Hypothalamic Inflammation in Obesity, Insulin Resistance and Ageing

Inaugural-Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von Eva Tsaousidou

aus Thessaloniki

Köln 2015

Berichterstatter: Prof. Dr. Jens C. Brüning
Prof. Dr. Sigrun I. Korsching

Tag der mündlichen Prüfung: 20. April 2015

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Abbreviations

aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AgRP	agouti-related peptide
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP	action potential
ApoE	apolipoprotein E
ARC	arcuate nucleus
ATM	adipose tissue macrophage
ATP	adenosine triphosphate
BAT	brown adipose tissue
BBB	blood-brain-barrier
BMI	body mass index
BSA	bovine serum albumin
CA	constitutively active
CNS	central nervous system
Cre	site-specific recombinase from phage 1 (causes recombination)
CRH	corticotropin-releasing hormone
DAPI	4', 6-diamidino-2-phenylindole
DIO	diet-induced obesity
DMH	dorsomedial nucleus of the hypothalamus
DNA	deoxyribonucleic acid
DREADD	designer receptors exclusively activated by designer drugs
GFP	green fluorescent protein
ER	endoplasmic reticulum
FFA	free fatty acid
fl	floxed
FoxO	forkhead box
g	gram
Gab	Grb2 protein-associated binder
GABA	γ-aminobutyric acid
GLP1R	glucagon-like peptide 1 receptor
GLUT	glucose transporter
Grb	growth factor receptor binding
GTP	guanosine triphosphate
GTT	glucose tolerance test
GWAS	genome-wide association studies
h	hour
HFD	high-fat diet
HPA	hypothalamic-pituitary-adrenal axis
HRP	horse-radish peroxidase
ICV	intracerebroventricular
IGF1	insulin-like growth factor 1
IκB	inhibitor of NFκB
IKK	IκB kinase
IKK2CA	IKK2 constitutively active
IL	interleukin

IR	insulin receptor
IRS	insulin receptor substrate
ITT	insulin tolerance test
JAK2	janus kinase 2
JNK	c-Jun N-terminal kinase
JNK1CA	JNK1 consitutively active
KATP	ATP-dependent potassium channel
L	liter
LepR	leptin receptor
LH	lateral hypothalamic area
LPS	lipopolysaccharide
m	milli
MAPK	mitogen-activated protein kinase
MC(1-5)R	melanocortin 1-5 receptor
min	minute
mTOR	mammalian target of rapamycin
n	nano
NCD	normal chow diet
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
PBN	parabrachial nucleus
PBS	phosphate buffered saline
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
PCK	protein kinase C
POMC	proopiomelanocortin
PVH	paraventricular nucleus of the hypothalamus
PTP1B	protein-tyrosince phosphatase 1B
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SEM	standard error of the mean
SFA	spike frequency adaptation
SFO	subfornical organ
SOCS3	suppressor of cytokine signalling 3
STAT	signal transducer of transcription
TLR	toll-like receptor
TNF	tumor necrosis factor
TRH	thyrotropin-releasing hormone
UPR	unfolded protein response
VMH	ventromedial nucleus of the hypothalamus
WHO	World Health Organization
α-MSH	α-melanocyte-stimulating hormone
µ	micro

Abstract

In this study, the role of hypothalamic inflammation in obesity, insulin resistance and the regulation of the ageing process is investigated. Activation of c-Jun N-terminal kinase (JNK)1- and inhibitor of nuclear factor kappa-B kinase (IKK)2-dependent signalling plays a crucial role in the development of obesity-associated insulin and leptin resistance not only in peripheral tissues but also in the CNS. This study demonstrates that constitutive JNK1 activation in agouti-related peptide (AgRP)-expressing neurons of the hypothalamus is sufficient to induce weight gain and adiposity in mice as a consequence of hyperphagia. JNK1 activation increases spontaneous action potential firing of AgRP cells and causes both neuronal leptin resistance in a molecular level and resistance in the anorexigenic and body weight regulating effects of leptin. Similarly, activation of IKK2 signalling in AgRP neurons also increases firing of these cells but fails to cause obesity and leptin resistance. In contrast to JNK1 activation, IKK2 activation blunts insulin signalling in AgRP neurons and impairs systemic glucose homeostasis. Collectively, these experiments reveal both overlapping and non-redundant effects of JNK- and IKK-dependent signalling in AgRP neurons, which cooperate in the manifestation of the metabolic syndrome.

JNK1 ablation in the CNS has been demonstrated to resemble the effects of caloric restriction, a dietary intervention that delays ageing. In this study, JNK1 ablation in the CNS results in extended median and maximum lifespan in mice, protection from high-fat diet induced insulin resistance and increased energy expenditure but also increased adiposity and decreased bone mineral density. Hypothalamic inflammation amelioration via JNK1 and/or IKK2 inhibition are potential future therapeutic targets to counteract obesity- and ageing-associated diseases.

Zusammenfassung

In dieser Studie wird die Rolle von hypothalamischer Entzündung bei Adipositas, Insulinresistenz und der Regulation von Alterungsprozessen untersucht. Die Aktivierung der c-Jun N-terminal Kinase (JNK)1 und die der Inhibitor der Nuklear-Faktor-Kappa-B Kinase (IKK)2-abhängigen Signalübertragung spielt bei der Entwicklung von adipositasassoziierter Insulin- und Leptinresistenz in peripheren Geweben aber auch im ZNS eine entscheidende Rolle. Diese Arbeit zeigt, dass konstitutive JNK1-Aktivierung in Agouti-assoziierten Peptid (AgRP)-exprimierenden Neuronen des Hypothalamus ausreichend ist um Gewichtszunahme und Adipositas in Mäusen aufgrund von Hyperphagie zu induzieren. Die Aktivierung von JNK1 erhöht spontane Aktionspotentialimpulse der AgRP-Zellen und verursacht sowohl neuronale Leptinresistenz auf molekularer Ebene als auch Resistenzen des appetitzügelnden sowie körpergewichtsregulierenen Effektes von Leptin. Ebenso erhöht die Aktivierung der IKK2-Signalübertragung das Feuern in AgRP-Neuronen, verursacht jedoch weder Adipositas noch Leptinresistenz. Im Gegensatz zur JNK1-Aktivierung dämpft die Aktivierung von IKK2 Insulinsignalübertragung in AgRP-Neuronen und beeinträchtigt die systemische Glucosehomöostase. Zusammengefasst zeigen die Experimente sowohl den überlappenden als auch nichtredundanten Effekt der JNK- und IKK abhängigen Signalübertragung in AgRP-Neuronen, welche kooperativ zur der Erscheinungsform des metabolischen Syndroms beitragen.

