Metabolic Programming of Hypothalamic Neurocircuits by Maternal High-Fat Feeding

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Merly Carlotta Vogt

aus Salzkotten

Köln, 2014

Berichterstatter/in: Prof. Dr. Jens C. Brüning
Prof. Dr. Carien M. Niessen

Tag der mündlichen Prüfung: 8.4.2014

Table of contents

Figure index

Table index

Abbreviations

(dd)H_2O	(double distilled) water
°C	degrees Celsius
3’	three prime end of DNA sequences
5’	five prime end of DNA sequences
A	adenosine
AAV	adeno-associated virus
ACTH	adrenocorticotropic hormone
AgRP	agouti-related peptide
ANOVA	analysis of variance
ANS	autonomic nervous system
ARH	arcuate nucleus of the hypothalamus
ATP	adenosine triphosphate
AUC	area under the curve
BMI	body mass index
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
C	cytosine
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CLIP	cotricotropin-like intermediate peptide
CNS	central nervous system
CPE	carboxypeptidase E
Cre	site-specific recombinase from phage 1 (causes recombination)
CRH	corticotropin-releasing hormone
DAPI	4’, 6-diamidino-2-phenylindole
DMH	dorsomedial nucleus of the hypothalamus
DMX	motor nucleus of the vagus
DNA	desoxyribonucleic acid
dNTP	deoxynucleoside triphosphate

DOB	day of birth
DVC	dorsal vagal complex
E	embryonic day
e.g.	exempli gratia
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FFA	free fatty acid
fl	floxed
FOXO	forkhead box
fx	fornix
g	gram
G	guanosine
GABA	γ-aminobutyric acid
GDM	gestational diabetes mellitus
GLP-1	glucagon-like peptide 1
GLUT	glucose transporter
GTT	glucose tolerance test
h	hour
HCl	hydrochloric acid
HFD	high-fat diet
HGP	hepatic glucose production
HOMA-IR	homeostatic model assessment of insulin resistance
HRP	horse-radish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
ICV	intracerebroventricular
Il6/1b	interleukin 6/1b
IML	intermediolateral nucleus
IR	insulin receptor
IRS	insulin receptor substrate
ITT	insulin tolerance test

JAK2	janus kinase 2
KATP	ATP-dependent potassium channel
L	liter
LepRb	long form of the leptin receptor
LGA	large for gestational age
LH	lateral hypothalamic area
LPH	lipotropin hormone
m	milli
MAP	mitogen-activated protein
MC(1-5)R	melanocortin 1-5 receptor
ME	median eminence
min	minute
mTOR	mammalian target of rapamycin
n	nano
NCD	normal chow diet
NEFA	non-esterified fatty acid
NHP	nonhuman primate
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
ob	leptin
p	pico
P	postnatal day
PAG	periaqueductal grey
PBN	parabrachial nucleus
PBS	phosphate buffered saline
PC1/2/3	prohormone convertase 1/2/3
PCR	polymerase chain reaction
PDK1	phosphoinositide-dependent kinase 1
PFA	paraformaldehyde
PI3K	phoshoinositol-3 kinase
PIP2	phosphatidyl-inositol-4,5-bisphosphate
PIP3	phosphatidyl-inositol-3,4,5-trisphosphate

POMC	proopiomelanocortin
PVH	paraventricular nucleus of the hypothalamus
PVT	paraventricular thalamus
RNA	ribonucleic acid
RT	room temperature
SEM	standard error of the mean
SH	Src-homology
SOCS3	suppressor of cytokine signaling 3
STAT3	signal transducer of transcription 3
STZ	streptozotocin
T	thymidine
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TH	tyrosin hydroxylase
TNF	tumor necrosis factor
TRH	thyrotropin-releasing hormone
TRPC	transient receptor potential cation
vACHT	vesicular acetylcholine transporter
VMH	ventromedial nucleus of the hypothalamus
VTA	ventral tegmental area
α-MSH	α-melanocyte-stimulating hormone
µ	micro

1 Introduction

1.1 Metabolic disorders of epidemic proportions in the 21st century

1.1.1 Obesity

Hardly any other epidemic in history has been as apparent as the current global rise in the prevalence of overweight and obesity (Figure 1.1). Over the last 30 years the worldwide incidences of obesity have nearly doubled to approximately 500 million obese and 1.4 billion overweight people in 2008 (WHO, 2013c).

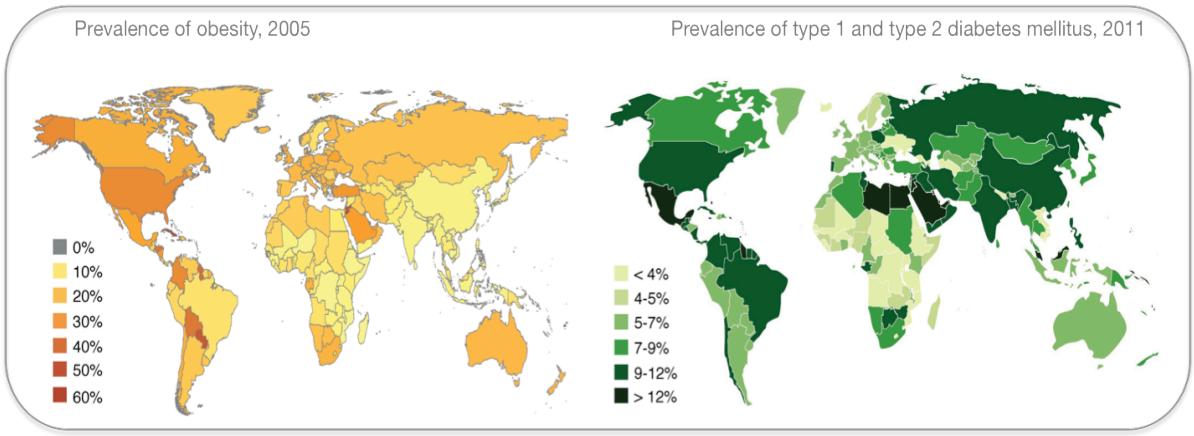


Figure 1.1: Schematic illustration of the global obesity and diabetes epidemic
Over the last decades, the prevalence of obesity has dramatically increased, affecting approximately 20 % of the adult population in Central Europe and 30 % in the United States of America (USA). Similarly, Diabetes mellitus is diagnosed in approximately 5-7 % and 7-9 % in Central Europe and the USA, respectively. Illustrations were adapted from the World Health Organization and the International Diabetes Federation (WHO and IDF).

Overweight and obesity are commonly assessed by the body mass index (BMI). The BMI is calculated by the body weight in kilogram divided by the square of the height in meter [kg/m^2] of an individual (WHO, 2013c). Whereas a normal BMI ranges from 18.5 to 24.9, a person with a BMI above or equal to 25 is considered overweight and even diagnosed with obesity, if the BMI is equal to, or exceeds the critical threshold of 30 (WHO, 2013c). However, the BMI as a measure of adiposity has been criticized as it neglects to distinguish between fat and lean mass, as well as between the distribution of body fat (Snijder et al., 2006). Intra-abdominal or visceral fat is considered to confer greater risk to develop obesity-associated diseases than subcutaneous fat (Bergman et al., 2006). Therefore, measures other

than the BMI, such as the waist-to-hip ratio, the waist circumference, or the body fat percentage have been applied to categorize an individual's state of adiposity (Snijder et al., 2006). Nevertheless, due to the inexpensive and simple measures needed to determine the BMI, as well as the large amount of epidemiological data available in the literature, the BMI is still the most commonly used measure to correlate an individual's state of adiposity with the associated risks to develop certain co-morbidities.

Generally, obesity develops when the overall caloric intake exceeds the calories expended. Such a chronic imbalance in energy homeostasis can have long-term consequences: Apart from psychological problems resulting e.g. from social exclusion and subsequent introversion, overweight and/or obesity can lead to the development of Type 2 Diabetes mellitus (T2DM), cardiovascular diseases, stroke and certain forms of cancer (i.e. endometrial, colon and breast cancer), linking overweight and obesity to the deaths of approximately 2.8 million people per year (WHO, 2013c). As a consequence, recent prognoses predict an overall decline in life expectancy for the first time in centuries (Olshansky et al., 2005).

In light of these developments, efforts have been made to identify the underlying causes responsible for the obesity epidemic. Although rare monogenetic mutations, such as mutations in key players involved in the central regulation of energy homeostasis, such as the *melanocortin 4 receptor* (*Mc4r*), *proopiomelanocortin* (*Pomc*), or *leptin* (*ob*) genes result in drastic obese phenotypes, they can only be found in a minority of patients, and can therefore not be held accountable for the epidemic dimensions of obesity (Farooqi, 2008; Krude et al., 1998; Montague et al., 1997; Santini et al., 2009). In contrast, complex polygenetic variations in combination with unfavorable environmental factors are thought to mainly contribute to the development of increased adiposity (Hetherington and Cecil, 2010). Importantly, the global shift to a more sedentary lifestyle and increased consumption of palatable, high-caloric, energy-dense food does not only increase an individual's risk to develop obesity, but also augments the susceptibility for future generations. Multiple human epidemiological, as well as animal studies have demonstrated that children of obese mothers are more susceptible to eventually develop metabolic disorders themselves, a phenomenon that is often referred to as "metabolic programming" (see also Chapter 1.1.3) (for review, see (Sullivan and Grove, 2010)).

Thus, further efforts have to be made to unravel molecular mechanisms contributing to the development and manifestation of obesity to not only find novel therapeutic strategies, but also preventative measures to effectively intervene with the obesity epidemic, as well as its associated diseases.

1.1.2 Type 2 diabetes mellitus

As one of the main consequences of overweight and obesity, T2DM has also evolved into a global epidemic. In 2012, the international diabetes federation estimated the total number of patients with diabetes mellitus to be 382 million worldwide (Figure 1.1) (IDF, 2013). Of these 382 million, approximately 10 % account for patients diagnosed with Type 1 diabetes mellitus (T1DM) (IDF, 2013), which is characterized by chronic hyperglycemia caused by the complete loss, or highly impaired production of insulin, secondary to an autoimmune-reaction-mediated destruction of pancreatic β-cells (WHO, 2013b). In contrast, T2DM is characterized by chronic hyperglycemia caused by the development of insulin resistance in peripheral organs, such as the muscle, white adipose tissue and the liver, as well as the central nervous system (CNS) (for review, see (Biddinger and Kahn, 2006)). As previously described, excessive caloric intake in combination with physical inactivity are the main reasons for the development of obesity, which is associated with chronic hyperglycemia. Under these pathological conditions, insulin is no longer able to efficiently stimulate glucose uptake into muscle and fat, and further fails to suppress hepatic glucose production, thereby additionally increasing blood glucose levels. Although hyperactivation of pancreatic β-cells is initially able to compensate for this insulin resistance, chronic hyperglycemia eventually leads to progressive deterioration of pancreatic β-cell function and finally, to exhaustion and loss of these cells, resulting in relative insulin deficiency in T2DM patients (Kahn et al., 1993; Polonsky et al., 1988; Prentki and Nolan, 2006).

The T2DM-associated hyperglycemia can have tremendous effects on the function of multiple organs as it increases the risk for microvascular damage (i.e. retinopathy, nephropathy and neuropathy) (WHO, 2006). Moreover, T2DM is associated with an increased risk of macrovascular complications and, – if left untreated, or poorly controlled – can eventually lead to organ failure, ischemic heart disease or stroke (Vasudevan et al., 2006;

WHO, 2006). Therefore, despite the growing knowledge about cellular and molecular changes associated with T2DM, the ever-increasing incidences of T2DM highlight the necessity to decipher all underlying aspects contributing to the development and progression of this disease.

1.1.3 Maternal obesity, maternal diabetes and the concept of metabolic programming

Coincident with the ever-increasing cases of obesity and T2DM, the number of obese and/or diabetic women of childbearing age has reached an all-time high. Depending on the country and ethnicity of the cohort analyzed, studies estimate that currently 15-40% of pregnancies are affected by maternal obesity, and 3-10% of pregnancies by maternal diabetes (i.e. T1DM, T2DM and gestational diabetes) (Anna et al., 2008; Yu et al., 2013). This epidemic development has been a matter of great concern, because maternal obesity and diabetes have been associated with a multitude of adverse pregnancy outcomes, both for the mother and the child (Catalano and Ehrenberg, 2006; Dabelea et al., 2000; Torloni et al., 2009a; Yu et al., 2013).

1.1.3.1 Origin of the metabolic programming hypothesis

The hypothesis that early life experience can determine the metabolic fate of an individual evolved as early as during the 1960s. Dubos and colleagues were the first to find a direct association between the nutritional content of maternal milk and the growth rate, as well as the lifelong efficacy of energy utilization of the offspring in mice (Dubos et al., 1966). In line with these results obtained in rodents, Ravelli and colleagues demonstrated that nutritional deprivation during distinct phases of pregnancy enhances the propensity to develop metabolic disorders during adulthood in humans. These findings demonstrated that metabolic programming occurs in response to hormonal insults during a critical time window of particular vulnerability: Exposure to the Dutch famine (which occurred in the winter of 1944-1945) during the first half of pregnancy significantly increased the rate of obesity later in life (Ravelli et al., 1976). In contrast, undernutrition during the third trimester of pregnancy decreased the rate of obesity, while at the same time increasing the incidences of insulin resistance and impaired glucose tolerance in adult men (Ravelli et al.,

1998). In similar retrospective studies, David Barker and his colleagues found a negative correlation between birth weight (as an indicator of poor maternal nutrition during pregnancy) and the risk of developing cardiovascular, as well as metabolic diseases during adult life in an independent human cohort (Barker et al., 1993; Barker and Osmond, 1986; Barker et al., 1989; Hales et al., 1991).

Although the ideas of latent health effects stemming from the perinatal period preceded the work of David Barker (Forsdahl, 1977; Ravelli et al., 1976), he is considered as one the pioneers of metabolic programming research. Based on his findings, David Barker postulated the "fetal and infant origin of adult disease" hypothesis (Barker, 1990) (later rephrased into "developmental origins of health and disease") (Barker, 2007), which was further extended and modified by the "thrifty phenotype hypothesis" (Hales and Barker, 1992, 2001), as well as the "developmental plasticity" (Barker, 2004) and "perinatal programming" theory ((Lucas, 1991), for review, see (McMillen and Robinson, 2005)). Together, these concepts suggest that environmental factors in response to maternal health and nutrition during a critical plastic phase of development trigger adaptive responses in the developing fetus, which result in persistent morphological modifications and altered sensitivity of organs involved in metabolic regulation. From an evolutionary perspective, these adaptations would enable a highly efficient use of metabolites and thereby increase the chances of survival in times of scarce food availability. However, upon an imbalance between the developmental and postnatal (nutritional) environment, these adaptations could prove detrimental, resulting in cardiovascular and/or metabolic diseases. These persistent developmental changes on the efficacy and tight control of metabolism in the offspring are often referred to as "metabolic programming". However, underlying mechanisms of how metabolic programming might be mediated still remain largely elusive.

1.1.3.2 Adverse pregnancy outcomes in response to maternal obesity and diabetes

Over the last decade, the main focus of metabolic programming research has expanded from studying undernutrition to also include deciphering the effects of over and/or malnutrition, as well as hyperglycemia during pregnancy on the future health of the offspring. Of note, studies analyzing the effects of maternal overnutrition on the offspring's metabolic health found a similar link between elevated birth weight and an increased risk to develop

metabolic diseases as previously described for children born with low birth weight (Pettitt and Jovanovic, 2001). These findings highlight the crucial importance of a well-balanced hormonal and nutritional milieu for the developing fetus. In more recent studies, maternal BMI has replaced birth weight as an indicator for future health risks of the offspring. Prepregnancy BMIs above 25 have been shown to increase the risk for complications during pregnancy, such as pre-eclampsia, extreme pre-term deliveries and need for caesarean sections, thereby further augmenting the risk for the necessity of neonatal intensive care and possibly neonatal death (Cnattingius et al., 2013; Doherty et al., 2006; Kalk et al., 2009). Moreover, even under normal pregnancy progression, children of overweight and/or obese women have an increased risk to be large for gestational age (LGA) and are more likely to be born macrosomic (Yu et al., 2013). In turn, these neonatal abnormalities are associated with higher levels of obesity and T2DM during childhood that often persist throughout lifetime, possibly resulting in the development of certain co-morbidities including hypertension, coronary heart disease, or stroke (Ahlsson et al., 2007; Boney et al., 2005; Pham et al., 2013; Salsberry and Reagan, 2005; Whitaker, 2004).

Apart from the direct effects of maternal adiposity on the offspring's health, several studies clearly show a positive correlation between maternal BMI and the incidence of gestational diabetes mellitus (GDM) (Torloni et al., 2009b). GDM is defined as a state of glucose intolerance with onset or first recognition during pregnancy (WHO, 2013a). Importantly, GDM also develops in normal weight women and has been shown to increase the risk factors for adverse pregnancy outcomes independent of the maternal prepregnancy BMI or weight gain during pregnancy (Jovanovic-Peterson et al., 1991; Schaefer-Graf et al., 2003). Thus, children born to diabetic mothers are more likely to be LGA, to be born with asymmetric macrosomia and to eventually develop obesity and T2DM irrespective of the maternal state of adiposity (Alberico et al., 2014; Dabelea and Pettitt, 2001; Schaefer-Graf et al., 2003; Silverman et al., 1998). However, dietary interventions in combination with well-controlled physical exercise (and in some cases insulin therapy) have been proven to work effectively against the chronic hyperglycemia in GDM patients, thereby protecting the offspring from adverse pregnancy outcomes (Crowther et al., 2005). Nevertheless, even slightly elevated glucose levels during pregnancy, which are below the official criteria to be diagnosed with GDM, can lead to macrosomia, neonatal hyperinsulinemia and childhood obesity (Metzger et al., 2008) (Deierlein et al., 2011). Together, these findings demonstrate

the necessity to unravel the molecular and cellular changes not only in response to pathological maternal conditions such as obesity or diabetes during pregnancy, but also in response to slightly elevated levels of hormones and nutrients, such as insulin and glucose, predisposing for metabolic disorders throughout lifetime. In the search of the cause for this predisposition, hypothalamic neurocircuits regulating energy and glucose homeostasis have been identified as primary targets of metabolic programming,

1.2 The role of the hypothalamus in the regulation of energy homeostasis

The hypothesis that a stable internal environment even under changing external conditions is essential for the health and survival of an organism was originally suggested by the French Physiologist Claude Bernard and further developed to the definition of "homeostasis" by Walter B. Cannon (Cannon, 1929; Gross, 1998). Accordingly, energy homeostasis is defined as the equilibrium of energy intake (food consumption) and energy expenditure (basal and exercise-induced metabolic rate) (WHO, 2013c). Similarly, the term glucose homeostasis has been used to describe all processes involved in maintaining blood glucose concentrations at relatively stable levels. The detrimental effects of an imbalance in energy and glucose homeostasis are evident by the current obesity and T2DM epidemics responsible for a multitude of health complications and exploding health care costs.

Whereas Claude Bernard suggested a significant role of the brain in the regulation of glucose homeostasis already in the middle of the 19th century, the notion of the central nervous system (CNS), specifically the hypothalamus, as being one of the most important regulators of energy homeostasis did not evolve until the beginning of the 20th century. Tumors in that particular brain region lead to hyperphagia, obesity and retarded puberty (known as the Babinski-Frohlich syndrome) (Bruch, 1993; Gross, 1998). Subsequent lesioning and electrical stimulation studies of distinct regions in the hypothalamus in rodents suggested the existence of specialized hypothalamic centers either mediating hunger, i.e. the lateral hypothalamic area (LH) or satiety, i.e. the arcuate nucleus of the hypothalamus (ARH), the ventromedial hypothalamus (VMH) and the paraventricular nucleus of the hypothalamus (PVH) (Anand and Brobeck, 1951; Gold, 1973; Hetherington and Ranson, 1940). Although the functions of these hypothalamic areas are not as black and white as

initially thought, but more complex and intertwined, these experiments provided direct evidence that an intact hypothalamus was essential for maintaining energy homeostasis. Since then, a completely new field of research has emerged, dedicated to understanding the mechanisms underlying an impaired efficiency of hypothalamic neurocircuits in regulating energy and glucose homeostasis and thereby contributing to the development and manifestation of metabolic disorders.

1.2.1 Structural organization of the hypothalamus

As the key site in the CNS for the integration and translation of multiple biological signals, the hypothalamus has an array of unique features that enable the mediation of adaptive endocrine, autonomic and behavioral responses to maintain homeostasis: First, the hypothalamus displays an extensive structural complexity. Apart from its 11 major nuclei and compartmental areas, which are situated symmetrically around the third ventricle, the hypothalamus harbors several small nuclei as well as large fiber bundles (Figure 1.2) (Simerly, 1995). Second, the hypothalamus, which occupies the ventral part of the diencephalon located beside the midbrain of the brainstem and below the thalamus, forms extensive bilateral neuronal connections within intra-, but also extra-hypothalamic sites (such as autonomic centers and the limbic system) (Hess, 1957; Simerly, 1995). Finally, its relative localization in the CNS adjacent to the third ventricle enables reciprocal communication with peripheral organs *via* blood-borne signals.

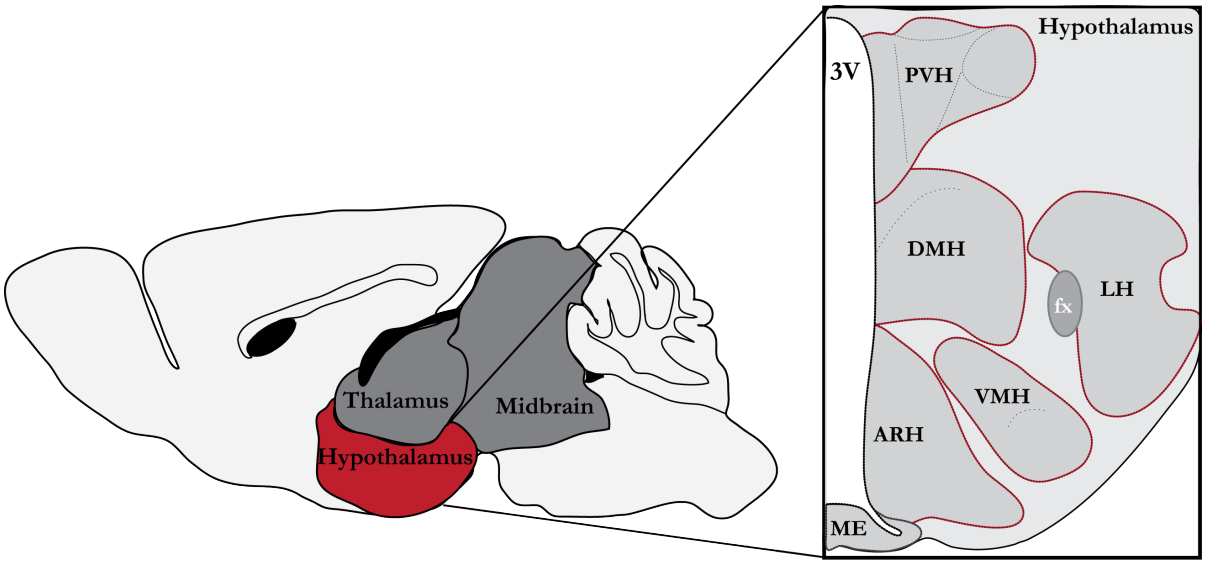


Figure 1.2: Schematic overview of the hypothalamus in the brain
The hypothalamus is located beside the midbrain of the brainstem and below the thalamus. The major hypothalamic nuclei are situated symmetrically around the third ventricle above the median eminence. ME, median eminence; ARH, arcuate nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; PVH, paraventricular nucleus of the hypothalamus; LH, lateral hypothalamic area; fx, fornix; 3V, third ventricle.

With regard to energy homeostasis, the ARH has evolved as one of the most intensively studied regions in the CNS. The ARH resides at the mediobasal hypothalamus adjacent to the third ventricle and the median eminence (ME). The ME is defined as a circumventricular organ with high permeability for blood-borne signals due to its fenestrated capillaries, high vascularization and a "leaky" blood-brain barrier (Ciofi et al., 2009; Rodriguez et al., 2010). This unique location allows the ARH to integrate peripheral nutritional and hormonal signals, which reflect the energy status of the body, into neuronal responses to ultimately adapt behavior. At more rostral areas, the PVH resides in the periventricular zone at the dorsal end of the third ventricle. The main functions of the PVH are the regulation of pituitary hormones, as well as the modulation of the autonomic nervous system in response to changes in energy status (Figure 1.2) (Biag et al., 2012). Additionally, neurons of the VMH, an area that lies just above the ARH, have been established as crucial regulators of feeding behavior and glucose homeostasis, e.g. in the detection and counter-regulation of hypoglycemia (Beverly et al., 1995; Levin et al., 2008). Residing dorsally of the

VMH, the dorsomedial hypothalamus (DMH) represents one of the main downstream target sites of neuronal projections from other hypothalamic nuclei and is therefore suggested to play an integrative role, for example as a relay center of ARH to PVH neuronal connections, thereby modulating appetite and emotional behavior (Chao et al., 2011; Thompson and Swanson, 1998). In contrast to the aforementioned hypothalamic nuclei, the LH is a loosely organized and heterogeneous zone that (as it name suggests) occupies most of the lateral area of the hypothalamus (Bernardis and Bellinger, 1993). Moreover, as the most-intensively interconnected areas of the hypothalamus, the LH has not only been implicated in the regulation of energy and glucose homeostasis via endocrine and autonomic mechanisms, but has further been shown to modulate cognitive and skeletal motor functions (Bernardis and Bellinger, 1993; Cui et al., 2012).

Although substantial progress has been made in unraveling the neuronal and hormonal circuits of these distinct hypothalamic nuclei controlling energy and glucose homeostasis, we are only starting to understand the complexity of their regulatory processes and how developmental and/or pathological insults may impair their efficiency to adapt energy balance to meet the prevailing demands of the organism.

1.2.2 The Arcuate nucleus of the hypothalamus (ARH)

1.2.2.1 ARH neuronal populations and the central melanocortin circuitry

Two functionally antagonistic neuronal populations that reside in the ARH orchestrate transduction of peripheral nutritional and hormonal signals into behavioral responses: the anorexigenic proopiomelanocortin (POMC)-expressing and the orexigenic agouti-related peptide/neuropeptide Y (AgRP/NPY)-coexpressing neurons (for review, see (Butler, 2006)).

To exert its anorexigenic effects, the pro-protein POMC has to undergo posttranslational modifications mediated by cell type-specific prohormone convertases resulting in the biologically active neurotransmitter α-melanocyte-stimulating-hormone (α-MSH) (for review, see (Wardlaw, 2011)). Initially, POMC is endoproteolytically cleaved by the prohormone convertase 1/3 (PC1/3) into two main derivatives: the adrenocorticotropic hormone (ACTH) and β-lipotropin hormone (β-LPH), as well as a 16 kDa N-terminal fragment. In the hypothalamus, further processing of ACTH by PC2 yields a truncated form

of ACTH ($ACTH_{1-17}$) and the corticotropin-like intermediate peptide (CLIP). Subsequently, carboxypeptidase E (CPE) endoproteolytically cleaves the C-terminal part of $ACTH_{1-17}$, which is then amidated to form desacetyl α-MSH, the predominant form of α-MSH in the rodent hypothalamus. Moreover, PC2 and CPE further process β-LPH into β-MSH and the opioid peptide β-endorphin, as well as the N-terminal part of POMC into γ-MSH, neuropeptides that have also been implicated in the modulation of energy balance (for review, see (Wardlaw, 2011)). Accordingly, mutations resulting in impaired sorting or processing of POMC have been associated with the development of obesity, both in humans and rodents (Creemers et al., 2008; Jackson et al., 1997; Lloyd et al., 2006).

Of note, POMC expression is not restricted to the ARH, but can also be found in extra-hypothalamic sites, such as the retina, spinal cord, the commissural part of the nucleus of the solitary tract (NTS) and the area postrema in the brainstem (Bronstein et al., 1992; Knigge et al., 1981; Lein et al., 2007). Moreover, POMC has critical functions in peripheral organs, including the pituitary and melanocytes of the skin, where it is essential for the production of ACTH and α-MSH-mediated hair follicle pigmentation, respectively (Bornstein and Chrousos, 1999; Rees, 2003). In contrast to POMC, AgRP can be modulated by, but does not depend on posttranslational modifications to exert its orexigenic effects, i.e. stimulation of feeding and inhibition of energy expenditure (Creemers et al., 2006). Whereas AgRP expression in the CNS is restricted to the ARH, the co-expressed orexigenic NPY is one of the most abundant neuropeptides and widely expressed throughout the brain (Allen et al., 1983; Broberger et al., 1998; Minth et al., 1984; Sahu et al., 1988). Consistently, NPY is not only involved in the regulation of energy homeostasis, but has also been implicated in the control of blood pressure and circadian rhythm in an AgRP-independent manner (Balasubramaniam, 1997).

POMC and AgRP/NPY neurons form the basis of the so-called "central melanocortin system", which is considered as one of the main neuronal circuits involved in the regulation of energy balance. Downstream neurons of the melanocortin system are defined by their expression of melanocortin receptors, a family of G-protein-coupled receptors (for review, see (Yang, 2011)). Although a total of five isoforms of the melanocortin receptors (MC1-5R) exist, only MC3Rs and MC4Rs are expressed in the brain, of which the MC4R seems to be the main isoform for the central melanocortin circuitry involved in the regulation of energy homeostasis (Cone, 2006; Yang, 2011). Second-order

neurons expressing the MC4Rs reside predominantly in the PVH, but can also be found in various other regions throughout the CNS (see Chapter 1.2.2.2 for further detail) (Balthasar et al., 2005; Warne and Xu, 2013). Upon feeding, binding of α-MSH to the MC4R stimulates neuronal activity, presumably by increasing intracellular cAMP levels in anorexigenic neurons in the PVH (Lee et al., 2001). However, in the fasted state, AgRP acts as an inverse agonist of the MC4Rs and prevents the anorectic effects mediated by α-MSH (Ollmann et al., 1997). In contrast to α-MSH and AgRP, NPY does not bind to MC4Rs, but inhibits the activity of MC4R-expressing neurons in the PVH by binding to NPYRs, a family of $G_{i/o}$-protein-coupled receptors, resulting in decreased cAMP levels (Gerald et al., 1996). Moreover, AgRP/NPY neurons further mediate their orexigenic effects by synaptic release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) directly onto neighboring POMC, as well as to other neurons throughout the CNS (see Chapter 1.2.2.2 for further detail) (Cowley et al., 2001; Horvath et al., 1997).

The importance of a functional central melanocortin system for maintaining energy homeostasis is supported by the fact that loss or mutations of POMC, MC3R, MC4R or ectopic expression of the MC3/4R antagonist Agouti in agouti viable yellow mutant mice (A^{vy}) result in hyperphagia, obesity, hyperinsulinemia and hyperglycemia, both in rodents and in humans (Chen et al., 2000; Huszar et al., 1997; Lu et al., 1994; Smart et al., 2007; Yaswen et al., 1999). Additionally, acute intracerebroventricular (ICV) injection of α-MSH decreases, whereas ICV injections of AgRP and NPY stimulate food intake (Clark et al., 1984; Millington et al., 2001; Rossi et al., 1998). However, genetic deletion of *Agrp* and/or *Npy* does not lead to any metabolic impairment and ablation of AgRP neurons early during neonatal development has only mild effects on body weight and feeding behavior, questioning the relative contributions of these orexigenic neurotransmitters in the regulation of energy homeostasis (Erickson et al., 1996; Luquet et al., 2005; Marsh et al., 1998; Qian et al., 2002). Nevertheless, acute ablation of AgRP neurons in adult mice induces extreme anorexia, eventually leading to starvation (Gropp et al., 2005; Luquet et al., 2005). These findings suggest that the central orexigenic feeding circuits remain highly plastic during development and that early loss of AgRP/NPY signaling can be compensated for by alternate signaling pathways to ensure survival of the organism. Importantly, specific restoration of GABA-signaling in one of the main extra-hypothalamic AgRP target sites, the parabrachial nucleus (PBN) of the brainstem, rescues the starvation phenotype in adult AgRP-ablated

mice (Wu et al., 2009). Thus, AgRP/NPY neurons of the ARH play a pivotal role in regulating energy homeostasis not only *via* modulation of MC4R-expressing neurons, but also *via* GABA-ergic signaling throughout the CNS (for review, see (Wu et al., 2009)).

Taken together, central regulation of energy and glucose homeostasis depends upon a functional melanocortin circuitry, as well as melanocortin-independent ARH neuronal signaling to efficiently coordinate feeding behavior.

1.2.2.2 Intra-and extra-hypothalamic ARH neuronal circuits

To mediate central regulation of energy and glucose homeostasis, the ARH POMC and AgRP/NPY neurons project to various sites within the hypothalamus, such as the PVH, DMH and LH. In the PVH, α-MSH and AgRP directly act (among other neuronal populations) on anorexigenic MC4R-expressing neurons defined by their expression of either thyrotropin-releasing hormone (TRH) or corticotropin-releasing hormone (CRH), which regulate hormonal release from the pituitary. In contrast, the neuronal identity of ARH downstream neurons of the DMH and LH are not fully elucidated, yet. However, it has been suggested that POMC and AgRP/NPY neurons modulate the activity of MC4R-expressing GABA-ergic neurons in the DMH. Under conditions with increased energy demand or energy intake (e.g. during lactation or obesity), distinct neurons in the DMH express NPY, which are a direct target of MC4R-expressing GABA-ergig neurons of the DMH (Chen et al., 2004). These DMH NPY neurons in turn modulate the activity of anorexigenic PVH neurons, providing an additional, but indirect ARH neuronal circuit regulating feeding behavior. Similarly, MC4R-expressing neurons in the LH have been demonstrated to be GABA-ergic and to synapse onto the orexigenic orexin-expressing neurons implicated not only in the homeostatic, but also the hedonic modulation of food intake (Cui et al., 2012). In line with these findings, long-term blockage of MC4R-signaling by adeno-associated virus (AAV)-mediated expression of the MC4R antagonist Agouti in these distinct hypothalamic ARH neuronal target sites all resulted in robust, but differential effects on obesity development (Kas et al., 2004). Whereas Agouti expression in the PVH led to rapid hyperphagia and weight gain, Agouti expression in the DMH was associated with a delayed onset of increased food intake and body weight starting after approximately 6 weeks. Moreover, LH-specific inhibition of MC4R-signaling had no effect on feeding behavior

under normal condition, but augmented diet-induced obesity. Together, these findings demonstrate the complexity of ARH neuronal mediated regulation of energy homeostasis with distinct roles of MC4R-dependent POMC and AgRP neuronal signaling in different intra-hypothalamic nuclei that differentially impact on obesity development.

In addition to MC4R-dependent effects of POMC and AgRP neurons on energy balance via intra-hypothalamic target sites, these ARH neurons further modulate energy and glucose homeostasis by acting on brain regions implicated in the regulation of i) the autonomic nervous system, such as the dorsal vagal complex (DVC) in the brainstem and the intermediolateral nucleus (IML) in the spinal cord, ii) the reward circuitry, such as the amygdala, ventral tegmental area (VTA), nucleus accumbens and the bed nucleus of the stria terminalis (BNST) and iii) analgesia, such as the PBN, the periaqueductal grey (PAG) and dorsal raphe nucleus (Figure 1.3) (Betley et al., 2013; King and Hentges, 2011; O'Donohue et al., 1979). Of note, although there is a great convergence between the innervation pattern of POMC and AgRP/NPY axons throughout the CNS, not all target areas are innervated by both ARH neuronal populations to the same extent.

Given the distinct functions of the aforementioned regions, they also diverge in their relative contribution in the POMC and AgRP/NPY-mediated regulation of energy balance. For example, while PVH- and amygdala-specific re-expression of the MC4R in an MC4R-deficient background prevented approximately 60 % of obesity development due to complete restoration of food intake to wildtype levels, these mice still displayed a decrease in energy expenditure. In contrast, re-expression of the MC4R exclusively in the DVC and IML restored energy expenditure and glucose homeostasis without affecting food intake (Balthasar et al., 2005; Sohn et al., 2013). Thus, dissection of neuronal circuits emerging from the very well defined POMC and AgRP/NPY neurons of the ARH throughout the CNS revealed distinct cell-type and region-specific divergence in the relative contribution to regulate different aspects of energy and glucose homeostasis.

