male mice. In order to dissect the role of FKBP51 in energy and glucose homeostasis, we assessed the metabolic phenotype of FKBP51 knockout (51KO) mice and wild-type (WT) littermates under control and high fat diet conditions. 51KO mice were protected from HFD-induced weight gain and glucose intolerance. In addition, 51KO mice showed a prolonged response to insulin. This phenotype correlated with heightened insulin signaling specifically within skeletal muscle of 51KO mice. In line with these findings, we found that glucose uptake was significantly increased by *FKBP5* knockdown in differentiated myotubes. Importantly, pharmacological inhibition of FKBP51 by the specific antagonist SAFit2 for either 10 or 30 days recapitulated the phenotype observed in 51KO mice. Finally, in C2C12 myotubes, we found that SAFit2 treatment increased glucose uptake. The improved metabolic phenotype arising from loss of FKBP51 cannot be explained by FKBP51 action in the hypothalamus as targeted hypothalamic FKBP51 overexpression in mice actually reduces body weight. Furthermore, this thesis identifies novel interaction partners of FKBP51, including AMPK and TSC2. Through these interactions, FKBP51 directs AMPK-dependent regulation of mTOR signaling, a major signaling pathway underlying cellular and whole body energy homeostasis. The collective findings of the current thesis provide physiological and mechanistic bases for the therapeutic potential of FKBP51 in the treatment of obesity and T2D.

Abbreviations

4EBP1	eIF-4E-binding protein 1
51KO	FKBP51 knockout
51OE	*Fkbp5* overexpression
AAV	Adeno-associated virus
ACTH	Adrenocorticotropic hormone
AEE	Activity-related energy expenditure
AGRP	Agouti related peptide
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ARC	Arcuate nucleus
AS160	AKT substrate 160
AUC	Area under the curve
AVP	Arginine-vasopressin
BAT	Brown adipose tissue
BMI	Body mass index
CAB39	Calcium-binding protein 39
CAMKK	Calmodulin-dependent kinase kinase
CART	Cocaine and amphetamine related transcript
Clomi-D3	Deuterated clomipramine
CNS	Central nervous system
coIP	Co-immunoprecipitation
CRH	Corticotropin-releasing hormone
CSDS	Chronic social defeat stress
CV	Coefficient of variation
CyPs	Cyclosporin-binding cyclophilins
DIG	Digoxigenin
DIO	Diet-induced obesity
DISH	Double in situ hybridization

DMEM	Dulbecco's modified Eagle's medium
DMH	Dorsomedial hypothalamic nucleus
EAT	Exercise activity thermogenesis
EDL	Extensor digitorum longus
eIF4E	eukaryotic initiation factor 4E
EPM	Elevated plus maze
eWAT	Epididymal white adipose tissue
FD	Food deprivation
FK1	FK506 binding domain
FK2	FKBP-like domain
FKBP51	FK506-binding protein 51
FKBP52	FK506-binding protein 52
FKBPs	FK506-binding proteins
FOX	Forkhead box protein
FST	Forced swim test
G18	Gestational day 18
GAP	GTPase activating protein
GC	Glucocorticoids
GLUT4	Glucose transporter protein 4
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
GSK-3	Glycogen synthase kinase 3
GSVs	GLUT4 storage vesicles
GTT	Glucose tolerance test
HEK	Human embryonic kidney
HF	High fat
HFD	High fat diet
HPA	Hypothalamic-pituitary-adrenal
HPA	Hypothalamic -pituitary-adrenal
HPLC/MS	High-performance liquid chromatography/mass spectrometry
Hsp90	Heat shock protein 90
IMM	Immunophilin
IPGTT	Intraperitoneal glucose tolerance test

IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
ISH	In situ hybridization
ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue
JAK2	Janus kinase-2
KRH	Krebs-Ringer-HEPES
LKB1	Liver kinase B1
LKB1	Liver Kinase B-1
LUT	Lookup table
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MC4RKO	Melanocortin 4 receptor knockout
MD	Major depression
ME	Metabolizable energy
MEF	Mouse embryonic fibroblast
MOI	Multiplicity of infection
MR	Mineralocorticoid receptor
mTOR	Mammalian target of rapamycin
NEAT	Non-exercise activity thermogenesis
NPY	Neuropeptide Y
OF	Open field
P24	Postnatal day 24
p70S6K1	p70S6 Kinase 1
PBS	Phosphate-buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PFA	Paraformaldehyde
PHLPP	PH domain and Leucine rich repeat Protein Phosphatases
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol-4,5-diphosphate
PIP3	Phosphatidylinositol -3,4,5-trisphosphate
PKB	Protein kinase B
POMC	Pro-opiomelanocortin

PPIase	Peptidyl-prolyl cis-trans isomerase
PVC	Polyvinyl chloride
PVN	Paraventricular nucleus
PVN	Paraventricular nucleus
pWAT	Perirenal white adipose tissue
RER	Respiratory exchange ratio
Rheb	Ras homolog enriched in brain
RMR	Resting metabolic rate
RTKs	Receptor tyrosine kinases
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
SI	Social interaction
SNPs	Single nucleotide polymorphisms
STAT-3	Signal transducer and activator of transcription 3
STRAD	Ste-20-related adaptor
T2D	Type 2 diabetes
TEE	Total energy expenditure
Thr	Threonine
TPR	Tetratricopeptide repeat
TSC	Tuberous sclerosis complex
TSC1	Hamartin
TSC2	Tuberin
UCP1	Uncoupling protein 1
VMH	Ventromedial hypothalamic nucleus
VTA	Ventral tegmental area
WAT	White adipose tissue
WHO	World Health Organization
WT	Wild-type
α-MSH	α-melanocyte-stimulating hormone

Publications

Chapter 2.1

<u>Balsevich G</u>, Uribe A, Wagner KV, Hartmann J, Santarelli S, Labermaier C, Schmidt MV. (2014). The interplay between diet-induced obesity and chronic stress in mice: potential role of FKBP51. J Endocrinol, 222, 15 – 26

Chapter 2.2

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Chapter 2.3

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Chapter 2.4

Gassen NC, **<u>Balsevich G</u>**, Dournes C, Hafner K, Chen A, Schmidt MV, Rein T. (2015). FKBP51 enhances AMPK inhibition of mTOR. Manuscript in preparation.

Chapter 2.5

<u>Balsevich G</u>, Häusl A, Dournes C, Santarelli S, Uribe A, Chen A, Schmidt MV. (2015). FKBP51 acts in the hypothalamus to regulate body weight. Manuscript in preparation.

Additional Contributions

Bellisario V, Panetta P, **<u>Balsevich G</u>**, Baumann V, Noble J, Raggi C, Nathan O, Berry A, Seckl J, Schmidt M, Holmes M, Cirulli F. (2015). Maternal high-fat diet acts as a stressor increasing maternal glucocorticoids' signaling to the fetus and disrupting maternal behavior and brain activation in C57BL/6J mice. Psychoneuroendocrinology, 60, 138 – 150.

Arloth J, Bogdan R, Weber P, Frishman G, Menke A, Wagner KV, **Balsevich G**, Schmidt MV, Karbalai N, Czamara D, Altmann A, Trümbach D, Wurst W, Mehta D, Uhr M, Klengel T, Erhardt A, Carey CE, Conley ED; Major Depressive Disorder Working Group of the Psychiatric Genomics Consortium (PGC), Ruepp A, Müller-Myhsok B, Hariri AR, Binder EB; Major Depressive Disorder Working Group of the Psychiatric Genomics Consortium PGC. (2015). Genetic differences in the immediate transcriptome response to stress predict risk-related brain function and psychiatric disorders. Neuron, 86(5), 1189-202.

Balsevich G, Namendorf C, Gerlach T, Uhr M, Schmidt MV. (2015). The bio-distribution of the antidepressant clomipramine is modulated by chronic stress in mice: effects on behavior. Front Behav Neurosci, 8, 445

Gaali S, Kirschner A, Cuboni S, Hartmann J, Kozany C, **Balsevich G**, Namendorf C, Fernandez-Vizarra P, Sippel C, Zannas AS, Draenert R, Binder EB, Almeida OF, Rühter G, Uhr M, Schmidt MV, Touma C, Bracher A, Hausch F. (2015). Selective inhibitors of the FK506-binding protein 51 by induced fit. Nat Chem Biol, 11(1), 33 – 37

Gassen NC, Hartmann J, Zschocke J, Stepan J, Hafner K, Zellner A, Kirmeier T, Kollmannsberger L, Wagner KV, Dedic N, **Balsevich G**, Deussing JM, Kloiber S, Lucae S, Holsboer F, Eder M, Uhr M, Ising M, Schmidt MV, Rein T. (2014). Association of FKBP51 with priming of autophagy pathways and mediation of antidepressant treatment response: evidence in cells, mice, and humans. PLoS Med, 11(11), e1001755

Iozzo P, Holmes M, Schmidt MV, Cirulli F, Guzzardi MA, Berry A, **Balsevich G**, Andreassi MG, Wesselink JJ, Liistro T, Gómez-Puertas P, Eriksson JG, Seckl J. (2014). Developmental ORIgins of healthy and unhealthy AgeiNg: the role of maternal obesity – Introduction to DORIAN. Obes Facts, 7(2), 130-151

Declaration of Contributions

I hereby certify that I contributed my own work to the current thesis, entitled ‘Identification of FKBP51 as a novel susceptibility gene for metabolic dysfunction’ in the following way:

Chapter 2.1

Designing and Planning the Study –
In collaboration with MVS

Conducting the Experiments –
In collaboration with AU, KVW, JH, SS, CL

Analysing the Data –
Independently executed

Preparing the Manuscript –
In collaboration with MVS

Chapter 2.2

Designing and Planning the Study –
In collaboration with MVS

Conducting the Experiments –
In collaboration with VB, AU

Analysing the Data –
Independently executed

Preparing the Manuscript –
In collaboration with MVS

Chapter 2.3

Designing and Planning the Study –
In collaboration with MVS

Conducting the Experiments –

In collaboration with NCG, AH, CWM, XF, CD, SS, AU, CN

Analysing the Data –

In collaboration with NCG, SK

Preparing the Manuscript –

In collaboration with MVS

Chapter 2.4

Designing and Planning the Study –

In collaboration with MVS, NCG

Conducting the Experiments –

In collaboration with NCG, CD, KH

Analysing the Data –

In collaboration with NCG

Preparing the Manuscript –

In collaboration with MVS

Chapter 2.5

Designing and Planning the Study –

In collaboration with MVS

Conducting the Experiments –

In collaboration with AH, CD, SS, AU

Analysing the Data –

Independently executed

Preparing the Manuscript –

In collaboration with MVS

Munich, September 2015

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München, September 2015

Mathias V. Schmidt

1. General Introduction

1.1. Obesity

In Europe overweight and obesity have reached epidemic proportions, emphasizing the need to decipher the complex regulatory mechanisms underlying energy balance (Berghofer et al., 2008). By definition, overweight is considered a body mass index (BMI, weight in kg/height in meters squared) between 25 and 30, and obesity is considered a BMI over 30 (WHO, 2000). Obesity is associated with increased morbidity and mortality and thus is recognized as a major health problem. The etiology of obesity, like all complex diseases, is heterogeneous and involves both environmental and genetic factors. It is well known that lifestyle, namely a poor diet and physical inactivity, is a central driver contributing to the relatively recent rise in obesity. However, it remains unclear as to why some individuals are resilient, whereas others are vulnerable to such an obesogenic environment. Regardless, the current trends in obesity prevalence highlight the need for improved treatment strategies to achieve and maintain a healthier BMI (Rodgers et al., 2012). Possible treatment strategies include either reductions in food/energy intake or increases in energy expenditure. Unfortunately, to-date effective treatment strategies are largely limited to invasive bariatric surgeries, and effective pharmacological treatments remain elusive.

1.2. Pathophysiologies Associated with Obesity

The disease burden associated with obesity is a global problem. Obesity increases the risk of premature mortality and morbidity, and is associated with a poorer health-related quality of life (Fontaine and Barofsky, 2001;Peeters et al., 2003). Not only is obesity a major health concern, but it is also an enormous economic burden (Wang et al., 2011b). The health and economic burdens arising from obesity are largely attributed to the increased rates of T2D and coronary heart disease and stroke. However the association between obesity and mental illness is gaining increasing recognition as a major health concern. The aim of the current thesis is not to comprehensively summarize the disease states associated with obesity. Instead, the focus will be centered on T2D and psychiatric disorders (namely depression), which are relevant to the thesis content.

1.2.1. Type 2 Diabetes (T2D)

T2D is a heterogeneous disease characterized by metabolic dysfunction resulting in hyperglycemia, defined as fasting blood glucose levels greater than 7.0 mM or two-hour blood glucose levels greater than 11.1 mM following a glucose tolerance test (Turner, 2013). A central feature to both obesity and T2D is insulin resistance, defined as a state of reduced responsiveness to normal levels of circulating insulin (Schwartz and Porte, Jr., 2005). According to the World Health Organization (WHO), 347 million people worldwide suffer from T2D (WHO, 2014a). Type 2 diabetes increases the risk of serious health complications including heart disease, stroke, kidney disease, and premature death. In fact in 2012, an estimated 1.5 million deaths were the direct consequence of diabetes (WHO, 2014b). Clinical management of established T2D involves the lowering of blood glucose levels with the application of oral anti-diabetic medications, such as metformin, and oftentimes also requires insulin administration. Although extensively studied, the exact mechanisms causing insulin resistance remain unclear. In addition there is an unmet need for the development of novel drugs for the treatment of T2D.

1.2.2. Depression

Depression is a common disorder and is the leading cause of disability worldwide (WHO, 2012). Interestingly, there is a strong association between major depression (MD), obesity, and T2D. Depressed patients show higher rates of general obesity and related diseases (Faith et al., 2002;Raikkonen et al., 2002;Skilton et al., 2007;Toker et al., 2008;Viinamaki et al., 2009). Likewise patients diagnosed with metabolic syndrome (a complex disorder characterized by a cluster of metabolic factors, namely insulin resistance, visceral obesity, hypertension, and atherogenic dyslipidemia) show increased rates of affective disorders. Similarly, the prevalence of depression is higher in patients suffering from T2D and furthermore depression constitutes a major risk factor for the development of T2D (Musselman et al., 2003). Overlapping psychosocial and pathophysiological etiologies have been suggested to underlie these two converging disabilities. Specifically, proposed candidate pathways at the interface between MD and obesity include chronic inflammation (Goldstein et al., 2009), the HPA axis (Bjorntorp and Rosmond, 2000), and adipokine signaling (Taylor and Macqueen, 2010). Nevertheless, accessory mediators and clear molecular targets underpinning the association between MD and obesity remain elusive.

1.3. Energy Balance

Although obesity is indeed a complex disease with both genetic and environmental determinants, it simply arises from a positive energy balance. Energy balance refers to the balance between energy consumed (through caloric intake) and energy expended (through heat or work) (Hall et al., 2012) (Figure 1.3.1). Indeed both energy intake and expenditure are tightly regulated by complex, redundant processes comprising thousands of genes and involving both hormonal and neuronal signaling networks (Lenard and Berthoud, 2008). Yet in recent decades we have nevertheless witnessed an enormous rise in overweight, obesity, and related metabolic disorders. This rise has been mainly attributed to our changing environment, namely the prevalence of energy-dense foods, increased social stress, and physical inactivity (Lattimore and Maxwell, 2004;Pandit et al., 2011;Wallis and Hetherington, 2009). An in-depth understanding of the components comprising energy balance is required in order to effectively combat the obesity epidemic.

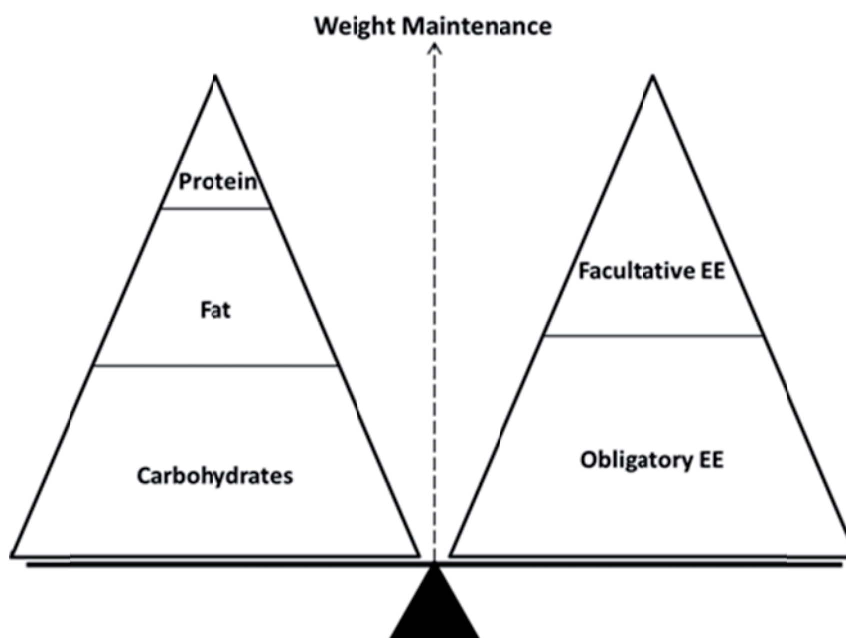

Figure 1.3.1 Energy balance

Energy balance consists of two sides. On one side food intake, comprising the 3 major macronutrients, delivers energy to the body. On the other side energy expenditure refers to the act of using energy in order to maintain physiological functions and can be further divided into obligatory and facultative energy expenditure.

1.3.1. Energy Intake

There are 3 major macronutrients that comprise energy intake: carbohydrate, protein, and fat. Multiple factors nevertheless contribute to long-term energy intake. For example, inter-individual differences in metabolizable energy (ME), referring to the net energy available after accounting for the energy losses found in feces and urine, contribute to energy intake. Factors such as meal composition and intestinal factors including gut motility are examples of components influencing ME. Feeding behaviors however, including food choices and amounts, undeniably have the most profound impact on energy intake.

Feeding behavior is essential for survival. However if feeding were simply driven by a homeostatic system able to respond to disturbances in energy balance, obesity would not be a global epidemic. Rather, the regulation of feeding behavior and thus energy intake is governed by two parallel systems: the homeostatic system, which senses and responds to changes in the energy status and the hedonic system, which is driven by the rewarding properties of food (Lutter and Nestler, 2009).

Homeostatic control of feeding requires the coordinated action of both peripheral and central pathways. Central hypothalamic neural circuits continuously monitor metabolic signals reflecting energy state to mediate behavioral and metabolic alterations in response to nutrient availability (Lam et al., 2005;Plum et al., 2006;Seeley and Woods, 2003). In particular, activity in the arcuate nucleus of the hypothalamus has been recognized to play a key role in the regulation of homeostatic feeding (Buettner et al., 2008;Elias et al., 1999;Hill et al., 2008;Koch et al., 2008;Morton et al., 2006;Nogueiras et al., 2007;Nogueiras et al., 2009;Sawchenko, 1998;Watts, 2000). The arcuate nucleus contains two principal neuronal populations whose actions are reciprocal. Specifically, the neuropeptide Y (NPY)/agouti related peptide (AGRP) –expressing neurons stimulate feeding whereas the pro-opiomelanocortin (POMC)/cocaine and amphetamine related transcript (CART) neurons suppress feeding (Elias et al., 1999;Hill et al., 2008;Morton et al., 2006). These neurons are able to sense a broad range of nutrient and hormonal signals (nutrients, insulin, and leptin), and their responses change according to the energy state (Cone et al., 2001) (Refer to Section 1.6.1). Such hormones reflect the energy status within the periphery, which subsequently signal to the brain to mount an appropriate response. Taken together, fluctuations in nutrient availability are sensed by neuropeptides within the arcuate nucleus, which respond to achieve a balance between energy intake and energy expenditure.

The central melanocortin pathway is one of the most essential neuronal pathways underlying energy homeostasis (Cone, 2005). By definition, the melanocortin system includes neurons that express POMC (originating in the arcuate nucleus of the hypothalamus or the commissural nucleus of the solitary tract) and neurons that express NPY/AGRP (originating in the arcuate nucleus) as well as their downstream targets. Specifically, in response to circulating metabolic signals (i.e. leptin), the melanocortin system regulates energy balance through agonist α-melanocyte-stimulating hormone (α-MSH), derived from POMC cleavage, and antagonist AgRP acting on melanocortin receptors 3 and 4 (MC3/4R) (Figure 1.3.2). Transgenic animal models and human genetic studies highlight the integral role of the central melanocortin system in the regulation of energy. For example, mice deficient in melanocortin-4 receptor (MC4RKO) exhibit an obese phenotype (Huszar et al., 1997). By contrast transgenic overexpression of MSH attenuates the effects induced by a high fat diet (Lee et al., 2007). Similar attributes are seen in humans with mutations in the genes that encode for POMC (Krude et al., 1998) and MC4R (Vaisse et al., 1998;Yeo et al., 1998).

The abundance of energy-rich foods combined with the positive effects of palatable food is a strong driver underlying today's obesity epidemic (Volkow and Wise, 2005). The drive to eat palatable foods is indeed able to override homeostatic signals leading to such increased ingestive behavior. It is believed that hedonic feeding is largely regulated by the brain reward systems, most notably the mesocorticolimbic circuitry (Liu and Borgland, 2015). Within the ventral tegmental area (VTA) of the midbrain, dopamine neurons send projections to the nucleus accumbens and prefrontal cortex, and the subsequent release of dopamine is believed to coordinate the rewarding aspects of food (Lutter and Nestler, 2009). Behavioral studies in rodent models reinforce the central role of dopamine in the motivation to obtain food (Kenny, 2011). Nevertheless the various intrinsic and extrinsic factors acting on the dopamine system to regulate hedonic feeding remain vague. It is known that the same metabolic signals regulating homeostatic feeding, are also acting on the brain reward systems, indicating that hedonic and homeostatic feeding are not mutually exclusive.

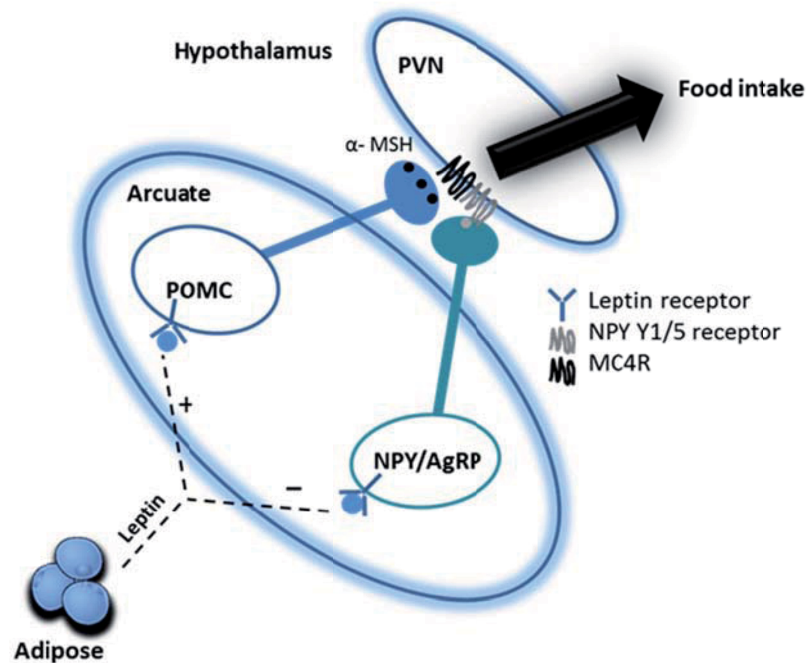

Figure 1.3.2 Homeostatic control of food intake

Leptin is secreted from adipose tissue. In the hypothalamus leptin binds to its receptors on orexigenic NPY/AgRP neurons and anorexigenic POMC neurons, which both send projections to the PVN. Leptin activates POMC neurons to stimulate the release of α-MSH which binds to and stimulates MC4R to trigger satiety. Leptin inhibits NPY neurons by inhibiting the expression of AgRP (MC4R antagonist) and NPY. Abbreviations: α-MSH, α-melanocyte stimulating hormone; AgRP, agouti-related protein; MC4R, melanocortin receptor 4; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus.

1.3.2. Energy Expenditure

Energy expenditure (i.e. output) comprises the other side of energy balance. Total energy expenditure is roughly divided into three components: (1) resting metabolic rate, referring to the energy required during a non-stressed, resting state; (2) activity-related energy expenditure; and (3) diet-induced thermogenesis (van Marken Lichtenbelt and Schrauwen, 2011). Alternatively, energy expenditure can be divided into obligatory energy expenditure (referring to the energy required for core body functions) and facultative energy expenditure, comprising cold-induced shivering and non-shivering thermogenesis, physical activity, and diet-induced thermogenesis. While obligatory energy expenditure is relatively fixed, facultative thermogenesis, and in particular activity thermogenesis and non-shivering thermogenesis, is highly variable.

Activity Thermogenesis

Activity thermogenesis refers to the increase in energy expenditure that accompanies physical activity (Levine, 2005). Importantly, activity thermogenesis is further divided into exercise (EAT) and non-exercise activity thermogenesis (NEAT). Whereas EAT encompasses purposeful or structured exercise activity, NEAT rather encompasses non-structured activity that comprise daily living (i.e. posture, fidgeting, lifestyle movement patterns, etc.). The majority of individuals do not regularly participate in exercise training, and therefore the energy expenditure from activity thermogenesis is predominantly determined by NEAT. Interestingly, activity thermogenesis is highly variable, both within subjects and between subjects, with studies estimating that its contribution to total energy expenditure ranges from 15% to 50% (Levine, 2004).

Non-Shivering Thermogenesis

Non-shivering thermogenesis refers to the production of heat that develops overtime when mammals are acclimated to cold (Cannon and Nedergaard, 2010). The ability to maintain a stable body temperature in response to prolonged cold exposure through the activation of non-shivering thermogenesis relies on the expansion &/or activation of brown adipose tissue (BAT) (Feldmann et al., 2009;Rothwell and Stock, 1983). In mammals, there are two major types of adipose tissue, BAT and white adipose tissue (WAT), which are both structurally and functionally distinct (Saely et al., 2012). Whereas WAT primarily acts as a storage site for lipids, BAT functions as a thermogenic tissue, dissipating energy as heat to mediate non-shivering thermogenesis. The expression of UCP1 (uncoupling protein 1) in BAT mediates non-shivering thermogenesis through its ability to separate fatty acid oxidation from ATP synthesis (Nicholls, 2006). Consequently, adipocytes in BAT have a relatively high metabolic rate (Cannon and Nedergaard, 2004), and studies have shown that human BAT significantly contributes to total energy expenditure. Specifically, it is estimated that cold-induced BAT activation leads to a 10-20% rise in the daily metabolic rate (Chen et al., 2013;Ouellet et al., 2012;Vijgen et al., 2013;Yoneshiro et al., 2011). Collectively, expansion and/or activation of brown fat increase energy expenditure.

1.4. Tissue-Specific Regulation of Glucose Homeostasis

The coordinated actions of multiple organs and tissues including the pancreas, liver, skeletal muscle, brain, and adipose tissue, are required for the maintenance of glucose homeostasis (Figure 1.4.1). Together, these tissues are able to sense whole body energy status,

communicate energy availability to the body, and respond to changes in energy levels. For example, the body regulates glucose utilization, production, and release in order to maintain glycaemia at ~5mM, which ensures sufficient glucose flux to meet the demands of the body (Saltiel and Kahn, 2001). These metabolic processes are tightly regulated in order to prevent loss of consciousness on account of hypoglycaemia and/or toxicity to peripheral tissues on account of chronic hyperglycaemia. In a disease state, such as obesity or T2D, glucose homeostasis is often impaired, and is associated with hyperglycemia (Huang and Czech, 2007).

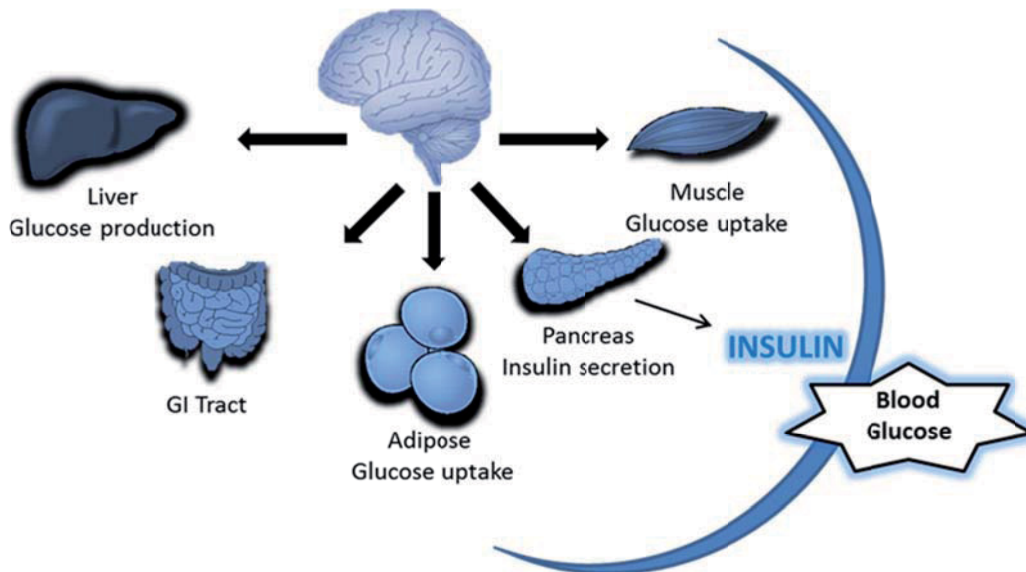

Figure 1.4.1 Tissue-specific regulation of blood glucose

Adapted from (Morton and Schwartz, 2011)

Glucose uptake into skeletal muscle and adipose tissue is an essential component of whole body glucose homeostasis. In general, glucose uptake into cells involves a family of structurally-related carrier proteins, collectively known as sugar transporter proteins, which act to accelerate facilitative membrane diffusion of sugars down their concentration gradient (Huang and Czech, 2007). Each member displays distinct tissue and substrate specificity which ultimately determines its function. The glucose transporter protein 4 (GLUT4), for example, is a hexose transporter expressed predominately in adipose and muscle tissues, and is responsible for insulin-stimulated glucose uptake. More specifically, in the absence of insulin, GLUT4 is localized to specialized intracellular structures that consist of GLUT4 storage vesicles (GSVs) (Leto and Saltiel, 2012). Only upon stimulation (in response to insulin or exercise) does GLUT4 acutely redistribute to the plasma membrane, which results in a 10-fold increase in glucose uptake. Indeed insulin-stimulated glucose uptake is the rate-

limiting step for both muscle and adipose tissue glucose metabolism, and is therefore very important to whole body glucose homeostasis. In fact defective GLUT4 translocation to the plasma membrane in response to insulin contributes to the development of insulin resistance and T2D (Leto and Saltiel, 2012).

Skeletal muscle is recognized as the principal site of insulin-stimulated glucose uptake, accounting for approximately 80% of total glucose disposal following glucose infusion (DeFronzo et al., 1985;Katz et al., 1983). In mouse models where GLUT4 is absent from skeletal muscle, a diabetic phenotype manifests, highlighting the importance of insulin-stimulated glucose uptake in the muscle (Zisman et al., 2000). Although adipose tissue is responsible for significantly less insulin-stimulated glucose uptake, it still plays an essential role in whole body glucose homeostasis. For example, an adipose-specific reduction in GLUT4 expression results in insulin resistance (Abel et al., 2001). Moreover, selective overexpression of GLUT4 in mouse adipose tissue rescues the insulin-resistant phenotype of mice lacking GLUT4 in skeletal muscle, suggesting that adipose-specific increased GLUT4 expression protects against whole-body insulin resistance (Carvalho et al., 2005). Finally, a cardinal feature of an insulin-resistant state, present in T2D and obesity, is the selective downregulation of GLUT4 expression in adipose tissue, but not muscle (Shepherd and Kahn, 1999). Taken together, insulin mediated glucose uptake through GLUT4 in both muscle and adipose tissue is critical for the maintenance of glucose homeostasis.

1.5. Genetics of Obesity

There is substantial evidence indicating that genetic factors play an important role in determining body weight and subsequently BMI (Barsh et al., 2000). Importantly, the influence of genetic factors is most evident in extremely obese individuals. For example, using mouse models as well as human population-based studies, central nodes in energy balance have been identified, which when mutated result in morbid obesity (Farooqi, 2014). Nevertheless, single point mutations in genes involved in energy homeostasis represent the exception, not the majority, of obesity cases. In reality, obesity is often the net cause of genetic, behavioral, and environmental factors. Yet the biological processes altering individual vulnerability or resilience to metabolic diseases remain poorly understood. For example, differences in body size between individuals raised in a common environment are considered mainly to arise by virtue of genetic factors (Allison et al., 1996;Ginsburg et al., 1998). By contrast, body weight differences amongst genetically identical populations (i.e. monozygotic twins and inbred mouse models) have been attributed to diverging

environmental stimuli, such as diet and stress (Balsevich et al., 2014;Bartolomucci et al., 2009;Segal et al., 2009;Segal and Allison, 2002). Genetic factors contributing to individual vulnerability or resilience to develop obesity are not easy to identify given the pleiotropic nature of body weight.

In the last few decades several strategies have been adopted in order to investigate genetic targets regulating body weight and energy balance (Barsh and Schwartz, 2002). Monozygotic and dizygotic twin studies have suggested that the genetic contribution to obesity is between 50% and 90% (Barsh et al., 2000). Nevertheless, environmental conditions determine whether the phenotype is expressed, which is reflected by the fact that obesity is largely a health concern unique to post-industrial societies. Moreover, identification of molecules regulating body size is not trivial on account that many genetic variants are likely involved and interact to produce only a modest effect. In addition, at an individual level, various genetic variants interact with various environmental cues to predict obesity outcome. Therefore this intricate gene by environment landscape complicates the search for novel targets. Nevertheless, the identity of several 'obesity genes' is now known, and has shed light onto various signaling pathways integral to energy balance regulation. In fact at least 430 genes, markers, and chromosomal regions have been associated with body weight regulation and an obese phenotype (Snyder et al., 2004). Interestingly, many of these genetic variants are directly linked to energy intake, expenditure, adipogenesis, and insulin resistance. For an extensive review of genetic variants contributing to an obese phenotype, the reader is referred to (van der Klaauw and Farooqi, 2015).

1.6. Signaling Molecules of Energy Homeostasis

Both peripheral and central pathways are important in whole body energy metabolism, and there is significant cross-talk between them (Morton et al., 2006). In general, the body must be able to sense the current energy status of an individual and further respond to changes in the nutrient status in order to maintain stability of body energy stores. Collectively, the brain integrates such incoming information in order to formulate a response which would offset changes in energy stores. Several circulating factors are able to signal between the periphery and the central nervous system (CNS) in order to mount an adaptive adjustment of energy homeostasis to stabilize body weight and fat stores, including leptin, insulin, nutrients, and glucocorticoids (Morton et al., 2006).

1.6.1. Leptin

Leptin is perhaps the most well characterized signaling molecule regulating energy balance. Secreted from adipocytes in proportion to body fat mass, leptin is a 16 kDa protein consisting of 167 amino acids (Cohen et al., 1996;Considine et al., 1996;Munzberg and Morrison, 2014). Given higher levels of circulating leptin reflect a higher degree of adiposity, it may come as no surprise that leptin acts to reduce food intake (Friedman, 2000) and stimulate sympathetic nerve activity to BAT to promote UCP-1 expression and BAT activation (Commins et al., 2000;Morrison and Madden, 2014). In obese patients and rodent models of obesity, leptin resistance often persists (Maffei et al., 1995;Munzberg et al., 2004;Pelleymounter et al., 1995). In both human and transgenic mouse models, where either leptin (ob gene) or the leptin receptor (db gene) is deficient, hyperphagia, obesity, and insulin resistance results, highlighting the importance of leptin signaling on body weight and glucose regulation (Clement et al., 1998;Coleman, 1973;Maffei et al., 1995;Montague et al., 1997;Munzberg et al., 2004;Tartaglia et al., 1995;Zhang et al., 1994).

Leptin action is mediated through its leptin receptors, which are highly expressed in the hypothalamus, a key metabolic regulatory center of the brain (Elmquist et al., 1998). Upon leptin binding to its receptor, Janus kinase-2 (JAK2) becomes activated, which in turn phosphorylates itself and 3 tyrosine residues in the leptin receptor tail region. Each of these phosphorylation sites activates distinct signaling pathways which ultimately determine the functional outcome of leptin signaling (Kloek et al., 2002) (Figure 1.6.1). Leptin receptors, for example, are expressed in at least 2 distinct neuronal populations in the arcuate nucleus of the hypothalamus, namely the NPY/AgRP-expressing neurons and the POMC/CART-expressing neurons (Cheung et al., 1997;Mercer et al., 1996). NPY/AgRP and POMC/CART –expressing neurons have opposing effects on food intake. Whereas NPY and AgRP are recognized as potent orexigenic signals, POMC and CART are rather known for their anorectic effects (refer to Section 1.3.1). Leptin signaling activates POMC/CART-expressing neurons whereas inhibits NPY/AgRP-expressing neurons both through the activation of the JAK/STAT signaling cascade (Kaelin et al., 2006;Xu et al., 2007). This opposing regulation is attributed to STAT-3 function, whereby activated STAT-3 binds to *Pomc* and *Agrp* promoter regions to stimulate POMC expression whereas inhibit AGRP expression. Interestingly, leptin action through phosphoinositide 3-kinases (PI3K) signaling has furthermore been shown to down-regulate NPY expression (Morrison et al., 2005). Of course, not all actions of either POMC or NPY are exclusively regulated by leptin, as only a subset of

each neuronal population express leptin receptors and furthermore both NPY and POMC-expressing neurons are responsive to many other circulating factors (Morton et al., 2006).

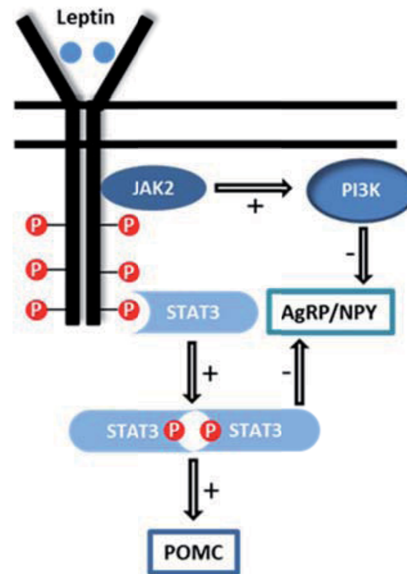

Figure 1.6.1 Leptin signaling

Upon leptin binding to the extracellular domain of its leptin receptor, janus kinase (JAK) is recruited and activated (Vaisse et al., 1996). JAK subsequently binds and phosphorylates the leptin receptor which then phosphorylates STAT-3 (signal transducer and activator of transcription 3). Activated STAT-3 stimulates POMC expression whereas inhibits AgRP expression. JAK2/PI3K signaling furthermore inhibits NPY expression.

Initially, leptin signaling in the ARC was the central focus, but more recently leptin signaling across other hypothalamic nuclei has gained attention (Munzberg and Morrison, 2014). Distinct hypothalamic and extra-hypothalamic populations of leptin receptors exist, which collectively contribute to leptin's diverse effects on energy homeostasis, including its effect on homeostatic feeding, hedonic feeding, BAT thermogenesis, and glucose homeostasis.

1.6.2. Insulin

Insulin is a peptide hormone comprised of 51 amino acids produced by β cells of the islets of Langerhans in the pancreas (Brange and Langkjoer, 1993). In response to elevated levels of blood glucose, insulin is secreted and acts through its membrane receptor tyrosine kinases (RTKs) to ultimately regulate diverse cellular processes (Taniguchi et al., 2006). Upon insulin binding, the RTK autophosphorylates at tyrosine residues and subsequently phosphorylates tyrosine residues of its immediate intracellular substrates, namely insulin receptor substrates (i.e. IRS-1, IRS-2, etc.) (Thirone et al., 2006). Phosphorylation of IRS proteins leads to the recruitment and activation of PI3K, which converts phosphatidylinositol

-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 serves as a docking site for phosphoinositide-dependent kinase 1 (PDK1) and AKT (protein kinase B, PKB) at the membrane, which is subsequently required for phosphorylation and activation of AKT. AKT, a serine-threonine (Ser/Thr) kinase, itself has several downstream targets, including mammalian target of rapamycin (mTOR) (Garami et al., 2003), forkhead box protein (FOXO) (Nakae et al., 1999), AKT substrate 160 (AS160) (Kane et al., 2002), and glycogen synthase kinase 3 (GSK-3) (Desbois-Mouthon et al., 2001). Together, the PI3K-AKT pathway is the primary pathway responsible for the metabolic effects of insulin (Figure 1.6.2).

Circulating insulin has several effects on metabolism, which collectively function to lower blood glucose levels. The actions of insulin on metabolism are tissue-specific, comprising rapid changes in protein phosphorylation states and activity, as well as changes in gene expression (Kahn and Flier, 2000). Insulin action, for example, stimulates glucose uptake into adipose tissue and skeletal muscle. Specifically, the AKT-dependent phosphorylation of AS160 favors GLUT4 translocation to the membrane and thus glucose uptake (Sano et al., 2003). By contrast, in the liver, insulin acts to suppress glucose production (Gabbay et al., 1996). Furthermore, insulin is considered an anabolic hormone on account of its abilities to promote the synthesis of glycogen, protein, and lipid, while inhibit glycogenolysis, protein degradation, and lipolysis (Dimitriadis et al., 2011). The ability of insulin to regulate so many diverse functions depends on its signaling cascade which consists of multiple points of divergence resulting in distinct end points as well as complex crosstalk with other signaling cascades (refer to Section 1.6.3 for more detail).

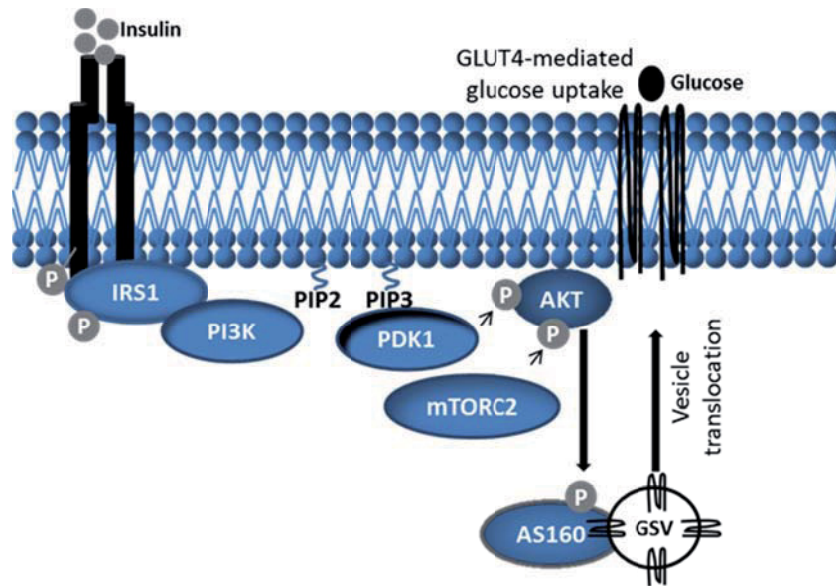

Figure 1.6.2 Insulin-stimulated glucose uptake

Insulin binds to its receptor to initiate the phosphoinositide 3-kinase (PI3K)-dependent signaling cascade which leads to the phosphorylation and activation of AKT. Activated AKT promotes glucose transporter 4 (GLUT4) storage vesicle (GSV) exocytosis in part by phosphorylating and inactivating AKT substrate 160 (AS160), which relieves the inhibitory effect on vesicle translocation. Adapted from (Leto and Saltiel, 2012).

1.6.3. Nutrients

Nutrients, including glucose and amino acids, were originally recognized simply for their roles as energy substrates and precursors for the synthesis of macromolecules. However, it is now well recognized that glucose and amino acids serve additionally as important signaling molecules, able to regulate energy metabolism, cell growth, proliferation, and survival (Marshall, 2006). Collectively nutrients and downstream nutrient-sensing signaling pathways are decisive components of the larger interconnected metabolic regulatory network which functions to maintain energy homeostasis. Two important, defining characteristics of nutrient signaling pathways include the ability to sense changes in nutrient/energy availability and to quickly respond to such changes by transducing the signal through the modification (i.e. phosphorylation) of regulatory proteins. The mTOR and adenosine monophosphate (AMP)-activated protein kinase (AMPK) signaling pathways are two of the best established nutrient signaling pathways within this complex network.

mTOR Signaling

mTOR is a highly conserved Ser/Thr protein kinase that exists in two distinct complexes, mTORC1 and mTORC2, to regulate cell growth and metabolism in response to nutrient and hormonal signals (Wullschleger et al., 2006). mTORC1 consists of mTOR in complex with raptor, mLST8, and PRAS40, and is sensitive to the immunosuppressive drug rapamycin. By contrast, mTORC2 consists of mTOR, rictor, mSIN1, and mLST8, and is not sensitive to rapamycin. Although both complexes have been implicated in whole body metabolism, mTORC1 is directly regulated by cellular energy and nutrient status whereas mTORC2 is not (Inoki et al., 2012). Furthermore, mTORC1 is much better characterized on account of the availability of the mTORC1-specific inhibitor rapamycin (Polak and Hall, 2009).

In response to growth factors and nutrients (i.e. amino acids and glucose), mTORC1 is activated and targets its downstream effectors, p70S6 Kinase 1 (p70S6K1) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP-1) (for review refer to (Manning, 2004), Figure 1.6.5). These two proteins are the best characterized targets of mTORC1 and are integrally involved in mTORC1-mediated increases in ribosomal biogenesis and translation of specific mRNA populations. In more detail, 4EBP1, which acts as a repressor of translation initiation, is inhibited by mTORC1, whereas p70S6K1, a Ser/Thr kinase that regulates diverse processes including translation initiation, is activated by mTORC1. Collectively, mTORC1 activation promotes cell growth, protein synthesis and furthermore down-regulates the catabolic process of autophagy. When energy levels are high, growth factors and nutrients activate mTORC1 and its downstream effectors primarily via the PI3K-AKT pathway. Specifically, mTORC1 is connected to the PI3K-AKT pathway through an AKT-mediated phosphorylation and inactivation of the tuberous sclerosis proteins TSC1 and TSC2, critical negative regulators of mTORC1 (Inoki et al., 2002).

The TSC1/TSC2 complex represents an important node in the mTOR pathway, determining the activity of mTORC1. For example, activated TSC1/TSC2 inhibits mTORC1 activity whereas inactive TSC1/2 enhances mTORC1 activity. This is nicely illustrated by the constitutive activation of mTORC1 in TSC-deficient cells (Kwiatkowski et al., 2002). The regulatory function of the TSC1/2 complex on mTORC1 depends on its role as a GTPase activating protein (GAP) to the small GTPase protein known as rheb (Ras homolog enriched in brain). In more detail, mTORC1 directly interacts with rheb, an established positive regulator of mTORC1 (Long et al., 2005;Yamagata et al., 1994). Yet rheb either exists in its inactive, GDP-bound form, or its active, GTP-bound form. In its active, GTP-bound state,

rheb is able to activate mTORC1. Importantly TSC2 acts as a GAP toward rheb, promoting GTP hydrolysis to GDP and inactivation of rheb (Inoki et al., 2003a). Importantly, the TSC1/2 complex is itself regulated by upstream signaling molecules and subsequent phosphorylation events. For example, AMPK phosphorylates TSC2 to promote its activity and subsequently suppress mTORC1 activity (Inoki et al., 2003b). By contrast, growth-related kinases, including AKT, phosphorylate TSC2 to inhibit its activity and thus enhance mTORC1 signaling (Inoki et al., 2002).

The mTORC1 signaling cascade plays an important role in whole body metabolism in a tissue-specific manner. The use of tissue-specific knockout models in which components of the mTORC1 signaling cascade have been deleted, has enriched our understanding of mTOR function (Polak and Hall, 2009). For example, loss of p70S6K1 in adipose tissue leads to a leaner phenotype, with mice presenting decreased lipid accumulation and increased mitochondrial respiration (Um et al., 2004). By contrast muscle-specific loss of p70S6K1 results in the downregulation of proteins involved in mitochondrial biogenesis and decreased mitochondrial respiration. The seemingly opposite phenotype on mitochondrial activity from either fat- or muscle-specific loss of mTORC1 signaling highlights the importance of tissue specificity (Figure 1.6.3). Tissue-specific gene expression profiles and thus different downstream effectors have been suggested to contribute to the distinct metabolic functions of mTORC1 signaling across various tissues (Polak and Hall, 2009).

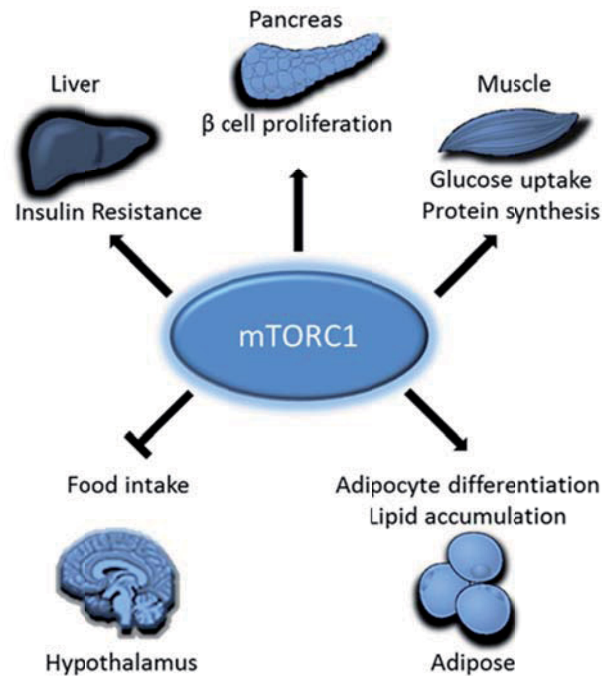

Figure 1.6.3 Tissue-specific mTORC1 regulation of energy metabolism

Activated mTORC1 promotes anabolic cellular responses, such as protein translation and lipid biosynthesis to stimulate cell growth and proliferation. In the hypothalamus, mTOR decreases food intake.

AMPK Signaling

AMPK is a highly conserved Ser/Thr kinase renowned for its role as an energy sensor of both cellular and whole body energy homeostasis (Kahn et al., 2005). In fact, AMPK has been defined as the 'metabolic master switch' due to its ability to sense and respond to changing energy supplies and demands. Naturally, this requires the coordinated regulation of multiple metabolic pathways. The AMPK holoenzyme is comprised of three subunits, α, β, and γ. The α-subunit is critical for the catalytic activity of the protein kinase whereas the β- and γ-subunits are important regulatory subunits (Inoki et al., 2012). Each subunit in turn has multiple isoforms, which show tissue specificity. Activation of AMPK in response to low energy conditions results in the phosphorylation of numerous substrates to enhance catabolism and inhibit anabolism (Towler and Hardie, 2007). Two upstream kinases, liver kinase B1 (LKB1) and calmodulin-dependent kinase kinase (CaMKK), have been identified as positive regulators of AMPK (Hurley et al., 2005;Woods et al., 2003). Both phosphorylate a conserved threonine residue (Thr172) within the activation loop of the alpha domain to activate AMPK. AMP also acts as an upstream activator of AMPK either by promoting Thr172 phosphorylation by LKB1 or by acting as an allosteric activator (Towler and Hardie,

2007). In addition, activation of AMPK has been associated with various therapeutic agents, including the first-line drug treatment for T2D, metformin, which present glucose-lowering capabilities (Foretz et al., 2014). Once activated, AMPK acts to restore energy balance in response to low energy conditions by enhancing ATP generation or rather inhibiting ATP-consuming processes. One of the major downstream targets of AMPK is mTORC1 through the direct phosphorylation of TSC2, in which a reciprocal relationship exists between them (Inoki et al., 2012) (discussed above).

AMPK has several downstream targets (for an exhaustive list please refer to (Hardie, 2007) which act to upregulate catabolic pathways and inhibit anabolic pathways. Whereas AMPK-mediated changes are initially achieved through post-translational modifications (namely phosphorylation) of metabolic enzymes, long-term alterations are the result of AMPK-dependent effects on gene expression. For example, AMPK regulation of glucose uptake in skeletal muscle is initially achieved through AMPK-mediated increases in GLUT4 translocation, in part by direct phosphorylation and activation of downstream effector AS160 (Sakamoto and Holman, 2008) (refer to section 1.6.2). However in the long-term, activation of AMPK promotes increased transcription of the GLUT4 gene (Zheng et al., 2001). Furthermore AMPK-mediated regulation of energy metabolism is highly tissue specific (Figure 1.6.4). Whereas AMPK predominantly acts to drive glucose uptake in skeletal muscle, in adipose tissue AMPK primarily functions to inhibit fatty acid synthesis. An in-depth discussion of AMPK signaling across various tissue types is beyond the scope of this thesis. However, it is evident that the ability of AMPK to respond to energy deficiency has broad effects on whole body energy homeostasis.

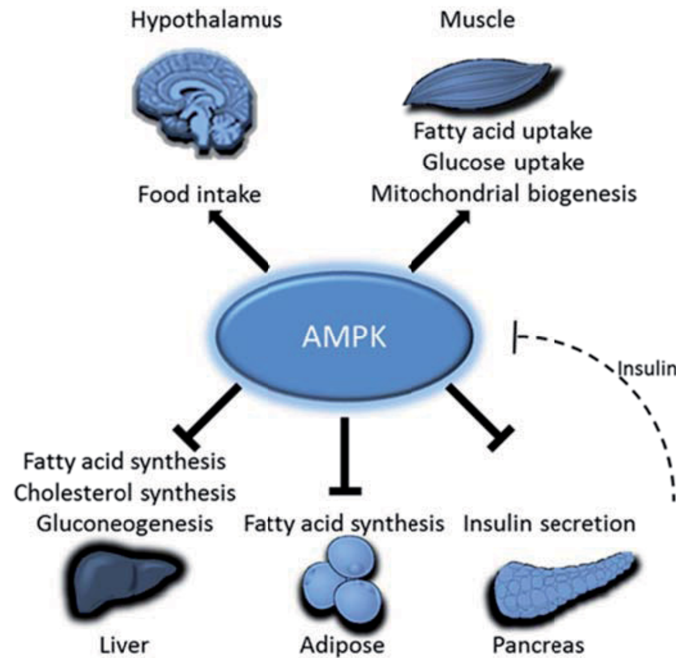

Figure 1.6.4 Tissue-specific AMP-activated protein kinase (AMPK) regulation of whole body energy homeostasis

Activated AMPK promotes cellular responses directed at generating ATP while inhibits energy-consuming processes in many tissue types. In the hypothalamus, AMPK increases food intake. Insulin inhibits AMPK within the hypothalamus, whereas has no effect on AMPK within skeletal muscle or adipose tissue. Adapted from (Kahn et al., 2005).

Crosstalk between Nutrient Signaling Pathways and the Insulin Signaling Pathway

Although it was initially thought that signaling cascades all operated independently of one another, unequivocal evidence has more recently demonstrated significant crosstalk and overlap between them and the entire network regulating energy homeostasis (Figure 1.6.5). In particular, the role of insulin signaling, mTOR signaling, and AMPK signaling in the context of a larger metabolic regulatory network has been well studied. Within each pathway there are critical nodes where crosstalk between pathways occurs. On the basis of such extensive interplay between signaling cascades, regulation along any one of these pathways may have indirect effects on many diverse processes. For example, it has been demonstrated using the AMPK agonist AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) that AMPK activation increases insulin sensitivity in skeletal muscle (Fisher et al., 2002;Iglesias et al., 2002). The mTOR signaling cascade is likewise known to regulate insulin sensitivity, albeit in an opposite manner. Specifically, phosphorylation of IRS-1 by the mTOR/p70S6K represents an important negative feedback loop in which insulin attenuates its own signaling in response to prolonged stimulation (Takano et al., 2001;Tremblay and Marette, 2001).

Interestingly, the increased phosphorylation of IRS-1 observed in animal models of obesity is markedly reduced in p70S6K-deficient mice fed a HFD (Um et al., 2004), suggesting that inhibition of this feedback loop increases insulin sensitivity. Finally there is also direct crosstalk between the AMPK and mTOR signaling pathways as reflected through the ability of AMPK to inhibit mTOR via the TSC1/2 complex (Section 1.6.3).

Besides the phosphorylation states of the proteins comprising the regulatory machinery of signal transduction pathways, subcellular localization of these various proteins has emerged as an important regulatory mechanism mediating both signal transduction specificity and crosstalk (Abraham, 2002;Scott and Pawson, 2009). The role of various scaffolding, adapter, and anchoring proteins in determining the specificity of signal transduction by positioning enzymes close to their substrates is well recognized. This is exemplified by the mTOR pathway in which mTOR exists in one of two complexes (mTORC1 and mTORC2). Substrate specificity is conveyed by the mTOR scaffolding proteins Raptor and Rictor as well as other mTOR binding proteins (Hoeffer and Klann, 2010). Scaffolding, adapter, and anchoring proteins may moreover confer crosstalk specificity through spatial control of the signal transduction proteins. Taken together, multiple levels of regulation determine the fate of the cell in response to various signals, which involve multiple signaling cascades and significant crosstalk between them (Table 1.6.1).