Es wurde gezeigt, dass JNK1-Ablation im ZNS den Effekten von Kalorienrestriktion, einer den Alterungsprozess verlangsamenden Ernährungsintervention gleicht. In dieser Studie führt JNK1-Ablation im ZNS zu erhöhter mittlerer und maximaler Lebensdauer, Schutz vor von fettreicher Ernährung induzierter Insulinresistenz sowie Energieverbrauch, jedoch auch zu erhöhter Adipositas sowie geringerer Knochendichte. Eine Verbesserung der hypothalamischen Entzündung durch Inhibierung von JNK1 und IKK2 stellen potentielle therapeutische Ziele dar um adipositas- und altersassoziierter Krankheiten entgegenzuwirken.

1 Introduction

1.1 Obesity

Overweight, defined as body mass index (BMI) greater or equal to 25 kg/m^2, and obesity, BMI greater or equal to 30 kg/m^2, are conditions of excess fat accumulation that affect more than one third of the global adult population [source: World Health Organization (WHO), 2015].

Obesity and its associated pathologies, such as type 2 diabetes and cardiovascular diseases (Katzmarzyk et al., 2003), have reached epidemic proportions enhancing the necessity to develop effective therapeutic strategies against them. Various environmental factors, including consumption of energy-dense food and sedentary lifestyle, have been held responsible for the obesity epidemic, however the genome-wide association studies (GWAS) and studies in families and twins (Farooqi and O'Rahilly, 2006) revealed that the truth is also lying in our genes (Locke et al., 2015) and the epigenetic modifications we carry (Slomko et al., 2012). In relatively similar environmental backgrounds some individuals are more prone to develop obesity and obesity-associated diseases and the effect of dietary interventions has been proven to be ineffective in the long-run for obese patients as 90% of them return to their initial body weight in a 5-year period (Safer, 1991).

Obesity is not only a major health concern but also an immense economic burden with the health care costs for obesity and its associated diseases expanding every year, reaching an annual impact of 2 trillion dollars globally, paralleling expenses associated with smoking and war according to the WHO. Collectively, obesity is a global threat and its initiation and manifestation arise from interactions between multiple genes and the environment that in turn affect behaviour and energy homeostasis.

1.2 Energy homeostasis

Life is a process that relies on energy. Energy homeostasis is the balance between energy intake and expenditure to ensure steady body weight and its dysregulation can lead to excessive fat accumulation, and eventually obesity.

In order to ensure steady body weight orexigenic, promoting appetite, and anorexigenic, suppressing appetite, hormones have been identified to communicate signals of energy status in mammals. Two of the most important hormones for energy homeostasis regulation are insulin and leptin.

Insulin is secreted by pancreatic β-cells in response to increased blood glucose levels to promote glucose uptake and suppress hepatic glucose production and also to regulate glycogenesis, lipogenesis and protein synthesis (Roth et al., 2012). Upon increased blood glucose concentrations, glucose enters the β-cells, it is metabolized into adenosine triphosphate (ATP) resulting in the closure of ATP-dependent potassium (KATP) channels, cell depolarization and subsequent influx of Ca^{2+} ions which in turn leads to the exocytosis of insulin-containing vesicles and the release of insulin into the circulation (Roth et al., 2012; Taniguchi et al., 2006). In the prediabetic state, the β-cells produce more insulin, resulting in hyperinsulinemia, to compensate for the decreased efficacy of insulin in exerting its effects, a condition termed insulin resistance. The β-cells cannot sustain the excessive insulin secretion for long, as it leads to their exhaustion, impaired glucose homeostasis and the development of type 2 diabetes (Muoio and Newgard, 2008).

Leptin is anorexigenic, secreted from adipocytes in proportion to the adiposity of an organism communicating the current energy status to the central nervous system (CNS) (Friedman and Mantzoros, 2015). Importantly, leptin deficiency (Halaas et al., 1995; Ozata et al., 1999) as well as lack of a functional leptin receptor (Clément et al., 1998; Montague et al., 1997; Zhang et al., 1994) in mice and humans results in morbid obesity. Although initially leptin was considered a potential treatment against obesity, it has been demonstrated that obese individuals have increased plasma leptin levels and develop resistance to its anorexigenic action (Friedman, 2011).

Insulin and leptin communicate their signals to the central nervous system to regulate energy and glucose homeostasis (Cummings and Overduin, 2007; Belgardt and Brüning, 2010). Leptin and insulin signalling in the regulation of energy and glucose homeostasis will be introduced further in detail.