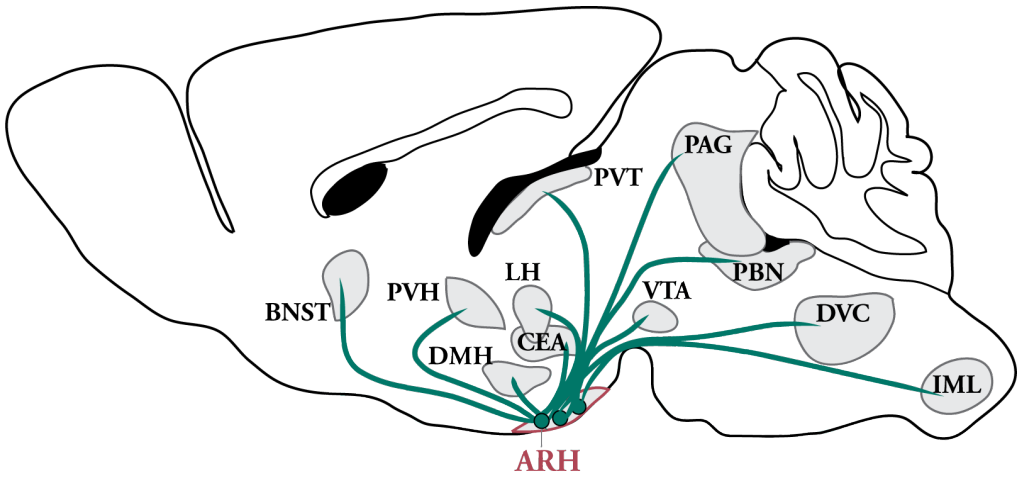


Figure 1.3: Schematic overview of ARH neuronal projections throughout the CNS
POMC and AgRP/NPY neurons residing in the ARH project to various intra-hypothalamic (i.e. PVH, DMH, LH) nuclei, as well as to extra-hypothalamic target sites involved in the regulation of energy and glucose homeostasis, the control of the autonomic nervous system, the mesolimbic dopaminergic neurocircuitry and analgesia. POMC, proopiomelanocortin; AgRP, agouti-related peptide; NPY, neuropeptide Y; ARH, arcuate nucleus of the hypothalamus; BNST, bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; DMH, dorsomedial nucleus of the hypothalamus; DVC, dorsal vagal complex; IML, intermediolateral column; LH, lateral hypothalamic area; PAG, periaqueductal grey; PBN, parabrachial nucleus; PVH, paraventricular nucleus of the hypothalamus; PVT, paraventricular thalamic nucleus; VTA, ventral tegmental area.

1.2.3 Hormonal signals regulating the melanocortin circuitry

ARH-residing POMC and AgRP/NPY neurons act on their designated downstream neurons in response to hormonal and nutritional changes in the periphery. Here, the pancreas-derived hormone insulin and the adipocyte-derived hormone leptin have been shown to be critically involved in conveying information about the current energy status and overall energy stores into centrally mediated adaptation of feeding behavior and metabolism (for review, see (Belgardt and Bruning, 2010)).

1.2.3.1 Insulin signaling

Insulin is secreted postprandially in response to elevated blood glucose levels from pancreatic β-cells. After meal-ingestion, glucose is imported into β-cells via glucose transporter (GLUT) 2. Subsequent metabolism of glucose via glycolysis and the citric-acid cycle rapidly increases intracellular adenosine triphosphate (ATP) levels, which in turn leads to closure of ATP-

sensitive potassium (K_{ATP}) channels and thus depolarization of the cell membrane. This depolarization opens voltage-gated Calcium (Ca^{2+}) channels, thereby facilitating diffusion of Ca^{2+} into the cell, which triggers additional Ca^{2+} release from the endoplasmatic reticulum (ER) and finally insulin secretion into the blood stream (for review, see (Komatsu et al., 2013)).

Upon its secretion from pancreatic β-cells, insulin regulates various aspects of carbohydrate and lipid metabolism, e.g. insulin facilitates glucose uptake in muscle and adipose tissue, stimulates lipid and glycogen synthesis and suppresses hepatic glucose production (HGP), as well as lipolysis (Bluher et al., 2002; Bruning et al., 1998; Claycombe et al., 1998; Cross et al., 1995; Kershaw et al., 2006; Puigserver et al., 2003). Although these functions used to be primarily attributed to direct systemic effects of insulin, the CNS has emerged as a critical target site for insulin-mediated regulation of energy and glucose homeostasis, as well as reproduction (see chapter 1.2.3.3 for further detail). Insulin receptors (IR) are widely distributed throughout the CNS with marked regional variation in receptor density, showing the highest levels of expression in the hypothalamus, as well as in the olfactory bulb, cerebral cortex, cerebellum, hippocampus, thalamus and midbrain (Havrankova et al., 1978; van Houten et al., 1979). Insulin passes through the blood-brain barrier via a saturable, receptor-mediated process that yields insulin levels in the CNS proportional to plasma insulin (Baura et al., 1993). Identical to insulin signaling in the periphery, binding of insulin to its receptors in the CNS leads to rapid autophosphorylation of the IR and thereby to further activation of its intrinsic kinase activity, resulting in tyrosine-phosphorylation of insulin receptor substrate (IRS) proteins (for review, see (Biddinger and Kahn, 2006). Unlike in the periphery, where IRS-1 is essential for signal-transduction, IRS-2 is the main mediator of insulin's intracellular responses in the brain (Araki et al., 1994). Phosphorylated IRS-2 serves as a docking platform for proteins harboring a Src-homology (SH) domain, e.g. the p85 regulatory subunit of the phosphatidylinositide-3 kinase (PI3K). Subsequent phosphorylation of the membrane-bound phosphatidyl-inositol-4,5-bisphosphate (PIP2) to phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) by the p110 catalytic subunit of PI3K results in a context-specific activation of several downstream molecules (for review, see (Cheatham and Kahn, 1995)). On the one hand, PIP3 activates K_{ATP} channels, leading to potassium outflow and thus, hyperpolarization and silencing of neurons (MacGregor et al., 2002; Plum et al., 2006). On

the other hand, PIP3 activates phosphoinositide-dependent kinase 1 (PDK1), which in turn phosphorylates and thus activates the kinase AKT to elicit downstream signaling events (for review, see (Vanhaesebroeck and Alessi, 2000)). Among other targets, AKT phosphorylates forkhead box-1 (FOXO-1), which results in nuclear-exclusion and thus inhibition of FOXO-1-mediated activation and/or repression of target genes (for review, see (Biddinger and Kahn, 2006)). Moreover, AKT phosphorylates the mammalian target of rapamycin (mTOR), a critical regulator of protein synthesis and inhibitor of autophagy (Figure 1.4B) (for review, see (Wullschleger et al., 2006)). Similar to insulin's action in peripheral target tissues, insulin signaling in the brain mediates its variety of effects not only via PI3K, but also via the mitogen-activated protein (MAPK) signaling pathways (Figure 1.4 A). However, the role of IR-mediated activation of the MAPK-signaling in the regulation of energy homeostasis is less well defined so far.

1.2.3.2 Leptin signaling

In addition to insulin, central leptin action is critically involved in the regulation of energy and glucose homeostasis. As early as the 1950s, Gordon C. Kennedy postulated the lipostatic theory suggesting that a humoral signal produced by adipose tissue in direct proportion to fat mass could act on the hypothalamus to decrease food intake and increase energy expenditure (Kennedy, 1953). The brain would thereby monitor fat levels and maintain them at certain fix points. With the discovery of leptin in 1994, a hormone that is secreted in direct proportion to fat mass and acts in the brain to modulate energy and glucose metabolism, this humoral factor was identified and proved Kennedy's theory correct in many aspects (Zhang et al., 1994).

Leptin signals via the long form of its receptor (LepRb) to induce multiple intracellular signaling cascades, one of them being the JAK/STAT pathway. Upon leptin binding, the Janus kinase 2 (JAK2) is recruited to the receptor, where it autophosphorylates itself, as well as multiple tyrosine residues of the LepRb, which serve as binding sites for signal transducer and activator of transcription 3 (STAT3) proteins (Banks et al., 2000; Tartaglia, 1997). Subsequent JAK2-mediated phosphorylation of STAT3 leads to STAT3 homodimerization, the exposure of its nuclear-localization domain and consequently to nuclear localization. In the nucleus, STAT3 serves as a transcription factor at various

promoter sites to regulate gene expression, e.g. upregulation of its own negative regulator suppressor of cytokine signaling 3 (SOCS3) (Bjorbaek et al., 1998). In addition to activating the JAK/STAT signaling cascade, leptin has also been described to signal via the PI3K pathway (Mirshamsi et al., 2004). Thus, the PI3K pathway resembles a point of convergence between insulin and leptin signaling. While studies have shown independent, as well as synergistic effects of insulin and leptin signaling in the regulation of energy and glucose homeostasis, we are only starting to understand the relative contribution of these hormones in adapting cellular and molecular processes in the CNS, to ultimately meet the immediate demands of the body.

1.2.3.3 Insulin and leptin action on ARH neurons

The notion that insulin acts in the brain to modulate feeding behavior has been demonstrated across species, as centrally administered insulin (either via ICV injection or intranasal application) decreases food intake, which is associated with loss of body weight in rodents, non-human primates and humans (Benedict et al., 2008; Woods et al., 1979; Zhang et al., 2008). Identical effects have been shown for central leptin injections, at least in rodents (Calapai et al., 1998; Schulz et al., 2012). Consistently, genetic deletion of the IR in the brain results in mild obesity, insulin resistance and infertility (Bruning et al., 2000); and neuron-specific LepRb ablation induces obesity and impaired neuroendocrine functions essential for energy and glucose homeostasis in mice (Cohen et al., 2001).

To decipher the relative contribution of insulin and leptin action on ARH neurons in mediating these effects, studies have genetically ablated the IR and LepRb from these distinct neuronal populations. Surprisingly, specific deletion of the IR in either of these neuronal subtypes did not affect feeding behavior, or energy expenditure (Konner et al., 2007). However, insulin-mediated suppression of HGP was blunted in mice lacking the IR specifically on AgRP neurons, demonstrating the role of central insulin in the regulation of glucose metabolism (Konner et al., 2007). In addition, specific reconstitution of the IR in AgRP neurons of L1 mice (mice that exclusively express the IR in liver, pancreatic cells and several regions in the brain, but which exhibit a 90 % reduction of IR expression in the ARH) further supported this finding. Here, HGP levels of L1 mice were restored to normal levels upon AgRP-specific reconstitution of the IR (Lin et al., 2010). By contrast, POMC-

specific IR deletion had no effect on HGP (Konner et al., 2007). However, reconstitution of the IR in POMC neurons of L1 mice further exacerbated their hepatic insulin resistance, while at the same time restoring locomotor activity, suggesting a role for insulin signaling in POMC neurons in the regulation of energy expenditure (Lin et al., 2010). Similarly, LepRb deficiency in POMC or AgRP/NPY neurons had only mild effects on body weight gain and adiposity, which was slightly augmented upon LepRb ablation from both neuronal populations at the same time (van de Wall et al., 2008).

Nonetheless, insulin and leptin have been shown to act via POMC and AgRP/NPY neurons to mediate central regulation of energy balance. On a molecular level, insulin and leptin are able to regulate *Pomc*, *Agrp* and *Npy* gene transcription. Whereas insulin increases POMC mRNA expression in a PI3K-dependent manner, upregulation of POMC mRNA expression via leptin depends on phosphorylation of STAT3 (pSTAT3) (Belgardt et al., 2008; Xu et al., 2007). In contrast, both insulin and leptin decrease NPY and AgRP expression in a PI3K-dependent manner. Crucial for both of these effects is FOXO-1, which acts as a transcriptional repressor of *Pomc* by recruiting histone deacetylases, and as a transcriptional activator of *Agrp* and *Npy*, respectively (Kim et al., 2006; Kitamura et al., 2006). Thus, in POMC neurons, PI3K-mediated phosphorylation and subsequent nuclear exclusion of FOXO-1 allows pSTAT3 to bind and induce *Pomc* transcription, whereas in AgRP/NPY neurons, PI3K-mediated phosphorylation of FOXO-1 indirectly inhibits *Agrp* and *Npy* expression, as subsequent nuclear exclusion allows for pSTAT3 to act as a transcriptional repressor of these orexigenic neuropeptides (Figure 1.4 B) (Fukuda et al., 2008; Morrison et al., 2005).

Moreover, insulin and leptin directly modulate the activity of both POMC and AgRP/NPY neurons. Central leptin administration depolarizes and thus increases POMC neuronal activity in a PI3K-dependent manner (presumably via transient receptor potential cation (TRPC) channels and, at the same time, hyperpolarizes and thus inhibits AgRP/NPY neurons by modulating K_{ATP} channel activity (Mirshamsi et al., 2004; Qiu et al., 2010; van den Top et al., 2004). Collectively, these changes in neuronal activity lead to an increased α-MSH and decreased NPY synaptic release, thereby mediating anorexigenic behavior. Insulin on the other hand leads to a PI3K-dependent activation of K_{ATP} channels and thus hyperpolarization of both POMC and AgRP/NPY neurons (Figure 1.4 B) (Konner et al., 2007; Plum et al., 2006). To date, this seemingly paradoxical situation, where insulin exerts

the same effect on neuronal activity of two functionally opposing neurons is not yet resolved. Moreover, the question arises how two "anorexigenic" hormones, i.e. insulin and leptin, which both require PI3K-mediated signaling cascades in POMC neurons to exert their functions, can result in opposite effects regarding the net outcome of POMC neuronal activity. Interestingly, insulin- and leptin-mediated changes in POMC neuronal activity occur in two distinct neuronal subpopulations (Williams et al., 2010). Furthermore, this segregation in POMC neuronal insulin and leptin responses show a neuroanatomical pattern, further supporting the existence of distinct POMC neuronal subpopulations. However, whether this selective responsiveness of POMC neurons to these differences in hormone sensitivity with regards to neuronal activity are due to 1) differential expression of their respective hormone receptors, 2) different ion channel distribution, 3) distinctive activation of specific signaling intermediates or 4) completely different mechanisms, remains to be elucidated.

In contrast, such heterogeneity in terms of hormone-mediated changes in neuronal activity has not been described for AgRP neurons so far. However, a recent paper from Betley and colleagues investigated the efficacy of distinct AgRP/NPY neurocircuits to stimulate feeding in response to channelrhodopsin 2 (ChR2)-mediated AgRP neuronal activation (Betley et al., 2013). Here, they could show that distinct AgRP/NPY neuronal subgroups located in distinct areas of the ARH innervate only one specific target area. Moreover, neuronal activation of AgRP/NPY circuits only from ARH->PVH, ARH->LH and ARH->BNST were sufficient to induce rapid feeding, whereas optogenetic activation of AgRP/NPY circuits from ARH->PBN, ARH->CEA and ARH->PAG were not associated with changes in feeding behavior. Thus, similar to the specialization of POMC neurons in terms of hormone-responsiveness, AgRP/NPY neurons can be divided into subgroups depending on their relative topographic distribution and their specific downstream target area of innervation, as well as their relative contribution to regulate feeding behavior. The findings that discrete subgroups of defined neuronal populations of the ARH segregate not only in their sensitivity towards hormonal and nutritional changes, but also in their innervation pattern, as well as their relative contribution to mediate different aspects of metabolism, highlight the complexity of hypothalamic regulated energy and glucose balance.

To further complicate matters, one hallmark of obesity is the development of insulin and leptin resistance not only in peripheral organs, such as liver, muscle and fat, but also in

the CNS. Thus, although insulin and leptin might be secreted in obese patients in response to glucose excursions and elevated fat mass, respectively, IR and LepRb-expressing target cells are incapable of converting these signals into adaptive behavioral and systemic metabolic changes. Thus, a better understanding of how cell-type specific central insulin and leptin signaling contribute to the regulation of energy and glucose homeostasis, in combination with a clearer picture of the mechanisms underlying hormonal resistance at the level of the CNS, is of utmost importance to develop effective therapeutics and preventative strategies in the fight against obesity.

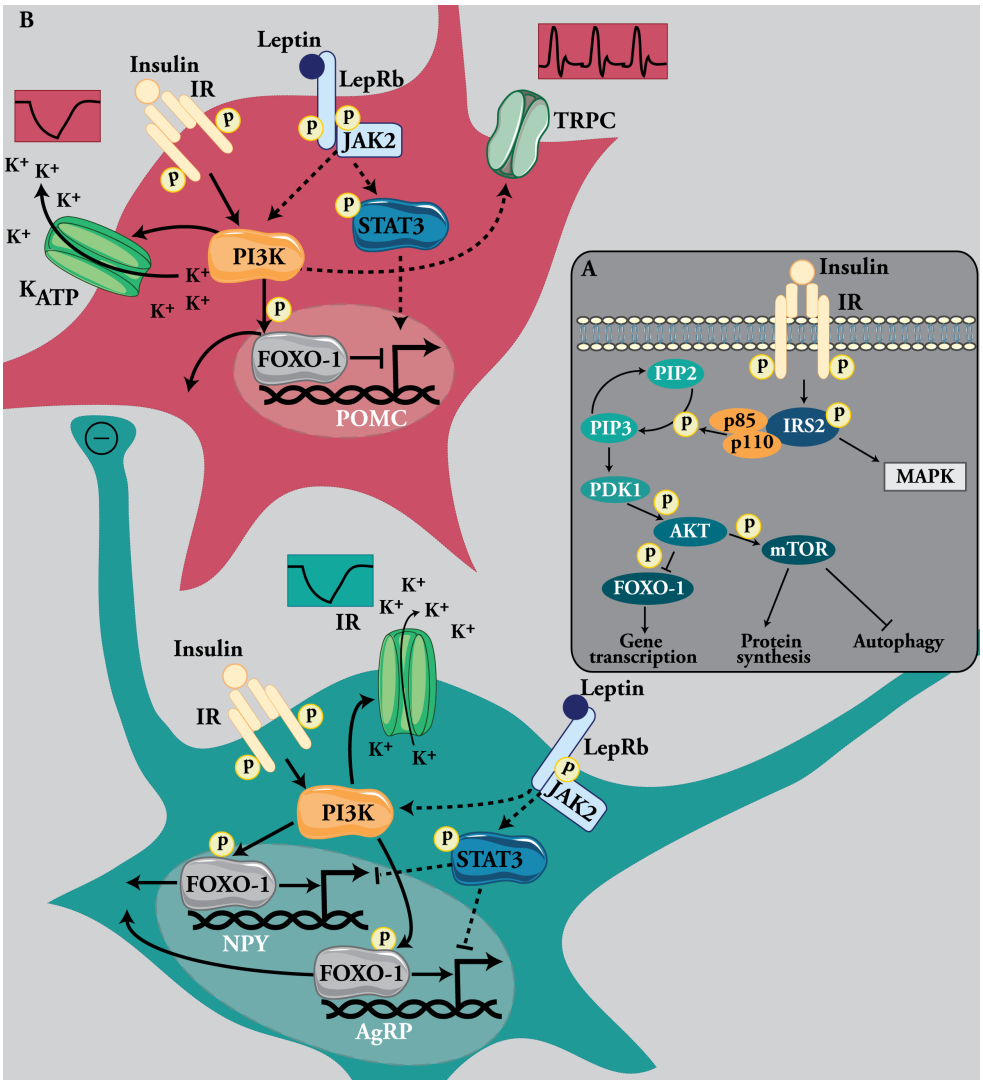


Figure 1.4: Insulin and leptin signaling in POMC and AgRP/NPY neurons
A) Binding of insulin to the insulin receptor (IR) results in a conformational change, activation of the membrane bound receptor tyrosine kinase and autophosphorylation of the IR. IRS2 is phosphorylated, binds to the p85-regulatory subunit of PI3K, leading to phosphorylation of PIP2 to PIP3 by the p110-catalytic domain of PI3K. PIP3 activates PDK1, which phosphorylates AKT,which activates several downstream signaling events, e.g. phosphorylation and nuclear exclusion of FOXO-1 and phosphorylation of mTOR. B) Insulin-mediated phosphorylation of FOXO-1 allows leptin-mediated phosphorylated STAT3 to bind to the promoter of the *Pomc*, as well as *Agrp* and *Npy* gene, resulting in their upregulation and downregulation, respectively. Moreover, insulin activates K_{ATP} channels in POMC (red) and AgRP (green) neurons, resulting in their hyperpolarization, whereas leptin depolarizes POMC in a TRPC-dependent and hyperpolarizes AgRP neurons in a JAK2/PI3K-depenendent manner. PI3K, phosphatidylinositol 3 kinase; IRS2, insulin receptor substrate 2; PIP2, phosphatidylinositol (4,5) bisphosphate; PIP3, phosphatidylinositol (3,4,5) trisphosphate; PDK1, protein-dependent kinase 1; AKT, protein kinase B; mTOR, mammalian target of rapamycin; FOXO-1, forkhead box 1; JAK2, janus kinase 2; STAT3, signal transducer and activator of transcription 3; KATP, ATP-dependent potassium channel; TRPC, transient receptor potential cation channel; POMC, proopiomelanocortin; AgRP, agouti-related peptide; NPY, neuropeptide Y; P, phosphorylation; dashed lines indicate leptin-mediated signaling.

1.3 The hypothalamus as a primary target site for metabolic programming

Given its crucial role in the regulation of energy and glucose homeostasis, the hypothalamus has been in the focus as one of the main target sites for metabolic programming in offspring of obese, diabetic, hyperglycemic or malnourished mothers. Moreover, both mother and child are highly sensitive towards nutritional and/or hormonal changes during pregnancy and development, respectively. Thus, alterations in the nutritional environment in response to impaired maternal health or quality of food during pregnancy have been demonstrated to have a direct impact on hypothalamic neurocircuit development, which is associated with a lifelong predisposition for metabolic disorders in the offspring.

1.3.1 Development of hypothalamic neurocircuits

Hypothalamic neurocircuit development follows a specific pattern of events that can be roughly divided into two major phases: 1) Determination of neuronal cell number, which involves neurogenesis, neuronal death, neuronal differentiation and migration and 2) Formation of functional circuits, which includes axonal outgrowth and synaptogenesis (for review, see (Bouret, 2012)). Whereas the sequence of events is thought to be identical between rodents and humans, the relative timing is highly distinct between these species: Unlike in humans, where both of these phases occur in utero, axon formation and synaptogenesis of hypothalamic neurocircuits occur during the first three weeks after birth in rodents (Figure 1.5) (Grove et al., 2005; Koutcherov et al., 2002) .

Hypothalamic neurons are generated predominantly in the neuroepithelium of the third ventricle. Extensive thymidine labeling and BrdU incorporation studies in the rodent could further pinpoint the period(s) of neurogenesis of distinct hypothalamic nuclei, which occurs in a rostral to caudal and lateral to medial pattern (thus, cells that have to migrate the farthest, are born the earliest) (Shimada and Nakamura, 1973). The peak of hypothalamic neurogenesis is considered to be E12, at which most LH neurons are born. Moreover, neurogenesis of PVH and DMH neurons extends from E12-E16 and that of ARH and VMH neurons from E12 to E17 (Ishii and Bouret, 2012; Padilla et al., 2010). Similarly, in non-human primates neurogenesis of distinct neuronal subgroups occurs in specific time

windows during the first trimester of gestation (Koutcherov et al., 2003). To date, little is known about the subsequent differentiation and migration processes of these newly generated neuronal progenitors. At least for the VMH, it has been described that neurons migrate along radial glial fibers to their final positions in the nucleus (Rakic et al., 1994). However, whether this holds true as a global hypothalamic neuronal migration pattern remains to be investigated.

The second phase of hypothalamic neurocircuit development is not initiated until the neurons have reached their final target area. In rodents, axonal projections of POMC and AgRP/NPY neurons innervate distinct intra-hypothalamic target areas between postnatal day (P) 6 and P16 (Figure 1.5) (Bouret et al., 2004a). Moreover, ARH neuronal projections to the extra-hypothalamic BSTN are established between P12 and P18 (Bouret et al., 2004a). Subsequently, synaptic connections are established and possibly pruned again to form functional neurocircuits, processes that gradually mature from birth until adulthood. As depicted before, contrarily to rodents, in humans and non-human primates, AgRP/NPY innervation of target areas starts at the end of the second and continues until the end of the last trimester of gestation (Figure 1.5) (Grayson et al., 2006; Koutcherov et al., 2002).

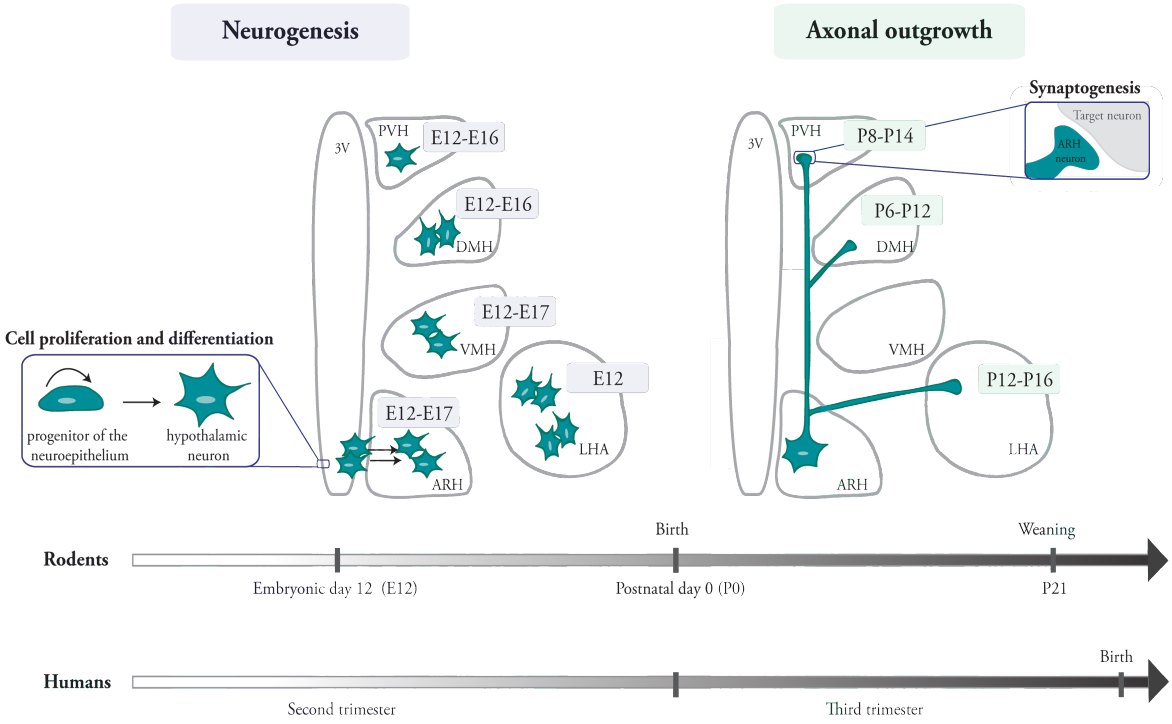


Figure 1.5: Development of hypothalamic neurocircuits in rodents and humans
The ontogeny of functional hypothalamic neurocircuits is composed of two major steps: 1) Determination of neuronal cell number (i.e. neurogenesis) directly followed by their differentiation and migration and 2) the formation of mature axonal projections that include axonal outgrowth and synaptogenesis. In rodents hypothalamic neurons originate from progenitor cells of the neuropithelium of the third ventricle that are generated between embryonic day 12 (E12) and E17 depending on the area. In rodents, development of axonal projections from arcuate neurons occurs during the first weeks of life. Once arcuate projections reach their target neurons, they develop synaptic contact. Hypothalamic development occurs during gestation and lactation in rodents and is not mature before weaning, whereas in humans, the hypothalamus is relatively mature already at birth as neurogenesis and axonal outgrowth take place during the second and third trimester. ARH, arcuate nucleus of the hypothalamus; PVH, paraventricular nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; LH, lateral hypothalamic area; E, embryonic day; P, postnatal day; 3V,third ventricle**.** Adapted from Steculorum, Vogt and Brüning, 2013 (Steculorum et al., 2013).

1.3.2 Different animal models of metabolic programming show defects in hypothalamic neurocircuit development

Although several human epidemiological studies have convincingly demonstrated that maternal obesity and diabetes result in an increased propensity for the unborn child to

develop metabolic diseases, further investigations of underlying cellular and molecular mechanisms responsible for this predisposition have been difficult to perform due to obvious ethical reasons. Therefore, several different animal models have been established to mimic this pathophysiological state, and could show that impaired maternal health and/or nutrition is directly associated with changes in hypothalamic neurocircuit formation in the offspring (for review, see (Plagemann, 2012)).

One of the most commonly used models to mimic maternal obesity is maternal HFD-feeding, which has repeatedly been shown to increase body weight and adiposity, as well as to impair insulin sensitivity and glucose tolerance in the offspring (Guo and Jen, 1995; Gupta et al., 2009; Kirk et al., 2009; Sun et al., 2012). On a cellular level, exposure to maternal HFD during gestation and lactation increases hypothalamic neurogenesis and neuronal migration of predominantly orexigenic neurons in the offspring (Chang et al., 2008). Moreover, several studies have reported altered hypothalamic gene expression of anorexigenic and orexigenic neuropeptides in response to maternal HFD-feeding. However, there have been inconsistencies between studies as to whether the hypothalamic neuropeptidergic profile shifts to a more orexigenic tone (potentially acting as a main driver of obesity development) or to a predominantly anorexigenic tone (possibly as a counter regulation) in offspring from mothers consistently fed a HFD throughout gestation and lactation (Beck et al., 2006; Chen et al., 2008; Gout et al., 2010; Gupta et al., 2009). In addition to affecting the prenatal phase of hypothalamic neurocircuit development, consistent maternal HFD-feeding throughout gestation and lactation also impairs AgRP/NPY neuronal projection formation to the PVH (Kirk et al., 2009).

To investigate the effects of maternal hyperglycemia on hypothalamic neurocircuit development and health outcome of the offspring, studies have made use of the β-cell toxin streptozotocin. Although streptozotocin destroys pancreatic β-cells, which results in complete insulin deficiency and therefore represents a state of maternal T1DM rather than GDM or T2DM, subsequent maternal hyperglycemia has the desired effects of hyperglycemia and hyperinsulinemia in the offspring (Pitkin and Van Orden, 1974). Similar to maternal obesity, maternal hyperglycemia also impairs both phases of hypothalamic neurocircuit development in the offspring, potentially contributing to the increased body weight and insulin resistance observed throughout lifetime in the offspring. However, different studies have led to conflicting results: Whereas Plagemann and colleagues observe

an increase in orexigenic NPY neurons with no effects on POMC neuronal cell number, Steculorum et al. find an increased POMC neuronal cell number without changes in the total count of AgRP/NPY neurons (Plagemann et al., 1998; Steculorum and Bouret, 2011). The latter finding has been confirmed in a genetic model of maternal diabetes (Carmody et al., 2011). Moreover, Steculorum et al. find a consistent decrease of ARH neuronal fiber densities in the PVH, suggesting impaired ARH neuronal axon formation in response to maternal diabetes, possibly adding to the impaired regulation of energy and glucose homeostasis of the offspring later in life (Steculorum and Bouret, 2011).

Taken together, different models of metabolic programming have concordantly shown that both major phases of hypothalamic neurocircuit development are impaired in response to maternal obesity and maternal hyperglycemia. However, given the differences in the effects on hypothalamic neurocircuit development, possibly due to differences in study design (in particular the use of different species, as well as duration and severity of environmental cues), the relative contribution of these hypothalamic developmental abnormalities in mediating the strong metabolic impairments in the offspring remains to be clarified.

1.3.3 Hypothalamic insulin and leptin signaling in metabolic programming

The crucial role of well-balanced hypothalamic leptin levels during hypothalamic neurocircuit development has been established in a series of outstanding studies (Bouret et al., 2004b; Bouyer and Simerly, 2013). Different models of metabolic programming lead to increased levels of leptin during lactation in the offspring, which has repeatedly been shown to induce central leptin resistance early in life (Kirk et al., 2009; Sun et al., 2012). Given the well-described axonotrophic effect of leptin on neurons of the ARH, particularly during a critical time window in the postnatal period, it is conceivable that impaired axon formation in response to neuronal leptin resistance results in impaired energy and glucose homeostasis (Bouret et al., 2004b). In addition, specific restoration of a distinct AgRP to PVH innervation pattern in response to intraperitoneal leptin injections during postnatal development partially rescues the drastically impaired metabolic phenotype of leptin-deficient mice (Bouyer and Simerly, 2013). These findings highlight the impact of abnormal

leptin levels on hypothalamic neurocircuit development, which is associated with an increased propensity to develop metabolic disorders in life.

In contrast, the role of abnormal insulin signaling during development in mediating metabolic programming is less well described so far. Insulin has been shown to exert neuroproliferative and axonotrophic effects, at least in vitro (Desai et al., 2011; Schechter et al., 1999; Toran-Allerand et al., 1988). Moreover, elevated hypothalamic insulin levels have been detected in newborn offspring of diabetic mothers in rats (Plagemann et al., 1998). Therefore, developmental hyperinsulinemia in response to maternal glucose excursions either as a result of maternal obesity, diabetes or postnatal overnutrition has been hypothesized to exert detrimental effects on hypothalamic neurocircuit formation. As depicted before, maternal diabetes affects hypothalamic neuronal cell number, as well as axon formation in the offspring. Of note, the observed architectural differences in the hypothalamus, i.e. decreased size of VMH and PVH, as well as upregulated levels of tyrosin-hydroxylase (TH)-expressing neurons in offspring of STZ-treated dams were abolished upon adaptation of insulin and glucose levels in the mother, pointing to a distinct role of these hormones in mediating these effects (Franke et al., 2005; Plagemann et al., 1999a). Moreover, in a well controlled, but non-physiological experimental setup, early postnatal hypothalamic administration of insulin in neonatal rats resulted in malformations of distinct hypothalamic nuclei, i.e. VMH and DMH, possibly contributing to the observed impairment of body weight regulation and glucose tolerance in the offspring (Plagemann et al., 1999c; Plagemann et al., 1992). Nonetheless, although there is growing evidence that substantiates the hypothesis that early-life hyperinsulinemia impairs hypothalamic neurocircuit development and thereby contributes to the predisposition for metabolic disorders in offspring of obese/diabetic mothers, to date there is no direct proof that insulin is responsible for these changes or mechanistic evidence of how insulin might mediate these effects *in vivo*.

1.4 Objectives

Obesity and type 2 diabetes mellitus (T2DM) still represent two of the major health burdens of our society today. Indeed, despite ongoing extensive research performed to decipher all aspects contributing to the development and manifestation of these epidemics and ultimately

to find effective therapies, the incidences of obesity and T2DM are still rising and increasingly affect people at younger ages (Sabin and Shield, 2008).

The concept of maternal or metabolic programming has received particular attention as one important contributing factor for this on-going trend: Women with altered metabolic homeostasis (e.g. as a result of overnutrition or hyperglycemia) during pregnancy are at increased risk to deliver children that are more prone to develop obesity, diabetes mellitus or cardiovascular diseases later in life, creating a futile cycle of metabolic disorder development (Sullivan and Grove, 2010). Given its crucial role in the regulation of energy and glucose homeostasis and its sensitivity towards environmental changes during development, the CNS, in particular the hypothalamus has evolved as one of the main target sites primarily affected in offspring of obese and/or hyperglycemic mothers. However, although various different mechanisms have been proposed to contribute to this metabolic programming, differences in study design, in particular in severity, duration and onset of abnormal environmental cues has led to different and even conflicting results, making it challenging to perform cross-comparisons between studies. Moreover, only a few studies could convincingly demonstrate that the observed malformations of hypothalamic neurocircuits in response to impaired maternal health or nutrition were directly associated with the impaired metabolic phenotype in the offspring throughout lifetime.