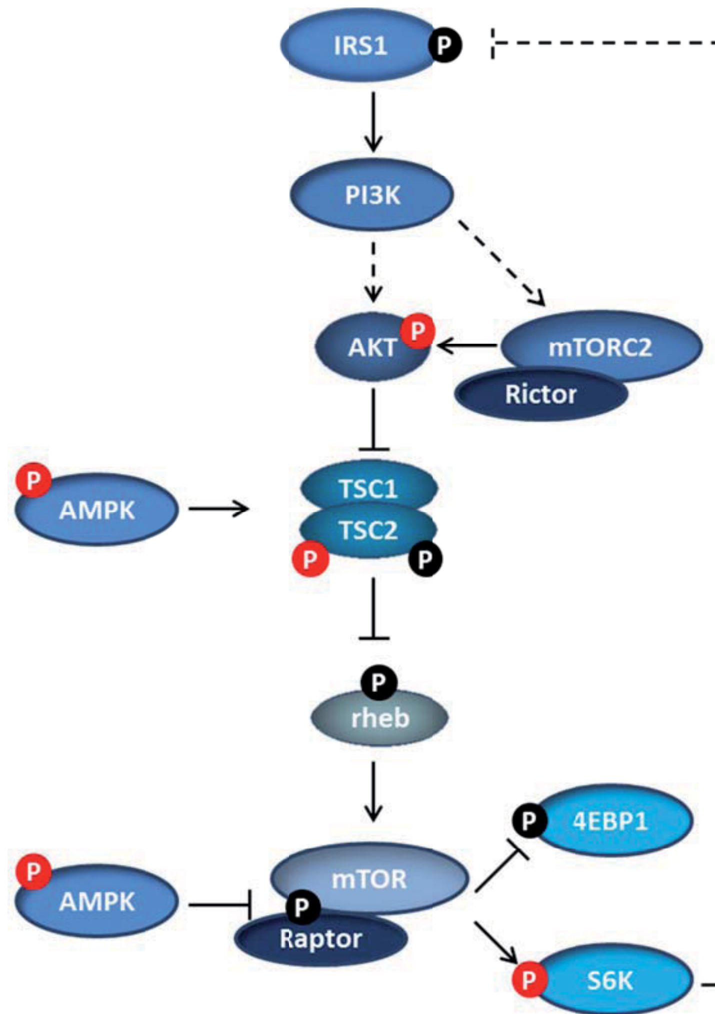

Figure 1.6.5 The interconnected metabolic regulatory network

The crosstalk between pathways comprising the larger interconnected metabolic regulatory network which functions to maintain energy homeostasis. Red = Activating phosphorylation; Black = Inhibiting phosphorylation. Abbreviations: 4EBP1, eIF4E-binding protein 1; AMPK, AMP-activated kinase; IRS1, insulin receptor substrate 1; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; rheb, Ras homolog enriched in brain; S6K, p70S6 Kinase 1; TSC1/2, tuberous sclerosis proteins 1/2. Adapted from (Inoki et al., 2012).

Table 1.6.1 The integrated metabolic regulatory network

Nutrient-sensing signaling pathways as constituents of an integrated metabolic regulatory network. Nutrient signaling pathways do not operate as individual entities but rather function cooperatively to coordinate energy homeostasis as an integrated response. Adapted from (Marshall, 2006).

<table>
<tr><th>Signaling Pathway</th><th>Function</th><th>Mechanism</th><th>Integrated Response</th></tr>
<tr><td>AMPK</td><td>Sense AMP/ATP ratio

Restore ATP levels</td><td>Stimulate catabolic metabolism

Inhibit anabolic metabolism</td><td rowspan="3">Maintain whole body energy and glucose homeostasis

Sense energy availability and changes in energy stores

Respond to changes in energy availability

Coordinate central regulation with peripheral metabolism</td></tr>
<tr><td>mTOR</td><td>Regulate cell growth and survival

Regulate tissue/organ growth</td><td>Stimulate protein synthesis

Stimulate ribosomal biogenesis

Inhibit autophagy</td></tr>
<tr><td>Insulin</td><td>Regulate glucose metabolism

Regulate lipid metabolism</td><td>Post-translational phosphorylation of regulatory proteins

Regulation of gene transcription</td></tr>
</table>

1.6.4. Glucocorticoids

Glucocorticoids (GCs) are steroid hormones produced by the adrenal cortex. The secretion of GCs is under the regulation of a neuroendocrine feedback system, known as the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis plays an integral role in the physiological stress response as well as metabolic control (de Kloet et al., 2005). In response to a stressor, including both metabolic and psychosocial stressors, the HPA axis is activated. Although different categories of stressors (i.e. physical vs. neurogenic stressors) require different brain networks, all stressors converge to activate the HPA axis (Herman et al., 2003). In response to a stressor the parvocellular neurosecretory neurons within the

paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP), which summate the excitatory and inhibitory inputs into a net output (Herman and Cullinan, 1997). CRH and AVP are released from the median eminence into the hypophyseal portal blood system, which bridges the hypothalamus and anterior part of the pituitary gland. At the pituitary gland, CRH and AVP act synergistically to stimulate corticotrophin cells to synthesize POMC, the precursor of adrenocorticotropic hormone (ACTH) (Papadimitriou and Priftis, 2009). When released into peripheral circulation, ACTH stimulates the release of GCs from the adrenal cortex. GCs (cortisol in humans or corticosterone in rodents) are recognized as the major end products of the HPA axis and subsequently act on various targets to modulate the effects of a wide range of physiological and developmental processes, exerting its effects on practically every system of the body (e.g. metabolic, endocrine, nervous, cardiovascular, immune) (Chrousos et al., 2004). Furthermore GCs are involved in a negative feedback circuit whereby they operate at different levels of the HPA axis and at the hippocampus to terminate the stress response (de Kloet et al., 1998;Ulrich-Lai and Herman, 2009).

GCs signal through two receptor systems, the type I, high affinity mineralocorticoid receptor (MR) and the type II, low affinity glucocorticoid receptor (GR) systems (Aronsson et al., 1988;Kretz et al., 2001;Morimoto et al., 1996;Reul and de Kloet, 1986). Based on differences in ligand affinities, MRs and GRs have distinct roles. Specifically, MRs and GRs are responsible for regulating basal HPA axis function and terminating the stress response, respectively (de Kloet et al., 1993;de Kloet and Reul, 1987;Reul and de Kloet, 1985;Veldhuis et al., 1982). MRs and GRs belong to the nuclear hormone receptor superfamily, and act as ligand-activated transcription factors (Evans, 1988). In the absence of ligand, steroid receptors are sequestered in the cytoplasm as a multimeric protein complex (Cheung and Smith, 2000). Upon binding GCs, MRs and GRs dissociate from this complex, and translocate into the nucleus in association with a group of nuclear transport proteins. Within the nucleus, homo- or heterodimer steroid receptors alter gene expression through the interaction with glucocorticoid response elements (GREs) (reviewed by (de Kloet et al., 2005)). The multimeric nature of the steroid receptor-protein complex has an important regulatory role, affecting steroid receptor function through regulation of receptor folding, hormone binding, and nuclear translocation (Grad and Picard, 2007;Pratt et al., 2006). The mature GR complex comprises a dimer of the heat shock protein 90 (hsp90) and additional components including hsp70, p23, and potentially an hsp90-binding immunophilin (IMM),

namely FK506-binding protein 51 (FKBP51) or FK506-binding protein 52 (FKBP52) (Pratt and Toft, 1997). The regulatory chaperones and co-chaperones are integral to HPA axis reactivity.

It is well recognized that GCs are intimately linked to metabolic regulation. For example, in response to a stressor the body elicits a specific physiological response. Not only does the physiological response to stress increase arousal and alertness, but peripheral mechanisms furthermore redirect energy, such that oxygen and nutrients are directed to the CNS as well as the stressed body site. Mechanisms to mobilize available energy resources are implemented, including enhanced gluconeogenesis, lipolysis, and protein degradation (Chrousos and Gold, 1992;Kyrou and Tsigos, 2009). By contrast, growth and reproduction are inhibited. The overall effects of GCs on whole body energy metabolism are the product of tissue-specific effects of GC action (Figure 1.6.6). These effects are very broad affecting insulin signaling and glucose homeostasis, lipid homeostasis, and food intake alike. For example, GC action regulates feeding neuropeptides in the hypothalamus, GLUT4 translocation in the muscle, and the activity of lipogenic and lipolytic enzymes in adipose tissue in order to promote food intake, decrease glucose uptake, and increase lipolysis, respectively (de Guia et al., 2014;Delaunay et al., 1997;Khan et al., 1992;Maniam and Morris, 2012;Weinstein et al., 1998;Xu et al., 2009). In the short-term, GC action is highly adaptive, however can become maladaptive if it persists. This can be evidenced in Cushing's syndrome, a disorder caused by excess GCs, which is characterized by visceral adiposity (Boscaro et al., 2001).

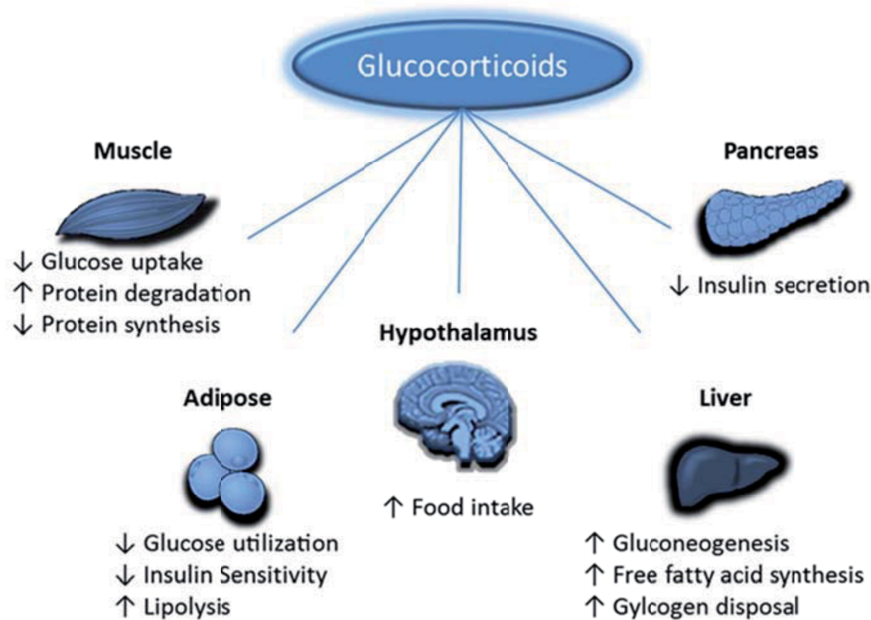

Figure 1.6.6 Tissue-specific effects of glucocorticoids on energy homeostasis

1.7. Therapeutic Targets/Pathways to Treat Obesity and Obesity-Related Disorders

Despite the in-depth characterization of the various components comprising energy balance as well as the underlying signaling pathways regulating them, effective pharmacological treatments of obesity and related complications remain elusive. There are many anti-obesity therapeutics targeting numerous mechanisms currently under investigation and in clinical trials (Rodgers et al., 2012). To-date anti-obesity strategies have chiefly attempted to restrict caloric intake, but have unfortunately proven largely ineffective because of the resulting decrease in energy expenditure associated with weight loss (Rodgers et al., 2012;Spiegelman and Flier, 2001). Emerging evidence suggests that enhanced energy expenditure may represent an attractive alternative anti-obesity strategy (Nedergaard and Cannon, 2010). Yet attempts to target voluntary physical activity have had limited success in the treatment of obesity (Hankey, 2010;Poirier and Despres, 2001;Westerterp, 2010). Increased energy expenditure by targeting non-shivering thermogenesis has recently been a prominent research avenue in the development of anti-obesity drugs (Cannon and Nedergaard, 2011). Based on the central role of β adrenergic signaling in BAT thermogenesis, sympathomimetic agents have been examined in the treatment of obesity. Non-selective β adrenergic receptor compounds however led to too many off-target, undesired side effects to be considered an effective treatment strategy. Therefore, it is critical to identify additional modifying factors

that may serve as novel therapeutic targets. For example, several transgenic mouse models present a favorable metabolic phenotype, despite an incomplete or lack of understanding of the underlying mechanisms (Arch, 2002). In this regard, characterization of transgenic mouse models presenting favorable metabolic outcomes may assist researchers in the discovery of novel targets for anti-obesity drug design.

1.8. Novel Molecular Targets in the Regulation of Whole Body Energy Metabolism

Despite remarkable progress in elucidating energy balance regulation, our understanding of the complex, interacting mechanisms that comprise the regulatory network is far from complete. The varying contributions of multiple environmental and genetic factors complicate the study of energy balance and consequently the pathophysiological states of obesity and T2D. The use of animal models has been a powerful tool considerably contributing to our current understanding. Parallel findings between transgenic mouse models of obesity and population-based studies indicate that the same underlying biological processes are operating in both mice and men to regulate body weight, and support the use of genetic mouse models in obesity research (Barsh et al., 2000). In the case of transgenic rodent models that are fed a high fat diet (HFD), it is further possible to elucidate whether the genotype interacts with the dietary environment to predict susceptibility or resilience to diet-induced weight gain and related phenotypes. Of course identification of novel candidates involved in energy metabolism from knockout, knockdown, or overexpression of specific genes, which result in a metabolic phenotype, will inevitably contribute to the search for novel anti-obesity drugs. Although target identification has improved with the development of transgenic animal models, target validation in the context of therapeutic efficacy remains limited. Preliminary research in our lab suggests that the FK506 binding protein 51 may be a novel regulator of whole body energy metabolism and furthermore a prospective anti-obesity drug target.

1.8.1. FK506 binding protein 51 (FKBP51) Characterization

FKBP51 (encoded by gene fkbp5) belongs to the IMM superfamily consisting of FK506-binding proteins (FKBPs) and cyclosporin-binding cyclophilins (CyPs) (Baughman et al., 1995). FKBPs were first identified by virtue of their ability to bind immunosuppressants rapamycin and FK506 (Schreiber, 1991). All members possess the signature PPIase domain, and thus display inherent peptidyl-prolyl cis-trans isomerase (PPIase) activity. The high

molecular weight FKBPs, FKBP51 and FKBP52, possess increased architectural complexity compared to low molecular weight IMMs, which contributes to the functional differences between the low and high molecular IMMs. In particular, FKBP51 and FKBP52 are not related to the immunosuppression process but are rather better known for their role as an Hsp90-associated co-chaperone, regulating steroid hormone receptor signaling (Galigniana et al., 2012;Storer et al., 2011).

FKBP51 consists of 3 important functional domains (Figure 1.8.1). First, a 34 amino acid tetratricopeptide repeat (TPR) domain, located at their C-terminus, facilitates Hsp90 binding within steroid receptor complexes (Pratt and Toft, 1997). The FK506 binding domain (FK1) with PPIase activity, located at the N-terminus, is recognized as the primary regulatory domain for steroid receptor signaling and furthermore as the binding domain of FK506 and rapamycin immunosuppressants (Riggs et al., 2003). The third functional domain is referred to as the FKBP-like domain (FK2), but little is known about this domain. Regardless, the multifunctional domain structure of FKBP51 facilitates multiple protein-protein interactions and underlies its regulatory function across diverse biological processes (Sinars et al., 2003).

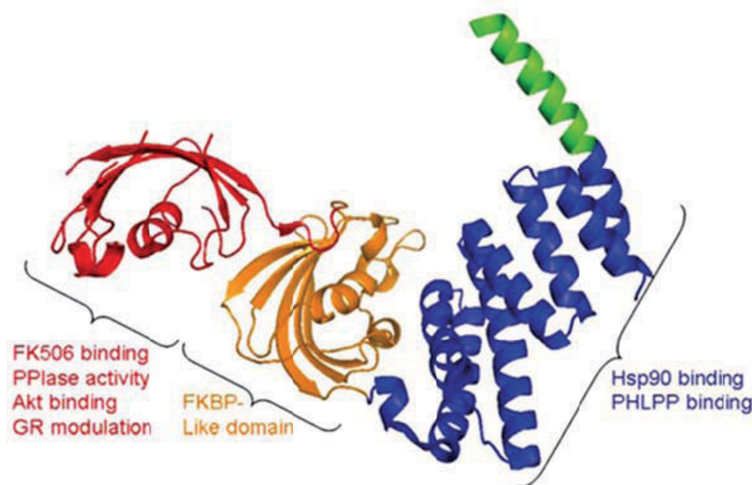

Figure 1.8.1 Structural domains of FKBP51

Reprinted with permission from (Schmidt et al., 2012).

1.8.2. Established Regulatory Functions of FKBP51

FKBP51 is best known for its role as a negative regulator of the GR (Gallo et al., 2007;Wochnik et al., 2005). In particular, FKBP51 decreases GR ligand-binding sensitivity and nuclear translocation efficiency (Binder et al., 2004;Denny et al., 2000;Scammell et al., 2001;Westberry et al., 2006). In addition, fkbp5 expression is induced by GR activation itself

and may represent a short-loop negative feedback mechanism to regulate GR sensitivity (Vermeer et al., 2003). Within the context of GR signaling, FKBP51 has been implicated in stress-related psychiatric disorders. Specifically, single nucleotide polymorphisms (SNPs) within the Fkbp5 gene have been connected to antidepressant response and the recurrence of depressive episodes (Binder et al., 2004). Most notably, Fkbp5 SNPs leading to increased FKBP5 expression following GR activation have been associated with GR resistance and confer a greater risk for the development of stress-related psychiatric disorders (Binder, 2009). It is postulated that increased *FKBP5* levels following GR activation favors a GR complex with lower binding affinity for GCs, reduced GR translocation, and thus reduced GR sensitivity.

Studies have furthermore focused on alternative FKBP51-mediated regulatory mechanisms. For example, beyond its effects on steroid receptor signaling, FKBP51 has been identified as a regulator of NF-κB signaling (Jiang et al., 2008;Komura et al., 2005), autophagy (Gassen et al., 2014), as well as AKT (PKB) signaling (Pei et al., 2009). Importantly, as a regulator of AKT, FKBP51 acts as a scaffolding protein between AKT and the negative regulator of AKT, phosphatase PHLPP, and thus ultimately inhibits AKT signaling (Pei et al., 2009). AKT promotes cell survival and cell growth and is a central node in cellular signaling pathways that lies downstream of various growth factors (Manning and Cantley, 2007). Regulation of AKT activation through protein-protein interactions is paramount, and aberrant AKT activation has been identified in various disease states. Through its scaffolding function, FKBP51 enhances the interaction between AKT and PHLPP to promote AKT dephosphorylation and subsequent inactivation. In line with its regulatory role over AKT, loss of FKBP51 expression and Akt hyperactivation are consistently reported in a number of pancreatic cancer and breast cancer cell lines (Pei et al., 2009).

More recently, preliminary findings have indicated that FKBP51 may additionally serve as a regulator of whole body energy metabolism. In this context, FKBP51 is highly expressed in metabolically active tissues (Baughman et al., 1997). Notably, according to the human data set 'GeneAtlas U133A, gcrma', human adipocytes, skeletal muscle, and lymphocytes show the strongest expression of FKBP51 compared to all other tissue types (Su et al., 2004). Indeed FKBP51 has been implicated in fine-tuning the expression of GR-regulated genes during adipocyte differentiation (Toneatto et al., 2013). Glucocorticoids are known to induce adipocyte differentiation. FKBP51 levels were shown to progressively increase and the interaction between FKBP51 and GR tighten when 3T3-L1 pre-adipocytes are induced to

differentiate. In addition, knockdown of FKBP51 in pre-adipocytes accelerated adipogenesis, suggesting that FKBP51 regulation of GR is important for the proper regulation of adipocyte differentiation. FKBP51 has likewise been implicated in insulin resistance. In particular, SNPs within the human *FKBP5* gene have been associated with type 2 diabetes and markers of insulin resistance (Pereira et al., 2014a). In support of these findings, the authors furthermore report that higher *FKBP5* gene expression in omental fat was associated with reduced insulin-mediated glucose uptake in both human subcutaneous and omental adipocytes. Despite such compelling findings involving FKBP51 in different aspects of metabolism, there has been no characterization of FKBP51 in whole body energy and/or glucose metabolism.

1.8.3. FKBP51 in Gene x Early Life Environment Interactions

FKBP5 gene expression is highly responsive to the environment. In fact, using a dexamethasone activation paradigm to mimic stress, it has been shown that *FKBP5* is one of the most stress-responsive genes in humans and rodents (Menke et al., 2012;Scharf et al., 2011), suggesting that *FKBP5* may be in a prime position to mediate gene x environment interactions. Along these lines, findings have shown that exposure to childhood trauma mediates FKBP5 allele-specific changes in DNA methylation (Klengel et al., 2013). Such allele-specific gene x environment interactions have subsequently been described to predict the adult risk of developing psychiatric disorders (reviewed by (Zannas and Binder, 2014)). Interestingly, this interaction is limited to early life trauma exposure given that there was no interaction between *FKBP5* haplotype and adult trauma exposure (Binder et al., 2008;Ising et al., 2008). Such findings are based on the notion that environmental-mediated epigenetic modifications must occur during specific critical developmental time windows in order to become a stable epigenetic modification able to impact adult phenotypes. Taken together, there is clear evidence suggesting that FKBP5 is able to respond to early life environmental conditions.

1.8.4. FKBP51 Knockout Studies

A conventional FKBP51 knockout (51KO) mouse line has been previously generated (Tranguch et al., 2005) and has greatly advanced our understanding of the diverse biological roles of FKBP51. Importantly, the phenotypes described in 51KO mice support human *FKBP5* studies. For example, human SNPs within the *FKBP5*, which are associated with increased induction of *FKBP5* mRNA and protein expression following GR activation, are

associated with GR resistance, a slower recovery of stress-induced cortisol levels, and are furthermore disproportionately over-represented in patients suffering from mood disorders (Binder et al., 2008;Binder, 2009;Ising et al., 2008). Along these lines, 51KO mice exhibit resilience to chronic social stress exposure (Hartmann et al., 2012) as well as improved stress-coping behavior (Touma et al., 2011). The 51KO mice have not yet been characterized for a metabolic phenotype, but Schmidt and colleagues have reported that 51KO mice have a significantly lower body weight compared to wild-type littermates (Hartmann et al., 2012). Taken together, the parallelisms between human and 51KO mouse phenotypes support its validity as a model system to study the molecular mechanisms by which FKBP51 regulates various biological processes and/or responds to various environmental cues.

1.8.5. FKBP51 as a Potential Therapeutic Target in the Treatment of Metabolic-Related Disorders

Animal and human data suggest that strategies aimed at attenuating FKBP51 may represent a possible basis for the development of novel therapeutics (Schmidt et al., 2012). The favorable stress resilient phenotype arising from loss of FKBP51 expression in mice along with the association between psychiatric disorders and functional *FKBP5* SNPs, justifies FKBP51 as a possible therapeutic target in stress-related mood disorders. Importantly, highly selective antagonists for FKBP51, referred to as SAFit and SAFit2, have recently been described to enhance neurite elongation in neuronal cultures and improve stress-coping behavior in mice (Gaali et al., 2014). The availability of such highly selective FKBP51 antagonists provides the opportunity to investigate additional therapeutic prospects for FKBP51 antagonism, beyond the realm of psychiatric disorders. The recent preliminary evidence implicating FKBP51 in energy metabolism (Section 1.8.2) and its ability to regulate AKT signaling provide compelling indication that FKBP51 antagonism may be a novel therapeutic strategy in the treatment of obesity and/or T2D.

1.9. Rationale and Thesis Objectives

Human and animal studies have shown that FKBP51 is able to modulate stress responsiveness through its ability to regulate various biological processes either as a co-chaperone to hsp90 or independently as a scaffolding protein. Recently preliminary studies have furthermore emerged suggesting a role of FKBP51 in metabolic regulation. Still, there has been no systematic characterization of FKBP51 in the context of whole body energy homeostasis. The main objective of the current thesis was to establish the role of FKBP51 in metabolic regulation. To this end, we formulated explicit research questions to address whether, and to what extent, FKBP51 is involved in energy balance regulation using different model systems.

Research Questions

1. Does FKBP51 mediate the crosstalk between stress regulation and energy balance? (Chapter 2.1)
2. Is the prenatal environmental dietary condition sufficient to modulate FKBP51 gene expression? (Chapter 2.2)
3. Does loss of FKBP51 impact whole body energy metabolism, and if so how? (Chapter 2.3 – 2.4)
4. Is FKBP51 a prospective drug target for the treatment of obesity and/or T2D? (Chapter 2.3)
5. Does FKBP51 act centrally to regulate energy homeostasis? (Chapter 2.5)

2. Research Articles

2.1. The Interplay between Diet-Induced Obesity and Chronic Stress in Mice: Potential Role of FKBP51

Balsevich G, Uribe A, Wagner KV, Hartmann J, Santarelli S, Labermaier C, Schmidt MV

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Research | G BALSEVICH and others | FKBP51 on stress and metabolic regulation | 222:1 | 15–26

Interplay between diet-induced obesity and chronic stress in mice: potential role of FKBP51

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Abstract

While it is known that stress promotes obesity, the effects of stress within an obesogenic context are not so clear and molecular targets at the interface remain elusive. The FK506-binding protein 51 (FKBP51, gene: *Fkbp5*) has been identified as a target gene implicated in the development of stress-related psychiatric disorders and is a possible candidate for involvement in stress and metabolic regulation. The aims of the current study are to investigate the interaction between chronic stress and an obesogenic context and to additionally examine whether FKBP51 is involved in this interaction. For this purpose, male C57BL/6 mice were exposed to a high-fat diet for 8 weeks before being challenged with chronic social defeat stress. Herein, we demonstrate that chronic stress induces hypophagia and weight loss, ultimately improving features arising from an obesogenic context, including glucose tolerance and levels of insulin and leptin. We show that *Fkbp5* expression is responsive to diet and stress in the hypothalamus and hippocampus respectively. Furthermore, under basal conditions, higher levels of hypothalamic *Fkbp5* expression were related to increased body weight gain. Our data indicate that *Fkbp5* may represent a novel target in metabolic regulation.

Key Words

- glucocorticoid receptor
- obesity
- HPA axis
- gene expression

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Introduction

The global prevalence of obesity is rising (Yach *et al.* 2006), emphasizing the need to decipher the complex regulatory mechanisms underlying energy balance. Evidence indicates that chronic stress is a risk factor for obesity development (Tamashiro 2011). Nevertheless, the relationship between stress and metabolic regulation is very complex and not easily interpreted. For example, in response to stress, some individuals lose weight, whereas others gain weight (Kivimaki *et al.* 2006, Dallman 2010). The seemingly opposite stress-induced metabolic outcomes in humans are also observed in animal models (Weninger *et al.* 1999, Kim *et al.* 2003, Michel *et al.* 2005, Moles *et al.* 2006, Kuo *et al.* 2008, Bartolomucci *et al.* 2009) and highlight the intricate relationship between stress and metabolic regulation. Stress and metabolic regulation share common regulatory pathways orchestrated by neural networks centered in the hypothalamus. The hypothalamic–pituitary–adrenal (HPA) axis is a central component of the stress response (de Kloet *et al.* 2005) and is also involved in energy balance (Akana *et al.* 1994).

In response to an acute stressor, changes in HPA axis activity are generally regarded as adaptive and aim to re-establish homeostasis. However, such changes may become maladaptive when the stressor persists

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(McEwen 2007). For example, alterations in HPA axis function on account of chronic stress are considered a major risk factor for both psychiatric and metabolic disorders (Holsboer 2000, Tamashiro 2011). Similarly, perturbations in glucocorticoid receptor (GR) signaling, which plays an important role in the glucocorticoid-mediated negative feedback loop for the termination of the stress response (Ulrich-Lai & Herman 2009), have been implicated in the development of obesity (Bjorntorp & Rosmond 2000, Pasquali *et al.* 2006, Grun & Blumberg 2007). Furthermore, it has been shown that the energy status of an individual itself, reflected in levels of circulating metabolic hormones, is able to directly affect HPA axis activation (Bagdade *et al.* 1967, Akana *et al.* 1994, Considine *et al.* 1996, Hallschmid & Schultes 2009, Roubos *et al.* 2012). Indeed, it is evident that both chronic stress and diet have the potential to interact in order to modulate both metabolic and neuroendocrine phenotypes.

Research on the interaction between stress and obesity has focused primarily on the role of chronic stress in mediating obesity. Although these studies are important for understanding the role of chronic stress in development of obesity, research is also required to identify mechanisms responsible for the emergence of various pathophysiologies once obesity has already been established. Despite the rising rates of both obesity and chronic social stress, relatively little research has addressed the question as to how an obese individual responds to chronic stress. One study investigated the behavioral outcomes of chronic stress exposure in a mouse model of diet-induced obesity (DIO) (Finger *et al.* 2011). The authors found that diet-induced obese mice were resistant to selective stress-induced anxiety- and depressive-like symptoms. However, there is a paucity of data identifying molecular targets involved in the interplay between DIO and chronic stress.

Emerging literature on possible mechanisms underlying stress-induced psychiatric disorders have identified the *Fkbp5* gene, encoding the FK506-binding protein 51 (FKBP51), as a novel candidate gene (Binder *et al.* 2004, 2008, Ising *et al.* 2008, Binder 2009, Zimmermann *et al.* 2011). Interestingly, mice deficient in FKBP51 display a moderately lean phenotype under basal conditions (Hartmann *et al.* 2012, Sanchez 2012). In this regard, *Fkbp5* is an interesting candidate gene to study in the context of stress and metabolic regulation.

In the brain, *Fkpb5* is expressed ubiquitously, with high expression levels in the hippocampus and the hypothalamus (Scharf *et al.* 2011). FKBP51 is well recognized for its ability to regulate GR sensitivity and HPA axis functioning (Touma *et al.* 2011, Hartmann *et al.* 2012). Most notably, FKBP51 acts as a negative regulator of GR by reducing nuclear translocation of the GR complex and ligand-binding sensitivity (Davies *et al.* 2002, Wochnik *et al.* 2005, Binder 2009). Interestingly, functional polymorphisms within *Fkbp5* interact with environmental cues, namely early life trauma, to predict the risk of developing various psychiatric disorders (Binder *et al.* 2008, Roy *et al.* 2010, Xie *et al.* 2010, Collip *et al.* 2013), indicating that *Fkbp5* is sensitive to the environment and may be an important mediator of other gene× environment interactions. In this context, studies demonstrated that food deprivation (FD) induces *Fkbp5* expression in the brain (Scharf *et al.* 2011, Yang *et al.* 2012). In the study by Scharf and colleagues, FD was used as a stressor, but intuitively, changes resulting from FD may also represent a response to the metabolic challenge.

Taken together, there is evidence to suggest that FKBP51 may be involved in the interplay between stress and metabolic regulation. Thus, in this study, we examined the interaction between chronic stress and an obesogenic context. Furthermore, we examined whether a possible interaction exists between FKBP51 and chronic stress in order to modulate metabolic-related readouts.

Materials and methods

Animals and animal housing

Initially, 5-week-old male C57BL/6 mice (Charles River Laboratories, Maastricht, The Netherlands) were housed in groups for 6 weeks. Mice were maintained on a 12 h light:12 h darkness cycle, with controlled temperature (22±2 °C) and humidity (55±5%). Mice were allowed to access high-fat (58% kcal from fat, D12331) or control chow diet *ad libitum* (10.5% kcal from fat, D12329, Research Diets, Inc., New Brunswick, NJ, USA), which, except for the fat content, were identical. After 6 weeks, mice were housed individually for an additional 2 weeks, maintained on their respective diets, before the onset of testing. Body weight and food intake were measured weekly. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental design

After 8 weeks on the dietary regimen, each diet group was randomly divided into stressed and control groups,

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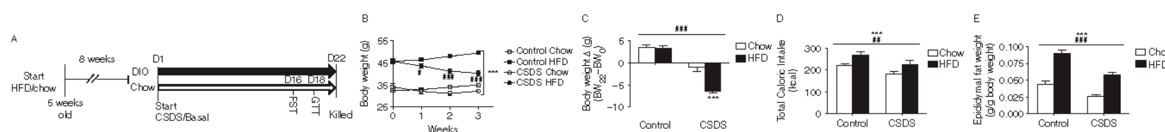

Figure 1
Experimental design and phenotypic features during CSDS. (A) Experimental time course. Mice were exposed to 21 days of CSDS or left under basal conditions following 8 weeks of exposure to a HFD or chow dietary regimen. Behavioral and metabolic readouts were carried out in the final week of the CSDS procedure. (B) Body weights of chow-fed and HFD-fed mice during the CSDS procedure. (C) Stress-induced changes in body weight and (D) total caloric intake in chow-fed and HFD-fed mice calculated as the average total caloric intake throughout the entire CSDS procedure. (E) Effect of CSDS on relative epididymal fat pad weight in mice fed on chow or HFD. Body weight during CSDS was analyzed by repeated measures two-way ANOVA. All other data were analyzed by two-way ANOVA followed by Student's *t*-test. $***P<0.001$, ## $P<0.01$, ### $P<0.001$; *significant diet effect and #significant stress effect.

balanced for body weight (chronic stress: high-fat diet (HFD) ($n=10$), chow ($n=10$) and control: HFD ($n=11$), chow ($n=10$)) (Fig. 1A). Mice were challenged with 3 weeks of chronic social defeat stress (CSDS) or left under control (basal) conditions. Body weight and food intake were recorded daily. The forced swim test (FST) was performed on day 16 of the 21-day stress procedure, and on day 18, the glucose tolerance test (GTT) was performed.

CSDS procedure

The CSDS paradigm lasted for 21 days and was conducted as described previously (Wagner *et al.* 2011, 2012). Briefly, experimental mice were placed in the home cage of a dominant CD1 resident mouse. Interaction between the mice was permitted until the experimental mouse was attacked and defeated by the CD1 aggressor. Mice were subsequently separated by a wire mesh divider that prevented physical contact but maintained sensory contact for 24 h. Each day, for 21 days, the procedure was repeated with a different unfamiliar CD1 aggressor mouse. The control mice were housed in their home cages throughout the CSDS procedure. Both control and stressed mice were handled daily during the course of the stress procedure.

Forced swim test

Mice were placed in a 2-l glass beaker filled with water (22±1 °C) to a height of 15 cm, so that the mouse could neither touch the bottom nor escape. The test lasted 6 min. Time immobile, time struggling, and latency to immobility were scored (Porsolt *et al.* 1977).

Acute stress response

The FST served as an acute stressor. At the conclusion of the FST, animals were towel dried and returned to their home cages to recover. At 30-min (stress response) and 90-min (stress recovery) after the onset of the FST, blood samples were collected by tail cut (Fluttert *et al.* 2000). Samples were collected in EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Nümbrecht-Elsenroth, Germany) and later centrifuged at 6000 ***g*** at 4 °C for 15 min. Plasma was collected and stored at −20 °C.

Intraperitoneal glucose tolerance test

Mice were fasted for 14 h and subsequently received intraperitoneal injections of 2 g/kg body weight of D-glucose. Blood glucose levels were assessed from blood collected by tail cut at 0, 30, 60, and 120 min intervals following the glucose load. Glucose levels were measured using a handheld Contour XT glucometer (Bayer Health Care, Basel, Switzerland).

Tissue collection and processing

Mice were anesthetized with isoflurane and then killed by decapitation. Basal trunk blood was collected and processed (as described above). Brains were removed, snap frozen, and stored at −80 °C until use. Thymus and adrenal glands were removed, pruned from fat, and weighed. Epididymal fat was collected and weighed. All tissue weights are expressed as relative weights (weight (mg)/body weight (g)).

In situ hybridization

Coronal whole-brain sections were cryosectioned at 18 μm thickness and directly thaw mounted onto Super Frost Plus Slides as eight sequential series. A single series of sections was selected for each ^{35}S UTP-labeled ribonucleotide probe (*Fkbp5*, *Gr* (*Nr3c1*), and *Mc4r*) or ^{35}S ATP-labeled oligonucleotide probe (*Pomc*). The antisense riboprobe

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was transcribed from a linear plasmid for *Fkbp5* (forward primer: 5′-CTTGGACCACGCTATGGTTT-3′; reverse primer: 5′-GGATTGACTGCCAACACCTT-3′), *Gr* (forward primer: 5′-AGGTCGACCAGCCGTCCAGA-3′; reverse primer: 5′-AAGCTTGCCTGGCAATAAAC-3′), *Mc4r* (forward primer: 5′-GCAAACAGCAGACTGGTCAA-3′; reverse primer: 5′-CACAGCCAGGCTACAGATGA-3′), and *Pomc* (forward primer: 5′-GGGTCCCTCCAATCTTGT-TT-3′; reverse primer: 5′-ACGTTGGGGTACACCTTCAC-3′). *In situ* hybridization was performed as described previously (Schmidt *et al*. 2007). For signal detection, the slides were exposed to a Kodak Biomax MR film (Eastman Kodak Co.) and developed. Autoradiographic densities were quantified using the NIH ImageJ Software (NIH, Bethesda, MD, USA). Regions of interest were traced from digitized autoradiograms and the mean optical density from two sections was calculated for each animal. The data were analyzed blindly, subtracting the background signal from the measurements.

Hormone quantification

Plasma corticosterone levels were determined by RIA using a commercially available kit (MP Biomedicals, Inc., Solon, OH, USA; sensitivity 12.5 ng/ml). Plasma insulin and leptin levels were determined using a mouse metabolic magnetic bead panel (Millipore Corp., Billerica, MA, USA; sensitivity: insulin 14 pg/ml and leptin 19 pg/ml).

Statistical analysis

All variables were evaluated using the IBM SPSS Statistics 18 Software (IBM SPSS Statistics). Body weight and glucose tolerance were analyzed by repeated measures two-way ANOVA with stress and diet conditions as the between-subject factors. Student's *t*-test was employed for comparison of two independent groups. All other data were analyzed by two-way ANOVA for significant overall effects. For instances where the initial test yielded a significant interaction, Student's *t*-tests (two-tailed) were conducted to locate the interaction effect using simple comparisons. Effect size was calculated for each significant effect. Cohen's *f*-statistic was used to estimate effect sizes for ANOVAs, in which an *f*-value of 0.10, 0.25, and 0.40 reflects a small, medium, and large effect size respectively (Cohen 1992). Cohen's *d*-statistic was similarly used to measure the effect size for Student's *t*-tests, using 0.20, 0.40, and 0.80 as a small, medium, and large effect size respectively. Finally, correlations between metabolic- and neuroendocrine parameters and mRNA expression were analyzed with the Pearson's product-moment test under basal and CSDS conditions. Fisher's z-transformation was then used to compare correlation coefficients between basal and CSDS conditions. Statistical significance was set at $P<0.05$. Data are expressed as mean±s.e.m.

Results

Effects of DIO and stress on body weight parameters

DIO had already been established in mice at the onset of the stress procedure ($T_{30.3}=-9.645$, $d=3.05$, $P<0.001$) on account of the preceding 8 weeks of HFD exposure. Over the course of the stress procedure, mice fed on a HFD were heavier than mice fed on chow (time×diet×stress: $F(1.3,49.1)=27.587$, $f=0.86$, $P<0.001$ and diet: $F(1,37)=124.982$, $f=1.84$, $P<0.001$) (Fig. 1B). At the end of the CSDS, all defeated animals exhibited a reduction in body weight gain (stress: $F(1,36)=127.7$, $f=1.88$, $P<0.001$), but the stress-induced weight loss was significantly greater among HFD-fed mice compared with chow-fed mice (stress×diet: $F(1,36)=16.296$, $f=0.67$, $P<0.001$) (Fig. 1C). Energy intake was also lowered on account of CSDS (stress: $F(1,37)=10.177$, $f=0.52$, $P=0.003$) and increased on account of the HFD (diet: $F(1,37)=12.335$, $f=0.58$, $P=0.001$) (Fig. 1D).

The stress-induced body weight loss was recapitulated in the quantification of epididymal fat, which reflected a reduction in relative epididymal fat weight in all defeated animals (stress: $F(1,37)=40.988$, $f=1.05$, $P<0.001$) as well as a relative increase from a HFD, independent of stress condition (diet: $F(1,37)=97.818$, $f=1.63$, $P<0.001$) (Fig. 1E).

Effects of DIO and stress on organ weights and corticosterone levels

Increased adrenal gland weight (stress: $F(1,37)=94.535$, $f=1.60$, $P<0.001$) and reduced thymus weight (stress: $F(1,33)=5.240$, $f=0.40$, $P=0.029$) resulted from CSDS independent of dietary condition (Fig. 2A and B). Furthermore, mice fed on a HFD tended to present reduced thymus size compared with chow-fed mice (diet: $F(1,33)=3.453$, $f=0.32$, $P=0.072$).

CSDS elevated basal corticosterone levels only in mice fed on a HFD (stress×diet: $F(1,35)=5.625$, $f=0.40$, $P=0.023$) (Fig. 2C). Furthermore, the response to an acute stressor was significantly elevated in mice exposed to chronic stress, regardless of the diet (stress: $F(1,36)=8{,}745$, $f=0.49$, $P=0.005$) (Fig. 2D). Corticosterone tended to remain elevated during stress recovery on account of

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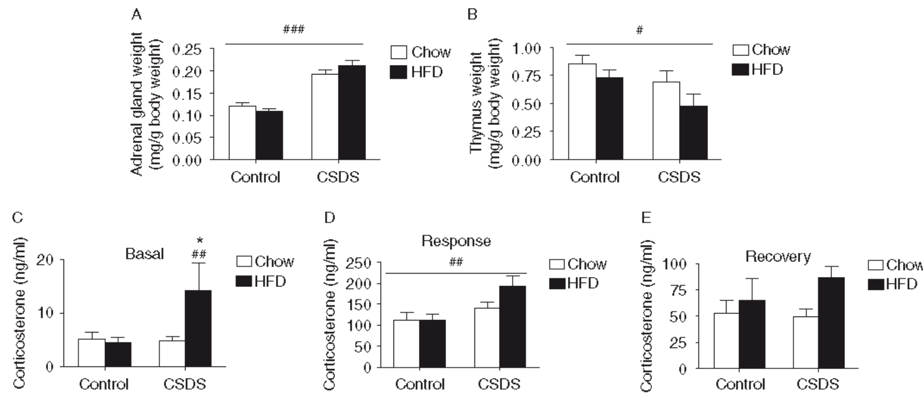

Figure 2
Physiological and neuroendocrine phenotypes in response to CSDS. (A) Relative adrenal gland weight and (B) thymus weight in chow-fed and HFD-fed mice after CSDS. (C) Basal morning corticosterone levels at the end of the CSDS procedure. (D) Corticosterone response and (E) recovery measured 30 and 90 min respectively, after the onset of the FST. All data were analyzed by two-way ANOVA followed by Student's *t*-test. $^{*}P<0.05$, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$; *significant diet effect and #significant stress effect.

the HFD regimen (diet: (1,36)=2.847, $f=0.28$, $P=0.100$) (Fig. 2E).

Effects of DIO and stress on depressive-like behavior

There was no effect of stress on any of the behavioral parameters analyzed in the FST (time spent struggling, floating, and latency to float) (Fig. 3). There was a significant effect of diet on the time spent struggling ($F(1,33)=5.066$, $f=0.39$, $P=0.031$) and the latency to float ($F(1,33)=5.069$, $f=0.39$, $P=0.031$), whereby mice fed on a HFD struggled less and began floating sooner compared with chow-fed mice.

Effects of DIO and stress on glucose tolerance and metabolic hormones

Under control conditions, the HFD significantly impaired glucose tolerance (time×stress: $F(1.7,64.4)=8.648$, $f=0.48$, $P=0.001$); diet×stress: $F(1,37)=6.637$, $f=0.42$, $P=0.014$); however, chronic stress exposure counteracted these DIO-induced effects on glucose tolerance (Fig. 4A). Furthermore, CSDS resulted in decreased insulin (stress: $F(1,35)=7.313$, $f=0.46$, $P=0.010$) and leptin ($F(1,36)=15.758$, $f=0.66$, $P<0.001$), whereas exposure to a HFD increased both circulating insulin (diet: $F(1,35)=6.250$, $f=0.42$, $P=0.017$) and leptin levels (diet: $F(1,36)=13.926$, $f=0.62$, $P=0.001$) (Fig. 4B and C).

Effects of DIO and stress on hypothalamic gene expression

A HFD (obesogenic context) significantly increased *Fkbp5* mRNA expression in the ventromedial hypothalamic nuclei (VMH) (diet: $F(1,34)=5.685$, $f=0.41$, $P=0.023$), an effect that was most robust among mice exposed to CSDS (Fig. 5A and B). There was no effect of chronic stress exposure on hypothalamic *Fkbp5* expression.

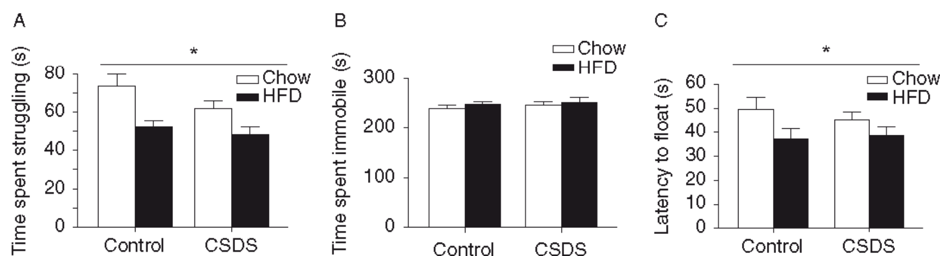

Figure 3
Behavioral response in the forced swim test. (A) Time spent struggling in the FST. (B) Time spent immobile in the FST. (C) Latency to first float in the FST. Data were analyzed by two-way ANOVA. *Significant diet effect, $P<0.05$.

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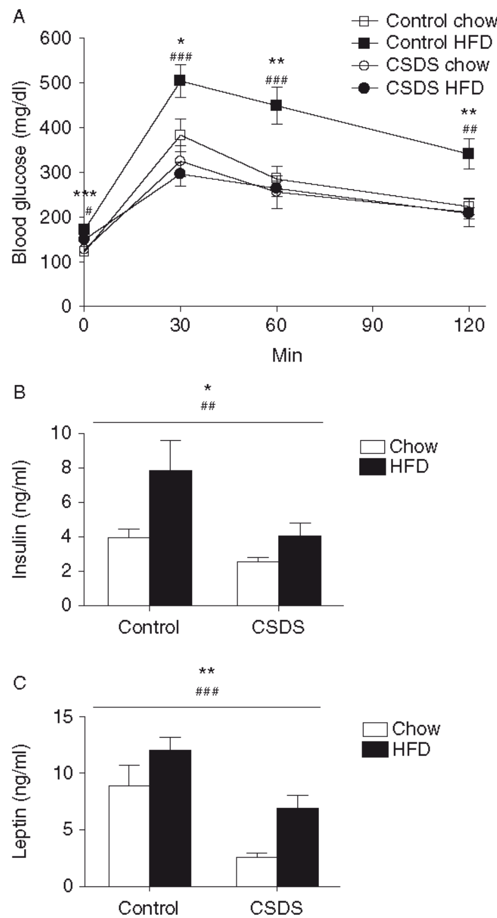

Figure 4
Metabolic profile in response to CSDS. (A) Blood glucose levels during an intraperitoneal glucose tolerance test performed after a 14 h fast. (B) Circulating levels of plasma insulin and (C) leptin collected after 3 weeks of CSDS. The glucose tolerance test was analyzed by repeated-measures two-way ANOVA. All other data were analyzed by two-way ANOVA followed by Student's *t*-test. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ##$P<0.01$, ###$P<0.001$; *significant diet effect and #significant stress effect.

As shown in Fig. 5C and D, VMH *Fkbp5* mRNA levels significantly correlated with total change in body weight ($r=0.481$, $P=0.032$) and total caloric intake ($r=0.551$, $P=0.001$) under basal conditions. In contrast, this association was lost or tended to reverse (body weight change: $r=-0.481$, $P=0.070$) under conditions of CSDS (Fig. 5E and F) A Fisher's z-transformation revealed that the correlations between basal and CSDS condition differed significantly for total body weight change ($z=2.51$, $P=0012$) but not for total caloric intake ($z=0.723$, $P=0.469$).

Gr expression was significantly decreased by an obesogenic environment in both the VMH (diet: $F(1,30)=9.323$, $f=0.56$, $P=0.005$) and arcuate nucleus (diet: $F(1,29)=4.346$, $f=0.39$, $P=0.046$) of the hypothalamus independent of CSDS exposure (Fig. 5G, H and I).

In the PVN, diet significantly elevated MC4R regardless of stress condition (diet: $F(1,36)=4.518$, $f=0.36$, $P=0.040$), but there was no effect of either stress or diet on POMC expression (data not shown).

Effects of DIO and stress on hippocampal gene expression

CSDS significantly increased *Fkbp5* mRNA expression in the CA3 region of the hippocampus (stress: $F(1,35)=5.186$, $f=0.38$, $P=0.029$) (Fig. 6A and E). There was no effect of feeding regimen on hippocampal *Fkbp5* expression.

CA3 *Fkbp5* mRNA levels negatively correlated with total caloric intake ($r=-0.494$, $P=0.037$) and mean caloric efficiency ($r=-0.472$, $P=0.048$) under conditions of chronic stress (Fig. 6C and D). In contrast, this association does not exist under basal conditions (data not shown). Fisher's z-transformation was used to compare the correlation coefficients between basal and CSDS conditions. The analyses revealed that the correlations were not significantly different between conditions (total caloric intake: $z=1.25$, $P=0.210$ and mean caloric intake: $z=0.787$, $P=0.431$).

Finally, under stressful conditions, higher *Fkbp5* mRNA expression was associated with an improved corticosterone response reflected in the negative correlation between CA3 *Fkbp5* expression and the level of corticosterone measured 30 min after an acute stressor (response corticosterone level) ($r=-0.489$, $P=0.046$) in the defeated mice (Fig. 6B). The Fisher's z-transformation revealed that the correlation between *Fkbp5* expression and corticosterone response differed significantly between basal and CSDS conditions ($z=2.52$, $P=0.011$).

Discussion

Little research has been carried out to improve understanding of the dynamic relationship between chronic stress and an obesogenic environment. This study aimed to investigate the direct effects of CSDS in an established diet-induced obese mouse model, and further to investigate FKBP51 regulation relative to metabolic regulation. We clearly demonstrated a body weight reduction on account of chronic stress exposure, which further led to improved glucose tolerance and lower levels of leptin and insulin. Additionally, *Fkbp5* expression was induced in

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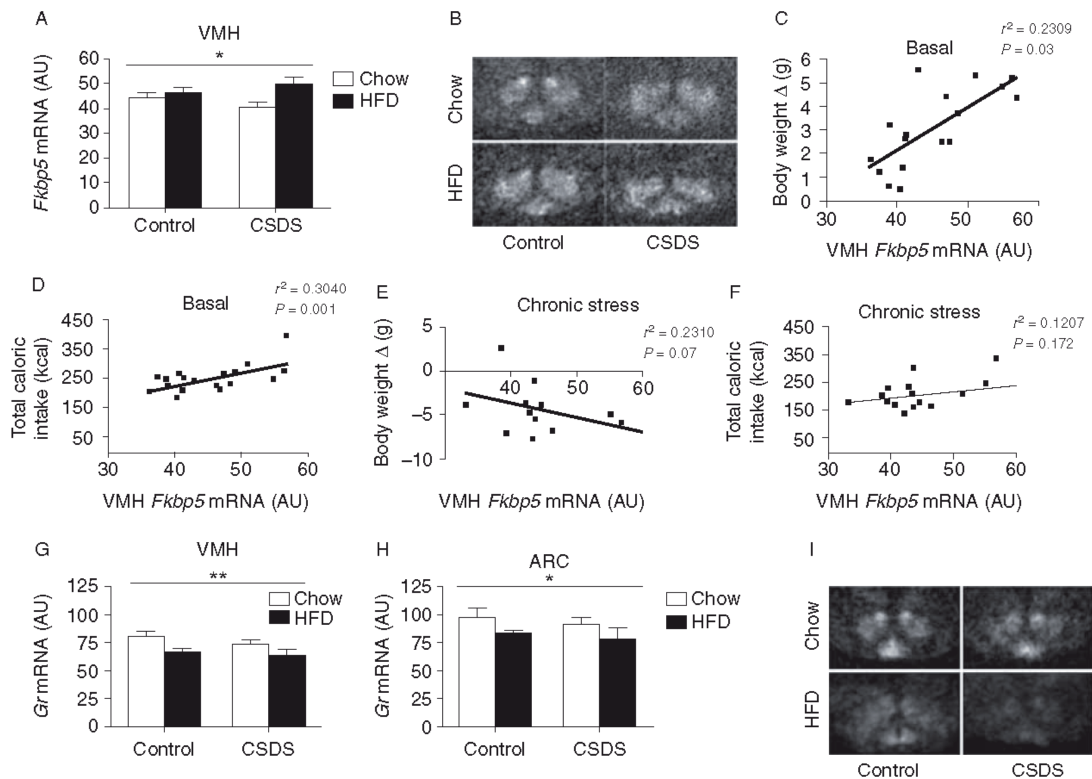

Figure 5
Hypothalamic gene expression and correlational data. Quantitative (A) and qualitative (B) *Fkbp5* mRNA expression in the VMH. (C) Correlational analysis under basal conditions between VMH *Fkbp5* mRNA expression and total body weight change as well as (D) total caloric intake. (E) Correlational analysis under chronic stress conditions between VMH *Fkbp5* mRNA expression and total body weight change as well as (F) total caloric intake. (G) Quantitative *Gr* mRNA expression in the VMH and (H) ARC of the hypothalamus. (I) Qualitative *Gr* mRNA expression in the VMH and ARC. Expression data were analyzed by two-way ANOVA; correlations were analyzed with the Pearson's product moment. **P*<0.05, ***P*<0.01; *significant diet effect.

response to diet and stress in the hypothalamus and hippocampus respectively. Correlational analyses revealed that under non-stressed conditions, higher hypothalamic expression of *Fkbp5* is associated with increased body weight gain. Collectively, our data indicate that *Fkbp5* may represent a novel target in metabolic regulation.

We investigated the interaction between chronic stress exposure and obesity by subjecting DIO mice to CSDS. Mice challenged to CSDS presented adrenal hypertrophy and thymus involution, which supports the validity of our stress paradigm (Schmidt *et al.* 2007, Wang *et al.* 2011). Furthermore, chronic stress exposure led to hypophagia. The stress-induced hypophagia probably contributed to the stress-induced body weight loss. In parallel, chronic stress exposure reduced the relative epididymal fat pad mass regardless of the dietary condition.

Glucose intolerance is a clinical feature of the metabolic syndrome and is a feature present in mouse models of DIO (Surwit *et al.* 1988, Ahren & Pacini 2002). As expected, we found impaired glucose tolerance in DIO mice under basal conditions. Interestingly, CSDS was able to offset the effects of the HFD on glucose tolerance. In addition, CSDS lowered circulating levels of leptin and insulin, whereas a HFD led to increased levels of both. Previous studies have also demonstrated that leptin and insulin levels decrease in response to stress (Chuang *et al.* 2010, Finger *et al.* 2011, Solomon *et al.* 2011) and increase following a HFD (Scarpace & Zhang 2007, Chuang *et al.* 2010, Finger *et al.* 2011, Solomon *et al.* 2011). Although our data clearly indicate that CSDS offsets the DIO-induced effects on glucose tolerance and levels of leptin and insulin, it is practically impossible to decipher whether such downstream metabolic phenotypes are a

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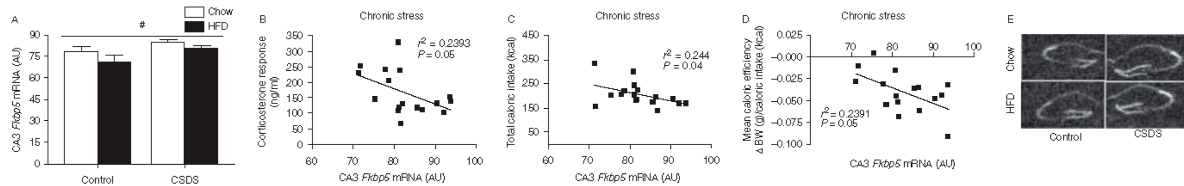

Figure 6

Hippocampal CA3 region gene expression and correlational data. (A) Quantitative *Fkbp5* mRNA expression in the hippocampal CA3 region. (B) Correlational analysis under chronic stress conditions between CA3 *Fkbp5* mRNA expression and corticosterone response. (C) Correlational analysis under chronic stress conditions between CA3 *Fkbp5* mRNA expression and total caloric intake (throughout the stress procedure) as well as (D) mean caloric efficiency (calculated as the average daily caloric efficiency throughout the entire stress procedure). (E) Qualitative *Fkbp5* mRNA expression in the hippocampus. Expression data were analyzed by two-way ANOVA; correlations were analyzed with the Pearson's product moment. #Significant stress effect, $P<0.05$.

direct outcome of stress or rather an indirect outcome of differences in body weight. Despite its limitations, this collection of phenotypes confirms stress-induced alterations in body weight regulation and related metabolic phenotypes.