1.2.1 Insulin signalling pathway

Upon binding of insulin to the insulin receptor (IR) the insulin signalling cascade is activated [reviewed in (Guo, 2014)]. The insulin receptor consists of two

heterodimers of the α- and β- subunits, which are products of a single gene and are derived from the same polypeptide that is subjected to proteolytic processing. The insulin-binding site is on the α-subunits and the insulin-regulated tyrosine kinase activity domain is on the β-subunits. Conformational changes upon binding of insulin to the α-subunits of the IR activate the tyrosine kinase activity of the β-subunits resulting in autophosphorylation of tyrosine residues and in turn recruitment of the insulin receptor substrates (IRS1-4) and the growth factor receptor binding (Grb)2 protein-associated binder (Gab) proteins. IRS proteins, when phosphorylated, are docking platforms for the phosphatidylinositol 3 kinase (PI3K), Grb2 and the SH2 domain containing phosphatase (Shp)-2. PI3K phosphorylates phosphatidylinositol-(4,5)-bisphophate (PIP2) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) and PIP3 in turn activates downstream targets including the Akt, which co-localizes with phosphoinositide-dependent protein kinase 1 (PDK1) leading to phosphorylation and activation of Akt. Akt, among other proteins, can phosphorylate FoxO1 thereby triggering its translocation out of the nucleus, thus regulating gene transcription (Figure 1). Activation of the IRS-PI3K cascade is crucial for most of insulin's well-known effects, including glucose transporter translocation, glycogen synthesis, protein synthesis and the regulation of gene transcription [reviewed in (Boucher et al., 2014)].

1.2.2 Leptin signalling pathway

Leptin signalling is initiated upon binding of leptin to its receptor (LepR) to initiate the JAK/STAT pathway, among others. Activation of LepRb, the long form of leptin receptor mediating its anorexigenic effects (de Luca et al., 2005), leads to recruitment of JAK2, which phosphorylates the receptor and itself. Phosphorylated LepRb recruits and phosphorylates STAT3, triggering STAT3 dimerization and nuclear localization, where it regulates gene expression (Figure 1). Leptin has been also demonstrated to trigger the mitogen-activated protein kinase (MAPK), adenosine monophosphate-activated protein kinase (AMPK) and PI3K pathways (Figure 1) [reviewed in (Bjørbaek and Kahn, 2004)].

1.3 CNS in energy homeostasis regulation

The major orchestrator of energy homeostasis is the hypothalamus. The importance of the hypothalamus in energy homeostasis regulation has been demonstrated since the 1940s with the first lesion experiments in rats from Hetherington and Ranson (Hetherington and Ranson, 1940). The hypothalamus is connected to brain areas that affect behaviours such as reward and food foraging either directly or through inter-neurons (Heisler et al., 2003; Leinninger et al., 2009).

The major hypothalamic areas responsible for energy homeostasis are situated around the third ventricle and above the median eminence and are the ventromedial hypothalamus (VMH), dorsomedial hypothalamic nucleus (DMH), paraventricular nucleus (PVN), lateral hypothalamic nucleus (LH) and the arcuate nucleus (ARC). The hypothalamus is a region sensitive to blood-born signals due to a permeable blood-brain-barrier (BBB), and to its position adjacent to the third ventricle, thus allowing hypothalamic communication with the periphery. The VMH is very important in the feeding response to insulin-induced hypoglycemia and is home to many glucose sensing neurons that can be glucose-excited or glucose-inhibited (Verberne et al., 2014). The DMH is a target site of projections from other hypothalamic regions and is pivotal to appetite regulation, circadian rhythm and thermogenesis (Chao et al., 2011; Chou et al., 2003). The PVN regulates the hypothalamic-pituitary-adrenal axis (HPA) by the release of corticotropin-releasing hormone (CRH) in response to stress and thyrotropin-releasing hormone (TRH) and also modulates the autonomic nervous system (Biag et al., 2012). Furthermore, the PVN harbours second-order neurons with melanocortin 4 receptors (MC4R) regulated by neurons of the arcuate nucleus. The LH is responsible for circadian rhythm (Goodless-Sanchez et al., 1991) and additionally for hedonic and reward-related behaviours (Sternson, 2013). The ARC is the best characterized hypothalamic region concerning energy homeostasis and will be discussed further in detail.

1.3.1 Arcuate nucleus in energy homeostasis regulation

Multiple neuronal populations regulate food intake and energy expenditure via tight-coordination in intra- and extra-hypothalamic brain areas and the most thoroughly studied hypothalamic region for energy homeostasis is the ARC adjacent to the third ventricle and over the median eminence. The ARC is considered a circumventricular

organ due to its position, a permeable blood-brain barrier (BBB) and extensive vasculature.

In the ARC two neuronal populations with opposing roles reside, the orexigenic neuropeptide Y (NPY) and Agouti-related peptide (AgRP)-co-expressing neurons and the anorexigenic pro-opiomelanocortin (POMC) neurons. The AgRP and POMC neurons are first-order neurons that receive diverse circulating signals relevant to energy homeostasis, such as hormones and nutrients, from the periphery. AgRP and POMC neurons act on second-order neurons with MC4R residing predominantly in the PVN forming the melanocortin system (Cowley et al., 1999; Elmquist et al., 1999). The anorexigenic POMC neurons activate MC4R-expressing neurons to decrease food intake and increase energy expenditure whereas the AgRP neurons increase food intake and decrease energy expenditure by antagonizing POMC action on MC4R (Kim et al., 2014b). Importantly, *Mc4r* mutations are one of the most common monogenic mutations resulting in obesity in humans (Yeo et al., 1998).

A variety of proteins, including α-, β- and γ-melanocyte stimulating hormone (MSH) and adrenocorticotrophin (ACTH), are generated by the *Pomc* gene encoding the protein precursor Pro-opiomelanocortin which undergoes post-translational modification processes and mutations of the gene result in obesity (Krude et al., 1998). The best-studied anorexigenic peptide mediating its effects on energy expenditure and food intake is α-MSH by binding and activating MC4Rs (Ollmann et al., 1997). POMC-deficient mice are obese (Yaswen et al., 1999) and acute ablation of POMC neurons in adult mice results in hyperphagia and obesity (Gropp et al., 2005). Interestingly, early postnatal ablation of POMC neurons results in decreased food intake but also decreased energy expenditure resulting in an obese phenotype (Greenman et al., 2013).