Thus, to shed new light on these matters, we aimed to establish a physiological and well-controlled mouse model of metabolic programming that would allow us to identify the most sensitive period of hypothalamic neurocircuit development in response to maternal high fat feeding. Importantly, we further employed this model to delineate the distinct role of neuron-specific insulin signaling in causing the lifelong predisposition for metabolic disorders in the offspring of obese and/or hyperglycemic mothers.

2 Materials and methods

2.1 Chemicals and biological material

Size markers for agarose gel electrophoresis (Gene Ruler DNA ladder mix) were obtained from Thermo Fisher Scientific, Inc., Germany. Solutions were prepared with double-distilled water (ddH_2O).

All chemicals and biological material used for this work are listed in table 2.1.1.

Table 2.1.1 Chemicals and enzymes used in this study

Chemical/Enzyme	Supplier (Company, Country)
0.9 % Sodium Chloride	Delta Select, Germany
Acetic Acid	Merck, Germany
Acetone	KMF Laborchemie, Germany
Actrapid® (Insulin)	Novo Nordisk, Denmark
Agarose (Ultra Pure)	Invitrogen, Germany
Avidin Biotin Complex-Vectastain Kit	Vector, USA
Bacillol	Bode Chemie, Germany
Calcium Chloride	Merck, Germany
Desoxy-ribonucleotide-triphosphate	Amersham, Germany
di-Sodiumhydrogenphosphate	Merck, Germany
Diaminobenzidin (DAB)	Dako, Germany
DNase, RNase-free	Qiagen, Germany
DPP4 Inhibitor	Merck Millipore, Germany
DreamTaq DNA Polymerase	Thermo Fisher Scientific, Inc., Germany
Ethanol, absolute	Roth, Germany
Ethidium bromide	Applichem, Germany

Chemical/Enzyme	**Supplier (Company, Country)**
Ethylendiamine tetraacetate (EDTA)	AppliChem, Germany
EuroScript Reverse Transcriptase	Eurogentec, Belgium
Glucose 20%	Delta Select, Germany
Glycine	Applichem, Germany
HEPES	Applichem, Germany
Hydrochloride Acid (37 %)	KMF Laborchemie, Germany
Hydrogen Peroxide	Sigma-Aldrich, Germany
Isopropanol (2-Propanol)	Roth, Germany
Kaisers Glycerin Gelatine	Merck, Germany
L-Arginine	Sigma-Aldrich, Germany
Magnesium Chloride	Merck, Germany
Nitrogen (liquid)	Linde, Germany
Octenisept	Schüle & Mayr, Germany
Paraformaldehyde (PFA)	Fluka, Sigma-Aldrich, Germany
Potassium Chloride	Merck, Germany
Potassium Hydroxide	Merck, Germany
Proteinase K	Roche, Germany
Sodium Acetate	AppliChem, Germany
Sodium Chloride	AppliChem, Germany
Sodium Hydroxide	Applichem, Germany
Sodium-dihydrogenphosphate	Sigma-Aldrich, Germany
Sodium tetraborate decahydrate	Sigma-Aldrich, Germany
Sucrose	Sigma-Aldrich, Germany

Chemical/Enzyme	Supplier (Company, Country)
Tissue Freezing Medium	Jung, Germany
Trishydroxymethylaminomethane	AppliChem, Germany
Triton X-100	AppliChem, Germany
Vectashield® Mounting Medium (DAPI)	Vector, USA
β-Mercaptoethanol	GIBCO (Invitrogen), UK

2.2 Molecular biology

2.2.1 Isolation of genomic DNA

Genomic DNA was isolated from tail-biopsies obtained between P14 and P20. Briefly, the samples were incubated overnight in Tail Lysis Buffer (10 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 150 mM NaCl; 0.2 % (w/v) SDS) containing 1 % Proteinase K (Roche, Germany) at 56°C. DNA was precipitated by adding an equal volume of isopropanol, mixed and pelleted by centrifugation. Subsequently, the DNA was washed with 70 % (v/v) Ethanol, dried at RT and resuspended in H_2O.

2.2.2 Quantification of nucleic acids

Nucleic acid concentration was assessed by measuring the sample absorption at 260 nm with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Peqlab, Germany). The 260/280 nm absorbance ratio was used as a measure of purity of nucleic acid samples. Ratios of approximately 1.8 and 2.0 were accepted as pure DNA and pure RNA, respectively.

2.2.3 PCR for genotyping

Genotyping was performed by PCR (Mullis and Faloona, 1987; Saiki et al., 1988) with customized primers (purchased from Eurogentec, Germany) listed in table 2.2.1. Reactions were performed in a Thermocycler iCycler PCR machine (Bio-Rad, Germany). All amplifications were performed in a total reaction volume of 25 µl containing a minimum of 50 ng DNA template, 25 pmol of each primer, 25 µM dNTP Mix (Genaxxon BioScience, Germany), 10 x DreamTaq reaction buffer (Thermo Fisher Scientific, Inc., Germany) and 1 unit of DreamTaq Polymerase (Thermo Fisher Scientific, Inc., Germany). Amplified DNA fragments were analyzed on 2 % (w/v) agarose (Sigma, Germany) gels (1 x TAE, 0.5 mg /ml ethidium bromide), which were electrophoresed at 120 mV.

Table 2.2.1 Oligonucleotides used for genotyping
All primer sequences are displayed in 5'-3' order. Primer orientation is designated "sense" when coinciding with transcriptional direction

Primer	Sequence (5'-3')	Annealing site	Orientation
POMCCre-5' (N16R)	TGGCTCAATGTCCTTCCTGG	*Pomc* promoter	sense
POMCCre-3' (N57R)	CACATAAGCTGCATCGTTAAG	endogenous *Pomc*	antisense
POMC-Cre-3' (AA03)	GAGATATCTTTAACCCTGATC	Cre	antisense
AgRPCre-5'	GGGCCCTAAGTTGAGTTTTCCT	*Agrp*	sense
AgRPCre-3'	GATTACCCAACCTGGGCAGAAC	*Agrp*	antisense
AgRPCre-3'	GGGTCGCTACAGACGTTGTTTG	mutan*t Agrp*	antisense
IR-5'	CTGAATAGCTGAGACCACAG	*Insr* floxed exon	sense
IR-delta-5'	GATGTGCACCCCATGTCTG	*Insr* deletion	sense
IR-3'	GGGTAGGAAACAGGATGG	*Insr* floxed exon	antisense
POMC-eGFP1-5'	TGGCTCAATGTCCTTCCTGG	*Pomc* promoter	sense
POMC-eGFP2-3'	CACATAAGCTGCATCGTTAAG	endogenous *Pomc*	antisense
POMC-eGFP3-3'	CAGGAACTCCAGCAGGACCATG	e*GFP*	antisense
tdTomato1-5'	AAGGGAGCTGCAGTGGAGTA	Rosa26	sense
tdTomato2-5'	CTGTTCCTGTACGGCATGG	mutant Rosa26	sense
tdTomato3-3'	CCGAAAATCTGTGGGAAGTC	Rosa26	antisense
tdTomato4-3'	GGCATTAAAGCAGCGTATCC	mutant Rosa26	antisense

2.2.4 Analysis of gene expression

Hypothalami were dissected using Brain Matrices (Braintree Scientific, Inc, USA). Isolated mRNA from this tissue was analyzed using quantitative real-time PCR. RNA was isolated using the Qiagen RNeasy Kit (Qiagen, Germany), which was combined with the RNase-Free DNase Set (Qiagen, Germany). RNA was reversely transcribed with High Capacity

cDNA RT Kit and amplified using TaqMan® Universal PCR-Master Mix, NO AmpErase UNG with TaqMan® Assay-on-demand kits (Applied Biosystems, USA; see Table 2.2.2) with the exception of the POMC mRNA: POMC sense 5'-GACACGTGGAAGATGCCGAG-3'; POMC antisense 5'-CAGCGAGAGGTCGAGTTTGC-3'; probe sequence, 5'-FAM-CAACCTGCTGGCTTGCATCCGG_TAMRA-3'. Relative expression of target mRNAs was adjusted for total RNA content by *hypoxanthine guanine phosphoribosyl transferase 1* (*Hprt1*) RNA. Calculations were performed by a comparative method ($2^{-\Delta\Delta CT}$). Quantitative PCR was performed on an ABI-PRISM 7900 HT Sequence Detection system (Applied Biosystems, USA).

Table 2.2.2: Probes used for quantitative real-time PCR
All probes were purchased from Applied Biosystems, USA

Probe	Catalogue N°
Hprt-1	Mm01545399_m1
Pomc	Mm00435874_m1
Agrp	Mm00475829_g1
Npy	Mm00445771_m1
Trh	Mm01182425_g1
Tnf	Mm00443258_m1
Il6	Mm00446190_m1
Il1b	Mm01336189_m1

2.3 Cellular biology

2.3.1 Immunohistochemistry

2.3.1.1 POMC and AgRP neuronal cell count

$POMC^{eGFP}$ and $AgRP^{tdTomato}$ mice were transcardially perfused with phosphate buffered saline (PBS), pH 7.5, followed by 4 % paraformaldehyde (PFA) in PBS, pH 7.5. Brains were dissected, postfixed for 4 h at 4°C in PBS containing 4% PFA and 20% Sucrose, pH 7.5 and cryoprotected overnight in PBS containing 20% Sucrose, pH 7.5. To determine neuronal cell number, 25 µm-thick free-floating coronal sections through the ARH were cut using a cryostat (Leica, Germany) and used for the respective staining. GFP-positive neurons were detected as previously described (Janoschek et al., 2007). Briefly, sections were incubated in rabbit anti-GFP antiserum (1:10000, Molecular Probes/Invitrogen). Incubation with secondary Biotin-labeled donkey antirabbit IgG (1:500, Jackson ImmunoResearch) was followed by incubation with the VECTASTAIN Elite ABC kit (Vector Laboratories) and 0.4 % DAB/0.01 % H_2O_2 for 1 h. Pictures were acquired using a Leica DM1000 LED microscope (Leica, Germany) equipped with a 10x objective. Pictures of red fluorescent protein in AgRP neurons were acquired using a confocal Leica TCS SP-8-X microscope (Leica, Germany) equipped with a 20x objective. For each animal, neurons were manually counted to obtain the total $POMC^{eGFP}$ and/or $AgRP^{tdTomato}$ neuronal number.

2.3.1.2 α-MSH and AgRP immunostaining

8- and 20-week-old male mice were anesthetized and transcardially perfused with phosphate buffered saline (PBS), pH 7.5, followed by 4 % paraformaldehyde (PFA) in borate buffer, pH 9.5 and post-fixed for 4 h in 20 % sucrose 4 % PFA in borate buffer and transferred to 20 % sucrose in PBS overnight. Brains were frozen, sectioned at 25 µm and processed for immunofluorescence as previously described (Bouret et al., 2004b; Bouret et al., 2008; Steculorum and Bouret, 2011). Briefly, sections were incubated in goat anti-AgRP (1:4000; Phoenix Pharmaceuticals) or sheep anti-α-MSH (1:20000, Millipore). Secondary Alexa Fluor 488 goat antirabbit IgG or Alexa Fluor 568 donkey anti-sheep IgG (1:200, Life Technologies™) were used to detect primary antibodies. Sections were mounted in DAPI-containing Vectashield (Vector Laboraties Inc.). Sections were only used for quantification if

the region could be unequivocally identified. Pictures were acquired using a confocal Leica TCS SP-8-X microscope equipped with a 20x objective (Leica, Germany). Image analysis was performed in a blind manner using ImageJ software (ImageJ1.45s; National Institutes of Health, USA), as previously described (Bouret et al., 2004b; Steculorum and Bouret, 2011). Briefly, the integrated density was calculated for each image, a figure that reflects the total number of pixels in a given region of the image and that is proportional to the total density of labeled fibers in this region (for a step-by-step illustration of the quantification process, please see Figure 2.3.1). If there were more than one section per animal in the correct region, the average integrated intensity was used to calculate the ratio to the average integrated density of control animals (i.e. NCD/NCD ctrl), which was used for further statistical analyses.

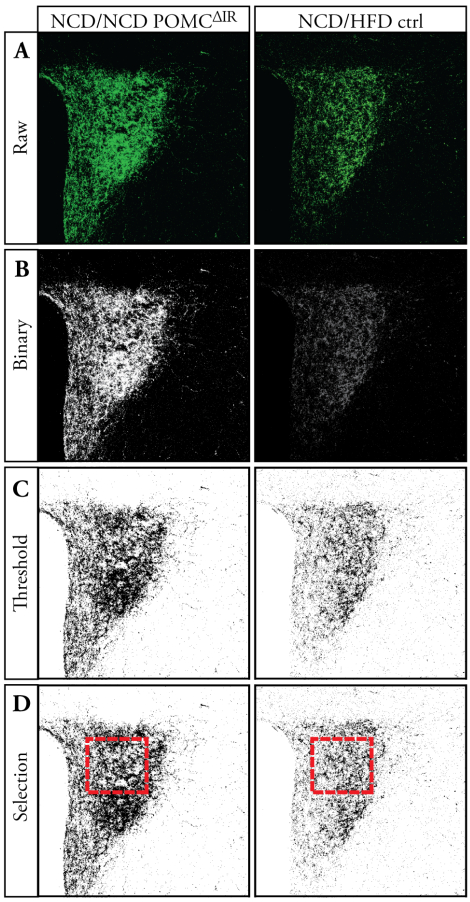


Figure 2.3.1 Representative step-by-step quantification of α-MSH and AgRP immunostaining
Pictures were quantified using the Image J software. A) Raw pictures were imported and B) transformed into 16-bit images and subsequently binarized. C) The thresholds of each image were adjusted to highlight specific staining. D) The integrated density from each picture was determined from a region of interest that represented the respective brain region of interest (here the anterior paraventricular nucleus of the hypothalamus). Agouti-related peptide (AgRP) immunostaining in green. The red box indicates area of quantification.

2.3.1.3 Insulin and vAChT fluorescent double-immunostaining

Frozen pancreata were sectioned at 10 µm at three different levels, collected on microscope slides and frozen at -20°C until further use. Microscope slides were warmed up to room temperature (RT) for 20 mins and fixed with 4 % PFA in PBS, pH 7.5 for 10 minutes. The slides were then rinsed in PBS and subjected to antigen retrieval. For this, 500 ml 1x Citrate Buffer (10 mM Citric Acid in ddH_2O, pH=6.0) were preheated in Tupperware (without lid and without slides) for 1 min at 900 W in a microwave. Subsequently, the slides were placed in a glass holder and put into the preheated solution (slides must be well covered with liquid) and heated for 5 mins at 900 W, 5 mins at 600 W and 10 mins at 360 W without lid. Slides cooled down slowly in the solution for 30 mins at RT and were subsequently washed twice in PBS for 5mins each. Pancreatic sections were then blocked in PBS containing 3% donkey serum and 0.3 % Triton X (Sigma) for 1 h at RT and subsequently incubated in goat anti-vACht (1:100, Millipore) and guinea-pig anti-Insulin (1:500, Dako) in PBS containing 3 % donkey serum and 0.3 % Triton X overnight at RT. Primary antibodies were detected with Alexa Fluor488 donkey anti-goat IgG (1:500, Life Technologies™, USA) and Cy3 donkey anti-guinea-pig (1:500, Jackson ImmunoResearch Laboratories, Inc., USA) for 1 h at RT. 10-15 islets per animal were acquired using a confocal Leica TCS SP-8-X microscope equipped with a 40x objective. Image analysis was performed using ImageJ software (ImageJ1.45s; National Institutes of Health, USA). Briefly, each vAChT-immunoreactive button that could clearly be distinguished from background signal was counted and set in relation to the insulin-immunoreactive islet size. The average relative number of vAChT-immunoreactive buttons of all islets from one animal was used to calculate its ratio to the average number of the relative vAChT-immunoreactive buttons of ctrl animals (i.e. NCD/NCD ctrl), which was used for further statistical analyses.

2.3.1.4 Analysis of pancreatic β-cell mass

Pancreatic sections were fixed with acetone for 10 minutes at 4°C. Subsequently, sections were first blocked in PBS containing 1 % BSA and 0.3 % H_2O_2 and, after washing 3x with PBS, blocked in Rotiblock (1:10 with PBS, Roth®, Germany) containing 0.1 % Triton X. Incubation with guinea pig anti-insulin (1:50, DAKO, Germany) for 60 minutes was followed by 3x washing with PBS and 60 minutes incubation with secondary horse-radish

peroxidase (HRP)-coupled anti-guinea-pig (HRP; 1:500, Jackson ImmunoResearch Laboratories, Inc., USA) in PBS containing 0.25 % Triton X. HRP was detected using liquid diaminobenzidine + chromogene Substrate (1:50, Dako) for 20 minutes. Sections were counter-stained with Mayer's Haematoxylin for 3 minutes. Pictures were acquired using the LeicaSCN400 slidescanner for brightfield images equipped with a 20x objective (Leica, Germany). For quantification, the size of the each islet per pancreatic section was determined using Image J (ImageJ1.45s; National Institute of Health, USA) and the average islet size, the total number of islets and β-cell mass in relation to overall size of the pancreatic section was calculated.

2.3.2 Electrophysiology

All electrophysiological experiments were performed by Dr. Lars Paeger, Dr. Simon Hess and Dr. Andreas Pippow from the laboratory of Prof. Dr. Kloppenburg from the Biocenter, Institute for Zoology, University of Cologne, Germany. POMC neurons were recorded from NCD/NCD and NCD/HFD $POMC^{eGFP}$ transgenic mice (Cowley et al., 2001) at room temperature (RT) under current- and voltage-clamp in the perforated patch and whole-cell patch-clamp configuration using an EPC10 patch-clamp amplifier (HEKA). The animals were anesthetized with halothane (B4388; Sigma-Aldrich, Germany) and decapitated. Coronal slices (250 – 300 µm) containing the arcuate nucleus (ARH) were cut with a vibration microtome (HM-650 V; Thermo Scientific, USA) under cold (4°C), carbogenated (95% O_2 and 5% CO_2), glycerol-based modified artificial cerebrospinal fluid (GaCSF; (Ye et al., 2006)), which contained (in mM): 250 Glycerol, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 10 HEPES, 21 $NaHCO_3$, and 5 Glucose adjusted to pH 7.2 with NaOH. If not mentioned otherwise, the brain slices were continuously superfused with carbogenated artificial cerebrospinal fluid (aCSF) at a flow rate of ~2 ml·min-1, which contained (in mM): 125 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 21 $NaHCO_3$, 10 HEPES, and 5 Glucose adjusted to pH 7.2 with NaOH.

Current-clamp recordings in the perforated patch configuration were performed using protocols modified from Horn and Marty (Horn and Marty, 1988), Rae et al. (Rae et al., 1991) and Akaike and Harata (Akaike and Harata, 1994). The recordings were performed with ATP and GTP free pipette solution containing (in mM): 128 K-gluconate, 10 KCl, 10

HEPES, 0.1 EGTA, 2 $MgCl_2$ and adjusted to pH 7.3 with KOH. The patch pipette was tip filled with internal solution and back filled with tetraethylrhodamine-dextran (D3308; Invitrogen, Eugene, OR, USA) and amphotericin B- (~200 $\mu g \cdot ml^{-1}$; A4888; Sigma) or nystatin-containing (~200 $\mu g \cdot ml^{-1}$; N6261; Sigma) internal solution to achieve perforated patch recordings. For the mean firing rate of POMC neurons, the measured frequency was averaged as soon as it was stable for ~5 mins after perforation reached stable series resistances. Cell input resistance and whole-cell conductance were calculated from voltage responses to hyperpolarizing current pulses.

Whole-cell recordings were performed following the methods of Hamill et al. (Hamill et al., 1981). For measurements of postsynaptic currents (PSCs), the patch pipette solution contained (in mM): 140 KCl, 10 HEPES, 0.1 EGTA, 5 $MgCl_2$, 5 K-ATP, 0.3 Na-GTP; adjusted to pH 7.3 with KOH). Cells were voltage clamped at -60 mV and the high intracellular chloride concentration shifted the chloride equilibrium potential to a more depolarized potential, which reversed the polarity of $GABA_A$ receptor mediated currents from outward to inward, and made their detection easier by increasing the driving force on the chloride ions. The contribution of excitatory (EPSCs) and inhibitory PSCs (IPSCs) to the synaptic input was determined in three steps. First, we measured the overall frequency of PSCs. Second, we blocked the glutamatergic EPSCs with 5 x 10^{-5} D-AP5 and 10^{-5} M CNQX to isolate the IPSCs, which were identified as GABAergic (inhibitory) PSCs by their sensitivity to 10^{-4} M PTX. The EPSC frequency was determined by subtracting the IPSC frequency from the overall frequency. The overall PSC frequency was determined after the recording had stabilized (>10 minutes after break in) for a 2 minutes interval. The IPSC frequency was measured after 10 – 15 minutes D-AP5/CNQX application for a 2 minutes interval. Ratios of IPSCs are relative to overall PSCs.

2.4 Biochemistry

2.4.1 Enzyme-linked immunosorbent assays (ELISA)

Serum insulin, leptin, C-peptide and GLP-1 levels were measured by enzyme-linked immunosorbent assays according to the manufacturer's instructions (Mouse/Rat Insulin

ELISA, Mouse Leptin ELISA, Crystal Chem Inc., USA; Mouse C-peptide (Mouse) ELISA Kit, ALPCO, USA; GLP-1 ELISA kit, MyBioSource, USA).

2.4.2 MALDI-TOF mass spectrometry

The ARH were carefully microdissected under visual guidance using a binocular microscope, then rinsed in distilled water for a few seconds and transferred with a glass capillary into 20 µl methanol/water (1/1) in 1 % formic acid. All following steps were performed by Dr. Susanne Neupert from the laboratory of Prof. Dr. Predel from the Biocenter, Institute for Zoology, University of Cologne, Germany. Dissected tissues were homogenized, sonicated and subsequently centrifuged for 15 min at 13.000 U/min. All steps were performed at 4°C; 0.3µl of the supernatant was pipetted on a stainless steel sample plate for MALDI-TOF mass spectrometry. Remaining supernatants were each filled with 40 µl 0.5 % acetic acid and then evaporated to about 10 µl to reduce the methanol concentration. Samples were refilled, again with 40 µl 0.5 % acetic acid, and loaded onto activated and equilibrated home-made Stage Tips (Rappsilber et al., 2007) filled with C18 discs from Empore™ filter material (Bioanalytical Technologies). After rinsing with 20 µl 0.5 % acetic acid, peptides were eluted using 4 µl of 10/20/25/30/40/50 % acetonitrile in 0.5 % acetic acid, respectively. Each eluate was divided in six portions and applied onto the sample plate for MALDI-TOF mass spectrometry. Dried samples were covered with a mixture of 0.5 µl α-cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich) dissolved in 50% methanol/water. After drying at room temperature, each spot was finally rinsed with water for a few seconds. Mass spectra were manually acquired in positive ion and reflectron mode on an ultrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Germany). Instrument calibration was performed using a peptide standard kit (Bruker, Germany) and, before sample analysis, laser power was adjusted to provide optimal signal-to-noise ratio. For mass fingerprints, brain samples were analyzed with a laser frequency of 1 kHz (3000 laser shots; m/z 600-4000). Ion signals mass-identical with products of the POMC-precursor were selected and fragmented by tandem mass spectrometry (LIFT technology, with acceleration set at 1 kV) using the same samples. Mass spectra obtained in these experiments were processed with the flexAnalysis 3.4 software (Bruker); deduced sequences were counterchecked using the ProteinProspector (http://prospector.ucsf.edu). ClinPro Tools

software 3.0 (Bruker, Germany) was used for comparison of signal intensities of *Pomc*-products; the results are presented as gel view in logarithmic gray scale display mode.

2.5 Mouse Experiments

2.5.1 Animal care

All animal procedures were conducted in compliance with protocols approved by local government authorities (Bezirksregierung Köln) and were in accordance with National Institutes of Health guidelines. All animals were housed in 3-4 animals per cage at 22-24°C on a 12 h light/dark cycle with lights on at 6 am. Unless otherwise stated, animals were allowed *ad libitum* access to either normal chow diet (#T.2018.R12, Harlan Teklad Global Rodent) containing 53.5 % carbohydrates, 18.5 % protein and 5.5 % fat (12 % fat calories from fat) or high-fat diet (#C1057, Altromin) containing 32.7 % carbohydrates, 20% protein and 35.5 % fat (55.2 % calories from fat) and drinking water.

2.5.2 Generation of POMC^{eGFP}, POMC$^{\Delta IR}$ and AgRP^{tdTomato} mice

Mice expressing the POMC^{eGFP} transgene were generated by crossing C57Bl/6 female to transgenic POMC^{eGFP} male mice (Cowley, 2001). To generate POMC$^{\Delta IR}$ mice, IR$^{fl/fl}$ female virgin mice were mated with POMCCre$^{pos/neg}$, IR$^{fl/fl}$ male mice (Balthasar et al., 2004; Bruning et al., 1998; Konner et al., 2007). AgRPCre^{tdTomato} mice were generated by crossing female ROSA-Red-Tomato$^{fl/fl}$ (B6;129S6-*Gt(ROSA)26Sortm9(CAG-tdTomato)Hze*/J); purchased from The Jackson Laboratories, USA) to male AgRPCre$^{pos/neg}$ mice (Tong et al., 2008).

2.5.3 Analysis of body composition

Nuclear magnetic resonance was employed to determine whole-body composition of 20 week-old live animals using the NMR Analyzer minispeq mq7.5 (Bruker Optik, Germany).

2.5.4 Serum analyses

Blood glucose levels were determined from whole venous tail blood using an automatic glucose monitor (GlucoMen Glycó, A.Menarini Diagnostics). To obtain serum, blood samples were stored on ice, centrifuged at 13.000 rpm at 4°C for 15 mins. Serum samples were stored at -20°C until further use and only thawn once for subsequent analyses. Serum insulin, leptin, C-peptide and GLP-1 levels were measured by enzyme-linked immunosorbent assays according to the manufacturer's instructions (Mouse/Rat Insulin ELISA, Mouse Leptin ELISA, Crystal Chem Inc., USA; Mouse C-peptide (Mouse) ELISA Kit, ALPCO, USA; GLP-1 ELISA kit, MyBioSource, USA). Serum non-esterified free fatty acids were quantified according to the manufacturer's instructions (WAKO Chemicals GmbH, Germany).

2.5.5 Determination of HOMA-IR

The HOMA-IR was calculated as follows: fasting serum insulin concentration (µU/ml) multiplied by fasting blood glucose levels (mg/dl) divided by 405 (Matthews, 1985).

2.5.6 Analysis of maternal milk composition

At P19, lactating mothers were separated from their pups for 16 h overnight prior to milk collection. Milk was manually collected from deeply anesthetized females for a total of 5 minutes and stored at -20°C until further use. For determination of milk glucose content, milk was diluted 1:2 (v/v) in H_2O and measured using the B-Glucose Analyzer (Hemocue, Germany). For all subsequent analyses, milk was diluted 1:10 (v/v) in H_2O. 2.5 µl of diluted milk was used to determine insulin and leptin levels by ELISA assays according to the manufacturer's instruction for serum analyses (Mouse/Rat Insulin ELISA, Mouse Leptin ELISA, Crystal Chem, USA). Non-esterified free fatty acids (NEFA) were determined from 3 µl of the diluted milk according to the manufacturer's instructions for serum analysis (WAKO Chemicals GmbH, Germany). All samples were determined in duplicates.

2.5.7 Glucose- and insulin tolerance tests

Insulin-tolerance tests were performed with 14-week-old animals fed *ad libitum*. After measuring basal blood glucose levels, each animal was intraperitoneally (i.p.) injected with 0.75 U kg^{-1} body weight of insulin (Actrapid; Novo Nordisk A/S). Blood glucose levels were recorded 15, 30 and 60 minutes after injection. Glucose-tolerance tests were carried out on 15-week-old mice after a 16 h overnight fast. After determination of fasted blood glucose levels, animals were injected i.p. with a bolus of 2 mg mg^{-1} body weight of glucose (20% glucose, Delta Select) and blood glucose levels were monitored 15, 30, 60 and 120 minutes after injection.

2.5.8 Determination of glucose-stimulated insulin, C-peptide, GLP-1 and FFA secretion

After a 16 h overnight fast, approximately 30 µl of blood was collected from all animals. Subsequently, all animals were intravenously (i.v.) injected with 1 mg g^{-1} body weight of glucose (20 % glucose, Delta Select, Germany) and blood samples were collected 2, 5, 15 and 30 mins after injection. For the analysis of glucose-stimulated C-peptide, GLP-1 and FFA levels, blood samples were collected only before and 5 mins after i.v. glucose injection. Samples used for determination of GLP-1 concentration were immediately treated with a soluble dipeptidyl peptidase 4 (DPP4) inhibitor (Merck Millipore, Germany).

2.5.9 Determination of L-arginine-stimulated insulin secretion

After a 16 h overnight fast, animals were i.p. injected with 3 mg g^{-1} body weight of L-arginine dissolved in 0.9 % NaCl solution. Blood samples were collected 0, 2, 5, 15 and 30 minutes after injection. Insulin was determined from serum using Mouse Insulin ELISA (Mouse/Rat Insulin ELISA, Crystal Chem, USA).

2.5.10 Maternal diet manipulation and analytical time-points of their offspring

3 week-old female C57Bl/6 mice were either fed a NCD or a HFD up to the age of 11 weeks. At the age of 10 weeks, blood glucose levels were determined and whole venous tail

blood was collected, after a 16 h overnight fast, to determine fasted serum insulin. Breedings with male C57Bl/6 mice were set up at 11 weeks of age. NCD-fed females were only used for breedings if their HOMA-IR was below or equal to the mean of all NCD-fed females and HFD-fed females were only used for breedings if their HOMA-IR was above this threshold. During gestation, mice were maintained on the same diet they had received before. During lactation, half of the mothers were kept on the same diet they had received pregestationally and the other half received the other respective diet. At the day of weaning, whole venous tail blood was collected both from the mothers, as well as from their offspring. Blood samples were collected from the offspring in the same manner at 8 weeks of age, and, after a 16 h fast, at 15 weeks of age before being subjected to a GTT. Body weight of the offspring was measured in the second and third week of postnatal life, as well as after week 8 on a weekly basis. Offspring for phenotypical analyses were sacrificed in a random-fed state at 20 weeks of age. $IR^{fl/fl}$ mothers and their offspring were treated the same way except that they were only fed a NCD prior to and during gestation. Every experimental group consists of offspring from at least three independent litters.

2.6 Computer analyses

2.6.1 Software

All figures and statistical analyses were conducted using Graphpad Prism 5.0. Illustrations were drawn in Adobe Illustrator CS4. This thesis was written in Micorosoft® Word 2011 for Mac.

2.6.2 Statistical analyses

Data sets with only two independent groups were analyzed for statistical significance using unpaired two-tailed Student's t-test. Data sets with more than two groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's posthoc test. For statistical analyses of body weight gain, the area-under-the-curve (AUC) from week 2 to week 20 was calculated for each mouse and used for further statistical analyses. For statistical analyses of GTTs and ITTs, we performed two-way ANOVAs followed by Bonferroni's

posthoc test. Statistically significant outliers were calculated using Grubb's test for outliers. All p-values below 0.05 were considered significant. All displayed values are presented as mean ± SEM; *p < 0.05. **p < 0.01. ***p < 0.001 versus all other groups within the same diet after week 8, unless otherwise indicated.

3 Results

3.1 Establishing a mouse model to define the critical timing of maternal high-fat feeding on metabolic programming in the offspring

To determine the most vulnerable time frame of hypothalamic neurocircuit development to hormonal insults in response to maternal high-fat diet (HFD)-feeding, we sought to establish a model of metabolic programming, which would allow us to independently and synergistically manipulate these distinct time-periods and analyze their respective effects on the metabolic phenotype in the offspring.

Female C57Bl/6 virgin mice were either fed a normal chow diet (NCD) or a HFD for 8 weeks prior to gestation. During gestation, the mice were maintained on the same diet that they received during the pre-gestational period. Importantly, at the day of birth (DOB), litter size was adapted to 6-7 pups per mother to assure the same quantitative nutritional availability for each litter, since it had previously been demonstrated that litter size reduction increases the propensity to develop metabolic diseases in the offspring during adulthood (Habbout et al., 2013). Additionally, at DOB, half of the NCD-fed mothers were exposed to a HFD (i.e. NCD/NCD and NCD/HFD), and vice versa, half of the mothers fed a HFD pre-gestationally and during gestation were exposed to a NCD during the period of lactation (i.e. HFD/NCD and HFD/HFD). After weaning, all offspring were fed a NCD until the age of 8 weeks, after which each group of offspring was again divided into a group that was exposed to either a NCD or a HFD for the following 12 weeks up to the age of 20 weeks. This strategy allowed us to directly assess whether an altered maternal diet modulates the propensity of the offspring to respond to exposure to HFD later in life. This experimental outline resulted in a total of 8 different groups of offspring that differed in the prenatal maternal diet, the postnatal maternal diet and in the diet of the offspring after 8 weeks of age (Figure 3.1.1).

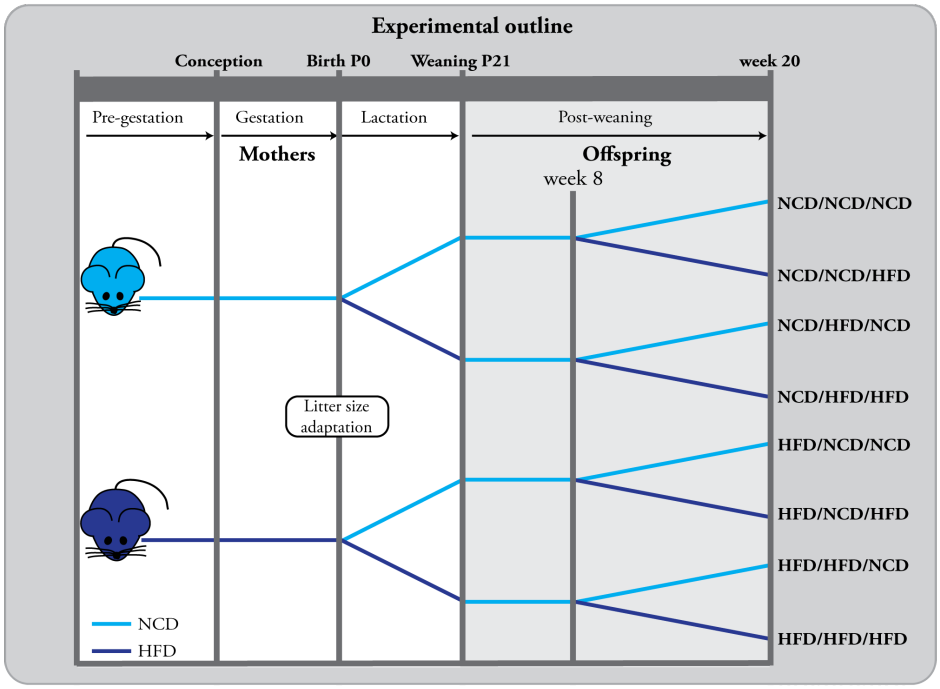


Figure 3.1.1: Experimental outline to determine the critical timing of maternal HFD-feeding on metabolic programming in the offspring
Female C57Bl/6 virgin mice were fed with either a normal chow diet (NCD) or a high-fat diet (HFD) from 4 to 11 weeks of age. During gestation, mice were maintained on the same diet that they had received before. On the day of birth (DOB) of the offspring, litter size was reduced to 6-7 animals per mother and half of the NCD-fed mothers were exposed to a HFD, while half of the HFD-fed mothers were exposed to a NCD during lactation. After weaning, all offspring was exposed to a NCD until 8 weeks of age, after which each group of offspring was divided into groups receiving either a NCD or a HFD until 20 weeks of age. Light blue lines indicate NCD-feeding and dark blue lines indicate HFD-feeding.