In addition to examining metabolic phenotypes resulting from CSDS exposure in DIO mice, we assessed stress responsiveness and depressive-like behavior. In line with previous findings (Bartolomucci *et al.* 2005, Wagner *et al.* 2012), CSDS caused disturbances in HPA axis function, as reflected in the heightened corticosterone release in response to an acute stressor in mice exposed to CSDS. Although CSDS had no effect on basal corticosterone levels in our chow-fed mice, there was a significant increase in basal corticosterone levels from CSDS in the HFD-fed mice. It is difficult to determine whether the HFD aggravated disturbances in HPA axis function or whether stress-induced body weight loss accounts for the aggravated phenotype. A prominent theory of emotional behavior explains that consumption of calorically dense food actually offsets the negative emotional effects of chronic stress exposure and HPA axis activation (Pasquali *et al.* 2006, Dallman 2010). In this context, our data showing significant weight reduction on account of chronic stress exposure may be analogous to a state of starvation (Leibel *et al.* 1991), which is accompanied by neuroendocrine counter-regulatory adjustments to reestablish the non-reduced state (Bjorntorp & Rosmond 2000). Therefore, the aggravated HPA axis dysfunction in the stress-exposed HFD-fed mice may be driven by a state of weight loss rather than a direct effect of the HFD.

In the FST, a HFD decreased time spent struggling and the latency to float, which is interpreted as increased depressive-like behavior (Porsolt *et al.* 1977). Again this would oppose the protective role of calorically dense food in stress-induced emotional despair. Nevertheless, the dietary effect observed in the FST may be a confounding effect of increased body fat, whereby the mice float more readily, because we observed no effect of stress on any depressive-like phenotype. Finally, it is difficult to disentangle whether the observed dietary effects are on account of an obesogenic (HFD) context or rather a starvation-like state (body weight differences, discussed above).

In addition to the *P*-statistics, we also reported effect sizes as proposed by Cohen (1992). Such analyses are useful to determine whether the observed effects are small, medium, or large and may give a stronger indication of the relevance of the observed data. Indeed, most of our results display large effect sizes, indicating that the effects are biologically meaningful.

It is important to identify markers mediating the crosstalk between stress regulation and energy balance. *Fkbp5* is a strong candidate gene for stress-related metabolic disorders on the basis that it has already been identified as a candidate gene in depression, which shares common overlapping pathways with obesity (Bornstein *et al.* 2006). Importantly, *Fkbp5* knockout (51KO) mice are less affected by CSDS and show a reduced body weight compared with WT mice (Hartmann *et al.* 2012). Therefore, we investigated the effects of HFD and CSDS on *Fkbp5* expression in the hypothalamus and hippocampus given their respective roles in metabolic and stress regulation. In the hypothalamus, chronic stress did not have any effect on *Fkbp5* expression, but HFD was found to have an effect selectively in hypothalamic nuclei. Specifically, we assessed *Fkbp5* expression in the arcuate nucleus, paraventricular nucleus, and VMH nucleus and found that *Fkbp5* expression was significantly regulated by diet within the VMH, whereby exposure to a HFD elevated *Fkbp5* expression. Additionally, we examined *Gr* mRNA expression on the basis that FKBP51 acts as a negative regulator of GR

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(Davies *et al.* 2005, Wochnik *et al.* 2005, Binder 2009). A HFD resulted in decreased GR expression in both the VMH and arcuate nucleus (ARC). Collectively, the resulting reduced *Gr* expression and elevated *Fkbp5* expression from a HFD would indicate to reduced GR signaling.

This study also investigated mRNA expression of proopiomelanocortin (*Pomc*) and melanocortin 4 receptor (*Mc4r*), which are the components of the central melanocortin system. The melanocortin signaling system is well known for its role in food intake, body weight regulation, and regulation of the stress response (Seeley *et al.* 2004, Liu *et al.* 2007, 2013). We wanted to determine whether the stress-induced hypophagia and weight loss were reflected at the level of gene expression. We report that neither *Pomc* nor *Mc4r* mRNA expression were regulated by stress. However, mice fed on a HFD showed increased *Mc4r* expression in the PVN, which is well recognized for its anorexigenic effects (Hinney *et al.* 2013).

Although *Fkbp5* expression increased in response to a HFD in the VMH, there was no effect of dietary condition on hippocampal *Fkbp5* expression. In contrast, in the hippocampal CA3 region *Fkbp5* expression increased on account of CSDS. This corroborates results from an earlier study that demonstrated an increase in *Fkbp5* expression in the hippocampus in response to CSDS (Wagner *et al.* 2012). Moreover, the results of this study also indicated an association between higher hippocampal *Fkbp5* mRNA expression and an improved corticosterone response following chronic stress exposure. Interestingly, our data also reveal an association between higher CA3 hippocampal *Fkbp5* expression and lower response corticosterone levels following CSDS.

The specific spatial pattern of *Fkbp5* induction by either diet or stress conditions is in accordance with each region's respective function. The VMH is integrally involved in energy balance and is known to respond to metabolic signaling hormones (King 2006). The hippocampus on the other hand is well recognized for its role in the stress response, most notably in its termination (Ulrich-Lai & Herman 2009). Although we proposed the hypothesis that FKBP51 may be involved in the interplay between stress and metabolic regulation, the lack of interaction between diet and stress on *Fkbp5* expression and the spatially distinct regulation of *Fkbp5* expression by diet and stress does not support this. Nevertheless, the present data provide support for results from previous studies defining a role of FKBP51 in stress regulation and indicate a possible role in energy homeostasis. Further investigation involving genetic manipulation of *Fkbp5* in the brain is required to establish causality.

Finally, we used correlational analyses to assess the association between VMH and hippocampal *Fkbp5* expression and metabolic readouts. In the basal state, the expression level of VMH *Fkbp5* mRNA was correlated with both total body weight change and total caloric intake. Our data indicate that under basal conditions, increased expression of *Fkbp5* mRNA is associated with increased weight gain and increased total caloric intake. This agrees with the 51KO mouse model, which is leaner compared with WT littermates (Hartmann *et al.* 2012, Sanchez 2012). When we compared the correlation coefficients between basal and stressed conditions, there was in fact no difference for *Fkbp5* and caloric intake between conditions. However, the correlation coefficients for *Fkbp5* and body weight change were significantly different between stress conditions. Taken together, it appears that stress-induced changes interfere with the normal association between energy balance and FKBP51 function.

Strikingly, in the hippocampal CA3 region, there were no associations between *Fkbp5* mRNA levels and metabolic readouts under basal conditions. However, when challenged with CSDS, low *Fkbp5* expression in the hippocampus is associated with increased total caloric intake and mean caloric efficiency. Nevertheless, the correlation coefficients did not significantly differ between basal and stressed conditions, which may be due to the fact that the study was underpowered to address this question adequately. In contrast, the correlation coefficients for *Fkbp5* and corticosterone response were significantly different, revealing that higher corticosterone response correlates with lower *Fkbp5* expression exclusively under CSDS conditions, which is consistent with previously published data (Wagner *et al.* 2012). Our correlation analyses unveiled a highly complex association between *Fkbp5* expression and metabolic readouts, reflecting not only the aforementioned spatial regulation but also a strong dependence on the environmental conditions.

The stress-dependent relationship between *Fkbp5* mRNA levels and metabolic readouts may be observed in the regulation of FKBP51 by the stress response itself. FKBP51 modulates GR sensitivity and HPA activation, whereby lower FKBP51 expression reflects a state of higher GR sensitivity (Binder 2009). However, FKBP51 expression is also induced by GR activation through an ultrashort negative feedback loop in order to regulate GR sensitivity (Vermeer *et al.* 2003). Accordingly, higher FKBP51 induction would reflect an initial state of higher GR sensitivity corresponding to lower FKBP51 expression. In effect, it is very difficult to differentiate high initial FKBP51 expression (reflecting a lower GR sensitivity) from efficient

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GR-induced FKBP51 expression (reflecting a higher GR sensitivity) even though they reflect two opposing conditions. Therefore, the seemingly contradictory results for FKBP51 expression under basal and chronic stress conditions may both represent the same initial condition, whereby high FKBP51 expression promotes metabolic phenotypes leading to a positive energy balance. In this case, the high initial FKBP51 expression following stress exposure would result in less efficient GR-activated FKBP51 induction, which would ultimately manifest as lower overall FKBP51 expression, masking the initial situation.

In summary, we show that adult exposure to CSDS results in hypophagia and weight loss. In parallel, FKBP51 is responsive to diet and stress conditions, as reflected in diet- and stress-induced *Fkbp5* expression changes. Moreover, higher levels of FKBP51 are closely related to higher food intake and body weight gain under basal conditions, which is consistent with the lean phenotype of 51KO mice. This collection of phenotypes indicates that there may be a novel role for FKBP51 in metabolic regulation. Nevertheless, genetic manipulation of *Fkbp5* in the brain is required to establish causality. Future investigation should focus on the exact role of FKBP51 in metabolism, which may have therapeutic implications for the treatment of metabolic disorders.

Declaration of interest

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

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Reference

Ahren B & Pacini G 2002 Insufficient islet compensation to insulin resistance vs. reduced glucose effectiveness in glucose-intolerant mice. *American Journal of Physiology. Endocrinology and Metabolism* **283** E738–E744. (doi:10.1152/ajpendo.00199.2002)

Akana SF, Strack AM, Hanson ES & Dallman MF 1994 Regulation of activity in the hypothalamo-pituitary–adrenal axis is integral to a larger hypothalamic system that determines caloric flow. *Endocrinology* **135** 1125–1134. (doi:10.1210/endo.135.3.8070356)

Bagdade JD, Bierman EL & Porte D Jr 1967 The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. *Journal of Clinical Investigation* **46** 1549–1557. (doi:10.1172/JCI105646)

Bartolomucci A, Palanza P, Sacerdote P, Panerai AE, Sgoifo A, Dantzer R & Parmigiani S 2005 Social factors and individual vulnerability to chronic stress exposure. *Neuroscience and Biobehavioral Reviews* **29** 67–81. (doi:10.1016/j.neubiorev.2004.06.009)

Bartolomucci A, Cabassi A, Govoni P, Ceresini G, Cero C, Berra D, Dadomo H, Franceschini P, Dell'Omo G, Parmigiani S *et al.* 2009 Metabolic consequences and vulnerability to diet-induced obesity in male mice under chronic social stress. *PLoS ONE* **4** e4331. (doi:10.1371/journal.pone.0004331)

Binder EB 2009 The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. *Psychoneuroendocrinology* **34**(Suppl 1) S186–S195. (doi:10.1016/j.psyneuen.2009.05.021)

Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Putz B, Papiol S, Seaman S, Lucae S, Kohli MA *et al.* 2004 Polymorphisms in *FKBP5* are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. *Nature Genetics* **36** 1319–1325. (doi:10.1038/ng1479)

Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, Tang Y, Gillespie CF, Heim CM, Nemeroff CB *et al.* 2008 Association of *FKBP5* polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *Journal of the American Medical Association* **299** 1291–1305. (doi:10.1001/jama.299.11.1291)

Bjorntorp P & Rosmond R 2000 Obesity and cortisol. *Nutrition* **16** 924–936. (doi:10.1016/S0899-9007(00)00422-6)

Bornstein SR, Schuppenies A, Wong ML & Licinio J 2006 Approaching the shared biology of obesity and depression: the stress axis as the locus of gene–environment interactions. *Molecular Psychiatry* **11** 892–902. (doi:10.1038/sj.mp.4001873)

Chuang JC, Krishnan V, Yu HG, Mason B, Cui H, Wilkinson MB, Zigman JM, Elmquist JK, Nestler EJ & Lutter M 2010 A β_3-adrenergic-leptin-melanocortin circuit regulates behavioral and metabolic changes induced by chronic stress. *Biological Psychiatry* **67** 1075–1082. (doi:10.1016/j.biopsych.2009.12.003)

Cohen J 1992 A power primer. *Psychological Bulletin* **112** 155–159. (doi:10.1037/0033-2909.112.1.155)

Collip D, Myin-Germeys I, Wichers M, Jacobs N, Derom C, Thiery E, Lataster T, Simons C, Delespaul P, Marcelis M *et al.* 2013 FKBP5 as a possible moderator of the psychosis-inducing effects of childhood trauma. *British Journal of Psychiatry* **202** 261–268. (doi:10.1192/bjp.bp.112.115972)

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ & Bauer TL 1996 Serum immunoreactive–leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine* **334** 292–295. (doi:10.1056/NEJM199602013340503)

Dallman MF 2010 Stress-induced obesity and the emotional nervous system. *Trends in Endocrinology and Metabolism* **21** 159–165. (doi:10.1016/j.tem.2009.10.004)

Davies TH, Ning YM & Sanchez ER 2002 A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. *Journal of Biological Chemistry* **277** 4597–4600. (doi:10.1074/jbc.C100531200)

Davies TH, Ning YM & Sanchez ER 2005 Differential control of glucocorticoid receptor hormone-binding function by tetra-tricopeptide repeat (TPR) proteins and the immunosuppressive ligand FK506. *Biochemistry* **44** 2030–2038. (doi:10.1021/bi048503v)

Finger BC, Dinan TG & Cryan JF 2011 High-fat diet selectively protects against the effects of chronic social stress in the mouse. *Neuroscience* **192** 351–360. (doi:10.1016/j.neuroscience.2011.06.072)

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Fluttert M, Dalm S & Oitzl MS 2000 A refined method for sequential blood sampling by tail incision in rats. *Laboratory Animals* **34** 372–378. (doi:10.1258/002367700780387714)

Grun F & Blumberg B 2007 Perturbed nuclear receptor signaling by environmental obesogens as emerging factors in the obesity crisis. *Reviews in Endocrine & Metabolic Disorders* **8** 161–171. (doi:10.1007/s11154-007-9049-x)

Hallschmid M & Schultes B 2009 Central nervous insulin resistance: a promising target in the treatment of metabolic and cognitive disorders? *Diabetologia* **52** 2264–2269. (doi:10.1007/s00125-009-1501-x)

Hartmann J, Wagner KV, Liebl C, Scharf SH, Wang XD, Wolf M, Hausch F, Rein T, Schmidt U, Touma C *et al.* 2012 The involvement of FK506-binding protein 51 (FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress. *Neuropharmacology* **62** 332–339. (doi:10.1016/j.neuropharm.2011.07.041)

Hinney A, Volckmar AL & Knoll N 2013 Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis. *Progress in Molecular Biology and Translational Science* **114** 147–191. (doi:10.1016/B978-0-12-386933-3.00005-4)

Holsboer F 2000 The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* **23** 477–501. (doi:10.1016/S0893-133X(00)00159-7)

Ising M, Depping AM, Siebertz A, Lucae S, Unschuld PG, Kloiber S, Horstmann S, Uhr M, Muller-Myhsok B & Holsboer F 2008 Polymorphisms in the FKBP5 gene region modulate recovery from psychosocial stress in healthy controls. *European Journal of Neuroscience* **28** 389–398. (doi:10.1111/j.1460-9568.2008.06332.x)

Kim H, Whang WW, Kim HT, Pyun KH, Cho SY, Hahm DH, Lee HJ & Shim I 2003 Expression of neuropeptide Y and cholecystokinin in the rat brain by chronic mild stress. *Brain Research* **983** 201–208. (doi:10.1016/S0006-8993(03)03087-7)

King BM 2006 The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. *Physiology & Behavior* **87** 221–244. (doi:10.1016/j.physbeh.2005.10.007)

Kivimaki M, Head J, Ferrie JE, Shipley MJ, Brunner E, Vahtera J & Marmot MG 2006 Work stress, weight gain and weight loss: evidence for bidirectional effects of job strain on body mass index in the Whitehall II study. *International Journal of Obesity* **30** 982–987. (doi:10.1038/sj.ijo.0803229)

de Kloet ER, Joels M & Holsboer F 2005 Stress and the brain: from adaptation to disease. *Nature Reviews. Neuroscience* **6** 463–475. (doi:10.1038/nrn1683)

Kuo LE, Czarnecka M, Kitlinska JB, Tilan JU, Kvetnansky R & Zukowska Z 2008 Chronic stress, combined with a high-fat/high-sugar diet, shifts sympathetic signaling toward neuropeptide Y and leads to obesity and the metabolic syndrome. *Annals of the New York Academy of Sciences* **1148** 232–237. (doi:10.1196/annals.1410.035)

Leibel RL, Berry EM & Hirsch J 1991 Metabolic and hemodynamic responses to endogenous and exogenous catecholamines in formerly obese subjects. *American Journal of Physiology* **260** R785–R791.

Liu J, Garza JC, Truong HV, Henschel J, Zhang W & Lu XY 2007 The melanocortinergic pathway is rapidly recruited by emotional stress and contributes to stress-induced anorexia and anxiety-like behavior. *Endocrinology* **148** 5531–5540. (doi:10.1210/en.2007-0745)

Liu J, Garza JC, Li W & Lu XY 2013 Melanocortin-4 receptor in the medial amygdala regulates emotional stress-induced anxiety-like behaviour, anorexia and corticosterone secretion. *International Journal of Neuropsychopharmacology* **16** 105–120. (doi:10.1017/S146114571100174X)

McEwen BS 2007 Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiological Reviews* **87** 873–904. (doi:10.1152/physrev.00041.2006)

Michel C, Duclos M, Cabanac M & Richard D 2005 Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice. *Hormones and Behavior* **48** 172–179. (doi:10.1016/j.yhbeh.2005.02.004)

Moles A, Bartolomucci A, Garbugino L, Conti R, Caprioli A, Coccurello R, Rizzi R, Ciani B & D'Amato FR 2006 Psychosocial stress affects energy balance in mice: modulation by social status. *Psychoneuroendocrinology* **31** 623–633. (doi:10.1016/j.psyneuen.2006.01.004)

Pasquali R, Vicennati V, Cacciari M & Pagotto U 2006 The hypothalamic–pituitary–adrenal axis activity in obesity and the metabolic syndrome. *Annals of the New York Academy of Sciences* **1083** 111–128. (doi:10.1196/annals.1367.009)

Porsolt RD, Bertin A & Jalfre M 1977 Behavioral despair in mice: a primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Thérapie* **229** 327–336.

Roubos EW, Dahmen M, Kozicz T & Xu L 2012 Leptin and the hypothalamo-pituitary–adrenal stress axis. *General and Comparative Endocrinology* **177** 28–36. (doi:10.1016/j.ygcen.2012.01.009)

Roy A, Gorodetsky E, Yuan Q, Goldman D & Enoch MA 2010 Interaction of *FKBP5*, a stress-related gene, with childhood trauma increases the risk for attempting suicide. *Neuropsychopharmacology* **35** 1674–1683. (doi:10.1038/npp.2009.23)

Sanchez ER 2012 Chaperoning steroidal physiology: lessons from mouse genetic models of Hsp90 and its cochaperones. *Biochimica et Biophysica Acta* **1823** 722–729. (doi:10.1016/j.bbamcr.2011.11.006)

Scarpace PJ & Zhang Y 2007 Elevated leptin: consequence or cause of obesity? *Frontiers in Bioscience* **12** 3531–3544. (doi:10.2741/2332)

Scharf SH, Liebl C, Binder EB, Schmidt MV & Muller MB 2011 Expression and regulation of the *Fkbp5* gene in the adult mouse brain. *PLoS ONE* **6** e16883. (doi:10.1371/journal.pone.0016883)

Schmidt MV, Sterlemann V, Ganea K, Liebl C, Alam S, Harbich D, Greetfeld M, Uhr M, Holsboer F & Muller MB 2007 Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. *Psychoneuroendocrinology* **32** 417–429. (doi:10.1016/j.psyneuen.2007.02.011)

Seeley RJ, Drazen DL & Clegg DJ 2004 The critical role of the melanocortin system in the control of energy balance. *Annual Review of Nutrition* **24** 133–149. (doi:10.1146/annurev.nutr.24.012003.132428)

Solomon MB, Jankord R, Flak JN & Herman JP 2011 Chronic stress, energy balance and adiposity in female rats. *Physiology & Behavior* **102** 84–90. (doi:10.1016/j.physbeh.2010.09.024)

Surwit RS, Kuhn CM, Cochrane C, McCubbin JA & Feinglos MN 1988 Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* **37** 1163–1167. (doi:10.2337/diab.37.9.1163)

Tamashiro KL 2011 Metabolic syndrome: links to social stress and socioeconomic status. *Annals of the New York Academy of Sciences* **1231** 46–55. (doi:10.1111/j.1749-6632.2011.06134.x)

Touma C, Gassen NC, Herrmann L, Cheung-Flynn J, Bull DR, Ionescu IA, Heinzmann JM, Knapman A, Siebertz A, Depping AM *et al.* 2011 FK506 binding protein 5 shapes stress responsiveness: modulation of neuroendocrine reactivity and coping behavior. *Biological Psychiatry* **70** 928–936. (doi:10.1016/j.biopsych.2011.07.023)

Ulrich-Lai YM & Herman JP 2009 Neural regulation of endocrine and autonomic stress responses. *Nature Reviews. Neuroscience* **10** 397–409. (doi:10.1038/nrn2647)

Vermeer H, Hendriks-Stegeman BI, van der Burg B, van Buul-Offers SC & Jansen M 2003 Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. *Journal of Clinical Endocrinology and Metabolism* **88** 277–284. (doi:10.1210/jc.2002-020354)

Wagner KV, Wang XD, Liebl C, Scharf SH, Muller MB & Schmidt MV 2011 Pituitary glucocorticoid receptor deletion reduces vulnerability to chronic stress. *Psychoneuroendocrinology* **36** 579–587. (doi:10.1016/j.psyneuen.2010.09.007)

Wagner KV, Marinescu D, Hartmann J, Wang XD, Labermaier C, Scharf SH, Liebl C, Uhr M, Holsboer F, Muller MB *et al.* 2012 Differences in FKBP51 regulation following chronic social defeat stress correlate with

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individual stress sensitivity: influence of paroxetine treatment. *Neuropsychopharmacology* **37** 2797–2808. (doi:10.1038/npp.2012.150)
Wang XD, Chen Y, Wolf M, Wagner KV, Liebl C, Scharf SH, Harbich D, Mayer B, Wurst W, Holsboer F *et al.* 2011 Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. *Neurobiological Disorders* **42** 300–310. (doi:10.1038/nn.3395)
Weninger SC, Muglia LJ, Jacobson L & Majzoub JA 1999 CRH-deficient mice have a normal anorectic response to chronic stress. *Regulatory Peptides* **84** 69–74. (doi:10.1016/S0167-0115(99)00070-1)
Wochnik GM, Ruegg J, Abel GA, Schmidt U, Holsboer F & Rein T 2005 FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. *Journal of Biological Chemistry* **280** 4609–4616. (doi:10.1074/jbc.M407498200)
Xie P, Kranzler HR, Poling J, Stein MB, Anton RF, Farrer LA & Gelernter J 2010 Interaction of *FKBP5* with childhood adversity on risk for post-traumatic stress disorder. *Neuropsychopharmacology* **35** 1684–1692. (doi:10.1038/npp.2010.37)
Yach D, Stuckler D & Brownell KD 2006 Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nature Medicine* **12** 62–66. (doi:10.1038/nm0106-62)
Yang L, Isoda F, Yen K, Kleopoulos SP, Janssen W, Fan X, Mastaitis J, Dunn-Meynell A, Levin B, McCrimmon R *et al.* 2012 Hypothalamic Fkbp51 is induced by fasting, and elevated hypothalamic expression promotes obese phenotypes. *American Journal of Physiology. Endocrinology and Metabolism* **302** E987–E991. (doi:10.1152/ajpendo.00474.2011)
Zimmermann P, Bruckl T, Nocon A, Pfister H, Binder EB, Uhr M, Lieb R, Moffitt TE, Caspi A, Holsboer F *et al.* 2011 Interaction of *FKBP5* gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study. *American Journal of Psychiatry* **168** 1107–1116. (doi:10.1176/appi.ajp.2011.10111577)

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2.2. Prenatal Exposure to Maternal Obesity Alters Anxiety and Stress-Coping Behaviors in Aged Mice

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Prenatal Exposure to Maternal Obesity Alters Anxiety and Stress Coping Behaviors in Aged Mice

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Key Words
Obesity · Anxiety · Stress coping behaviors · Mice

Abstract
Background: There is growing evidence that maternal obesity and prenatal exposure to a high-fat diet program fetal development to regulate the physiology and behavior of the offspring in adulthood. Yet the extent to which the maternal dietary environment contributes to adult disease vulnerability remains unclear. In the current study we tested whether prenatal exposure to maternal obesity increases the offspring's vulnerability to stress-related psychiatric disorders. ***Methods:*** We used a mouse model of maternal diet-induced obesity to investigate whether maternal obesity affects the response to adult chronic stress exposure in young adult (3-month-old) and aged adult (12-month-old) offspring. ***Results:*** Long-lasting, delayed impairments to anxiety-like behaviors and stress coping strategies resulted on account of prenatal exposure to maternal obesity. Although maternal obesity did not change the offspring's behavioral response to chronic stress per se, we demonstrate that the behavioral outcomes induced by prenatal exposure to maternal obesity parallel the deleterious effects of adult chronic stress exposure in aged male mice. We found that the glucocorticoid receptor (GR, *Nr3c1*) is upregulated in various hypothalamic nuclei on account of maternal obesity. In addition, gene expression of a known regulator of the GR, FKBP51, is increased specifically within the paraventricular nucleus. ***Conclusions:*** These findings indicate that maternal obesity parallels the deleterious effects of adult chronic stress exposure, and furthermore identifies GR/FKBP51 signaling as a novel candidate pathway regulated by maternal obesity.

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Introduction

The prevalence of maternal obesity is on the rise, emphasizing the need to understand the effects of maternal nutritional status on the offspring. There is growing evidence from both epidemiological studies and animal models indicating that maternal health and diet composition during pregnancy programs fetal development to ultimately regulate the physiology and behavior of the offspring [1]. The concept of fetal programming, whereby the early life environment later contributes to adult disease vulnerability [2], has been strongly reinforced by findings demonstrating that suboptimal maternal nutritional status is associated with an increased risk of devel-

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oping chronic diseases. In particular, substantial evidence demonstrates that maternal obesity increases the offspring's risk of later developing obesity and metabolism-related disorders [3–6]. Beyond fetal programming of metabolic disturbances, maternal obesity has been associated with adverse effects on offspring's brain development [1]. Specifically, animal studies have noted alterations in hippocampal and hypothalamic regions, alterations in serotonergic, dopaminergic, and opioid neurotransmitter systems, and resulting alterations in cognitive and affective behaviors in offspring born to obese mothers [1, 7].

Few studies have investigated the effects of maternal obesity on stress reactivity, and those which have examined hypothalamic-pituitary-adrenal (HPA) axis reactivity have mainly reported short-term effects during development [8, 9]. Importantly, Trottier et al. [9] found that maternal hypernutrition blunts the stress response in neonatal pups while increasing stress reactivity during adolescence. Similarly, an animal model of maternal food restriction revealed that maternal undernutrition is associated with reduced HPA axis activity during preadolescence but HPA axis hyperactivity during late adulthood [10]. Collectively, these data reinforce the notion that the HPA axis is highly susceptible to fetal programming by maternal nutritional status, but they do not address whether the resulting changes are associated with the development of stress-related diseases.

Chronic stress is a risk factor for both the development of metabolic disturbances and affective disorders [11, 12]. Chronic or repeated stress exposure challenges homeostasis, potentially resulting in a dysregulation of the HPA axis and an increased risk of disease development [13]. For example, heightened stress reactivity is among the most consistent features in patients suffering from major depression [14]. As both maternal diet and chronic stress potentially regulate HPA axis reactivity, it is important to investigate these two conditions in parallel. To our knowledge, only one study has examined the effects of maternal nutritional status on stress reactivity following repeated stress exposure during adulthood. Using a repeated tail pinch stress, which represents a mixed physical/emotional stressor, perinatal exposure to a high-fat (HF) diet impaired adaptations in the HPA axis response, a phenomenon which typically follows repeated stress [15]. However, the effects of maternal obesity following chronic psychosocial stress exposure on stress-related behaviors and pathophysiologies have not been clearly defined despite representing the most common type of stress encountered by humans [16].

The HPA axis is regulated by glucocorticoid receptors (GRs) through a negative feedback loop [11]. Genetic polymorphisms of the GR *Nr3c1* gene and the GR regulator protein FK506-binding protein 51 (*Fkbp5* gene) have been associated with the pathophysiology of mood disorders within the human population [17]. Interestingly, human studies have furthermore identified both GR and FKBP51 as mediators of 'gene' × 'early life environment' interactions to predict the risk of developing metabolic dysfunction and psychiatric disorders, respectively [18–21]. In this context, GR and FKBP51 are shown to program the persistent effects of early life experiences. In animal studies, it has been demonstrated that *Nr3c1* and *Fkbp5* gene expression are regulated by exposure to an HF diet during adulthood [22, 23]. Taken together, the HPA axis and, in particular, GR and FKBP51 are prime candidates for linking maternal obesity and persistent behavioral changes in adult offspring.

Given the high prevalence of both obesity and chronic stress in present-day society, it is pertinent to understand how maternal obesity shapes stress reactivity and disease susceptibility in the adult progeny under both basal and stressful conditions. To this end, we investigated the long-lasting effects of maternal obesity on stress-related behavioral responses in a mouse model of HF diet-induced obesity (DIO) under control conditions and conditions of chronic social defeat stress (CSDS), representing a preclinical model of chronic stress possessing features of many psychiatric disorders [24].

Materials and Methods

Animals and Animal Housing

All mice were maintained on a 12:12-hour light/dark cycle, with controlled temperature (22 ± 2°C) and humidity (55 ± 5%). Five-week-old female C57BL/6N mice (Charles River Laboratories, Maastricht, The Netherlands) were group housed (4 per cage) for 6 weeks with ad libitum access to water and food. Female mice were initially split into two groups: one group (n = 16) received a control chow diet (10.5% kcal from fat, D12329; Research Diets, New Brunswick, N.J., USA), and the other group (n = 48) received an HF diet (58% kcal from fat, D12331). Body weight and average daily food intake per cage were measured weekly. After 6 weeks, female mice fed with the HF diet were subsequently stratified based on their body weight. Mice within the top 33% of the body weight range were classified as DIO mice (n = 16) and were used for subsequent experiments. For mating, each chow-fed and DIO female was paired with a single control C57BL/6N male. Chow-fed mice and DIO mice were kept on their respective diets from 6 weeks prior to mating until gestational day 18 ± 2 days, at which point all mice were switched to standard laboratory chow. Therefore, dams were exposed to their respective diets for a total of 9 weeks. In total there were 11 chow-fed litters and 8 HF diet-fed

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Table 1. Summary of sample sizes and the number of litters included in each experimental group

Age, months	Diet	Condition	Individual pups, n	Litters represented, n
3	chow	control	12	10
		CSDS	12	8
	DIO	control	12	7
		CSDS	12	7
12	chow	control-CSDS	12	9
	DIO		8	8

litters; no more than 2 animals per group were drawn from any single mother. All of the resulting offspring stayed with their respective mother, under standard rearing conditions, until they were weaned at postnatal day 24. Only male mice were used for subsequent experiments. These male offspring were raised under standard conditions until they reached adulthood. Male offspring were divided into 2 separate cohorts, counterbalanced by litter, in order to assess the effects of maternal DIO at 2 different life stages (table 1). In the first cohort, behavioral, endocrine, and molecular characterization was performed at 3 months (young adults), whereas the other cohort was tested at 12 months (aged adults), in order to assess the long-lasting effects of maternal obesity. The body weights of offspring were recorded regularly from weaning until they reached 3 months or 12 months. All experiments complied with the ethics guidelines of the European Communities' Council Directive 2010/63/EU. The protocols were approved by the Committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Experimental Design

3-Months

In the 1st cohort, male offspring from chow-fed mothers and DIO mothers were examined at 3 months of age, representing young adulthood. At this time, offspring from chow-fed dams (n = 24) and DIO dams (n = 24) were divided into stress and control mice, and mice within each dietary group were counterbalanced for body weight [CSDS DIO (n = 12), CSDS chow (n = 12), control DIO (n = 12), and control chow (n = 12)]. The mice were challenged to 3 weeks of CSDS or left under control (basal) conditions. Body weight and food intake were recorded daily. In the final week of the CSDS procedure, from day 15 (D15) to D19, the mice underwent behavioral testing (1 test per day); the tests were performed in the following order: open field (OF), social avoidance, elevated plus maze (EPM), glucose tolerance, and forced swim. The animals were sacrificed on D22, following the CSDS paradigm.

12-Months

In the 2nd cohort, male offspring from chow-fed mothers and DIO mothers were examined at 12 months of age, representing older adults. At this time, the offspring from chow-fed dams (n = 12) and DIO dams (n = 8) were first characterized under control (basal) conditions; thereafter, the mice were challenged to 3 weeks of CSDS. Body weight and food intake were recorded daily. In the final week of the CSDS procedure, from D15 to D19, the mice underwent behavioral testing in order to assess whether maternal nutritional status affects behavioral outcomes in situations of chronic stress. The behavioral tests under both basal and CSDS conditions were identical to those performed at the 3-month time point.

Chronic Social Defeat Stress

The CSDS paradigm lasted for 21 days and was conducted as described previously [23]. Briefly, experimental mice were placed in the cage of a male dominant aggressor (CD1) mouse. The mice were allowed to interact until the aggressor attacked and defeated the experimental mouse, at which point the mice were separated into opposite sides of the caging using a wired mesh divider. The division prevented any physical contact between the animals but allowed sensory contact. This procedure was repeated daily for 21 days using unfamiliar CD1 aggressors. Resident CD1 mice were trained beforehand to be highly aggressive such that they attacked and defeated the test mice within 60 s of the initial encounter. The control mice were housed in their home cages throughout the entire stress procedure. Both defeated mice and control mice were handled daily throughout the CSDS paradigm.

Behavioral Tests

All behavioral tests were performed between 08:00 and 13:00 h. They were recorded using a video tracking system (ANY-maze 4.20; Stoelting, Wheat Lane, Ill., USA). All behavioral equipment was cleaned with water at the beginning of testing and between each animal.

Open Field Test

Mice were placed in one corner of a 50 × 50 × 50 cm arena made of plastic. Fifteen-minute trials were video recorded by an overhead camera. The test was performed under conditions of dim light (15 lx), which was specifically chosen to investigate locomotor behavior. Total distance travelled was taken as a measure of locomotor activity.

Social Avoidance Test

The social avoidance test was performed as previously described [25]. Briefly, mice were placed into the same OF arenas that were used for the OF test. The mice were allowed to explore this arena containing an empty (no target) wire cage (height × width: 10.8 × 10.2 cm; Galaxy Cup; Spectrum Diversified Designs Inc., Streetsboro, Ohio, USA) for 2.5 min. Immediately following this initial (no target) stage, the empty wire cage was replaced with an identical wire cage containing an unfamiliar CD1 resident mouse (target). The mice were again allowed to explore the OF for an additional 2.5 min with the target (CD1 mouse) present. Therefore, the test lasted 5 min. Exploratory behavior during testing was transformed into social interaction (SI) ratios [time spent in interaction zone $(s)_{target}$/time spent in interaction zone $(s)_{no\ target}$] to assess social avoidance behavior.

Elevated Plus Maze

The EPM was performed as previously described [26]. Briefly, the apparatus, synthesized from gray polyvinyl chloride, consisted of 2 open arms (length: 30 cm; width: 5 cm; height: 0.5 cm) and 2 closed arms (length: 30 cm; width: 5 cm; height: 15 cm). The apparatus was elevated 50 cm above the ground. At the onset of the

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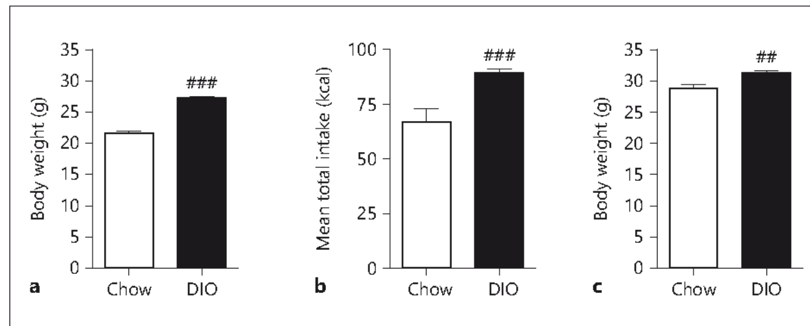

Fig. 1. Validation of maternal DIO mouse model. **a** Body weight of C57BL/6N mothers at the onset of pregnancy. **b** Mean total caloric intake throughout the 6-week period to induce obesity in dams fed an HF diet. **c** Body weight of C57BL/6N mothers at the time of weaning. All data are represented as the mean ± SEM. ## $p < 0.01$, ### $p < 0.001$ (significant diet effect).

test, rodents were placed at the junction of the 4 arms, facing a closed arm, and the exploratory pattern was examined over 10 min. An increase in open arm activity (duration and/or entries) is interpreted as a decrease in anxiety-like behavior.

Forced Swim Test

Mice were placed in a 2-liter glass beaker (diameter: 13 cm; height: 24 cm) filled with room-temperature water to a height of 15 cm so that the animals stayed afloat without their hind legs or tails touching the bottom. The test lasted 6 min. The trials were captured as side-view video recordings. Time spent floating, time spent struggling, and latency to float were scored. Floating is interpreted as despair, whereas struggling is interpreted as escape-orientated behavior [27].

Intraperitoneal Glucose Tolerance Test

Mice were fasted for 14 h and were subsequently injected intraperitoneally with 2 g/kg body weight with D-glucose. Blood glucose levels were assessed using a handheld glucometer (Bayer, Contour, N.Y., USA) from blood taken by tail cut at 0, 30, 60, and 120 min following the glucose load.

Acute Stress Response

The forced swim test (FST) additionally served as an acute stressor in order to examine the stress response. At the conclusion of the FST, the animals were allowed to recover in their home cages for 30 min, at which point blood was collected by tail cut in order to examine response corticosterone levels. At 90 min following the onset of the FST, blood samples were again collected by tail cut in order to assess corticosterone recovery levels. Plasma was collected and stored at –20°C. Levels of plasma corticosterone were subsequently determined using a commercially available radioimmunoassay kit with ^{125}I-labeled anti-corticosterone antibody [MP Biomedicals Inc.; sensitivity: 12.5 ng/ml; intra-assay coefficient of variation (CV): 7%; inter-assay CV: 7%].

Tissue Collection and Processing

Following CSDS at 3 months and 12 months, mice were anesthetized on D22 with isoflurane and sacrificed by decapitation. Trunk blood was collected and processed (as described above). Brains were removed, snap frozen, and stored at –80°C until processed. The brains of those mice that had undergone behavioral testing were subsequently used for gene expression analyses by in situ hybridization (ISH). Epididymal fat was collected and weighed.

Hormone Quantification

Determination of leptin and insulin levels was performed on plasma obtained from trunk blood collected at the time of sacrifice. Levels were determined using a mouse metabolic magnetic bead panel [Millipore Corp., Billerica, Mass., USA; sensitivity: insulin 14 pg/ml and leptin 19 pg/ml; intra-assay CV: 5% (insulin and leptin); inter-assay CV: 7% (insulin and leptin)].

In situ Hybridization

Hypothalamic *Fkbp5* and *Nr3c1* gene expression was examined in both 3-month-old and 12-month-old offspring by ISH. Coronal whole-brain slices were cryosectioned at a thickness of 18 μm and then directly mounted onto Superfrost Plus Slides as 8 sequential series. A single series was selected for each [^{35}S]UTP-labeled ribonucleotide probe. A cRNA anti-sense riboprobe was transcribed from linearized plasmid DNA for *Fkbp5* (forward primer: 5′-CTTGGACCACGCTATGGTTT; reverse primer: 5′-GGATTGACTGCCAACACCTT) and *Nr3c1* (forward primer: 5′-AGGTCGACCAGCCGTCCAGA; reverse primer: 5′-AAGCTTGCCTGGCAATAAAC). ISH was performed as previously described [28]. Slides were exposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, N.Y., USA) and later developed for signal detection. NIH ImageJ software (NIH, Bethesda, Md., USA) was used to quantify autoradiographic densities. Anatomical boundaries of the regions of interest were delineated manually from the digitized autoragiograms. The mean optical density from 2 sequential sections was calculated for each animal subtracting the background signal from the measurements. Hypothalamic gene expression data are only reported when there were significant differences between experimental groups.

Data Analysis

All variables were evaluated using IBM SPSS Statistics 18 software (IBM, Chicago, Ill., USA). The dam served as the statistical unit for analysis of maternal phenotype and pregnancy parameters. Individual pups served as the statistical unit for physiological, behavioral, and gene expression analyses. Student's t test was employed for comparison of 2 independent groups. Two-way ANOVA was used for all other parameters. Body weight and glucose tolerance were always analyzed by repeated-measures ANOVA. Where the initial test yielded a significant interaction, the Bonferroni post hoc test was conducted to locate the interaction effect. In the case of the FST and total body weight change, a post hoc test was used in the absence of significant ANOVA effects according

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to the indications given by Wilcox [29]. The area under the curve (AUC) for the glucose tolerance test and the stress response was calculated by the trapezoidal rule for the observed measurements. Statistical significance was set at $p < 0.05$. Data are presented as means ± SEM.

Results

Maternal Phenotype and Pregnancy Parameters

At the onset of mating, female mice in the DIO dietary group weighed significantly more than chow-fed control mice on account of 6 weeks of exposure to an HF diet (diet: $T_{30} = -14.668$, $p < 0.001$; fig. 1a), which was accompanied by a significantly higher caloric intake (diet: $T_{14} = -10.333$, $p < 0.001$; fig. 1b). The elevated body mass in DIO dams was still present at weaning (diet: $T_{17} = -3.164$, $p = 0.006$; fig. 1c). The dietary condition had no effect on either the litter size (chow: 6.82 ± 0.54 pups; DIO: 7.50 ± 0.68 pups) or the sex ratio (chow: 0.88 ± 0.16 male:female ratio; HF diet: 1.21 ± 0.27 male:female ratio) of the offspring. Moreover, there was no effect of dietary condition on the rate of pregnancy failure, although a maternal HF diet tended to increase their offspring's perinatal death rate (diet: $T_{19.3} = -1.845$, $p = 0.080$).

Offspring's Body Weight Phenotype

Offspring's Body Weight Progression

At weaning, maternal DIO had no effect on the body weight of the offspring. Similarly, there was no effect of maternal DIO on body weight examined at the onset of behavioral characterization at the 3-month time point. Nevertheless, body weight progression from 1 to 12 months later diverged on account of maternal DIO, whereby adult offspring from DIO dams weighed significantly less than offspring from chow-fed mothers, which was interpreted from the AUC (diet: $T_{18} = 2.097$, $p = 0.050$; fig. 2a, b).

Offspring's Body Weight Phenotype in Response to CSDS

A 2-way ANOVA revealed main effects of maternal diet [diet: $F(1, 42) = 4.577$, $p = 0.038$] and stress [stress: $F(1, 42) = 8.308$, $p = 0.004$] on total body weight change following exposure to CSDS in 3-month-old offspring (fig. 2c). Follow-up analyses revealed that only 3-month-old offspring of DIO dams were vulnerable to stress-induced weight gain as reflected in the stress effect in the maternal DIO offspring ($T_{21} = -3.323$, $p = 0.003$) as well as the diet effect in chronically stressed offspring ($T_{20} = -2.128$, $p = 0.046$). Despite presenting a significant increase in body weight gain, adiposity was nevertheless reduced on account of stress exposure in 3-month-old offspring [stress: $F(1, 43) = 68.928$, $p < 0.001$; fig. 2d]. Regardless, food intake was increased by stress exposure independent of the maternal dietary condition [stress: $F(1, 44) = 28.595$, $p < 0.001$; fig. 2e].

Our data indicate that the stress-induced effects on body weight are age dependent. In particular, although CSDS resulted in a significant weight gain in the 3-month-old offspring, at 12 months, the offspring presented a weight loss on account of exposure to chronic stress (fig. 2f). In addition, 12-month-old offspring from DIO mothers presented reduced epididymal white adipose tissue (eWAT) compared to control offspring ($T_{17} = 2.468$, $p = 0.025$; fig. 2g), which accompanied their lower body weight progression up to this point (see Offspring's Body Weight Progression). There was, however, no effect of maternal DIO on food intake in 12-month-old offspring (fig. 2h).

Behavioral Characterization under Basal and Chronic Stress Conditions

Open Field Test

Maternal obesity induced hyperactivity in 3-month-old offspring independent of the stress condition, as reflected in the OF test [diet: $F(1, 44) = 7.678$, $p = 0.008$; fig. 3a]. Stress, by contrast, had no effect on locomotor behavior.

There was no continuing effect of maternal obesity on locomotor activity, as interpreted from the loss of the maternal dietary effect in the 12-month-old offspring on locomotor activity. Nevertheless, aged offspring were vulnerable to stress-induced hypoactivity independent of the maternal dietary condition [stress: $F(1, 17) = 85.040$, $p < 0.001$; fig. 3f].

Social Avoidance Test

In young adult mice, CSDS induced social avoidance regardless of the maternal dietary condition. Specifically, CSDS reduced the SI ratio, defined as the time spent in the interaction zone when the target (CD1 mouse) was present compared to the time spent there when no target was present [interaction zone $(s)_{target}$/interaction zone $(s)_{no\ target}$; stress: $F(1, 35) = 10.726$, $p = 0.002$; fig. 3b]. The effect of stress exposure on the absolute time spent exploring the interaction zone when the target was present did not reach statistical significance (stress: $p = 0.114$). Likewise, aged adult mice were also vulnerable to increased social avoidance behavior on account of chronic

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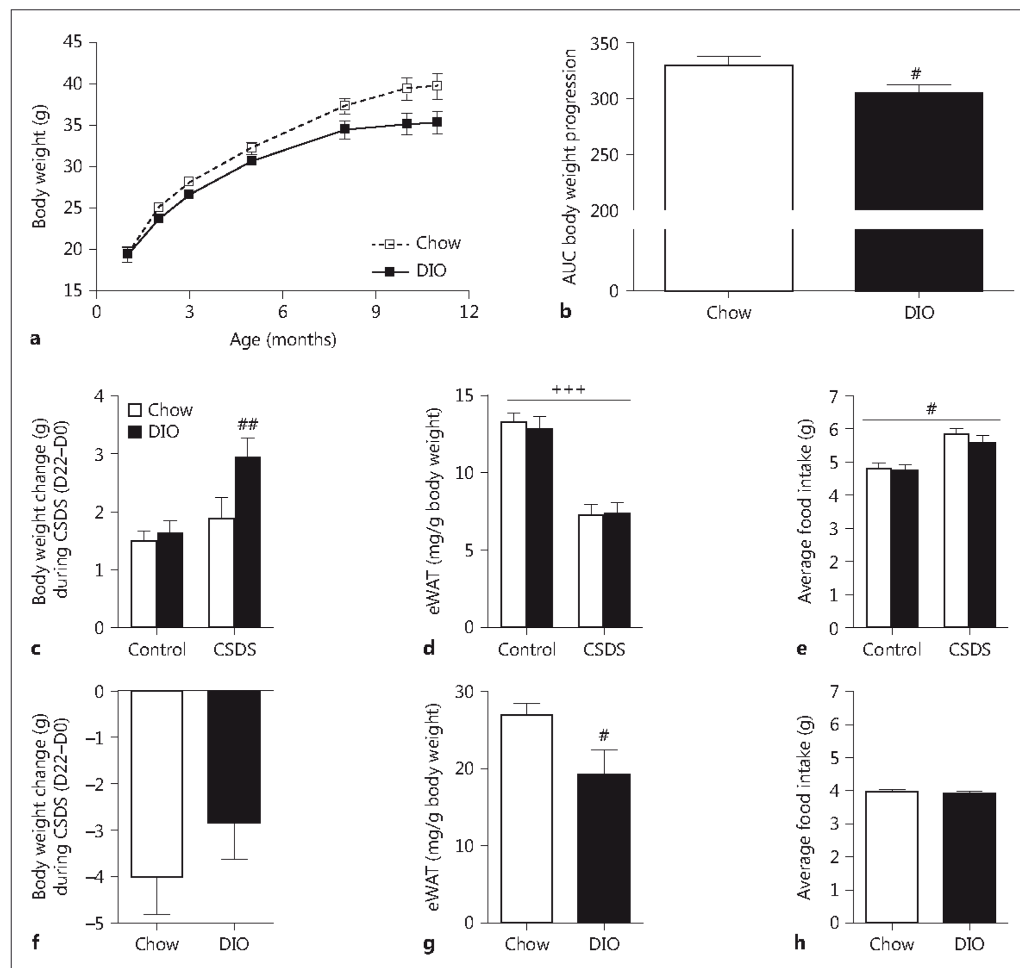

Fig. 2. Effects of maternal obesity on body weight and food intake. **a** Body weight progression in offspring of chow-fed (control) dams or DIO dams from weaning until 12 months. **b** AUC for body weight progression from weaning until 12 months. **c** Total body weight gain following the 3-week CSDS paradigm in 3-month-old offspring. **d** Relative eWAT weight in 3-month-old offspring following the CSDS paradigm. **e** Average food intake in 3-month-old offspring during the CSDS paradigm. **f** Total body weight change following the 3-week CSDS paradigm in 12-month-old offspring. **g** Relative eWAT weight in 12-month-old offspring who had undergone 3 weeks of CSDS. **h** Average food intake in 12-month-old offspring during the 3-week CSDS paradigm. All data are represented as the mean ± SEM. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ (significant diet effect); $^{+++}$ $p < 0.001$ (significant stress effect).

stress exposure, which was interpreted from the SI ratio [stress: $F(1, 14) = 8.356$, $p = 0.012$; fig. 3g] and the absolute time spent exploring the interaction zone when the target was present [stress: $F(1, 17) = 18.206$, $p < 0.001$].

Elevated Plus Maze

To determine whether maternal obesity leaves a long-lasting impression on anxiety-like behaviors, young adult and aged adult offspring were tested in the EPM. In young adult mice, a significant diet effect and a diet × stress interaction for the time spent in the open arms resulted [diet: $F(1, 39) = 6.951$, $p = 0.012$; stress × diet: $F(1, 39) = 4.950$, $p = 0.032$]. Post hoc analysis showed that 3-month-old offspring exposed to maternal obesity spent significantly more time in the open arms compared to offspring from chow-fed dams under basal conditions (fig. 3c).

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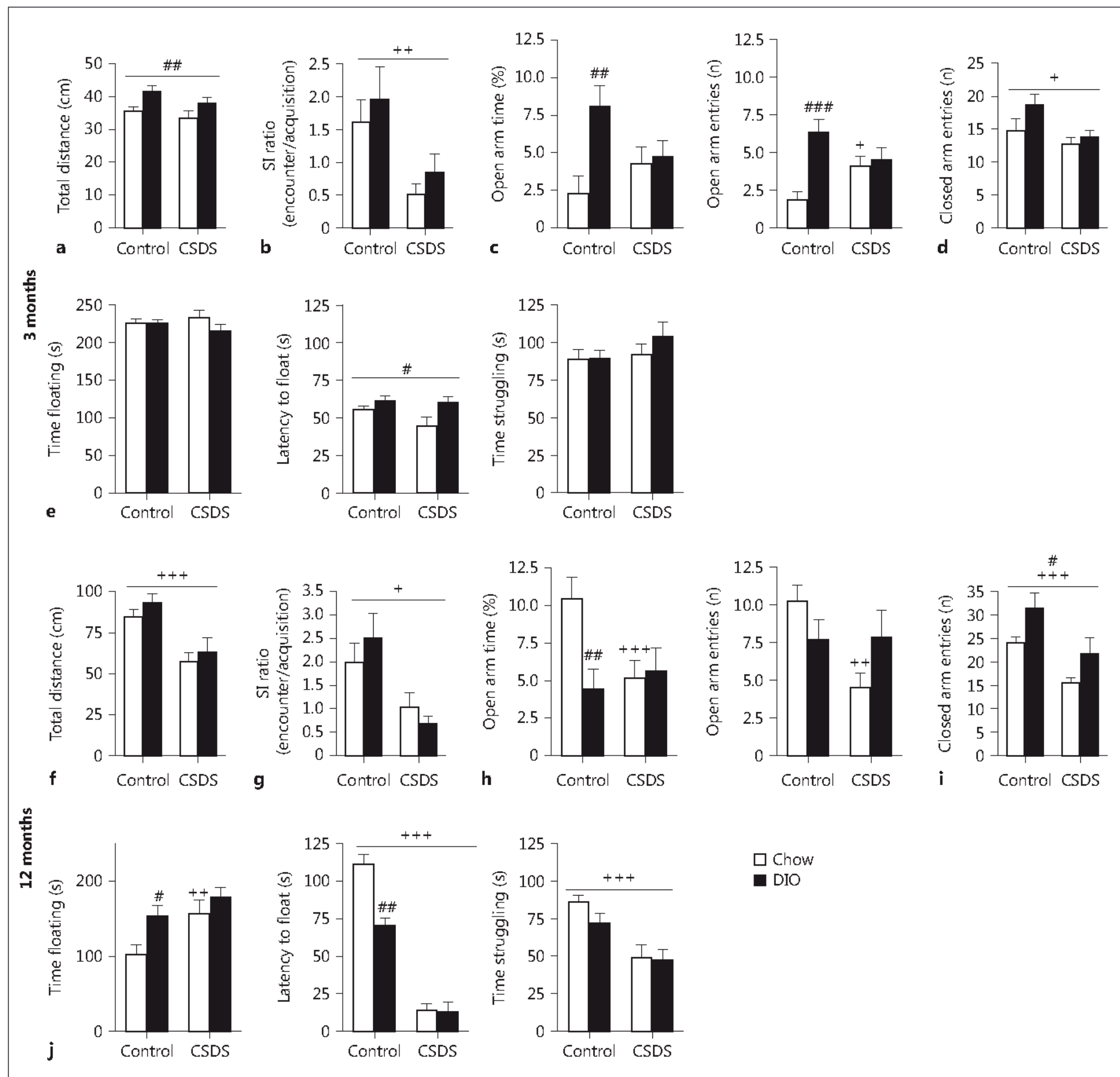

Fig. 3. Behavioral outcomes in 3-month-old (**a–e**) and 12-month-old (**f–j**) offspring under basal and CSDS conditions. **a**, **f** Total distance travelled in a 15-min OF test in 3-month-old (**a**) and 12-month-old (**f**) offspring. **b**, **g** SI ratio for the interaction zone assessed during the social avoidance test in 3-month-old (**b**) and 12-month-old (**g**) offspring. **c**, **h** Percent time spent in the open arms and the number of entries into the open arms in the EPM in 3-month-old (**c**) and 12-month-old (**h**) offspring. **d**, **i** Number of closed arm entries in the EPM in 3-month-old (**d**) and 12-month-old (**i**) offspring. **e**, **j** Time spent floating, latency to begin floating, and time spent struggling in the FST in 3-month-old (**e**) and 12-month-old (**j**) offspring. All data are represented as the mean ± SEM. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ (significant diet effect); $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ (significant stress effect).

Neuroendocrinology
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Similarly, a significant effect of diet [diet: $F(1, 39) = 10.249$, $p = 0.003$] and a stress × diet interaction [stress × diet: $F(1, 39) = 7.062$, $p = 0.011$] were observed for the number of open arm entries. Specifically, stress significantly increased the number of open arm entries in 3-month-old offspring from chow-fed mothers, whereas maternal DIO increased the number of open arm entries under control conditions.

By contrast, maternal DIO resulted in increased anxiety-like behavior in aged mice. Repeated-measures ANOVA revealed a significant diet × stress interaction for the time spent in the open arms [stress × diet: $F(1, 16) = 7.246$, $p = 0.016$; fig. 3h]. Post hoc analysis indicated that while aged offspring of chow-fed mothers are susceptible to stress-induced anxiogenic effects [stress: $F(1, 10) = 19.575$, $p < 0.001$], offspring of DIO dams show increased anxiety-like behavior already under control (nonstressed) conditions (diet: $T_{17} = 2.915$, $p = 0.010$), which was comparable to the stress-induced anxiogenic effects exhibited in offspring of chow-fed dams (fig. 3h). Similarly, investigation of open arm entries revealed an anxiogenic effect of CSDS [stress: $F(1, 15) = 13.276$, $p = 0.002$]. Closer examination indicated that the stress-induced anxiogenic effects on the number of open arm entries were limited to aged offspring of chow-fed mothers [stress: $F(1, 10) = 21.893$, $p = 0.001$]. Collectively, our data unveiled that aged adult offspring were susceptible to maternal DIO-mediated anxiogenic effects in the EPM.