The orexigenic AgRP neurons integrate peripheral signals and release NPY, AgRP and the inhibitory neurotransmitter γ-aminobutyric acid (GABA) to mediate their effects on other neuronal populations and in turn exert behavioural responses. AgRP and NPY bind to MC4Rs and NPYRs, respectively, to inhibit the activity of MC4R-expressing neurons (Gerald et al., 1996; Cowley et al., 1999) through G-protein dependent and independent pathways (Ghamari-Langroudi et al., 2015). Ablation of AgRP neurons in adult mice induces hypophagia and starvation (Gropp et al., 2005; Luquet et al., 2005; Luquet et al., 2007) but surprisingly, early postnatal ablation or *AgRP* mutations result in unchanged food intake and body weight

(Erickson et al., 1996; Gropp et al., 2005; Luquet et al., 2005; Qian et al., 2002). In order to succumb the absence of functional AgRP neurons compensatory mechanisms are proposed to be taking place during development (Wu and Palmiter, 2011; Wu et al., 2012) and the importance of GABA as a pivotal inhibitory neurotransmitter is pointed out. GABAergic signalling by AgRP neurons through direct synaptic innervations in the ARC inhibits POMC neurons (Cowley et al., 2001; Horvath et al., 1997) and the parabranchial nucleus (PBN) in the hindbrain (Wu et al., 2009). Additionally, the oxytocin (OXT) and Sim1 neurons in the PVN receive GABAergic input from the AgRP neurons (Atasoy et al., 2012; Krashes et al., 2014). Furthermore, AgRP neuron-specific deletion of vesicular GABA transporter genes results in a lean phenotype and protection from HFD-induced obesity demonstrating the importance of GABAergic signalling (Tong et al., 2008). Although the AgRP neuropeptide is able to antagonize the melanocortin pathway, the orexigenic effects of AgRP neurons are not affected by MC4R deletion (Krashes et al., 2011) again proposing the main role of GABAergic signalling to exert the orexigenic effects of AgRP neurons.

Acute neuronal activation of AgRP neurons, by approaches such as the designer receptors exclusively activated by designer drugs (DREADD) and optogenetic stimulation, resulted in robust hyperphagia and provided strong evidence for the orexigenic role of AgRP neurons (Aponte et al., 2011; Atasoy et al., 2012; Krashes et al., 2011). Of note, the AgRP neurons are not only tightly-wired to the PVN and intra-hypothalamic areas as the DMH and LH but also to extra-hypothalamic areas, as demonstrated by anatomical and functional analyses of AgRP neuronal projections (Betley et al., 2013; Broberger et al., 1998) adding up to the complexity of neuronal circuits regulating feeding behaviour. Collectively, the AgRP neurons are potent regulators of energy homeostasis, participating in the melanocortin pathway, with GABAergic signalling playing a crucial role in exerting their orexigenic effects.

1.3.2 Insulin and leptin signalling in the CNS

Insulin's functions are not only profound in the periphery, regulating glycogenesis, lipogenesis and protein synthesis (Roth et al., 2012), but also in the CNS, where insulin receptors (IR) are widely expressed (Havrankova et al., 1978). Brain-specific IR-deficient mice have increased food intake and fat mass, developing mild obesity

(Brüning et al., 2000). Furthermore, CNS insulin signalling is important for the control of peripheral fat metabolism (Koch et al., 2008; Scherer et al., 2011). Although IR deletion in AgRP and POMC neurons does not affect energy homeostasis in mice, IR deletion in AgRP neurons results in defective suppression of hepatic glucose production (Könner et al., 2007). Additionally, in a study using mice with hypothalamic deficiency of IRs, re-expression of IR in AgRP and POMC neurons had distinct effects, with insulin signalling in AgRP neurons decreasing hepatic glucose production whereas in POMC neurons increasing hepatic glucose production, energy expenditure and locomotor activity (Lin et al., 2010).

Insulin signalling not only modulates neuronal gene expression by activation of the PI3K pathway (Niswender et al., 2003) but also PIP3 has been shown to bind to KATP channels antagonizing the action of ATP, hyperpolarizing and thus silencing the neurons (MacGregor et al., 2002; Plum et al., 2006). Insulin further exerts its anorexigenic role in AgRP and POMC neurons by triggering *Pomc* expression and suppressing *Agrp* by phosphorylating and excluding FoxO1 from the nucleus (Kim et al., 2006). FoxO1 was also demonstrated to activate Gpr17 to activate AgRP neurons and regulate food intake (Ren et al., 2012) but recently Gpr17 deficient mice were reported to have unaltered food intake, body weight and glucose homeostasis compared to their control littermates (Mastaitis et al., 2015).

Leptin signalling in the CNS has been extensively studied, especially its role in AgRP and POMC neurons. Neuron-specific leptin deficient mice are obese (de Luca et al., 2005) together with POMC- and AgRP-restricted leptin deficient mice (Balthasar et al., 2004; van de Wall et al., 2008). Leptin is able to depolarize POMC neurons and simultaneously hyperpolarize AgRP neurons (Cowley et al., 2001) being also able to modulate synaptic plasticity in the hypothalamus (Pinto et al., 2004). Furthermore, leptin has been demonstrated to increase POMC and decrease AgRP mRNA levels (Mizuno and Mobbs, 1999; Mizuno et al., 1998). Collectively, leptin's regulatory functions are diverse and broad affecting not only gene expression but also synaptic plasticity and neuronal activity.

Importantly, there are distinct leptin and insulin responsive POMC neuronal subpopulations (Williams et al., 2010) adding to the complexity of the hypothalamic regulation of energy homeostasis.