Pregestational HFD-feeding for the period of 7 weeks resulted in a moderate, but significantly increased body weight (Figure 3.1.2 A), as well as elevated fasting blood glucose concentrations (Figure 3.1.2 B) and an approximately 7-fold-increase in the homeostatic model assessment indices of insulin resistance (HOMA-IR) (Figure 3.1.2 C) in HFD-fed females at 10 weeks of age, indicating that pre-gestational HFD-feeding causes insulin resistance. Interestingly, HFD-exposure during lactation, independent of the prenatal maternal diet, resulted in a slight elevation of serum insulin concentrations in the mothers (Figure 3.1.2 D), but most importantly, in a significant increase in serum insulin levels in the offspring at 3 weeks of age (Figure 3.1.2 E). Together, these data demonstrate that maternal diet changes after parturition affect the metabolic phenotype of the mother, which results in significant changes in the offspring's insulin levels. Thus, altering maternal diet

during gestation and lactation is a valid model to study the independent and synergistic effects of maternal HFD-feeding during distinct periods of hypothalamic neurocircuit formation on the metabolic phenotype in the offspring.

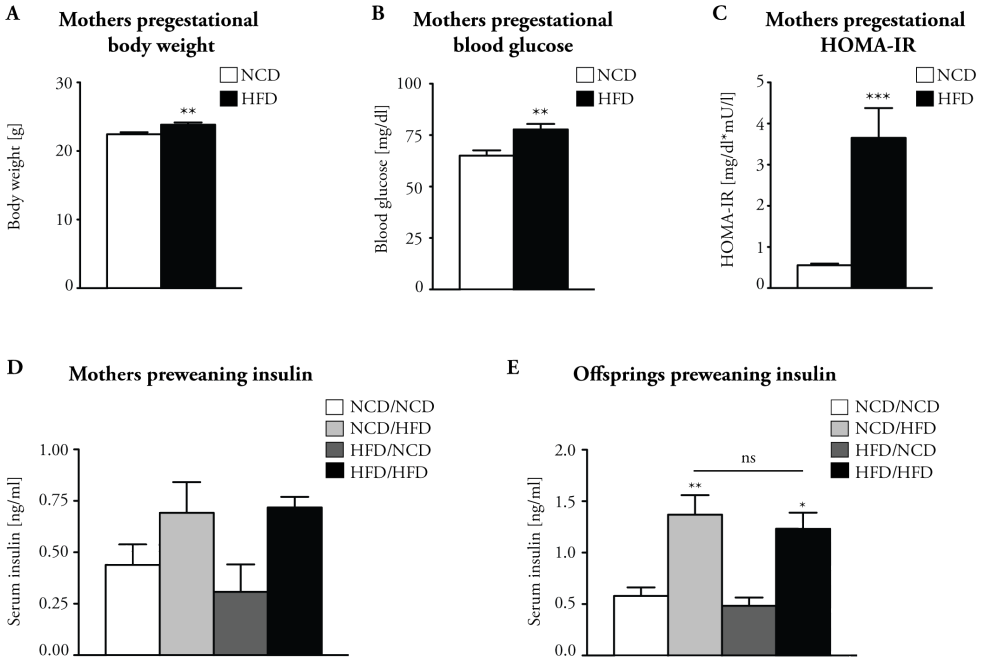


Figure 3.1.2 Maternal HFD-feeding induces pregestational metabolic abnormalities and hyperinsulinemia during lactation in the offspring
Maternal pregestational (A) body weight, (B) fasted blood glucose levels and (C) homeostatic model assessment indices of insulin resistance (HOMA-IR) at 10 weeks of age. Preweaning (A) maternal serum insulin levels in the fed state at the age of 17 weeks. (E) Preweaning serum insulin levels in the offspring in the fed state. NCD, normal chow diet; HFD, high fat diet. Data are presented as mean +/- SEM, **p<0.01, ***p<0.001 versus all other groups.

3.2 Maternal HFD-feeding exclusively during lactation predisposes the offspring for metabolic disorders

We first subjected all groups of offspring to a thorough metabolic characterization. When male offspring were fed a NCD, only mice whose mothers were fed a HFD exclusively

during lactation (NCD/HFD) displayed significantly increased body weight throughout their adult life compared to all other groups (Figure 3.2.1 A). Consistently, NCD/HFD mice showed elevated body fat content (Figure 3.2.1 B), as well as significantly increased perigonadal fat pad weight at 20 weeks of age (Figure 3.2.1 C) compared to NCD/NCD mice on a NCD. This increase in adiposity in NCD/HFD mice exposed to NCD throughout adulthood was further accompanied by significantly elevated serum leptin levels at 15 weeks of age (Figure 3.2.1 D). Moreover, NCD/HFD mice showed enhanced susceptibility to develop insulin resistance (Figure 3.2.1 E) and glucose intolerance (Figure 3.2.1 F) when compared to any other group of offspring. In contrast, male offspring consistently exposed to maternal HFD-feeding (HFD/HFD) only showed elevated body fat content when exposed to a HFD after 8 weeks of age, which however did not exceed the adiposity seen in NCD/HFD mice on a HFD (Figure 3.2.1 B). Surprisingly, male HFD/NCD offspring displayed significantly decreased body weight on a HFD throughout lifetime, suggesting a partial resistance against diet-induced obesity (Figure 3.3.1 A).

Notably, although most metabolic abnormalities were only seen in male NCD/HFD mice when exposed to a NCD, female offspring from NCD/HFD mothers displayed a similar obese phenotype, only when challenged with a HFD after 8 weeks of age (Figure 3.2.2). NCD/HFD female offspring on a HFD displayed significantly increased body weight gain (Figure 3.2.2 A), which was due to increased overall body fat content (Figure 3.2.2.B) that was also reflected in elevated perigonadal fat pad weight (Figure 3.2.2 C), as well as in their serum leptin levels (Figure 3.2.2 D). Moreover, NCD/HFD females showed a tendency towards impaired insulin sensitivity (Figure 3.2.2 E) and significantly impaired glucose tolerance (Figure 3.2.2 F).

Taken together, our model of metabolic programming consistently demonstrates that exposure to a HFD exclusively during the lactation phase in mice exerts the strongest effects on alterations in energy and glucose homeostasis in both male and female offspring.

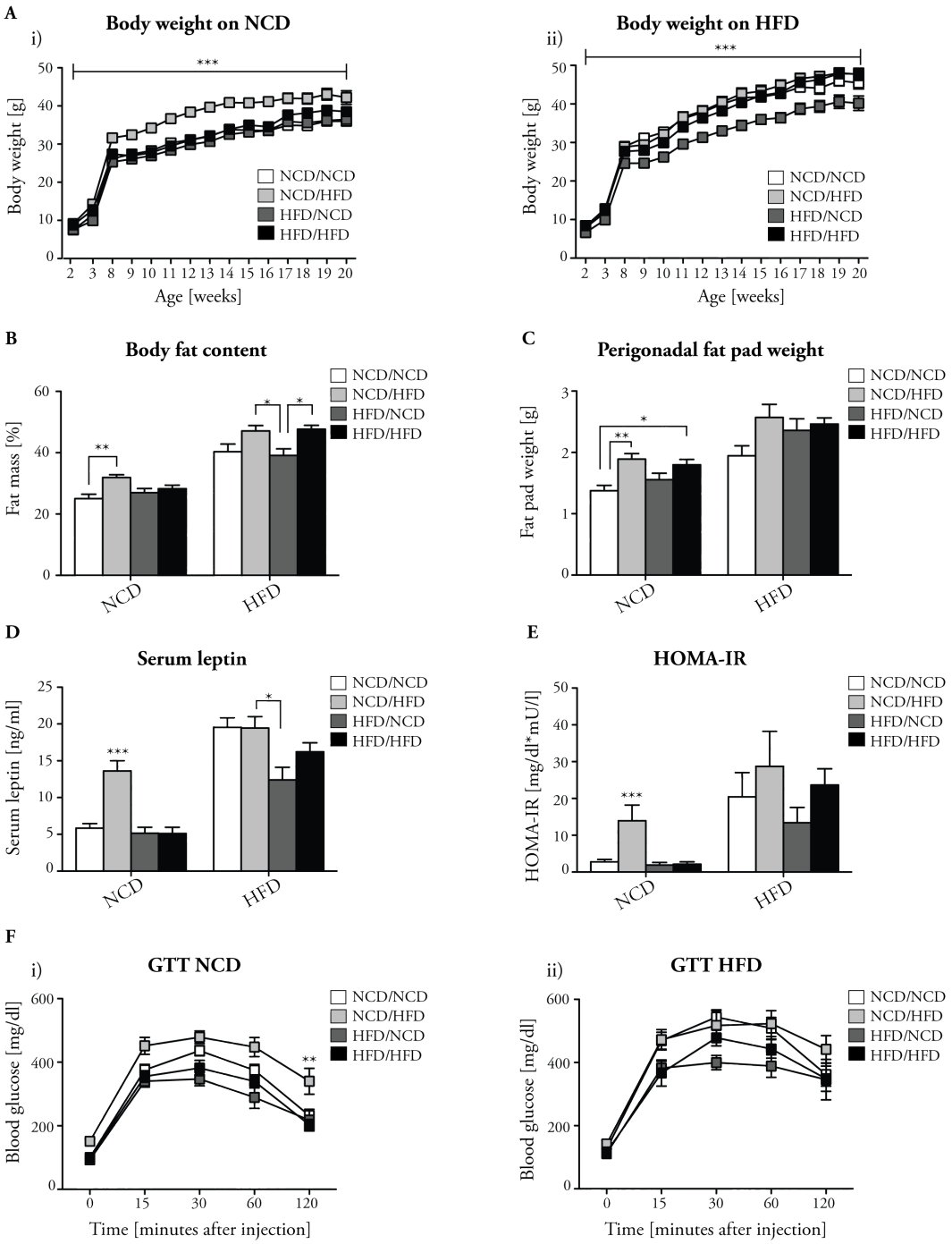


Figure 3.2.1 Maternal HFD-feeding exclusively during lactation predisposes the offspring for metabolic disorders

The following metabolic parameters were analyzed in all eight groups of male offspring. (A) Body weight on (i) normal chow diet (NCD) or (ii) high fat diet (HFD), (B) body fat content and (C) perigonadal fat pad weight at 20 weeks, (D) fasted serum leptin levels and (E) homeostatic model assessment indices of insulin resistance (HOMA-IR) at 15 weeks and (F) glucose tolerance tests (GTT) at 15 weeks of age on i) NCD and ii) HFD. Data are presented as mean +/- SEM, *p<0.05, **p<0.01, ***p<0.001 versus all other groups within the same diet after the age of 8 weeks, if not indicated otherwise.

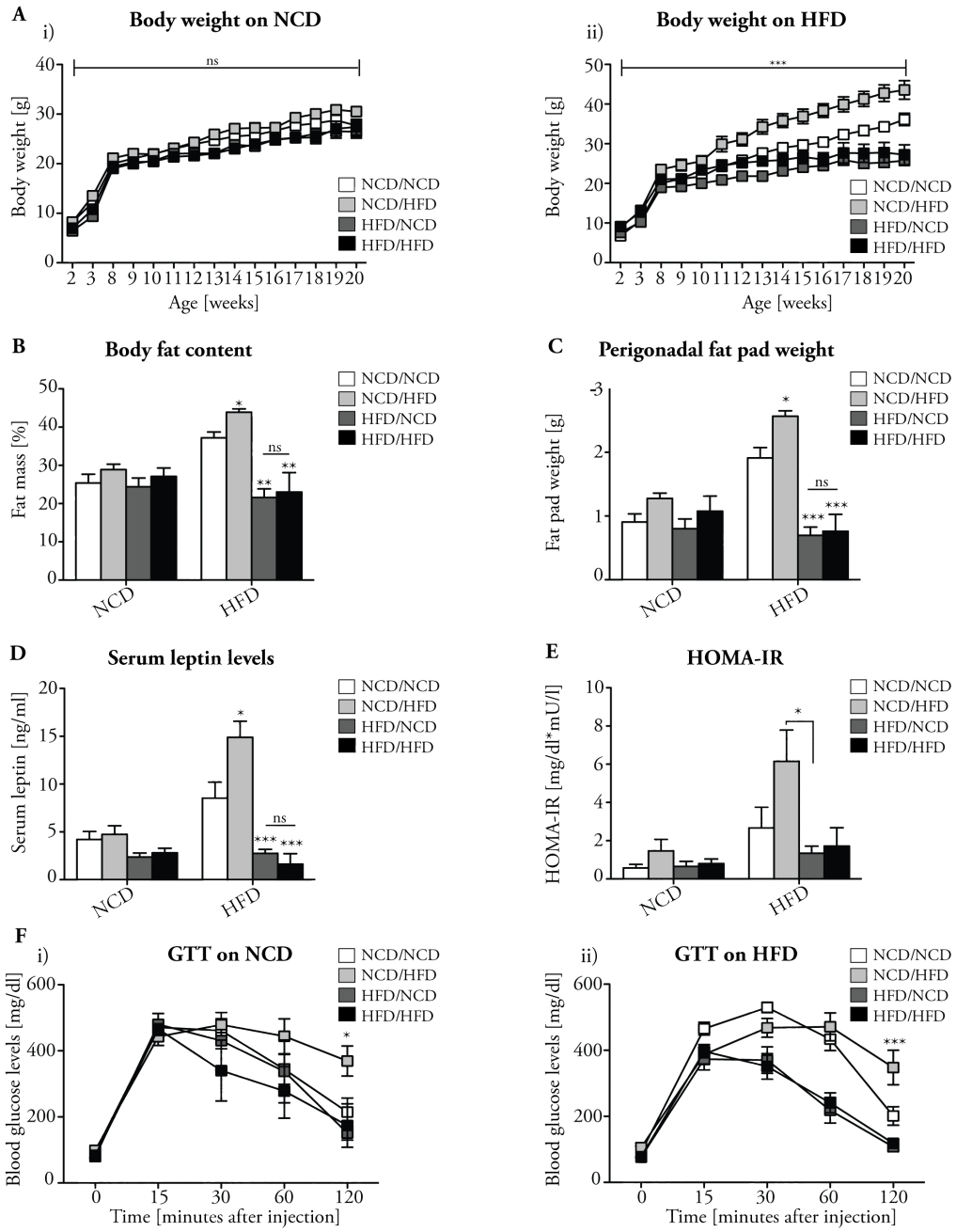


Figure 3.2.2 Maternal HFD-feeding exclusively during lactation predisposes the offspring for metabolic disorders - also in females

The following metabolic parameters were analyzed in all eight groups of female offspring. (A) Body weight on (i) normal chow diet (NCD) or (ii) high fat diet (HFD), (B) body fat content and (C) perigonadal fat pad weight at 20 weeks, (D) fasted serum leptin levels and (E) homeostatic model assessment indices of insulin resistance (HOMA-IR) at 15 weeks and (F) glucose tolerance tests (GTT) at 15 weeks of age on i) NCD and ii) HFD. Data are presented as mean +/- SEM, *p<0.05, **p<0.01, ***p<0.001 versus all other groups within the same diet after the age of 8 weeks, if not indicated otherwise.

3.3 Effects of maternal HFD-feeding during lactation on hypothalamic neurocircuits

Upon identifying the lactation phase as the most critical period where altered maternal diet impairs metabolic homeostasis in the offspring, we focused on comparing NCD/NCD and NCD/HFD male offspring to define the molecular mechanism(s) underlying the obese and glucose intolerant phenotype of NCD/HFD mice. First, we determined mRNA expression of hypothalamic neuropeptides critically involved in the regulation of energy and glucose homeostasis. Surprisingly, although there was no difference in expression of any ARH neuropeptide genes, i.e. *Pomc*, *Agrp* and *Npy* (Figure 3.3.1 A) between NCD/NCD and NCD/HFD-mice, mRNA expression of one of their anorexigenic downstream targets thyrotropin-releasing hormone (*Trh*), which is predominantly but not exclusively expressed in the PVH (Segerson, 1987), was significantly lower in NCD/HFD offspring both at 3 and 20 weeks of age (Figure 3.3.1 B). These experiments indicate that in the absence of alterations of *Pomc* and *Agrp/Npy*-expression in the ARH, expression of one of the melanocortin-effector pathways in the PVH is impaired.

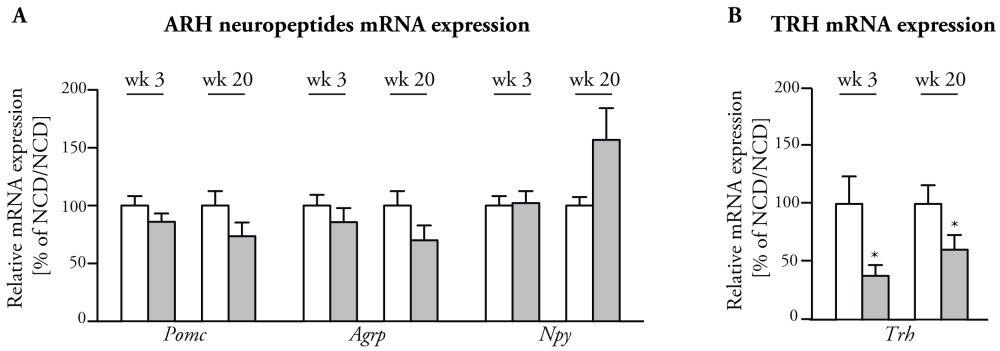


Figure 3.3.1 Hypothalamic neuropeptide expression profile reveals alterations only in ARH downstream neurons
Quantitative real-time PCR analysis of hypothalamic (A) *pro-opiomelanocortin* (*Pomc*), *agouti-related peptide* (*Agrp*) and *neuropeptide Y* (*NPY*) and (B) *thyrotropin-releasing hormone* (*Trh*) mRNA expression in NCD/NCD and NCD/HFD offspring at 3 and 20 weeks of age. NCD, normal chow diet; HFD, high fat diet; Data are presented as mean +/- SEM, *p<0.05.

Thus, we further investigated mechanisms, which could result in an impairment of POMC- and/or AgRP-neuron function as a consequence of maternal HFD-feeding during lactation. To determine whether hypothalamic inflammation in response to postnatal maternal HFD-feeding contributed to the impairment of the melanocortin circuitry, we analyzed mRNA expression of classical inflammatory markers, such as *Tnf*, *Il6* and *Il1b*, in the hypothalamus. However, we could not detect differences in the hypothalamic expression of any of these genes between NCD/NCD and NCD/HFD offspring at 3 and 20 weeks of age (Figure 3.3.2).

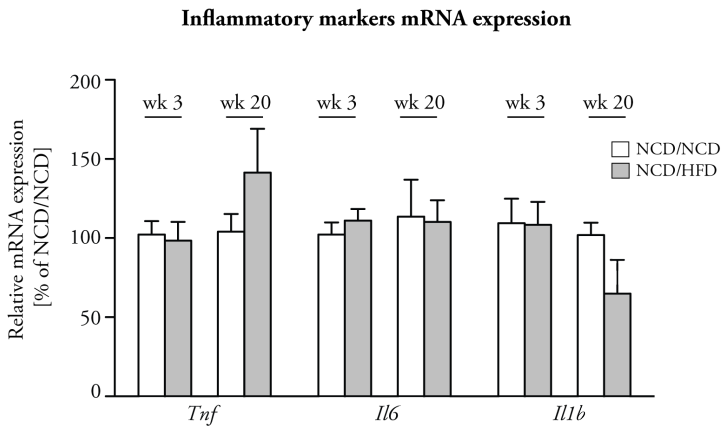


Figure 3.3.2 Maternal HFD-feeding during lactation does not induce hypothalamic inflammation in the offspring
Quantitative real-time PCR analysis of hypothalamic *tumor necrosis factor* (*Tnf*), *interleukin 6* (*Il6*) and *interleukin 1 beta* (*Il1b*) mRNA expression of NCD/NCD and NCD/HFD offspring at 3 and 20 weeks of age on a NCD. NCD, normal chow diet; HFD, high fat diet. Data are presented as mean +/- SEM.

Next, we analyzed the effect of postnatal maternal HFD-feeding on the cell number of ARH neurons by employing our postnatal feeding paradigm to female C57Bl/6 mice that were crossed to male transgenic mice expressing the enhanced green fluorescent protein (eGFP) under the transcriptional control of the POMC promoter (POMC^{eGFP}) (Cowley et al., 2001); and further to females homozygously carrying a floxed Rosa26-tdTomato allele encoding red fluorescent protein (Jackson Laboratories, Bar Har, ME, USA) that were crossed to male mice expressing Cre recombinase under the transcriptional control of the AgRP promoter (Tong et al., 2008) to generate AgRP^{tdTomato} mice. Consistent with unaltered *Pomc*- and *Agrp*/*Npy* expression, there was no difference in the number of eGFP-positive POMC, or tdTomato-positive AgRP neurons between NCD/NCD and NCD/HFD offspring (Figure 3.3.3). Thus, maternal high fat feeding appears neither to affect neurogenesis/survival of ARH neurons, nor mRNA-expression of key neuropeptides in the ARH, which control energy homeostasis.

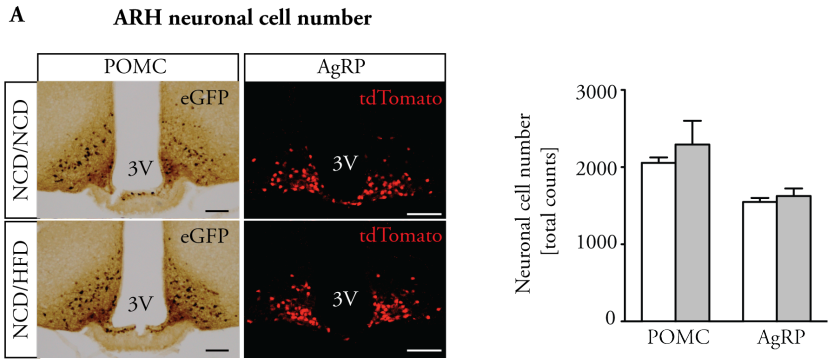


Figure 3.3.3 Maternal HFD-feeding during lactation does not affect ARH neuronal cell number
Analysis of POMC (left) and AgRP (right) neurons in the arcuate nucleus of the hypothalamus (ARH) in POMC^{eGFP} and AgRP^{tdTomato} mice, respectively, at 8 weeks of age (scale bar = 100 µm). NCD, normal chow diet; HFD, high fat diet.

Of note, AgRP can be modulated by, but does not depend on posttranslational modifications to decrease TRH expression in the PVH (Creemers et al., 2006; Fekete et al., 2001). However, POMC has to undergo proprotein-convertase (PC)-1, -2 and carboxypeptidase (CPE)-mediated processing to generate the active neuropeptide alpha-melanocyte-stimulating-hormone (α-MSH), which exerts its anorexigenic functions, in part

via upregulation of TRH (Fekete et al., 2000; Pritchard and White, 2007). Thus, we investigated whether POMC processing might be impaired in NCD/HFD offspring. Hypothalamic mRNA expression of *Pcsk1*, *Pcsk 2* (respectively PC-1 and PC-2) and *Cpe* did not show any differences between groups of offspring (Figure 3.3.4 A). Moreover, MALDI-TOF mass spectrometry of dissected ARH samples showed nearly identical peptide signals, including ions that are mass-identical with products of the POMC precursor protein (i.e. α-MSH, di-acetylated α-MSH and joining peptide) (Pritchard and White, 2007) between NCD/NCD and NCD/HFD offspring (Figure 3.3.4 B). Together, these experiments exclude an impairment of POMC processing to α-MSH as the underlying cause for the obese phenotype in NCD/HFD offspring.

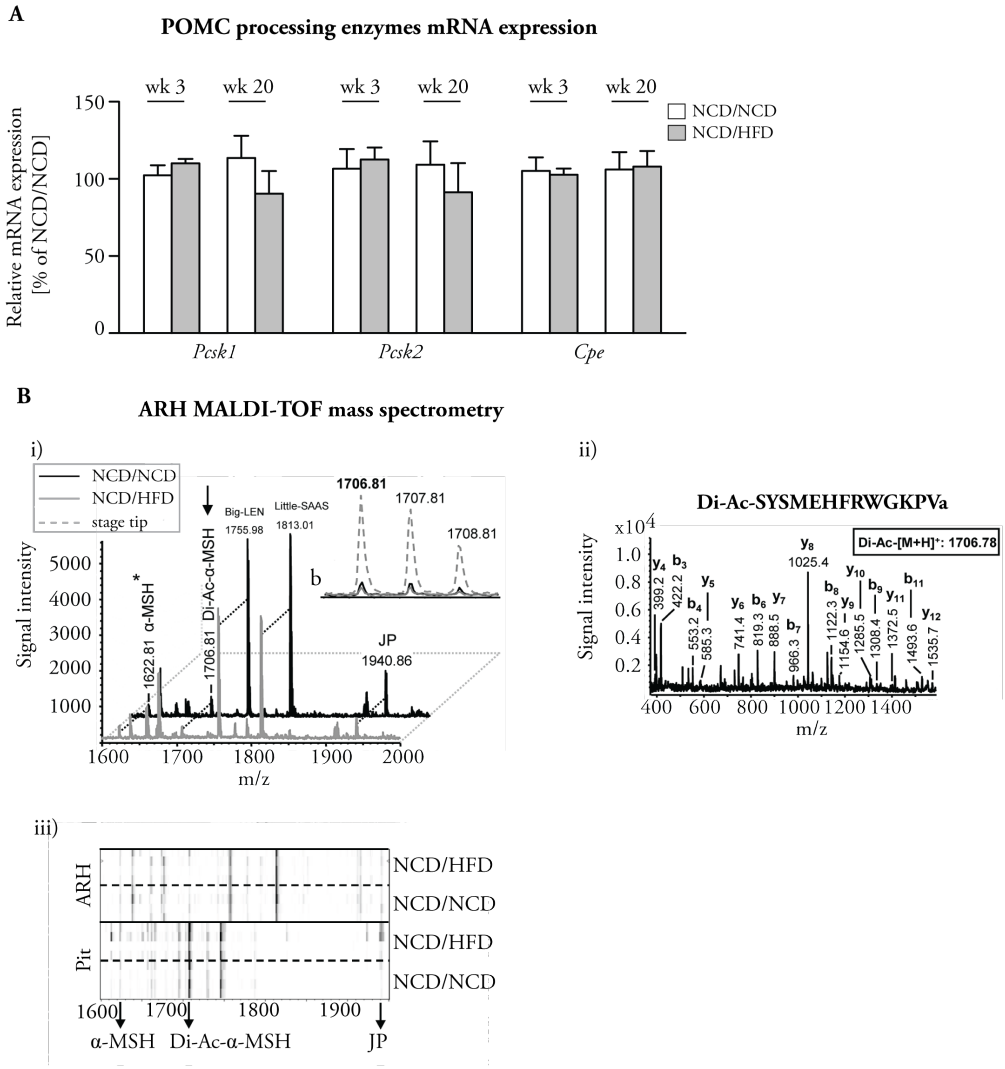


Figure 3.3.4 POMC processing to α-MSH is not impaired in response to postnatal HFD-exposure

(A) Quantitative real-time PCR analysis of hypothalamic *proprotein convertase subtilisin/kexin type 1* (*Pcsk1*), *proprotein convertase subtilisin/kexin type 2* (*Pcsk2*) and *carboxypeptidase E* (*Cpe*) mRNA expression at 3 and 20 weeks of age on NCD. (B) MALDI-TOF mass spectra obtained by profiling extracts of the arcuate nucleus of the hypothalamus (ARH) at 20 weeks of age. Prominent ion signals are labeled. i) Comparison of mass fingerprints of products of the POMC precursor (α-MSH, Di-Ac-MSH, joining peptide (JP)). The ion-signal at 1622.81 (asterisk) is composed of two substances (including α-MSH). The arrow marks processed and biologically more potent di-acetylated α-MSH. b) Isotopic pattern and signal intensity of di-acetylated α-MSH before (lower traces) and after Stage Tip concentration. ii) MALDI-TOF/TOF fragment spectrum of di-acetylated α-MSH purified and concentrated with Stage Tips. Y- and b-type fragment ions are labeled, which confirmed the amino acid sequence of di-acetylated α-MSH. iii) Gel view of mass spectra from preparation of ARH and pituitary gland (Pit) demonstrating identical processing of Pomc-products in all samples. NCD, normal chow diet; HFD, high fat diet. Data are presented as mean +/- SEM.

Next, we analyzed whether maternal HFD-feeding during lactation had an effect on the electrophysiological properties of POMC neurons in the offspring. Whole-cell and perforated patch clamp recordings on POMC^{eGFP} transgenic NCD/NCD and NCD/HFD offspring indicated that maternal HFD-feeding during lactation did not result in any differences in spontaneous firing frequency of POMC-neurons (Figure 3.3.5 A), POMC-neuron resting membrane potential (Figure 3.3.5 B), or in the relative synaptic input onto POMC neurons (Figure 3.3.5 C).

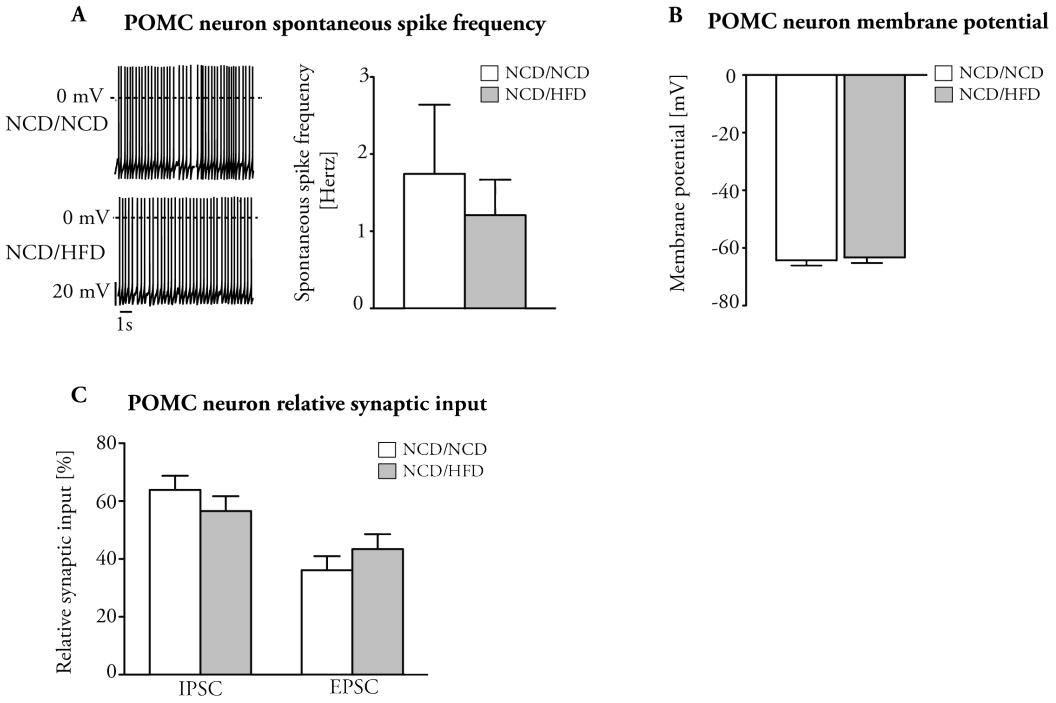


Figure 3.3.5 POMC electrophysiological properties are unaltered in offspring from postnatally HFD-fed mothers

(A) Spontaneous spike frequency and (B) membrane potential of POMC^{eGFP} neurons obtained by perforated patch clamp recordings (n=13 neurons obtained from n=3 mice). (C) Whole-cell recordings showing the relative synaptic input on POMC^{eGFP} neurons (n=13vs14 neurons obtained from n=5vs8 mice). NCD, normal chow diet; HFD, high fat diet. Data are presented as mean +/- SEM.

Collectively, these data demonstrate that maternal HFD-feeding exclusively during lactation permanently decreases anorexigenic TRH expression in the PVH, which is a target of POMC- and AgRP-neurons in the ARH, without altering ARH neuropeptide gene expression, POMC neuronal cell number, neuropeptide processing of POMC to di-acetylated α-MSH and/or electrophysiological properties of POMC neurons.

3.4 Maternal HFD-feeding exclusively during lactation impairs axonal projections of ARH neurons to intra-hypothalamic target sites

Considering that altering maternal diet selectively during lactation, the phase of hypothalamic neurocircuit development in which axonal projections are formed in rodents, had the strongest impact on the metabolic fate of the offspring, we next analyzed the immunoreactivity of α-MSH- and AgRP-containing fibers in three of the main ARH downstream hypothalamic projection areas: the PVH, DMH and LH. Of note, the PVH consists of distinct functional subcompartments that regulate neuroendocrine, behavioral and autonomic responses to control energy and glucose homeostasis (Biag et al., 2012). Neuroendocrine neurons, such as TRH neurons, reside mainly in the anterior two thirds of the PVH (referred to as PVH_{ant}), whereas preautonomic neurons are predominantly found in the posterior part of the PVH (referred to as PVH_{post}) (Biag et al., 2012). Due to this distinct compartmentalization and the associated diverse functions of the PVH, we differentiated both, the PVH_{ant} and the PVH_{post}, in our analysis. Quantification of the fiber density in the PVH_{ant} (Figure 3.4.1 A), the PVH_{post} (Figure 3.4.1 B), the DMH (Figure 3.4.1 C) and the LH (Figure 3.4.1 D) revealed robust reductions in both, the α-MSH and AgRP fiber densities in NCD/HFD offspring compared to NCD/NCD offspring.

Thus, maternal HFD-feeding results in a consistent decrease of ARH neuronal fiber densities in hypothalamic areas critically involved in the neuroendocrine and autonomic regulation of energy homeostasis, likely due to impaired axon formation in the offspring.

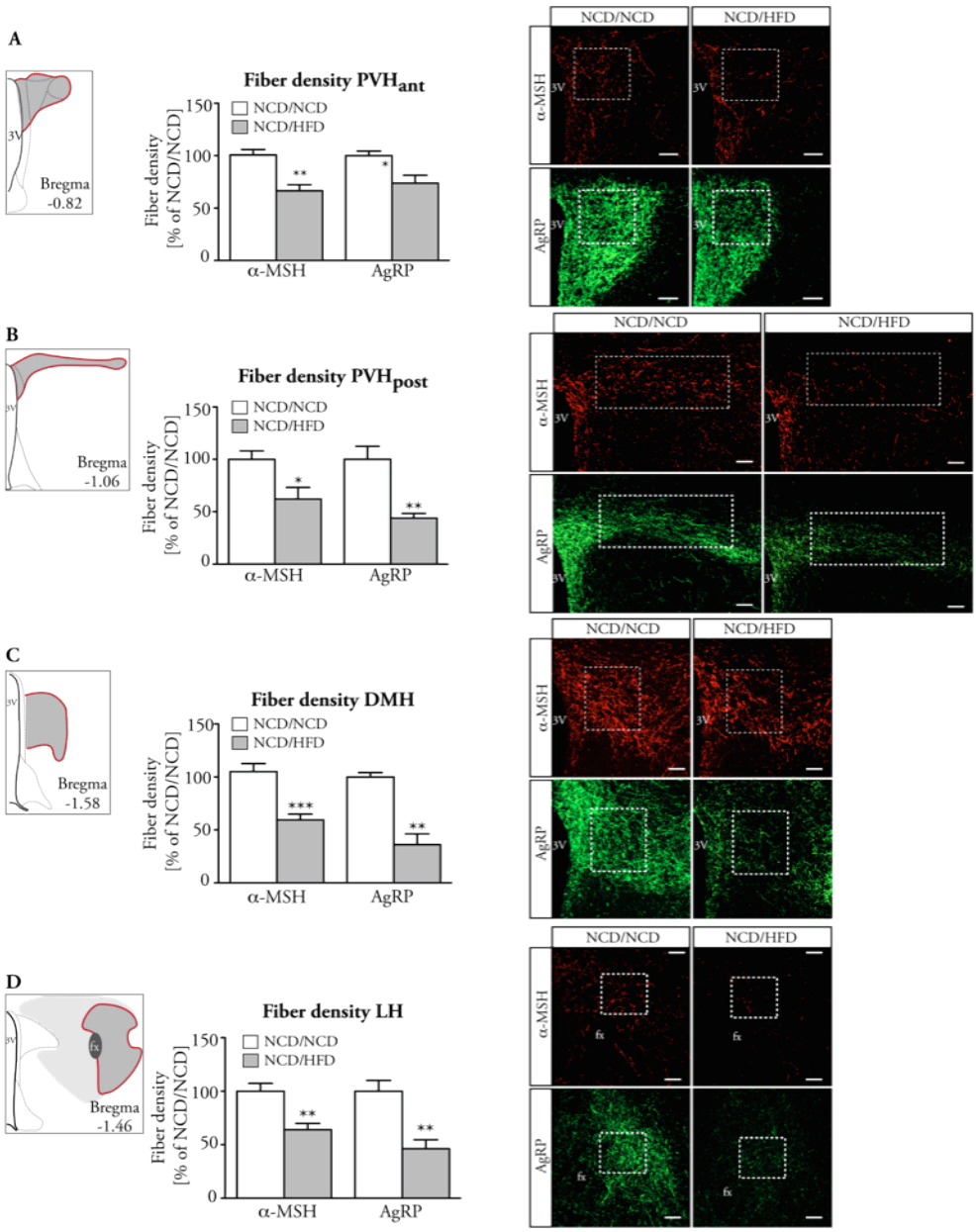


Figure 3.4.1 Maternal HFD-feeding exclusively during lactation impairs axonal projections of ARH neurons to intra-hypothalamic target sites
Images and quantification of α-melanocyte-stimulating hormone (α-MSH) and agouti-related peptide (AgRP) immunoreactive fibers innervating (A) the anterior endocrine paraventricular nucleus of the hypothalamus (PVH_{ant}), (B) the posterior preautonomic PVH_{post}, (C) the dorsomedial nucleus of the hypothalamus (DMH) and (D) the lateral hypothalamic area (LH) at 8 weeks of age. White boxes indicate area of quantification. 3V, third ventricle; fx, fornix; Scale bar = 100 µm. NCD, normal chow diet; HFD, high fat diet. Data are presented as mean +/- SEM, *p<0.05, **p<0.01.