To assess general activity, the number of closed arm entries was also evaluated. Whereas stress significantly reduced closed arm entries [stress: $F(1, 40) = 6.701$, $p = 0.013$], maternal DIO tended to increase the number of closed arm entries [diet: $F(1, 40) = 3.543$, $p = 0.067$] in 3-month-old offspring (fig. 3d). Likewise, in 12-month-old offspring, stress decreased closed arm entries [stress: $F(1, 16) = 44.851$, $p < 0.001$], while closed arm entries were increased on account of exposure to maternal DIO [diet: $F(1, 16) = 8.065$, $p = 0.012$; fig. 3i]. Therefore, general activity as assessed in the EPM supports our OF data, collectively demonstrating that maternal DIO resulted in hyperactivity in adult offspring.

Forced Swim Test

The FST was used to investigate stress coping behavior. In young adult mice, maternal DIO increased the latency to first float, which suggests improved stress coping behavior [diet: $F(1, 44) = 6.721$, $p = 0.013$; fig. 3e]. There was no effect of CSDS at the 3-month characterization time point on any of the scored behaviors (time struggling, time floating, and latency to floating onset).

Interestingly, the effects of maternal obesity were much more robust in aged adult mice. Specifically, exposure to chronic stress resulted in a significant increase in time spent floating [stress: $F(1, 15) = 12.491$, $p = 0.003$] and a between-subjects factor of maternal diet approached significance [diet: $F(1, 15) = 3.194$, $p = 0.094$; fig. 3j]. Follow-up analyses revealed that stress only significantly increased time spent floating in offspring of chow-fed mothers [stress: $F(1, 11) = 19.198$, $p = 0.001$] given that under control conditions offspring of DIO mothers already showed an increased time spent floating (diet: $T_{16} = -2.345$, $p = 0.032$). In a similar manner, examination of the latency to begin floating revealed that CSDS significantly reduced the time to floating onset [stress: $F(1, 15) = 134.168$, $p < 0.001$]. Importantly, a diet × stress interaction [$F(1, 15) = 7.660$, $p = 0.014$] and a main effect of diet [$F(1, 15) = 9.514$, $p = 0.008$] revealed that maternal obesity also affected the latency to floating onset. In particular, offspring from maternal DIO dams presented significantly lowered latencies to floating onset compared to offspring of chow-fed dams under control conditions (diet: $T_{17} = 4.241$, $p = 0.001$). Nevertheless, the effect of CSDS was so robust that there was no difference between maternal dietary conditions following CSDS. Finally, CSDS significantly decreased the time spent struggling independently of maternal nutritional status [stress: $F(1, 14) = 35.638$, $p < 0.001$]. Taken together, although young adult mice did not present any overt alterations in stress coping behavior resulting from maternal nutritional status, maternal obesity impaired stress coping behavior in aged adult offspring.

Stress Response under Basal and Chronic Stress Conditions

The FST was used as an acute stressor in order to induce the stress response. CSDS led to HPA axis hyperactivity in young adult offspring whereby the AUC for corticosterone was increased on account of stress exposure regardless of maternal dietary condition [stress: $F(1, 41) = 29.424$, $p < 0.001$; fig. 4a].

In 12-month-old offspring, the stress response was only assessed following CSDS, because the experimental design, in which the basal characterization preceded the CSDS paradigm using the same animals, prevented investigation of both. Therefore, we were unable to assess the effects of CSDS on the stress response but rather only examined the effect of maternal obesity. Nevertheless, there was no significant effect of maternal obesity on stress responsiveness (fig. 4b).

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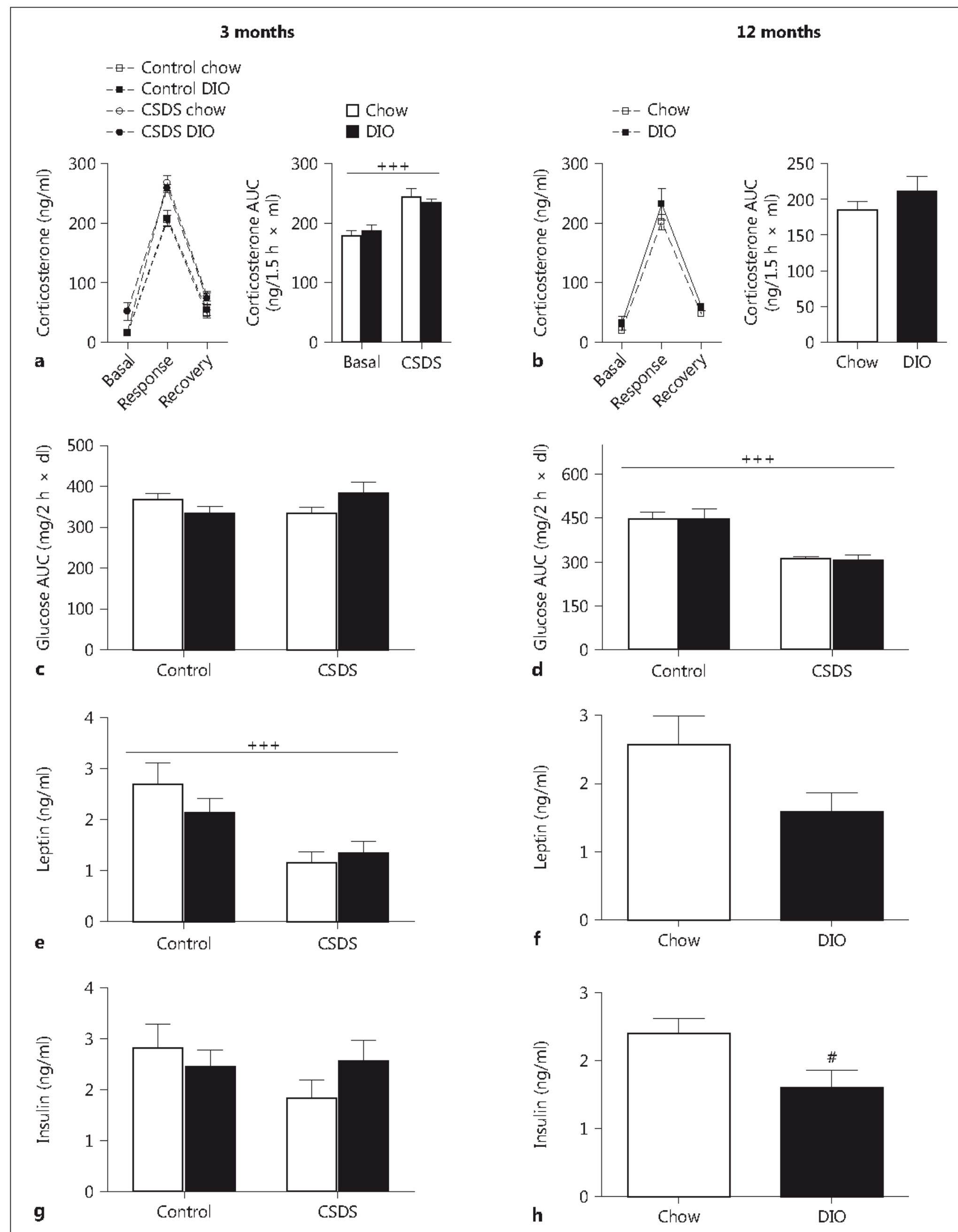

Fig. 4. Effects of maternal obesity on stress response, glucose tolerance, and levels of circulating metabolic hormones. **a**, **b** AUC calculated for corticosterone from basal corticosterone plasma levels and response and recovery plasma corticosterone assessed 30 and 90 min following an acute stressor in 3-month-old (**a**) and chronically defeated, 12-month-old (**b**) offspring. **c**, **d** AUC following a glucose tolerance test calculated from blood glucose examined 0, 30, 60, and 120 min after a 2 g/kg intraperitoneal injection of glucose in 3-month-old (**c**) and 12-month-old (**d**) offspring. **e**, **f** Plasma leptin levels in 3-month-old (**e**) and 12-month-old (**f**) offspring. **g**, **h** Plasma insulin levels in 3-month-old (**g**) and 12-month-old (**h**) offspring following CSDS. All data are represented as the mean ± SEM. $^{\#}$ $p < 0.05$ (significant diet effect); $^{+++}$ $p < 0.001$ (significant stress effect).

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Metabolic Characterization under Basal and Chronic Stress Conditions

Glucose Tolerance

Glucose tolerance was assessed from the AUC for blood glucose measured 0, 30, 60, and 120 min following delivery of 2 mg/kg glucose. At the 3-month time point, there was a significant diet × stress interaction [diet × stress: $F(1, 41) = 4.318$, $p = 0.044$; fig. 4c]. Nevertheless, post hoc analysis revealed no significant effects.

In 12-month-old offspring, CSDS significantly lowered the AUC for glucose independently of the maternal dietary condition [$F(1, 15) = 35.059$, $p < 0.001$; fig. 4d]. Overall, maternal obesity produced no long-lasting adaptation in glucose tolerance.

Plasma Insulin and Leptin

In order to further characterize the effects of maternal DIO on the metabolic phenotype of offspring under basal and chronic stress conditions, we evaluated levels of both circulating leptin and insulin. Three-month-old offspring presented significantly reduced circulating leptin following CSDS [stress: $F(1, 41) = 15.959$, $p < 0.001$; fig. 4e]. Nevertheless, the maternal nutritional status had no effect on leptin, and, furthermore, insulin was neither affected by stress nor maternal dietary conditions (fig. 4g). By contrast, following chronic stress exposure, 12-month-old offspring presented lowered levels of circulating insulin on account of maternal DIO (diet: $T_{17} = 2.245$, $p = 0.038$; fig. 4h). At the 12-month time point, leptin levels remained unaffected by maternal nutritional status (fig. 4f).

Molecular Characterization under Basal and Chronic Stress Conditions

GR and FKBP51 have been shown to program the persistent effects of early life experiences; yet, the role of GR and FKBP51 in the context of maternal DIO remains elusive. To test whether GR and FKBP51 are regulated by maternal DIO, we examined both *Nr3c1* (GR) and *Fkbp5* (FKBP51) gene expression in the hypothalamic nuclei [arcuate nucleus (ARC), paraventricular nucleus (PVN), and ventromedial hypothalamic nucleus (VMH)]. In 3-month-old offspring, there was a significant diet × stress interaction [$F(1, 39) = 5.085$, $p = 0.030$] for *Fkbp5* expression in the PVN. Post hoc analysis revealed that under control conditions, maternal DIO elevated FKBP51 expression in the PVN ($T_{21} = -2.223$, $p = 0.037$; fig. 5a). Furthermore, CSDS tended to increase *Fkbp5* expression in control offspring ($T_{9.70} = -2.065$, $p = 0.067$). There was, however, no effect of maternal nutritional status on *Fkbp5* expression in any other regions examined at the 3-month time point. By contrast, exposure to maternal DIO had a significant effect on *Nr3c1* expression in the VMH and ARC of young adult offspring. Interestingly, the maternal effects were dependent on the adult stress condition, in which maternal DIO significantly decreased *Nr3c1* expression under basal conditions while significantly increasing *Nr3c1* expression following CSDS [diet × stress interaction: VMH $F(1, 29) = 8.741$, $p = 0.006$; ARC $F(1, 29) = 11.949$, $p = 0.002$; fig. 5b).

Molecular changes in 12-month-old offspring were examined exclusively following CSDS, due to the experimental design at the 12-month time point. Maternal DIO significantly increased *Fkbp5* gene expression (fig. 5c) in the PVN ($T_{13} = -2.507$, $p = 0.026$) and ARC ($T_{16} = -2.468$, $p = 0.025$). Examination of *Nr3c1* expression in 12-month-old offspring indicated that *Nr3c1* expression is also susceptible to fetal programming by maternal DIO in the hypothalamus (PVN: $T_{17} = -3.294$, $p = 0.004$; ARC: $T_{13.7} = -2.060$, $p = 0.021$; VMH: $T_{16} = -2.245$, $p = 0.039$; fig. 5d).

Discussion

It is believed that environmental conditions during fetal development program permanent changes in biological processes to ultimately determine the adult phenotype [2]. In the current study we provide a detailed characterization of the effects of maternal DIO on young adult and aged adult male offspring. We furthermore determined whether the effects of maternal obesity were modulated (alleviated or exacerbated) by chronic stress exposure during adulthood. Our results indicate that maternal obesity has stress-dependent effects on body weight control in young adult offspring and long-lasting effects on anxiety-like and stress coping behaviors in aged adult offspring.

Metabolic Phenotype in Young Adult and Aged Adult Offspring

Epidemiological findings provide strong evidence that maternal obesity is a risk factor for offspring obesity [30]. Along these lines, animal studies have attempted to dissect the relative contribution of the pre- and postnatal dietary environments on the metabolic outcomes of the offspring. Using cross-fostering techniques, some studies have shown that HF diet exposure exclusively during pregnancy is sufficient to impair the metabolic phenotype of offspring [31]. By contrast, other studies have reported that maternal HF diet throughout both pregnancy and

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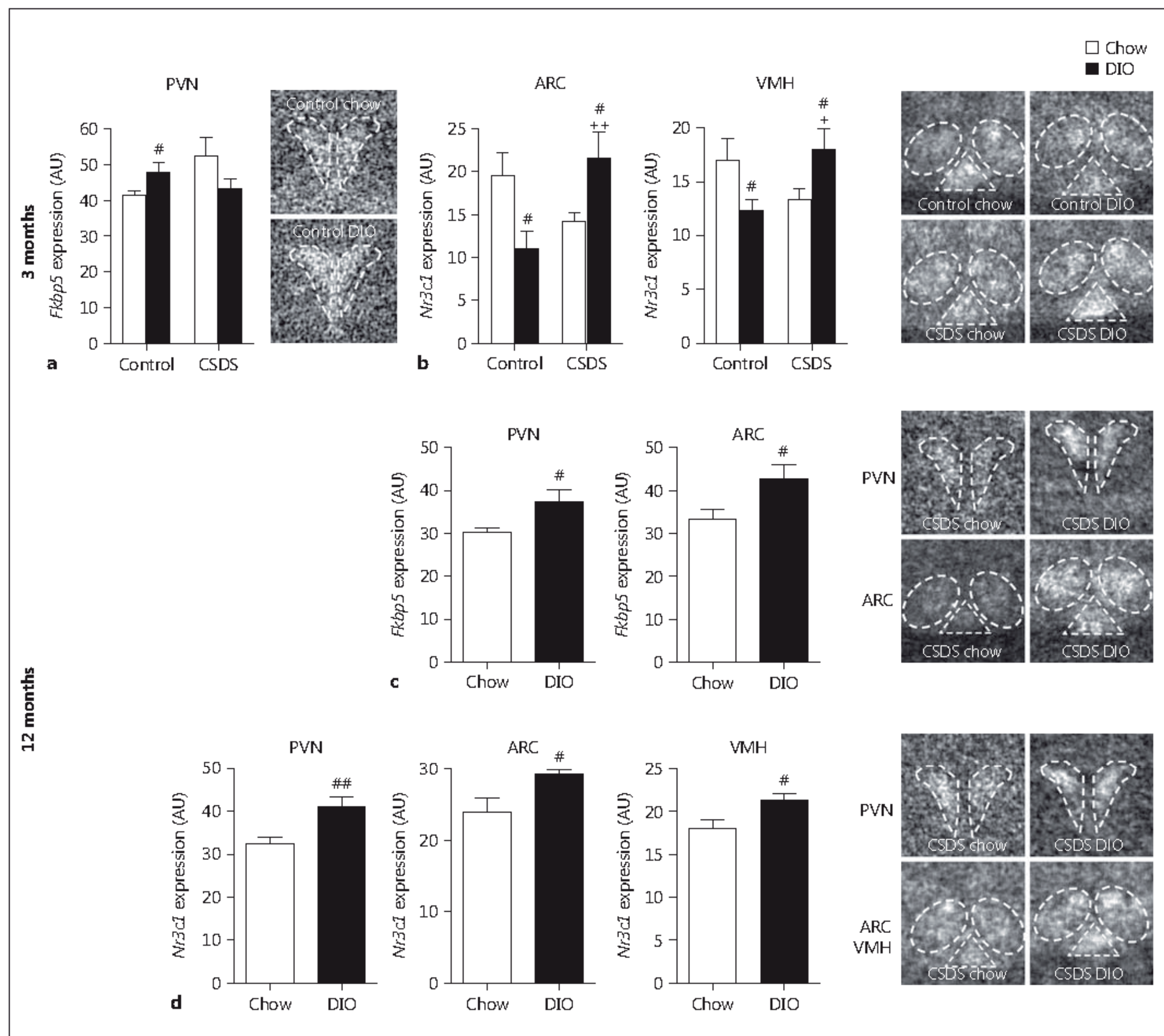

Fig. 5. Gene expression analysis. **a** *Fkbp5* gene expression in the PVN of 3-month-old offspring and representative inverted LUT autoradiograms. **b** *Nr3c1* gene expression in the ARC and VMH of the hypothalamus of 3-month-old offspring and representative inverted LUT autoradiograms. **c** *Fkbp5* gene expression in the PVN and ARC of the hypothalamus of 12-month-old offspring and representative inverted LUT autoradiograms. **d** *Nr3c1* gene expression in the PVN, ARC, and VMH nuclei of the hypothalamus in chronically defeated, 12-month-old offspring and representative inverted LUT autoradiograms. All data are represented as the mean ± SEM. # $p < 0.05$, ## $p < 0.01$ (significant diet effect); + $p < 0.05$, ++ $p < 0.01$ (significant stress effect). LUT = Look-up table.

lactation is essential for predisposing offspring toward adverse metabolic outcomes [32]. Our data suggest that maternal HF diet exposure exclusively during pregnancy has little effect on body weight control in offspring at weaning or during young adulthood. However, body weight was lowered in aged adults on account of maternal HF feeding, which was further accompanied by reduced adiposity and lowered levels of circulating insulin. Importantly, although the maternal nutritional status had no effect on body weight under basal conditions at 3

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months of age, under conditions of CSDS, the offspring born to DIO dams were more vulnerable to stress-induced weight gain.

Besides the effect of the maternal dietary environment on body weight, we also analyzed the effects of adult stress exposure. Specifically, while young adult offspring (at 3 months) experienced stress-induced weight gain, aged adult offspring (at 12 months) experienced stress-induced weight loss and improved glucose tolerance. Moreover, despite increased stress-induced weight gain at the 3-month time point, offspring presented decreased adiposity and circulating leptin levels on account of stress exposure. This paradoxical phenotype has previously been reported in a model of CSDS [33] and supports the notion that CSDS leads to the redistribution of energy stores.

In the human population, stress exposure is closely linked to metabolic disturbances whereby some individuals gain weight while others present weight loss when faced with stress [34–36]. Yet, the biological underpinnings associated with such metabolic phenotypes remain elusive. The current finding that body weight regulation is dependent on the interaction of the early life environment (i.e. maternal obesity) and later environmental challenges (chronic stress exposure/ageing) is therefore of critical importance.

Behavioral Outcomes in Young Adult and Aged Adult Offspring

By definition, fetal programming leads to permanent alterations in biological processes. For example, maternal nutritional status, glucocorticoid overexposure, and prenatal stress have all been implicated in the programming of the HPA axis [37, 38]. Nevertheless, the effects of fetal programming may manifest differently in an age-dependent manner. Differential and age-dependent effects of early life experiences on HPA axis function have been reported [39–41]. For this purpose, we examined the effects of maternal DIO in both young adult (3-month-old) and aged adult (12-month-old) offspring. Maternal DIO had no prominent effects on behavioral readouts in young adult offspring under basal or stress conditions, with the exception of hyperactivity observed in the OF test and EPM. The hyperactivity arising from prenatal exposure to maternal obesity supports previous findings [42]. Furthermore, examination of the 3-month-old offspring revealed unexpected anxiety-like behavior assessed in the EPM in offspring from control chow-fed dams. It is uncertain whether the unexpected findings are related to the composition of the control diet, thus acting as a stressor itself, or rather a technical issue of the test. Additional experiments are needed to determine whether this is truly an effect of the control diet.

The behavioral consequences of prenatal exposure to maternal obesity were, in general, much more robust in aged adult (12-month-old) offspring, suggesting that the postnatal environment and/or the intrinsic aging process interact with the early life experience to shape behavioral outcomes. In particular, prenatal exposure to maternal obesity increased the vulnerability to developing anxiety-like behaviors as well as impaired stress coping in aged adult offspring. In fact, prenatal exposure to maternal DIO mimicked many of the effects of chronic stress exposure on anxiety-like behavior and stress coping behavior assessed in the EPM and FST, respectively. In this regard, offspring from DIO mothers presented a similar phenotype before and after CSDS. Nevertheless, social avoidance behavior in 12-month-old offspring was resistant to the effects of maternal DIO, and only a strong effect of CSDS on social avoidance was observed. These results demonstrate that prenatal exposure to maternal obesity mediates delayed impairments of anxiety-like behavior and stress coping behavior, paralleling the effects of adult chronic stress exposure. Given the lasting functional consequences associated with the structural remodeling of any system, it is possible that morphological changes induced by maternal obesity may underlie the delayed behavioral outcomes. Future studies are required to investigate whether structural alterations arising from maternal obesity underlie the delayed behavioral deficits observed in the current study.

Previous studies have already shown that maternal obesity is associated with increased anxiety [43–45]. Our data nicely support these previous findings and further provide novel data illustrating that exposure to maternal obesity alone is sufficient to imitate the deleterious effects of chronic stress exposure on anxiety-like behaviors in aged adult offspring. In the human population, there is high comorbidity between obesity and increased anxiety and depression; yet, at present, the direction of their relationship is unclear. In other words, is obesity a risk factor for developing depression, or is it rather that depression predisposes an individual to obesity? Our data would suggest that obesity plays a role in the etiology of depression, although a bidirectional relationship may also exist.

Despite the clear effects of maternal obesity on behavioral outcomes in aged adult offspring, we cannot decipher the nature of the relationship between them. For example, does maternal obesity lead directly to the phenotypic outcomes observed in the offspring, or is it rather that exposure to an HF diet induces maternal behavioral

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changes which are indirectly responsible for the behavioral outcomes? As maternal behavior was not measured in the present study, this cannot be clearly determined. Future studies should address whether exposure to an HF diet throughout gestation affects maternal behavior. Regardless, maternal obesity resulted in lasting alterations in anxiety and stress coping behaviors in aged mice.

Gene Expression Analysis

Despite tremendous efforts to understand the biological processes by which maternal obesity modulates complex behaviors, research has only begun to disentangle the various mechanisms involved. Changes within the HPA axis as well as serotonergic and dopaminergic pathways have all been shown to be targeted by maternal obesity [7, 15, 43, 46, 47]. Here, we extend the current understanding by identifying specific genetic targets as well as targeted regions that regulate HPA axis activity and that are sensitive to maternal obesity. Specifically, we provide a detailed analysis of the effects of maternal obesity on *Fkbp5* (FKBP51) and *Nr3c1* (GR) gene expression in the hypothalamus under control and chronic stress conditions.

In young adult offspring, elevated *Fkbp5* mRNA expression was detected in the PVN on account of maternal obesity under control conditions. Likewise, in aged adult offspring, there was an upregulation of *Fkbp5* gene expression in the PVN and ARC nuclei following CSDS on account of exposure to maternal obesity. Previous rodent studies have nicely illustrated that *Fkbp5* expression is increased in select brain regions on account of both acute and chronic stress exposure [48, 49]. The parallel effects of stress exposure and maternal dietary condition on *Fkbp5* gene expression may contribute to the paralleled effects of CSDS and maternal diet on stress coping and anxiety-like behaviors reported here.

The effect of maternal DIO on *Nr3c1* (GR) expression in 3-month-old offspring was highly dependent on adult stress exposure. For example, whereas maternal DIO significantly downregulated *Nr3c1* expression under basal conditions in the VMH and ARC, it rather upregulated *Nr3c1* expression following CSDS. Our data clearly indicate that hypothalamic *Nr3c1* is susceptible to maternal obesity-directed environmental reactivity, which highlights the importance of early life experiences in dictating the molecular response to later adult life experiences. Investigation of *Nr3c1* expression in aged adult offspring assessed following chronic exposure to social defeat stress also revealed a strong, long-lasting regulatory role of maternal obesity. In aged adult offspring, exposure to maternal DIO resulted in the upregulation of *Nr3c1* gene expression across several hypothalamic nuclei. Indeed, the direction of regulation by maternal obesity of *Nr3c1* expression was consistent between the 3-month and 12-month time points.

Programming of GR expression by maternal nutritional status has previously been reported. Maternal undernutrition alters the epigenetic phenotype of the GR gene in the hypothalamus, and subsequently its expression [50]. Elevations in *Nr3c1* expression in the PVN are typically associated with heightened GR sensitivity, decreased stress reactivity, and favorable behavioral outcomes [51]. In the current study, *Nr3c1* was also found to be upregulated in the PVN following CSDS in 12-month-old offspring from obese mothers despite presenting impaired anxiety-like behaviors and stress coping strategies. Yet, it is important to recognize that the environmental context determines the adaptive value of increased or decreased stress reactivity. Furthermore, although we report impaired behavioral outcomes in the EPM and FST, the corticosterone stress response was not overtly affected by prenatal exposure to maternal obesity. In this context, the upregulation of both *Fkbp5* and *Nr3c1* within the PVN in aged adults may offset any change in stress reactivity given that FKBP51 is a known negative regulator of GR [52, 53].

Limitations

A limitation to the current study design is that in the aged adult cohort, the basal and stress conditions were investigated successively, and therefore tissue was only available for the stressed condition. Hence, the effects of stress could not be interpreted. Caution is furthermore advised in the interpretation of the effects in the 12-month-old offspring. The effect of training history may influence the behavioral responses, and therefore the direct comparison between the two age groups should be interpreted carefully. Finally, the nature of the current study limits the ability to establish a direct link between changes in glucocorticoid-relevant gene expression and the behavioral phenotype. Nonetheless, it opens new avenues of research and identifies FKBP51 and GR as novel targets in the hypothalamus involved in programming the response to maternal obesity in adult offspring. Future studies are required to elucidate a direct role of GR/FKBP51 signaling in the fetal programming of adulthood disease.

Conclusions

We provided a detailed analysis of the effects of maternal obesity on metabolic, behavioral, and gene expression outcomes in young adult and aged adult offspring. We further examined whether adult exposure to stress influ-

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ences the effects of maternal obesity. In general, our behavioral data suggest that there are long-lasting, delayed impairments to anxiety-like behaviors and stress coping strategies. Specifically, although maternal obesity did not change the offspring's behavioral response to chronic stress per se, we demonstrated that prenatal exposure to maternal obesity mediates delayed impairments to anxiety-like behavior and stress coping behavior, paralleling the deleterious effects of adult chronic stress exposure. In addition, we identify GR/FKBP51 signaling as a novel candidate pathway regulated by maternal obesity.

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Disclosure Statement

The authors declare no conflict of interest.

References

1 Sullivan EL, Nousen EK, Chamlou KA: Maternal high fat diet consumption during the perinatal period programs offspring behavior. Physiol Behav 2014;123:236–242.
2 Barker DJ, Bagby SP, Hanson MA: Mechanisms of disease: in utero programming in the pathogenesis of hypertension. Nat Clin Pract Nephrol 2006;2:700–707.
3 Khan IY, Taylor PD, Dekou V, Seed PT, Lakasing L, Graham D, Dominiczak AF, Hanson MA, Poston L: Gender-linked hypertension in offspring of lard-fed pregnant rats. Hypertension 2003;41:168–175.
4 Dunn GA, Bale TL: Maternal high-fat diet promotes body length increases and insulin insensitivity in second-generation mice. Endocrinology 2009;150:4999–5009.
5 Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L: Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol 2004;561:355–377.
6 Simmons R: Perinatal programming of obesity. Semin Perinatol 2008;32:371–374.
7 Vucetic Z, Kimmel J, Totoki K, Hollenbeck E, Reyes TM: Maternal high-fat diet alters methylation and gene expression of dopamine and opioid-related genes. Endocrinology 2010; 151:4756–4764.
8 d'Asti E, Long H, Tremblay-Mercier J, Grajzer M, Cunnane SC, Di Marzo V, Walker CD: Maternal dietary fat determines metabolic profile and the magnitude of endocannabinoid inhibition of the stress response in neonatal rat offspring. Endocrinology 2010;151: 1685–1694.
9 Trottier G, Koski KG, Brun T, Toufexis DJ, Richard D, Walker CD: Increased fat intake during lactation modifies hypothalamic-pituitary-adrenal responsiveness in developing rat pups: a possible role for leptin. Endocrinology 1998;139:3704–3711.
10 Vieau D, Sebaai N, Leonhardt M, Dutriez-Casteloot I, Molendi-Coste O, Laborie C, Breton C, Deloof S, Lesage J: HPA axis programming by maternal undernutrition in the male rat offspring. Psychoneuroendocrinology 2007;32(suppl 1):S16–S20.
11 de Kloet ER, Joels M, Holsboer F: Stress and the brain: from adaptation to disease. Nat Rev Neurosci 2005;6:463–475.
12 Björntorp P: Do stress reactions cause abdominal obesity and comorbidities? Obes Rev 2001;2:73–86.
13 McEwen BS: Stress, adaptation, and disease. Allostasis and allostatic load. Ann NY Acad Sci 1998;840:33–44.
14 Holsboer F: The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 2000;23:477–501.
15 Naef L, Gratton A, Walker CD: Exposure to high fat during early development impairs adaptations in dopamine and neuroendocrine responses to repeated stress. Stress 2013;16: 540–548.
16 Björkqvist K: Social defeat as a stressor in humans. Physiol Behav 2001;73:435–442.
17 Binder EB: The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology 2009;34(suppl 1):S186–S195.
18 Koenen KC, Saxe G, Purcell S, Smoller JW, Bartholomew D, Miller A, Hall E, Kaplow J, Bosquet M, Moulton S, Baldwin C: Polymorphisms in FKBP5 are associated with peritraumatic dissociation in medically injured children. Mol Psychiatry 2005;10:1058–1059.
19 Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, Tang Y, Gillespie CF, Heim CM, Nemeroff CB, Schwartz AC, Cubells JF, Ressler KJ: Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. JAMA 2008;299:1291–1305.
20 Rautanen A, Eriksson JG, Kere J, Andersson S, Osmond C, Tienari P, Sairanen H, Barker DJ, Phillips DI, Forsén T, Kajantie E: Associations of body size at birth with late-life cortisol concentrations and glucose tolerance are modified by haplotypes of the glucocorticoid receptor gene. J Clin Endocrinol Metab 2006; 91:4544–4551.
21 Appel K, Schwahn C, Mahler J, Schulz A, Spitzer C, Fenske K, Stender J, Barnow S, John U, Teumer A, Biffar R, Nauck M, Völzke H, Freyberger HJ, Grabe HJ: Moderation of adult depression by a polymorphism in the *FKBP5* gene and childhood physical abuse in the general population. Neuropsychopharmacology 2011;36:1982–1991.
22 Auvinen HE, Romijn JA, Biermasz NR, Pijl H, Havekes L, Smit JW, Rensen PC, Pereira AM: The effects of high fat diet on the basal activity of the hypothalamus-pituitary-adrenal axis in mice. J Endocrinol 2012;214:191–197.
23 Balsevich G, Uribe A, Wagner KV, Hartmann J, Santarelli S, Labermaier C, Schmidt MV: The interplay between diet-induced obesity and chronic stress in mice: potential role of FKBP51. J Endocrinol 2014;222:15–26.
24 Krishnan V, Berton O, Nestler E: The use of animal models in psychiatric research and treatment. Am J Psychiatry 2008;165:1109.
25 Golden SA, Covington HE 3rd, Berton O, Russo SJ: A standardized protocol for repeated social defeat stress in mice. Nat Protoc 2011;6:1183–1191.
26 Santarelli S, Lesuis SL, Wang XD, Wagner KV, Hartmann J, Labermaier C, Scharf SH, Müller MB, Holsboer F, Schmidt MV: Evidence supporting the match/mismatch hypothesis of psychiatric disorders. Eur Neuropsychopharmacol 2014;24:907–918.
27 Ohl F: Animal models of anxiety. Handb Exp Pharmacol 2005;169:35–69.
28 Schmidt MV, Sterlemann V, Ganea K, Liebl C, Alam S, Harbich D, Greetfeld M, Uhr M, Holsboer F, Müller MB: Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. Psychoneuroendocrinology 2007;32:417–429.
29 Wilcox RR: New Statistical Procedures for the Social Sciences: Modern Solutions to Basic Problems. Psychology Press, 1987.

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30 Rooney K, Ozanne SE: Maternal over-nutrition and offspring obesity predisposition: targets for preventative interventions. Int J Obes (Lond) 2011;35:883–890.
31 Khan IY, Dekou V, Douglas G, Jensen R, Hanson MA, Poston L, Taylor PD: A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. Am J Physiol Regul Integr Comp Physiol 2005;288:R127–R133.
32 Bayol SA, Farrington SJ, Stickland NC: A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. Br J Nutr 2007;98:843–851.
33 Chuang JC, Krishnan V, Yu HG, Mason B, Cui H, Wilkinson MB, Zigman JM, Elmquist JK, Nestler EJ, Lutter M: A β_3-adrenergic-leptin-melanocortin circuit regulates behavioral and metabolic changes induced by chronic stress. Biol Psychiatry 2010;67:1075–1082.
34 Block JP, He Y, Zaslavsky AM, Ding L, Ayanian JZ: Psychosocial stress and change in weight among US adults. Am J Epidemiol 2009;170:181–192.
35 Gibson EL: Emotional influences on food choice: sensory, physiological and psychological pathways. Physiol Behav 2006;89:53–61.
36 Torres SJ, Nowson CA: Relationship between stress, eating behavior, and obesity. Nutrition 2007;23:887–894.
37 Harris A, Seckl J: Glucocorticoids, prenatal stress and the programming of disease. Horm Behav 2011;59:279–289.
38 Bertram CE, Hanson MA: Animal models and programming of the metabolic syndrome. Br Med Bull 2001;60:103–121.
39 Workel JO, Oitzl MS, Fluttert M, Lesscher H, Karssen A, de Kloet ER: Differential and age-dependent effects of maternal deprivation on the hypothalamic-pituitary-adrenal axis of Brown Norway rats from youth to senescence. J Neuroendocrinol 2001;13:569–580.
40 Sebaai N, Lesage J, Breton C, Vieau D, Deloof S: Perinatal food deprivation induces marked alterations of the hypothalamo-pituitary-adrenal axis in 8-month-old male rats both under basal conditions and after a dehydration period. Neuroendocrinology 2004;79:163–173.
41 Sapolsky RM, Krey LC, McEwen BS: The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocr Rev 1986;7:284–301.
42 Fernandes C, Grayton H, Poston L, Samuelsson AM, Taylor PD, Collier DA, Rodriguez A: Prenatal exposure to maternal obesity leads to hyperactivity in offspring. Mol Psychiatry 2012;17:1159–1160.
43 Sullivan EL, Grayson B, Takahashi D, Robertson N, Maier A, Bethea CL, Smith MS, Coleman K, Grove KL: Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. J Neurosci 2010;30:3826–3830.
44 Peleg-Raibstein D, Luca E, Wolfrum C: Maternal high-fat diet in mice programs emotional behavior in adulthood. Behav Brain Res 2012;233:398–404.
45 Bilbo SD, Tsang V: Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. FASEB J 2010;24:2104–2115.
46 Sasaki A, de Vega W, Sivanathan S, St-Cyr S, McGowan PO: Maternal high-fat diet alters anxiety behavior and glucocorticoid signaling in adolescent offspring. Neuroscience 2014;272:92–101.
47 Naef L, Moquin L, Dal Bo G, Giros B, Gratton A, Walker CD: Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring. Neuroscience 2011;176:225–236.
48 Scharf SH, Liebl C, Binder EB, Schmidt MV, Müller MB: Expression and regulation of the *Fkbp5* gene in the adult mouse brain. PLoS One 2011;6:e16883.
49 Wagner KV, Marinescu D, Hartmann J, Wang XD, Labermaier C, Scharf SH, Liebl C, Uhr M, Holsboer F, Müller MB, Schmidt MV: Differences in FKBP51 regulation following chronic social defeat stress correlate with individual stress sensitivity: influence of paroxetine treatment. Neuropsychopharmacology 2012;37:2797–2808.
50 Stevens A, Begum G, Cook A, Connor K, Rumball C, Oliver M, Challis J, Bloomfield F, White A: Epigenetic changes in the hypothalamic proopiomelanocortin and glucocorticoid receptor genes in the ovine fetus after periconceptional undernutrition. Endocrinology 2010;151:3652–3664.
51 Meaney MJ: Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 2001;24:1161–1192.
52 Davies TH, Ning YM, Sanchez ER: A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem 2002;277:4597–4600.
53 Wochnik GM, Ruegg J, Abel GA, Schmidt U, Holsboer F, Rein T: FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. J Biol Chem 2005;280:4609–4616.

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2.3. Loss or Inhibition of FKBP51 Protects Against Diet-Induced Metabolic Disorders by Shaping Insulin Signaling

Balsevich G, Gassen NC, Häusl A, Meyer CW, Karamihalev S, Feng X, Dournes C, Santarelli S, Uribe A, Theodoropoulou M, Namendorf C, Uhr M, Paez-Pereda M, Hausch F, Chen A, Tschöp MH, Rein T, Schmidt MV

Manuscript in preparation

2.3.1. Abstract

A genome wide association study demonstrated that polymorphisms within the FKBP51 gene (*FKBP5*) loci are associated with type 2 diabetes (T2D) and markers of insulin resistance (Pereira et al., 2014b). In line with this, the FK506 binding protein 51 (FKBP51) is a known negative regulator of the serine/threonine protein kinase, AKT. AKT is a central node within the insulin signaling pathway, and deregulation of AKT activation has been linked to the pathogenenesis of diabetes and obesity. In this context, FKBP51 may be an important regulator of insulin signaling and consequently energy and glucose homeostasis. To this end, we characterized the metabolic phenotype arising from FKBP51 deletion in mice (51KO) and addressed whether FKBP51 represents a novel therapeutic target for obesity and/or T2D using an FKBP51 antagonist, SAFit2. 51KO mice resisted high fat (HF) diet-induced body weight gain and independently presented improved glucose tolerance. Importantly, pharmacological inhibition of FKBP51 for 10 days improved glucose tolerance, whereas 30 day inhibition completely recapitulated the phenotype observed in 51KO mice. Finally, we probed the mechanism of action by which FKBP51 affects glucose metabolism and could show that FKBP51 knockdown and FKBP51 blockade similarly enhance glucose uptake in skeletal myotubes. These data directly implicate FKBP51 in metabolic regulation and provide physiological and mechanistic evidence for the therapeutic potential of FKBP51 in the treatment of obesity and T2D.

2.3.2. Introduction

There is an unmet need for therapeutic strategies targeting both obesity and T2D. Identification of novel multifunctional molecules, which regulate multiple key metabolic pathways, offers promise to overcome the current limitations in conventional therapeutic strategies. FKBP51 is an immunophilin protein best known as a regulator of the glucocorticoid receptor and consequently the physiological stress response (Ratajczak et al., 2015). Additionally, FKBP51 regulates AKT activation, and subsequently the response to chemotherapy (Pei et al., 2009). Emerging evidence has linked levels of *FKBP5* mRNA in adipose tissue to T2D-related traits (Pereira et al., 2014b). Nevertheless, whether FKBP51 plays a critical role in whole body energy and glucose metabolism remains to be elucidated. For this purpose, we aimed to characterize the role of FKBP51 in energy and glucose homeostasis using a combination of FKBP51 knockout (51KO) mice, pharmacological manipulations, and mechanistic studies.

2.3.3. Methods

Animals & Animal Housing

The FKBP51 knockout (51KO) mouse line, used in experiments 1, 2, and 3, had been previously generated (Tranguch et al., 2005). C57BL/6 mice were used in experiment 4 for the pharmacological blockade of FKBP51 (Charles River Laboratories, Maastricht, The Netherlands). For all experiments, male mice between 3-4 months old were used. During each experiment, mice were singly-housed. Mice were maintained on a 12:12hr light/dark cycle, with controlled temperature (22 +/- 2°C) and humidity (55+/- 5%) conditions. Mice received ad libitum access to water and standard lab chow, unless otherwise specified. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental Design

Experiment 1

Cohort 1: In the first experiment, the direct effects of FKBP51 deficiency on metabolic parameters were investigated in 51KO (n = 16) and WT (n = 18) mice. Body composition (fat and lean mass) was assessed using whole body magnetic resonance imaging (Echo-MRI, Houston, TX). Thereafter, mice were surgically implanted with a telemetric transponder (E-mitter, Mini-mitter Inc., Bend, OR). Mice were allowed to recover for approximately 2 weeks

before any metabolic recordings were performed. Indirect calorimetry and telemetry were performed on mice initially under chow conditions for the assessment of energy intake, energy expenditure, body temperature, and home-cage activity (TSE PhenoMaster, TSE Systems, Bad Homburg, Germany). Each genotype groups was subsequently divided into a chow diet and high fat diet (HFD) (58% kcal from fat, D12331, Research Diets, New Brunswick, NJ, USA) group, counterbalanced for body weight in order to assess the response to HFD exposure. Body weight was measured throughout the experiment. After 8 weeks on the respective diets, 51KO and WT mice were sacrificed. Epididymal (e), inguinal (i), and perirenal (p) white adipose tissue (WAT) was harvested and weighed; brown adipose tissue (BAT) was harvested and weighed.

Cohort 2: In a second cohort of 51KO and WT mice (n = 8 per genotype), body weight and body composition were examined under HF diet conditions at 30°C to minimize the effects of thermal stress.

Cohort 3: A third cohort of 51KO and WT mice were exposed to 6 h of cold exposure (4°C) to assess cold-induced thermoregulation under both control and HF diet conditions. Briefly, 51KO and WT males were divided into a control diet (10.5%kcal fromfat,D12329, ResearchDiets, Inc., New Brunswick, NJ, USA) and HFD (D12331) group (n = 11/group). After 5 weeks on their respective diets, cold-induced thermoregulation was monitored. Initially rectal body temperature was measured for 4 days prior to the cold exposure paradigm to habituate mice to the rectal thermocouple probe. On the 5th day, rectal temperatures were monitored at 0, 2, 4, and 6 h following exposure to 4°C using an Oakton Acorn Temp JKT Thermocouple Thermometer (Oakton Instruments, IL, USA).

Experiment 2

To assess the contribution of food intake on body weight regulation in 51KO and WT mice, a pair-feeding experiment was performed. Mice were initially singly-housed one week prior to the experimental onset. On day one of the pair-feeding paradigm, 51KO mice (n = 9, 51KO) and WT mice (n = 11, WT) received ad libitum access to HFD (D12331, Research Diets, New Brunswick, NJ, USA). A second group of WT mice (WT-PF) were pair-fed to the 51KO mice. Each day for 6 weeks, mice in the WT-PF group received restricted access to the HFD, defined as the amount consumed by the 51KO mice 2 days earlier. Food was weighed and replaced daily at 08:00. If residual food remained in the cages of WT-PF mice, it was weighed and removed prior to the delivery of the daily food portion.

Experiment 3

In experiment 3, glucose tolerance and insulin tolerance were investigated in 51KO (n = 25) and WT mice (n = 18). Briefly, 51KO and WT mice were initially divided into a control diet group and a HF diet group counterbalanced for body weight. After 8 weeks on the dietary treatment, mice were subjected to a glucose tolerance test (GTT). Additionally, blood was collected to assess fasting insulin and glucose-stimulated insulin levels. Mice were allowed to recover for one week before being subjected to an insulin tolerance test (ITT). Body weight and food intake were measured regularly throughout the experiment. After 9.5 weeks on the respective diets, mice were sacrificed and tissues were collected and stored at -80°C until needed.

Experiment 4

To determine whether inhibition of FKBP51 may be an effective anti-obesity and/or diabetic therapeutic strategy, we treated mice for with a highly selective antagonist of FKBP51, known as SAFit2. Briefly, SAFit2 (20 mg/kg body weight) or vehicle were administered by intraperitoneal injections twice daily. SAFit2 was solubilized in vehicle containing 4% ethanol, 5% Tween80, 5% PEG400 in 0.9 % saline. Body weight and food intake were measure daily throughout the treatment periods.

Cohort 1, sub-chronic administration: Male C57BL/6 mice were singly-housed for 2 weeks prior to the treatment onset. One day before the treatment period, mice were divided into a vehicle-treated group and a SAFit2-treated group counterbalanced for body weight (n = 8/group). On treatment day 7 locomotor activity was assessed in the open field. On treatment day 8 a GTT was performed. SAFit2 levels were assessed in plasma from blood taken from at the time of sacrifice. Animals were sacrificed on day 10 following treatment onset. Body weight and food intake were measured regularly throughout the 10-day treatment schedule.

Cohort 2, chronic administration: Four weeks before treatment onset, male C57BL/6 mice were divided into a control diet group (n = 25) and a HF diet group (n = 25) counterbalanced for body weight. One day before the treatment period, mice of each dietary group were further subdivided into a vehicle-treated group and a SAFit2-treated group counterbalanced for body weight. SAFit2 (20 mg/kg body weight) or vehicle were administered by intraperitoneal injections twice daily for 30 days. On treatment day 10 and again at the end of the treatment period (day 30), SAFit2 levels were assessed in plasma from blood taken from tail cut and decapitation, respectively (see below). The open field, dark-light transition, and elevated plus maze behavioral tests were performed on treatment days 15, 16, and 17,

respectively. The GTT was performed on treatment day 25 and the ITT on treatment day 29. Animals were sacrificed on day 31 following treatment onset; tissues were harvested and stored at -80°C for further analyses.

Indirect Calorimetry

Energy expenditure was assessed using indirect calorimetry (TSE PhenoMaster, TSE Systems, Bad Homburg, Germany). Briefly, animals were allowed to habituate to the indirect calorimetry cages for 48h before data were collected. Following 48 h of acclimatization, O2 consumption and CO2 production were measured every 5 min for a total of 68.5 h. Indirect calorimetry was performed at room temperature (experiment 1, cohort 1) and at 30°C (experiment 1, cohort 2). O2 consumption (VO2, [ml/h]), CO2 production (VCO2, [ml/h]), and heat production (referred to as total energy expenditure (TEE), [kcal/24h]) were calculated based on the Weir equations (WEIR, 1949). The respiratory exchange ratio (RER) was calculated as the ratio of CO2 produced to O2 consumed (CO2/ O2). Home-cage locomotor activity was assessed by beam breaks using an ActiMot infrared light beam system within the calorimetry system.

Assessment of Energy Expenditure Components

The delay in acquisition between indirect calorimetry measurements and instantaneous locomotor activity measurements was corrected using the deconvolution procedure (Speakman, 2013) and was performed using a two-compartment gas diffusion model (Van Klinken et al., 2012), which takes into account chamber washout characteristics. Following deconvolution, total energy expenditure (TEE, kcal/24) was decomposed into its activity-related energy expenditure (AEE) and resting metabolic rate (RMR). RMR was modelled in a time-dependent manner using a method based on penalized spline regression, allowing for the detection of up to four RMR frequency components per 24h (8 knots/24h)(Van Klinken et al., 2012). Cases were excluded if the correlation between the convoluted activity and TEE did not reach an arbitrary cutoff of $r \geq 0.7$. Model fit was assessed by visual inspection of the component analysis residuals.

Intraperitoneal Glucose Tolerance Test (GTT)

An intraperitoneal injection of D- glucose (2 g/kg body weight) was delivered to fasted mice (14 h fast). Blood glucose levels were measured on blood collected by tail cut at 0, 30, 60, and 120 min intervals following the glucose load. Glucose levels were measured using a handheld Contour XT glucometer (Bayer Health Care, Basel, Switzerland). Plasma was also

collected from blood at 0 and 30 min to assess fasting insulin and glucose-stimulated insulin levels, respectively.

Intraperitoneal Insulin Tolerance Test (ITT)

Mice were fasted for 14 h and subsequently received intraperitoneal injections of 0.5 IU/kg body weight of D-glucose. Blood glucose levels were assessed from tail cuts at 0, 30, 60, and 120 min following the insulin load. Glucose levels were measured using a handheld Contour XT glucometer (Bayer Health Care, Basel, Switzerland).

Behavioral Analyses

All behavioral tests were performed during 08:00 and 12:00 (Experiment 4). General locomotor activity was examined in an empty open field arena over 15 min under 15 lux illumination. Anxiety-related behaviors were assessed using the elevated plus maze and dark-light transition tests as previously described (Schmidt et al., 2009). Each test was videotaped by an overhead camera and analyzed using the automated video-tracking software ANYmaze4.9 (Stoelting, Wood Dale, IL, USA).

Tissue Collection

Mice were anesthetized with isoflurane and immediately sacrificed by decapitation. Basal trunk blood was collected and subsequently processed (plasma was collected and stored at -20°C). Skeletal muscle (extensor digitorum longus (EDL) and soleus), WAT (inguinal (i), epididymal (e), and perirenal (p)), liver, and hypothalamus were collected and stored at -80°C until used.

Hormone Quantification

Plasma insulin levels were determined using a mouse metabolic magnetic bead panel (Millipore Corp. Billerica, Massachusetts; sensitivity: insulin 14pg/ml). For the assessment of fasting insulin levels, blood was collected 14 h following an overnight fast by tail cut. Similarly, glucose-stimulated insulin levels were measured from plasma taken from blood collected 30 min following glucose load during the glucose tolerance test.

SAFit2 Quantification

The concentration of the FKBP51 antagonist SAFit2 on day 10 and 30 of treatment was quantified from plasma by LCMS/ MS. Briefly, plasma was analyzed using the combined high-performance liquid chromatography/mass spectrometry (HPLC/MS-MS) technique. Analysis was performed using an Agilent 1100 Series (Agilent, Waldbronn, Germany) liquid

chromatograph which was interfaced to the ESI source of an Applied Biosystems API 4000 (ABSciex, Darmstadt, Germany) triple quadrupole mass spectrometer. All samples were added to Ostro protein precipitation and phospholipid removal plates (Waters, Eschborn, Germany). Deuterated clomipramine (Clomi-D3) was used as internal standard. Chromatography was performed using a gradient elution in an Accucore RP-MS 2,6µm column (2.1 x 50 mm, Thermo Scientific, Dreieich, Germany) at a flow rate of 0.3ml/min and 30 °C.

Cell Lines

C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1x penicillin streptomycin antibiotics at 37 °C in a humidified atmosphere with 5% CO_2. Once the cells reached ~90% confluency, C2C12 myoblasts were infected with adeno-associated viral (AAV) vectors expressing either GFP control or shRNA against FKBP51. A multiplicity of infection (MOIs) of 5 x 10^4 was used. Cells were induced to differentiate 8 h after infection for 3 days before the examination of glucose uptake. Differentiation was induced by switching the growth medium to DMEM containing 2% horse serum for 3 days.

Glucose Uptake

Basal and insulin-stimulated glucose uptake in differentiated C2C12 myotubes was examined. Briefly, C2C12 myotubes were serum-starved in low glucose (1000 mg/L) DMEM for 4h, and then incubated in Krebs-Ringer-HEPES (KRH) buffer (136 mM NaCl, 4.7 mM KCl, 10 mM sodium phosphate buffer, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4, 0.2% BSA) for 10 min. Cells were stimulated with insulin (100 nM) or left unstimulated for 1h. Glucose uptake was induced by the addition of KRH buffer containing 100 µM 2-deoxy-D-[1,2-3H]glucose, 2 µCi/ml (Perkin Elmer) to each well. 4 minutes thereafter, the reactions were terminated by washing the cells with ice-cold PSB containing 10µM cytochalasin B (inhibitor of membrane transporter-dependent glucose transport), and then 2 additional washes with ice-cold 1x PBS. Cells were lysed with 0.1 M NaOH for 30 min, and the incorporated radioactivity was determined by liquid scintillation counting. 2-deoxy-D-[1,2-3H]glucose uptake was furthermore normalized to total protein content assessed by the BCA assay (BCA Protein Assay Kit, Life Technologies, Darmstadt, Germany).

Western Blot Analysis

Tissues were homogenized in lysis buffer containing 62.5 mM Tris-HCl, 2% SDS, and 10% sucrose supplemented with protease (Sigma, P2714) and phosphatase (Roche, 04906837001) inhibitor cocktails, and subsequently centrifuged at 12,00 x g to remove cell debris. Lysates were sonicated three times, and protein concentrations were measured using the BCA assay (BCA Protein Assay Kit, Life Technologies, Darmstadt, Germany). After dilution, protein samples (40 µg) were heated for 5 min at 95°C in loading buffer. Equal amounts of proteins were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. Non-specific binding was blocked in Tris-buffered saline, supplemented with 0.05% Tween (Sigma, P2287) and 5% non-fat milk for 1 h at room temperature and subsequently blots were incubated with primary antibody (diluted in TBS/0.05% Tween) overnight at 4 ºC. For a list of primary antibodies used, please refer to Supplementary Table S 1. The following day, blots were washed and probed with the respective horseradish peroxidase secondary antibody for 1 h at room temperature. Immuno-reactive bands were visualized using ECL detection reagent (Millipore, Billerica, MA, USA, WBKL0500). Band intensities were evaluated with the ChemiDoc Imaging System (Bio-Rad).

Quantification of Protein Data

The level of each phosphorylated protein was normalized to its respective non-phosphorylated protein. For total protein content, actin was used as an internal control.

Statistical Analysis

Data were analyzed by Student's t-tests or ANOVA, where appropriate, using IBM SPSS Statistics 18 software (IBM SPSS Statistics, IBM, Chicago, IL, USA). Data were transformed for ANOVA to meet the assumption of homogeneity of variance when required (Supplementary Table S 2). Repeated measures ANOVA was used for all body weight progression, GTT, and ITT data, in which the degrees of freedom were corrected for deviance from sphericity (Greenhouse–Geisser) for within-subjects comparisons. A Bonferroni post hoc test was used for ANOVA tests to determine statistical significance between individual groups. The decomposition of TEE into AEE and RMR was performed in MATLAB (The MathWorks, Natick, MA, USA) using a custom-designed toolbox graciously provided by JB van Klinken (Bioinformatics Center of Expertise, Leiden University Medical Center, Leiden, The Netherlands). Body weight was included as a covariate in the analyses of energy expenditure (Arch et al., 2006). Statistical analyses for all energy expenditure outcome

variables, RER, home-cage activity, food intake, water intake, and body temperature were performed on either 24-hour averages. Statistical significance was set at $p < 0.05$; a statistical tendency was set at $p < 0.1$. For interactions at $p < 0.1$, we also examined lower order main effects. Data are presented as the mean +/- S.E.M.

2.3.4. Results

In order to examine the role of FKBP51 in energy and glucose homeostasis we initially characterized the metabolic outcomes arising in FKBP51 knockout (51KO) mice. We found that 51KO mice fed a chow diet showed a modest body weight phenotype, and furthermore presented reduced adiposity and increased lean mass compared to wild-type (WT) littermates (Figure 1.3.1a). Strikingly, when challenged with HF diet exposure for 8 weeks, 51KO mice were protected from both HF diet-induced weight gain and increased adiposity (Figure 2.3.1b & c). Loss of FKBP51 likewise counteracted diet-induced obesity under thermoneutral conditions (30°C), arguing against a thermoregulatory basis of the phenotype (Supplementary Figure S 1). Indirect calorimetry revealed that the HF diet resistant phenotype observed in 51KO mice was accompanied by a modest increase in total energy expenditure, as a result of an increased resting metabolic rate (RMR) (Supplementary Figure S 2). In addition, 51KO mice presented a modest decrease in their RER and an increase in their home-cage activity. By contrast, food intake was not affected by total loss of FKBP51. To confirm a lack of FKBP51 effect on feeding behavior, a separate pair-feeding experiment was performed, in which a cohort of WT mice was pair-fed to 51KO mice. This experiment again revealed no genotype effect on energy intake (Supplementary Figure S 3). No additional outcomes of FKBP51 on energy homeostasis were identified using the comprehensive lab animal monitoring system (Supplementary Figure S 2).