1.4 Insulin and leptin resistance

Obesity and type 2 diabetes are underlined by a deteriorated efficacy of leptin and insulin to exert their anorexigenic and glucoregulatory functions, conditions termed leptin and insulin resistance (Könner and Brüning, 2012). Leptin and insulin resistance develop despite the high plasma concentrations of both hormones in obese individuals through different mechanisms, such as metabolic inflammation, which will be introduced later.

Chronic leptin signalling induces high levels of suppressor of cytokine signalling 3 (SOCS3), which has been shown to blunt leptin signalling by inhibiting the leptin-induced tyrosine phosphorylation of JAK2 (Bjørbaek et al., 1999). High-fat feeding can also induce the protein-tyrosine phosphatase 1B (PTP1B) (Zabolotny et al., 2008) and protein kinase C (PKC) contributing to insulin resistance (Figure 1) (Benoit et al., 2009). Furthermore, activation of inflammatory signalling which in turn induces, among others, c-Jun N-terminal kinase 1 (JNK1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) has been implicated in leptin and insulin resistance and will be introduced further in detail.

1.5 Metabolic Inflammation, or Metaflammation

The last two decades metabolic inflammation, or metaflammation, has been in focus as a mechanism to explain the pathophysiology of obesity and its hallmarks, insulin and leptin resistance (Gregor and Hotamisligil, 2011). Differing from classical inflammation, which is activated by pathogens and trauma, metaflammation is chronic and low-grade. Metaflammation is observed after high-fat diet (HFD) consumption and during obesity in multiple organs, as the adipose tissue, liver, pancreas, muscle and brain and results in insulin resistance and impaired energy homeostasis (Hotamisligil et al., 1993; Cai et al., 2005; Ehses et al., 2007; Saghizadeh et al., 1996; De Souza et al., 2005). The mechanisms and time-course of metaflammation initiation are of great importance and will be introduced further.

1.5.1 Metabolic inflammation: Where and when?

Metaflammation was first described in the adipose tissue (Hotamisligil et al., 1993) and gained a lot of attention with multiple studies demonstrating its apparent role in

obesity-associated pathologies (Sam and Mazzone, 2014), as the manifestation of insulin resistance after HFD consumption (Xu et al., 2003). The role of immune cells (Mraz and Haluzik, 2014) and especially adipose tissue macrophages (ATMs) in metaflammation is essential (McNelis and Olefsky, 2014). ATMs are either resident and get activated or infiltrate the adipose tissue attracted by signals from dying adipocytes, which reached their oxygen diffusion limit. ATMs are shown to foster the remodelling of the adipose tissue when it reaches its capacity limits during obesity (McNelis and Olefsky, 2014). Interestingly, obese people can remain healthy and insulin sensitive when they can safely store their excess energy, keeping the levels of 'toxic' circulating FFA low and avoiding ectopic lipid accumulation in liver and muscle (Blüher, 2010). Furthermore, the polarization of ATMs, into M1 or M2, is important in the regulation of insulin sensitivity, affected by the primary inflammatory response, but is hard to define because ATMs can simultaneously express M1 and M2 markers (Bourlier et al., 2008). Collectively, the impact of metaflammation in obesity and insulin resistance is being thoroughly studied (Gregor and Hotamisligil, 2011), making it an important target for therapeutics.

Metaflammation is detected after some hours of HFD consumption or lipid infusion with changes in the circulating mononuclear cells, liver, muscle and brain (Ghanim et al., 2009; Aljada et al., 2004; Watt et al., 2006; Thaler et al., 2012). Inflammatory pathways are activated involving, most commonly NFκB and JNK pointing to the direction that inflammation is triggered by nutrients long before obesity arises. Indeed, fatty acids (Shi et al., 2006; Schaeffler et al., 2009) can directly activate TLR4 signalling to induce insulin resistance and mice lacking TLR4 are protected from HFD-induced insulin resistance. HFD can induce gut microbiota to exacerbate inflammation in mice via the TLR4 signalling pathway changing also the permeability of the gut (Kim et al., 2012; Schaeffler et al., 2009). Changes in gut permeability enhance endotoxemia, seen as increases in circulating lipopolysaccharide (LPS) and tumour necrosis factor α (TNFα) concentrations to initiate insulin resistance and obesity (Cani et al., 2007; Cani et al., 2008). It is evident that HFD-induced metaflammation is initiated before obesity arises and it is a rapid and multifaceted process with multiple organs participating in this nutrient response.

To understand the complex interplay between metabolic organs during metaflammation, their endocrine role has to be considered. Leptin and insulin

communicate the energy status to the CNS comprising the two master regulators of energy and glucose homeostasis (Myers and Simerly, 2010). Changes in circulating leptin and insulin levels but also nutrients and cytokines are sensed by defined hypothalamic areas and importantly the arcuate hypothalamic nucleus (Vogt and Brüning, 2013; Milanski et al., 2009; Thaler et al., 2012), where the orexigenic AgRP and the anorexigenic POMC neurons reside. The AgRP neurons are the first to sense small fluctuations in plasma metabolic signals to regulate food intake and short term high-fat feeding increases SOCS3 to cause leptin resistance, already after 48h of HFD consumption (Olofsson et al., 2013).

Collectively, the importance of metaflammation in obesity-associated complications is highly appreciated. Importantly, as metaflammation is initiated so fast, it can be defined as a nutrient response. The role of CNS, particularly the hypothalamus, is vital in orchestrating energy homeostasis since it can sense slight changes in the circulation and is rapidly affected by metaflammation. CNS metaflammation is an important target for the development of therapies against obesity and obesity–associated pathologies.

1.5.2 Hypothalamic inflammation

It has been a decade since the first observations of hypothalamic metabolic inflammation after HFD consumption (De Souza et al., 2005). There are still a lot of open questions regarding the fine mechanisms that regulate energy homeostasis which are affected by the activated inflammatory pathways leading to vast amounts of research conducted in order to define them. Hypothalamic metaflammation is associated with multiple stimuli/signalling pathways like ER stress and activated inflammatory receptors resulting in central insulin and leptin resistance. The focus here will be on the hypothalamus, although metaflammation is also induced in other regions as the amygdala of DIO rats, related to insulin resistance and ER stress (Castro et al., 2013) and the subfornical organ (SFO), a brain region regulating blood pressure (de Kloet et al., 2014).