3.5 Deciphering the role of neuronal insulin signaling in the predisposition for metabolic disorders in NCD/HFD offspring

Although maternal HFD-feeding during lactation did not affect body weight (Figure 3.5.1 A), it significantly increased the glucose, insulin, leptin and non-esterified fatty acid content of the milk in NCD/HFD mothers (Figure 3.5.1 B-E), which was associated with distinct hyperinsulinemia in the NCD/HFD offspring at 3 weeks of age (Figure 3.1.2 E). Moreover, given the well-defined axonotrophic role of leptin on ARH neurons during development (Bouret, 2004, Bouyer 2013), we sought to identify the potential contribution of neuronal insulin signaling during the early postnatal phase in impairing melanocortin projections in response to maternal HFD-feeding.

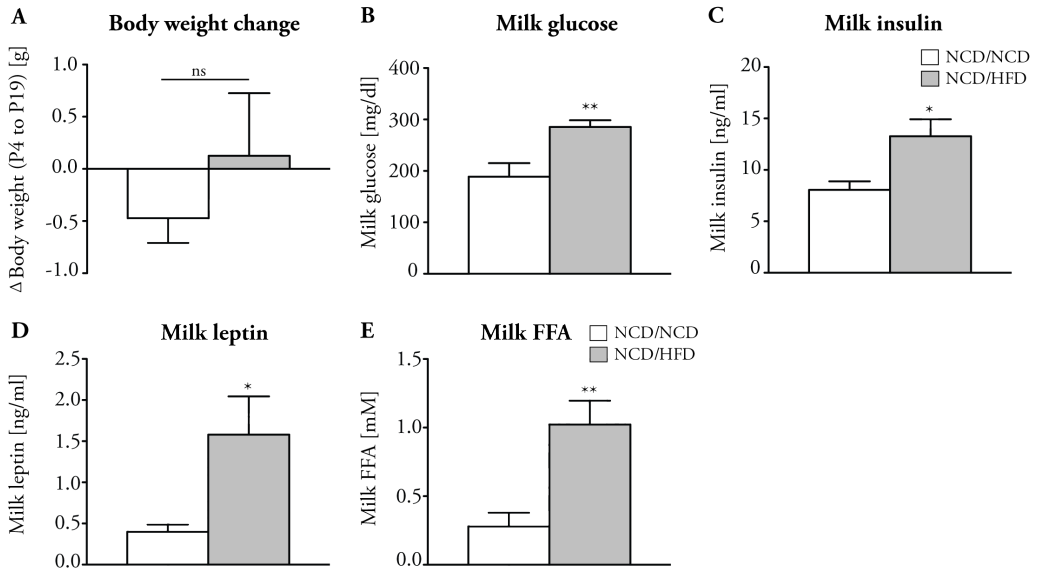


Figure 3.5.1 Milk composition is highly enriched in mothers exposed to HFD during lactation

(A) Maternal body weight changes between postnatal day 4 (P4) and P19, (B) glucose, (C) insulin and (D) leptin levels, as well as (E) non-esterified free fatty acid (FFA) content in the milk at P19 in mothers consistently exposed to a NCD during gestation and lactation (NCD/NCD) and mothers fed a HFD only during lactation (NCD/HFD). NCD, normal chow diet; HFD, high fat diet. Data are presented as mean +/- SEM, p*<0.05, **p>0.01

To this end, we specifically inactivated the insulin receptor (IR) from POMC neurons in NCD/NCD and NCD/HFD offspring by crossing female IR floxed/floxed ($IR^{fl/fl}$) mice with male $IR^{fl/fl}$ mice expressing Cre recombinase under the control of the POMC promoter (Balthasar et al., 2004; Bruning et al., 1998; Konner et al., 2007) and further subjected the mothers to our postnatal feeding paradigm. All groups of offspring were challenged with a HFD after 8 weeks of age, resulting in 4 groups of offspring differing in both, the maternal diet during lactation and in the presence (NCD/NCD ctrl and NCD/HFD ctrl) and absence (NCD/NCD $POMC^{\Delta IR}$ and NCD/HFD $POMC^{\Delta IR}$) of IR-expression on POMC neurons.

Similar to our previous results, maternal HFD-feeding exclusively during lactation did not alter body weight between NCD/NCD ctrl and NCD/HFD ctrl offspring on HFD, and also had no effect on the respective POMC-specific IR-deficient offspring (Figure 3.5.2 A). Despite showing no increases in body weight, NCD/HFD offspring developed greater adiposity independent of their genotype, as revealed by elevated body fat content (Figure 3.5.2 B), increased perigonadal fat pad weight (Figure 3.5.2 C) and elevated serum leptin levels (Figure 3.5.2 D). Similarly, both measures of insulin sensitivity, i.e. the HOMA-IR (Figure 3.5.2 E) and insulin tolerance tests (ITT) (Figure 3.5.2 F) showed tendencies towards an impaired insulin sensitivity in both NCD/HFD ctrl and in NCD/HFD $POMC^{\Delta IR}$ offspring. Thus, POMC-specific inactivation of IR-signaling did not affect adiposity, nor the impaired insulin sensitivity in offspring from mothers fed a HFD exclusively during lactation.

However, when subjected to a glucose tolerance test (GTT), NCD/HFD ctrl mice displayed a pronounced glucose intolerance, which was rescued to NCD/NCD levels in NCD/HFD $POMC^{\Delta IR}$ offspring (Figure 3.5.2 G). Together, these results point to a distinct role for abnormal neuronal insulin signaling in response to maternal postnatal HFD-feeding in predisposing the offspring for an impaired glucose tolerance throughout lifetime.

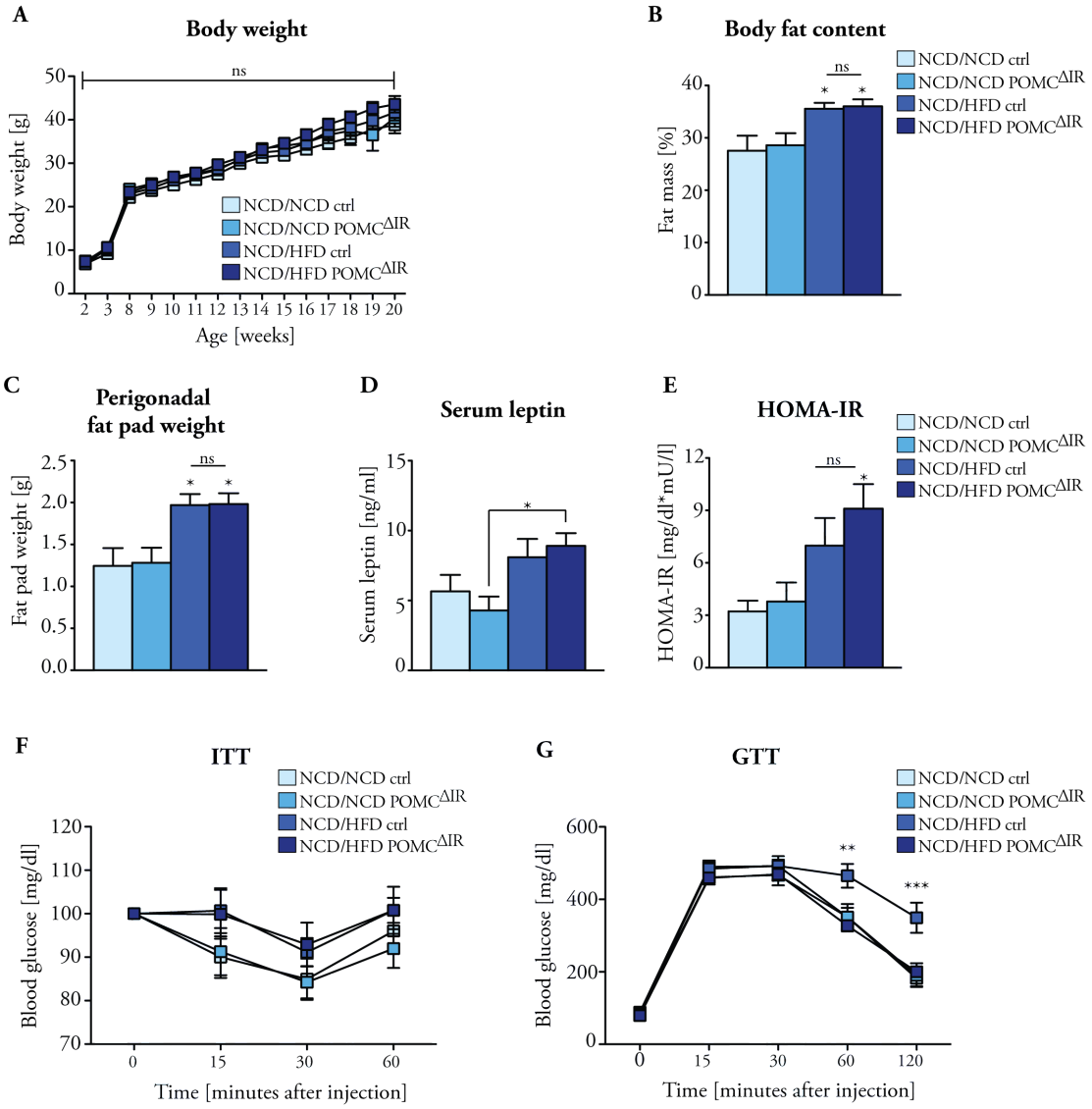
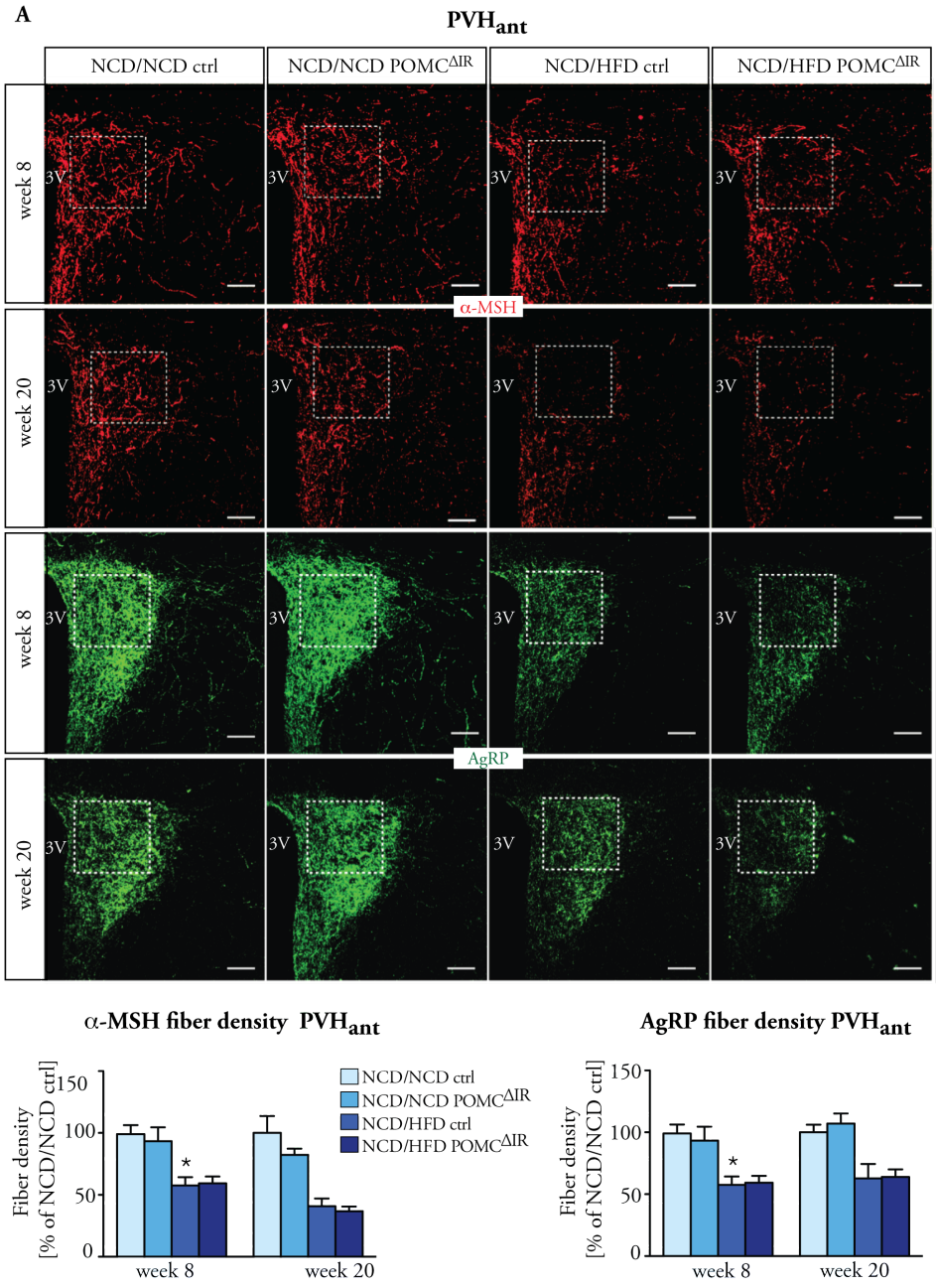


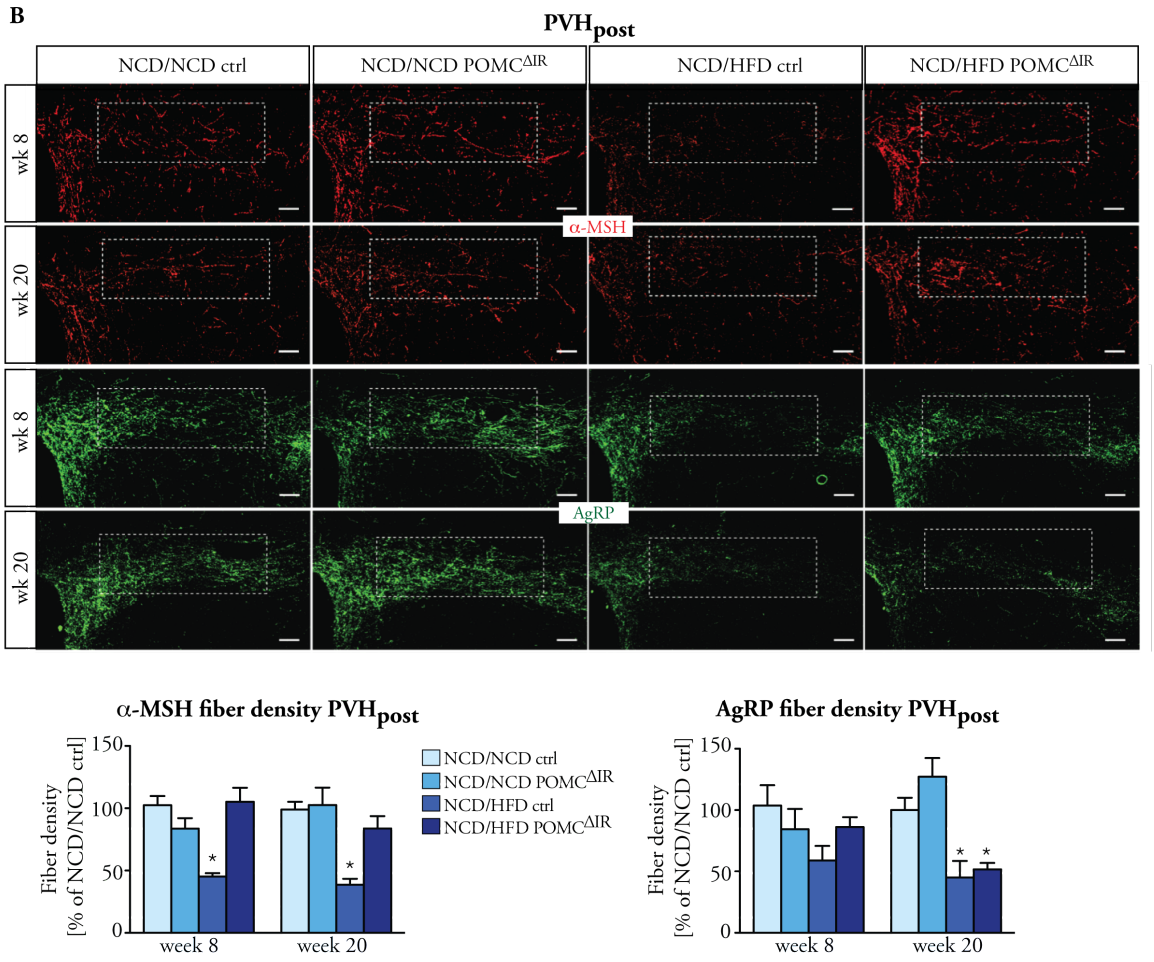
Figure 3.5.2 POMC-specific IR-deficiency does not protect from adiposity or insulin resistance, but improves glucose tolerance in NCD/HFD offspring
(A) Body weight, (B) body fat content and (C) perigonadal fat pad weight at 20 weeks of age. (D) Fasted serum leptin levels and (E) homeostatic model assessment indices of insulin resistance (HOMA-IR) at 15 weeks of age, (F) insulin tolerance tests (ITT) and (G) glucose tolerance tests (GTT) at 15 weeks of age. NCD, normal chow diet; HFD, high fat diet. Data are presented as mean +/- SEM, *p<0.05, **p<0.01, ***p<0.001 versus all other groups of offspring, unless otherwise indicated.

3.6 POMC-specific IR-deficiency in NCD/HFD offspring prevents altered axonal projections of POMC neurons to preautonomic PVH

To decipher persistent hypothalamic cellular changes responsible for the metabolic rescue of glucose tolerance in NCD/HFD POMC$^{\Delta IR}$ mice, we analyzed fiber densities of ARH neurons to the distinct subcompartments of the PVH at 8 and 20 weeks of age, as well as to the DMH and LH at 20 weeks of age. Loss of the IR specifically on POMC neurons did not change α-MSH or AgRP fiber densities to any hypothalamic target site in NCD/NCD offspring at any age (Figure 3.6.1). Moreover, maternal HFD-feeding exclusively during lactation resulted in a decrease of α-MSH fiber density in the neuroendocrine PVH$_{ant}$ in young and old NCD/HFD animals (Figure 3.6.1 A), as well as in the DMH (Figure 3.6.1 C) and LH (Figure 3.6.1 D), independent of their genotype. However, although NCD/HFD POMC$^{\Delta IR}$ mice displayed a similar decrease in α-MSH fiber density in the neuroendocrine PVH$_{ant}$ region as their control littermates (Figure 3.6.1 A), specific inactivation of the IR on POMC neurons protected against a decrease in the α-MSH fiber density in the preautonomic PVH$_{post}$ compartment resulting in a persistent restoration of α-MSH fiber density in NCD/HFD POMC$^{\Delta IR}$ offspring close to NCD/NCD levels both at 8 and 20 weeks of age (Figure 3.6.1 B).

In contrast, AgRP fiber densities were significantly reduced in NCD/HFD offspring independent of their genotype in the PVH$_{ant,}$ DMH, LH and most importantly, also the PVH$_{post}$ at the age of 20 weeks (Figure 3.6.1). Collectively, our results suggest that hyperinsulinemia in response to maternal HFD-feeding during lactation impairs axonal outgrowth of POMC neurons specifically to the preautonomic compartment of the PVH.


A
PVHant
NCD/NCD ctrl
NCD/NCD POMCΔIR
NCD/HFD ctrl
NCD/HFD POMCΔIR
week 8
week 20
3V
α-MSH
AgRP
α-MSH fiber density PVHant
AgRP fiber density PVHant
Fiber density [% of NCD/NCD ctrl]
150
100
50
0
*
week 8
week 20


B
PVHpost
NCD/NCD ctrl
NCD/NCD POMCΔIR
NCD/HFD ctrl
NCD/HFD POMCΔIR
wk 8
wk 20
α-MSH
AgRP
α-MSH fiber density PVHpost
Fiber density [% of NCD/NCD ctrl]
150
100
50
0
week 8
week 20
NCD/NCD ctrl
NCD/NCD POMCΔIR
NCD/HFD ctrl
NCD/HFD POMCΔIR
AgRP fiber density PVHpost

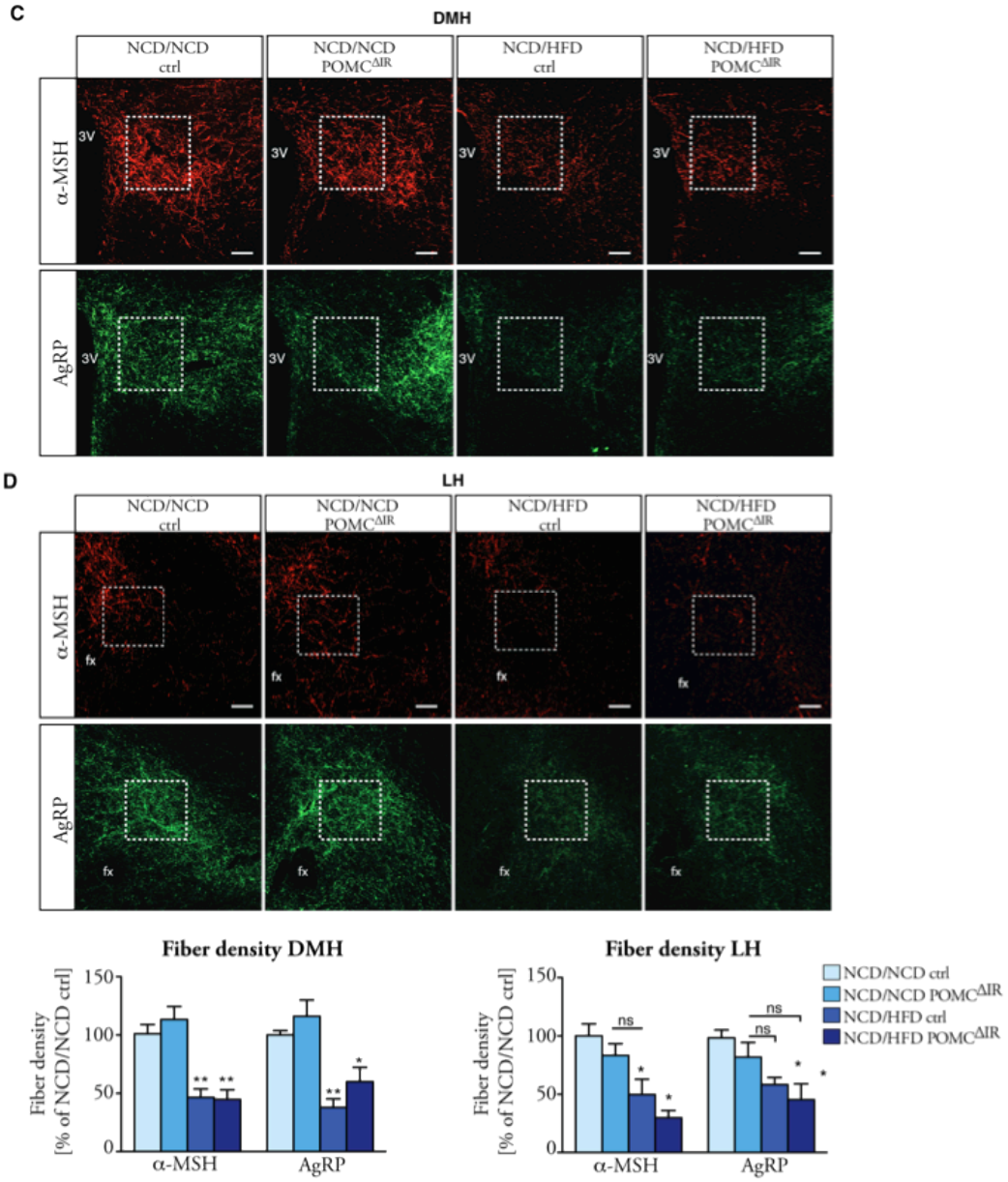


Figure 3.6.1 POMC-specific IR-deficiency in NCD/HFD offspring rescues POMC axonal projections to preautonomic regions in the PVH

Images and quantification of α-melanocyte-stimulating hormone (α-MSH) and agouti-related peptide (AgRP) immunoreactive fibers innervating (A) the anterior neuroendocrine paraventricular nucleus of the hypothalamus (PVH_{ant}) and (B) the posterior preautonomic PVH_{post} at 8 and 20 weeks age, as well as (C) the dorsomedial nucleus of the hypothalamus (DMH) and (D) lateral hypothalamic area (LH) at 20 weeks of age. White boxes indicate area of quantification. NCD, normal chow diet; HFD, high fat diet; IR, insulin receptor; 3V, third ventricle; fx, fornix; Scale bar = 100 µl. Data are presented as mean +/- SEM, *p<0.05, **p<0.01 versus all other groups of offspring, unless otherwise indicated.

3.7 Effects of maternal HFD-feeding during lactation and POMC-specific IR-deficiency on pancreatic β-cells

Reciprocal neural connections between the hypothalamus and preganglionic motor neurons of the autonomic nervous system play an important role in the regulation of energy and glucose homeostasis (Marino et al., 2011). Therefore, we aimed to identify changes in the autonomic tone in peripheral organs that could possibly be linked to the specific restoration of α-MSH axonal projections to the preautonomic compartment of the PVH_{post} in NCD/HFD $POMC^{\Delta IR}$ mice. Given the distinct rescue of glucose tolerance in POMC-specific IR-deficient NCD/HFD offspring in the absence of alterations in insulin sensitivity, we analyzed the parasympathetic innervation of pancreatic β-cells by staining for vesicular acetylcholine transporter (vAChT) (Rossi et al., 2005). Strikingly, the number of vAChT-immunoreactive buttons per islet area was significantly reduced in NCD/HFD ctrl offspring, but rescued to NCD/NCD levels in NCD/HFD $POMC^{\Delta IR}$ mice (Figure 3.7.1).

In line with the decreased parasympathetic innervation of pancreatic β-cells in NCD/HFD ctrl mice, glucose-stimulated insulin secretion was significantly decreased compared to NCD/HFD $POMC^{\Delta IR}$ offspring (Figure 3.7.2 A). Consistently, C-peptide levels were decreased 5 minutes after intravenous glucose injection in NCD/HFD ctrl offspring (Figure 3.7.2 C). In contrast, this defect in insulin secretion in NCD/HFD ctrl mice was not seen upon L-arginine stimulation (Figure 3.7.2 B) and was not associated with glucose-stimulated alterations in levels of free fatty acids (FFA) or glucagon-like peptide 1 (GLP-1) (Figure 3.7.2 D). Moreover, neither maternal HFD-feeding during lactation, nor POMC-specific IR-deficiency had any effect on the average pancreatic β-cell-mass or the average islet size of the pancreas (Figure 3.7.2 E). Taken together, our results indicate that POMC-specific IR-deficiency improves glucose-stimulated insulin secretion presumably in part via modulation of the parasympathetic tone in offspring from postnatally HFD-fed mothers.

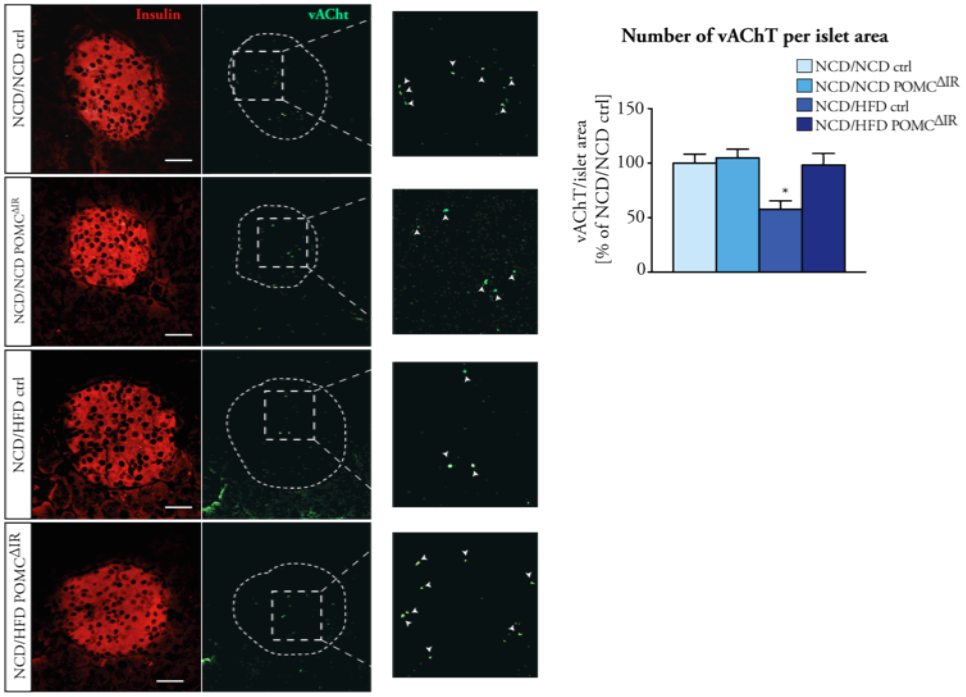


Figure 3.7.1 Increased parasympathetic innervation of pancreatic β-cells upon POMC-specific IR-deficiency in NCD/HFD offspring

Images and quantification of the parasympathetic marker vesicular acetylcholine transporter (vAChT, green) on pancreatic β-cells (insulin, red) at 20 weeks of age. NCD, normal chow diet; HFD, high fat diet. Scale bar = 50 µm. Data are presented as mean +/- SEM, *p < 0.05 versus all other groups of offspring.

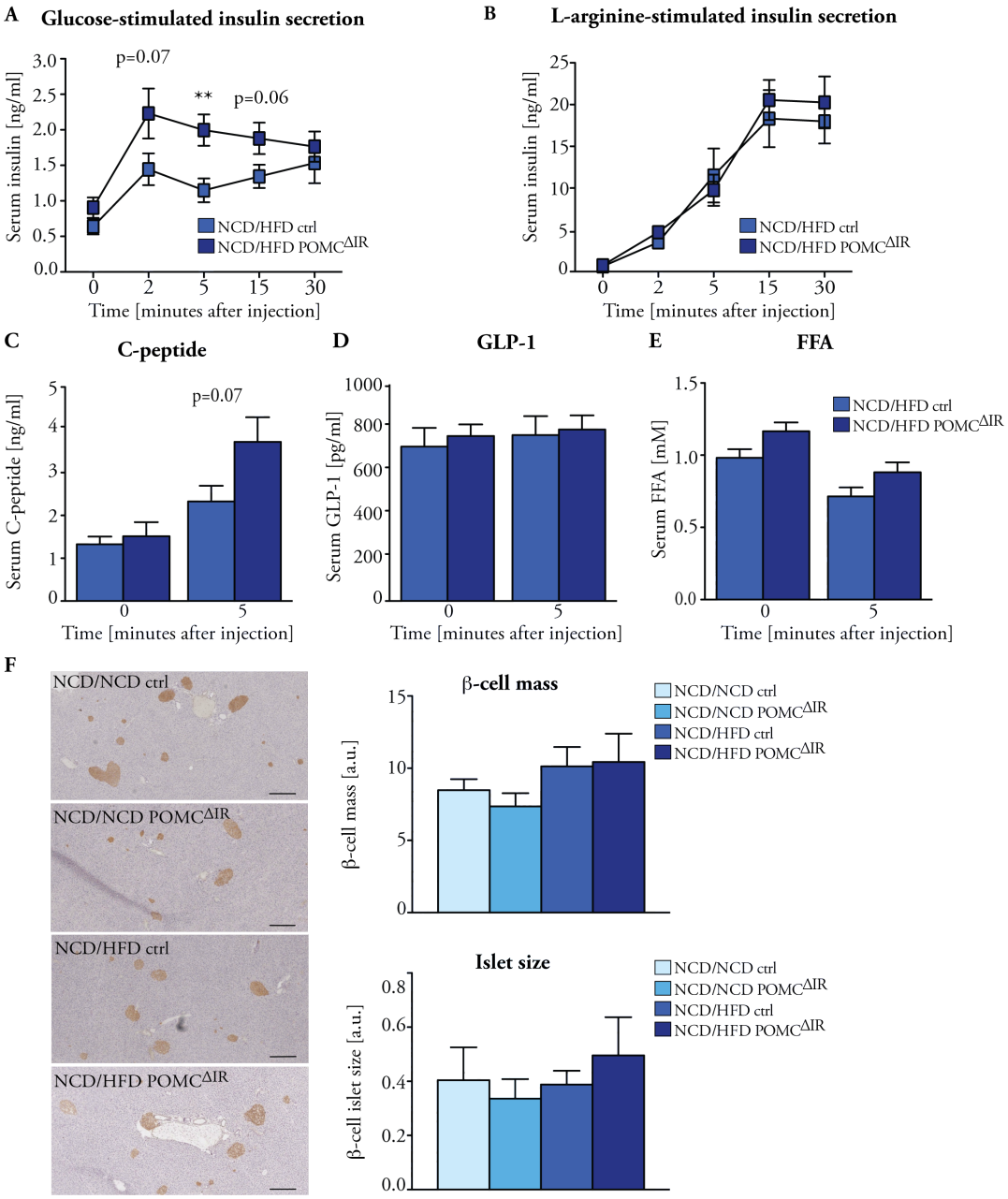


Figure 3.7.2 POMC-specific IR-deficiency protects from impaired glucose-stimulated insulin secretion in offspring from postnatally HFD-fed mothers
(A) Glucose-stimulated insulin secretion, (B) L-arginine-stimulated insulin secretion, (C) C-peptide, (D) Glucagon-like peptide 1 and (E) non-esterified fatty acid (FFA) concentrations 0 and 5 minutes after intravenous glucose injection at 15 weeks of age. (E) Images and quantification of total β-cell mass and average β-cell islet size at 20 weeks of age. NCD, normal chow diet; HFD, high fat diet. Scale bar = 300 µm, *p<0.05, **p<0.01 versus all other groups of offspring, unless otherwise indicated.