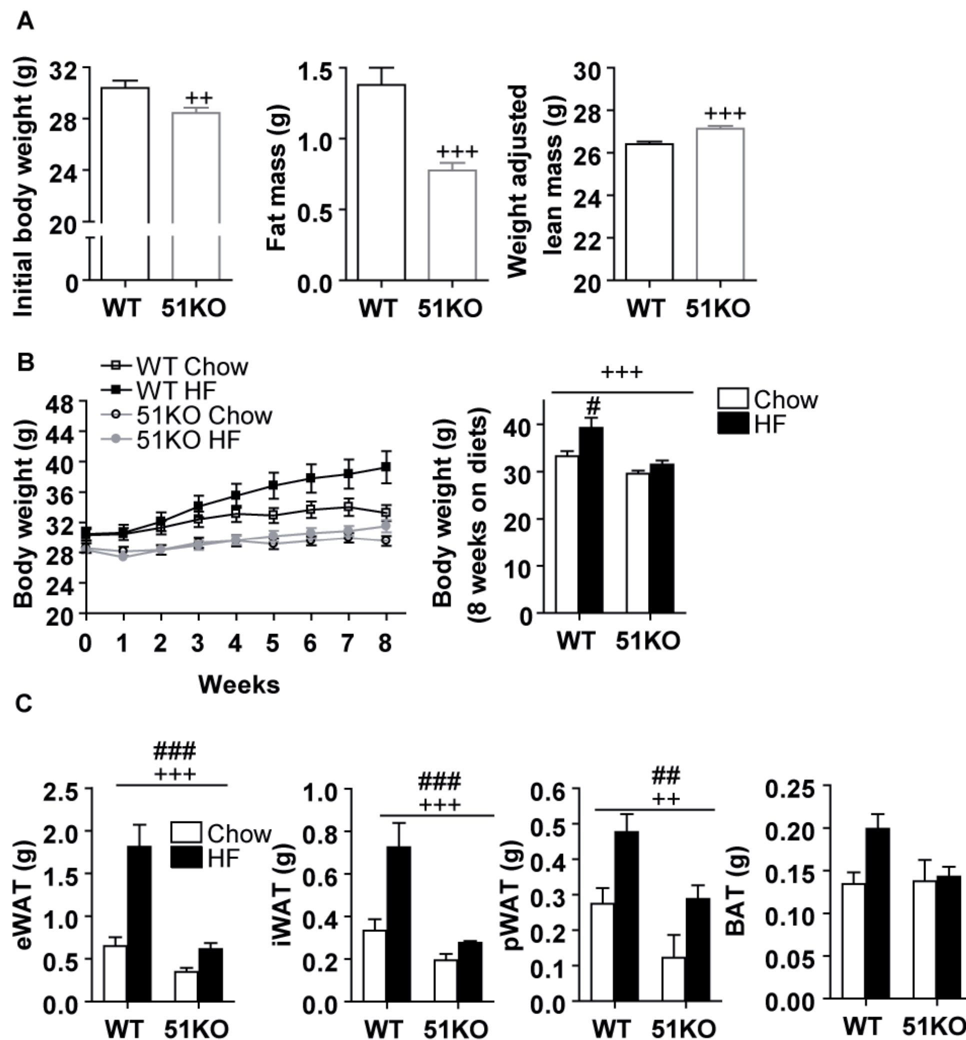


Figure 2.3.1 Genetic ablation of FKBP51 prevents HF diet-induced weight gain

(a) 51KO mice presented lowered body weight (T_{36} = 2.577, p = 0.014), decreased fat mass ($T_{26.7}$ = 4.584, p < 0.001), and increased lean mass (T_{36} = -3.870, p < 0.001) compared to WT littermates at the onset of the dietary feeding period (b) 51KO mice weighed significantly less than WT mice throughout the 8 week dietary treatment (Time x Genotype: F(1, 50.7) = 16.328, p < 0.001; Genotype: F(1, 30) = 17.924, p < 0.001) and at the experimental end (Genotype: F(1, 30) = 19.658, p < 0.001). Interestingly, whereas WT mice were susceptible to HF diet-induced weight gain, 51KO mice were not as interpreted from weight progression (Time x Genotype x Diet: F(1, 50.7) = 3.370, p = 0.050) and final body weight (following 8 weeks on respective diets) (Diet: F(1, 30) = 9.116, p = 0.005, Bonferroni correction). (c) After 8 weeks on the dietary treatment, 51KO mice presented decreased fat pad weights for eWAT (Genotype: F(1, 30) = 23.195, p < 0.001), iWAT (Genotype: F(1, 30) = 22.979, p < 0.001), and pWAT (Genotype: F(1, 22) = 10.456, p = 0.004) compared to WT counterparts. Nevertheless, HF diet exposure significantly increased fat pad mass, regardless of genotype (Diet for eWAT: F(1, 30) = 25.607, p < 0.001; iWAT: F(1, 30) = 16.942, p < 0.001; pWAT: F(1, 22) = 12.255). $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001; + significant genotype effect; # significant diet effect.

To determine the effects of FKBP51 on glucose metabolism and insulin sensitivity, we performed glucose tolerance and insulin tolerance tests in a separate cohort of 51KO and WT mice. Body weight data were consistent with our previous experiments (Supplementary Figure S 4). FKBP51 deletion lowered fasting glucose (Figure 2.3.2a) and remarkably improved glucose tolerance under control and HF dietary conditions (Figure 2.3.2b – c). Interestingly, the levels of fasted insulin and glucose-stimulated insulin were not different between 51KO and WT mice (Figure 2.3.2d – e), indicating that differences in insulin secretion do not contribute to the improved glucose tolerance phenotype. During an insulin tolerance test, 51KO mice presented a prolonged response to insulin under control and HF dietary conditions, despite the fact that both 51KO and WT mice remained vulnerable to HF diet-induced insulin intolerance (Figure 2.3.2f – g).

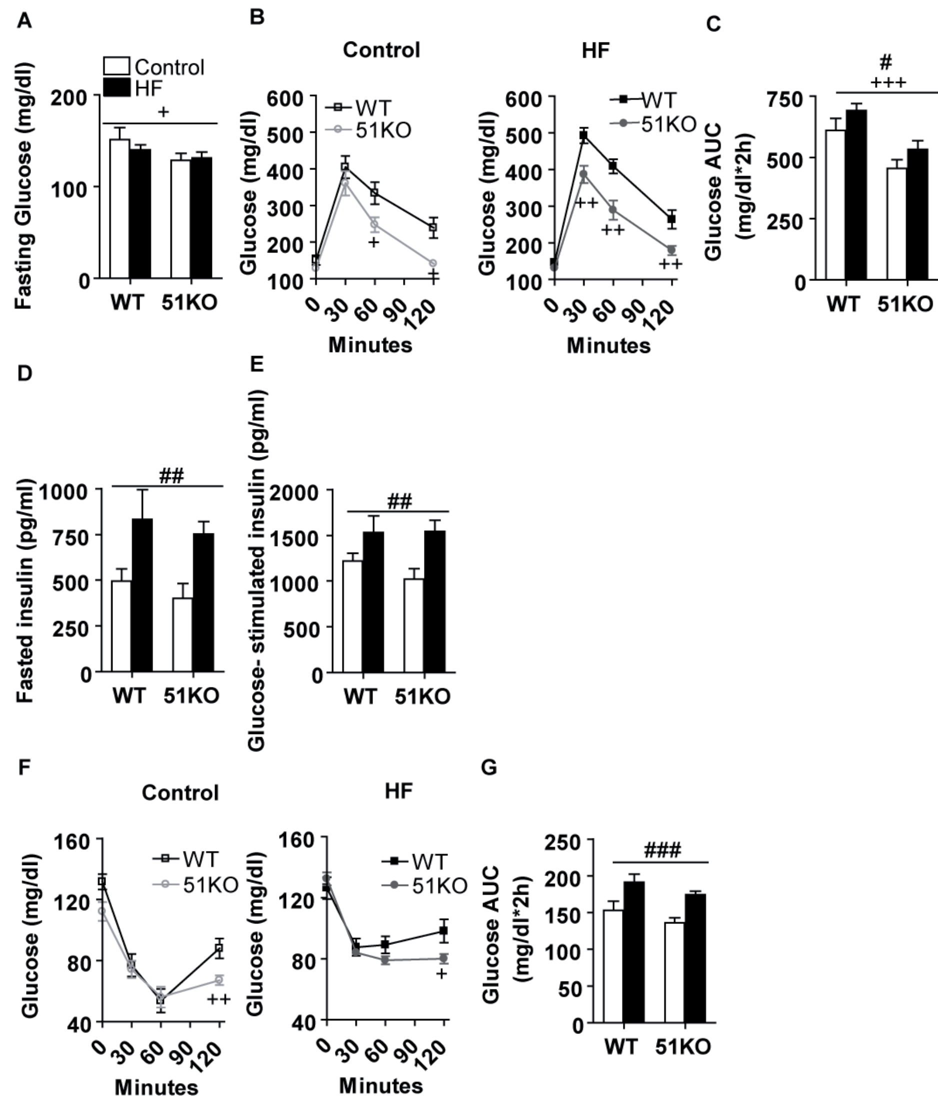


Figure 2.3.2 Genetic ablation of FKBP51 improves glucose tolerance

(a) Blood glucose following a 14 h fast was significantly lower in 51KO mice compared to WT mice (Genotype: $F(1, 37) = 5.197$, $p = 0.028$). (b) In the GTT, a HF diet impaired glucose tolerance in WT mice but not in 51KO mice (HF group: Time x Genotype: $F(1, 38.9) = 4.440$, $p = 0.018$, Genotype: $F(1, 19) = 10.971$, $p = 0.004$; Chow group: Genotype: ($F(1, 17) = 6.567$, $p = 0.020$). (c) The area under the glucose curve illustrates the effect of genotype ($F(1, 36) = 17.004$, $p < 0.001$) and diet ($F(1, 36) = 4.628$, $p = 0.038$) on glucose tolerance. (d, e) Fasted insulin and glucose-stimulated insulin were significantly elevated from HF diet exposure (Fasted: $F(1, 35) = 10.653$, $p = 0.004$; Stimulated: $F(1, 37) = 9.228$, $p = 0.004$) independent of genotype. (f) In HF diet-fed mice, loss of FKBP51 significantly reduced insulin tolerance (Time x Genotype: $F(1, 60) = 6.031$, $p = 0.004$). Importantly blood glucose remained significantly lower 120 min following insulin administration on account of FKBP51 deletion under both chow conditions ($T16 = 3.286$, $p = 0.005$) and HF diet conditions ($T20 = 2.466$, $p = 0.023$). (g) The Area under the glucose curve for ITT demonstrates the strong diet effect ($F(1, 34) = 19.877$, $p < 0.001$) and a trend for genotype ($F(1, 34) = 3.851$, $p = 0.058$). +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$; + significant genotype effect; # significant diet effect.

Due to the improved metabolic phenotype of 51KO mice, we subsequently assessed the therapeutic efficacy of pharmacological blockade of FKBP51. Importantly, a highly selective antagonist for FKBP51, known as SAFit2, has recently been developed (Gaali et al., 2015;Hartmann et al., 2015). The authors described improved neurite outgrowth in neuronal cultures and improved stress coping behavior in mice from SAFit2 treatment, with possible therapeutic potential in the treatment of stress-related psychiatric disorders. In the current study, we determined the therapeutic efficacy of FKBP51 antagonism in mice on metabolic parameters. At first, SAFit2 (20mg/kg) was administered twice daily for 10 days to adult C57BL/6 mice by intraperitoneal injections under chow-fed conditions. Although 10 days of FKBP51 antagonism yielded no body weight phenotype, there was a marked lowering of glucose tolerance assessed on treatment day 8 (Figure 2.3.3a – b). To determine whether a longer treatment period was required to achieve a body weight phenotype, a second cohort of C57BL/6 mice were treated with SAFit2, this time for 30 days under both control and HF diet conditions. This injection schedule resulted in high SAFit2 plasma levels and minimal inter-animal variability (Supplementary Figure S 5). At the onset of treatment, following 4 weeks of dietary exposure, there was no difference in body weight between treatment groups (Supplementary Figure S 6). However, we found that 30 days of SAFit2 administration led to a reduction in body weight under both control and HF diet conditions (Figure 2.3.3c). Furthermore FKBP51 antagonism protected against HF diet-mediated glucose intolerance (Figure 2.3.3d). There was however no effect of SAFit2 on insulin tolerance or on locomotor activity tested in the open field (Supplementary Figure S 7). Importantly, there were no unwanted side-effects of FKBP51 antagonism on behavioral readouts tested in the elevated plus maze and dark-light transition tests (Supplementary Figure S 8). Taken together, these results clearly demonstrate that pharmacological blockade of FKBP51 phenocopies the effects of FKBP51 genetic ablation and furthermore demonstrate the therapeutic potential of SAFit2 in the treatment of obesity and T2D.

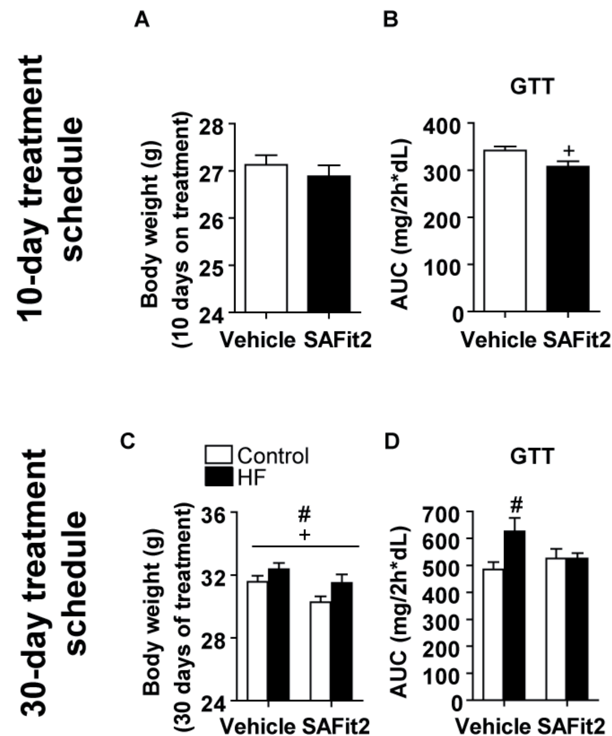


Figure 2.3.3 Pharmacological inhibition of FKBP51 parallels the metabolic effects resulting from genetic ablation of FKBP51

(a) 10-day SAFit2 treatment had no significant effect on body weight. (b) Despite no effect on body weight, SAFit2 treatment significantly lowered glucose tolerance as reflected in the glucose AUC for the GTT measured on treatment day 8 ($T_{13} = 2.469$, $p = 0.028$). (c) At the experimental end point (following 30 days of treatment), mice treated with SAFit2 weighed significantly less than their diet counterparts (Treatment: $F(1, 44) = 6.148$, $p = 0.017$). Nevertheless mice fed the HF diet remained significantly heavier independent of treatment (Diet: $F(1, 44) = 5.380$, $p = 0.025$). (d) The extended SAFit2 treatment schedule furthermore protected against HF diet-induced impaired glucose tolerance as reflected in the glucose AUC measured on day 25 (Diet x Treatment: $F(1, 42) = 2.954$, $p = 0.093$, Bonferroni correction). $^{+}P < 0.05$; $^{\#}P < 0.05$; + significant treatment effect; # significant diet effect.

To determine the mechanism by which FKBP51 may regulate glucose homeostasis and consequently glucose tolerance, we initially examined the phosphorylation status of critical nodes along the insulin signaling cascade as a marker of insulin signaling activation. As we found a strong effect of FKBP51 loss on glucose tolerance without any alterations of circulating insulin levels, we hypothesized that intracellular insulin signaling is enhanced following total loss of FKBP51. We found that insulin sensitivity (as reflected through the phosphorylation status of p70S6K, IRS-1, and AKT) was markedly increased in skeletal muscle of 51KO mice, whereas insulin activation in adipose tissue remained unchanged (Figure 2.3.4a – b). Indeed, skeletal muscle accounts for an estimated 80% of postprandial glucose disposal and is regarded as a principal site responsible for the maintenance of glucose

homeostasis (DeFronzo et al., 1985;Zierath and Wallberg-Henriksson, 2002). Therefore, as a natural next step we examined glucose uptake in differentiated C2C12 myotubes. Knockdown of FKBP51 in differentiated skeletal myotubes enhanced glucose uptake (Figure 2.3.4c). Importantly, inhibition of FKBP51 using SAFit2 similarly increased glucose uptake in C2C12 cells (Figure 2.3.4d). Thus the beneficial effects of FKBP51 inhibition on glucose homeostasis require enhanced insulin signaling.

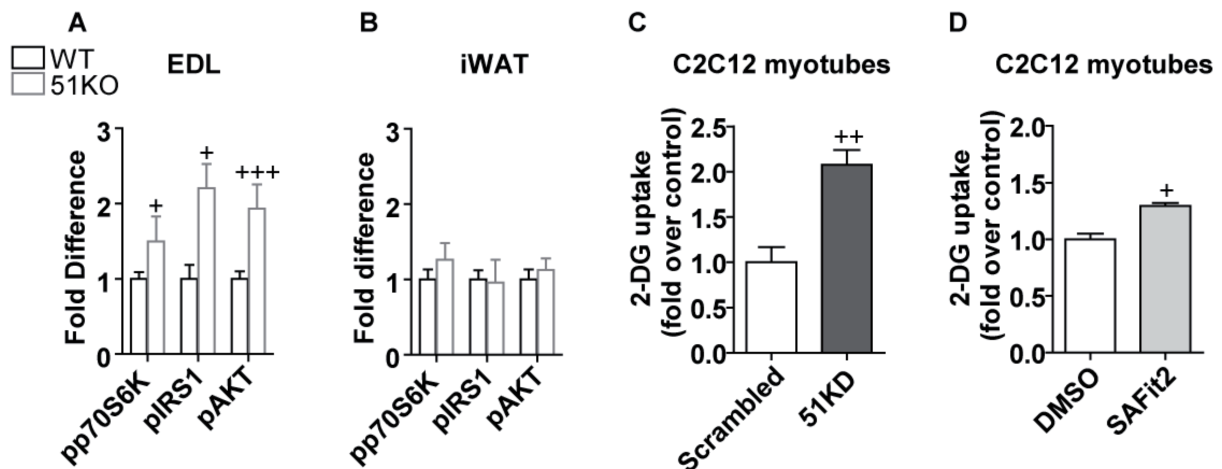


Figure 2.3.4 Loss of FKBP51 sensitizes insulin signaling and enhances glucose uptake

(a) In EDL skeletal muscle of 51KO mice, insulin signaling was enhanced compared to WT mice as assessed by pAKT ($T_{8.7}$ = -3.005, p = 0.015), pIRS1 (T_{16} = -2.660, p = 0.017), and pp70S670 (T_{16} = -4.491, p < 0.001) phosphorylated protein expression. (b) This outcome was specific for skeletal muscle, as phosphorylated protein expression was not changed in white adipose tissue (WAT) depots on account of genotype. (c) 2-deoxyglucose uptake is significantly enhanced in C2C12 myotubes in which FKBP51 has been knocked down (T_4 = 4.674, p = 0.009). (d) Similarly, FKBP51 blockade with SAFit2 significantly enhanced 2-deoxyglucose uptake in C2C12 myotubes (T_3 = 4.446, p = 0.021). Data are expressed as relative fold change compared to wild-type condition. $^{+}P < 0.05$, $^{++}P < 0.01$ $^{+++}P < 0.001$; + significant genotype/treatment effect.

2.3.5. Discussion

Here we describe for the first time that loss of FKBP51 in mice protects against diet-induced obesity and markedly lowers glucose tolerance under both control and HF diet conditions. The effects of FKBP51 on glucose homeostasis are driven by enhanced insulin signaling within skeletal muscle and consequently heighted glucose disposal. Moreover, administration of a recently developed selective antagonist of FKBP51 mimicked the metabolic phenotype arising from total genetic loss of FKBP51. FKBP51 blockade produced a modest body weight phenotype and a strong effect on glucose tolerance as early as 8 days following treatment onset. From these data, we conclude that FKBP51 is an integral component of energy and glucose homeostatic regulation, particularly in response to nutritional changes. The improved

metabolic outcomes following systemic administration of a selective antagonist of FKBP51, SAFit2, offers new opportunities for drug development.

The effects of FKBP51 on body weight are likely driven by increases in RMR without compensatory increases in energy intake. Nevertheless, the present study does not allow one to identify the mechanism underlying the increased RMR. Rather the focus of the current study was to characterize the striking association in humans between single nucleotide polymorphisms with the *FKBP5* gene loci and traits related to T2D (Pereira et al., 2014b). Our data highlight a fundamental role for FKBP51 in the regulation of glucose homeostasis. Glucose tolerance in 51KO is improved even in the absence of a strong body weight phenotype, under control diet settings. This suggests that the FKBP51-dependent improvement of glucose homeostasis is not secondary to body weight. In line with these findings, we found that loss of FKBP51 sensitizes the insulin signaling pathway. Interestingly, FKBP51-dependent effects on insulin signaling was highly tissue-specific, in which FKBP51-dependent increases in insulin signaling was limited to skeletal muscle. In this context, we also found that glucose uptake in mouse myotubes is enhanced on account of either FKBP51 knockdown or FKBP51 pharmacological blockade.

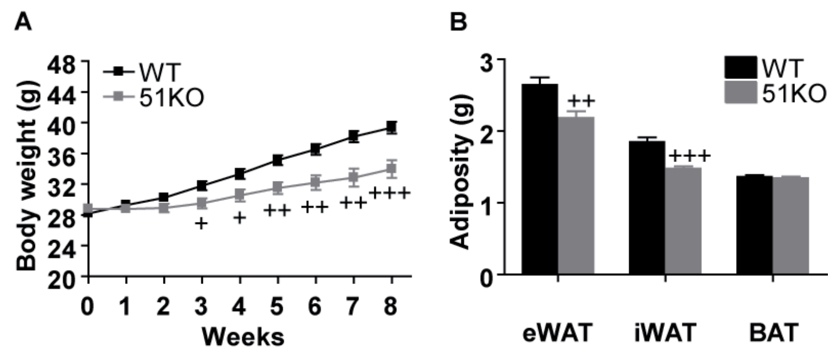
It has been previously shown that FKBP51 binds to AKT, a serine-threonine kinase within the insulin signaling cascade, to ultimately favor AKT inactivation (Pereira et al., 2014b). Through this interaction, FKBP51 was associated with tumor growth and chemotherapy responsiveness. We have extended such previous findings by demonstrating that not only are AKT and downstream targets of AKT regulated in an FKBP51-dependent manner (reflected by the phosphorylation states of AKT downstream effector proteins), but we have also demonstrated that FKBP51 exerts an effect on upstream signaling, whereby 51KO mice displayed enhanced activation of the insulin receptor substrate 1 (IRS-1). Within the insulin signaling pathway, the IRS-1 represents a critical node for the regulation of insulin sensitivity, and dysregulation at IRS-1 is often featured in T2D (Taniguchi et al., 2006). Tissue responsiveness to insulin in fact requires IRS-1 and IRS-2, which function to couple insulin receptor activation to the recruitment and activation of downstream signaling molecules including AKT (Copps and White, 2012). IRS-1 contains over 50 possible serine-phosphorylation sites, whose phosphorylation status governs IRS-1 signaling. We specifically examined phosphorylation of serine-307 (S307), which has been shown to promote insulin sensitivity in mice (Copps et al., 2010). Our data support this notion such that 51KO mice

present increased S307 IRS-1 phosphorylation in skeletal muscle which is accompanied by enhanced downstream insulin signaling and improved glucose tolerance.

An important question raised by this study is how exactly FKBP51 acts within distinct cellular/tissue environments to affect whole body energy and glucose homeostasis. Previous studies have already alluded to the importance of tissue-specific actions of FKBP51 (Hartmann et al., 2015;Toneatto et al., 2013). Here we extend our current understanding of the tissue-specific actions of FKBP51 to include FKBP51-dependent regulation of the insulin signaling exclusively within skeletal muscle. Despite microarray-based data demonstrating that skeletal muscle shows the second strongest expression profile of FKBP51 across all tissues examined (Su et al., 2004), to our knowledge, the current study is the first to define a skeletal muscle-specific role of FKBP51. These data, along with our FKBP51-dependent glucose uptake findings in cultured myotubes, suggest the possibility that targeted disruption of FKBP51 specifically within skeletal muscle may be sufficient to improve glucose homeostasis.

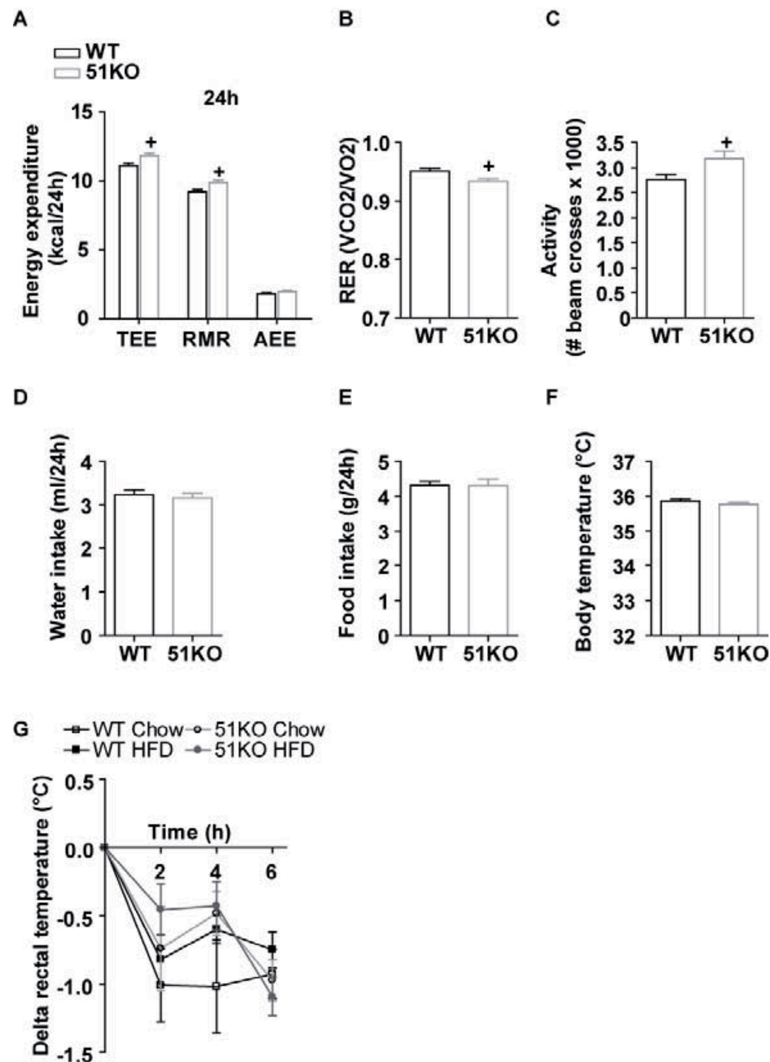
Together, these findings define a novel role of FKBP51-dependent regulation of glucose uptake and whole body glucose homeostasis. In addition, the positive effects of FKBP51 pharmacological blockade on both body weight and glucose tolerance suggests a potential opportunity to employ FKBP51 antagonists for the treatment of obesity and T2D.

2.3.6. Supplementary Data



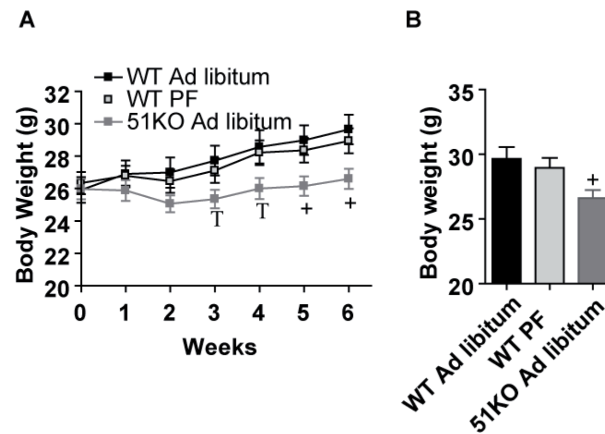
Supplementary Figure S 1 Genetic ablation of FKBP51 protects against diet-induced obesity under thermoneutral conditions

(a) Over 8 weeks of HF diet exposure at 30°C, 51KO mice are still resistant to HF diet-induced weight gain (Time x Genotype: $F(1, 21.2) = 18.567$, $p < 0.01$; Genotype: $F(1, 14) = 9.346$, $p = 0.009$) and (b) adiposity (eWAT: $T_{14} = 5.054$, $p < 0.001$; iWAT: $T_{14} = 3.313$, $p = 0.005$). Data are expressed as means ± s.e.m. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$; + significant genotype effect



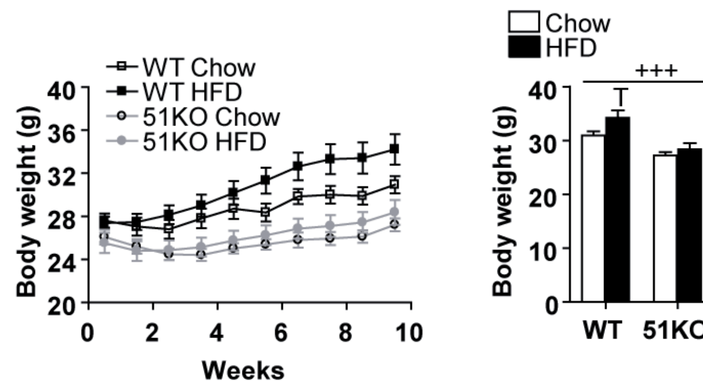
Supplementary Figure S 2 Metabolic phenotype in 51KO transgenic mice

Initially metabolic readouts were assessed in WT mice (n = 17) and 51KO mice (n = 16) under chow conditions in CLAMS (Comprehensive Lab Animal Monitor Systems). (a) When bodyweight was held constant, total energy expenditure (TEE) was higher in the chow-fed 51KO animals measured across 24h (Genotype: $F(1, 28) = 7.275$, $p = 0.012$). Decomposition of TEE into its resting (RMR) and activity-related (AEE) components revealed that the observed TEE difference was due to increased RMR in the 51KO animals measured over 24h (Genotype: $F(1, 27) = 7.495$, $p = 0.011$). Activity-related energy expenditure did not differ between genotypes as assessed across 24h (Genotype: $F(1, 27) = 1.288$, ns). (b) Loss of FKBP51 furthermore decreased the average respiratory exchange ratio (RER) activity (Genotype: $T25 = -2.507$, $p = 0.019$) and (c) increased the average home-cage (Genotype: $T27 = -2.301$, $p = 0.029$) assessed across 24h. There was however no effect of genotype on either water consumption (d), food intake (e) or body temperature (f) assessed across 24 h. (g) In order to ensure there was no effect on body temperature regulation, a separate cohort of mice were examined for cold-induced rectal temperature changes following exposure to 4°C under control and HF diet conditions. Repeated measures ANOVA revealed neither a genotype effect nor a diet effect on the ability to regulate body temperature following short-term (6h) cold exposure. +$P < 0.05$; + significant treatment effect.



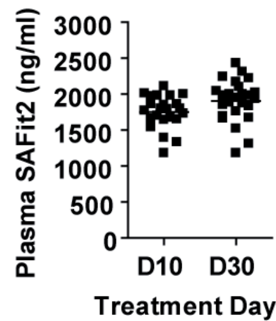
Supplementary Figure S 3 Food intake does not contribute to the diet-resistant phenotype in 51KO mice

A pair-feeding experiment was performed over 6 weeks to determine whether genotype-dependent differences in food intake exist. (a) At the experiment onset, all mice were placed on a HF diet. 51KO control and WT ad libitum mice had free access to food, whereas WT PF were pair-fed to the 51KO mice. The WT PF group continued to gain weight despite pair-feeding (Time x Experimental group: $F(1, 45.5) = 4.528$, $p = 0.006$). (b) At the end of the pair-feeding paradigm 51KO mice weighed significantly less compared to WT mice (Experimental group: $F(1, 28) = 4.410$, $p = 0.022$, Bonferroni correction). Data are expressed as means ± s.e.m. $^{+}P < 0.05$, $^{T}P < 0.1$; $^{+}$ 51KO mice are significantly different from WT control mice.



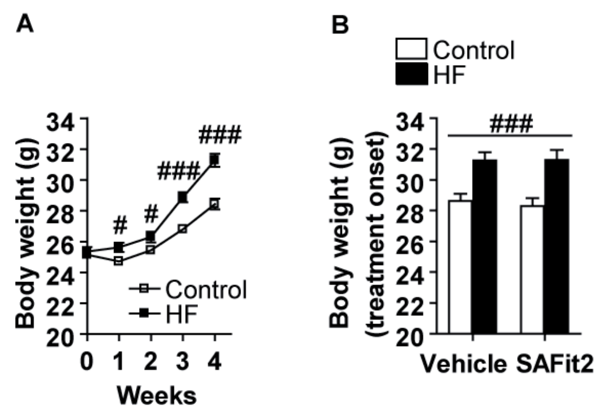
Supplementary Figure S 4 Genetic ablation of FKBP51 prevents HF diet-induced weight gain observed across 2 independent experiments

In an independent experiment used to assess glucose and insulin tolerance, 51KO mice presented the same body weight phenotype compared to WT mice as reported in Experiment 1. (a) 51KO mice weighed significantly less than WT mice throughout the 8 week dietary treatment (Time x Genotype: $F(1, 93.0) = 9.807$, $p < 0.001$; Genotype: $F(1, 41) = 8.057$, $p = 0.001$) and (b) at the experimental end (Genotype: $F(1, 42) = 10.262$, $p < 0.001$). Furthermore, a tendency was observed indicating that WT mice were susceptible to HF diet-induced weight gain compared to 51KO mice as interpreted from body weight progression (Time x Genotype x Diet: $F(1, 93.0) = 2.040$, $p = 0.087$). TP < 0.1, + ++P < 0.001; + significant treatment effect; T significant trend for diet.



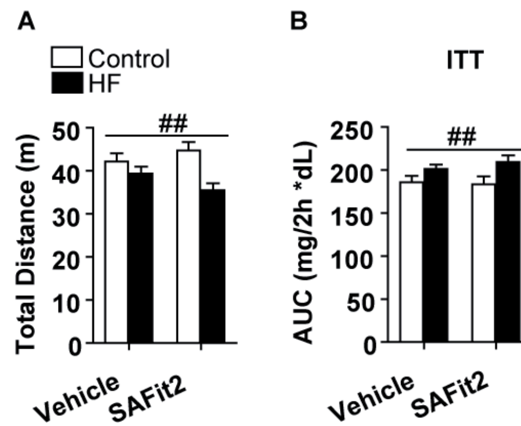
Supplementary Figure S 5 Plasma SAFit2 levels

Administration of SAFit2 by intraperitoneal injections resulted in high SAFit2 plasma levels and minimal inter-animal variability. SAFit2 was delivered twice daily at 20 mg/kg. Blood was collected by tail cut 5 h post-injection for the measurement of SAFit2 in all SAFit-treated mice (n = 24) Measurement was performed on day 10 and day 30 of treatment schedule. SAFit2-treated mice showed meaningful levels of plasma SAFit2.



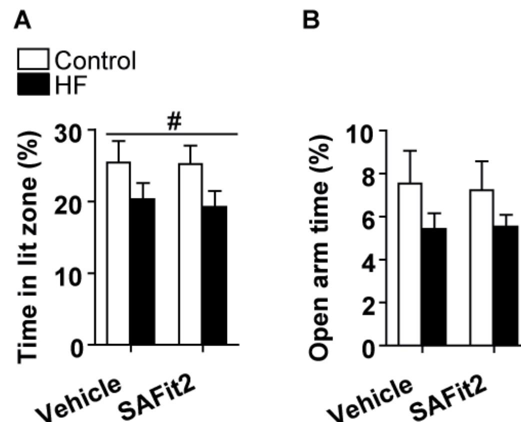
Supplementary Figure S 6 Body weight progression preceding SAFit2 treatment

4 weeks before SAFit2 treatment onset, mice were randomly assigned to either the control diet or HF diet group. (a) Exposure to the HF diet resulted in a significant increase in body weight progression (Time x Diet: $F(1, 121.2) = 10.250$, $p < 0.001$; Diet: $F(1, 47) = 22.397$, $p < 0.001$). (b) At treatment onset, mice were subdivided into treatment groups counterbalanced by body weight, and therefore within each dietary group there was no difference in body weight. Nevertheless, mice exposed to 4 weeks of HF diet weighed significantly more than control diet-fed mice (Diet: $F(1, 45) = 25.341$, $p < 0.001$). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$; # significant diet effect.



Supplementary Figure S 7 Administration of SAFit2 by intraperitoneal injections had no effect on locomotor activity or insulin tolerance

(a) SAFit2 treatment had no effect on total distance traveled in an open field, although HF diet exposure significantly reduced locomotor activity (Diet: $F(1, 45) = 10.942$, $p = 0.002$). (b) Insulin tolerance is expressed as the area under the glucose curve (AUC). Although insulin tolerance was significantly impaired by HF diet exposure, there was no improvement on account of SAFit2 treatment (Diet: $F(1, 43) = 8.376$, $p = 0.006$). Data are expressed as means ± s.e.m. $^{\#\#}P < 0.01$; # significant diet effect.



Supplementary Figure S 8 Administration of SAFit2 by intraperitoneal injections had no undesirable behavioural side effects

30-day treatment of SAFit2 had no effect on anxiety-like behavior examined in both the dark-light transition test (**a**) and the elevated plus maze test (**b**). By contrast, exposure to a HF decreased the time spent in the lit compartment of the dark-light test (Diet: $F(1, 45) = 4.817$, $p = 0.033$). Altogether these findings indicate that there are no undesirable behavioral outcomes on account of SAFit2 treatment. $^{\#}P < 0.05$; # significant diet effect.

Primary Antibody	Dilution	Phosphorylation site	Company
AKT2	1:1000	/	CST, Akt2 (D6G4) Rabbit mAb #3063
IRS1	1:1000	/	CST, IRS-1 (59G8) Rabbit mAb #2390
p70S6K	1:1000	/	CST, p70 S6 Kinase Antibody #9202
pAKT2	1:1000	S473	Cell Signaling, # 4058
pIRS1	1:1000	S307	CST, Phospho-IRS-1 (Ser307) Antibody #2381
pp70S6K	1:1000	T389	CST, Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb #

Supplementary Table S 1 List of all primary antibodies

Measurement	Figure	Experiment	Transformation
24h RER	S2	1 (cohort 1)	Reciprocal
24h Fecal Energy	S2	1 (cohort 1)	Lg10
Final body weight	1	1 (cohort 1)	Lg10
eWAT	1	1 (cohort 1)	Lg10
iWAT	1	1 (cohort 1)	Lg10
Fasted insulin	2	3	Lg10

Supplementary Table S 2 Data transformations

Data transformations used to generate data with homogeneous variance. Data represented in all Figures reflect the non-transformed data.

2.4. FKBP51 Enhances AMPK Inhibition of mTOR: A Preliminary Report

Gassen NC, Balsevich G, Dournes C, Hafner K, Chen A, Schmidt MV, Rein T

Manuscript in preparation

2.4.1. Abstract

Although 5'-AMP-activated protein kinase (AMPK) is an established regulator of cellular and whole body energy metabolism, its own regulation is incompletely understood. FK506 binding protein 51 (FKBP51) is known to interact with several serine/threonine kinases but whether it interacts with AMPK is unknown. Given the recently identified role of FKBP51 in whole body energy metabolism, we investigated whether FKBP51 regulates AMPK, and furthermore addressed whether FKBP51 is required for AMPK-mediated regulation of metabolic outcomes by employing metformin (an AMPK agonist) in wild-type (WT) and FKBP51 knockout (51KO) mice. We demonstrate that FKBP51 binds to various AMPK subunits and to the direct downstream target of AMPK, tuberin (TSC2), which is a negative regulator of mTOR. Interestingly, loss of FKBP51 impairs the ability of metformin to suppress downstream mTOR signaling in both in vitro and in vivo models. Nevertheless, metformin was equally effective at lowering fasting blood glucose in WT and 51KO mice. In conclusion, we establish FKBP51 as a novel constituent of the AMPK heterocomplex and consequently as a novel regulator of the AMPK-mTOR signaling axis.

2.4.2. Introduction

5'-AMP-activated protein kinase (AMPK) is a highly conserved serine (ser)/threonine (thr) kinase that acts as an energy sensor to regulate cellular and whole body energy metabolism (Hardie, 2015). As a heterotrimeric complex containing a catalytic α-subunit and regulatory β and γ subunits, AMPK is activated by an increase in the cellular AMP-to-ATP ratio and thus signals low energy status. Any stimulus, including stressors, glucose deprivation, muscle contraction, and exercise, that increases ATP consumption activates AMPK. Full activation of AMPK not only requires allosteric activation by 5'-AMP, but also requires phosphorylation on Thr-172 within the α-subunit by upstream kinases, including the ser/thr protein kinase LKB1 (liver kinase B1) (Lizcano et al., 2004). Once activated AMPK phosphorylates multiple downstream targets, whose concerted actions promote catabolic (ATP-generating) pathways, while inhibit anabolic (ATP-consuming) pathways. Therefore the appropriate regulation of AMPK and the upstream LKB1 kinase activity is critical for health and disease (Steinberg and Kemp, 2009).

LKB1 is active when in complex with Ste-20-related kinase adaptor (STRAD) and calcium-binding protein 39 (CAB39, also known as MO25α). Within the LKB1-STRAD-MO25 complex, LKB1 phosphorylates AMPK, increasing AMPK activation over 50-fold compared to AMP allosteric activation alone (Lizcano et al., 2004). The LKB1 complex is known to possess high basal activity, and thus its capacity to phosphorylate Thr-172 within the α-subunit of AMPK is regulated by the binding of AMP to AMPK, which induces a conformational change that favors LKB1-dependent Thr-172 phosphorylation (Hardie, 2015). However it is only recently that studies have identified that both the LKB1 complex itself and the LKB1-AMPK interaction are regulated by additional extrinsic proteins. In particular, it was demonstrated that Skp2-SCF ubiquitin ligase governs LKB1 activity by maintaining the LKB1-STRAD-MO25 complex integrity (Lee et al., 2015). Furthermore it has been shown that an adapter protein, axin, facilitates the interaction between the LKB1 complex and AMPK (Zhang et al., 2013). Whether additional, unidentified extrinsic proteins regulate LKB1-AMPK signaling remains unclear.

The mammalian target of rapamycin (mTOR) is a ser/thr protein kinase that lies downstream of AMPK signaling (Inoki et al., 2012). The mTOR signaling cascade acts as a key integrator for nutrient and growth factor signals. In contrast to AMPK, mTOR activity favors catabolic metabolism. The AMPK-mTOR signaling pathways are intimately connected through the tuberous sclerosis complex (TSC), comprising TSC1 (hamartin) and TSC2 (tuberin)

(Wullschleger et al., 2006). AMPK phosphorylates Ser-1387 within the TSC2 protein to activate the TSC1/2 complex (Inoki et al., 2003b). In turn the activated complex acts indirectly to inhibit mTOR (Inoki et al., 2003a). Taken together, the balance between AMPK and mTOR signaling is important for maintaining cellular and whole body energy homeostasis.

We (unpublished data, Chapter 2.3) and others (Pereira et al., 2014b) have identified the FK506 binding protein 51 (FKBP51) as a novel molecule in the regulation of whole body energy metabolism. FKBP51 is an immunophilin protein that is best characterized as a co-chaperone to the Hsp90 complex and a regulator of steroid receptor signaling (Riggs et al., 2003;Wochnik et al., 2005). FKBP51 has also been shown to directly bind several ser/thr protein kinases to ultimately affect their signaling cascade (Gassen et al., 2015;Pei et al., 2009). For example, Pei et al. reported that FKBP51 acts as a scaffolding protein between the ser/thr protein kinase, AKT, and a negative regulator of AKT, PHLPP to ultimately inhibit AKT signaling (Pei et al., 2009). Interestingly, systematic characterization of the interaction network of various co-chaperone proteins, including FKBP51, described LKB1 as one of the strongest binding partners of FKBP51 (Taipale et al., 2014). Nevertheless, this interaction was not characterized any further. Based on the ability of FKBP51 to interact with LKB1, several additional ser/thr protein kinases, and due to its association with whole body energy metabolism, we sought to examine whether FKBP51 regulates the AMPK-mTOR signaling axis.

2.4.3. Methods

Cell lines and Culture Conditions

Human embryonic kidney cells (HEK-293; ATCC, CRL-1573) and mouse embryonic fibroblasts (MEFs) were cultured in DMEM (Gibco) supplemented with 10% FCS and 100 units/ml penicillin and streptomycin at 37°C and 5% CO_2. FKBP51 knockout (51KO) MEFs were derived from 51KO mice and have been previously described (Touma et al., 2011). Cells were passaged by trypsinization once the cells had reached ~80% confluency.

Co-Immunoprecipitation (coIP)

Immunoprecipitations of endogenous proteins were performed in HEK-293 cells. Briefly, the cells were lysed in coIP buffer containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1mM EDTA, 0.5% Igepal complemented with protease inhibitor cocktail (Sigma, P2714), followed by incubation for 20 min at 4 °C while shaking on an overhead shaker. To clear the lysate,

samples were centrifuged (14,000xg for 20 min at 4°C) and protein concentrations were determined. 500 µg of lysate was incubated overnight with 2 µg of the appropriate IP-antibody (antibodies for each AMPK-subunit) (See Table 1) at 4 °C. 20 µL of protein G dynabeads (Invitrogen, 100-03D) was blocked with bovine serum albumin and subsequently added to the lysate-antibody mix and allowed to incubate at 4 °C for 3 h in order to mediate binding between the dynabeads and the antibody-antigen complex of interest. The beads were washed three times with ice-cold phosphate-buffered saline (PBS). The protein-antibody complexes were eluted with 60 µL Laemmli loading buffer. Thereafter the eluate was boiled for 5 min at 95 °C. 2-5 µL of each immunoprecipitate reaction product was separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. For assessing protein complexes, immunoblotting against FKBP51 was performed.

Co-immunoprecipitations (CoIPs) of FLAG-tagged TSC2 with endogenous FKBP51 was performed in HEK-293 cells. $5x10^6$ cells were electroporated with 5 µg of a FLAG-tagged TSC2 expression plasmid using a GenePulser (Bio-Rad) at 350 V/700 mF in 400 ml of electroporation buffer (50 mM, K2HPO4/KH2PO4, 20 mM KAc [pH 7.35], 25 mM MgSO4). Untransfected cells (without ectopically expressed FLAG-tagged protein) were processed in parallel as a control. Following 3 d of cultivation in DMEM/10% FCS, cells were lysed in CoIP buffer, and co-immunoprecipitation experiments using the FLAG antibody were performed followed by Western blot detection with anti-FKBP51 and anti-FLAG antibodies as described above.

Western Blot Analysis

Protein extracts were obtained by lysing cells or tissue in lysis buffer containing 62.5 mM Tris, 2% sodium dodecyl sulfate and 10% sucrose, completed with protease inhibitor cocktail (Sigma, P2714) and phosphatase inhibitor (Roche, 04906837001). After sonication and heating for 5 min at 95 °C, samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes by electrotransfer. Membranes were blocked in Tris-buffered saline, supplemented with 0.05% Tween (Sigma, P2287) and 5% non-fat milk for 1 h at room temperature. Incubation with primary antibodies was carried over night at 4 °C. Subsequently, membranes were washed and probed with respective fluorophore- or horseradish peroxidase-conjugated secondary antibody for 3-5 h at room temperature. Detectable, immune-reactive bands were visualized either by excitation of the respective fluorophore or by using ECL reagent (Millipore, WBKL0500). Detection and quantification of band intensities were performed

with ChemiDoc MP (BioRad). For quantification of phospho-specific antibody signals, the intensity was always referred to the signal intensity of total antibodies.

Antibodies

For a complete list of primary antibodies, refer to Table 1. Secondary antibodies were donkey anti-goat immunoglobulin G, Alexa Flour 488 (Life Technologies, A-11055, 1:7000), anti-rabbit immunoglobulin G, horseradish peroxidase-linked (Cell Signaling Technologies, #7074, 1:10000) and anti-mouse immunoglobulin G, horseradish peroxidase-linked (Cell Signaling Technologies, #7076, 1:10000).

Table 2.4.1 List of all primary antibodies

Primary Antibody	Dilution	Phosphorylation site	Company
4EBP1		/	CST, 4E-BP1 (53H11) Rabbit mAb #9644
AMPK-alpha1	1:1000	/	CST, AMPK Subunit Antibody Sampler Kit #9839
AMPK-alpha2	1:1000	/	CST, AMPK Subunit Antibody Sampler Kit #9839
AMPK-beta1	1:1000	/	CST, AMPK Subunit Antibody Sampler Kit #9839
AMPK-beta2	1:1000	/	CST, AMPK Subunit Antibody Sampler Kit #9839
AMPK-gamma1	1:1000	/	CST, AMPK Subunit Antibody Sampler Kit #9839
AMPK-gamma2	1:1000	/	CST, AMPK Subunit Antibody Sampler Kit #9839
AMPK-gamma3	1:1000	/	CST, AMPK Subunit Antibody Sampler Kit #9839
Beclin1	1:1000	/	CST, Beclin-1 (D40C5) Rabbit mAb #3495
FKBP51	1:1000	/	Bethyl, A301-430A
FLAG	1:7000	/	Rockland, 600-401-383
LC3B-I/II	1:1000	/	Cell Signaling, # 2010
p4EBP1	1:1000	T37/46	CST, Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb #2855
p70S6K	1:1000	/	CST, p70 S6 Kinase Antibody #9202
pBeclin1	1:1000	S234 and S295	Phosphosolutions, #p117-234 and #p117-295
pp70S6K	1:1000	T389	CST, Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb #9234
pTSC2	1:1000	S1387	CST, Phospho-Tuberin/TSC2 (Ser1387) Antibody #5584
TSC2	1:1000	/	CST, Tuberin/TSC2 (D57A9) Rabbit mAb #3990

Metformin Treatment in MEF Cells

MEFs derived from 51KO and WT mice were cultivated in a 6-well plate until they reached 90% confluency. 2 mM metformin hydrochloride (Sigma–Aldrich, St. Louis, USA) was subsequently added to the culture media for 48 h. Cells were lysed in and protein extracts were prepared and analyzed by Western blot with pTSC2, p4EBP1, and Beclin1 antibodies.

Quantification of Protein Data

Protein data were normalized to actin for cell culture experiments and to HSC70 for tissue lysates. For normalization actin or HSC70 were detected on the same blot. Data are reported as fold change relative to wild-type condition.

Animal Studies

To determine whether FKBP51 affects AMPK-dependent regulation of mTOR signaling *in vivo*, metformin, an agonist of AMPK (Zhou et al., 2001), was administered for 6 weeks to wild-type (WT) and FKBP51 knockout (51KO) mice. Briefly, 51KO (n = 5) and WT (n = 5) mice had ad libitum access to standard lab chow and tap water with metformin. Metformin hydrochloride (Enzo Life Sciences, Lörrach, Germany) was chronically administered to mice orally through drinking water (2mg/ml, ~300 mg/kg daily). The mice received fresh metformin solution twice weekly. Following 6 weeks of metformin treatment, tissues (hypothalamus, epididymal (e) white adipose tissue (WAT) inguinal WAT (iWAT), and soleus muscle) were collected for protein analyses.

To determine whether FKBP51 is required for metformin's glucose-lowering effects, metformin was administered for 6 weeks to an independent cohort of WT and 51KO mice in a 2x2 experimental design. Briefly, 51KO and WT mice were subdivided into a control and metformin treatment group (n=11-13 per group). Mice had ad libitum access to standard lab chow and tap water with or without metformin. Metformin was chronically administered to mice orally as described above. In the final (6th) week of metformin treatment, fasting blood glucose was measured. Body weight, food intake, and fluid intake were measured regularly throughout the entire treatment schedule.

Fasting Blood Glucose

In order to assess fasting blood glucose, mice were fasted overnight for 14 h. The following morning, a tail cut was made, and blood glucose was measured using a handheld Contour XT glucometer (Bayer Health Care, Basel, Switzerland).

Statistical Analysis

Data were analyzed using IBM SPSS Statistics 18 software (IBM SPSS Statistics, IBM, Chicago, IL, USA). Univariate ANOVA with genotype and metformin treatment as fixed factors was used to analyze fasting blood glucose. Student's T-tests were used to assess the genotype effect on $pTSC2^{S1387}$ expression across tissues. Repeated measures ANOVA was used for all body weight progression, food intake, and fluid intake data, in which the degrees of freedom were corrected for deviance from sphericity (Greenhouse–Geisser) for within-subjects comparisons. Statistical significance was set at $p < 0.05$. Data are presented as the mean +/- S.E.M.

2.4.4. Results

FKBP51 Interacts with AMPK Subunits and TSC2

Based on the combined observations that FKBP51 interacts with various ser/thr kinases (Gassen et al., 2015;Pei et al., 2009;Taipale et al., 2014) and FKBP51 regulates energy homeostasis (Chapter 2.3), we initially examined whether FKBP51 interacts with AMPK, the principal energy sensor of the cell. The current study demonstrates that FKBP51 binds to multiple subunits of AMPK, with the strongest interaction detected between FKBP51 and the β2 regulatory subunit (Figure 2.4.1a). Furthermore, FKBP51 additionally binds to TSC2 (Figure 2.4.1b), a direct downstream target of AMPK and the major negative regulator of mTOR (Inoki et al., 2003b).

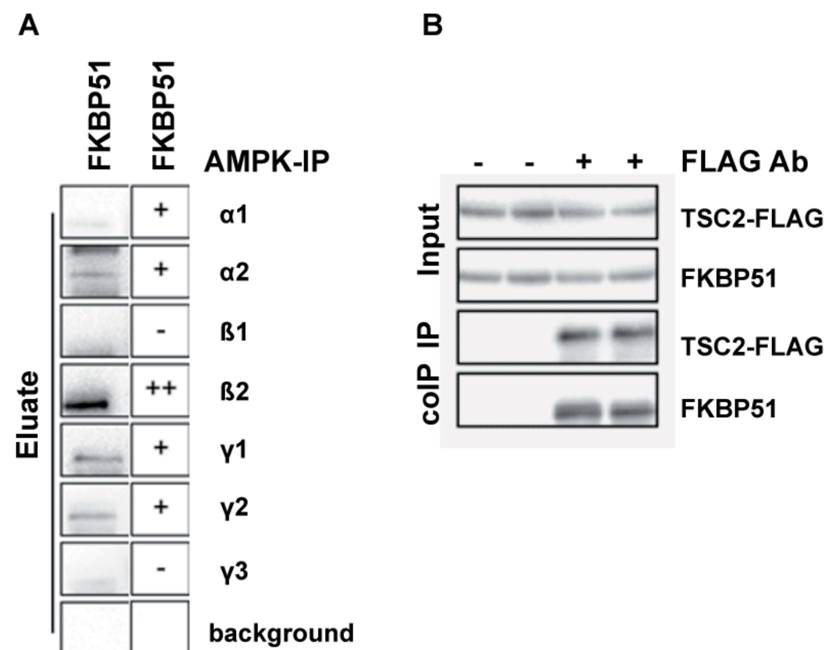


Figure 2.4.1 FKBP51 interacts with AMPK subunits and TSC2

(a) Immunoprecipitation (IP) of protein complexes was performed using antibodies for each of the AMPK subunits (α1, α2, β1, β2, γ1, γ2, and γ3). Co-precipitated proteins were visualized by Western blotting. FKBP51 co-precipitated with select AMPK subunits (+) but not with others (-). (b) HEK cells were transfected with vector control or a FLAG-tagged TSC2-expressing plasmid and lysed 72 h later. After immunoprecipitation (IP) of protein complexes using a FLAG antibody, input and co-precipitated proteins were visualized by Western blotting. FKBP51 co-precipitated with TSC2-FLAG.

Metformin Action on mTOR Signaling Requires FKBP51

In order to establish whether the interaction between FKBP51, AMPK subunits, and TSC2 is functionally relevant, we sought to examine mTOR signaling in response to metformin,

whose mode of action includes the activation of AMPK (Foretz et al., 2014). We assessed mTOR activity in MEFs derived either from 51KO or WT mice in order to determine whether FKBP51 directs AMPK regulation of mTOR signaling. We found that metformin treatment led to an apparent increase in the level of $pTSC2^{S1387}$, an AMPK-specific phosphorylation site within TSC2 in WT MEFs but not in 51KO MEFs (Figure 2.4.2). More striking was the FKBP51-dependent metformin effect on downstream signaling. $p4EBP1^{T37/46}$ and Beclin1 expression served as readouts for mTOR activity given mTOR directly phosphorylates 4EBP1 at Thr- 37 and 46, whereas inhibits autophagy as reflected in the levels of the autophagy initiator protein, Beclin1 (Figure 2.4.3) (Wullschleger et al., 2006). In particular, Thr-37/46 phosphorylation on 4EBP1 was drastically decreased whereas Beclin1 expression was drastically increased in WT but not 51KO MEFs from metformin treatment. In fact, in 51KO-derived MEFs, the expression of (phospho-) proteins along the mTOR signaling pathway was rather uniform between metformin-treated and untreated cells.