De Souza et al. were the first to demonstrate that hyperlipidic diet leads to increased pro-inflammatory cytokines and inflammatory responsive proteins in the hypothalamus, such as JNK and NFκB (De Souza et al., 2005). In the same year Zhang et al. reported increased NFκB activity and neural oxidative stress in the brain

after HFD consumption linking it to increased risk for dementia (Zhang et al., 2005). Here, the role of ER stress, different inflammatory mediators and cell types that mediate hypothalamic metaflammation will be introduced.

1.5.2.1 ER stress in hypothalamic inflammation

The importance of ER stress and the unfolded protein response (UPR) in metabolic regulation in the periphery is well appreciated (Hotamisligil, 2008) and it was first demonstrated in the hypothalamus by Ozcan et al. 2009 (Ozcan et al., 2009). Reduced ER capacity was shown to lead to a significant augmentation of obesity on a HFD (Ozcan et al., 2009). ER stress and UPR can be activated to a different degree by a variety of stimuli. For example, LPS can stimulate ER stress and UPR whereas TNFα activates ER stress to a certain degree but fails to induce a complete UPR in the hypothalamus (Denis et al., 2010) and palmitate is able to induce ER stress in the neuronal cell model mHypoE44 (Mayer and Belsham, 2010). Induction of ER stress in the hypothalamus by thapsigargin inhibits the anorexigenic effects of leptin and insulin, whereas treatment with the chemical chaperone 4-phenyl butylic acid significantly improves leptin and insulin sensitivity in diet-induced obese mice (Won et al., 2009). Furthermore, even short-term brain ER stress is sufficient to induce glucose intolerance, systemic insulin resistance, increased blood pressure and elevated sympathetic tone (Purkayastha et al., 2011).

Lipids are important regulators of hypothalamic inflammation and a recent study revealed that hypothalamic ER stress can be activated by ceramides, leading to sympathetic inhibition, reduced brown adipose tissue (BAT) thermogenesis and weight gain (Contreras et al., 2014). The ceramide action is abolished by genetic overexpression of GRP78/BiP in the VMH. GRP78 overexpression reduces hypothalamic ER stress and increases BAT thermogenesis, leading to weight loss and enhanced leptin and insulin sensitivity (Contreras et al., 2014). Inactivation of fatty acid synthase in the hypothalamus prevents diet-induced obesity (DIO) and systemic inflammation and neuron-specific deletion of PPARδ -a lipid sensor that regulates genes involved in lipid metabolism- leads to increased susceptibility to DIO, leptin resistance and hypothalamic inflammation in low-fat diet (Kocalis et al., 2012). Stearic acid, a saturated fatty acid, is able to cause hypothalamic inflammation and results in reduced oxygen consumption and blunted peripheral insulin signal

transduction (Arruda et al., 2011) and impaired insulin secretion from the pancreas (Calegari et al., 2011).

To understand the mechanistic basis of ER stress-induction, the significance of ER and mitochondria interactions has been investigated. Depletion of Mfn2, a protein vital for ER-mitochondria interactions, in POMC neurons results in ER stress-induced leptin resistance, hyperphagia, reduced energy expenditure and defective POMC processing, effects that are reversed by inhibition of ER stress by chemical chaperone treatment (Schneeberger et al., 2013).

Collectively, ER stress in CNS metaflammation is activated to a different degree by a variety of stimuli resulting in insulin and leptin resistance and diminishing BAT thermogenesis. ER-mitochondria interactions, pivotal for regulating energy balance and chaperone treatment, can reverse the detrimental effects of hypothalamic ER stress.

1.5.2.2 Pathways and mediators of hypothalamic inflammation

Obesity is accompanied by increased concentrations of circulating cytokines, pro-inflammatory interleukins and lipids, which can reach the brain and initiate inflammatory pathways, modulating hypothalamic function and regulation of energy homeostasis, a condition termed hypothalamic inflammation (Thaler et al., 2013). Hypothalamic inflammation involves multiple pathways converging in the activation of inflammatory mediator proteins, as JNK and NFκB. Here, important players of inflammatory pathways in the hypothalamus will be introduced.

TNFα

TNFα is a pleiotropic cytokine showing increased levels in obesity that is implicated in metabolic inflammation in many tissues (Gregor and Hotamisligil, 2011). TNFα in the hypothalamus can induce insulin and leptin resistance activating NFκB, JNK and SOCS3 (Romanatto et al., 2007). TNFα acts on the hypothalamus to increase PTP1B expression and activity via the NFκB pathway and cause insulin and leptin resistance (Ito et al., 2012) having also a dose dependent effect in regulating energy balance. Furthermore, although low doses of ICV administration of TNF induce leptin resistance (Arruda et al., 2011) and a dysfunctional increase in insulin secretion and markers of apoptosis in pancreatic islets (Calegari et al., 2011), higher doses

reproduce features of cancer-induced cachexia including reduced food intake (Arruda et al., 2011). Of note, hypothalamic administration of TNFα has been also demonstrated to reduce the expression of thermogenic genes in BAT (Arruda et al., 2011). Taken together, TNFα has dose-dependent orexigenic and anorexigenic effects in the hypothalamus and mediates insulin and leptin resistance.