4 Discussion

4.1 Altering maternal diet during hypothalamic neurocircuit development as a valuable model to study metabolic programming

The findings that have led to the concept of metabolic programming convincingly presented an additional contributor to the obesity and T2DM epidemic that we are facing today. The fact that maternal health and even nutrition strongly impact on the development of key organs, such as the brain, thereby predisposing the unborn child to metabolic disorders throughout lifetime has received considerable amount of attention over the last decades. However, despite the multitude of studies already carried out in this field of research, to date we still know very little about the cellular and molecular alterations that are responsible for an increased propensity towards an impaired regulation of energy homeostasis. This lack of knowledge mainly results from the huge variation of animal models used to study these effects, which differed not only in the use of species, but also in the duration and magnitude of environmental insults to the offspring, giving rise to inconsistent and even conflicting results. These variations made it difficult to dissociate between morphological alterations that are actually causal and not just coincidental to the obese phenotype seen in offspring of obese and/or diabetic mothers. Given the fact that in contrast to humans, the main two phases of hypothalamic neurocircuit development are separated into a prenatal and postnatal period in rodents, we used this discrepancy between these species to our advantage. We established a mouse model of metabolic programming in which we did not only assess the effect of consistent, but also alternating maternal HFD-feeding between gestation and lactation on the offspring's health, which allowed us to identify the most sensitive phase of hypothalamic neurocircuit development to the hormonal changes in response to maternal HFD-exposure. Moreover, the utilization of mice instead of rats further enabled us to make use of genetic tools to clearly decipher the role of abnormal insulin signaling in metabolic programming.

4.1.1 Environmental insults during the postnatal phase of development result in the strongest metabolic impairments in the offspring in rodents

Using our model of metabolic programming, we could show that maternal metabolic changes in response to acute HFD-feeding starting during lactation is sufficient to induce metabolic disorders, both in male and female offspring. To our knowledge, so far only one study has manipulated the maternal diet both independently and synergistically during these distinct phases of hypothalamic neurocircuit development in a similar manner (Sun et al., 2012). Despite clear differences in study design: 1) rats versus mice, 2) maternal HFD-feeding not pregestationally, but only starting during gestation or lactation and 3) cross fostering instead of postnatal diet switch, Sun et al. also identified the lactation phase as the most sensitive period to hormonal insults in response to maternal HFD-feeding. However, in contrast to our study, in which we do not see gross metabolic impairments of offspring from consistently HFD-fed mothers, Sun et al. report that offspring from mothers fed HFD both pre- and postnatally share the same metabolic impairments with offspring cross-fostered to HFD-fed mothers. Of note, due to their cross-fostering paradigm, in their model of metabolic programming, maternal metabolic homeostasis, as well as milk composition do not differ between these two groups of offspring. Thus, considering the results obtained from our study, i.e. that hormonal alterations during the postnatal phase are critical in the predisposition for metabolic disorders, it is not surprising that these two groups share the same phenotype, especially since these mothers had not developed obesity or insulin resistance prior to or during gestation (Sun et al., 2012). Moreover, other models of postnatal overnutrition, such as litter size adaptation and cross-fostering experiments further support our findings by demonstrating that an altered metabolic milieu during early postnatal development in rodents is not only sufficient to predispose for metabolic disorder, but can even partially override prenatal factors and genetic predisposition to develop obesity (Glavas et al., 2010; Gorski et al., 2006).

4.1.2 Impairment of ARH neuronal axon formation might be the common and main driver of metabolic programming

In the search of hypothalamic cellular changes underlying the obese and glucose intolerant phenotype in the offspring, we investigated developmental processes that occur postnatally and could possibly be affected in response to maternal HFD-feeding during lactation. Postnatal maternal HFD-feeding neither induced neuronal cell death of ARH neurons nor affected synaptogenesis (at least not onto POMC neurons). Moreover, our results repeatedly showed no changes in ARH neuropeptide gene expression, POMC processing to α-MSH or hypothalamic inflammation. In contrast, fiber densities of POMC and AgRP/NPY neurons were severely reduced in all major intrahypothalamic target sites, i.e. PVH, DMH and LH in offspring from postnatally HFD-fed mothers, suggesting an impairment of ARH neuronal axon formation. Interestingly, a variety of models such as postnatal overnutrition due to litter size reduction, streptozotocin-induced maternal hyperglycemia during gestation and lactation, maternal obesity in response to consistent maternal HFD-feeding, as well as neonatal nutritional deprivation have demonstrated decreased POMC and/or AgRP/NPY innervation of the PVH in the offspring (Bouret, 2012; Coupe et al., 2010; Delahaye et al., 2008; Kirk et al., 2009; Steculorum and Bouret, 2011). Moreover, rats genetically predisposed for diet-induced obesity (DIO) and obese leptin-deficient mice also display decreased PVH innervation of POMC and AgRP/NPY neurons (Bouret et al., 2004b; Bouret et al., 2008). Thus, these consistent findings of impaired ARH neuronal axon formation across studies suggest that this defect may be a common driver for metabolic programming and thus critical for the impaired regulation of energy and glucose homeostasis in offspring from obese, diabetic and even malnourished mothers. Nevertheless, we cannot exclude that impairment of other neurodevelopmental processes specifying neuronal cell number or affecting neuropeptide expression – as described in other models of metabolic programming – adds to the dysregulated metabolism in offspring of obese or diabetic mothers (for review, see (Plagemann, 2012)). However, most studies describing alterations in the number of anorexigenic or orexigenic neurons, as well as malformations of hypothalamic nuclei, did not assess the effects on ARH projection formation in the same model (Chang et al., 2008; Chen et al., 2008; Gout et al., 2010; Plagemann et al., 1999a;

Plagemann et al., 1999b). This lack of information clearly exacerbates the possibility to draw any conclusions about the role of the observed modifications in metabolic programming. Our results however, as well as the severe inconsistencies concerning the effects on prenatal developmental processes between other studies therefore question the overall contribution of alterations in neuronal cell number or neuropeptide expression and suggest that in contrast to impaired ARH axon formation, they play a minor or additive role in the predisposition for metabolic disorders.

Of note, so far, the effects of an abnormal developmental environment have mainly been studied on ARH neuronal innervation of intra-hypothalamic target sites of POMC and AgRP/NPY neurons. However, these neurons project to various other sites throughout the CNS implicated in the regulation of energy balance, for example via modulation of the autonomic nervous or mesolimbic reward system. One of the main contributors of the worldwide obesity epidemic is considered to be the ready-availability of palatable, high-fat food in westernized societies (WHO, 2013c). Given that the desire to eat is not only driven by the current energy or nutritional status, but also by the rewarding or hedonistic aspect of food, alterations in the efficacy and sensitivity of the central mesolimbic reward pathway have been associated with the development and manifestation of obesity (for review, see (Palmiter, 2007)). Along these lines, several studies have demonstrated that the perinatal nutritional environment has long-lasting effects on these neurocircuits leading to an impaired regulation of this reward-based feeding behavior and thereby predisposes for metabolic disorders throughout lifetime. Offspring from mothers exposed to a “junk food” or “cafeteria” diet (i.e. a dietary mixture of a high fat and high sugar diet with a normal chow diet) during gestation and lactation display an accelerated preference for fatty, sugary and salty food along with an increased frequency of feeding episodes throughout lifetime (Bayol et al., 2007; Ong and Muhlhausler, 2011; Wright et al., 2011b). Moreover, maternal perinatal HFD-feeding leads to an altered responsiveness of dopaminergic neurons (Naef et al., 2008). On a molecular level, these behavioral changes are associated with elevated levels of tyrosine hydroxylase and dopamine in distinct regions of the mesolimbic system, including in the VTA and nucleus accumbens (NAc) (Naef et al., 2008). Although it has not been investigated whether impaired maternal nutrition affects neuronal axon formation in the mesolimbic system directly, development of projections from the VTA to the NAc continues during the postnatal life in rodents and therefore represents a likely target for

developmental environmental insults (Antonopoulos et al., 2002; Kalsbeek et al., 1988). In addition to impaired regulation of hedonic feeding, altered maternal metabolic homeostasis has been associated with the development of mental health and behavioral disorders including anxiety, depression and attention deficit hyperactivity disorder, which are common co-morbidities of obese children (for review, see (Sullivan et al., 2012);(Rofey et al., 2009a; Rofey et al., 2009b; Waring and Lapane, 2008)). Although further studies are required to reliably demonstrate this causality in humans, animal models could convincingly show a direct association between maternal nutrition and development of abnormal social and aggressive behavior in rats and non-human primates (Bilbo and Tsang, 2010; Maniam and Morris, 2010; Raygada et al., 1998; Sullivan et al., 2010; Van Lieshout et al., 2011; Wright et al., 2011a). These behavioral deficits were partially attributed to perturbations of the serotonergic system (Sullivan et al., 2010). The serotonergic neurons originate in the dorsal raphe nuclei and project throughout most regions of the brain, such as the hypothalamus, amygdala and brainstem to name a few. Apart from its role in the regulation of energy balance, abnormal serotonergic signaling has been implicated in diverse functions, including in the development and manifestation of the aforementioned behavioral and mental disorders (for review, see (Marston et al., 2011)). Nevertheless, similar to the inconsistencies of reported hypothalamic changes in response to impaired maternal health and nutrition, described cellular modifications of the serotonergic system diverge and are even conflicting between studies (for review, see (Sullivan et al., 2012)).

In light of these findings, future studies of metabolic programming should not only incorporate analyses of ARH neuronal fiber densities in these extra-hypothalamic brain areas implicated in the regulation of hedonic aspects of food consumption and in the modulation of social behavior; but also decipher putative cellular changes, in particular alterations in axonal formation of these “higher” neuronal circuits independent of the central melanocortin system to fully understand the magnitude of environmental insults during development on central neurocircuit formation and phenotype development.

4.1.3 Hormonal, cellular and molecular alterations possibly affecting ARH neuronal axon formation

Reviewing the different studies conducted in the field of metabolic programming that have consistently led to an impaired ARH axon formation, the question arises which of the hormonal and/or nutritional alterations in response to diverse environmental insults might be uniformly implicated in mediating developmental defects that result in an impaired metabolic homeostasis. Comparison of the milk composition between control and postnatally HFD-fed mothers represent likely candidates responsible for this effect. In our model, the relatively short period of postnatal maternal HFD-exposure was sufficient to highly increase leptin, glucose, insulin and lipid content in the milk – factors that have all been implicated in brain development (for reviews, see (Bouret, 2010; Chiu and Cline, 2010; Sakayori et al., 2013)). Similarly, maternal obesity and overweight are associated with alterations in insulin and leptin levels, as well as the lipid composition in the milk in humans, as well as genetically predisposed obese DIO or HFD-fed rats (Ahuja et al., 2011; Gorski et al., 2006; Houseknecht et al., 1997; Kirk et al., 2009).

Although we did not directly assess the early postnatal serum leptin concentration in the offspring, the elevated milk leptin content in addition to results from other studies suggest that maternal HFD-feeding during the postnatal phase significantly increases circulating leptin in the offspring also in our model (Rother et al., 2012; Sun et al., 2012). The importance of well-balanced leptin levels during the postnatal phase of hypothalamic neurocircuit development in rodents has been convincingly demonstrated. Using obese leptin-deficient mice, Bouret and colleagues showed the necessity for a so-called neonatal "leptin surge" required for proper ARH axon formation and ultimately regulation of energy and glucose homeostasis (Bouret et al., 2004b). Here, the direct neurotrophic effects of leptin were shown to be critical in promoting neurite outgrowth to establish functional hypothalamic neurocircuits. Consistently, developmental hypoleptinemia, leptin resistance or even a prolonged leptin surge, were associated with impaired PVH innervation of ARH neurons (Bouret et al., 2004b; Kirk et al., 2009; Steculorum and Bouret, 2011). To date, the source of this critical leptin surge during development remains controversial. While it is likely that maternal leptin could be directly transferred from the milk to the offspring, other

data suggests that leptin originates mainly from the offspring's adipose tissue itself even early on during development (Ahima et al., 1998; Bouret et al., 2004b; Cottrell et al., 2009; Rayner et al., 1997). In addition, increased milk fatty acid concentration and alterations in milk fatty acid composition in response to maternal HFD-feeding increase leptin levels in the offspring (Korotkova et al., 2002a; Korotkova et al., 2002b). In combination with the possible direct effects of elevated fatty acid concentration in the milk, the lipid-mediated rise in leptin levels could further affect hypothalamic neurocircuit development. Thus, based on these findings, it is highly likely that also in our model of metabolic programming, elevated leptin levels in the offspring interfered with the establishment of hypothalamic neurocircuits in response to maternal HFD-feeding during lactation.

One striking physiological finding in our model of metabolic programming was the pronounced hyperinsulinemia in three-week old offspring from postnatally HFD-fed mothers, which was independent of the prenatal maternal diet. This drastic increase of serum insulin levels could originate from consumption of the hyperglycemic milk of HFD-fed mothers, which in turn stimulates pancreatic insulin release in the neonate. Moreover, it has been shown that maternal milk insulin retains biological activity upon ingestion and can cross the intestinal mucosal-blood barrier thereby directly contributing to serum insulin levels in the offspring (Kinouchi et al., 1998; Mosinger et al., 1959; Shulman, 1990). Similar to our observations, insulin levels have been described to be upregulated in offspring from postnatally HFD-fed DIO rats, which also display impaired formation of ARH neuronal circuits (Bouret et al., 2008; Gorski et al., 2007). Since perinatal hyperinsulinemia has been proposed to be involved in mediating metabolic programming as early as the 1970s (Pedersen, 1971), our results suggest that it might do so in part by impairing hypothalamic neurocircuit formation. In line with this hypothesis, Plagemann and colleagues could show that hypothalamic hyperinsulinemia during exactly this critical postnatal period resulted in an impaired metabolism of the offspring throughout lifetime (Plagemann et al., 1992). Although the approach used by the authors to induce hypothalamic hyperinsulinemia in the offspring was not physiological and highly unspecific, it was the first evidence that central insulin signaling during the period of hypothalamic neurocircuit formation either directly or indirectly leads to the development of metabolic disorders throughout lifetime (Plagemann et al., 1992). However, if abnormal levels of insulin during the postnatal phase of hypothalamic neurocircuit development did play a role in predisposing for metabolic

disorders by interfering with ARH axon formation, why does the offspring from mothers consistently exposed to HFD in our model not show any gross metabolic impairments? In line with the occurrence of early neuronal leptin resistance, studies in humans and rodents have shown that offspring from obese mothers display altered insulin sensitivity, including in the CNS (Catalano et al., 2009; Chen et al., 2008; Gupta et al., 2009; Morris and Chen, 2009). Although maternal insulin cannot cross the placental barrier during gestation, maternal glucose can permeate and stimulate fetal pancreatic insulin secretion during early life. Considering the hyperglycemic and insulin resistant phenotype of pregestationally HFD-fed mothers in our model, these maternal blood glucose excursions could have led to an early neuronal insulin resistance in the offspring, dampening the potentially detrimental effects of hyperinsulinemia on hypothalamic neurocircuit formation in postnatal life. However, whether neuronal insulin resistance actually occurs in response to consistent maternal HFD-feeding in our model still needs to be addressed. Moreover, based on previous publications demonstrating that maternal HFD-feeding and hyperglycemia during gestation lead to increased neurogenesis in the hypothalamus, we also hypothesized that HFD-feeding during gestation would increase neurogenesis of anorexigenic POMC neurons, thereby partially protecting the offspring from metabolic impairments (Chang et al., 2008; Steculorum and Bouret, 2011). However, analysis of the POMC neuronal cell number did not show any differences between offspring (data not shown). Nevertheless, as depicted before, in some models of metabolic programming, offspring from consistently HFD-fed obese mothers display strong metabolic impairments (for review, see (Plagemann, 2012)). These discrepancies between studies clearly highlight the necessity to thoroughly characterize metabolic and hormonal changes both in the mother and in the offspring in response to the distinct environmental insults when using any animal model to study metabolic programming. This thorough characterization would not only facilitate cross-comparisons between studies, but also allow the molecular driver(s) of the observed metabolic phenotype to be identified, thereby protecting the authors from drawing the wrong conclusions.

Taken together, in addition to the well described effects of abnormal leptin levels during early postnatal life on the establishment of ARH neuronal projections, the distinct development of hyperinsulinemia prompted us to decipher whether abnormal insulin signaling would contribute to the impaired axon formation of POMC and AgRP/NPY neurons in offspring of postnatally HFD-fed mothers.

4.2 Abnormal neuronal insulin signaling disrupts POMC axon formation and predisposes for impaired glucose metabolism

Insulin has been implicated in the regulation of various aspects of brain development and function, including synaptic plasticity, neuronal survival, proliferation and differentiation (for review, see (Chiu and Cline, 2010)). In addition, insulin exerts axonotrophic effects resulting in accelerated neurite outgrowth and increased average neurite length in cultured rodent fetal neuronal and human neuroblastoma cells (Recio-Pinto and Ishii, 1984; Recio-Pinto et al., 1986; Toran-Allerand et al., 1988). However, whether these effects of insulin on axon formation are relevant *in vivo* has yet to be demonstrated. To decipher the role of neuron-specific insulin signaling in ARH neuronal projection formation and the relative contribution of hyperinsulinemia in response to postnatal maternal HFD-feeding in mediating metabolic programming, we genetically ablated the insulin receptor (IR) from POMC-neurons in offspring from control and postnatally HFD-fed mothers ($POMC^{\Delta IR}$). In contrast to $POMC^{\Delta IR}$ mice, which do not have a baseline phenotype, mice lacking the IR specifically on AgRP-neurons display impaired regulation of hepatic glucose production (Konner et al., 2007). Thus, employing our feeding paradigm to $POMC^{\Delta IR}$ rather than $AgRP^{\Delta IR}$ offspring largely facilitated the interpretation of results and conclusions that could be drawn – as a proof of principle – for the role of hypothalamic insulin signaling during development under pathological conditions. Nevertheless, this model also displays one technical limitation, which warrants caution when interpreting the data. Some POMC progenitor cells differentiate into AgRP/NPY neurons later during development (Padilla et al., 2010). Consequently, IR signaling will also be genetically deleted in a minor subpopulation of these orexigenic neurons, possibly contributing to the observed metabolic and cellular effects.

In offspring from control NCD-fed mothers, POMC-specific insulin receptor deficiency did not lead to any gross hypothalamic anatomical malformations, nor did it affect POMC or AgRP/NPY innervation to any intra-hypothalamic target site. In contrast, postnatal maternal HFD-feeding decreased POMC and AgRP/NPY neuronal fiber densities in all major intra-hypothalamic target areas, which was associated with strong metabolic disorders in the offspring. Under these pathological developmental conditions, POMC-specific insulin receptor deficiency in the offspring restored POMC neuronal fiber density

exclusively in the posterior part of the PVH. These results suggest that as opposed to the effects seen *in vitro*, IR signaling is not essential for POMC axonal organization under normal developmental conditions. However, abnormal insulin action in response to maternal HFD-feeding exerts detrimental effects on POMC neurocircuit formation, which can partially be prevented by specific loss of the IR on these neurons. Our laboratory found similar adverse effects of aberrant hypothalamic insulin signaling in response to HFD-exposure on energy metabolism in adult mice. Here, activation of insulin signaling in the VMH contributed to the diet-induced inhibition of POMC neurons and subsequent manifestation of metabolic disorders (Klockener et al., 2011). Of note, this effect of insulin signaling in the VMH was only seen under NCD-conditions (Klockener et al., 2011). Moreover, whereas low concentrations of insulin (i.e. 10 nM) promoted neurite outgrowth, chronic exposure (i.e. 500 pM for 5 days) or stimulation with high doses of insulin (i.e. 1 µM) suppressed neurite outgrowth in a cell culture system of adult sensory neurons (Singh et al., 2012). Accordingly, it is conceivable that in our model, neonatal hyperinsulinemia in the offspring could result in an abnormal activation of insulin signaling in POMC neurons, thereby contributing to the disruption of melanocortin projections. Importantly, the sustained impairment of ARH neurocircuits throughout lifetime in the offspring from mothers exposed to HFD-feeding during lactation clearly highlights the apparent lack of plasticity of the CNS during adulthood needed to compensate for this developmental defect.

The discrepancy between the *in vitro* findings and our results in POMC neurons on insulin's role in axon formation under normal developmental conditions could originate from potential compensatory effects of type 1 insulin-like growth factor receptor (IGF1R) signaling. Insulin-like growth factor 1 and 2 (from now on called IGF-related peptides) signal *via* the IGF1R, which is expressed throughout the CNS, including in neurons of the mediobasal hypothalamus (Daftary and Gore, 2004; Lesniak et al., 1988). Although insulin and IGF-related peptides mainly display distinct physiological functions, similar to IR signaling, ligand binding to the IGF1R leads to IRS-mediated activation of PI3K and MAPK signaling (for review, see (Werner and Leroith, 2014)). Moreover, insulin and IGF-related peptides not only bind to their own, but they can also bind to and activate each other's receptors. Accordingly, they are also implicated in the regulation of the same processes, such as neurogenesis or neuron axon formation during CNS development. Thus, one could speculate that potential defects caused by IR signaling deficiency on neuronal

development could have been masked by compensatory action of insulin and/or IGF-related peptide signaling via the IGF1R under normal developmental conditions. Moreover, *in vitro* studies have shown that both insulin and IGF-related peptides enhance neurite growth independently, but may act in concert to exert their full effects on axon formation and to regulate other aspects of neuronal development (Recio-Pinto and Ishii, 1984, 1988; Recio-Pinto et al., 1986). In line with these findings, Toran-Allerand and colleagues demonstrated that in their hypothalamic fetal neuronal cell culture system only supraphysiological levels of insulin were able to induce neurite formation (Toran-Allerand et al., 1988). Hence, the authors hypothesized that insulin may activate the receptor of different, but closely related molecules, such as the IGF1R, to elicit the observed axonotrophic effect in neurons (Toran-Allerand et al., 1988).

To fully understand the role of insulin signaling in hypothalamic neurocircuit formation under normal, as well as pathological conditions *in vivo*, complete abrogation of neuronal insulin signaling by specifically deleting IR and IGF1R expression in POMC neurons could shed new light on these matters. However, there are some possible limitations to this approach, which need to be considered when interpreting the metabolic phenotype and (possible) hypothalamic alterations in the offspring. First, whether POMC neurons express the IGF1R has not yet been investigated. Second, Cre-mediated ablation of IR and IGF1R signaling (often referred to as "IIS" signaling) in POMC neurons could lead to detrimental outcomes in the offspring. Cre expression under the transcriptional control of the POMC promoter starts around embryonic day 12.5 and would therefore result in complete loss of IIS signaling during both phases of hypothalamic neurocircuit development possibly leading to impaired differentiation into POMC neurons or defects in neuronal migration (Padilla et al., 2012). Finally, some AgRP/NPY neurons express *Pomc* early during development, which is sufficient to induce Cre-mediated IR/IGF1R ablation also in these orexigenic neurons (Padilla et al., 2010).

The molecular mechanisms how insulin and/or IGF-related peptides mediate neurite formation remains unknown. Some experiments indicate that both insulin and IGF-related peptides promote synthesis of proteins, such as neurofilament subunits or the presynaptic growth-associated protein 43, which are implicated in neuron axon formation, growth and stabilization in a PI3K/MAPK-dependent manner (Liu et al., 2012; Wang et al., 1992). However, further studies are needed to decipher downstream targets of insulin and/or IGF-

related peptides critical for neuron axon formation that might be impaired upon abnormal hormonal signaling in response to maternal HFD-feeding.

Although we initially decided against studying the contribution of AgRP/NPY neuron-specific insulin signaling in our model, the specificity of both the cellular and metabolic improvements in POMC$^{\Delta IR}$ offspring from postnatally HFD-fed mice demands further investigation of cell-type specific alterations in response to distinct hormonal insults during development to fully understand processes underlying metabolic programming. Interestingly, a recent study published by Bouyer and Simerly demonstrated specific restoration of AgRP/NPY innervation of the posterior PVH upon intraperitoneal leptin injections during the postnatal period in leptin-deficient mice (Bouyer and Simerly, 2013). However, whether abnormal levels of insulin have identical effects on the axonal projections of AgRP neurons as we described for POMC neurons or whether insulin action impacts on an AgRP neuronal subpopulation that innervates other intra-or extrahypothalamic target sites possibly responsible for different metabolic impairments in offspring from postnatally HFD-fed mothers remains to be identified.

Taken together, despite the multitude of studies demonstrating an axonotrophic effect of insulin on neurons *in vitro*, based on our data, IR signaling *per se* does not seem to be essential for neuronal axon formation of POMC neurons under normal developmental conditions *in vivo*. Nevertheless, hyperinsulinemia in response to maternal postnatal HFD-feeding specifically contributes to impaired POMC innervation to a distinct compartment of the PVH implicated in the regulation of the autonomic nervous system. Our findings provide further evidence for the existence of distinct subpopulations of POMC neurons that seem to diverge in their projection sites and thus in their relative contribution to regulate different aspects of energy and glucose homeostasis. These results highlight the necessity to further dissect the effect of specific hormonal alterations on individual neuronal populations in response to developmental insults.

4.3 A novel brain-pancreas circuit regulating glucose homeostasis?

The site-specificity in the rescue of POMC axonal formation to the preautonomic, but not to the neuroendocrine compartment of the PVH or any other intra-hypothalamic target site in POMC-specific IR-deficient offspring from postnatally HFD-fed mothers might result from the cellular heterogeneity of this neuronal population. Only a subset of POMC neurons is insulin-responsive and this subpopulation of neurons is distinct from leptin-responsive POMC neurons in the ARH (Williams et al., 2010). Thus, loss of IR-signaling under hyperinsulinemic conditions might only lead to a beneficial effect on a subpopulation of POMC neurons, which might predominantly target the posterior part of the PVH. However, future studies will clearly have to address the mechanistic basis for the selective effect of IR-signaling on projection formation to the PVH.

Strikingly, the specific restoration of the α-MSH fiber density to the posterior PVH was associated with an improvement of glucose homeostasis without affecting adiposity or insulin resistance in $POMC^{\Delta IR}$ offspring from postnatally HFD-fed mothers. The posterior PVH harbors some neuroendocrine neurons expressing vasopressin, oxytocin or TRH implicated in the regulation of pituitary hormones, but mainly contains preautonomic neurons that project to various regions in the brainstem, including to parasympathetic and sympathetic preganglionic neurons located in the dorsal motor nucleus of the vagus (DMX) and the intermediolateral column (IML), respectively (Geerling et al., 2010; Lakke and Hinderink, 1989). Parasympathetic and sympathetic nerve terminals innervate peripheral organs, such as the pancreas, liver and white adipose tissue to modulate cellular processes adapting behavior and metabolism to the current energetic and environmental demand (for review see (Marino et al., 2011)). In the pancreas, parasympathetic vagal nerve activity stimulates, whereas sympathetic activation inhibits insulin secretion from pancreatic β-cells (for review, see (Ahren, 2000)). This autonomic control of pancreatic insulin secretion is essential for the so-called “cephalic”, as well as “first” phase of glucose-stimulated insulin secretion. The cephalic phase of insulin secretion, which is triggered by sensory mechanisms and not by absorbed nutrients, primarily depends on direct neuronal connections between the taste buds and the brain stem (Berthoud et al., 1981; Berthoud and Jeanrenaud, 1982; Berthoud et al., 1980). In contrast, neuronal circuits responsible for the regulation of first phase insulin secretion are not completely elucidated, yet. However, there are studies clearly

demonstrating a role for ARH-residing neurons in mediating this response. First, ARH neurons form direct, as well as multisynaptic contacts via the PVH with neurons of the DMX that innervate the pancreas (Buijs et al., 2001; Jansen et al., 1997; King and Hentges, 2011). Second, ICV application of NPY induces insulin secretion – an effect that can be suppressed by vagatomy (Sainsbury et al., 1997; Zarjevski et al., 1993). Third, intracarotid glucose injections, which exclusively increase glucose levels in the brain, but not in the periphery, stimulate pancreatic insulin secretion within one minute. This central response to glucose is mediated at least in part by neurons of the ARH and PVH, as assessed by analysis of neuronal activation via c-Fos staining subsequent to intracarotid glucose injections (Guillod-Maximin et al., 2004).

Accordingly, the improved glucose-stimulated insulin secretion in POMC$^{\Delta IR}$ mice compared to their wildtype littermates from mothers exposed to HFD postnatally in our model suggests that the restored insulin-responsive neuronal circuit from ARH POMC neurons to the preautonomic compartment of the PVH plays a critical role in adapting autonomic nervous system activity in response to glucose excursions. To clearly decipher whether this neuronal circuit plays a general role in adapting glucose-stimulated insulin secretion, or whether this effect is exclusively due to the effects of abnormal insulin signaling on POMC neuronal axon formation, glucose-stimulated insulin secretion should be investigated in control and POMC$^{\Delta IR}$ offspring exposed to normal developmental conditions. In line with our hypothesis that the POMC ARH circuit to the preautonomic PVH is critical for adapting autonomic nervous system activity to regulate insulin secretion was the finding that the decreased parasympathetic innervation of pancreatic β-cells in response to postnatal HFD-feeding was restored to normal levels upon POMC-specific IR-deletion. However, it is possible that the observed decrease in parasympathetic innervation of the pancreas is not directly linked to the central changes in offspring from postnatally HFD-fed mothers. Given that these animals display various metabolic impairments and hormonal alterations during postnatal development, any of these changes could have led to direct modifications in pancreatic morphology and/or altered sensitivity to external stimuli, as previously described in other models of metabolic programming (for review, see (Reusens and Remacle, 2006)). A recent study published by Tarussio et al. demonstrated a critical role for glucose sensing in the brain in the control of parasympathetic activity that defines a CNS/pancreas axis critical for β-cell mass establishment in the postnatal period and for long-

term β-cell maintenance (Tarussio et al., 2014). Although we did not see changes in total β-cell mass or insulin content in response to maternal HFD-feeding or POMC-specific IR-deficiency, we cannot exclude that alterations in glucose and/or leptin levels during postnatal development impacted on β-cell sensitivity or innervation resulting in the observed phenotype. In addition, analyses of pancreatic parasympathetic innervation were performed in adult animals and not before the onset of drastic metabolic impairments. Thus, improved glucose-stimulated insulin secretion and parasympathetic innervation of the pancreas in $POMC^{\Delta IR}$ offspring from postnatally HFD-fed mothers could have developed secondary to their partially improved metabolic milieu. To clearly decipher whether the observed pancreatic changes are due to neuronal hyperinsulinemia during postnatal development, analysis of pancreatic autonomic innervation should be conducted in much younger animals. Moreover, alterations in the activity of the vagal nerve and sympathetic nerve fibers should be investigated, as well as the relative contribution of changes in the autonomic nervous system to the impaired glucose tolerance. These experiments would aid in the definition of the molecular and cellular contributors of metabolic disorders. In addition, as depicted before, POMC neurons form functional neurocircuits with the DMX, directly. These ARH->brain stem projections are of particular importance for glucose homeostasis, as re-expression of the MC4R specifically in the DMX and IML in an MC4R-deficient background restores central regulation of insulin secretion (Sohn et al., 2013). Thus, postnatal maternal HFD-feeding and POMC-specific IR-deficiency could directly impact on the formation of these neurocircuits and thereby program the offspring for altered autonomic nervous system activity. However, given the scarcity of ARH-neurons that project to the brainstem (approximately 50-100 POMC neurons) (Zheng et al., 2005), sophisticated retrograde tracing experiments in combination with additional transgenic neuronal markers would be necessary to reliably detect putative cellular changes in these brain areas. Although these experiments were beyond the scope of this study so far, the undisputable importance of this direct melanocortin-dependent ARH->brain stem circuit regulating autonomic nervous system activity demands further investigation to decipher whether they may contribute to the observed phenotypes.

Of note, apart from the ARH, the nucleus of the solitary tract (NTS) in the brainstem harbors approximately 200 POMC-expressing neurons, which form reciprocal connections to other brain areas, including the PVH and regions implicated in the

regulation of the autonomic nervous system (Joseph and Michael, 1988; King and Hentges, 2011). Hence, it is possible that postnatal maternal HFD-feeding and/or POMC-specific IR-deficiency affected NTS POMC neuronal projections and thereby contributed to the metabolic and cellular phenotype in response to developmental insults. However, to date it is not known whether these neurons are actually insulin-responsive, during which developmental stage they form axonal projections and whether these innervate the DMX and/or IML. In addition to the relatively small number of NTS POMC neurons, this missing information rather excludes a major contribution of these neurons to the observed phenotypes in $POMC^{\Delta IR}$ offspring from postnatally HFD-fed mothers. Moreover, the fact that efferent PVH projections to the brainstem are already mature at embryonic day 20 and do therefore not represent a target for environmental insults during the postnatal phase, further support the hypothesis that POMC ARH axon formation to the posterior PVH itself is critical for the control of glucose homeostasis at least in part by modulation of the autonomic nervous system.

Obesity has previously been associated with disturbed functions of the autonomic nervous system even in obese children (Baum et al., 2013; Greenfield and Campbell, 2008). In addition to our study, findings from other models of metabolic programming suggest that these defects may be programmed during early development. Offspring from undernourished mothers, which share several metabolic impairments with offspring from obese mothers, display decreased pancreatic parasympathetic activity in rodents (de Oliveira et al., 2011; Gravena et al., 2007). Moreover, perinatal HFD-exposure reduces hepatic sympathetic innervation in non-human primates (Grant et al., 2012). Although these studies did not analyze putative changes in the melanocortin circuitry, both of these models of metabolic programing have been associated with alterations in hypothalamic neurocircuit development (Grayson et al., 2010; Orozco-Solis et al., 2010). In light of these findings it is conceivable that alterations of the plasticity and development of peripheral organs, as described for the pancreas, liver, skeletal muscle and white adipose tissue in response to environmental insults during development, might be intimately linked to changes in the developing brain (for review, see (McMillen and Robinson, 2005)). Thus, our results underline the importance to broaden our perspectives when analyzing morphological and cellular changes in offspring from obese, diabetic or malnourished mothers to both

peripheral organs and central neurocircuits to eventually draw a more complete picture of developmental defects underlying metabolic programming.

4.4 Nice study in rodents, but how is this all relevant to the human situation?

At present – due to technical and ethical limitations – there is no data in humans referring to the effects of altered maternal homeostasis during pregnancy on the development of hypothalamic neurocircuits in the fetus. Despite the lack of knowledge concerning malformations of hypothalamic neurocircuits in humans, epidemiological studies have demonstrated the existence of critical time windows of particular vulnerability for metabolic phenotype development in the offspring similar to what we report in rodents. Nutritional deprivation during different phases of pregnancy results in distinct metabolic impairments in children during adulthood. In retrospective studies conducted by Ravelli and colleagues, maternal malnutrition during the first trimester was shown to increase the offspring's incidences of obesity, whereas food scarcity during the third trimester were associated with impaired glucose tolerance and insulin resistance (Ravelli et al., 1998; Ravelli et al., 1976). Moreover, analyzing the effects of gestational diabetes mellitus (GDM) on the metabolic outcome of the offspring, independent of maternal BMI, provides the possibility to decipher the relative contribution of environmental insults on developmental processes occurring during the third trimester of pregnancy in humans, because GDM frequently manifests during exactly this period (Mumtaz, 2000). Although treatment of GDM protects the offspring from adverse pregnancy outcomes, poorly controlled GDM and maternal hyperglycemia below the official threshold for GDM diagnosis, are associated with increased incidences of neonatal macrosomia (Crowther et al., 2005; Metzger et al., 2008). Importantly, macrosomia is an early indicator for an increased risk to develop metabolic disorders later in life. These children further display neonatal hyperinsulinemia, pointing to a critical role of abnormal insulin in mediating these adverse pregnancy outcomes (Metzger et al., 2008). Newborns from obese mothers are insulin resistant as estimated by umbilical cord glucose and insulin concentrations (Catalano et al., 2009). This finding further supports the idea that abnormal insulin signaling during fetal development might also impact on organ plasticity and sensitivity in humans as it does in rodents.