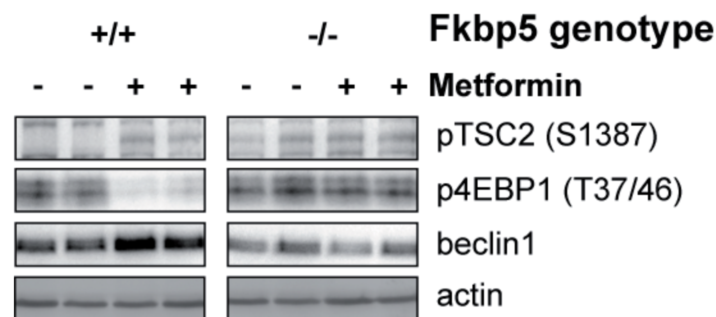


Figure 2.4.2 FKBP51 is required for metformin action on mTOR signaling in mouse embryonic fibroblasts (MEFs)

mTOR signaling was examined in MEFs derived from WT or 51KO mice with or without 48 h treatment with metformin. Metformin treatment significantly enhanced S1387 phosphorylation of TSC2 in WT but not 51KO MEFs. More striking, metformin treatment significantly lowered the phosphorylation of T37/46 on 4EBP1 (which are directly phosphorylated by mTOR) exclusively in WT MEFs. Metformin-mediated inhibition of mTOR signaling is equally observed downstream of mTOR at the level of autophagy (reflected by Beclin1 expression). Again this is exclusively observed in WT MEFs. 51KO MEFs are not sensitive to metformin effects at any level examined

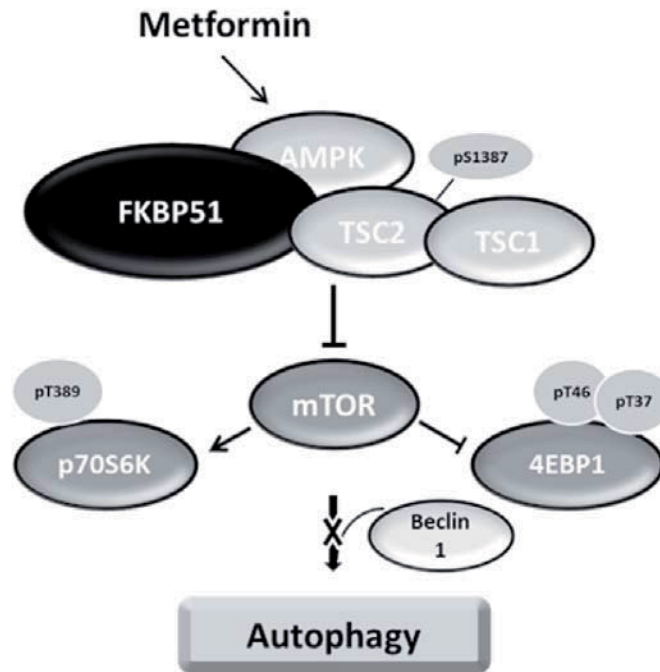


Figure 2.4.3 Model of FKBP51-dependent regulation of the AMPK-mTOR signaling axis

FKBP51 interacts with AMPK and TSC2 to govern downstream mTOR signaling. The interactions between FKBP51-AMPK and FKBP51-TSC2 favor AMPK-mediated TSC2 phosphorylation at S1387 and thus TSC2 activation. Activated TSC2, within the TSC1-TSC2 complex, inhibits mTOR signaling, which is reflected in the phosphorylation states of mTOR direct targets, p70S6K (at residue T389) and 4EBP1 (at residues T37 and T46). FKBP51-dependent regulation of the AMPK-mTOR signaling is equally evident downstream of mTOR at the level of autophagy in which inhibition of mTOR ultimately favors autophagic events, as indicated by increased Beclin1 levels, a known autophagy initiator protein.

In order to investigate whether FKBP51 affects AMPK-dependent regulation of mTOR signaling in vivo, we stimulated AMPK signaling using metformin in WT and 51KO mice. Analysis of $pTSC2^{S1387}$ in tissues collected from metformin-treated WT and 51KO mice revealed that 51KO mice had significantly reduced levels of $pTSC2^{S1387}$ in comparison to WT mice in all tissues examined except for eWAT (eWAT: $T_8 = 1.540$, $p = 0.162$; iWAT: $T_8 = 2.441$, $p = 0.040$; Hypothalamus: $T_8 = 3.038$, $p = 0.016$; Soleus muscle: $T_8 = 3.969$, $p = 0.004$) (Figure 2.4.4).

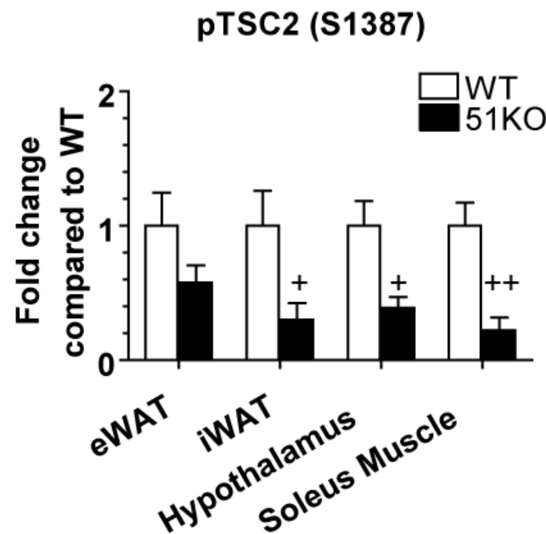


Figure 2.4.4 FKBP51 affects AMPK-dependent regulation of mTOR signaling in vivo

WT and 51KO mice were treated with metformin in order to stimulate AMPK. $pTSC2^{S1387}$ was assessed as a readout for AMPK-dependent regulation of mTOR. With the exception of epididymal white adipose tissue (eWAT), 51KO mice presented significantly reduced $pTSC2^{S1387}$ expression across all tissues examined. Data are presented as the mean +/- s.e.m. +P < 0.05, ++P < 0.01; + significant genotype effect.

Metformin Glucose-Lowering Action is Independent of FKBP51

Based on our findings suggesting that FKBP51 is required for metformin action on mTOR signaling, we sought to examine the efficacy of metformin treatment in 51KO and WT mice. Metformin had no effect on body weight gain, food intake, or fluid intake measured over the entire treatment period, independent of genotype (Figure 2.4.5a – c). Nevertheless, a significant Time x Genotype interaction revealed that 51KO mice presented slower body weight gain compared to WT over the 6 week experimental period (Time x Genotype: $F(1, 113.1) = 2.941$, $p = 0.044$). By contrast, metformin treatment significantly decreased fasting glucose (Treatment: $F(1,41) = 5.101$, $p = 0.029$) (Figure 2.4.5d). The effect of metformin on fasting glucose was independent of genotype.

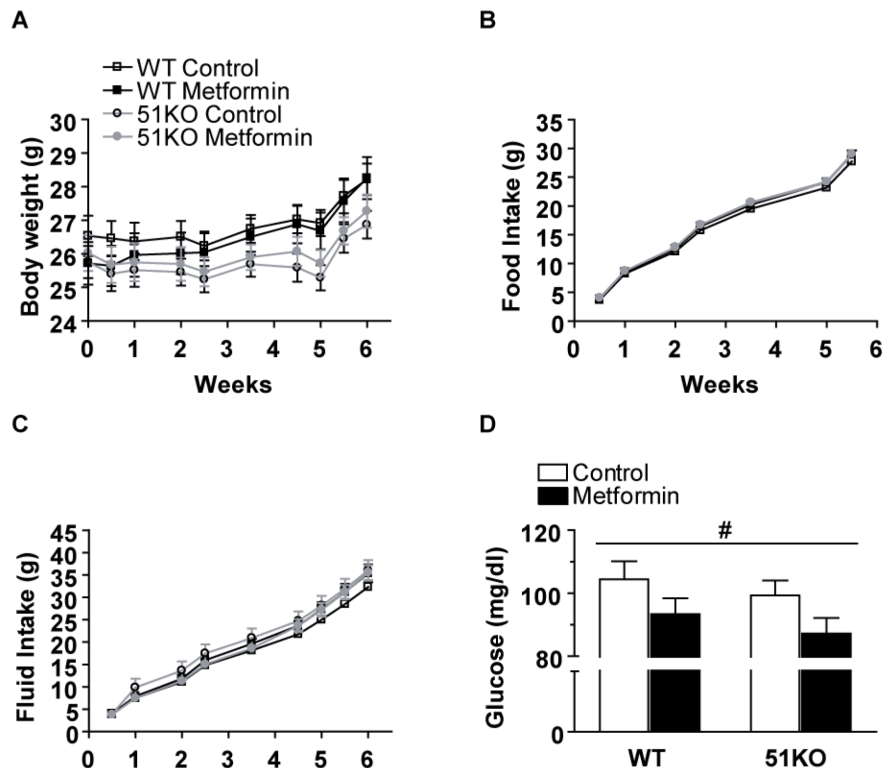


Figure 2.4.5 Metformin lowers fasting blood glucose independent of genotype

Oral administration of metformin for 6 weeks to WT and 51 KO mice had no effect on body weight gain (a), food intake (b) or fluid intake (c), independent of genotype. 51KO mice nevertheless presented slower body weight gain compared to WT mice. Finally, metformin treatment significantly lowered fasting blood glucose, regardless of genotype (d). Data are presented as the mean +/- s.e.m. #P < 0.05; # significant metformin treatment effect.

2.4.5. Discussion

The results from the current study demonstrate that FKBP51 is important for AMPK-dependent regulation of mTOR signaling. We have demonstrated that FKBP51 is found in complex with AMPK and TSC2. Interestingly this interaction appears to have functional consequences. Specifically, MEFs lacking FKBP51 were unresponsive to metformin-mediated regulation of mTOR signaling. In fact, the FKBP51-dependent effects of metformin were evident all the way down to the level of autophagy. In line with these findings, metformin-treated 51KO mice presented less activation of TSC2 ($pTSC2^{S1387}$), the principal negative regulator of mTOR, and consequently increased activation of mTORC signaling (assessed from levels of phosphorylated p70 ribosomal S6 kinase (pp70S6K) at thr-389 ($pp70S6K^{T389}$)) compared to metformin-treated WT mice. Nevertheless, chronic metformin treatment reduced fasting blood glucose in both WT and 51KO mice, which suggests that metformin is still able to mediate its glucose-lowering effects independent of FKBP51. Taken

together we identify FKBP51 as a novel constituent of the AMPK heterocomplex, a novel regulator of the AMPK-mTOR signaling axis, and an important determinant in the cellular response to the widely used anti-diabetic drug metformin.

AMPK is a heterotrimeric complex, in which all three subunits are essential for significant kinase activity (Dyck et al., 1996). In mammals several isoforms for each subunit exist. In particular, there are two α-subunits, three γ-subunits, and two β-subunits (α1, α2, γ1, γ2, γ3, β1, and β2) (Krishan et al., 2015). Each isoform shows tissue-specific expression patterns. Importantly, the various isoforms present different substrate preferences, suggesting that distinct isoforms may confer distinct functional properties (Sinnett and Brenman, 2014;Woods et al., 1996). Interestingly, our co-immunoprecipitation experiments revealed that FKBP51 preferentially binds certain AMPK subunits. For example, FKBP51 showed no binding to β1 or γ3 subunits. These results support the literature that has found substrate preference between isoforms (Sinnett and Brenman, 2014;Woods et al., 1996). Importantly, the specificity of interaction between FKBP51 and distinct AMPK isoforms suggests that FKBP51 is able to modulate specific AMPK signaling pathways while leaving others unperturbed. If we consider the β regulatory subunit, FKBP51 strongly binds β2, whereas does not bind the β1 subunit. Interestingly, whereas β1 is highly expressed in liver, β2 is highly expressed in skeletal muscle (Thornton et al., 1998). This suggests that FKBP51 may be able to modulate muscle-specific AMPK signaling cascades, whereas leave liver-specific AMPK signaling unperturbed. In support of the current hypothesis, FKBP51 is highly expressed in mouse skeletal muscle, but is not expressed in mouse liver (unpublished data).

LKB1 is recognized as a key upstream AMPK-activating kinase. As a ser/thr protein kinase, LKB1 phosphorylates AMPK at Thr-172, which in turn phosphorylates numerous substrates regulating diverse metabolic processes. A previous study identified a strong interaction between LKB1 and FKBP51 (Taipale et al., 2014), but no further investigation was performed. Here we show that FKBP51 exists in complex with AMPK and downstream effectors of AMPK, thereby determining the signaling fate of AMPK activation. Given the multiple downstream effectors which AMPK targets, the ability to direct AMPK action towards select targets and to furthermore organize specific protein complexes (including both upstream regulators and downstream effectors) has significant implications. It is important to establish whether additional downstream effectors of AMPK are also interacting partners of FKBP51. Regardless, the past study identifying LKB1 as an interaction partner of FKBP51

and the current study identifying AMPK and TSC2 as interaction partners of FKBP51, indicate an important regulatory role for FKBP51 along this signaling axis.

FKBP51 gene expression is highly responsive to the environment. For example, FKBP5 expression is responsive to stress, dexamethasone administration, as well as to the dietary condition (Balsevich et al., 2014;Guidotti et al., 2013;Pereira et al., 2014b;Scharf et al., 2011;Vermeer et al., 2003). In this regard, it is tempting to speculate that FKBP51 may facilitate spatio-temporal signaling cascades in response to environmental challenges in order to re-establish homeostasis. Indeed the environmental context is also very important for AMPK, which is recognized as the cellular sensor of energy status. Taken together, we hypothesize that FKBP51 governs downstream AMPK targets in response to specific environmental stimuli. Future work should address to what extent the FKBP51-dependent regulation of AMPK relies on the environmental context.

Metformin is the first-line oral treatment for patients with type 2 diabetes. Although its mode of action is not entirely understood, metformin is known to activate AMPK, and metformin-induced AMPK activation is an essential entity to its therapeutic effects (Foretz et al., 2014). As an antidiabetic agent, metformin is known to combat hyperglycemia without inducing hypoglycemia or weight gain. Therefore we investigated whether the glucose-lowering capacity of metformin depends on FKBP51. We observed no effect of metformin on body weight gain or food intake. However, we observed the expected metformin-mediated reduction in fasting glucose. Interestingly, this effect was independent of the FKBP51 genotype, suggesting that metformin is able to act independent of FKBP51 to regulate glucose homeostasis. Based on our findings that FKBP51 does not regulate AMPK per se, but rather facilitates the interaction between AMPK and its downstream target TSC2 to ultimately govern mTOR signaling, it is not surprising that metformin is still able to reduce fasting glucose levels. In fact the ability of metformin to counteract hyperglycemia has been largely ascribed to non-mTOR signaling pathways (Foretz et al., 2014). Nevertheless, FKBP51-directed AMPK regulation of mTOR signaling is highly relevant and warrants further investigation into its impact on (patho-) physiological outcomes.

Limitations

The current study is largely observational and serves as a starting point for continued analysis of FKBP51-dependent regulation of the AMPK-mTOR signaling axis. In particular, quantification analyses of AMPK-mTOR signaling are required with additional controls to show the specificity of metformin action on the AMPK-mTOR signaling axis and not simply

on all downstream targets. Another limitation is the design of the AMPK stimulation in WT and 51KO mice using metformin in order to assess the AMPK-mTOR signaling axis. Specifically, we did not include a non-stimulated (metformin-free) condition. Studies are currently underway in order to overcome these limitations. Nevertheless the current study warrants further investigation into the novel FKBP51-dependent regulation of the AMPK-mTOR signaling axis.

Conclusion

In the past few years, studies have identified a number of novel interacting partners of FKBP51. In this sense, FKBP51 acts as a scaffolding protein to facilitate the interaction between signaling molecules and their regulators in order to concentrate and thus functionally regulate such molecules. The current study establishes a novel interaction between FKBP51, AMPK, and the regulator of mTOR, TSC2. Furthermore, it was already known that an upstream regulator of AMPK, LKB1 is one of the strongest interacting partners of FKBP51. Taken together, FKBP51 is in a prime location to regulate AMPK signalling, both upstream and downstream, through direct protein-protein interactions.

2.5. FKBP51 Acts in the Hypothalamus to Regulate Body Weight

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Manuscript in preparation

2.5.1. Abstract

FK506 binding protein 51 (FKBP51, encoded by the *FKBP5* gene) has been identified as a susceptibility gene for stress-related disorders. We and others have recently found a novel role for FKBP51 in whole body energy homeostasis. However, the broad expression profile of FKBP51 across peripheral tissues and the central nervous system has hampered attempts to define the exact mode of action in which FKBP51 contributes to energy homeostasis. In order to isolate the hypothalamic action of FKBP51 on metabolic regulation, we overexpressed FKBP51 within the hypothalamus using adeno-associated viral vector-mediated *Fkbp5* gene transfer. FKBP51 overexpression within the hypothalamus resulted in decreased body weight gain as early as 3 weeks post-surgery compared to control viral-injected mice. The reduction in body weight gain arising from hypothalamic FKBP51 overexpression persisted for an additional 6 weeks until the end of the experiment under both chow and high fat diet conditions. The lowered body weight phenotype observed in hypothalamic FKBP51-overxpressing mice was accompanied by hypophagia. Double in situ hybridization revealed that *Fkbp5* mRNA is co-expressed in both *Pomc*- and *Npy*-containing neurons, suggesting possibly multiple sites of action for FKBP51 across the hypothalamus. Collectively, the current findings implicate FKBP51 action within the hypothalamus as a determinant of body weight and food intake, in which NPY- and POMC-containing neurons are both plausible sites of FKBP51 action.

2.5.2. Introduction

Obesity is a major health concern, and is a recognized risk factor for cardiovascular disease, atherosclerosis, and type 2 diabetes (Friedman, 2009). Although significant progress has been made to elucidate biological mechanisms of energy balance and related diseases, its entirety is poorly understood. Body weight regulation is a polygenic trait, and involves the interplay between environmental and genetic factors (Barsh et al., 2000). Identification of novel genetic targets provides a means to understand novel regulatory mechanisms in energy homeostasis and new therapeutic potentials.

FK506 binding protein (FKBP51, encoded by the *FKBP5* gene) is a member of the immunophilin protein family, and was first identified in HeLa cells as a component of the Hsp90-progesterone complex (Smith et al., 1993). Since then, additional functions of FKBP51 have been identified and include the regulation of steroid hormone receptors, distinct kinases and transcription factors, the microtubule-associated protein Tau, as well as autophagic events (Gassen et al., 2014;Jiang et al., 2008;Jinwal et al., 2010;Pei et al., 2009;Storer et al., 2011). Through its pleiotropic regulatory properties, FKBP51 has been implicated in the etiology of stress-related psychiatric disorders, various cancers, and neurodegenerative disorders (Schmidt et al., 2012). Convincing evidence suggests that tissue/cell-type specific expression may be associated with specialized physiological roles for FKBP51. For example, FKBP51 is upregulated in certain cancer types, namely melanoma, brain cancers, prostate cancer, lymphoma, and head and neck cancer, whereas downregulated in other cancer types, including pancreatic cancer, colon cancer, and testicular cancer (Li et al., 2011). The differential FKBP51 expression profile amongst various cancer types has been attributed to tissue-specific FKBP51-dependent regulation of distinct signaling pathways. Recently, we and others have furthermore demonstrated a novel role for FKBP51 in metabolic regulation (unpublished data, refer to Chapter 2.3, (Pereira et al., 2014b)). Specifically, we have shown that genetic ablation of FKBP51 in mice protects against diet-induced obesity and systemic glucose intolerance (unpublished data, refer to Chapter 2.3). Nevertheless, the broad expression profile of FKBP51 across various tissues (Su et al., 2004) has hindered the ability to define the exact mode of action in which FKBP51 contributes to disease etiology. Taken together, FKBP51 has several potential sites of regulation within the context of whole body energy metabolism.

Regulation of whole body energy homeostasis requires the concerted actions of multiple tissues. There is significant crosstalk between peripheral organs, which convey peripheral

information regarding the energy status of the body, and the central nervous system, which is responsible for sensing and integrating these peripheral signals, in order to mount a response (Dietrich and Horvath, 2013).Within the central nervous system, the hypothalamus contains cell populations intimately involved in the maintenance of energy intake and energy expenditure and consequently the regulation of body weight (Gao and Horvath, 2007). In more detail, the hypothalamus is comprised of several nuclei, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the ventromedial hypothalamic nucleus (VMH), and the dorsomedial hypothalamic nucleus (DMH) which have all been implicated in the regulation of body weight (Gao and Horvath, 2007). Perhaps the most recognized network involved in body weight regulation is the melanocortin system, consisting of two subsets of neurons, POMC- and NPY- containing neurons within the arcuate nucleus and their projections. Both sets of neurons are able to respond to peripheral cues which reflect energy status. For example, leptin, which circulates at levels proportional to the degree of adiposity, activates POMC neurons whereas inhibits NPY activity. Interestingly, POMC and NPY have opposing effects on food intake and body weight regulation, whereby POMC is a recognized anorexigenic neuropeptide whereas NPY is rather an orexigenic neuropeptide. Therefore, given the fundamental role of the hypothalamus in energy homeostasis, we sought to identify the specific role of hypothalamic FKBP51 on body weight regulation.

2.5.3. Methods

Animals & Animal Housing

Male C57BL/6N mice between 3-4 months were used (Charles River Laboratories, Maastricht, Netherlands). Mice were maintained on a 12:12hr light/dark cycle, with controlled temperature (22 +/- 2°C) and humidity (55+/- 5%) conditions. Mice had ad libitum access to water and standard lab chow, unless otherwise specified. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental Design

To assess the contribution of hypothalamic FKBP51 on energy homeostasis, adeno-associated viral (AAV) vector-mediated *Fkbp5* gene transfer into the hypothalamus was performed (Figure 2.5.1a). Mice were initially singly-housed one week prior to the experimental onset. Bilateral microinjections targeting the hypothalamus were performed

from experiment day 1 to experiment day 4 for AAV vector delivery of *Fkbp5* or empty control. Mice selected for AAV vector delivery of *Fkbp5* overexpression (51OE, n = 28) or empty control (Empty, n = 26) were counterbalanced for body weight. Mice were allowed to recover for 4 weeks following the surgeries with minimal disturbance. Following the 4-week recovery period, mice from each genotype group (51OE and Control) were divided into chow-fed and high fat diet (HFD)-fed groups, balanced by body weight. For an additional 6 weeks, mice received ad libitum access to either HFD (58% kcal from fat, D12331, Research Diets, Inc., New Brunswick, NJ, USA) or control chow diet (10.5% kcal from fat,D12329). Food intake and body weight were carefully monitored over the 6 week dietary period. In the final week of the experiment, mice were assessed for locomotor activity in the open field.

Micro-Injections

AAV vector-mediated overexpression of FKBP51 was performed using an adeno-associated bicistronic AAV1/2 vector as previously described (Schmidt et al., 2011). AAV vectors were designed and obtained by GeneDetect (Auckland, New Zealand). An injection volume of 0.5 µl was used to deliver AAV vectors expressing either the human FKBP51 cDNA sequence (CAG-HA-tagged-FKBP51-WPRE-BGH-polyA expression cassette; titer: $1.2x10^{12}$ GP/ml) or an empty vector construct (CAG-Null/Empty-WPRE-BGH-polyA). For bilateral microinjections into the hypothalamus, mice were anesthetized with isoflurane and fixed in a stereotaxic frame in order to target the following coordinates relative to Bregma: posterior 1.5 mm; 0.5 mm lateral; 5.2 mm ventral. Injections were carried out using glass capillaries with a tip resistance of 2–4MΩ over 10 min (0.05 µl/min). Successful AAV vector targeting was later confirmed on brain sections by in situ hybridization.

Fasting Blood Glucose Levels

Mice were fasted overnight (14 h). Blood glucose levels were subsequently measured on blood collected by tail cut using a handheld Contour XT glucometer (Bayer Health Care, Basel, Switzerland).

Open Field

In order to assess locomotor activity, mice were placed in one corner of a 50 cm x 50 cm x 50 cm arena under dim light (15 Lux) conditions. Thirty-minute trials were video-recorded by an overhead camera. Tests were recorded using a video-tracking system (Anymaze 4.20; Stoelting). All behavioral equipment was cleaned with water at the beginning of testing and between each animal. Total distance travelled was taken as a measure of locomotor activity.

Tissue Collection

On the day of sacrifice, mice were deeply anesthetized with isoflurane. Blood was collected by cardiac puncture. Immediately thereafter, mice were transcardially perfused with 0.1M phosphate-buffered saline (PBS) followed by 4% (v/v) paraformaldehyde (PFA) fixative in PBS. Brains were rapidly removed and post-fixed in 4% PFA overnight at 4°C. The following day, post-fixed brains were transferred to a cryoprotectant solution (30% (w/v) sucrose in 0.1M PBS) for an additional overnight incubation at 4°C. Brains were stored at -80°C until used. Liver, epididymal adipose tissue, and inguinal adipose tissue were also collected, immediately frozen on dry ice, and stored at -80°C.

Hormone Quantification

Blood samples were collected in EDTA-coated tubes and stored on ice until processed. Plasma was separated from collected blood by centrifugation at 6000 *g* at 4°C for 15 min. Plasma insulin and leptin levels were determined using a mouse metabolic magnetic bead panel (Millipore Corp. Billerica, Massachusetts; sensitivity: insulin 14pg/ml; leptin 19pg/ml). Corticosterone levels were determined using a commercially available RIA kit (MP Biomedicals, Inc., Solon, OH, USA; sensitivity 12.5 ng/ml).

In Situ Hybridization (ISH)

ISH was performed on coronal brain sections (25 µm thick) that were cut on a cryostat as a 1:6 series as previously described (Schmidt et al., 2007). Specifically, a ^{35}S-UTP-labeled antisense cRNA probe for *Fkbp5* (Forward primer: 5'-CTTGGACCACGCTATGGTTT; Reverse primer: 5'- ATTGACTGCCAACACCTT) was used. For signal detection, slides were exposed to a Kodak Biomax MR film (Eastman Kodak Co). Autoradiographic densities were quantified using the NIH ImageJ Software (NIH, Bethesda, MD, USA) to both validate and quantify hypothalamic *Fkbp5* overexpression.

Double In Situ Hybridization (DISH)

In a separate cohort of test-naïve C57BL/6N male mice, coronal brain sections were cryosectioned at 20µm thickness and directly thaw mounted onto Super Frost Plus Slides. For the co-localization of *Fkbp5* mRNA with *Pomc* and *Npy,* ^{35}S UTP-labelled *Fkbp5* riboprobe was used with either a digoxigenin (DIG)-labelled *Pomc* riboprobe or DIG-labelled *Npy* riboprobe. The antisense riboprobes were transcribed from a linear plasmid for *Fkbp5* (forward

primer: 5'- CTT GGA CCA CGC TAT GGT TT -3'; reverse primer: 5'-GGA TTG ACT GCC AAC ACC TT-3'), *Pomc* , (forward primer: 5'- TTA CGG TGG CTT CAT GAC CT – 3'; reverser primer: 5' – AGA GCC GAC TGT GAA ATC TGA – 3'), and *Npy* (forward primer: 5' – AGG AAA GCA CAG AAA ACG CC-3'; reverse primer: 5'- AAC AAC AAG GGA AAT GGG GC - 3'). Double in situ hybridization (DISH) was performed as previously described (Refojo et al., 2011). Briefly, sections were fixed in 4 % PFA. After several washing steps, endogenous peroxidase was quenched in 1% H202. Background was reduced in 0.2 M HCl, followed by 2 additional washing steps (1 x PBS). The slides were acetylated in 0.1 M triethanolamine, washed (1 x PBS) and dehydrated through increasing concentrations of ethanol. Tissue sections were saturated with 90 µl of hybridization buffer containing approximately 50,000 cpm/µl ^{35}S-labelled riboprobe and 0.2µg/ml DIG-labelled riboprobe. Brain sections were cover-slipped and incubated overnight at 55 °C. The following day, coverslips were removed and sections were washed several times in decreasing concentrations of SSC/formamide buffers under stringent temperature settings. After SSC washes, sections were treated with RNAse A in 1 x NTE at 37°C and washed in 1x NTE/0.05% Tween20 (2x times) followed by a blocking step in 4% BSA for 1 hour. After additional washing steps, sections were blocked in NEN-TNB for 30 min. In a final step, slides were incubated with Roche's anti-DIG (FAB) (1:400, Roche Molecular Diagnostics) at 4°C overnight. On the last day, sections were washed several times in TNT at 30°C followed by a signal amplification step in which sections were incubated for 15 min in tyramide-biotin. Thereafter, additional washing steps were performed (Roche washing buffer, Roche Molecular Diagnostics). Sections were then incubated for 1 h with Roche streptavidin-AP (1:400, Roche Molecular Diagnostics). Sections were washed in Roche washing buffer and were subsequently prepared for Vector red staining in 100mM Tris/HCL (Vector Laboratories, Burlingame, CA, USA). Slides were immersed in Vector red solution under unlit conditions for 15-30 min depending on staining. When staining was sufficient, the reaction was stopped in 1 x PBS followed by a fixation step in 2.5% glutaraldehyde. Finally, sections were washed in 0.1 x SSC and dehydrated in a graded series of ethanol solutions (30, 50, 70, and 96%).

Statistical Analysis

Data were analyzed using IBM SPSS Statistics 18 software (IBM SPSS Statistics, IBM, Chicago, IL, USA). For the comparison of two groups, independent Student's t-tests were used. A one-way repeated measures ANOVA was used for longitudinal analysis of body

weight progression, in which the degrees of freedom were corrected for deviance from sphericity (Greenhouse–Geisser) for within-subjects comparisons. A Bonferroni post hoc test was used for ANOVA tests to determine statistical significance between individual groups. Finally, correlations were analyzed with the Pearson product-moment test. Statistical significance was set at $p < 0.05$ and a statistical trend at $p < 0.1$. Data are presented as the mean +/- S.E.M.

2.5.4. Results

Validation of AAV Vector-Mediated Overexpression of FKBP51 in the Hypothalamus

Viral-mediated gene transfer to the hypothalamus was used to selectively overexpress FKBP51. In situ hybridization of coronal hypothalamic sections was later used to quantify the level of overexpression across hypothalamic nuclei and was additionally used to exclude mice who presented off-target injection sites. Mice that were not infected bilaterally in the hypothalamus were excluded from all analyses. In total, 13 empty and 9 51OE mice under chow diet conditions and 11 empty and 14 51OE mice under HFD were used for further analyses. AAV vector-mediated delivery of FKBP51 resulted in a significant increase in FKBP51 mRNA expression within the PVN ($T_{20.4} = -19.063$, $p < 0.001$), ARC ($T_{23.5} = -7.594$, $p < 0.001$), VMH ($T_{24.0} = -18.171$, $p < 0.001$), and DMH ($T_{22.3} = -17.782$, $p < 0.001$) compared to empty control animals (Figure 2.5.1b).

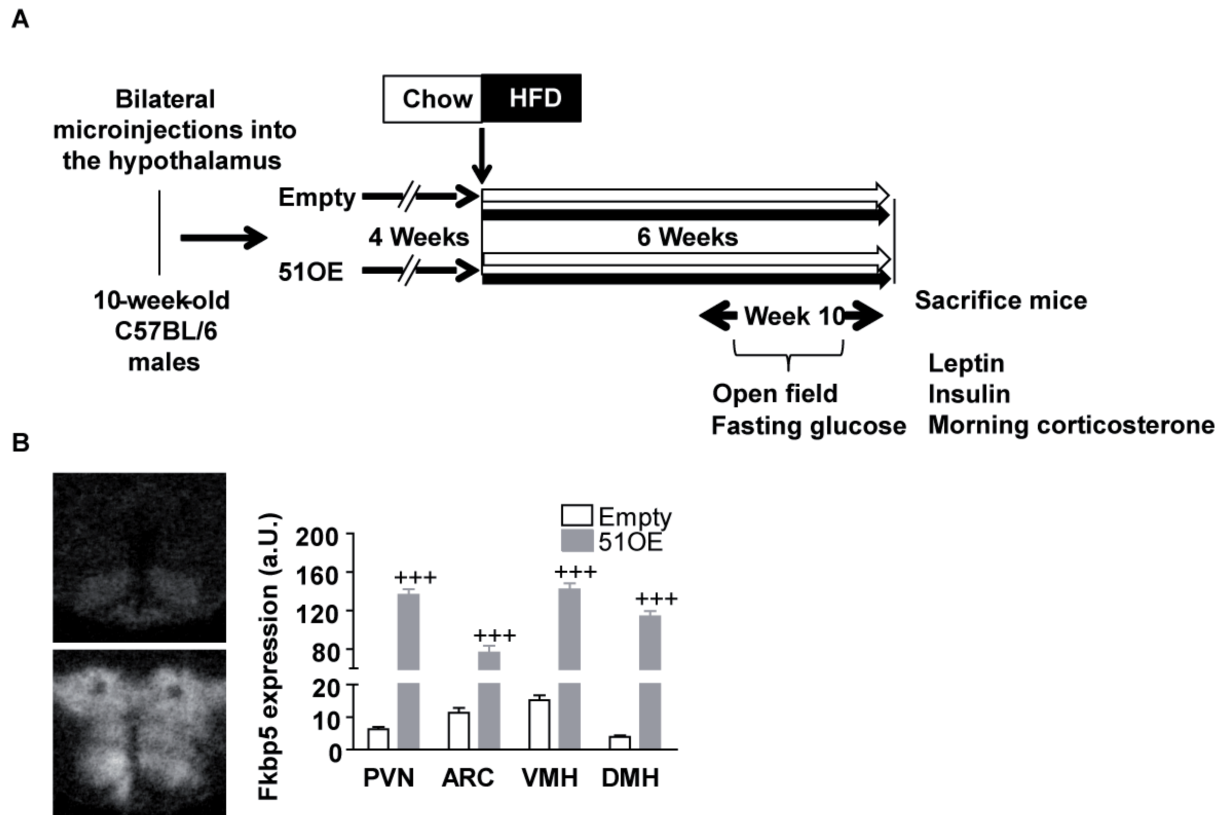

Figure 2.5.1 Experimental design and validation of hypothalamic Fkbp5 overexpression

(A) Experimental time course. Brain-site specific microinjections were performed on 10-week -old C57BL/6 male mice for the delivery of Fkbp5 or an empty control construct to the hypothalamus. Following 4 weeks of recovery, mice from each genotype group were divided into a dietary group and were subsequently exposed to either chow diet or HFD for an additional 6 weeks. Metabolic readouts were carried out in the final week of the dietary regime. (B) Representative autoradiographs of viral Fkbp5 mRNA expression in the PVN, ARC, VMH, and DMH of the hypothalamus and quantification of Fkbp5 mRNA levels across the PVN, ARC, VMH, and DMH. +++P < 0.001; + significant viral effect.

Effects of Hypothalamic FKBP51 Overexpression on Body Weight and Food Intake

Body weight was monitored regularly during the recovery period following the microinjections and thereafter during the 6-week dietary period. At the time of microinjections, mice were divided into viral groups counterbalanced for body weight, and therefore on the day of surgery there was no difference in body weight between the 2 viral expression groups (Figure 2.5.2a). A one-way repeated measures ANOVA performed for body weight gain from 1 week post-surgery to 4 weeks post-surgery revealed that as early as 3 weeks post-surgery, FKBP51 overexpression led to a significant reduction in body weight gain (Time x Virus: $F(1, 97.2) = 15.741$, $p < 0.001$; Virus: $F(1, 45) = 4.698$, $p = 0.036$)

(Figure 2.5.2b). Following 4 weeks recovery, mice of each viral expression group were further divided into chow-fed and HFD-fed mice. Mice fed either chow or HFD, presented reduced body weight gain on account of hypothalamic FKBP51 overexpression (Chow: Time x Virus: $F(1, 46.9) = 4.483$, $p = 0.011$, Virus: $F(1, 19) = 8.343$, $p = 0.009$; HFD: Time x Virus: $F(1, 35.9) = 6.195$, $p = 0.008$, Virus: $F(1, 23) = 9.040$, $p = 0.006$) (Figure 2.5.2c). Accordingly, the final body weight assessed at the end of the experiment was significantly lower in 51OE mice compared to empty control mice, although consumption of a HFD significantly increased final body weight, independent of genotype (Virus: $F(1, 42) = 15.400$, $p < 0.001$; Diet: $F(1, 42) = 36.038$, $p < 0.001$). This was also observed using the Pearson product-moment test to assess the relationship between Fkbp5 expression and body weight. The Pearson product-moment test revealed negative associations between final body weight and Fkbp5 expression within the PVN (Chow: $r = -0.504$, $p = 0.028$; HFD: $r = -0.485$, $p = 0.026$), the ARC (Chow: $r = -0.267$, $p = 0.255$; HFD: $r = -0.523$, $p = 0.010$), the VMH (Chow: $r = -0.522$, $p = 0.018$; HFD: $r = -0.671$, $p < 0.001$), and the DMH (Chow: $r = -0.537$, $p = 0.015$; HFD: $r = -0.622$, $p = 0.002$) (Figure 2.5.2d). Additionally, FKBP51 hypothalamic overexpression significantly reduced caloric intake (Virus: $F(1, 43) = 6.022$, $p = 0.018$), whereas HFD exposure significantly increased caloric intake (Diet: $F(1, 43) = 72.832$, $p < 0.001$) (Figure 2.5.2e). In fact, in chow-fed animals the reduced body weight phenotype arising from hypothalamic FKBP51 overexpression is explained by the significant reduction in caloric intake as shown by ANCOVA for final body weight using caloric intake as a covariate (caloric intake: $F(1, 18) = 13.637$, $p = 0.002$; Virus: $F(1, 18) = 1.789$, $p = 0.198$). However, in HFD-fed mice additional factors besides only caloric intake contribute to the effects of hypothalamic FKBP51 overexpression on body weight regulation (caloric intake: $F(1, 22) = 7.005$, $p = 0.015$; Virus: $F(1, 22) = 6.830$, $p = 0.016$). Finally, our data indicate that changes in locomotor activity do not underlie the lean phenotype given that hypothalamic overexpression of FKBP51 had no effect on locomotor activity examined in the open field over 30 min in either chow-fed mice or HFD-fed mice (Figure 2.5.2f).

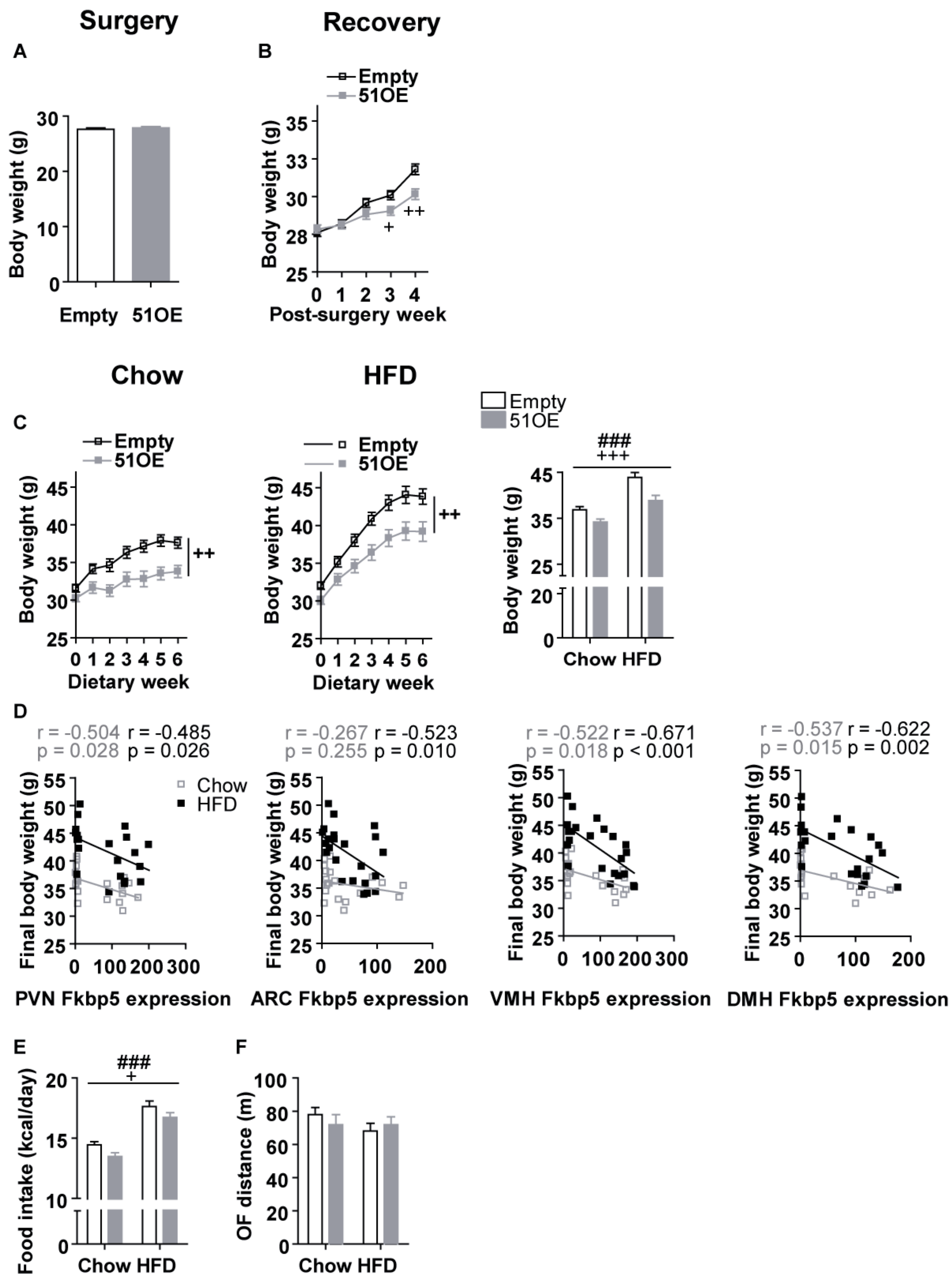

Figure 2.5.2 Effects of hypothalamic FKBP51 overexpression on body weight and food intake

(A) At the onset of AAV vector-mediated FKBP51 overexpression within the hypothalamus, there was no difference in body weight between groups. (B) Hypothalamic overexpression of FKBP51 decreased the progression of body weight, which was already evident during the 4-week recovery stage. (C) 51OE mice presented reduced body weight throughout the entire dietary regime under both chow and HFD conditions, which is reflected in the body weight at the experimental endpoint.

(D) Correlational analyses between the final body weight and Fkbp5 expression across hypothalamic nuclei (PVN, ARC, VMH, and DMH) revealed strong negative correlations between Fkbp5 expression levels and final body weight. (E) A reduction in food intake (kcal/day) was observed in 51OE mice, whereas both genotype groups presented HFD-mediated increased food intake. (F) Examination of locomotor activity in the open field over a 30 min period revealed no effect of either genotype or diet. +P < 0.05, ++P < 0.01, +++P <0.001, ###P < 0.001; + significant viral effect; # significant diet effect. Correlations were analyzed with the Pearson product-moment test with statistical significance set at p < 0.05.

Effects of Hypothalamic FKBP51 Overexpression on Circulating Levels of Glucose and Signaling Hormones

In order to examine whether AAV viral-mediated hypothalamic FKBP51 overexpression impacts fasting blood glucose, we fasted the mice for 14 h and then collected blood by tail cut. Overall, FKBP51 overexpression lowered fasting blood glucose (Virus: F(1, 43) = 4.702, p = 0.036) (Figure 2.5.3a). Likewise, hypothalamic overexpression of FKBP51 lowered circulating leptin levels (Virus: F(1, 41) = 4.233, p = 0.046) (Figure 2.5.3b). In addition, HFD-fed mice presented elevated levels of leptin compared to chow-fed counterparts (Diet: F(1, 41) = 7.079, p = 0.011). Interestingly, although there was a significant diet effect on circulating insulin levels (Diet: F(1, 41) = 11.971, p = 0.001), we observed no effect of genotype (Figure 2.5.3c). Finally, we also measured corticosterone levels, and we found that both HFD (Diet: F(1, 40) = 4.810, p = 0.034) and hypothalamic FKBP51 overexpression (Virus: F(1,40) = 12.789, p = 0.001) increased circulating corticosterone levels (Figure 2.5.3d). The Pearson product-moment test to assess the relationship between Fkbp5 expression and corticosterone also revealed strong tendencies for positive associations between corticosterone and Fkbp5 expression within the PVN (Chow: r = 0.418, p = 0.075; HFD: r = 0.446, p = 0.055), the ARC (Chow: r = 0.561, p = 0.010; HFD: r = 0.239, p = 0.296), the VMH (Chow: r = 0.397, p = 0.083; HFD: r = 0.400, p = 0.072), and the DMH (Chow: r = 0.375, p = 0.103; HFD: r = 0.479, p = 0.028) (Figure 2.5.3e).

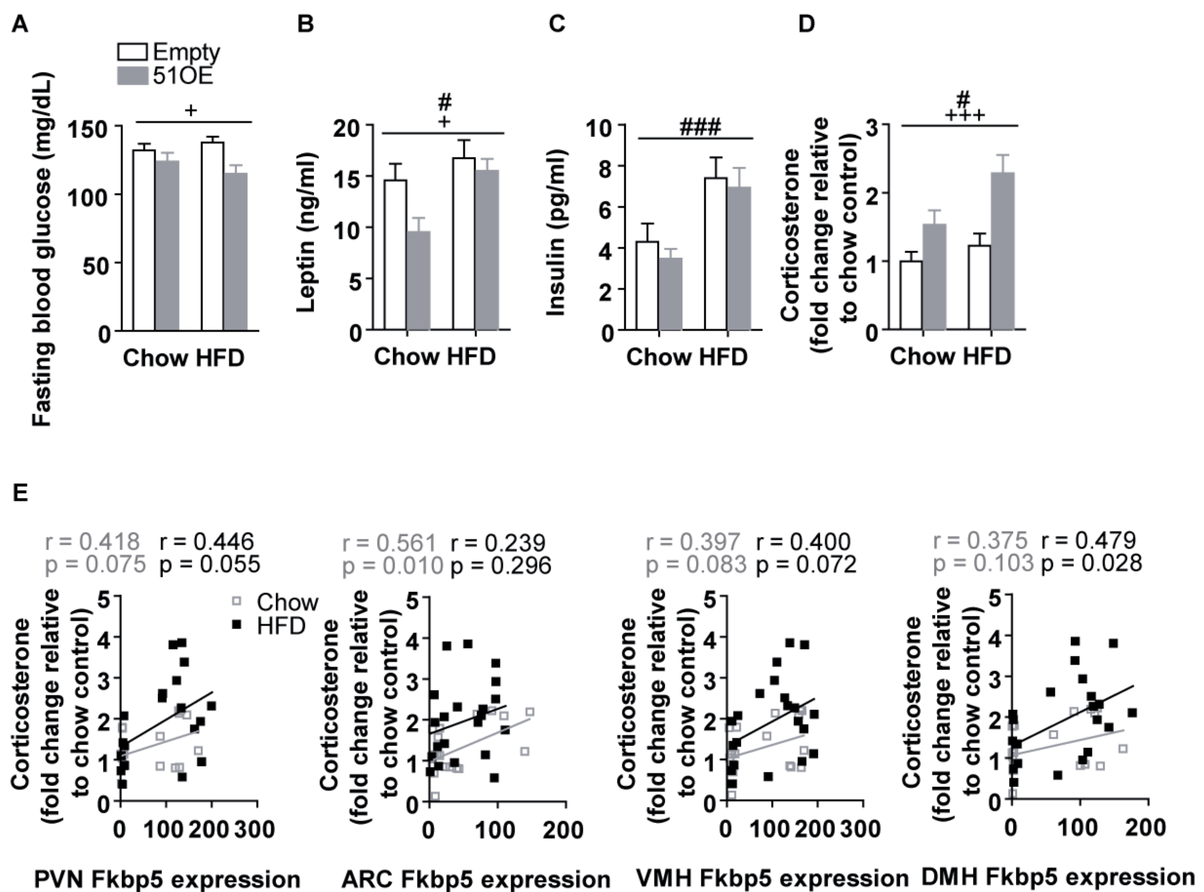

Figure 2.5.3 Effects on hypothalamic FKBP51 overexpression on fasting glucose and circulating hormone levels

(A) 14h-fasting glucose was significantly lowered on account of hypothalamic overexpression of FKBP51. (B) Circulating leptin levels were found to be decreased by hypothalamic overexpression of FKBP51, whereas were found to be increased by HFD exposure. (C) Circulating levels of insulin were insensitive to hypothalamic FKBP51 overexpression, but were nevertheless significantly increased by HFD exposure. (D) Morning corticosterone was significantly increased by both hypothalamic FKBP51 overexpression and HFD exposure. (E) Correlational analyses between plasma corticosterone and Fkbp5 expression across hypothalamic nuclei (PVN, ARC, VMH, and DMH) revealed positive correlations between Fkbp5 expression levels and plasma corticosterone. +P < 0.05, +++P <0.001, #P < 0.05, ###P < 0.001; + significant viral effect; # significant diet effect. Correlations were analyzed with the Pearson product-moment test with statistical significance set at $p < 0.05$ and a statistical trend at $p < 0.1$.

Cellular FKBP51 Expression in the Arcuate Nucleus

The cellular distribution of FKBP51 across the hypothalamus, and specifically within the ARC, is unknown. For this purpose we performed double in situ hybridization in order to co-localize the mRNA of *Fkbp5* with either *Npy* or *Pomc* mRNA. As expected, *Pomc* expression

was detected within the ARC. *Fkbp5* expression was dispersedly distributed across the hypothalamus. Importantly, our results demonstrated that *Fkbp5* mRNA co-localized with *Pomc* within the ARC (Figure 2.5.4). Likewise, *Npy* expression was detected primarily in the ARC, but was also detected to a degree in the VMH. Within the ARC, we found that *Fkbp5* largely co-localized with *Npy*. Interestingly, although the greater extent of Npy-expressing neurons co-expressed *Fkbp5*, there was a sub-population of *Npy*-positive cells that did not co-express *Fkbp5* (Figure 2.5.5).

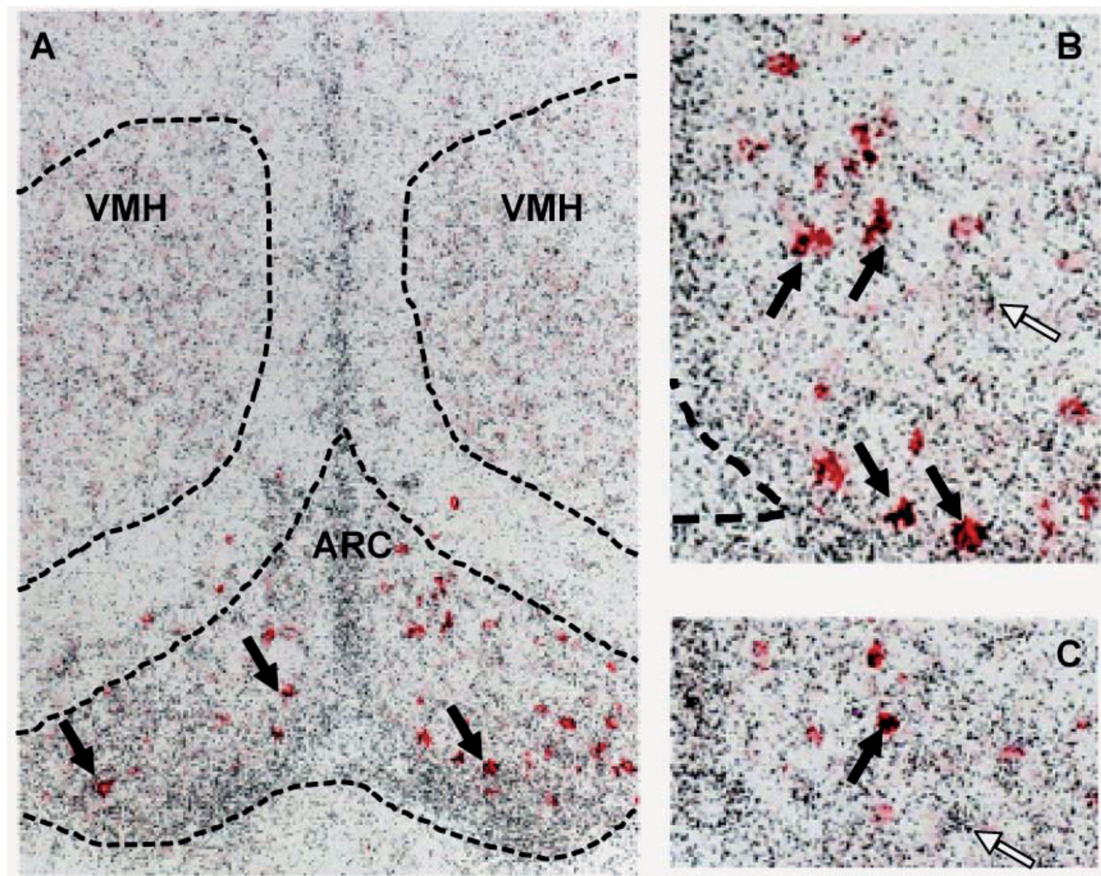

Figure 2.5.4 Fkbp5 is co-expressed with pomc-containing neurons

(A – C) Representative bright-field photomicrographs of brain sections derived from C57BL/6 mice showing double in situ hybridization of Fkbp5 mRNA (silver grains) and pomc mRNA (red staining) within the ARC. Dashed lines delineate the VMH and ARC nuclei within the hypothalamus. Black arrows indicate cells co-expressing Fkbp5 and Pomc, whereas white arrows indicate cells only expressing Fkbp5.

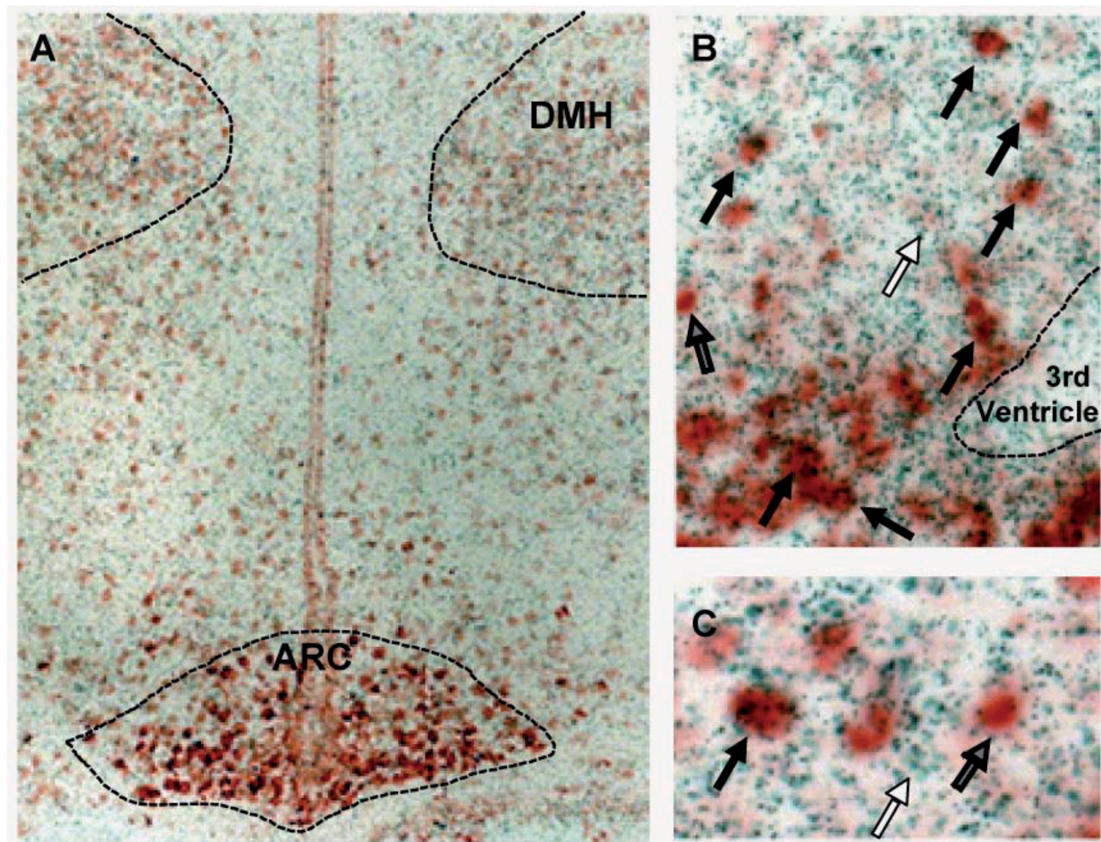

Figure 2.5.5 Fkbp5 is co-expressed with npy-containing neurons

(A – C) Representative bright-field photomicrographs of brain sections derived from C57BL/6 mice showing double in situ hybridization of Fkbp5 mRNA (silver grains) and npy mRNA (red staining) within the ARC. Dashed lines delineate the DMH and ARC nuclei within the hypothalamus. Black arrows indicate cells co-expressing Fkbp5 and npy, whereas grey arrows indicate cells only expressing Npy, whereas white arrow indicate cells only expressing Fkbp5.

2.5.5. Discussion

The major finding of the current study was that FKBP51 overexpression within the hypothalamus reduces body weight gain and food intake, independent of dietary context. In addition, 51OE mice presented lower levels of circulating leptin, whereas elevated levels of circulating morning corticosterone. We found that *Fkbp5* is expressed in two types of peptide-secreting neurons within the ARC, *Npy*-expressing and *Pomc*-expressing neurons, which are critical regulators of feeding. In conclusion, the current findings implicate FKBP51 action within the hypothalamus as a determinant of body weight and food intake. Future studies will be able to build on these findings, to decipher the exact role of FKBP51 within subpopulations of hypothalamic neurons on whole body energy homeostasis.