Interleukins

Pro-inflammatory interleukins (ILs), as IL6, are found in obese patients with increased plasma concentrations. The role of diet-induced pro-inflammatory IL signalling in the brain is highly complex. Central administration of IL4 during HFD increases pro-inflammatory cytokine gene expression and causes excess weight gain (Oh-I et al., 2010). Interestingly, transgenic mice secreting human IL6 predominantly from the brain and lung are more insulin sensitive, with human IL6 enhancing central leptin action (Sadagurski et al., 2010). In human patients massive reduction of body mass after bariatric surgery promotes a partial reversal of hypothalamic dysfunction accompanied by increased IL6 concentration in the CSF (van de Sande-Lee et al., 2011). Furthermore, IL6 and IL1 have been demonstrated to mediate the anorexigenic effects of glucagon-like peptide 1 receptor (GLP1R) (Shirazi et al., 2013), with IL1 signalling being important in mediating glucose-induced anorexia (Mizuno et al., 2013). Pharmacological inhibition of IL1 and IL6 attenuates central exendin 4 effects on reducing food intake and body weight (Shirazi et al., 2013). Collectively, IL signalling in the hypothalamus exerts pro- and anti-inflammatory effects, which can suppress or enhance insulin sensitivity, respectively.

Toll-like Receptors

Inflammatory responses associated with high-fat feeding are also mediated by Toll-like Receptors (TLRs), key players in innate immunity. Saturated fatty acids can signal through TLR4 resulting in ER stress in the hypothalamus and obesity (Milanski et al., 2009). TLR4 pharmacological inhibition or loss of function mutation, protect mice from DIO (Milanski et al., 2009). Furthermore, genetic ablation of the downstream mediator of TLR4 signalling, MyD88, in the brain also protects mice from DIO and leptin resistance, caused either by HFD or central palmitate administration (Kleinridders et al., 2009). In contrast, intact TLR4 signalling protects cells from diet-induced apoptotic signals (Moraes et al., 2009) and TLR2-deficient

mice exhibit mature onset obesity and susceptibility to HFD-induced weight gain, with TLR2 being increased with age or HFD in POMC neurons (Shechter et al., 2013). Collectively, hypothalamic TLR signalling is pleiotropic and is not always connected to positive energy balance.

NFκB

NFκB is a transcription factor family acting as an integral immune response regulator. NFκB consists of five members, which form homo- and heterodimers (RelA or p65, RelB, c-Rel, p50 and p52). NFκB dimers are associating with proteins of the inhibitor of NFκB family (IκB) and kept inactive. Activation of NFκB is mediated by the IκB kinase (IKK) complex, which induces polyubiquytilation and degradation of the IκB proteins, allowing NFκB activation and translocation to the nucleus for the regulation of gene expression [reviewed in (Pasparakis, 2009)]. IKK signalling has been demonstrated to induce insulin resistance by serine phosphorylation of IRS proteins and increased NFκB activation in the liver results in insulin resistance and glucose intolerance (Cai et al., 2005). Haploinsufficient IKK2 knockout mice as well as hepatocyte- and myeloid-cell specific IKK2 deficient mice, are protected against systemic insulin resistance and glucose intolerance (Yuan et al., 2001; Arkan et al., 2005; Cai et al., 2005).

Activation of NFκB/IKK2 in the brain results in central insulin and leptin resistance (Zhang et al., 2008) and conversely, inactivation of NFκB in the mediobasal hypothalamus and AgRP neurons protects against obesity and glucose intolerance (Zhang et al., 2008). IKK2 is activated in the brain by HFD (Posey et al., 2009) being downstream of IL4 action and central administration of IL4 increases cytokine gene expression and causes excess weight gain, effects that are blocked by PS1145, an IKK2 inhibitor (Oh-I et al., 2010). NFκB is also linked to defective hypothalamic autophagy caused by high-fat feeding, resulting in hyperphagia and reduced energy expenditure (Meng and Cai, 2011). Defective autophagy in AgRP and POMC neurons has been linked to increased food intake and impaired lipolysis, respectively (Kaushik et al., 2011; Kaushik et al., 2012).

Maternal perinatal high-fat feeding and intake of trans fatty acids during lactation also leads to NFκB activation and impaired glucose homeostasis (Pimentel et al., 2012; Rother et al., 2012; Melo et al., 2014).

Neuronal apoptosis and neurogenesis are two mechanisms that are also implicated in the CNS metaflammation and diet-induced changes in the hypothalamus. It has been demonstrated that HFD induces apoptosis in neurons and a reduction of synaptic inputs in the ARC and the LH (Moraes et al., 2009). High-fat feeding has been shown to suppress neurogenesis by increasing apoptosis in new neurons (McNay et al., 2012). NFκB was identified as a critical mediator of stress with antineurogenic actions (Koo et al., 2010). Similarly, high-fat feeding has been shown to impair differentiation and result in depletion of hypothalamic stem cells upon NFκB activation (Li et al., 2012). Of note, leptin deficiency also results in partial loss of hypothalamic stem cells (McNay et al., 2012). Pierce et al. contributed to the deeper understanding of neurogenesis in metaflammation by demonstrating that the AgRP neurons are capable of de novo neurogenesis under neurodegenerative conditions suggesting this as a potential compensatory mechanism contributing to a more plastic control of energy balance (Pierce and Xu, 2010). Furthermore, it was demonstrated that NFκB-dependent gene expression establishes a growth inhibition in the post-lesioned brain that limits structural regeneration of neuronal circuits, pointing to a similar role in diet-induced hypothalamic changes (Engelmann et al., 2014).

Of note, neuronal androgen receptors regulate hypothalamic insulin signalling by repressing NFκB-mediated induction of PTP1B (Yu et al., 2013) and ERα protects premenopausal females from metabolic complications of inflammation and obesity-related diseases (Morselli et al., 2014).