In addition to this human epidemiological data, studies in non-human primates (NHP) provide substantial evidence that the developmental defects in the CNS seen in our model of metabolic programming might also be relevant for the accelerated propensity to develop metabolic disorders in response to impaired maternal health and nutrition in offspring from higher mammals. Apart from differences in the total duration of pregnancy between humans and NHPs, both phases of hypothalamic neurocircuit development occur in utero and follow the same pattern in terms of sequential events (Grove et al., 2005; Koutcherov et al., 2002). Similar to our findings in rodents, maternal HFD-feeding in NHPs is sufficient to predispose the offspring for metabolic disorders and is independent of maternal obesity (McCurdy et al., 2009). Moreover, maternal HFD-feeding during gestation in the NHP induces alterations in the central melanocortin circuitry and importantly, ARH neuronal axon formation (Grayson et al., 2010). However, due to the very low density of α-MSH fibers throughout the hypothalamus in both groups of offspring, the authors could not reliably quantify POMC neuronal innervation of the PVH. This technical limitation was a result of the early stage of pregnancy when the authors performed their analysis. Nevertheless, similar to our observations in rodents, AgRP fiber density was significantly decreased in the PVH and equally reduced throughout the hypothalamus in NHP offspring from HFD-fed mothers (Grayson et al., 2006). Based on these findings, it is conceivable that impaired ARH neuronal axon formation might be a common phenomenon underlying metabolic programming across species.

Due to the developmental differences between humans and rodents, a direct transfer of our results to the human situation warrants caution. Nevertheless, the existing human epidemiological data, as well as studies carried out in the NHP suggest that our findings concerning 1) the relative time-frame of particular developmental sensitivity, 2) the critical role of insulin in mediating these effects and 3) hypothalamic neurocircuit formation as a primary target for metabolic programming could also be used as a stepping stone in the battle to understand and prevent metabolic programming of obesity and T2DM in humans.

4.5 Conclusions

"Heavy mothers have heavy babies" – is one oversimplified conclusion from studies demonstrating that maternal obesity, diabetes and hyperglycemia during pregnancy and lactation have long-term effects on the offspring's future health prognoses (Plagemann, 2012; Sullivan and Grove, 2010). However, the cellular and molecular processes critically involved in this phenomenon often referred to as "metabolic programming" remain largely unknown. Our study not only shows that the lactation period in rodents is most sensitive to the altered developmental environment in response to maternal HFD-feeding, but importantly demonstrates that the short exposure to HFD during this distinct developmental phase is sufficient to predispose the offspring for metabolic disorders. This occurs, at least in part, by impairing ARH neuronal innervation of intrahypothalamic target areas. The critical impact of abnormal insulin signaling particularly on ARH neuronal axon formation and glucose homeostasis in the offspring of postnatally HFD-fed mothers further provides first in-depth mechanistic evidence underlining the critical role of hyperinsulinemia in the predisposition for metabolic disorders as suggested by Jorgen Pedersen already in 1971 (Pedersen, 1971). Moreover, the present study substantiates previous reports demonstrating the existence of highly specialized POMC neuronal subpopulations (Williams et al., 2010). According to our results, POMC neurons might not only diverge in their hormonal sensitivity, but may also differ in their specific target area innervation pattern and thus, also in their relative contribution to regulate different aspects of energy and glucose homeostasis. Since the increased adiposity and insulin resistance in offspring from postnatally HFD-fed mothers was not affected by POMC-specific IR-deficiency, one could speculate that these effects are likely attributable to developmental defects in response to abnormal insulin signaling in other neuronal populations or pathophysiological levels of other nutritional and/or hormonal signals, such as free fatty acids, leptin or ghrelin.

The multitude of studies including our own that clearly demonstrate that hypothalamic neurocircuit formation is impaired in response to diverse environmental insults, question the general validity of the "thrifty phenotype hypothesis" postulated by Barker and Hales. This concept suggests that key organs regulating energy homeostasis are developmentally programmed in response to the perinatal environment to provide the organism with the most efficient phenotype to survive under the same conditions

throughout lifetime that they had faced during development. However, in light of the aforementioned findings, it seems that these cellular and metabolic changes are developmental defects that prohibit an efficient regulation of energy and glucose homeostasis rather than developmental adaptations, as they would not be beneficial under any circumstances.

To eventually break through the vicious circle of impaired maternal metabolic homeostasis during pregnancy that in turn predisposes their children for metabolic and possibly mental health disorders, and who will eventually become parents themselves, more sensitive glucose tolerance screenings should be developed and performed in all pregnant women irrespective of their body mass index. Along with nutritional guidelines and well-controlled anti-diabetic therapies, these measures could prove valuable to combat the obesity and diabetes epidemics at their roots.

5 Summary

In light of the ever-increasing incidences of obesity and type 2 diabetes mellitus, development of novel therapeutic or preventative measures to combat these epidemics is of utmost importance. Identification of a causal relationship between an altered maternal metabolic homeostasis and an increased propensity for the unborn child to develop metabolic disorders throughout lifetime has shifted considerable amount of attention towards understanding the underlying cellular and molecular alterations. However, despite the multitude of studies already carried out in this field of research, to date we still know very little about the developmental alterations responsible for the increased propensity towards an impaired regulation of energy homeostasis.

By employing a thorough and physiological maternal high-fat diet-(HFD) feeding paradigm in mice, we could demonstrate that nutritional and hormonal alterations specifically during lactation predispose the offspring for obesity and impaired glucose homeostasis. These metabolic defects are associated with malformations of the hypothalamic melanocortin circuitry in the offspring. Whereas the number and neuropeptide expression of anorexigenic proopiomelanocortin (POMC) and orexigenic agouti-related peptide (AgRP) neurons, electrophysiological properties of POMC neurons and posttranslational processing of POMC to the active neurotransmitter α-MSH remain unaffected in response to maternal HFD-feeding during lactation, the formation of POMC and AgRP projections to hypothalamic target sites is severely impaired. Moreover, abrogating insulin action in POMC neurons of the offspring prevents altered POMC projections specifically to the preautonomic paraventricular nucleus of the hypothalamus (PVH), restores pancreatic parasympathetic innervation and improves glucose-stimulated insulin-secretion in response to maternal overnutrition.

Taken together, these experiments reveal a critical developmental period of particular vulnerability towards altered maternal metabolic homeostasis. An abnormal developmental environment during exactly this period impairs hypothalamic neuronal projections at least in part by abnormal neuronal insulin signaling and thereby contributes to the increased propensity to develop obesity and impaired glucose homeostasis.

6 Zusammenfassung

Angesichts der stetig wachsenden Zahl von Adipositas und Typ-2-Diabetes mellitus Erkrankungen steigt ebenfalls die Nachfrage bezüglich neuartiger, präventiver Behandlungsmethoden. Mit der Entdeckung eines kausalen Zusammenhangs zwischen diesen Stoffwechselkrankheiten, ungesunder Ernährung der Mutter während der Schwangerschaft und eines erhöhten Erkrankungsrisikos des ungeborenen Kindes wuchs auch das wissenschaftliche Interesse an einer Aufklärung der zugrundeliegenden Faktoren, die eine solche Prädisposition konstituieren. Trotz der Vielzahl an Studien, die sich dieser Aufklärung widmeten, sind die zellulären und molekularen Mechanismen, die für die beeinträchtigte Regulation der Energie- und Glukosehomöostase der Kinder adipöser und diabetischer Mütter verantwortlich sind, noch nicht vollständig identifiziert und verstanden worden. In der vorliegenden Arbeit konnten wir durch die Anwendung eines stringenten und physiologischen maternalen Fütterungsparadigmas in Mäusen zeigen, dass ein erhöhter Fettgehalt im Futter während der Stillphase ausreichend ist, um die Energiehomöostase des Nachwuchses nachhaltig zu stören. Des Weiteren konnten wir demonstrieren, dass den beobachteten metabolischen Veränderungen eine starke Fehlbildung des zentralen Melanocortin-Systems im Hypothalamus, das eine wichtige Rolle in der Regulation der Energie- und Glukosehomöostase spielt, zugrundeliegt. Obwohl sich ein erhöhter Fettgehalt im Futter während der postnatalen Entwicklungsphase weder auf die Anzahl noch auf die Neuropeptid-Expressionsprofile der hypothalamischen anorexigenen POMC und orexigenen AgRP/NPY Neurone auswirkt, findet die Innervierung dieser beiden Neuronenpopulationen von hypothalamischen Zielregionen nicht vollständig statt. Dieser Entwicklungsdefekt ist teilweise auf eine veränderte neuronale Insulin-vermittelte Zellantwort zurückzuführen, da eine spezifische Deletion des Insulin Rezeptors von POMC Neuronen die Dichte von POMC Nervenfasern im prä-autonomischen nucleus paraventricularis des Hypothalamus, sowie die parasympathische Innervierung des Pankreas wiederherstellt und die Glukose-stimulierte Insulin Sekretion verbessert. Zusammenfassend lässt sich aus unserer Studie schließen, dass eine Veränderung des hormonellen Milieus in Folge einer fettreichen Ernährung während einer kritischen Phase der hypothalamischen Entwicklung mit der Etablierung des zentralen Melanocortin-Systems interferiert. Ein Defekt dieser Entwicklungsprozesse, die sich im Menschen während des dritten Trimesters der Schwangerschaft ereignen, trägt zu einer lebenslangen Prädisposition für metabolische Krankheiten des Nachwuchses bei.

7 References

Ahima, R.S., Prabakaran, D., and Flier, J.S. (1998). Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. The Journal of clinical investigation *101*, 1020-1027.

Ahlsson, F., Gustafsson, J., Tuvemo, T., and Lundgren, M. (2007). Females born large for gestational age have a doubled risk of giving birth to large for gestational age infants. Acta Paediatr *96*, 358-362.

Ahren, B. (2000). Autonomic regulation of islet hormone secretion--implications for health and disease. Diabetologia *43*, 393-410.

Ahuja, S., Boylan, M., Hart, S., Román-Shriver, C., Spallholz, J., Pence, B., and Sawyer, B. (2011). Glucose and Insulin levels are increased in obese and overweight mothers' breast milk. Food and Nutrition Sciences *2*, 201-206.

Akaike, N., and Harata, N. (1994). Nystatin perforated patch recording and its applications to analyses of intracellular mechanisms. The Japanese journal of physiology *44*, 433-473.

Alberico, S., Montico, M., Barresi, V., Monasta, L., Businelli, C., Soini, V., Erenbourg, A., Ronfani, L., and Maso, G. (2014). The role of gestational diabetes, pre-pregnancy body mass index and gestational weight gain on the risk of newborn macrosomia: results from a prospective multicentre study. BMC pregnancy and childbirth *14*, 23.

Allen, Y.S., Adrian, T.E., Allen, J.M., Tatemoto, K., Crow, T.J., Bloom, S.R., and Polak, J.M. (1983). Neuropeptide Y distribution in the rat brain. Science *221*, 877-879.

Anand, B.K., and Brobeck, J.R. (1951). Localization of a "feeding center" in the hypothalamus of the rat. Proc Soc Exp Biol Med *77*, 323-324.

Anna, V., van der Ploeg, H.P., Cheung, N.W., Huxley, R.R., and Bauman, A.E. (2008). Sociodemographic correlates of the increasing trend in prevalence of gestational diabetes mellitus in a large population of women between 1995 and 2005. Diabetes care *31*, 2288-2293.

Antonopoulos, J., Dori, I., Dinopoulos, A., Chiotelli, M., and Parnavelas, J.G. (2002). Postnatal development of the dopaminergic system of the striatum in the rat. Neuroscience *110*, 245-256.

Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B., 3rd, Johnson, R.S., and Kahn, C.R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature *372*, 186-190.

Balasubramaniam, A.A. (1997). Neuropeptide Y family of hormones: receptor subtypes and antagonists. Peptides *18*, 445-457.

Balthasar, N., Coppari, R., McMinn, J., Liu, S.M., Lee, C.E., Tang, V., Kenny, C.D., McGovern, R.A., Chua, S.C., Jr., Elmquist, J.K., *et al.* (2004). Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron *42*, 983-991.

Balthasar, N., Dalgaard, L.T., Lee, C.E., Yu, J., Funahashi, H., Williams, T., Ferreira, M., Tang, V., McGovern, R.A., Kenny, C.D., *et al.* (2005). Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell *123*, 493-505.

Banks, A.S., Davis, S.M., Bates, S.H., and Myers, M.G., Jr. (2000). Activation of downstream signals by the long form of the leptin receptor. The Journal of biological chemistry *275*, 14563-14572.

Barker, D.J. (1990). The fetal and infant origins of adult disease. BMJ *301*, 1111.

Barker, D.J. (2004). Developmental origins of adult health and disease. Journal of epidemiology and community health *58*, 114-115.

Barker, D.J. (2007). The origins of the developmental origins theory. Journal of internal medicine *261*, 412-417.

Barker, D.J., Gluckman, P.D., Godfrey, K.M., Harding, J.E., Owens, J.A., and Robinson, J.S. (1993). Fetal nutrition and cardiovascular disease in adult life. Lancet *341*, 938-941.

Barker, D.J., and Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet *1*, 1077-1081.

Barker, D.J., Winter, P.D., Osmond, C., Margetts, B., and Simmonds, S.J. (1989). Weight in infancy and death from ischaemic heart disease. Lancet *2*, 577-580.

Baum, P., Petroff, D., Classen, J., Kiess, W., and Bluher, S. (2013). Dysfunction of autonomic nervous system in childhood obesity: a cross-sectional study. PloS one *8*, e54546.

Baura, G.D., Foster, D.M., Porte, D., Jr., Kahn, S.E., Bergman, R.N., Cobelli, C., and Schwartz, M.W. (1993). Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. The Journal of clinical investigation *92*, 1824-1830.

Bayol, S.A., Farrington, S.J., and 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. The British journal of nutrition *98*, 843-851.

Beck, B., Kozak, R., Moar, K.M., and Mercer, J.G. (2006). Hypothalamic orexigenic peptides are overexpressed in young Long-Evans rats after early life exposure to fat-rich diets. Biochemical and biophysical research communications *342*, 452-458.

Belgardt, B.F., and Bruning, J.C. (2010). CNS leptin and insulin action in the control of energy homeostasis. Annals of the New York Academy of Sciences *1212*, 97-113.

Belgardt, B.F., Husch, A., Rother, E., Ernst, M.B., Wunderlich, F.T., Hampel, B., Klockener, T., Alessi, D., Kloppenburg, P., and Bruning, J.C. (2008). PDK1 deficiency in POMC-expressing cells reveals FOXO1-dependent and -independent pathways in control of energy homeostasis and stress response. Cell metabolism *7*, 291-301.

Benedict, C., Kern, W., Schultes, B., Born, J., and Hallschmid, M. (2008). Differential sensitivity of men and women to anorexigenic and memory-improving effects of intranasal insulin. The Journal of clinical endocrinology and metabolism *93*, 1339-1344.

Bergman, R.N., Kim, S.P., Catalano, K.J., Hsu, I.R., Chiu, J.D., Kabir, M., Hucking, K., and Ader, M. (2006). Why visceral fat is bad: mechanisms of the metabolic syndrome. Obesity (Silver Spring) *14 Suppl 1*, 16S-19S.

Bernardis, L.L., and Bellinger, L.L. (1993). The lateral hypothalamic area revisited: neuroanatomy, body weight regulation, neuroendocrinology and metabolism. Neuroscience and biobehavioral reviews *17*, 141-193.

Berthoud, H.R., Bereiter, D.A., Trimble, E.R., Siegel, E.G., and Jeanrenaud, B. (1981). Cephalic phase, reflex insulin secretion. Neuroanatomical and physiological characterization. Diabetologia *20 Suppl*, 393-401.

Berthoud, H.R., and Jeanrenaud, B. (1982). Sham feeding-induced cephalic phase insulin release in the rat. The American journal of physiology *242*, E280-285.

Berthoud, H.R., Trimble, E.R., Siegel, E.G., Bereiter, D.A., and Jeanrenaud, B. (1980). Cephalic-phase insulin secretion in normal and pancreatic islet-transplanted rats. The American journal of physiology *238*, E336-340.

Betley, J.N., Cao, Z.F., Ritola, K.D., and Sternson, S.M. (2013). Parallel, redundant circuit organization for homeostatic control of feeding behavior. Cell *155*, 1337-1350.

Beverly, J.L., Beverly, M.F., and Meguid, M.M. (1995). Alterations in extracellular GABA in the ventral hypothalamus of rats in response to acute glucoprivation. The American journal of physiology *269*, R1174-1178.

Biag, J., Huang, Y., Gou, L., Hintiryan, H., Askarinam, A., Hahn, J.D., Toga, A.W., and Dong, H.W. (2012). Cyto- and chemoarchitecture of the hypothalamic paraventricular

nucleus in the C57BL/6J male mouse: a study of immunostaining and multiple fluorescent tract tracing. The Journal of comparative neurology *520*, 6-33.

Biddinger, S.B., and Kahn, C.R. (2006). From mice to men: insights into the insulin resistance syndromes. Annual review of physiology *68*, 123-158.

Bilbo, S.D., and Tsang, V. (2010). Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *24*, 2104-2115.

Bjorbaek, C., Elmquist, J.K., Frantz, J.D., Shoelson, S.E., and Flier, J.S. (1998). Identification of SOCS-3 as a potential mediator of central leptin resistance. Molecular cell *1*, 619-625.

Bluher, M., Michael, M.D., Peroni, O.D., Ueki, K., Carter, N., Kahn, B.B., and Kahn, C.R. (2002). Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. Developmental cell *3*, 25-38.

Boney, C.M., Verma, A., Tucker, R., and Vohr, B.R. (2005). Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics *115*, e290-296.

Bornstein, S.R., and Chrousos, G.P. (1999). Clinical review 104: Adrenocorticotropin (ACTH)- and non-ACTH-mediated regulation of the adrenal cortex: neural and immune inputs. The Journal of clinical endocrinology and metabolism *84*, 1729-1736.

Bouret, S.G. (2010). Neurodevelopmental actions of leptin. Brain research *1350*, 2-9.

Bouret, S.G. (2012). Nutritional programming of hypothalamic development: a critical periods and windows of opportunity. International Journal of Obesity Supplements *2*, S19-S24.

Bouret, S.G., Draper, S.J., and Simerly, R.B. (2004a). Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 2797-2805.

Bouret, S.G., Draper, S.J., and Simerly, R.B. (2004b). Trophic action of leptin on hypothalamic neurons that regulate feeding. Science *304*, 108-110.

Bouret, S.G., Gorski, J.N., Patterson, C.M., Chen, S., Levin, B.E., and Simerly, R.B. (2008). Hypothalamic neural projections are permanently disrupted in diet-induced obese rats. Cell metabolism *7*, 179-185.

Bouyer, K., and Simerly, R.B. (2013). Neonatal leptin exposure specifies innervation of presympathetic hypothalamic neurons and improves the metabolic status of leptin-deficient mice. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 840-851.

Broberger, C., Johansen, J., Johansson, C., Schalling, M., and Hokfelt, T. (1998). The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proceedings of the National Academy of Sciences of the United States of America *95*, 15043-15048.

Bronstein, D.M., Schafer, M.K., Watson, S.J., and Akil, H. (1992). Evidence that beta-endorphin is synthesized in cells in the nucleus tractus solitarius: detection of POMC mRNA. Brain research *587*, 269-275.

Bruch, H. (1993). The Frohlich syndrome: report of the original case. 1939. Obesity research *1*, 329-331.

Bruning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone, W., Muller-Wieland, D., and Kahn, C.R. (2000). Role of brain insulin receptor in control of body weight and reproduction. Science *289*, 2122-2125.

Bruning, J.C., Michael, M.D., Winnay, J.N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L.J., and Kahn, C.R. (1998). A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Molecular cell *2*, 559-569.

Buijs, R.M., Chun, S.J., Niijima, A., Romijn, H.J., and Nagai, K. (2001). Parasympathetic and sympathetic control of the pancreas: a role for the suprachiasmatic nucleus and other hypothalamic centers that are involved in the regulation of food intake. The Journal of comparative neurology *431*, 405-423.

Butler, A.A. (2006). The melanocortin system and energy balance. Peptides *27*, 281-290.

Calapai, G., Corica, F., Allegra, A., Corsonello, A., Sautebin, L., De Gregorio, T., Di Rosa, M., Costantino, G., Buemi, M., and Caputi, A.P. (1998). Effects of intracerebroventricular leptin administration on food intake, body weight gain and diencephalic nitric oxide synthase activity in the mouse. British journal of pharmacology *125*, 798-802.

Cannon, W.B. (1929). Organization for Physiological Homeostasis. Physiological reviews *9*.

Carmody, J.S., Wan, P., Accili, D., Zeltser, L.M., and Leibel, R.L. (2011). Respective contributions of maternal insulin resistance and diet to metabolic and hypothalamic phenotypes of progeny. Obesity (Silver Spring) *19*, 492-499.

Catalano, P.M., and Ehrenberg, H.M. (2006). The short- and long-term implications of maternal obesity on the mother and her offspring. BJOG : an international journal of obstetrics and gynaecology *113*, 1126-1133.

Catalano, P.M., Presley, L., Minium, J., and Hauguel-de Mouzon, S. (2009). Fetuses of obese mothers develop insulin resistance in utero. Diabetes care *32*, 1076-1080.

Chang, G.Q., Gaysinskaya, V., Karatayev, O., and Leibowitz, S.F. (2008). Maternal high-fat diet and fetal programming: increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 12107-12119.

Chao, P.T., Yang, L., Aja, S., Moran, T.H., and Bi, S. (2011). Knockdown of NPY expression in the dorsomedial hypothalamus promotes development of brown adipocytes and prevents diet-induced obesity. Cell metabolism *13*, 573-583.

Cheatham, B., and Kahn, C.R. (1995). Insulin action and the insulin signaling network. Endocrine reviews *16*, 117-142.

Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Guan, X.M., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L.*, et al.* (2000). Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nature genetics *26*, 97-102.

Chen, H., Simar, D., Lambert, K., Mercier, J., and Morris, M.J. (2008). Maternal and postnatal overnutrition differentially impact appetite regulators and fuel metabolism. Endocrinology *149*, 5348-5356.

Chen, P., Williams, S.M., Grove, K.L., and Smith, M.S. (2004). Melanocortin 4 receptor-mediated hyperphagia and activation of neuropeptide Y expression in the dorsomedial hypothalamus during lactation. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 5091-5100.

Chiu, S.L., and Cline, H.T. (2010). Insulin receptor signaling in the development of neuronal structure and function. Neural development *5*, 7.

Ciofi, P., Garret, M., Lapirot, O., Lafon, P., Loyens, A., Prevot, V., and Levine, J.E. (2009). Brain-endocrine interactions: a microvascular route in the mediobasal hypothalamus. Endocrinology *150*, 5509-5519.

Clark, J.T., Kalra, P.S., Crowley, W.R., and Kalra, S.P. (1984). Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology *115*, 427-429.

Claycombe, K.J., Jones, B.H., Standridge, M.K., Guo, Y., Chun, J.T., Taylor, J.W., and Moustaid-Moussa, N. (1998). Insulin increases fatty acid synthase gene transcription in human adipocytes. The American journal of physiology *274*, R1253-1259.

Cnattingius, S., Villamor, E., Johansson, S., Edstedt Bonamy, A.K., Persson, M., Wikstrom, A.K., and Granath, F. (2013). Maternal obesity and risk of preterm delivery. JAMA : the journal of the American Medical Association *309*, 2362-2370.

Cohen, P., Zhao, C., Cai, X., Montez, J.M., Rohani, S.C., Feinstein, P., Mombaerts, P., and Friedman, J.M. (2001). Selective deletion of leptin receptor in neurons leads to obesity. The Journal of clinical investigation *108*, 1113-1121.

Cone, R.D. (2006). Studies on the physiological functions of the melanocortin system. Endocrine reviews *27*, 736-749.

Cottrell, E.C., Cripps, R.L., Duncan, J.S., Barrett, P., Mercer, J.G., Herwig, A., and Ozanne, S.E. (2009). Developmental changes in hypothalamic leptin receptor: relationship with the postnatal leptin surge and energy balance neuropeptides in the postnatal rat. American journal of physiology Regulatory, integrative and comparative physiology *296*, R631-639.

Coupe, B., Amarger, V., Grit, I., Benani, A., and Parnet, P. (2010). Nutritional programming affects hypothalamic organization and early response to leptin. Endocrinology *151*, 702-713.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature *411*, 480-484.

Creemers, J.W., Lee, Y.S., Oliver, R.L., Bahceci, M., Tuzcu, A., Gokalp, D., Keogh, J., Herber, S., White, A., O'Rahilly, S.*, et al.* (2008). Mutations in the amino-terminal region of proopiomelanocortin (POMC) in patients with early-onset obesity impair POMC sorting to the regulated secretory pathway. The Journal of clinical endocrinology and metabolism *93*, 4494-4499.

Creemers, J.W., Pritchard, L.E., Gyte, A., Le Rouzic, P., Meulemans, S., Wardlaw, S.L., Zhu, X., Steiner, D.F., Davies, N., Armstrong, D.*, et al.* (2006). Agouti-related protein is posttranslationally cleaved by proprotein convertase 1 to generate agouti-related protein (AGRP)83-132: interaction between AGRP83-132 and melanocortin receptors cannot be influenced by syndecan-3. Endocrinology *147*, 1621-1631.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Crowther, C.A., Hiller, J.E., Moss, J.R., McPhee, A.J., Jeffries, W.S., and Robinson, J.S. (2005). Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. The New England journal of medicine *352*, 2477-2486.

Cui, H., Sohn, J.W., Gautron, L., Funahashi, H., Williams, K.W., Elmquist, J.K., and Lutter, M. (2012). Neuroanatomy of melanocortin-4 receptor pathway in the lateral hypothalamic area. The Journal of comparative neurology *520*, 4168-4183.

Dabelea, D., Hanson, R.L., Lindsay, R.S., Pettitt, D.J., Imperatore, G., Gabir, M.M., Roumain, J., Bennett, P.H., and Knowler, W.C. (2000). Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships. Diabetes *49*, 2208-2211.

Dabelea, D., and Pettitt, D.J. (2001). Intrauterine diabetic environment confers risks for type 2 diabetes mellitus and obesity in the offspring, in addition to genetic susceptibility. Journal of pediatric endocrinology & metabolism : JPEM *14*, 1085-1091.

Daftary, S.S., and Gore, A.C. (2004). The hypothalamic insulin-like growth factor-1 receptor and its relationship to gonadotropin-releasing hormones neurones during postnatal development. Journal of neuroendocrinology *16*, 160-169.

de Oliveira, J.C., Scomparin, D.X., Andreazzi, A.E., Branco, R.C., Martins, A.G., Gravena, C., Grassiolli, S., Rinaldi, W., Barbosa, F.B., and Mathias, P.C. (2011). Metabolic imprinting by maternal protein malnourishment impairs vagal activity in adult rats. Journal of neuroendocrinology *23*, 148-157.

Deierlein, A.L., Siega-Riz, A.M., Chantala, K., and Herring, A.H. (2011). The association between maternal glucose concentration and child BMI at age 3 years. Diabetes care *34*, 480-484.

Delahaye, F., Breton, C., Risold, P.Y., Enache, M., Dutriez-Casteloot, I., Laborie, C., Lesage, J., and Vieau, D. (2008). Maternal perinatal undernutrition drastically reduces postnatal leptin surge and affects the development of arcuate nucleus proopiomelanocortin neurons in neonatal male rat pups. Endocrinology *149*, 470-475.

Desai, M., Li, T., and Ross, M.G. (2011). Fetal hypothalamic neuroprogenitor cell culture: preferential differentiation paths induced by leptin and insulin. Endocrinology *152*, 3192-3201.

Doherty, D.A., Magann, E.F., Francis, J., Morrison, J.C., and Newnham, J.P. (2006). Pre-pregnancy body mass index and pregnancy outcomes. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics *95*, 242-247.

Dubos, R., Savage, D., and Schaedler, R. (1966). Biological Freudianism. Lasting effects of early environmental influences. Pediatrics *38*, 789-800.

Erickson, J.C., Clegg, K.E., and Palmiter, R.D. (1996). Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. Nature *381*, 415-421.

Farooqi, I.S. (2008). Monogenic human obesity. Frontiers of hormone research *36*, 1-11.

Fekete, C., Kelly, J., Mihaly, E., Sarkar, S., Rand, W.M., Legradi, G., Emerson, C.H., and Lechan, R.M. (2001). Neuropeptide Y has a central inhibitory action on the hypothalamic-pituitary-thyroid axis. Endocrinology *142*, 2606-2613.

Fekete, C., Legradi, G., Mihaly, E., Huang, Q.H., Tatro, J.B., Rand, W.M., Emerson, C.H., and Lechan, R.M. (2000). alpha-Melanocyte-stimulating hormone is contained in nerve terminals innervating thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and prevents fasting-induced suppression of prothyrotropin-releasing hormone gene expression. The Journal of neuroscience : the official journal of the Society for Neuroscience *20*, 1550-1558.

Forsdahl, A. (1977). Are poor living conditions in childhood and adolescence an important risk factor for arteriosclerotic heart disease? British journal of preventive & social medicine *31*, 91-95.

Franke, K., Harder, T., Aerts, L., Melchior, K., Fahrenkrog, S., Rodekamp, E., Ziska, T., Van Assche, F.A., Dudenhausen, J.W., and Plagemann, A. (2005). 'Programming' of orexigenic and anorexigenic hypothalamic neurons in offspring of treated and untreated diabetic mother rats. Brain research *1031*, 276-283.

Fukuda, M., Jones, J.E., Olson, D., Hill, J., Lee, C.E., Gautron, L., Choi, M., Zigman, J.M., Lowell, B.B., and Elmquist, J.K. (2008). Monitoring FoxO1 localization in chemically identified neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 13640-13648.

Geerling, J.C., Shin, J.W., Chimenti, P.C., and Loewy, A.D. (2010). Paraventricular hypothalamic nucleus: axonal projections to the brainstem. The Journal of comparative neurology *518*, 1460-1499.

Gerald, C., Walker, M.W., Criscione, L., Gustafson, E.L., Batzl-Hartmann, C., Smith, K.E., Vaysse, P., Durkin, M.M., Laz, T.M., Linemeyer, D.L., *et al.* (1996). A receptor subtype involved in neuropeptide-Y-induced food intake. Nature *382*, 168-171.

Glavas, M.M., Kirigiti, M.A., Xiao, X.Q., Enriori, P.J., Fisher, S.K., Evans, A.E., Grayson, B.E., Cowley, M.A., Smith, M.S., and Grove, K.L. (2010). Early overnutrition results in early-onset arcuate leptin resistance and increased sensitivity to high-fat diet. Endocrinology *151*, 1598-1610.

Gold, R.M. (1973). Hypothalamic obesity: the myth of the ventromedial nucleus. Science *182*, 488-490.

Gorski, J.N., Dunn-Meynell, A.A., Hartman, T.G., and Levin, B.E. (2006). Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance. American journal of physiology Regulatory, integrative and comparative physiology *291*, R768-778.

Gorski, J.N., Dunn-Meynell, A.A., and Levin, B.E. (2007). Maternal obesity increases hypothalamic leptin receptor expression and sensitivity in juvenile obesity-prone rats. American journal of physiology Regulatory, integrative and comparative physiology *292*, R1782-1791.

Gout, J., Sarafian, D., Mutel, E., Vigier, M., Rajas, F., Mithieux, G., Begeot, M., and Naville, D. (2010). Metabolic and melanocortin gene expression alterations in male offspring of obese mice. Molecular and cellular endocrinology *319*, 99-108.

Grant, W.F., Nicol, L.E., Thorn, S.R., Grove, K.L., Friedman, J.E., and Marks, D.L. (2012). Perinatal exposure to a high-fat diet is associated with reduced hepatic sympathetic innervation in one-year old male Japanese macaques. PloS one *7*, e48119.

Gravena, C., Andreazzi, A.E., Mecabo, F.T., Grassiolli, S., Scantamburlo, V.M., and Mathias, P.C. (2007). Protein restriction during lactation alters the autonomic nervous system control on glucose-induced insulin secretion in adult rats. Nutritional neuroscience *10*, 79-87.

Grayson, B.E., Allen, S.E., Billes, S.K., Williams, S.M., Smith, M.S., and Grove, K.L. (2006). Prenatal development of hypothalamic neuropeptide systems in the nonhuman primate. Neuroscience *143*, 975-986.

Grayson, B.E., Levasseur, P.R., Williams, S.M., Smith, M.S., Marks, D.L., and Grove, K.L. (2010). Changes in melanocortin expression and inflammatory pathways in fetal offspring of nonhuman primates fed a high-fat diet. Endocrinology *151*, 1622-1632.

Greenfield, J.R., and Campbell, L.V. (2008). Role of the autonomic nervous system and neuropeptides in the development of obesity in humans: targets for therapy? Current pharmaceutical design *14*, 1815-1820.

Gropp, E., Shanabrough, M., Borok, E., Xu, A.W., Janoschek, R., Buch, T., Plum, L., Balthasar, N., Hampel, B., Waisman, A.*, et al.* (2005). Agouti-related peptide-expressing neurons are mandatory for feeding. Nature neuroscience *8*, 1289-1291.

Gross, C.G. (1998). Claude Bernard and the Constrancy of the Internal Environment. History of Neuroscience *4*.

Grove, K.L., Grayson, B.E., Glavas, M.M., Xiao, X.Q., and Smith, M.S. (2005). Development of metabolic systems. Physiology & behavior *86*, 646-660.

Guillod-Maximin, E., Lorsignol, A., Alquier, T., and Penicaud, L. (2004). Acute intracarotid glucose injection towards the brain induces specific c-fos activation in hypothalamic nuclei: involvement of astrocytes in cerebral glucose-sensing in rats. Journal of neuroendocrinology *16*, 464-471.

Guo, F., and Jen, K.L. (1995). High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. Physiology & behavior *57*, 681-686.

Gupta, A., Srinivasan, M., Thamadilok, S., and Patel, M.S. (2009). Hypothalamic alterations in fetuses of high fat diet-fed obese female rats. The Journal of endocrinology *200*, 293-300.

Habbout, A., Li, N., Rochette, L., and Vergely, C. (2013). Postnatal overfeeding in rodents by litter size reduction induces major short- and long-term pathophysiological consequences. The Journal of nutrition *143*, 553-562.

Hales, C.N., and Barker, D.J. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia *35*, 595-601.

Hales, C.N., and Barker, D.J. (2001). The thrifty phenotype hypothesis. British medical bulletin *60*, 5-20.

Hales, C.N., Barker, D.J., Clark, P.M., Cox, L.J., Fall, C., Osmond, C., and Winter, P.D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. BMJ *303*, 1019-1022.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Archiv : European journal of physiology *391*, 85-100.

Havrankova, J., Roth, J., and Brownstein, M. (1978). Insulin receptors are widely distributed in the central nervous system of the rat. Nature *272*, 827-829.

Hess, W.R. (1957). The Functional Organization of the Diencephalon (New York: Grune & Stratton
).

Hetherington, A.W., and Ranson, S.W. (1940). Hypothalamic lesions and adipocity in the rat. Anat Record *78*.

Hetherington, M.M., and Cecil, J.E. (2010). Gene-environment interactions in obesity. Forum of nutrition *63*, 195-203.

Horn, R., and Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. The Journal of general physiology *92*, 145-159.

Horvath, T.L., Bechmann, I., Naftolin, F., Kalra, S.P., and Leranth, C. (1997). Heterogeneity in the neuropeptide Y-containing neurons of the rat arcuate nucleus: GABAergic and non-GABAergic subpopulations. Brain research *756*, 283-286.

Houseknecht, K.L., McGuire, M.K., Portocarrero, C.P., McGuire, M.A., and Beerman, K. (1997). Leptin is present in human milk and is related to maternal plasma leptin concentration and adiposity. Biochemical and biophysical research communications *240*, 742-747.

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.*, et al.* (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell *88*, 131-141.

IDF (2013). Diabetes: Facts and Figures. IDF Diabetes Atlas Sixth Edition, International Diabetes Federation 2013.

Ishii, Y., and Bouret, S.G. (2012). Embryonic birthdate of hypothalamic leptin-activated neurons in mice. Endocrinology *153*, 3657-3667.

Jackson, R.S., Creemers, J.W., Ohagi, S., Raffin-Sanson, M.L., Sanders, L., Montague, C.T., Hutton, J.C., and O'Rahilly, S. (1997). Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. Nature genetics *16*, 303-306.