We hypothesized that hypothalamic overexpression of FKBP51 would lead to obesity based on our previous (unpublished) data that total loss of FKBP51 protects against HFD-induced obesity (Chapter 2.3). To our surprise, hypothalamic FKBP51 overexpression paradoxically led to a slower progression of body weight gain. Nevertheless, unlike FKBP51 knockout (51KO) mice, 51OE mice were not protected from HFD-induced weight gain. Instead, under both chow and HFD conditions, 51OE mice presented reduced body weight over the entire 6 week dietary regimen. Furthermore, 51OE mice under both chow and HFD conditions presented reduced food intake, suggesting that FKBP51-mediated hypophagia partly underlies the body weight phenotype. ANCOVA using caloric intake as a covariate revealed that this is indeed the case in chow-fed mice, since the effect of hypothalamic overexpression on final body weight is lost when caloric intake is accounted for. However, in HFD-fed mice hypothalamic FKBP51 overexpression remained significant even after controlling for caloric intake suggesting that additional mechanisms underlie the hypothalamic FKBP51-mediated effects on body weight regulation under HFD conditions. In addition to the body weight phenotype in 51OE mice, hypothalamic FKBP51 overexpression furthermore reduced circulating levels of leptin. This agrees with the lower body weight of 51OE mice, given that leptin circulates at concentrations proportional to body fat mass (Considine et al., 1996). In summary, although at first glance the effects of hypothalamic 51OE appear paradoxical in view of the 51KO mouse experiments (in which both 51KO and hypothalamic 51OE induced weight loss), a more detailed analysis revealed different underlying mechanisms. Specifically, whereas hypothalamic FKBP51 overexpression had a strong effect on food intake, total loss of FKBP51 had no effect on food intake but rather had a strong effect on energy expenditure and glucose metabolism. Examination of circulating insulin levels revealed a strong effect of HFD exposure, but no effect of genotype. Interestingly, genetic ablation of FKBP51 in mice also has no effect on circulating concentrations of insulin, despite an impressive effect on body weight control (unpublished data). Collectively, such data imply that FKBP51 is not involved in the regulation of insulin secretion at the level of the pancreas. Despite no effect on insulin secretion, hypothalamic FKBP51 overexpression was found to reduce fasting blood glucose levels, suggesting that other mechanisms regulating whole body glucose homeostasis are influenced by FKBP51 hypothalamic action. Additional studies should examine fasting insulin levels and glucose-stimulated insulin levels in hypothalamic 51OE mice in order to reinforce the current findings.

Evaluation of circulating morning corticosterone levels revealed a robust genotype effect in which hypothalamic FKBP51 overexpression significantly increased corticosterone levels. This is in strong agreement with the known role of FKBP51 as an hsp90 co-chaperone of the glucocorticoid receptor (GR) and its subsequent regulation of GR sensitivity (Vermeer et al., 2003;Wochnik et al., 2005). Interestingly, elevated levels of corticosterone are typically associated with central obesity, insulin resistance, and symptoms related to the metabolic syndrome as reflected in patients suffering from Cushing's disease or patients receiving glucocorticoid therapy (Newell-Price et al., 2006). In the present study, we rather found elevated levels of corticosterone, decreased body weight, and lowered fasting blood glucose. Based on these findings, we deduce that the hypothalamic actions of FKBP51 on body weight regulation are not driven by FKBP51-dependent increases in glucocorticoids. A detailed characterization of the molecular events arising from manipulation of hypothalamic FKBP51 expression is warranted.

Previous studies have already reported tissue-specific effects of FKBP51 activity. For example, FKBP51 has been implicated in fine-tuning the expression of GR-regulated genes during adipocyte differentiation (Toneatto et al., 2013). In the brain, FKBP51 activity has been implicated in stress reactivity, especially at the level of the amygdala (Hartmann et al., 2015). It is clear from such previous findings that it is essential to delineate the tissue/cell-specific effects of FKBP51 in order to understand how FKBP51 is able to contribute to whole body energy homeostasis. In this regard, the current study has isolated the hypothalamic actions of FKBP51 from its actions in peripheral tissues or other brain centers. Although the hypothalamus is itself a broad brain region comprised of multiple subpopulations, each with distinct functions, we directed gene transfer to the hypothalamus based on the endogenous expression profile of FKBP51 across the VMH, PVN, and ARC (Scharf et al., 2011). Indeed our AAV viral-mediated overexpression of FKBP51 was very robust, and resulted in a pronounced body weight phenotype, partly mediated by hypophagia (see above).

Hypophagic effects are largely mediated by the activation of POMC-containing neurons within the ARC (Cone et al., 2001). By contrast, NPY-containing neurons within the ARC are primarily responsible for hyperphagic effects. In our hypothalamic FKBP51 overexpression experiment, FKBP51 was placed under the control of a constitutive CAG promoter, which drives gene expression across all cell types (Niwa et al., 1991;Okabe et al., 1997;Pratt et al., 2000). Therefore it was important to establish the cell-specific endogenous (physiological) expression of FKBP51 within NPY-containing neurons and POMC-

containing neurons. Using double in situ hybridization we sought to co-localize *Fkbp5* mRNA with either *Npy* or *Pomc* mRNA. As validation for our methodology, we found strong agreement between the observed and published (Allen brain atlas) *Npy* and *Pomc* expression profiles. Our co-expression analysis revealed that *Fkbp5* is endogenously expressed in both NPY- and POMC-containing neuronal populations. Interestingly, our results furthermore indicated that there is a subpopulation of NPY neurons that do not express FKBP51. The functional consequence of these distinct *Fkbp5*-expressing subpopulations is unknown.

Given the opposing roles of NPY- and POMC-containing neurons on body weight regulation, it is important to understand whether FKBP51 acts distinctly between each neuronal population. Indeed multiple regulatory roles and interacting partners of FKBP51 have been identified (Schmidt et al., 2012). In fact, it has been proposed that FKBP51 achieves pathway and network specificity through direct protein-protein interactions between critical nodes of signaling pathways and specific regulator proteins. In this manner, different functional outcomes may result depending on the predominant pathway regulated by FKBP51. For example, FKBP51 is found to be upregulated in melanoma samples where it acts as a tumor promoter by increasing NFκB signaling (Romano et al., 2011), whereas is found to be downregulated in pancreatic tumor samples where it acts as a tumor suppressor by inhibiting AKT signaling (Pei et al., 2009). It is possible that FKBP51 likewise has distinct actions in NPY- and POMC-containing neurons, whose concerted actions ultimately favor a lower body weight phenotype. Otherwise, it is possible that endogenous FKBP51 action is stronger in one population over the other, in which the balance of activity promotes slower body weight gain. Future studies are required to understand the full extent of FKBP51 action across different hypothalamic cellular populations.

In summary, we provide evidence suggesting that FKBP51 acts as a homeostatic regulator of body weight, in part through the regulation of food intake. First, we demonstrate that hypothalamic overexpression of FKBP51 reduces progression of body weight gain under chow and HFD conditions. Second, 51OE mice present reduced food intake compared to their control dietary counterparts. Finally FKBP51 is endogenously expressed within NPY- and POMC-containing neurons, two critical populations regulating energy expenditure and intake. It will be interesting to investigate whether cell-specific modulation of FKBP51 within the hypothalamus produces similar effects to those shown here.

3. General Discussion

3.1. Summary

The main objective of the current thesis was to investigate the role of FKBP51 in the regulation of whole body energy metabolism. In particular, we initially addressed the question whether FKBP51 is responsive to the dietary and/or stress condition by examining *Fkbp5* gene expression under changing environmental conditions (Chapter 2.1 & 2.2). In more detailed analyses, we addressed the questions whether loss or overexpression of FKBP51 leads to metabolic changes, and then proceeded to characterize the molecular changes underlying the resulting metabolic phenotypes (Chapters 2.3, 2.4, and 2.5). Finally, we tested the hypothesis that FKBP51 represents a promising therapeutic target in obesity and T2D (Chapter 2.3). These data are the first to comprehensively examine FKBP51-dependent metabolic regulation and the metabolic consequences following treatment with a selective inhibitor of FKBP51. Our findings indicate that body weight and glucose homeostasis are dependent on FKBP51 regulation and furthermore provide concrete evidence for the therapeutic potential of FKBP51 in the treatment of obesity and T2D.

3.1.1. *Fkbp5* Expression is Modulated by the Dietary Context

In our first study (Chapter 2.1), we tested the hypothesis that FKBP51 mediates the crosstalk between the regulation of the stress response and energy homeostasis. This hypothesis was based on the notion that FKBP51 is already known to regulate the stress response (Hartmann et al., 2012;Touma et al., 2011) and preliminary evidence suggests it may furthermore be involved in metabolic and glucose homeostasis (Pei et al., 2009;Pereira et al., 2014b). In addition, despite mounting evidence linking chronic stress exposure to obesity and glucose intolerance (Bartolomucci et al., 2009;Dallman, 2010), exact targets at the interface between stress and energy regulation remain poorly defined. Therefore, as a preliminary study, we measured whether *Fkbp5* gene expression is responsive to the dietary and/or stress condition in adult male mice, and furthermore addressed whether the dietary composition interacts with the stress environment to predict the expression level of *Fkbp5*. We focused our attention on the hypothalamus given its central role in both stress reactivity (Gold, 2015) and energy homeostasis (Saper and Lowell, 2014). Using a validated model of chronic social stress under both HFD-and control (chow) conditions, we found that although *Fkbp5* is responsive to both

stress and dietary conditions, there is no interaction effect (Chapter 2.1). Although these findings do not establish FKBP51 as a target at the interface between stress reactivity and energy balance regulation per se, they highlight the fact that *Fkbp5* is responsive to the adult dietary condition.

In our second study (Chapter 2.2), we continued to examine gene – environment interactions at the *Fkbp5* locus using a mouse model of maternal diet-induced obesity. We tested the hypothesis that in utero exposure to maternal HFD results in long-lasting alterations, which ultimately shape the adult phenotype. In this regard, early life dietary conditions impact adult gene expression of FKBP51 and thus function. This hypothesis was based on earlier findings that FKBP51 is a mediator of gene x early life trauma interactions to predict the risk of developing psychiatric disorders (Zannas and Binder, 2014). For this purpose, we examined the effects of prenatal exposure to maternal obesity in adult offspring on anxiety-like and depressive-like behaviors. In parallel we examined whether *Fkbp5* gene expression is altered on account of in utero exposure to maternal diet-induced obesity. We report that the behavioral outcomes induced by prenatal exposure to maternal obesity mimic the deleterious effects of adult chronic stress exposure in aged male mice. This was accompanied by an increase in *Fkbp5* gene expression within the paraventricular nucleus of the hypothalamus. Further studies are needed to determine a causal role of FKBP51 in mediating adverse behavioral outcomes resulting from prenatal exposure to maternal HFD. Nevertheless, this study extends the findings from our previous study, and together they demonstrate that not only is FKBP51 responsive to the adult dietary condition, but is likewise responsive to the in utero dietary condition.

3.1.2. FKBP51 Impacts Body Weight, Glucose Homeostasis, and Metabolic Signaling Cascades: Possible Therapeutic Implications

In our third study (Chapter 2.3), we asked whether the loss of FKBP51 impacts whole body energy metabolism, and if so how. To address this question, we characterized conventional FKBP51 knockout (51KO) mice under chow and HFD conditions. For this purpose, mice were subjected to indirect calorimetry. In additional studies body weight progression, food intake, glucose tolerance, and insulin tolerance were investigated. Following extensive metabolic phenotyping, we observed that loss of FKBP51 modestly lowers body weight under standard conditions and strikingly protects against HFD-induced obesity. The lower body weight phenotype in 51KO mice was accompanied by reduced fat pad mass and increased lean mass. This phenotype was very robust and was reproduced in separate

experiments. In order to determine the mechanistic underpinnings of the body weight phenotype, we assessed components of energy intake and energy expenditure. No differences in nutrient absorption (bioavailability) or food intake were detected between WT and 51KOmice, indicating that the improved body weight phenotype arising from genetic ablation of FKBP51 does not result from modulation of energy intake. A pair-feeding experiment, in which WT mice were given the exact amount of HFD consumed by 51KO mice the day before, confirmed that complete loss of FKBP51 has no effect on energy intake. Furthermore, there was no genotype effect on the respiratory exchange ratio (RER), which reflects the relative contributions of fat and carbohydrates to oxidative metabolism, suggesting that a preference for fat oxidation cannot explain the reduced adiposity present in 51KO mice. Finally, examination of energy expenditure by indirect calorimetry revealed an increase in total energy expenditure (TEE) in 51KO mice. Decomposition of TEE into its resting (RMR, resting metabolic rate) and activity-related (AEE, activity energy expenditure) components revealed that the observed TEE difference was due to an increase in RMR in 51KO animals. We therefore conclude that the improved body weight phenotype presented in 51KO mice is on account of an increase in RMR.

In order to assess whether FKBP51 acts on glucose homeostasis, a glucose tolerance test was performed to assess levels of circulating glucose at regular time intervals following glucose administration. Improved (lowered) glucose tolerance reflects either improved insulin secretion from the pancreas or enhanced actions of insulin to accelerate glucose uptake (especially in adipose tissue and skeletal muscle) and inhibit hepatic glucose production (Bowe et al., 2014). In our model, genetic deletion of FKBP51 lowered fasting glucose and improved glucose tolerance regardless of dietary (HFD or standard chow) condition. Examination of fasting insulin and glucose-stimulated insulin levels revealed no genotype effect, suggesting that the improved glucose tolerance phenotype results from improved insulin sensitivity rather than enhanced insulin release from the pancreas. In agreement with these findings, 51KO mice also showed a prolonged response to insulin examined in an insulin tolerance test, which assesses insulin-stimulated glucose disposal. Given the fact that FKBP51 is not expressed in mouse liver, the improved glucose tolerance phenotype is likely on account of the enhanced actions of insulin to accelerate glucose uptake. Taken together these data implicate FKBP51 in body weight regulation and glucose metabolism.

In a next step, we sought to identify metabolic signaling pathways regulated by FKBP51. Based on the earlier finding that FKBP51 acts as a negative regulator to AKT, a central node

within the insulin signaling pathway (Pei et al., 2009) and the current finding that 51KO mice present improved glucose homeostasis, we examined the activity of the insulin signaling cascade. We found that genetic ablation of FKBP51 resulted in a tissue-specific increase in insulin signaling, selectively within skeletal muscle. To assess the functional consequence of FKBP51-dependent increased insulin signaling, we examined glucose uptake in the presence of AAV vector-mediated silencing of FKBP51 in cultured C2C12 myotubes. We report that AAV vector-mediated silencing of FKBP51 significantly enhanced glucose uptake in cultured myotubes.

Finally, based on our earlier findings, we hypothesized that blockade of FKBP51 may improve obesity and/or insulin resistance. To determine whether FKBP51 inhibition represents a promising strategy in the treatment of obesity and/or T2D, we delivered a highly selective inhibitor of FKBP51 (SAFit2) to mice for 10 and 30 days. 10-day SAFit2 treatment significantly improved glucose tolerance, whereas 30-day SAFit2 treatment not only improved glucose tolerance but furthermore reduced body weight gain. At a functional level, SAFit2 treatment significantly augmented glucose uptake in C2C12 myotubes. These data are the first to demonstrate both physiologically and mechanistically the therapeutic potential of FKBP51 in the treatment of obesity and T2D.

In our fourth study (Chapter 2.4), we sought to establish novel signaling pathways contributing to FKBP51-dependent regulation of whole body energy and glucose metabolism. To-date several interacting partners of FKBP51 have been reported, including several serine-threonine kinases (Gassen et al., 2015;Pei et al., 2009;Taipale et al., 2014). Therefore using a candidate approach, we identified a novel regulatory role for FKBP51 in the AMPK (serine-threonine) signaling cascade. Specifically, we identified a novel interaction between FKBP51, AMPK, and the direct downstream target of AMPK, TSC1-TSC2. Interestingly, the tuberous sclerosis complex (TSC), comprising TSC1 and TSC2, is one of the most well characterized regulators of mTOR. In this context, we observed an FKBP51-dependent regulation of the mTOR signaling cascade, in which loss of FKBP51 leads to heightened mTOR activity. In support of these findings, wild-type mouse embryonic fibroblasts (MEFs) were responsive to metformin treatment, an activator of AMPK and a known anti-diabetic agent. By contrast MEFs lacking FKPP51 were unresponsive to metformin, assessed by the phosphorylation states of proteins downstream of AMPK and mTOR. Collectively, these data are the first to clearly demonstrate that AMPK regulation of mTOR is dependent on FKBP51 function. Considering the central role of AMPK in whole body energy metabolism (Kahn et

al., 2005), the regulatory role of FKBP51 on AMPK signaling is likely not without consequence. Future studies are required to determine the specific physiological context in which AMPK regulates mTOR signaling in a FKBP51-dependent manner.

3.1.3. Hypothalamic FKBP51 Overexpression Paradoxically Improves the Metabolic Outcome in Mice

In our fifth study (Chapter 2.5), we characterized the metabolic outcome arising specifically from hypothalamic overexpression of FKBP51. We hypothesized that FKBP51 hypothalamic overexpression would induce body weight gain and glucose intolerance based on our earlier findings that total loss of FKBP51 reduces body weight gain and glucose tolerance. To our surprise, FKBP51 overexpression within the hypothalamus resulted in lower body weight gain and glucose tolerance, resembling the metabolic phenotype of total 51KO mice.

Despite the unexpected metabolic phenotype arising from targeted hypothalamic overexpression of FKBP51, it is nevertheless clear that FKBP51 acts within the hypothalamus to regulate whole body energy metabolism. Indeed the hypothalamus is a crucial area within the central nervous system regulating feeding behavior, body weight, energy metabolism, and glucose metabolism, and therefore FKBP51 has the potential to modulate multiple functional outcomes. However, the questions of how and where FKBP51 acts within the hypothalamus remain elusive. Given the cellular and functional heterogeneity of the hypothalamus, this concern needs to be addressed. We overexpressed FKBP51 within the hypothalamus across the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamic nucleus (VMH), and dorsomedial hypothalamic nucleus (DMH). We initially aimed to broadly target the hypothalamus because we had no a priori knowledge as to whether or not hypothalamic overexpression would have any effect on the metabolic outcome whatsoever. Furthermore, based on the known expression profile of FKBP5 across the ARC, PVN, and VMH, we wanted to target each of these nuclei. In mice, the DMH does not express FKBP51, however FKBP5 was nevertheless found overexpressed in our study. From this experiment, we can conclude that FKBP51 acts broadly within the hypothalamus to decrease body weight, partly through its effect on food intake.

Within the hypothalamus, there are multiple neuronal types that act in concert to sense, integrate, and respond to hormonal and nutritional signals (Refer to General Introduction). Such concerted actions often require reciprocal actions across distinct neuronal populations. The integrated response of the summated actions dictates the phenotypic expression. For example, within the ARC two populations of neurons exist which have opposing effects on

food intake (Refer to General Introduction). Activation of POMC-expressing neurons favors reduced food intake, whereas activation of NPY-expressing neurons favors increased food intake. The level of activation of each neuronal population governs feeding behavior. Therefore in the context of FKBP51, it was important to determine the cell type-specific expression profile of FKBP51. Suppose FKBP51 is exclusively expressed in NPY-containing neurons, but in the current study we overexpressed FKBP51 across all cell types using a strong synthetic promoter (CAG) to drive high levels of gene expression. Our findings may be confounded by poor spatial and/or cell type-specific resolution. Therefore, as an initial first step, we established whether FKBP5 is expressed in NPY-containing neurons, POMC-containing neurons or both using double in situ hybridization. We are the first to identify that FKBP5 is expressed in both neuronal populations within the ARC. Future studies should target discrete neuronal populations within discrete hypothalamic nuclei in order to resolve the role of FKBP51 in each subpopulation.

Based on our past findings (Chapter 2.3) and current findings (Chapter 2.5), we conclude that the metabolic phenotype arising from total loss of FKBP51 is not mediated centrally through the hypothalamus, and we postulate that FKBP51 action in the periphery plays a more prominent role in whole body energy metabolism. Indeed the larger energy balance system encompasses multiple tissues, including many peripheral tissues presenting high levels of Fkbp5 expression. For example *FKBP5* shows the highest mRNA expression profile in human skeletal muscle, adipose tissue, and immune cells (Su et al., 2004). Interestingly, adipose-specific functions of FKBP51 in adipogenesis have already been reported (Toneatto et al., 2013). Our findings that FKBP51 selectively regulates the insulin signaling cascade within skeletal muscle, furthermore strengthens the notion that FKBP51 action in peripheral tissues is an important regulatory component for whole body energy and glucose homeostasis. Conditional transgenic mouse models with high spatial and temporal resolution are required to fully address the question as to where FKBP51 acts to regulate whole body energy and glucose homeostasis. At a physiological level, it is likely that the concerted action of FKBP51 across multiple tissues is required.

3.2. Significance to the Research Field

3.2.1. FKBP51: A Genetic Risk Factor in Metabolic Diseases

The current thesis provides a strong framework for understanding the relationship between FKBP51 and whole body energy homeostasis. We demonstrate that the dietary environment

alters FKBP51 expression. In turn, fluctuations in FKBP51 expression lead to diverging metabolic phenotypes. For example, based on our findings that complete loss of FKBP51 improves the metabolic phenotype of mice, one could speculate that physiological differences in FKBP51 may contribute to an individual's susceptibility to develop obesity or T2D. This is in line with an earlier study which reported that *FKBP5* gene expression in subcutaneous adipose tissue positively correlates with HOMA-IR (an index of insulin resistance (Pereira et al., 2014b). In parallel, this same study used data from genome-wide association studies to demonstrate that a number of genetic variants within the FKBP5 gene loci associate with traits of T2D.

Environmental factors are just as important as genetic factors in predicting susceptibility to obesity and/or T2D. In other words, individuals become obese when they are genetically susceptible to the obesogenic environment of Western society. In the context of the genetic risk factor FKBP51, 51KO mice are only moderately leaner than WT mice under standard diet conditions. It is not until they are challenged with a HFD that a diverging metabolic phenotype manifests between 51KO and WT mice. Along these same lines, obesity has only recently reached epidemic proportions, suggesting that genetic susceptibility and/or resilience to obesity is only realized when challenged by environmental factors (i.e. Western diets). This highlights the complexity of body weight regulation and glucose homeostasis. Many environmental conditions (besides HFD) and additional genetic factors (besides FKBP51) are indeed involved in predicting disease (obesity and T2D) vulnerability. Nevertheless, the in-depth metabolic characterization of FKBP51 knockout mice identifies FKBP51 as a novel genetic risk factor for metabolic diseases and also extends our current understanding of the detailed genetic profile relevant to metabolic regulation (Figure 3.2.1).

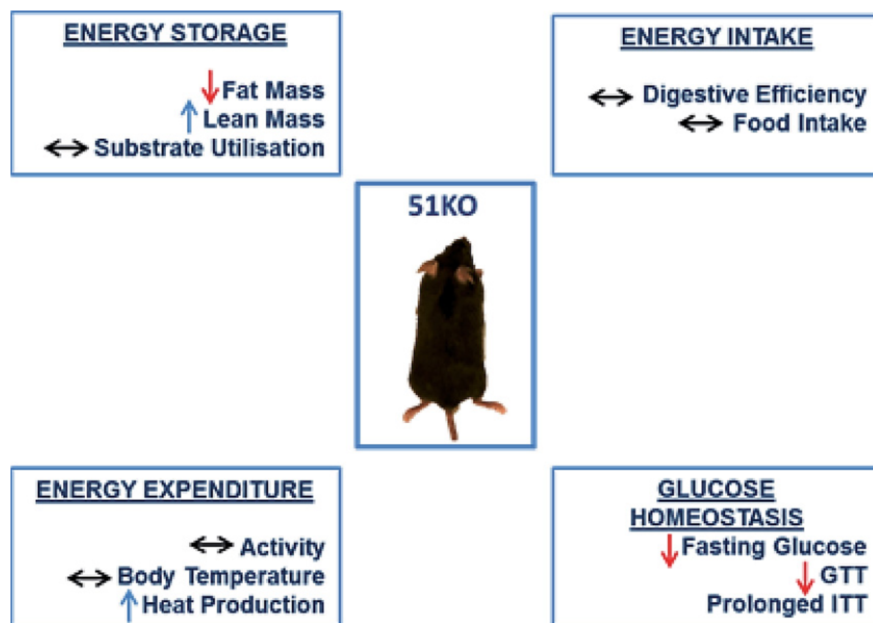

Figure 3.2.1 The metabolic phenotype arising from genetic ablation of FKBP51

Metabolic studies were conducted on 51KO and WT mice. 51KO mice presented decreased fat pad mass and increased lean mass under both HFD and control conditions, and were furthermore protected against HFD-induced weight gain. To examine the mechanism underlying the body weight phenotype, parameters comprising energy storage, energy intake, and energy expenditure were monitored. Loss of FKBP51 had no effect on home cage activity level, body temperature, substrate utilization, digestive efficiency (nutrient absorption), or feeding. However increased energy expenditure (heat production) may explain the lower body weight of 51KO mice compared to WT mice. Finally investigation into parameters of glucose homeostasis revealed that loss of FKBP51 decreased fasting glucose, lowered glucose tolerance, and prolonged the response to insulin during an ITT.

3.2.2. FKBP51: Novel and Established Molecular Mechanisms Governing Energy and Glucose Metabolism

Novel Molecular Mechanisms

Identification of underlying mechanisms by which newly identified genetic factors function to modulate metabolic regulation strengthens the existing, known molecular framework. In our case we identify FKBP51 as an important regulator of insulin signaling in the context of glucose homeostasis and furthermore as a novel regulator of AMPK-mTOR signaling pathways. The finding that FKBP51 regulates glucose homeostasis by modulating the insulin signaling cascade at the AKT locus identifies a previously unrecognized consequence of FKBP51-dependent AKT regulation. In general, the insulin signaling cascade stimulates glucose transport in skeletal muscle and adipose tissue. More precisely, it has been shown

that Akt is both necessary and sufficient for insulin-stimulated glucose transport (Sakamoto and Holman, 2008). Of the 3 AKT isoforms, AKT2 shows the highest expression in insulin sensitive tissues and likewise shows the strongest association to glucose uptake (Vasudevan and Garraway, 2010). Importantly, AKT2 knockout mice present glucose intolerance and insulin resistance owing to impaired insulin-stimulated glucose uptake in skeletal muscle and adipose tissue (Cho et al., 2001). This is in agreement with our findings in which 51KO mice present improved glucose and insulin tolerance owing to enhanced AKT activation (reduced inhibition by PHLPP) and increased glucose uptake in skeletal muscle. Therefore, while AKT is already well recognized to play a pivotal role in glucose transport, the regulation of AKT, and the identity of specific modulator proteins, is yet to be completely understood. In this sense, the interaction between AKT and FKBP51 resulting in functional changes at the level of glucose transport is highly relevant, especially concerning AKT regulation of glucose transport specifically within skeletal muscle.

Using a candidate approach, we furthermore identified a novel interaction between AMPK, TSC1/2, and FKBP51. AMPK is a conserved serine-threonine kinase which functions as a cellular and whole body energy sensor (Towler and Hardie, 2007). Under low energy conditions, activation of AMPK restores intracellular ATP levels by enhancing catabolic metabolism while inhibiting anabolic metabolism (Section 1.6.3.). Although well-recognized for its regulation of whole body energy metabolism, its own regulation is incompletely understood. Therefore the novel interaction between FKBP51, AMPK, and TSC1/2 is highly significant. Importantly, this novel interaction is functionally linked to mTOR activation, in which we demonstrate that AMPK inhibition of mTOR depends on FKBP51. Interestingly, our findings, reported here, compliment an earlier study which mapped protein-protein interactions using a proteomic approach (Taipale et al., 2014). In this earlier study, FKBP51 was identified as an interacting partner of LKB1, an upstream serine-threonine kinase that directly phosphorylates and activates AMPK (Shackelford and Shaw, 2009). Thus our study contributes important novel information to the growing research on AMPK regulation, suggesting that FKBP51 acts to direct AMPK action towards select targets and to furthermore organize the AMPK heterocomplex, including both upstream regulators (LKB1) and downstream effectors (TSC1/2).

Insulin signaling and AMPK-mTOR signaling are not mutually exclusive. Rather their concerted actions are integral to the larger interconnected metabolic regulatory network, which functions to maintain energy and glucose homeostasis (see Section 1.6.3). We and

others (Pei et al., 2009) have demonstrated that FKBP51 interacts with several proteins along the integrated pathway to ultimately regulate the activity of various nodes. Interestingly, although FKBP51 binds to distinct binding partners along this metabolic regulatory network, its overall action appears somewhat redundant. For example, in complex with AMPK and TSC1/2, FKBP51 increases the inhibition efficiency of AMPK-TSC1/2 on downstream mTOR signaling. Similarly, in complex with AKT and PHLPP, FKBP51 decreases the activation efficiency of AKT, which subsequently also favors mTOR inactivation (Figure 3.2.2). Taken together, these data suggest that FKBP51 is an important regulator of this integrated metabolic regulatory network, acting at multiple levels to regulate redundant or related homeostatic functions.

A major focus of research related to signal transduction pathways is to understand how protein kinases are able to achieve functional specificity in order to regulate multiple cellular functions in response to different environmental stimuli. Protein-protein interactions are inevitably very important determinants for recruiting and concentrating specific signaling molecules to discrete regulatory proteins. Targeting such protein-protein interactions that govern the specificity between binding partners improves our overall understanding of how signaling cascades achieve specificity. Importantly, in order to achieve functional specificity in response to specific environmental contexts, it is important that molecular targets are able to sense and furthermore respond to the environment. AMPK, mTOR, and FKBP51 all have the capacity to sense the environment. In particular, AMPK responds to low energy conditions (Hardie, 2015), mTOR responds to nutritional status (i.e. amino acids) (Wullschleger et al., 2006), and FKBP51 responds to both stress (Guidotti et al., 2013;Scharf et al., 2011) and dietary (Balsevich et al., 2014) conditions. Therefore, based on previous findings and the current work, we believe that FKBP51 may function as a scaffolding protein coordinating the signal transduction between AMPK, AKT, and mTOR by attracting various proteins required for the correct signaling cascade in response to the environmental stimuli.

Established Molecular Mechanisms

Besides the novel molecular mechanisms involving FKBP51 characterized in the current thesis, the well-established role of FKBP51 in the stress response and glucocorticoid signaling cannot be neglected. As a co-chaperone to the hsp90, FKBP51 decreases GR ligand-binding sensitivity and nuclear translocation efficiency (Section 1.8.) (Denny et al., 2000;Scammell et al., 2001;Westberry et al., 2006;Wochnik et al., 2005). Importantly, through a short-loop negative feedback system, fkbp5 expression is itself induced by GR

activation (Vermeer et al., 2003). This has important implications because of the association between high levels of FBP51 expression and adverse metabolic outcomes (current thesis; (Pereira et al., 2014b)). Therefore, does chronic stress predispose individuals to body weight gain, partly through the upregulation of FKBP51? In support of this, 51KO mice are less susceptible to stress-induced body weight gain examined in the chronic social defeat stress paradigm (Hartmann et al., 2012). Indeed, chronic stress is a known risk factor for metabolic disorders, namely obesity and T2D (Marcovecchio and Chiarelli, 2012). Yet there is a lack of known molecular targets governing this relationship. Further investigation is required to systematically characterize whether FKBP51 is involved in stress-induced body weight gain. In addition, it is important to delineate how chronic stress exposure governs the diverse roles of FKBP51 and to identify whether stress exposure favors specific protein-protein interactions involving FKBP51 over other interactions to ultimately dictate functional specificity.

Collective FKBP51-Mediated Mechanisms

In summary, the current dataset strongly suggests that FKBP51 may provide functional specificity to signaling cascades through multiple protein-protein interactions. Not only is FKBP51 a multi-domain protein whose structure is suitable for protein-protein interactions, but also FKBP51 is highly responsive to environmental cues, allowing it to mount a particular response to various stimuli. Our data indicate that reduced FKBP51 expression is associated with favorable metabolic outcomes. However, this is founded in the context of an obesogenic context. Given the pleiotropic functions of FKBP51, it is likely that FKBP51 expression and function may have both beneficial and disadvantageous outcomes depending on the context.

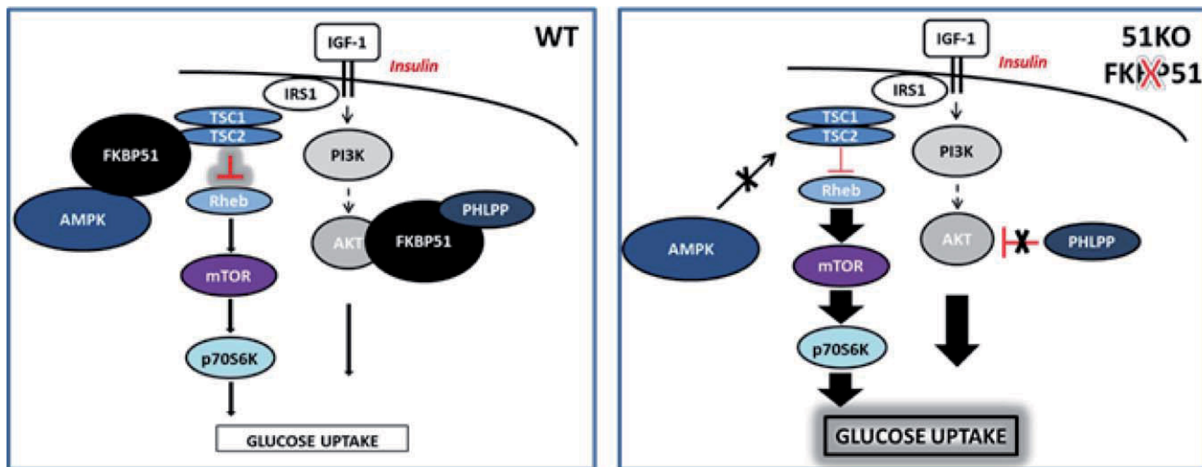

Figure 3.2.2 Working model of FKBP51 action on the metabolic regulatory network

Working model showing how the larger metabolic regulatory network may operate under conditions where FKBP51 is present (i.e. WT conditions) or lost (i.e. 51KO conditions or pharmacological blockade). The efficacy of signaling cascades depend on protein-protein interactions between critical signaling nodes and their respective regulators. Under WT conditions, FKBP51 acts as a scaffolding protein between AKT and PHLPP thereby down-regulating AKT signaling. Likewise, FKBP51 is in complex with AMPK, TSC1 and TSC2 thereby down-regulating mTOR signaling. Functionally, the resulting specific pattern of network connectivity favors reduced glucose uptake in the presence of FKBP51. Under 51KO conditions, loss of FKBP51 results in reduced inhibition of AKT and subsequently increased AKT downstream signaling. Likewise, AMPK-dependent inhibition of mTOR signaling is reduced under 51KO conditions. Functionally, loss of FKBP51 results in a specific pattern of network connectivity that favors increased glucose uptake.

3.2.3. FKBP51: Implications for the Treatment of Obesity and Type 2 Diabetes

Effective pharmacological treatment strategies for obesity and related complications remain elusive. Importantly, most currently available treatment options are able to address, at best, one disease mechanism (Grundy, 2006). For example, for the treatment of T2D, treatment often includes combination drug therapy in which separate agents are used to treat insulin deficiency and insulin resistance. Similarly, despite the high comorbidity between obesity and T2D, first-line pharmacological intervention strategies are not able to overcome both disease states. Therefore, research is currently exploring possible novel candidates that are able to target multiple risk factors more effectively. This requires the discovery of new, multifunctional molecules that act on numerous key pathways, whose concerted action governs energy balance and glucose homeostasis.

We identified FKBP51 as a novel genetic factor involved in metabolic regulation. Importantly, our mechanistic investigation demonstrated that FKBP51 indeed functions as a multifunctional molecule able to interact with multiple proteins at critical nodes along key pathways involved in energy balance and glucose homeostasis. To translate the current findings into a therapeutic context, we also established whether pharmacological blockade affects body weight and/or glucose homeostasis. We report that highly selective blockade of FKBP51 (using SAFit2) indeed has therapeutic promise. In fact, a 30-day treatment schedule reduced both body weight gain and glucose tolerance. Our comprehensive data set directly implicates FKBP51 in metabolic regulation and provides physiological and mechanistic evidence for the therapeutic potential of FKBP51 in the treatment of both obesity and T2D.

3.3. Limitations and Future Directions

We present compelling data that identify FKBP51 as a novel molecule involved in energy balance and glucose homeostasis. This opens a new avenue of research, which has the potential to advance current medical practices. Despite our in-depth characterization of FKBP51 function in the context of whole body metabolic regulation, there remain outstanding research questions. Two of the most pressing issues regarding the metabolic functions of FKBP51 are the following:

a. Which tissues mediate the metabolic actions of FKBP51?
b. How does FKBP51 respond to changing environmental contexts in order to regulate energy balance and glucose homeostasis?

3.3.1. Tissue-Specificity of FKBP51

In order to explore the metabolic function of FKBP51, we characterized the metabolic phenotype in total FKBP51 knockout mice. Although conventional 51KO mice provided a means to establish whether FKBP51 is involved in metabolic regulation or not, there are many shortcomings associated with any conventional knockout mouse line. For example, there is inevitably a lack of spatial and temporal control of gene expression. Furthermore the possibility exists that compensatory changes during development by other molecules may occur in the absence of the deleted gene. Therefore temporal and spatial transgenic animal models are preferred. In the specific case of FKBP51, tissue specificity is certainly an important consideration. For example, *FKBP5* is highly expressed across multiple tissues (Su et al., 2004), many of which are integral to whole body energy balance and glucose homeostasis. The contribution of individual tissue types, or specific cell types, must be

addressed in the future. Our data already highlight the tissue- specific effects of FKBP51, whereby FKBP51-dependent regulation of the insulin signaling cascade, and consequently glucose transport, is restricted to skeletal muscle. Likewise, across the whole hypothalamus, overexpression of FKBP51 resulted in reduced body weight gain, accompanied by a reduction in food intake. Previous studies have also highlighted tissue-specific actions of FKBP51, most notably in adipose tissue where it is involved in the regulation of adipogenesis (Toneatto et al., 2013). In addition, Pereira et al. also showed tissue-specific effects of FKBP51 whereby the positive association between *FKBP5* mRNA and HOMA-IR was restricted to subcutaneous adipose tissue and was not significant in the other examined adipose depots (Pereira et al., 2014b). Although the current work advances our understanding of the tissue-specific effects of FKBP51, (summarized in Figure 3.3.1**),** systematic characterization of the tissue-specific contributions of FKBP51 is still lacking.

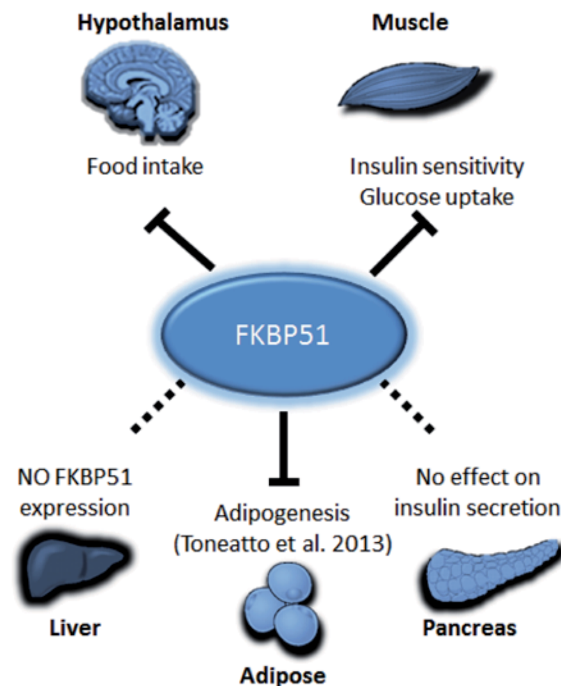

Figure 3.3.1 Tissue-specific FKBP51 regulation of whole body energy and glucose homeostasis

Across the whole hypothalamus, FKBP51 acts to decrease food intake (Chapter 2.5.). In skeletal muscle, FKBP51 acts to increase insulin sensitivity and glucose uptake (Chapter 2.3.). We found no effect of FKBP51 on insulin secretion from the pancreas, while FKBP51 is not expressed in mouse hepatocytes (Chapter 2.3.). Finally a previous study implicates FKBP51 in the regulation of adipogenesis in adipose tissue (Toneatto et al., 2013). Collectively, FKBP51 is able to act at multiple levels in order to modulate metabolic outcomes.

3.3.2. Responsiveness of FKBP51 to Environmental Cues

Although FKBP51 has been studied in the context of stress reactivity and as a stress-induced gene (Guidotti et al., 2013;Pereira et al., 2014b;Scharf et al., 2011;Vermeer et al., 2003), little research has been devoted to understanding how FKBP51 responds to additional environmental cues. Here we only begin to dissect how energy status and nutrient conditions influence FKBP51 expression and function. For example, HFD exposure increases hypothalamic expression of *Fkbp5*. Moreover, only under HFD conditions, does loss of FKBP51 produce a strong body weight phenotype. Nevertheless, it is important to understand the tissue-specific responsivity of *Fkbp5* as well as to understand how the nutrient environment affects FKBP51 function. For example, in conditions of high or low energy status, does FKBP51 preferentially bind to specific binding partners? This will shed light into the mechanisms underlying the HFD-resistant body weight phenotype presented in 51KO mice. Finally, it is highly relevant to understand the interaction between FKBP51, stress exposure, and dietary conditions. Specifically, could FKBP51 act at the interface between stress-related psychiatric disorders and metabolic disorders? Indeed there is a high comorbidity between psychiatric and metabolic disorders despite a lack of clear molecular targets responsible for this relationship. Although our preliminary study (Chapter 2.1) did not reveal that FKBP51 governs gene x environment interactions mediating the shared biology of psychiatric and metabolic disorders, it was a targeted, observational study. Based on the responsiveness of FKBP5 to the environment and its association to both psychiatric and metabolic diseases, examination into the role of FKBP51 on stress-dependent metabolic outcomes is warranted.

3.4. Closing remarks

We provide new insights into the regulation of energy balance and glucose homeostasis, which opens a new avenue of research, not yet widespread in literature. Future studies will be able to build on these findings, to provide an even clearer picture in order to understand the exact mechanisms underlying the FKBP51-dependent mechanisms mediating the metabolic effects on body weight regulation and glucose homeostasis.

4. Bibliography

Abel,E.D., Peroni,O., Kim,J.K., Kim,Y.B., Boss,O., Hadro,E., Minnemann,T., Shulman,G.I., Kahn,B.B., 2001. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 409, 729-733.

Abraham,R.T., 2002. Identification of TOR signaling complexes: more TORC for the cell growth engine. Cell 111, 9-12.

Ahren,B., Pacini,G., 2002. Insufficient islet compensation to insulin resistance vs. reduced glucose effectiveness in glucose-intolerant mice. Am J Physiol Endocrinol Metab 283, E738-E744.

Akana,S.F., Strack,A.M., Hanson,E.S., Dallman,M.F., 1994. Regulation of activity in the hypothalamo-pituitary-adrenal axis is integral to a larger hypothalamic system that determines caloric flow. Endocrinology 135, 1125-1134.

Allison,D.B., Kaprio,J., Korkeila,M., Koskenvuo,M., Neale,M.C., Hayakawa,K., 1996. The heritability of body mass index among an international sample of monozygotic twins reared apart. Int J Obes Relat Metab Disord 20, 501-506.

Appel,K., Schwahn,C., Mahler,J., Schulz,A., Spitzer,C., Fenske,K., Stender,J., Barnow,S., John,U., Teumer,A., Biffar,R., Nauck,M., Volzke,H., Freyberger,H.J., Grabe,H.J., 2011. Moderation of adult depression by a polymorphism in the FKBP5 gene and childhood physical abuse in the general population. Neuropsychopharmacology 36, 1982-1991.

Arch,J.R., 2002. Lessons in obesity from transgenic animals. J Endocrinol Invest 25, 867-875.

Arch,J.R., Hislop,D., Wang,S.J., Speakman,J.R., 2006. Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. Int J Obes (Lond) 30, 1322-1331.

Armitage,J.A., Khan,I.Y., Taylor,P.D., Nathanielsz,P.W., Poston,L., 2004. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol 561, 355-377.

Aronsson,M., Fuxe,K., Dong,Y., Agnati,L.F., Okret,S., Gustafsson,J.A., 1988. Localization of glucocorticoid receptor mRNA in the male rat brain by in situ hybridization. Proc Natl Acad Sci U S A 85, 9331-9335.

Auvinen,H.E., Romijn,J.A., Biermasz,N.R., Pijl,H., Havekes,L., Smit,J.W., Rensen,P.C., Pereira,A.M., 2012. The Effects of High Fat Diet on the Basal Activity of the Hypothalamus-Pituitary-Adrenal Axis in Mice. J Endocrinol.

Bagdade,J.D., Bierman,E.L., Porte,D., Jr., 1967. The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. J Clin Invest 46, 1549-1557.

Balsevich,G., Uribe,A., Wagner,K.V., Hartmann,J., Santarelli,S., Labermaier,C., Schmidt,M.V., 2014. The interplay between diet-induced obesity and chronic stress in mice: potential role of FKBP51. J Endocrinol.

Barker,D.J., Bagby,S.P., Hanson,M.A., 2006. Mechanisms of disease: in utero programming in the pathogenesis of hypertension. Nat Clin Pract Nephrol 2, 700-707.

Barsh,G.S., Farooqi,I.S., O'Rahilly,S., 2000. Genetics of body-weight regulation. Nature 404, 644-651.

Barsh,G.S., Schwartz,M.W., 2002. Genetic approaches to studying energy balance: perception and integration. Nat Rev Genet 3, 589-600.

Bartolomucci,A., Cabassi,A., Govoni,P., Ceresini,G., Cero,C., Berra,D., Dadomo,H., Franceschini,P., Dell'Omo,G., Parmigiani,S., Palanza,P., 2009. Metabolic consequences and vulnerability to diet-induced obesity in male mice under chronic social stress. PLoS One 4, e4331.

Bartolomucci,A., Palanza,P., Sacerdote,P., Panerai,A.E., Sgoifo,A., Dantzer,R., Parmigiani,S., 2005. Social factors and individual vulnerability to chronic stress exposure. Neurosci Biobehav Rev 29, 67-81.

Baughman,G., Wiederrecht,G.J., Campbell,N.F., Martin,M.M., Bourgeois,S., 1995. FKBP51, a novel T-cell-specific immunophilin capable of calcineurin inhibition. Mol Cell Biol 15, 4395-4402.

Baughman,G., Wiederrecht,G.J., Chang,F., Martin,M.M., Bourgeois,S., 1997. Tissue distribution and abundance of human FKBP51, and FK506-binding protein that can mediate calcineurin inhibition. Biochem Biophys Res Commun 232, 437-443.

Bayol,S.A., Farrington,S.J., Stickland,N.C., 2007. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. Br J Nutr 98, 843-851.

Berghofer,A., Pischon,T., Reinhold,T., Apovian,C.M., Sharma,A.M., Willich,S.N., 2008. Obesity prevalence from a European perspective: a systematic review. BMC Public Health 8, 200.

Bertram,C.E., Hanson,M.A., 2001. Animal models and programming of the metabolic syndrome. Br Med Bull 60, 103-121.

Bilbo,S.D., Tsang,V., 2010. Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. FASEB J 24, 2104-2115.

Binder,E.B., 2009. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology 34 Suppl 1, S186-S195.

Binder,E.B., Bradley,R.G., Liu,W., Epstein,M.P., Deveau,T.C., Mercer,K.B., Tang,Y., Gillespie,C.F., Heim,C.M., Nemeroff,C.B., Schwartz,A.C., Cubells,J.F., Ressler,K.J., 2008. Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. JAMA 299, 1291-1305.

Binder,E.B., Salyakina,D., Lichtner,P., Wochnik,G.M., Ising,M., Putz,B., Papiol,S., Seaman,S., Lucae,S., Kohli,M.A., Nickel,T., Kunzel,H.E., Fuchs,B., Majer,M., Pfennig,A., Kern,N., Brunner,J., Modell,S., Baghai,T., Deiml,T., Zill,P., Bondy,B., Rupprecht,R., Messer,T., Kohnlein,O., Dabitz,H., Bruckl,T., Muller,N., Pfister,H., Lieb,R., Mueller,J.C., Lohmussaar,E., Strom,T.M., Bettecken,T., Meitinger,T., Uhr,M., Rein,T., Holsboer,F., Muller-Myhsok,B., 2004. Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. Nat Genet 36, 1319-1325.

Bjorkqvist,K., 2001. Social defeat as a stressor in humans. Physiol Behav 73, 435-442.

Bjorntorp,P., 2001. Do stress reactions cause abdominal obesity and comorbidities? Obes Rev 2, 73-86.

Bjorntorp,P., Rosmond,R., 2000. Obesity and cortisol. Nutrition 16, 924-936.

Block,J.P., He,Y., Zaslavsky,A.M., Ding,L., Ayanian,J.Z., 2009. Psychosocial stress and change in weight among US adults. Am J Epidemiol 170, 181-192.

Bornstein,S.R., Schuppenies,A., Wong,M.L., Licinio,J., 2006. Approaching the shared biology of obesity and depression: the stress axis as the locus of gene-environment interactions. Mol Psychiatry 11, 892-902.

Boscaro,M., Barzon,L., Fallo,F., Sonino,N., 2001. Cushing's syndrome. Lancet 357, 783-791.

Bowe,J.E., Franklin,Z.J., Hauge-Evans,A.C., King,A.J., Persaud,S.J., Jones,P.M., 2014. Metabolic phenotyping guidelines: assessing glucose homeostasis in rodent models. J Endocrinol 222, G13-G25.

Brange,J., Langkjoer,L., 1993. Insulin structure and stability. Pharm Biotechnol 5, 315-350.

Buettner,C., Muse,E.D., Cheng,A., Chen,L., Scherer,T., Pocai,A., Su,K., Cheng,B., Li,X., Harvey-White,J., Schwartz,G.J., Kunos,G., Rossetti,L., Buettner,C., 2008. Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. Nat Med 14, 667-675.

Cannon,B., Nedergaard,J., 2004. Brown adipose tissue: function and physiological significance. Physiol Rev 84, 277-359.

Cannon,B., Nedergaard,J., 2010. Metabolic consequences of the presence or absence of the thermogenic capacity of brown adipose tissue in mice (and probably in humans). Int J Obes (Lond) 34 Suppl 1, S7-16.

Cannon,B., Nedergaard,J., 2011. Nonshivering thermogenesis and its adequate measurement in metabolic studies. J Exp Biol 214, 242-253.

Carvalho,E., Kotani,K., Peroni,O.D., Kahn,B.B., 2005. Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle. Am J Physiol Endocrinol Metab 289, E551-E561.

Chen,K.Y., Brychta,R.J., Linderman,J.D., Smith,S., Courville,A., Dieckmann,W., Herscovitch,P., Millo,C.M., Remaley,A., Lee,P., Celi,F.S., 2013. Brown fat activation mediates cold-induced thermogenesis in adult humans in response to a mild decrease in ambient temperature. J Clin Endocrinol Metab 98, E1218-E1223.

Cheung,C.C., Clifton,D.K., Steiner,R.A., 1997. Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology 138, 4489-4492.

Cheung,J., Smith,D.F., 2000. Molecular chaperone interactions with steroid receptors: an update. Mol Endocrinol 14, 939-946.

Cho,H., Mu,J., Kim,J.K., Thorvaldsen,J.L., Chu,Q., Crenshaw,E.B., III, Kaestner,K.H., Bartolomei,M.S., Shulman,G.I., Birnbaum,M.J., 2001. Insulin resistance and a

diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292, 1728-1731.

Chrousos,G.P., Charmandari,E., Kino,T., 2004. Glucocorticoid action networks--an introduction to systems biology. J Clin Endocrinol Metab 89, 563-564.

Chrousos,G.P., Gold,P.W., 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267, 1244-1252.

Chuang,J.C., Krishnan,V., Yu,H.G., Mason,B., Cui,H., Wilkinson,M.B., Zigman,J.M., Elmquist,J.K., Nestler,E.J., Lutter,M., 2010. A beta3-adrenergic-leptin-melanocortin circuit regulates behavioral and metabolic changes induced by chronic stress. Biol Psychiatry 67, 1075-1082.

Clement,K., Vaisse,C., Lahlou,N., Cabrol,S., Pelloux,V., Cassuto,D., Gourmelen,M., Dina,C., Chambaz,J., Lacorte,J.M., Basdevant,A., Bougneres,P., Lebouc,Y., Froguel,P., Guy-Grand,B., 1998. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392, 398-401.

Cohen,J., 1992. A power primer. Psychol Bull 112, 155-159.

Cohen,S.L., Halaas,J.L., Friedman,J.M., Chait,B.T., Bennett,L., Chang,D., Hecht,R., Collins,F., 1996. Human leptin characterization. Nature 382, 589.

Coleman,D.L., 1973. Effects of parabiosis of obese with diabetes and normal mice. Diabetologia 9, 294-298.

Collip,D., Myin-Germeys,I., Wichers,M., Jacobs,N., Derom,C., Thiery,E., Lataster,T., Simons,C., Delespaul,P., Marcelis,M., van,O.J., van,W.R., 2013. FKBP5 as a possible moderator of the psychosis-inducing effects of childhood trauma. Br J Psychiatry 202, 261-268.

Commins,S.P., Watson,P.M., Levin,N., Beiler,R.J., Gettys,T.W., 2000. Central leptin regulates the UCP1 and ob genes in brown and white adipose tissue via different beta-adrenoceptor subtypes. J Biol Chem 275, 33059-33067.

Cone,R.D., 2005. Anatomy and regulation of the central melanocortin system. Nat Neurosci 8, 571-578.

Cone,R.D., Cowley,M.A., Butler,A.A., Fan,W., Marks,D.L., Low,M.J., 2001. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int J Obes Relat Metab Disord 25 Suppl 5, S63-S67.

Considine,R.V., Sinha,M.K., Heiman,M.L., Kriauciunas,A., Stephens,T.W., Nyce,M.R., Ohannesian,J.P., Marco,C.C., McKee,L.J., Bauer,T.L., ., 1996. Serum

immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334, 292-295.

Copps,K.D., Hancer,N.J., Opare-Ado,L., Qiu,W., Walsh,C., White,M.F., 2010. Irs1 serine 307 promotes insulin sensitivity in mice. Cell Metab 11, 84-92.

Copps,K.D., White,M.F., 2012. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia 55, 2565-2582.

d'Asti,E., Long,H., Tremblay-Mercier,J., Grajzer,M., Cunnane,S.C., Di,M., V, Walker,C.D., 2010. Maternal dietary fat determines metabolic profile and the magnitude of endocannabinoid inhibition of the stress response in neonatal rat offspring. Endocrinology 151, 1685-1694.

Dallman,M.F., 2010. Stress-induced obesity and the emotional nervous system. Trends Endocrinol Metab 21, 159-165.

Davies,T.H., Ning,Y.M., Sanchez,E.R., 2002. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem 277, 4597-4600.

Davies,T.H., Ning,Y.M., Sanchez,E.R., 2005. Differential control of glucocorticoid receptor hormone-binding function by tetratricopeptide repeat (TPR) proteins and the immunosuppressive ligand FK506. Biochemistry 44, 2030-2038.

de Guia,R.M., Rose,A.J., Herzig,S., 2014. Glucocorticoid hormones and energy homeostasis. Horm Mol Biol Clin Investig 19, 117-128.

de Kloet,E.R., Joels,M., Holsboer,F., 2005. Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6, 463-475.

de Kloet,E.R., Oitzl,M.S., Joels,M., 1993. Functional implications of brain corticosteroid receptor diversity. Cell Mol Neurobiol 13, 433-455.

de Kloet,E.R., Reul,J.M., 1987. Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. Psychoneuroendocrinology 12, 83-105.

de Kloet,E.R., Vreugdenhil,E., Oitzl,M.S., Joels,M., 1998. Brain corticosteroid receptor balance in health and disease. Endocr Rev 19, 269-301.

DeFronzo,R.A., Gunnarsson,R., Bjorkman,O., Olsson,M., Wahren,J., 1985. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest 76, 149-155.

Delaunay,F., Khan,A., Cintra,A., Davani,B., Ling,Z.C., Andersson,A., Ostenson,C.G., Gustafsson,J., Efendic,S., Okret,S., 1997. Pancreatic beta cells are important targets for the diabetogenic effects of glucocorticoids. J Clin Invest 100, 2094-2098.

Denny,W.B., Valentine,D.L., Reynolds,P.D., Smith,D.F., Scammell,J.G., 2000. Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding. Endocrinology 141, 4107-4113.

Desbois-Mouthon,C., Cadoret,A., Blivet-Van Eggelpoel,M.J., Bertrand,F., Cherqui,G., Perret,C., Capeau,J., 2001. Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation. Oncogene 20, 252-259.

Dietrich,M.O., Horvath,T.L., 2013. Hypothalamic control of energy balance: insights into the role of synaptic plasticity. Trends Neurosci 36, 65-73.

Dimitriadis,G., Mitrou,P., Lambadiari,V., Maratou,E., Raptis,S.A., 2011. Insulin effects in muscle and adipose tissue. Diabetes Res Clin Pract 93 Suppl 1, S52-S59.

Dunn,G.A., Bale,T.L., 2009. Maternal high-fat diet promotes body length increases and insulin insensitivity in second-generation mice. Endocrinology 150, 4999-5009.

Dyck,J.R., Gao,G., Widmer,J., Stapleton,D., Fernandez,C.S., Kemp,B.E., Witters,L.A., 1996. Regulation of 5'-AMP-activated protein kinase activity by the noncatalytic beta and gamma subunits. J Biol Chem 271, 17798-17803.

Elias,C.F., Aschkenasi,C., Lee,C., Kelly,J., Ahima,R.S., Bjorbaek,C., Flier,J.S., Saper,C.B., Elmquist,J.K., 1999. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron 23, 775-786.

Elmquist,J.K., Bjorbaek,C., Ahima,R.S., Flier,J.S., Saper,C.B., 1998. Distributions of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol 395, 535-547.

Evans,R.M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240, 889-895.

Faith,M.S., Matz,P.E., Jorge,M.A., 2002. Obesity-depression associations in the population. J Psychosom Res 53, 935-942.

Farooqi,S.I., 2014. Genetic, molecular and physiological mechanisms involved in human obesity: Society for Endocrinology Medal Lecture 2012. Clin Endocrinol (Oxf).

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

Fernandes,C., Grayton,H., Poston,L., Samuelsson,A.M., Taylor,P.D., Collier,D.A., Rodriguez,A., 2012. Prenatal exposure to maternal obesity leads to hyperactivity in offspring. Mol Psychiatry 17, 1159-1160.

Finger,B.C., Dinan,T.G., Cryan,J.F., 2011. High-fat diet selectively protects against the effects of chronic social stress in the mouse. Neuroscience 192, 351-360.

Fisher,J.S., Gao,J., Han,D.H., Holloszy,J.O., Nolte,L.A., 2002. Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. Am J Physiol Endocrinol Metab 282, E18-E23.

Fluttert,M., Dalm,S., Oitzl,M.S., 2000. A refined method for sequential blood sampling by tail incision in rats. Lab Anim 34, 372-378.

Fontaine,K.R., Barofsky,I., 2001. Obesity and health-related quality of life. Obes Rev 2, 173-182.

Foretz,M., Guigas,B., Bertrand,L., Pollak,M., Viollet,B., 2014. Metformin: From Mechanisms of Action to Therapies. Cell Metab 20, 953-966.

Friedman,J.M., 2000. Obesity in the new millennium. Nature 404, 632-634.

Friedman,J.M., 2009. Obesity: Causes and control of excess body fat. Nature 459, 340-342.

Gaali,S., Kirschner,A., Cuboni,S., Hartmann,J., Kozany,C., Balsevich,G., Namendorf,C., Fernandez-Vizarra,P., Sippel,C., Zannas,A.S., Draenert,R., Binder,E.B., Almeida,O.F., Ruhter,G., Uhr,M., Schmidt,M.V., Touma,C., Bracher,A., Hausch,F., 2014. Selective inhibitors of the FK506-binding protein 51 by induced fit. Nat Chem Biol.

Gaali,S., Kirschner,A., Cuboni,S., Hartmann,J., Kozany,C., Balsevich,G., Namendorf,C., Fernandez-Vizarra,P., Sippel,C., Zannas,A.S., Draenert,R., Binder,E.B., Almeida,O.F., Ruhter,G., Uhr,M., Schmidt,M.V., Touma,C., Bracher,A., Hausch,F., 2015. Selective inhibitors of the FK506-binding protein 51 by induced fit. Nat Chem Biol 11, 33-37.

Gabbay,R.A., Sutherland,C., Gnudi,L., Kahn,B.B., O'Brien,R.M., Granner,D.K., Flier,J.S., 1996. Insulin regulation of phosphoenolpyruvate carboxykinase gene expression does not require activation of the Ras/mitogen-activated protein kinase signaling pathway. J Biol Chem 271, 1890-1897.

Galigniana,N.M., Ballmer,L.T., Toneatto,J., Erlejman,A.G., Lagadari,M., Galigniana,M.D., 2012. Regulation of the glucocorticoid response to stress-related disorders by the Hsp90-binding immunophilin FKBP51. J Neurochem 122, 4-18.

Gallo,L.I., Ghini,A.A., Piwien,P.G., Galigniana,M.D., 2007. Differential recruitment of tetratricorpeptide repeat domain immunophilins to the mineralocorticoid receptor influences both heat-shock protein 90-dependent retrotransport and hormone-dependent transcriptional activity. Biochemistry 46, 14044-14057.

Gao,Q., Horvath,T.L., 2007. Neurobiology of feeding and energy expenditure. Annu Rev Neurosci 30, 367-398.

Garami,A., Zwartkruis,F.J., Nobukuni,T., Joaquin,M., Roccio,M., Stocker,H., Kozma,S.C., Hafen,E., Bos,J.L., Thomas,G., 2003. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell 11, 1457-1466.

Gassen,N.C., Hartmann,J., Zannas,A.S., Kretzschmar,A., Zschocke,J., Maccarrone,G., Hafner,K., Zellner,A., Kollmannsberger,L.K., Wagner,K.V., Mehta,D., Kloiber,S., Turck,C.W., Lucae,S., Chrousos,G.P., Holsboer,F., Binder,E.B., Ising,M., Schmidt,M.V., Rein,T., 2015. FKBP51 inhibits GSK3beta and augments the effects of distinct psychotropic medications. Mol Psychiatry.

Gassen,N.C., Hartmann,J., Zschocke,J., Stepan,J., Hafner,K., Zellner,A., Kirmeier,T., Kollmannsberger,L., Wagner,K.V., Dedic,N., Balsevich,G., Deussing,J.M., Kloiber,S., Lucae,S., Holsboer,F., Eder,M., Uhr,M., Ising,M., Schmidt,M.V., Rein,T., 2014. Association of FKBP51 with Priming of Autophagy Pathways and Mediation of Antidepressant Treatment Response: Evidence in Cells, Mice, and Humans. PLoS Med 11, e1001755.

Gibson,E.L., 2006. Emotional influences on food choice: sensory, physiological and psychological pathways. Physiol Behav 89, 53-61.

Ginsburg,E., Livshits,G., Yakovenko,K., Kobyliansky,E., 1998. Major gene control of human body height, weight and BMI in five ethnically different populations. Ann Hum Genet 62, 307-322.

Gold,P.W., 2015. The organization of the stress system and its dysregulation in depressive illness. Mol Psychiatry 20, 32-47.

Golden,S.A., Covington,H.E., III, Berton,O., Russo,S.J., 2011. A standardized protocol for repeated social defeat stress in mice. Nat Protoc 6, 1183-1191.

Goldstein,B.I., Kemp,D.E., Soczynska,J.K., McIntyre,R.S., 2009. Inflammation and the phenomenology, pathophysiology, comorbidity, and treatment of bipolar disorder: a systematic review of the literature. J Clin Psychiatry 70, 1078-1090.

Grad,I., Picard,D., 2007. The glucocorticoid responses are shaped by molecular chaperones. Mol Cell Endocrinol 275, 2-12.

Grun,F., Blumberg,B., 2007. Perturbed nuclear receptor signaling by environmental obesogens as emerging factors in the obesity crisis. Rev Endocr Metab Disord 8, 161-171.

Grundy,S.M., 2006. Drug therapy of the metabolic syndrome: minimizing the emerging crisis in polypharmacy. Nat Rev Drug Discov 5, 295-309.

Guidotti,G., Calabrese,F., Anacker,C., Racagni,G., Pariante,C.M., Riva,M.A., 2013. Glucocorticoid receptor and FKBP5 expression is altered following exposure to chronic stress: modulation by antidepressant treatment. Neuropsychopharmacology 38, 616-627.

Hall,K.D., Heymsfield,S.B., Kemnitz,J.W., Klein,S., Schoeller,D.A., Speakman,J.R., 2012. Energy balance and its components: implications for body weight regulation. Am J Clin Nutr 95, 989-994.

Hallschmid,M., Schultes,B., 2009. Central nervous insulin resistance: a promising target in the treatment of metabolic and cognitive disorders? Diabetologia 52, 2264-2269.

Hankey,C.R., 2010. Session 3 (Joint with the British Dietetic Association): Management of obesity: Weight-loss interventions in the treatment of obesity. Proc Nutr Soc 69, 34-38.

Hardie,D.G., 2007. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol 8, 774-785.

Hardie,D.G., 2015. AMPK: positive and negative regulation, and its role in whole-body energy homeostasis. Curr Opin Cell Biol 33, 1-7.

Harris,A., Seckl,J., 2011. Glucocorticoids, prenatal stress and the programming of disease. Horm Behav 59, 279-289.

Hartmann,J., Wagner,K.V., Gaali,S., Kirschner,A., Kozany,C., Ruhter,G., Dedic,N., Hausl,A.S., Hoeijmakers,L., Westerholz,S., Namendorf,C., Gerlach,T., Uhr,M., Chen,A., Deussing,J.M., Holsboer,F., Hausch,F., Schmidt,M.V., 2015. Pharmacological Inhibition of the Psychiatric Risk Factor FKBP51 Has Anxiolytic Properties. J Neurosci 35, 9007-9016.

Hartmann,J., Wagner,K.V., Liebl,C., Scharf,S.H., Wang,X.D., Wolf,M., Hausch,F., Rein,T., Schmidt,U., Touma,C., Cheung-Flynn,J., Cox,M.B., Smith,D.F., Holsboer,F., Muller,M.B., Schmidt,M.V., 2012. The involvement of FK506-binding protein 51

(FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress. Neuropharmacology 62, 332-339.

Herman,J.P., Cullinan,W.E., 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 20, 78-84.

Herman,J.P., Figueiredo,H., Mueller,N.K., Ulrich-Lai,Y., Ostrander,M.M., Choi,D.C., Cullinan,W.E., 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24, 151-180.

Hill,J.W., Elmquist,J.K., Elias,C.F., 2008. Hypothalamic pathways linking energy balance and reproduction. Am J Physiol Endocrinol Metab 294, E827-E832.

Hinney,A., Volckmar,A.L., Knoll,N., 2013. Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis. Prog Mol Biol Transl Sci 114, 147-191.

Hoeffer,C.A., Klann,E., 2010. mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci 33, 67-75.

Holsboer,F., 2000. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 23, 477-501.

Huang,S., Czech,M.P., 2007. The GLUT4 glucose transporter. Cell Metab 5, 237-252.

Hurley,R.L., Anderson,K.A., Franzone,J.M., Kemp,B.E., Means,A.R., Witters,L.A., 2005. The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. J Biol Chem 280, 29060-29066.

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

Iglesias,M.A., Ye,J.M., Frangioudakis,G., Saha,A.K., Tomas,E., Ruderman,N.B., Cooney,G.J., Kraegen,E.W., 2002. AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. Diabetes 51, 2886-2894.

Inoki,K., Kim,J., Guan,K.L., 2012. AMPK and mTOR in cellular energy homeostasis and drug targets. Annu Rev Pharmacol Toxicol 52, 381-400.

Inoki,K., Li,Y., Xu,T., Guan,K.L., 2003a. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 17, 1829-1834.

Inoki,K., Li,Y., Zhu,T., Wu,J., Guan,K.L., 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 4, 648-657.

Inoki,K., Zhu,T., Guan,K.L., 2003b. TSC2 mediates cellular energy response to control cell growth and survival. Cell 115, 577-590.

Ising,M., Depping,A.M., Siebertz,A., Lucae,S., Unschuld,P.G., Kloiber,S., Horstmann,S., Uhr,M., Muller-Myhsok,B., Holsboer,F., 2008. Polymorphisms in the FKBP5 gene region modulate recovery from psychosocial stress in healthy controls. Eur J Neurosci 28, 389-398.

Jiang,W., Cazacu,S., Xiang,C., Zenklusen,J.C., Fine,H.A., Berens,M., Armstrong,B., Brodie,C., Mikkelsen,T., 2008. FK506 binding protein mediates glioma cell growth and sensitivity to rapamycin treatment by regulating NF-kappaB signaling pathway. Neoplasia 10, 235-243.

Jinwal,U.K., Koren,J., III, Borysov,S.I., Schmid,A.B., Abisambra,J.F., Blair,L.J., Johnson,A.G., Jones,J.R., Shults,C.L., O'Leary,J.C., III, Jin,Y., Buchner,J., Cox,M.B., Dickey,C.A., 2010. The Hsp90 cochaperone, FKBP51, increases Tau stability and polymerizes microtubules. J Neurosci 30, 591-599.

Kaelin,C.B., Gong,L., Xu,A.W., Yao,F., Hockman,K., Morton,G.J., Schwartz,M.W., Barsh,G.S., MacKenzie,R.G., 2006. Signal transducer and activator of transcription (stat) binding sites but not stat3 are required for fasting-induced transcription of agouti-related protein messenger ribonucleic acid. Mol Endocrinol 20, 2591-2602.

Kahn,B.B., Alquier,T., Carling,D., Hardie,D.G., 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 1, 15-25.

Kahn,B.B., Flier,J.S., 2000. Obesity and insulin resistance. J Clin Invest 106, 473-481.

Kane,S., Sano,H., Liu,S.C., Asara,J.M., Lane,W.S., Garner,C.C., Lienhard,G.E., 2002. A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. J Biol Chem 277, 22115-22118.

Katz,L.D., Glickman,M.G., Rapoport,S., Ferrannini,E., DeFronzo,R.A., 1983. Splanchnic and peripheral disposal of oral glucose in man. Diabetes 32, 675-679.

Kenny,P.J., 2011. Reward mechanisms in obesity: new insights and future directions. Neuron 69, 664-679.

Khan,A., Ostenson,C.G., Berggren,P.O., Efendic,S., 1992. Glucocorticoid increases glucose cycling and inhibits insulin release in pancreatic islets of ob/ob mice. Am J Physiol 263, E663-E666.

Khan,I.Y., Dekou,V., Douglas,G., Jensen,R., Hanson,M.A., Poston,L., Taylor,P.D., 2005. A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. Am J Physiol Regul Integr Comp Physiol 288, R127-R133.

Khan,I.Y., Taylor,P.D., Dekou,V., Seed,P.T., Lakasing,L., Graham,D., Dominiczak,A.F., Hanson,M.A., Poston,L., 2003. Gender-linked hypertension in offspring of lard-fed pregnant rats. Hypertension 41, 168-175.

Kim,H., Whang,W.W., Kim,H.T., Pyun,K.H., Cho,S.Y., Hahm,D.H., Lee,H.J., Shim,I., 2003. Expression of neuropeptide Y and cholecystokinin in the rat brain by chronic mild stress. Brain Res 983, 201-208.

King,B.M., 2006. The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. Physiol Behav 87, 221-244.

Kivimaki,M., Head,J., Ferrie,J.E., Shipley,M.J., Brunner,E., Vahtera,J., Marmot,M.G., 2006. Work stress, weight gain and weight loss: evidence for bidirectional effects of job strain on body mass index in the Whitehall II study. Int J Obes (Lond) 30, 982-987.

Klengel,T., Mehta,D., Anacker,C., Rex-Haffner,M., Pruessner,J.C., Pariante,C.M., Pace,T.W., Mercer,K.B., Mayberg,H.S., Bradley,B., Nemeroff,C.B., Holsboer,F., Heim,C.M., Ressler,K.J., Rein,T., Binder,E.B., 2013. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat Neurosci 16, 33-41.

Kloek,C., Haq,A.K., Dunn,S.L., Lavery,H.J., Banks,A.S., Myers,M.G., Jr., 2002. Regulation of Jak kinases by intracellular leptin receptor sequences. J Biol Chem 277, 41547-41555.

Koch,L., Wunderlich,F.T., Seibler,J., Konner,A.C., Hampel,B., Irlenbusch,S., Brabant,G., Kahn,C.R., Schwenk,F., Bruning,J.C., 2008. Central insulin action regulates peripheral glucose and fat metabolism in mice. J Clin Invest 118, 2132-2147.

Koenen,K.C., Saxe,G., Purcell,S., Smoller,J.W., Bartholomew,D., Miller,A., Hall,E., Kaplow,J., Bosquet,M., Moulton,S., Baldwin,C., 2005. Polymorphisms in FKBP5 are associated with peritraumatic dissociation in medically injured children. Mol Psychiatry 10, 1058-1059.

Komura,E., Tonetti,C., Penard-Lacronique,V., Chagraoui,H., Lacout,C., Lecouedic,J.P., Rameau,P., Debili,N., Vainchenker,W., Giraudier,S., 2005. Role for the nuclear factor

kappaB pathway in transforming growth factor-beta1 production in idiopathic myelofibrosis: possible relationship with FK506 binding protein 51 overexpression. Cancer Res 65, 3281-3289.

Kretz,O., Schmid,W., Berger,S., Gass,P., 2001. The mineralocorticoid receptor expression in the mouse CNS is conserved during development. Neuroreport 12, 1133-1137.

Krishan,S., Richardson,D.R., Sahni,S., 2015. Adenosine monophosphate-activated kinase and its key role in catabolism: structure, regulation, biological activity, and pharmacological activation. Mol Pharmacol 87, 363-377.

Krishnan,V., Berton,O., Nestler,E., 2008. The use of animal models in psychiatric research and treatment. Am J Psychiatry 165, 1109.

Krude,H., Biebermann,H., Luck,W., Horn,R., Brabant,G., Gruters,A., 1998. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nat Genet 19, 155-157.

Kuo,L.E., Czarnecka,M., Kitlinska,J.B., Tilan,J.U., Kvetnansky,R., Zukowska,Z., 2008. Chronic stress, combined with a high-fat/high-sugar diet, shifts sympathetic signaling toward neuropeptide Y and leads to obesity and the metabolic syndrome. Ann N Y Acad Sci 1148, 232-237.

Kwiatkowski,D.J., Zhang,H., Bandura,J.L., Heiberger,K.M., Glogauer,M., el-Hashemite,N., Onda,H., 2002. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. Hum Mol Genet 11, 525-534.

Kyrou,I., Tsigos,C., 2009. Stress hormones: physiological stress and regulation of metabolism. Curr Opin Pharmacol 9, 787-793.

Lam,T.K., Schwartz,G.J., Rossetti,L., 2005. Hypothalamic sensing of fatty acids. Nat Neurosci 8, 579-584.

Lattimore,P., Maxwell,L., 2004. Cognitive load, stress, and disinhibited eating. Eat Behav 5, 315-324.

Lee,M., Kim,A., Chua,S.C., Jr., Obici,S., Wardlaw,S.L., 2007. Transgenic MSH overexpression attenuates the metabolic effects of a high-fat diet. Am J Physiol Endocrinol Metab 293, E121-E131.

Lee,S.W., Li,C.F., Jin,G., Cai,Z., Han,F., Chan,C.H., Yang,W.L., Li,B.K., Rezaeian,A.H., Li,H.Y., Huang,H.Y., Lin,H.K., 2015. Skp2-dependent ubiquitination and activation

of LKB1 is essential for cancer cell survival under energy stress. Mol Cell 57, 1022-1033.

Leibel,R.L., Berry,E.M., Hirsch,J., 1991. Metabolic and hemodynamic responses to endogenous and exogenous catecholamines in formerly obese subjects. Am J Physiol 260, R785-R791.

Lenard,N.R., Berthoud,H.R., 2008. Central and peripheral regulation of food intake and physical activity: pathways and genes. Obesity (Silver Spring) 16 Suppl 3, S11-S22.

Leto,D., Saltiel,A.R., 2012. Regulation of glucose transport by insulin: traffic control of GLUT4. Nat Rev Mol Cell Biol 13, 383-396.

Levine,J.A., 2004. Nonexercise activity thermogenesis (NEAT): environment and biology. Am J Physiol Endocrinol Metab 286, E675-E685.

Levine,J.A., 2005. Measurement of energy expenditure. Public Health Nutr 8, 1123-1132.

Li,L., Lou,Z., Wang,L., 2011. The role of FKBP5 in cancer aetiology and chemoresistance. Br J Cancer 104, 19-23.

Liu,J., Garza,J.C., Li,W., Lu,X.Y., 2013. Melanocortin-4 receptor in the medial amygdala regulates emotional stress-induced anxiety-like behaviour, anorexia and corticosterone secretion. Int J Neuropsychopharmacol 16, 105-120.

Liu,J., Garza,J.C., Truong,H.V., Henschel,J., Zhang,W., Lu,X.Y., 2007. The melanocortinergic pathway is rapidly recruited by emotional stress and contributes to stress-induced anorexia and anxiety-like behavior. Endocrinology 148, 5531-5540.

Liu,S., Borgland,S.L., 2015. Regulation of the mesolimbic dopamine circuit by feeding peptides. Neuroscience.

Lizcano,J.M., Goransson,O., Toth,R., Deak,M., Morrice,N.A., Boudeau,J., Hawley,S.A., Udd,L., Makela,T.P., Hardie,D.G., Alessi,D.R., 2004. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J 23, 833-843.

Long,X., Lin,Y., Ortiz-Vega,S., Yonezawa,K., Avruch,J., 2005. Rheb binds and regulates the mTOR kinase. Curr Biol 15, 702-713.

Lutter,M., Nestler,E.J., 2009. Homeostatic and hedonic signals interact in the regulation of food intake. J Nutr 139, 629-632.

Maffei,M., Halaas,J., Ravussin,E., Pratley,R.E., Lee,G.H., Zhang,Y., Fei,H., Kim,S., Lallone,R., Ranganathan,S., ., 1995. Leptin levels in human and rodent: measurement

of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1, 1155-1161.

Maniam,J., Morris,M.J., 2012. The link between stress and feeding behaviour. Neuropharmacology 63, 97-110.

Manning,B.D., 2004. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. J Cell Biol 167, 399-403.

Manning,B.D., Cantley,L.C., 2007. AKT/PKB signaling: navigating downstream. Cell 129, 1261-1274.

Marcovecchio,M.L., Chiarelli,F., 2012. The effects of acute and chronic stress on diabetes control. Sci Signal 5, t10.

Marshall,S., 2006. Role of insulin, adipocyte hormones, and nutrient-sensing pathways in regulating fuel metabolism and energy homeostasis: a nutritional perspective of diabetes, obesity, and cancer. Sci STKE 2006, re7.

McEwen,B.S., 1998. Stress, adaptation, and disease. Allostasis and allostatic load. Ann N Y Acad Sci 840, 33-44.

McEwen,B.S., 2007. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 87, 873-904.

Meaney,M.J., 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 24, 1161-1192.

Menke,A., Arloth,J., Putz,B., Weber,P., Klengel,T., Mehta,D., Gonik,M., Rex-Haffner,M., Rubel,J., Uhr,M., Lucae,S., Deussing,J.M., Muller-Myhsok,B., Holsboer,F., Binder,E.B., 2012. Dexamethasone stimulated gene expression in peripheral blood is a sensitive marker for glucocorticoid receptor resistance in depressed patients. Neuropsychopharmacology 37, 1455-1464.

Mercer,J.G., Hoggard,N., Williams,L.M., Lawrence,C.B., Hannah,L.T., Trayhurn,P., 1996. Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Lett 387, 113-116.

Michel,C., Duclos,M., Cabanac,M., Richard,D., 2005. Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice. Horm Behav 48, 172-179.

Moles,A., Bartolomucci,A., Garbugino,L., Conti,R., Caprioli,A., Coccurello,R., Rizzi,R., Ciani,B., D'Amato,F.R., 2006. Psychosocial stress affects energy balance in mice: modulation by social status. Psychoneuroendocrinology 31, 623-633.

Montague,C.T., Farooqi,I.S., Whitehead,J.P., Soos,M.A., Rau,H., Wareham,N.J., Sewter,C.P., Digby,J.E., Mohammed,S.N., Hurst,J.A., Cheetham,C.H., Earley,A.R., Barnett,A.H., Prins,J.B., O'Rahilly,S., 1997. Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387, 903-908.

Morimoto,M., Morita,N., Ozawa,H., Yokoyama,K., Kawata,M., 1996. Distribution of glucocorticoid receptor immunoreactivity and mRNA in the rat brain: an immunohistochemical and in situ hybridization study. Neurosci Res 26, 235-269.

Morrison,C.D., Morton,G.J., Niswender,K.D., Gelling,R.W., Schwartz,M.W., 2005. Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling. Am J Physiol Endocrinol Metab 289, E1051-E1057.

Morrison,S.F., Madden,C.J., 2014. Central nervous system regulation of brown adipose tissue. Compr Physiol 4, 1677-1713.

Morton,G.J., Cummings,D.E., Baskin,D.G., Barsh,G.S., Schwartz,M.W., 2006. Central nervous system control of food intake and body weight. Nature 443, 289-295.

Morton,G.J., Schwartz,M.W., 2011. Leptin and the central nervous system control of glucose metabolism. Physiol Rev 91, 389-411.

Munzberg,H., Flier,J.S., Bjorbaek,C., 2004. Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 145, 4880-4889.

Munzberg,H., Morrison,C.D., 2014. Structure, production and signaling of leptin. Metabolism.

Musselman,D.L., Betan,E., Larsen,H., Phillips,L.S., 2003. Relationship of depression to diabetes types 1 and 2: epidemiology, biology, and treatment. Biol Psychiatry 54, 317-329.

Naef,L., Gratton,A., Walker,C.D., 2013. Exposure to high fat during early development impairs adaptations in dopamine and neuroendocrine responses to repeated stress. Stress 16, 540-548.

Naef,L., Moquin,L., Dal,B.G., Giros,B., Gratton,A., Walker,C.D., 2011. Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring. Neuroscience 176, 225-236.

Nakae,J., Park,B.C., Accili,D., 1999. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. J Biol Chem 274, 15982-15985.

Nedergaard,J., Cannon,B., 2010. The changed metabolic world with human brown adipose tissue: therapeutic visions. Cell Metab 11, 268-272.

Newell-Price,J., Bertagna,X., Grossman,A.B., Nieman,L.K., 2006. Cushing's syndrome. Lancet 367, 1605-1617.

Nicholls,D.G., 2006. The physiological regulation of uncoupling proteins. Biochim Biophys Acta 1757, 459-466.

Niwa,H., Yamamura,K., Miyazaki,J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108, 193-199.

Nogueiras,R., Perez-Tilve,D., Veyrat-Durebex,C., Morgan,D.A., Varela,L., Haynes,W.G., Patterson,J.T., Disse,E., Pfluger,P.T., Lopez,M., Woods,S.C., DiMarchi,R., Dieguez,C., Rahmouni,K., Rohner-Jeanrenaud,F., Tschop,M.H., 2009. Direct control of peripheral lipid deposition by CNS GLP-1 receptor signaling is mediated by the sympathetic nervous system and blunted in diet-induced obesity. J Neurosci 29, 5916-5925.

Nogueiras,R., Wiedmer,P., Perez-Tilve,D., Veyrat-Durebex,C., Keogh,J.M., Sutton,G.M., Pfluger,P.T., Castaneda,T.R., Neschen,S., Hofmann,S.M., Howles,P.N., Morgan,D.A., Benoit,S.C., Szanto,I., Schrott,B., Schurmann,A., Joost,H.G., Hammond,C., Hui,D.Y., Woods,S.C., Rahmouni,K., Butler,A.A., Farooqi,I.S., O'Rahilly,S., Rohner-Jeanrenaud,F., Tschop,M.H., 2007. The central melanocortin system directly controls peripheral lipid metabolism. J Clin Invest 117, 3475-3488.

Ohl,F., 2005. Animal models of anxiety. Handb Exp Pharmacol 35-69.

Okabe,M., Ikawa,M., Kominami,K., Nakanishi,T., Nishimune,Y., 1997. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407, 313-319.

Ouellet,V., Labbe,S.M., Blondin,D.P., Phoenix,S., Guerin,B., Haman,F., Turcotte,E.E., Richard,D., Carpentier,A.C., 2012. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. J Clin Invest 122, 545-552.

Pandit,R., de Jong,J.W., Vanderschuren,L.J., Adan,R.A., 2011. Neurobiology of overeating and obesity: the role of melanocortins and beyond. Eur J Pharmacol 660, 28-42.

Papadimitriou,A., Priftis,K.N., 2009. Regulation of the hypothalamic-pituitary-adrenal axis. Neuroimmunomodulation 16, 265-271.

Pasquali,R., Vicennati,V., Cacciari,M., Pagotto,U., 2006. The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome. Ann N Y Acad Sci 1083, 111-128.

Peeters,A., Barendregt,J.J., Willekens,F., Mackenbach,J.P., Al,M.A., Bonneux,L., 2003. Obesity in adulthood and its consequences for life expectancy: a life-table analysis. Ann Intern Med 138, 24-32.

Pei,H., Li,L., Fridley,B.L., Jenkins,G.D., Kalari,K.R., Lingle,W., Petersen,G., Lou,Z., Wang,L., 2009. FKBP51 affects cancer cell response to chemotherapy by negatively regulating Akt. Cancer Cell 16, 259-266.

Peleg-Raibstein,D., Luca,E., Wolfrum,C., 2012. Maternal high-fat diet in mice programs emotional behavior in adulthood. Behav Brain Res 233, 398-404.

Pelleymounter,M.A., Cullen,M.J., Baker,M.B., Hecht,R., Winters,D., Boone,T., Collins,F., 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269, 540-543.

Pereira,M.J., Palming,J., Svensson,M.K., Rizell,M., Dalenback,J., Hammar,M., Fall,T., Sidibeh,C.O., Svensson,P.A., Eriksson,J.W., 2014a. FKBP5 expression in human adipose tissue increases following dexamethasone exposure and is associated with insulin resistance. Metabolism 63, 1198-1208.

Pereira,M.J., Palming,J., Svensson,M.K., Rizell,M., Dalenback,J., Hammar,M., Fall,T., Sidibeh,C.O., Svensson,P.A., Eriksson,J.W., 2014b. FKBP5 expression in human adipose tissue increases following dexamethasone exposure and is associated with insulin resistance. Metabolism 63, 1198-1208.

Plum,L., Belgardt,B.F., Bruning,J.C., 2006. Central insulin action in energy and glucose homeostasis. J Clin Invest 116, 1761-1766.

Poirier,P., Despres,J.P., 2001. Exercise in weight management of obesity. Cardiol Clin 19, 459-470.

Polak,P., Hall,M.N., 2009. mTOR and the control of whole body metabolism. Curr Opin Cell Biol 21, 209-218.

Porsolt,R.D., Bertin,A., Jalfre,M., 1977. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 229, 327-336.

Pratt,T., Sharp,L., Nichols,J., Price,D.J., Mason,J.O., 2000. Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein. Dev Biol 228, 19-28.

Pratt,W.B., Morishima,Y., Murphy,M., Harrell,M., 2006. Chaperoning of glucocorticoid receptors. Handb Exp Pharmacol 111-138.

Pratt,W.B., Toft,D.O., 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18, 306-360.

Raikkonen,K., Matthews,K.A., Kuller,L.H., 2002. The relationship between psychological risk attributes and the metabolic syndrome in healthy women: antecedent or consequence? Metabolism 51, 1573-1577.

Ratajczak,T., Cluning,C., Ward,B.K., 2015. Steroid Receptor-Associated Immunophilins: A Gateway to Steroid Signalling. Clin Biochem Rev 36, 31-52.

Rautanen,A., Eriksson,J.G., Kere,J., Andersson,S., Osmond,C., Tienari,P., Sairanen,H., Barker,D.J., Phillips,D.I., Forsen,T., Kajantie,E., 2006. Associations of body size at birth with late-life cortisol concentrations and glucose tolerance are modified by haplotypes of the glucocorticoid receptor gene. J Clin Endocrinol Metab 91, 4544-4551.

Refojo,D., Schweizer,M., Kuehne,C., Ehrenberg,S., Thoeringer,C., Vogl,A.M., Dedic,N., Schumacher,M., von,W.G., Avrabos,C., Touma,C., Engblom,D., Schutz,G., Nave,K.A., Eder,M., Wotjak,C.T., Sillaber,I., Holsboer,F., Wurst,W., Deussing,J.M., 2011. Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. Science 333, 1903-1907.

Reul,J.M., de Kloet,E.R., 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505-2511.

Reul,J.M., de Kloet,E.R., 1986. Anatomical resolution of two types of corticosterone receptor sites in rat brain with in vitro autoradiography and computerized image analysis. J Steroid Biochem 24, 269-272.

Riggs,D.L., Roberts,P.J., Chirillo,S.C., Cheung-Flynn,J., Prapapanich,V., Ratajczak,T., Gaber,R., Picard,D., Smith,D.F., 2003. The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo. EMBO J 22, 1158-1167.

Rodgers,R.J., Tschop,M.H., Wilding,J.P., 2012. Anti-obesity drugs: past, present and future. Dis Model Mech 5, 621-626.

Romano,S., Mallardo,M., Romano,M.F., 2011. FKBP51 and the NF-kappaB regulatory pathway in cancer. Curr Opin Pharmacol 11, 288-293.

Rooney,K., Ozanne,S.E., 2011. Maternal over-nutrition and offspring obesity predisposition: targets for preventative interventions. Int J Obes (Lond) 35, 883-890.

Rothwell,N.J., Stock,M.J., 1983. Luxuskonsumption, diet-induced thermogenesis and brown fat: the case in favour. Clin Sci (Lond) 64, 19-23.

Roubos,E.W., Dahmen,M., Kozicz,T., Xu,L., 2012. Leptin and the hypothalamo-pituitary-adrenal stress axis. Gen Comp Endocrinol 177, 28-36.

Roy,A., Gorodetsky,E., Yuan,Q., Goldman,D., Enoch,M.A., 2010. Interaction of FKBP5, a stress-related gene, with childhood trauma increases the risk for attempting suicide. Neuropsychopharmacology 35, 1674-1683.

Saely,C.H., Geiger,K., Drexel,H., 2012. Brown versus white adipose tissue: a mini-review. Gerontology 58, 15-23.

Sakamoto,K., Holman,G.D., 2008. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. Am J Physiol Endocrinol Metab 295, E29-E37.

Saltiel,A.R., Kahn,C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799-806.

Sanchez,E.R., 2012. Chaperoning steroidal physiology: lessons from mouse genetic models of Hsp90 and its cochaperones. Biochim Biophys Acta 1823, 722-729.

Sano,H., Kane,S., Sano,E., Miinea,C.P., Asara,J.M., Lane,W.S., Garner,C.W., Lienhard,G.E., 2003. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem 278, 14599-14602.

Santarelli,S., Lesuis,S.L., Wang,X.D., Wagner,K.V., Hartmann,J., Labermaier,C., Scharf,S.H., Muller,M.B., Holsboer,F., Schmidt,M.V., 2014. Evidence supporting the match/mismatch hypothesis of psychiatric disorders. Eur Neuropsychopharmacol 24, 907-918.

Saper,C.B., Lowell,B.B., 2014. The hypothalamus. Curr Biol 24, R1111-R1116.

Sapolsky,R.M., Krey,L.C., McEwen,B.S., 1986. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocr Rev 7, 284-301.

Sasaki,A., de,V.W., Sivanathan,S., St-Cyr,S., McGowan,P.O., 2014. Maternal high-fat diet alters anxiety behavior and glucocorticoid signaling in adolescent offspring. Neuroscience 272, 92-101.

Sawchenko,P.E., 1998. Toward a new neurobiology of energy balance, appetite, and obesity: the anatomists weigh in. J Comp Neurol 402, 435-441.

Scammell,J.G., Denny,W.B., Valentine,D.L., Smith,D.F., 2001. Overexpression of the FK506-binding immunophilin FKBP51 is the common cause of glucocorticoid resistance in three New World primates. Gen Comp Endocrinol 124, 152-165.

Scarpace,P.J., Zhang,Y., 2007. Elevated leptin: consequence or cause of obesity? Front Biosci 12, 3531-3544.

Scharf,S.H., Liebl,C., Binder,E.B., Schmidt,M.V., Muller,M.B., 2011. Expression and regulation of the Fkbp5 gene in the adult mouse brain. PLoS One 6, e16883.

Schmidt,M.V., Paez-Pereda,M., Holsboer,F., Hausch,F., 2012. The prospect of FKBP51 as a drug target. ChemMedChem 7, 1351-1359.

Schmidt,M.V., Schulke,J.P., Liebl,C., Stiess,M., Avrabos,C., Bock,J., Wochnik,G.M., Davies,H.A., Zimmermann,N., Scharf,S.H., Trumbach,D., Wurst,W., Zieglgansberger,W., Turck,C., Holsboer,F., Stewart,M.G., Bradke,F., Eder,M., Muller,M.B., Rein,T., 2011. Tumor suppressor down-regulated in renal cell carcinoma 1 (DRR1) is a stress-induced actin bundling factor that modulates synaptic efficacy and cognition. Proc Natl Acad Sci U S A 108, 17213-17218.

Schmidt,M.V., Sterlemann,V., Ganea,K., Liebl,C., Alam,S., Harbich,D., Greetfeld,M., Uhr,M., Holsboer,F., Muller,M.B., 2007. Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. Psychoneuroendocrinology 32, 417-429.

Schmidt,M.V., Sterlemann,V., Wagner,K., Niederleitner,B., Ganea,K., Liebl,C., Deussing,J.M., Berger,S., Schutz,G., Holsboer,F., Muller,M.B., 2009. Postnatal glucocorticoid excess due to pituitary glucocorticoid receptor deficiency: differential short- and long-term consequences. Endocrinology 150, 2709-2716.

Schreiber,S.L., 1991. Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 251, 283-287.

Schwartz,M.W., Porte,D., Jr., 2005. Diabetes, obesity, and the brain. Science 307, 375-379.

Scott,J.D., Pawson,T., 2009. Cell signaling in space and time: where proteins come together and when they're apart. Science 326, 1220-1224.

Sebaai,N., Lesage,J., Breton,C., Vieau,D., Deloof,S., 2004. Perinatal food deprivation induces marked alterations of the hypothalamo-pituitary-adrenal axis in 8-month-old

male rats both under basal conditions and after a dehydration period. Neuroendocrinology 79, 163-173.

Seeley,R.J., Drazen,D.L., Clegg,D.J., 2004. The critical role of the melanocortin system in the control of energy balance. Annu Rev Nutr 24, 133-149.

Seeley,R.J., Woods,S.C., 2003. Monitoring of stored and available fuel by the CNS: implications for obesity. Nat Rev Neurosci 4, 901-909.

Segal,N.L., Allison,D.B., 2002. Twins and virtual twins: bases of relative body weight revisited. Int J Obes Relat Metab Disord 26, 437-441.

Segal,N.L., Feng,R., McGuire,S.A., Allison,D.B., Miller,S., 2009. Genetic and environmental contributions to body mass index: comparative analysis of monozygotic twins, dizygotic twins and same-age unrelated siblings. Int J Obes (Lond) 33, 37-41.

Shackelford,D.B., Shaw,R.J., 2009. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer 9, 563-575.

Shepherd,P.R., Kahn,B.B., 1999. Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. N Engl J Med 341, 248-257.

Simmons,R., 2008. Perinatal programming of obesity. Semin Perinatol 32, 371-374.

Sinars,C.R., Cheung-Flynn,J., Rimerman,R.A., Scammell,J.G., Smith,D.F., Clardy,J., 2003. Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. Proc Natl Acad Sci U S A 100, 868-873.

Sinnett,S.E., Brenman,J.E., 2014. Past strategies and future directions for identifying AMP-activated protein kinase (AMPK) modulators. Pharmacol Ther 143, 111-118.

Skilton,M.R., Moulin,P., Terra,J.L., Bonnet,F., 2007. Associations between anxiety, depression, and the metabolic syndrome. Biol Psychiatry 62, 1251-1257.

Smith,D.F., Albers,M.W., Schreiber,S.L., Leach,K.L., Deibel,M.R., Jr., 1993. FKBP54, a novel FK506-binding protein in avian progesterone receptor complexes and HeLa extracts. J Biol Chem 268, 24270-24273.

Snyder,E.E., Walts,B., Perusse,L., Chagnon,Y.C., Weisnagel,S.J., Rankinen,T., Bouchard,C., 2004. The human obesity gene map: the 2003 update. Obes Res 12, 369-439.

Solomon,M.B., Jankord,R., Flak,J.N., Herman,J.P., 2011. Chronic stress, energy balance and adiposity in female rats. Physiol Behav 102, 84-90.

Speakman,J.R., 2013. Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. Front Physiol 4, 34.

Spiegelman,B.M., Flier,J.S., 2001. Obesity and the regulation of energy balance. Cell 104, 531-543.

Steinberg,G.R., Kemp,B.E., 2009. AMPK in Health and Disease. Physiol Rev 89, 1025-1078.

Stevens,A., Begum,G., Cook,A., Connor,K., Rumball,C., Oliver,M., Challis,J., Bloomfield,F., White,A., 2010. Epigenetic changes in the hypothalamic proopiomelanocortin and glucocorticoid receptor genes in the ovine fetus after periconceptional undernutrition. Endocrinology 151, 3652-3664.

Storer,C.L., Dickey,C.A., Galigniana,M.D., Rein,T., Cox,M.B., 2011. FKBP51 and FKBP52 in signaling and disease. Trends Endocrinol Metab 22, 481-490.

Su,A.I., Wiltshire,T., Batalov,S., Lapp,H., Ching,K.A., Block,D., Zhang,J., Soden,R., Hayakawa,M., Kreiman,G., Cooke,M.P., Walker,J.R., Hogenesch,J.B., 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A 101, 6062-6067.

Sullivan,E.L., Grayson,B., Takahashi,D., Robertson,N., Maier,A., Bethea,C.L., Smith,M.S., Coleman,K., Grove,K.L., 2010. Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. J Neurosci 30, 3826-3830.

Sullivan,E.L., Nousen,E.K., Chamlou,K.A., 2014. Maternal high fat diet consumption during the perinatal period programs offspring behavior. Physiol Behav 123, 236-242.

Surwit,R.S., Kuhn,C.M., Cochrane,C., McCubbin,J.A., Feinglos,M.N., 1988. Diet-induced type II diabetes in C57BL/6J mice. Diabetes 37, 1163-1167.

Taipale,M., Tucker,G., Peng,J., Krykbaeva,I., Lin,Z.Y., Larsen,B., Choi,H., Berger,B., Gingras,A.C., Lindquist,S., 2014. A quantitative chaperone interaction network reveals the architecture of cellular protein homeostasis pathways. Cell 158, 434-448.

Takano,A., Usui,I., Haruta,T., Kawahara,J., Uno,T., Iwata,M., Kobayashi,M., 2001. Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Mol Cell Biol 21, 5050-5062.

Tamashiro,K.L., 2011. Metabolic syndrome: links to social stress and socioeconomic status. Ann N Y Acad Sci 1231, 46-55.

Taniguchi,C.M., Emanuelli,B., Kahn,C.R., 2006. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 7, 85-96.

Tartaglia,L.A., Dembski,M., Weng,X., Deng,N., Culpepper,J., Devos,R., Richards,G.J., Campfield,L.A., Clark,F.T., Deeds,J., Muir,C., Sanker,S., Moriarty,A., Moore,K.J., Smutko,J.S., Mays,G.G., Wool,E.A., Monroe,C.A., Tepper,R.I., 1995. Identification and expression cloning of a leptin receptor, OB-R. Cell 83, 1263-1271.

Taylor,V.H., Macqueen,G.M., 2010. The Role of Adipokines in Understanding the Associations between Obesity and Depression. J Obes 2010.

Thirone,A.C., Huang,C., Klip,A., 2006. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. Trends Endocrinol Metab 17, 72-78.

Thornton,C., Snowden,M.A., Carling,D., 1998. Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. J Biol Chem 273, 12443-12450.

Toker,S., Shirom,A., Melamed,S., 2008. Depression and the metabolic syndrome: gender-dependent associations. Depress Anxiety 25, 661-669.

Toneatto,J., Guber,S., Charo,N.L., Susperreguy,S., Schwartz,J., Galigniana,M.D., Piwien-Pilipuk,G., 2013. Dynamic mitochondrial-nuclear redistribution of the immunophilin FKBP51 is regulated by the PKA signaling pathway to control gene expression during adipocyte differentiation. J Cell Sci 126, 5357-5368.

Torres,S.J., Nowson,C.A., 2007. Relationship between stress, eating behavior, and obesity. Nutrition 23, 887-894.

Touma,C., Gassen,N.C., Herrmann,L., Cheung-Flynn,J., Bull,D.R., Ionescu,I.A., Heinzmann,J.M., Knapman,A., Siebertz,A., Depping,A.M., Hartmann,J., Hausch,F., Schmidt,M.V., Holsboer,F., Ising,M., Cox,M.B., Schmidt,U., Rein,T., 2011. FK506 binding protein 5 shapes stress responsiveness: modulation of neuroendocrine reactivity and coping behavior. Biol Psychiatry 70, 928-936.

Towler,M.C., Hardie,D.G., 2007. AMP-activated protein kinase in metabolic control and insulin signaling. Circ Res 100, 328-341.

Tranguch,S., Cheung-Flynn,J., Daikoku,T., Prapapanich,V., Cox,M.B., Xie,H., Wang,H., Das,S.K., Smith,D.F., Dey,S.K., 2005. Cochaperone immunophilin FKBP52 is critical to uterine receptivity for embryo implantation. Proc Natl Acad Sci U S A 102, 14326-14331.

Tremblay,F., Marette,A., 2001. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem 276, 38052-38060.

Trottier,G., Koski,K.G., Brun,T., Toufexis,D.J., Richard,D., Walker,C.D., 1998. Increased fat intake during lactation modifies hypothalamic-pituitary-adrenal responsiveness in developing rat pups: a possible role for leptin. Endocrinology 139, 3704-3711.

Turner,N., 2013. Mitochondrial Metabolism and Insulin Action. Masuo,K. (Ed.) Type 2 Diabetes. InTech.

Ulrich-Lai,Y.M., Herman,J.P., 2009. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10, 397-409.

Um,S.H., Frigerio,F., Watanabe,M., Picard,F., Joaquin,M., Sticker,M., Fumagalli,S., Allegrini,P.R., Kozma,S.C., Auwerx,J., Thomas,G., 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 431, 200-205.

Vaisse,C., Clement,K., Guy-Grand,B., Froguel,P., 1998. A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat Genet 20, 113-114.

Vaisse,C., Halaas,J.L., Horvath,C.M., Darnell,J.E., Jr., Stoffel,M., Friedman,J.M., 1996. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat Genet 14, 95-97.

van der Klaauw,A.A., Farooqi,I.S., 2015. The Hunger Genes: Pathways to Obesity. Cell 161, 119-132.

Van Klinken,J.B., van den Berg,S.A., Havekes,L.M., Willems Van,D.K., 2012. Estimation of activity related energy expenditure and resting metabolic rate in freely moving mice from indirect calorimetry data. PLoS One 7, e36162.

van Marken Lichtenbelt,W.D., Schrauwen,P., 2011. Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol 301, R285-R296.

Vasudevan,K.M., Garraway,L.A., 2010. AKT signaling in physiology and disease. Curr Top Microbiol Immunol 347, 105-133.

Veldhuis,H.D., Van,K.C., Van,I.M., de Kloet,E.R., 1982. Specificity of the adrenal steroid receptor system in rat hippocampus. Endocrinology 110, 2044-2051.

Vermeer,H., Hendriks-Stegeman,B.I., van der Burg,B., van Buul-Offers,S.C., Jansen,M., 2003. Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. J Clin Endocrinol Metab 88, 277-284.

Vieau,D., Sebaai,N., Leonhardt,M., Dutriez-Casteloot,I., Molendi-Coste,O., Laborie,C., Breton,C., Deloof,S., Lesage,J., 2007. HPA axis programming by maternal undernutrition in the male rat offspring. Psychoneuroendocrinology 32 Suppl 1, S16-S20.

Viinamaki,H., Heiskanen,T., Lehto,S.M., Niskanen,L., Koivumaa-Honkanen,H., Tolmunen,T., Honkalampi,K., Saharinen,T., Haatainen,K., Hintikka,J., 2009. Association of depressive symptoms and metabolic syndrome in men. Acta Psychiatr Scand 120, 23-29.

Vijgen,G.H., Sparks,L.M., Bouvy,N.D., Schaart,G., Hoeks,J., van Marken Lichtenbelt,W.D., Schrauwen,P., 2013. Increased oxygen consumption in human adipose tissue from the "brown adipose tissue" region. J Clin Endocrinol Metab 98, E1230-E1234.

Volkow,N.D., Wise,R.A., 2005. How can drug addiction help us understand obesity? Nat Neurosci 8, 555-560.

Vucetic,Z., Kimmel,J., Totoki,K., Hollenbeck,E., Reyes,T.M., 2010. Maternal high-fat diet alters methylation and gene expression of dopamine and opioid-related genes. Endocrinology 151, 4756-4764.

Wagner,K.V., Marinescu,D., Hartmann,J., Wang,X.D., Labermaier,C., Scharf,S.H., Liebl,C., Uhr,M., Holsboer,F., Muller,M.B., Schmidt,M.V., 2012. Differences in FKBP51 Regulation Following Chronic Social Defeat Stress Correlate with Individual Stress Sensitivity: Influence of Paroxetine Treatment. Neuropsychopharmacology 37, 2797-2808.

Wagner,K.V., Wang,X.D., Liebl,C., Scharf,S.H., Muller,M.B., Schmidt,M.V., 2011. Pituitary glucocorticoid receptor deletion reduces vulnerability to chronic stress. Psychoneuroendocrinology 36, 579-587.

Wallis,D.J., Hetherington,M.M., 2009. Emotions and eating. Self-reported and experimentally induced changes in food intake under stress. Appetite 52, 355-362.

Wang,X.D., Chen,Y., Wolf,M., Wagner,K.V., Liebl,C., Scharf,S.H., Harbich,D., Mayer,B., Wurst,W., Holsboer,F., Deussing,J.M., Baram,T.Z., Muller,M.B., Schmidt,M.V., 2011a. Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. Neurobiol Dis 42, 300-310.

Wang,Y.C., McPherson,K., Marsh,T., Gortmaker,S.L., Brown,M., 2011b. Health and economic burden of the projected obesity trends in the USA and the UK. Lancet 378, 815-825.

Watts,A.G., 2000. Understanding the neural control of ingestive behaviors: helping to separate cause from effect with dehydration-associated anorexia. Horm Behav 37, 261-283.

Weinstein,S.P., Wilson,C.M., Pritsker,A., Cushman,S.W., 1998. Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal muscle. Metabolism 47, 3-6.

WEIR,J.B., 1949. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol 109, 1-9.

Weninger,S.C., Muglia,L.J., Jacobson,L., Majzoub,J.A., 1999. CRH-deficient mice have a normal anorectic response to chronic stress. Regul Pept 84, 69-74.

Westberry,J.M., Sadosky,P.W., Hubler,T.R., Gross,K.L., Scammell,J.G., 2006. Glucocorticoid resistance in squirrel monkeys results from a combination of a transcriptionally incompetent glucocorticoid receptor and overexpression of the glucocorticoid receptor co-chaperone FKBP51. J Steroid Biochem Mol Biol 100, 34-41.

Westerterp,K.R., 2010. Physical activity, food intake, and body weight regulation: insights from doubly labeled water studies. Nutr Rev 68, 148-154.

WHO, 2000. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser 894, i-253.

WHO. Depression: Fact sheet N°369. WHO . 2012. 19-12-2014.

WHO. Fact sheet: Diabetes. World Health Organization . 2014a. 19-12-2014a.

WHO. Global Health Estimates: Deaths by Cause, Age, Sex and Country, 2000-2012. 2014b. Geneva.

Wilcox,R.R., 1987. New statistical procedures for the social sciences: Modern solutions to basic problems. Psychology Press.

Wochnik,G.M., Ruegg,J., Abel,G.A., Schmidt,U., Holsboer,F., Rein,T., 2005. FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. J Biol Chem 280, 4609-4616.

Woods,A., Johnstone,S.R., Dickerson,K., Leiper,F.C., Fryer,L.G., Neumann,D., Schlattner,U., Wallimann,T., Carlson,M., Carling,D., 2003. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol 13, 2004-2008.

Woods,A., Salt,I., Scott,J., Hardie,D.G., Carling,D., 1996. The alpha1 and alpha2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro. FEBS Lett 397, 347-351.

Workel,J.O., Oitzl,M.S., Fluttert,M., Lesscher,H., Karssen,A., de Kloet,E.R., 2001. Differential and age-dependent effects of maternal deprivation on the hypothalamic-pituitary-adrenal axis of brown norway rats from youth to senescence. J Neuroendocrinol 13, 569-580.

Wullschleger,S., Loewith,R., Hall,M.N., 2006. TOR signaling in growth and metabolism. Cell 124, 471-484.

Xie,P., Kranzler,H.R., Poling,J., Stein,M.B., Anton,R.F., Farrer,L.A., Gelernter,J., 2010. Interaction of FKBP5 with childhood adversity on risk for post-traumatic stress disorder. Neuropsychopharmacology 35, 1684-1692.

Xu,A.W., Ste-Marie,L., Kaelin,C.B., Barsh,G.S., 2007. Inactivation of signal transducer and activator of transcription 3 in proopiomelanocortin (Pomc) neurons causes decreased pomc expression, mild obesity, and defects in compensatory refeeding. Endocrinology 148, 72-80.

Xu,C., He,J., Jiang,H., Zu,L., Zhai,W., Pu,S., Xu,G., 2009. Direct effect of glucocorticoids on lipolysis in adipocytes. Mol Endocrinol 23, 1161-1170.

Yach,D., Stuckler,D., Brownell,K.D., 2006. Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. Nat Med 12, 62-66.

Yamagata,K., Sanders,L.K., Kaufmann,W.E., Yee,W., Barnes,C.A., Nathans,D., Worley,P.F., 1994. rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. J Biol Chem 269, 16333-16339.

Yang,L., Isoda,F., Yen,K., Kleopoulos,S.P., Janssen,W., Fan,X., Mastaitis,J., Dunn-Meynell,A., Levin,B., McCrimmon,R., Sherwin,R., Musatov,S., Mobbs,C.V., 2012. Hypothalamic Fkbp51 is induced by fasting, and elevated hypothalamic expression promotes obese phenotypes. Am J Physiol Endocrinol Metab 302, E987-E991.

Yeo,G.S., Farooqi,I.S., Aminian,S., Halsall,D.J., Stanhope,R.G., O'Rahilly,S., 1998. A frameshift mutation in MC4R associated with dominantly inherited human obesity. Nat Genet 20, 111-112.

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

Zannas,A.S., Binder,E.B., 2014. Gene-environment interactions at the FKBP5 locus: sensitive periods, mechanisms and pleiotropism. Genes Brain Behav 13, 25-37.

Zhang,Y., Proenca,R., Maffei,M., Barone,M., Leopold,L., Friedman,J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425-432.

Zhang,Y.L., Guo,H., Zhang,C.S., Lin,S.Y., Yin,Z., Peng,Y., Luo,H., Shi,Y., Lian,G., Zhang,C., Li,M., Ye,Z., Ye,J., Han,J., Li,P., Wu,J.W., Lin,S.C., 2013. AMP as a low-energy charge signal autonomously initiates assembly of AXIN-AMPK-LKB1 complex for AMPK activation. Cell Metab 18, 546-555.

Zheng,D., MacLean,P.S., Pohnert,S.C., Knight,J.B., Olson,A.L., Winder,W.W., Dohm,G.L., 2001. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol (1985) 91, 1073-1083.

Zhou,G., Myers,R., Li,Y., Chen,Y., Shen,X., Fenyk-Melody,J., Wu,M., Ventre,J., Doebber,T., Fujii,N., Musi,N., Hirshman,M.F., Goodyear,L.J., Moller,D.E., 2001. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108, 1167-1174.

Zierath,J.R., Wallberg-Henriksson,H., 2002. From receptor to effector: insulin signal transduction in skeletal muscle from type II diabetic patients. Ann N Y Acad Sci 967, 120-134.

Zimmermann,P., Bruckl,T., Nocon,A., Pfister,H., Binder,E.B., Uhr,M., Lieb,R., Moffitt,T.E., Caspi,A., Holsboer,F., Ising,M., 2011. Interaction of FKBP5 gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study. Am J Psychiatry 168, 1107-1116.

Zisman,A., Peroni,O.D., Abel,E.D., Michael,M.D., Mauvais-Jarvis,F., Lowell,B.B., Wojtaszewski,J.F., Hirshman,M.F., Virkamaki,A., Goodyear,L.J., Kahn,C.R., Kahn,B.B., 2000. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. Nat Med 6, 924-928.

Curriculum Vitae

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2012 – present	**Selected member** of the competitive IMPRS-LS graduate program, Munich Germany
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2009 – 2012	**Recipient** of Neuroscience-CIHR Training Program Scholarship, University scholarship awarded for academic distinction, $50,000
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Assertion/ Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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Erklärung

Hiermit erkläre ich, *

- [x] dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.
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