Jansen, A.S., Hoffman, J.L., and Loewy, A.D. (1997). CNS sites involved in sympathetic and parasympathetic control of the pancreas: a viral tracing study. Brain research *766*, 29-38.

Joseph, S.A., and Michael, G.J. (1988). Efferent ACTH-IR opiocortin projections from nucleus tractus solitarius: a hypothalamic deafferentation study. Peptides *9*, 193-201.

Jovanovic-Peterson, L., Peterson, C.M., Reed, G.F., Metzger, B.E., Mills, J.L., Knopp, R.H., and Aarons, J.H. (1991). Maternal postprandial glucose levels and infant birth weight: the Diabetes in Early Pregnancy Study. The National Institute of Child Health and Human Development--Diabetes in Early Pregnancy Study. American journal of obstetrics and gynecology *164*, 103-111.

Kahn, S.E., Prigeon, R.L., McCulloch, D.K., Boyko, E.J., Bergman, R.N., Schwartz, M.W., Neifing, J.L., Ward, W.K., Beard, J.C., Palmer, J.P.*, et al.* (1993). Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function. Diabetes *42*, 1663-1672.

Kalk, P., Guthmann, F., Krause, K., Relle, K., Godes, M., Gossing, G., Halle, H., Wauer, R., and Hocher, B. (2009). Impact of maternal body mass index on neonatal outcome. European journal of medical research *14*, 216-222.

Kalsbeek, A., Voorn, P., Buijs, R.M., Pool, C.W., and Uylings, H.B. (1988). Development of the dopaminergic innervation in the prefrontal cortex of the rat. The Journal of comparative neurology *269*, 58-72.

Kas, M.J., Tiesjema, B., van Dijk, G., Garner, K.M., Barsh, G.S., ter Brake, O., Verhaagen, J., and Adan, R.A. (2004). Induction of brain-region-specific forms of obesity by agouti. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 10176-10181.

Kennedy, G.C. (1953). The role of depot fat in the hypothalamic control of food intake in the rat. Proc R Soc Lond B Biol Sci *140*, 578-596.

Kershaw, E.E., Hamm, J.K., Verhagen, L.A., Peroni, O., Katic, M., and Flier, J.S. (2006). Adipose triglyceride lipase: function, regulation by insulin, and comparison with adiponutrin. Diabetes *55*, 148-157.

Kim, M.S., Pak, Y.K., Jang, P.G., Namkoong, C., Choi, Y.S., Won, J.C., Kim, K.S., Kim, S.W., Kim, H.S., Park, J.Y., *et al.* (2006). Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nature neuroscience *9*, 901-906.

King, C.M., and Hentges, S.T. (2011). Relative number and distribution of murine hypothalamic proopiomelanocortin neurons innervating distinct target sites. PloS one *6*, e25864.

Kinouchi, T., Koizumi, K., Kuwata, T., and Yajima, T. (1998). Crucial role of milk-borne insulin in the development of pancreatic amylase at the onset of weaning in rats. The American journal of physiology *275*, R1958-1967.

Kirk, S.L., Samuelsson, A.M., Argenton, M., Dhonye, H., Kalamatianos, T., Poston, L., Taylor, P.D., and Coen, C.W. (2009). Maternal obesity induced by diet in rats permanently influences central processes regulating food intake in offspring. PloS one *4*, e5870.

Kitamura, T., Feng, Y., Kitamura, Y.I., Chua, S.C., Jr., Xu, A.W., Barsh, G.S., Rossetti, L., and Accili, D. (2006). Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. Nature medicine *12*, 534-540.

Klockener, T., Hess, S., Belgardt, B.F., Paeger, L., Verhagen, L.A., Husch, A., Sohn, J.W., Hampel, B., Dhillon, H., Zigman, J.M., *et al.* (2011). High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons. Nature neuroscience *14*, 911-918.

Knigge, K.M., Joseph, S.A., and Nocton, J. (1981). Topography of the ACTH-immunoreactive neurons in the basal hypothalamus of the rat brain. Brain research *216*, 333-341.

Komatsu, M., Takei, M., Ishii, H., and Sato , Y. (2013). Glucose-stimulated insulin secretion: A newer perspective. Journal of DIabetes Investiagation *4*.

Konner, A.C., Janoschek, R., Plum, L., Jordan, S.D., Rother, E., Ma, X., Xu, C., Enriori, P., Hampel, B., Barsh, G.S., *et al.* (2007). Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. Cell metabolism *5*, 438-449.

Korotkova, M., Gabrielsson, B., Hanson, L.A., and Strandvik, B. (2002a). Maternal dietary intake of essential fatty acids affects adipose tissue growth and leptin mRNA expression in suckling rat pups. Pediatric research *52*, 78-84.

Korotkova, M., Gabrielsson, B., Lonn, M., Hanson, L.A., and Strandvik, B. (2002b). Leptin levels in rat offspring are modified by the ratio of linoleic to alpha-linolenic acid in the maternal diet. Journal of lipid research *43*, 1743-1749.

Koutcherov, Y., Mai, J.K., Ashwell, K.W., and Paxinos, G. (2002). Organization of human hypothalamus in fetal development. The Journal of comparative neurology *446*, 301-324.

Koutcherov, Y., Mai, J.K., and Paxinos, G. (2003). Hypothalamus of the human fetus. Journal of chemical neuroanatomy *26*, 253-270.

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

Lakke, E.A., and Hinderink, J.B. (1989). Development of the spinal projections of the nucleus paraventricularis hypothalami of the rat: an intra-uterine WGA-HRP study. Brain research Developmental brain research *49*, 115-121.

Lee, E.J., Lee, S.H., Jung, J.W., Lee, W., Kim, B.J., Park, K.W., Lim, S.K., Yoon, C.J., and Baik, J.H. (2001). Differential regulation of cAMP-mediated gene transcription and ligand selectivity by MC3R and MC4R melanocortin receptors. European journal of biochemistry / FEBS *268*, 582-591.

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J., *et al.* (2007). Genome-wide atlas of gene expression in the adult mouse brain. Nature *445*, 168-176.

Lesniak, M.A., Hill, J.M., Kiess, W., Rojeski, M., Pert, C.B., and Roth, J. (1988). Receptors for insulin-like growth factors I and II: autoradiographic localization in rat brain and comparison to receptors for insulin. Endocrinology *123*, 2089-2099.

Levin, B.E., Becker, T.C., Eiki, J., Zhang, B.B., and Dunn-Meynell, A.A. (2008). Ventromedial hypothalamic glucokinase is an important mediator of the counterregulatory response to insulin-induced hypoglycemia. Diabetes *57*, 1371-1379.

Lin, H.V., Plum, L., Ono, H., Gutierrez-Juarez, R., Shanabrough, M., Borok, E., Horvath, T.L., Rossetti, L., and Accili, D. (2010). Divergent regulation of energy expenditure and hepatic glucose production by insulin receptor in agouti-related protein and POMC neurons. Diabetes *59*, 337-346.

Liu, Z., Cai, H., Zhang, P., Li, H., Liu, H., and Li, Z. (2012). Activation of ERK1/2 and PI3K/Akt by IGF-1 on GAP-43 expression in DRG neurons with excitotoxicity induced by glutamate in vitro. Cellular and molecular neurobiology *32*, 191-200.

Lloyd, D.J., Bohan, S., and Gekakis, N. (2006). Obesity, hyperphagia and increased metabolic efficiency in Pc1 mutant mice. Human molecular genetics *15*, 1884-1893.

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

Lucas, A. (1991). Programming by early nutrition in man. Ciba Foundation symposium *156*, 38-50; discussion 50-35.

Luquet, S., Perez, F.A., Hnasko, T.S., and Palmiter, R.D. (2005). NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. Science *310*, 683-685.

MacGregor, G.G., Dong, K., Vanoye, C.G., Tang, L., Giebisch, G., and Hebert, S.C. (2002). Nucleotides and phospholipids compete for binding to the C terminus of KATP channels. Proceedings of the National Academy of Sciences of the United States of America *99*, 2726-2731.

Maniam, J., and Morris, M.J. (2010). Palatable cafeteria diet ameliorates anxiety and depression-like symptoms following an adverse early environment. Psychoneuroendocrinology *35*, 717-728.

Marino, J.S., Xu, Y., and Hill, J.W. (2011). Central insulin and leptin-mediated autonomic control of glucose homeostasis. Trends in endocrinology and metabolism: TEM *22*, 275-285.

Marsh, D.J., Hollopeter, G., Kafer, K.E., and Palmiter, R.D. (1998). Role of the Y5 neuropeptide Y receptor in feeding and obesity. Nature medicine *4*, 718-721.

Marston, O.J., Garfield, A.S., and Heisler, L.K. (2011). Role of central serotonin and melanocortin systems in the control of energy balance. European journal of pharmacology *660*, 70-79.

McCurdy, C.E., Bishop, J.M., Williams, S.M., Grayson, B.E., Smith, M.S., Friedman, J.E., and Grove, K.L. (2009). Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. The Journal of clinical investigation *119*, 323-335.

McMillen, I.C., and Robinson, J.S. (2005). Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiological reviews *85*, 571-633.

Metzger, B.E., Lowe, L.P., Dyer, A.R., Trimble, E.R., Chaovarindr, U., Coustan, D.R., Hadden, D.R., McCance, D.R., Hod, M., McIntyre, H.D.*, et al.* (2008). Hyperglycemia and adverse pregnancy outcomes. The New England journal of medicine *358*, 1991-2002.

Millington, G.W., Tung, Y.C., Hewson, A.K., O'Rahilly, S., and Dickson, S.L. (2001). Differential effects of alpha-, beta- and gamma(2)-melanocyte-stimulating hormones on hypothalamic neuronal activation and feeding in the fasted rat. Neuroscience *108*, 437-445.

Minth, C.D., Bloom, S.R., Polak, J.M., and Dixon, J.E. (1984). Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine. Proceedings of the National Academy of Sciences of the United States of America *81*, 4577-4581.

Mirshamsi, S., Laidlaw, H.A., Ning, K., Anderson, E., Burgess, L.A., Gray, A., Sutherland, C., and Ashford, M.L. (2004). Leptin and insulin stimulation of signalling pathways in arcuate nucleus neurones: PI3K dependent actin reorganization and KATP channel activation. BMC neuroscience *5*, 54.

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.*, et al.* (1997). Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature *387*, 903-908.

Morris, M.J., and Chen, H. (2009). Established maternal obesity in the rat reprograms hypothalamic appetite regulators and leptin signaling at birth. Int J Obes (Lond) *33*, 115-122.

Morrison, C.D., Morton, G.J., Niswender, K.D., Gelling, R.W., and Schwartz, M.W. (2005). Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling. American journal of physiology Endocrinology and metabolism *289*, E1051-1057.

Mosinger, B., Placer, Z., and Koldovsky, O. (1959). Passage of insulin through the wall of the gastro-intestinal tract of the infant rat. Nature *184(Suppl 16)*, 1245-1246.

Mullis, K.B., and Faloona, F.A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in enzymology *155*, 335-350.

Mumtaz, M. (2000). Gestational diabetes mellitus. The Malaysian journal of medical sciences : MJMS *7*, 4-9.

Naef, L., Srivastava, L., Gratton, A., Hendrickson, H., Owens, S.M., and Walker, C.D. (2008). Maternal high fat diet during the perinatal period alters mesocorticolimbic dopamine in the adult rat offspring: reduction in the behavioral responses to repeated amphetamine administration. Psychopharmacology *197*, 83-94.

O'Donohue, T.L., Miller, R.L., and Jacobowitz, D.M. (1979). Identification, characterization and stereotaxic mapping of intraneuronal alpha-melanocyte stimulating hormone-like immunoreactive peptides in discrete regions of the rat brain. Brain research *176*, 101-123.

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

Olshansky, S.J., Passaro, D.J., Hershow, R.C., Layden, J., Carnes, B.A., Brody, J., Hayflick, L., Butler, R.N., Allison, D.B., and Ludwig, D.S. (2005). A potential decline in life expectancy in the United States in the 21st century. The New England journal of medicine *352*, 1138-1145.

Ong, Z.Y., and Muhlhausler, B.S. (2011). Maternal "junk-food" feeding of rat dams alters food choices and development of the mesolimbic reward pathway in the offspring. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *25*, 2167-2179.

Orozco-Solis, R., Matos, R.J., Guzman-Quevedo, O., Lopes de Souza, S., Bihouee, A., Houlgatte, R., Manhaes de Castro, R., and Bolanos-Jimenez, F. (2010). Nutritional programming in the rat is linked to long-lasting changes in nutrient sensing and energy homeostasis in the hypothalamus. PloS one *5*, e13537.

Padilla, S.L., Carmody, J.S., and Zeltser, L.M. (2010). Pomc-expressing progenitors give rise to antagonistic neuronal populations in hypothalamic feeding circuits. Nature medicine *16*, 403-405.

Padilla, S.L., Reef, D., and Zeltser, L.M. (2012). Defining POMC neurons using transgenic reagents: impact of transient Pomc expression in diverse immature neuronal populations. Endocrinology *153*, 1219-1231.

Palmiter, R.D. (2007). Is dopamine a physiologically relevant mediator of feeding behavior? Trends in neurosciences *30*, 375-381.

Pedersen, J. (1971). Diabetes mellitus and pregnancy: present status of the hyperglycaemia--hyperinsulinism theory and the weight of the newborn baby. Postgraduate medical journal, Suppl:66-67.

Pettitt, D.J., and Jovanovic, L. (2001). Birth weight as a predictor of type 2 diabetes mellitus: the U-shaped curve. Current diabetes reports *1*, 78-81.

Pham, M.T., Brubaker, K., Pruett, K., and Caughey, A.B. (2013). Risk of childhood obesity in the toddler offspring of mothers with gestational diabetes. Obstetrics and gynecology *121*, 976-982.

Pitkin, R.M., and Van Orden, D.E. (1974). Fetal effects of maternal streptozotocin-diabetes. Endocrinology *94*, 1247-1253.

Plagemann, A. (2012). Perinatal programming : the state of the art (Berlin ; Boston: De Gruyter).

Plagemann, A., Harder, T., Janert, U., Rake, A., Rittel, F., Rohde, W., and Dorner, G. (1999a). Malformations of hypothalamic nuclei in hyperinsulinemic offspring of rats with gestational diabetes. Developmental neuroscience *21*, 58-67.

Plagemann, A., Harder, T., Melchior, K., Rake, A., Rohde, W., and Dorner, G. (1999b). Elevation of hypothalamic neuropeptide Y-neurons in adult offspring of diabetic mother rats. Neuroreport *10*, 3211-3216.

Plagemann, A., Harder, T., Rake, A., Janert, U., Melchior, K., Rohde, W., and Dorner, G. (1999c). Morphological alterations of hypothalamic nuclei due to intrahypothalamic hyperinsulinism in newborn rats. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience *17*, 37-44.

Plagemann, A., Harder, T., Rake, A., Melchior, K., Rittel, F., Rohde, W., and Dorner, G. (1998). Hypothalamic insulin and neuropeptide Y in the offspring of gestational diabetic mother rats. Neuroreport *9*, 4069-4073.

Plagemann, A., Heidrich, I., Gotz, F., Rohde, W., and Dorner, G. (1992). Lifelong enhanced diabetes susceptibility and obesity after temporary intrahypothalamic hyperinsulinism during brain organization. Experimental and clinical endocrinology *99*, 91-95.

Plum, L., Ma, X., Hampel, B., Balthasar, N., Coppari, R., Munzberg, H., Shanabrough, M., Burdakov, D., Rother, E., Janoschek, R.*, et al.* (2006). Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. The Journal of clinical investigation *116*, 1886-1901.

Polonsky, K.S., Given, B.D., Hirsch, L.J., Tillil, H., Shapiro, E.T., Beebe, C., Frank, B.H., Galloway, J.A., and Van Cauter, E. (1988). Abnormal patterns of insulin secretion in non-insulin-dependent diabetes mellitus. The New England journal of medicine *318*, 1231-1239.

Prentki, M., and Nolan, C.J. (2006). Islet beta cell failure in type 2 diabetes. The Journal of clinical investigation *116*, 1802-1812.

Pritchard, L.E., and White, A. (2007). Neuropeptide processing and its impact on melanocortin pathways. Endocrinology *148*, 4201-4207.

Puigserver, P., Rhee, J., Donovan, J., Walkey, C.J., Yoon, J.C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D.*, et al.* (2003). Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. Nature *423*, 550-555.

Qian, S., Chen, H., Weingarth, D., Trumbauer, M.E., Novi, D.E., Guan, X., Yu, H., Shen, Z., Feng, Y., Frazier, E.*, et al.* (2002). Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. Molecular and cellular biology *22*, 5027-5035.

Qiu, J., Fang, Y., Ronnekleiv, O.K., and Kelly, M.J. (2010). Leptin excites proopiomelanocortin neurons via activation of TRPC channels. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 1560-1565.

Rae, J., Cooper, K., Gates, P., and Watsky, M. (1991). Low access resistance perforated patch recordings using amphotericin B. Journal of neuroscience methods *37*, 15-26.

Rakic, P., Cameron, R.S., and Komuro, H. (1994). Recognition, adhesion, transmembrane signaling and cell motility in guided neuronal migration. Current opinion in neurobiology *4*, 63-69.

Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nature protocols *2*, 1896-1906.

Ravelli, A.C., van der Meulen, J.H., Michels, R.P., Osmond, C., Barker, D.J., Hales, C.N., and Bleker, O.P. (1998). Glucose tolerance in adults after prenatal exposure to famine. Lancet *351*, 173-177.

Ravelli, G.P., Stein, Z.A., and Susser, M.W. (1976). Obesity in young men after famine exposure in utero and early infancy. The New England journal of medicine *295*, 349-353.

Raygada, M., Cho, E., and Hilakivi-Clarke, L. (1998). High maternal intake of polyunsaturated fatty acids during pregnancy in mice alters offsprings' aggressive behavior, immobility in the swim test, locomotor activity and brain protein kinase C activity. The Journal of nutrition *128*, 2505-2511.

Rayner, D.V., Dalgliesh, G.D., Duncan, J.S., Hardie, L.J., Hoggard, N., and Trayhurn, P. (1997). Postnatal development of the ob gene system: elevated leptin levels in suckling fa/fa rats. The American journal of physiology *273*, R446-450.

Recio-Pinto, E., and Ishii, D.N. (1984). Effects of insulin, insulin-like growth factor-II and nerve growth factor on neurite outgrowth in cultured human neuroblastoma cells. Brain research *302*, 323-334.

Recio-Pinto, E., and Ishii, D.N. (1988). Insulin and related growth factors: effects on the nervous system and mechanism for neurite growth and regeneration. Neurochemistry international *12*, 397-414.

Recio-Pinto, E., Rechler, M.M., and Ishii, D.N. (1986). Effects of insulin, insulin-like growth factor-II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *6*, 1211-1219.

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

Reusens, B., and Remacle, C. (2006). Programming of the endocrine pancreas by the early nutritional environment. The international journal of biochemistry & cell biology *38*, 913-922.

Rodriguez, E.M., Blazquez, J.L., and Guerra, M. (2010). The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: the former opens to the portal blood and the latter to the cerebrospinal fluid. Peptides *31*, 757-776.

Rofey, D.L., Kolko, R.P., Iosif, A.M., Silk, J.S., Bost, J.E., Feng, W., Szigethy, E.M., Noll, R.B., Ryan, N.D., and Dahl, R.E. (2009a). A longitudinal study of childhood depression and anxiety in relation to weight gain. Child psychiatry and human development *40*, 517-526.

Rofey, D.L., Szigethy, E.M., Noll, R.B., Dahl, R.E., Lobst, E., and Arslanian, S.A. (2009b). Cognitive-behavioral therapy for physical and emotional disturbances in adolescents with polycystic ovary syndrome: a pilot study. Journal of pediatric psychology *34*, 156-163.

Rossi, J., Santamaki, P., Airaksinen, M.S., and Herzig, K.H. (2005). Parasympathetic innervation and function of endocrine pancreas requires the glial cell line-derived factor family receptor alpha2 (GFRalpha2). Diabetes *54*, 1324-1330.

Rossi, M., Kim, M.S., Morgan, D.G., Small, C.J., Edwards, C.M., Sunter, D., Abusnana, S., Goldstone, A.P., Russell, S.H., Stanley, S.A., *et al.* (1998). A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. Endocrinology *139*, 4428-4431.

Rother, E., Kuschewski, R., Alcazar, M.A., Oberthuer, A., Bae-Gartz, I., Vohlen, C., Roth, B., and Dotsch, J. (2012). Hypothalamic JNK1 and IKKbeta activation and impaired early

postnatal glucose metabolism after maternal perinatal high-fat feeding. Endocrinology *153*, 770-781.

Sabin, M.A., and Shield, J.P. (2008). Childhood obesity. Frontiers of hormone research *36*, 85-96.

Sahu, A., Kalra, S.P., Crowley, W.R., and Kalra, P.S. (1988). Evidence that NPY-containing neurons in the brainstem project into selected hypothalamic nuclei: implication in feeding behavior. Brain research *457*, 376-378.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science *239*, 487-491.

Sainsbury, A., Rohner-Jeanrenaud, F., Cusin, I., Zakrzewska, K.E., Halban, P.A., Gaillard, R.C., and Jeanrenaud, B. (1997). Chronic central neuropeptide Y infusion in normal rats: status of the hypothalamo-pituitary-adrenal axis, and vagal mediation of hyperinsulinaemia. Diabetologia *40*, 1269-1277.

Sakayori, N., Kimura, R., and Osumi, N. (2013). Impact of lipid nutrition on neural stem/progenitor cells. Stem cells international *2013*, 973508.

Salsberry, P.J., and Reagan, P.B. (2005). Dynamics of early childhood overweight. Pediatrics *116*, 1329-1338.

Santini, F., Maffei, M., Pelosini, C., Salvetti, G., Scartabelli, G., and Pinchera, A. (2009). Melanocortin-4 receptor mutations in obesity. Advances in clinical chemistry *48*, 95-109.

Schaefer-Graf, U.M., Kjos, S.L., Kilavuz, O., Plagemann, A., Brauer, M., Dudenhausen, J.W., and Vetter, K. (2003). Determinants of fetal growth at different periods of pregnancies complicated by gestational diabetes mellitus or impaired glucose tolerance. Diabetes care *26*, 193-198.

Schechter, R., Abboud, M., and Johnson, G. (1999). Brain endogenous insulin effects on neurite growth within fetal rat neuron cell cultures. Brain research Developmental brain research *116*, 159-167.

Schulz, C., Paulus, K., Johren, O., and Lehnert, H. (2012). Intranasal leptin reduces appetite and induces weight loss in rats with diet-induced obesity (DIO). Endocrinology *153*, 143-153.

Shimada, M., and Nakamura, T. (1973). Time of neuron origin in mouse hypothalamic nuclei. Experimental neurology *41*, 163-173.

Shulman, R.J. (1990). Oral insulin increases small intestinal mass and disaccharidase activity in the newborn miniature pig. Pediatric research *28*, 171-175.

Silverman, B.L., Rizzo, T.A., Cho, N.H., and Metzger, B.E. (1998). Long-term effects of the intrauterine environment. The Northwestern University Diabetes in Pregnancy Center. Diabetes care *21 Suppl 2*, B142-149.

Simerly, R.B. (1995). Anatomical Substrates of hypothalamic integration. Academic Press.

Singh, B., Xu, Y., McLaughlin, T., Singh, V., Martinez, J.A., Krishnan, A., and Zochodne, D.W. (2012). Resistance to trophic neurite outgrowth of sensory neurons exposed to insulin. Journal of neurochemistry *121*, 263-276.

Smart, J.L., Tolle, V., Otero-Corchon, V., and Low, M.J. (2007). Central dysregulation of the hypothalamic-pituitary-adrenal axis in neuron-specific proopiomelanocortin-deficient mice. Endocrinology *148*, 647-659.

Snijder, M.B., van Dam, R.M., Visser, M., and Seidell, J.C. (2006). What aspects of body fat are particularly hazardous and how do we measure them? International journal of epidemiology *35*, 83-92.

Sohn, J.W., Harris, L.E., Berglund, E.D., Liu, T., Vong, L., Lowell, B.B., Balthasar, N., Williams, K.W., and Elmquist, J.K. (2013). Melanocortin 4 receptors reciprocally regulate sympathetic and parasympathetic preganglionic neurons. Cell *152*, 612-619.

Steculorum, S.M., and Bouret, S.G. (2011). Maternal diabetes compromises the organization of hypothalamic feeding circuits and impairs leptin sensitivity in offspring. Endocrinology *152*, 4171-4179.

Steculorum, S.M., Vogt, M.C., and Bruning, J.C. (2013). Perinatal programming of metabolic diseases: role of insulin in the development of hypothalamic neurocircuits. Endocrinology and metabolism clinics of North America *42*, 149-164.

Sullivan, E., Nousen, E., Chamlou, K., and Grove, K. (2012). The impact of maternal high-fat diet consumption on neural development and behavior of offspring. International Journal of Obesity Supplements *2*.

Sullivan, E.L., Grayson, B., Takahashi, D., Robertson, N., Maier, A., Bethea, C.L., Smith, M.S., Coleman, K., and 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. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 3826-3830.

Sullivan, E.L., and Grove, K.L. (2010). Metabolic imprinting in obesity. Forum of nutrition *63*, 186-194.

Sun, B., Purcell, R.H., Terrillion, C.E., Yan, J., Moran, T.H., and Tamashiro, K.L. (2012). Maternal high-fat diet during gestation or suckling differentially affects offspring leptin sensitivity and obesity. Diabetes *61*, 2833-2841.

Tartaglia, L.A. (1997). The leptin receptor. The Journal of biological chemistry *272*, 6093-6096.

Tarussio, D., Metref, S., Seyer, P., Mounien, L., Vallois, D., Magnan, C., Foretz, M., and Thorens, B. (2014). Nervous glucose sensing regulates postnatal beta cell proliferation and glucose homeostasis. The Journal of clinical investigation *124*, 413-424.

Thompson, R.H., and Swanson, L.W. (1998). Organization of inputs to the dorsomedial nucleus of the hypothalamus: a reexamination with Fluorogold and PHAL in the rat. Brain research Brain research reviews *27*, 89-118.

Tong, Q., Ye, C.P., Jones, J.E., Elmquist, J.K., and Lowell, B.B. (2008). Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. Nature neuroscience *11*, 998-1000.

Toran-Allerand, C.D., Ellis, L., and Pfenninger, K.H. (1988). Estrogen and insulin synergism in neurite growth enhancement in vitro: mediation of steroid effects by interactions with growth factors? Brain research *469*, 87-100.

Torloni, M.R., Betran, A.P., Daher, S., Widmer, M., Dolan, S.M., Menon, R., Bergel, E., Allen, T., and Merialdi, M. (2009a). Maternal BMI and preterm birth: a systematic review of the literature with meta-analysis. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet *22*, 957-970.

Torloni, M.R., Betran, A.P., Horta, B.L., Nakamura, M.U., Atallah, A.N., Moron, A.F., and Valente, O. (2009b). Prepregnancy BMI and the risk of gestational diabetes: a systematic review of the literature with meta-analysis. Obesity reviews : an official journal of the International Association for the Study of Obesity *10*, 194-203.

van de Wall, E., Leshan, R., Xu, A.W., Balthasar, N., Coppari, R., Liu, S.M., Jo, Y.H., MacKenzie, R.G., Allison, D.B., Dun, N.J., *et al.* (2008). Collective and individual functions of leptin receptor modulated neurons controlling metabolism and ingestion. Endocrinology *149*, 1773-1785.

van den Top, M., Lee, K., Whyment, A.D., Blanks, A.M., and Spanswick, D. (2004). Orexigen-sensitive NPY/AgRP pacemaker neurons in the hypothalamic arcuate nucleus. Nature neuroscience *7*, 493-494.

van Houten, M., Posner, B.I., Kopriwa, B.M., and Brawer, J.R. (1979). Insulin-binding sites in the rat brain: in vivo localization to the circumventricular organs by quantitative radioautography. Endocrinology *105*, 666-673.

Van Lieshout, R.J., Taylor, V.H., and Boyle, M.H. (2011). Pre-pregnancy and pregnancy obesity and neurodevelopmental outcomes in offspring: a systematic review. Obesity reviews : an official journal of the International Association for the Study of Obesity *12*, e548-559.

Vanhaesebroeck, B., and Alessi, D.R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. The Biochemical journal *346 Pt 3*, 561-576.

Vasudevan, A.R., Burns, A., and Fonseca, V.A. (2006). The effectiveness of intensive glycemic control for the prevention of vascular complications in diabetes mellitus. Treatments in endocrinology *5*, 273-286.

Wang, C., Li, Y., Wible, B., Angelides, K.J., and Ishii, D.N. (1992). Effects of insulin and insulin-like growth factors on neurofilament mRNA and tubulin mRNA content in human neuroblastoma SH-SY5Y cells. Brain research Molecular brain research *13*, 289-300.

Wardlaw, S.L. (2011). Hypothalamic proopiomelanocortin processing and the regulation of energy balance. European journal of pharmacology *660*, 213-219.

Waring, M.E., and Lapane, K.L. (2008). Overweight in children and adolescents in relation to attention-deficit/hyperactivity disorder: results from a national sample. Pediatrics *122*, e1-6.

Warne, J.P., and Xu, A.W. (2013). Metabolic transceivers: in tune with the central melanocortin system. Trends in endocrinology and metabolism: TEM *24*, 68-75.

Werner, H., and Leroith, D. (2014). Insulin and insulin-like growth factor receptors in the brain: Physiological and pathological aspects. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology.

Whitaker, R.C. (2004). Predicting preschooler obesity at birth: the role of maternal obesity in early pregnancy. Pediatrics *114*, e29-36.

WHO (2006). Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia. Report of WHO/IDF consultation.

WHO (2013a). Diabetes Fact Sheet No 312.

WHO (2013b). Diabetes Fact Sheet No. 312 World Health Organization.

WHO (2013c). Overweight and Obesity Fact Sheet No. 311.

Williams, K.W., Margatho, L.O., Lee, C.E., Choi, M., Lee, S., Scott, M.M., Elias, C.F., and Elmquist, J.K. (2010). Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 2472-2479.

Woods, S.C., Lotter, E.C., McKay, L.D., and Porte, D., Jr. (1979). Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature *282*, 503-505.

Wright, T., Langley-Evans, S.C., and Voigt, J.P. (2011a). The impact of maternal cafeteria diet on anxiety-related behaviour and exploration in the offspring. Physiology & behavior *103*, 164-172.

Wright, T.M., Fone, K.C., Langley-Evans, S.C., and Voigt, J.P. (2011b). Exposure to maternal consumption of cafeteria diet during the lactation period programmes feeding behaviour in the rat. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience *29*, 785-793.

Wu, Q., Boyle, M.P., and Palmiter, R.D. (2009). Loss of GABAergic signaling by AgRP neurons to the parabrachial nucleus leads to starvation. Cell *137*, 1225-1234.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.

Xu, A.W., Ste-Marie, L., Kaelin, C.B., and 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.

Yang, Y. (2011). Structure, function and regulation of the melanocortin receptors. European journal of pharmacology *660*, 125-130.

Yaswen, L., Diehl, N., Brennan, M.B., and Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nature medicine *5*, 1066-1070.

Ye, J.H., Zhang, J., Xiao, C., and Kong, J.Q. (2006). Patch-clamp studies in the CNS illustrate a simple new method for obtaining viable neurons in rat brain slices: glycerol replacement of NaCl protects CNS neurons. Journal of neuroscience methods *158*, 251-259.

Yu, Z., Han, S., Zhu, J., Sun, X., Ji, C., and Guo, X. (2013). Pre-pregnancy body mass index in relation to infant birth weight and offspring overweight/obesity: a systematic review and meta-analysis. PloS one *8*, e61627.

Zarjevski, N., Cusin, I., Vettor, R., Rohner-Jeanrenaud, F., and Jeanrenaud, B. (1993). Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity. Endocrinology *133*, 1753-1758.

Zhang, X., Zhang, G., Zhang, H., Karin, M., Bai, H., and Cai, D. (2008). Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. Cell *135*, 61-73.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature *372*, 425-432.

Zheng, H., Patterson, L.M., Phifer, C.B., and Berthoud, H.R. (2005). Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. American journal of physiology Regulatory, integrative and comparative physiology *289*, R247-258.

8 Acknowledgements

Thank you to the whole AG Brüning and AG Wunderlich for your expertise, help and fun times – you have made the last 4 (and a half...) years truly special!
Of course, there are some people that I particularly would like to thank:

Prof. Dr. Jens Brüning *Thank you* for – first of all – accepting me as a Bachelor/Masters/PhD student and for trusting me with the best project that I could have ever asked for, but most importantly *Thank you* for your endless support, contagious enthusiasm for science and encouragement over these last years!

Prof. Dr. Peter Kloppenburg *Thank you* for being in my thesis committee, for a great collaboration and very interesting discussions!

Prof. Dr. Carien Niessen *Thank you* for being in my thesis committee and for supporting me throughout these last years!

Hayley, Claudia, Johan, Sarah, Maite, Justus, Wundi and Beatrice *Thank you* for fun and happy times inside and outside the lab!

Brigitte *Danke* für Deine wunderschönen Stainings und dafür, dass Du mich in die dunklen (bzw. fluoreszierenden) Geheimnisse der Immunohistochemie eingeweiht hast! Deine Unterstützung und Freundschaft haben mir sehr oft geholfen!

Chrissie *Danke* dafür, dass Du mich sogar bei den einfachsten wissenschaftlichen Fragen nicht ausgelacht hast, für Deine Unterstützung, und Dein Verständnis, das keine Worte brauchte. Ich bin unendlich dankbar, dass Du meine Laborpartnerin warst und sich daraus eine so tolle Freundschaft entwickelt hat!

Sophie *Thank you* for endless scientific and personal discussions and your willingness to teach me everything you know (which is quite a lot), without you, this project would have

never blossomed the way it did. You are a true friend and I hope we will always stay in touch!

Jesse *Thank you* for still being the best friend one could ever ask for although you basically know everything about me. You've been there for me throughout these years and I will never forget that! Also, thank you for your impeccable editing and correcting skills (I would have actually needed it for this section as well...).

Mama und Papa, ich hoffe Ihr wisst, wie unendlich dankbar ich für Eure kompromisslose Unterstützung bin! *Danke*, dass Ihr mir jegliche Freiheiten gelassen habt und doch immer für mich da gewesen seid, wenn ich Euch gebraucht habe. Danke, dass Ihr mir immer das Gefühl (und die nötige finanzielle Unterstützung) gegeben habt alles was ich mir vorgenommen habe schaffen zu können. Ohne Euren Rückhalt hätte ich das hier alles nicht geschafft.

Malte und Moritz, *Danke* dafür, dass Ihr seid wie Ihr seid und ich mich wirklich immer auf Euch verlassen kann! Ohne Euch wäre alles so viel langweiliger, trister, komplizierter und schwieriger. Ich hoffe Ihr wisst, wie einzigartig und wichtig Ihr für mich seid! Ich warte übrigens täglich auf den ersten Comic oder die erste Serie über Euch....

Und natürlich Janus... *Danke* für Deine unglaubliche Geduld mit mir, Dein Verständnis, Deine grenzenlose Unterstützung und Dein Vertrauen in mich. Danke für Deine Fähigkeit mich jederzeit lauthals zum Lachen zu bringen und dafür, dass Du mich aus meinen wirren Gedanken zurück in die Realität holen kannst. Aber vor allem Danke für Deine liebevolle Art und Zuneigung! Ich freue mich schon so sehr auf unsere gemeinsame Zukunft – wo auch immer wir hingehen werden...

9 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mit vorgelegte Dissertation ist von Prof. Dr. Jens C. Brüning betreut worden.

Köln, März 2014 Merly Carlotta Vogt

Teilpublikation

Vogt, M.C., Paeger, L., Hess, S., Steculorum, S.M., Awazawa, M., Hampel, B., Neupert, S., Nicholls, H.T., Mauer, J., Hausen, A.C., *et al.* (2014). Neonatal Insulin Action Impairs Hypothalamic Neurocircuit Formation in Response to Maternal High-Fat Feeding. Cell *156*, 495-509.