NFκB also demonstrates anorexigenic effects in the brain, making its action more complicated and difficult to define in the context of metabolic diseases. NFκB attenuates the glucocorticoid effect to stimulate the expression of AgRP and NPY under ER stress in mouse hypothalamic cultures (Hagimoto et al., 2013). Furthermore, RelA is able to bind to the POMC promoter region and activate transcription (Shi et al., 2013). Under HFD-induced chronic inflammation, the POMC promoter gets methylated and RelA cannot bind to it in order to activate the anorexigenic peptide transcription (Shi et al., 2013). Lastly, NFκB is tightly connected to illness- and leptin-induced anorexia and weight loss (Jang et al., 2010).

Collectively, NFκB activation is implicated in insulin and leptin resistance in the hypothalamus linked also to defective autophagy and neuronal apoptosis but has been also shown to mediate anorexigenic effects.

JNK

The c-Jun N-terminal kinase (JNK) family consists of three proteins, JNK1-3, with several isoforms due to alternative splicing (Weston and Davis, 2007). The JNK proteins are serine/threonine kinases that are activated by a variety of stimuli, including cytokines, resulting in the phosphorylation of c-Jun and the induction of the transcription factor activator protein 1 (AP1) (Weston and Davis, 2007). JNK is activated in obesity and it has been shown to interfere with insulin signalling by phosphorylating and inhibiting IRS1 (Aguirre et al., 2002). JNK1 null mice are protected against diet-induced obesity and mice with ablation of JNK1 in myeloid cells are protected from diet-induced insulin resistance (Bogoyevitch, 2006; Vallerie et al., 2008).

Hypothalamic JNK, similar to NFκB, is activated by maternal high-fat feeding during pregnancy and trans fatty acid intake during lactation resulting in impaired glucose metabolism in adult mice (Pimentel et al., 2012; Rother et al., 2012; Melo et al., 2014). Genetic JNK inactivation in the brain results in improved insulin sensitivity, protection from hepatic steatosis after high-fat feeding (Belgardt et al., 2010) and suppression of DIO by increasing energy expenditure connected with the HPA axis (Sabio et al., 2010). Acute inhibition of central JNK1 improves impaired glucose homeostasis and is associated with sensitization to hypothalamic insulin signalling independent of leptin levels (Benzler et al., 2013). JNK is also tightly connected to leptin resistance as it was demonstrated that JNK inhibition in the ARC reinstates the anorexigenic effects of leptin, in DIO leptin resistant mice (Koch et al., 2014). Interestingly, in this study it was also shown that even leptin deficient mice acquire leptin resistance upon HFD consumption pointing to the role of activated inflammatory pathways, independent of hyperleptinemia to be able to cause leptin resistance (Koch et al., 2014). Palmitate is an upstream mediator of JNK activation and can cause ER stress through a JNK-dependent pathway that activates eIF2 and XBP1 (Mayer and Belsham, 2010).

JNK has been also shown to have a pleiotropic role in the central regulation of energy homeostasis, demonstrating specific anorexigenic effects. JNK inhibition in hypothalamic explants stimulates AgRP and NPY expression (Unger et al., 2010). JNK inhibits AgRP and NPY antagonizing the orexigenic effects of glucocorticoids (Unger et al., 2010). Similar to NFκB, JNK has orexigenic and anorexigenic effects depending on the level of activation and upstream stimuli.

1.5.2.3 Complex environment: Microglia, astrocytes and endothelial cells

The role of different brain cell types that participate in metabolic inflammation has been extensively studied the last years. Microglia, astrocytes, endothelial cells interact with each other and the neurons to regulate energy homeostasis and are all distinctly affected by diet-induced obesity.

Microglia, the macrophages of the brain, have been demonstrated for the first time to participate in CNS metaflammation by Tapia-Gonzalez et al. who showed that neonatal overnutrition results in microglial activation not only in hypothalamic areas but also the cerebellum of rats (Tapia-González et al., 2011). Thaler et al. determined that already 1 to 3 days after HFD consumption reactive gliosis and markers of neuronal injury become evident in the arcuate nucleus of rodents and that gliosis is also present in obese humans, as assessed by MRI studies (Thaler et al., 2012). HFD-associated microglia activation is reversible with exercise as demonstrated by Yi et al. assessed in HFD-fed $LDL1R^{-/-}$ mice after treadmill running (Yi et al., 2012a). Reversal of microglial activation is also possible after a switch to normal chow diet (NCD) for 4 weeks, following a 16-week HFD-feeding (Berkseth et al., 2014). Recently the origin of microglia was determined, by Buckman et al. who demonstrated the recruitment of peripheral immune cells in the brain, most of them being CD45+ and CD11b+, characteristics of macrophages/microglia (Buckman et al., 2014).

Astrogliosis, as a marker of neuronal injury in the metabolic syndrome, has been observed after HFD consumption in brain areas including the medial preoptic, PVN and DMH and less in VMH, LH and AHA (Buckman et al., 2013). Furthermore, astrogliosis was detected in obese Zucker rats (Tomassoni et al., 2013) and as a result of neonatal overnutrition (Fuente-Martín et al., 2013) but already in 1999 Plagemann et al. observed astrogliosis due to hyperinsulinemia in the rat brain, pointing out the relationship between insulin signalling and astrocyte activation (Plagemann et al., 1999). Astrocytes can also be activated by saturated long chain fatty acids, such as palmitic acid, independent of the presence of microglia (Gupta et al., 2012). Furthermore, astrocyte activation is regulated by glucolipids, such as lactosylceramide (Mayo et al., 2014) and TLR4 signalling in astrocytes can induce pro-inflammatory signalling by NFκB, MAPK and JAK/STAT1 pathways with the crosstalk signal capable of modulating the response of surrounding cells (Gorina et al., 2011).

The role of endothelial cells and angiogenesis in diet-induced obesity was demonstrated by Yi et al., who observed that high-fat high-sucrose diet results in increased length and density of the blood vessels in the ARC and increased formation of new arterial vessels (Yi et al., 2012b). Similarly, type 2 diabetes patients have more arterioles, suggesting that the same mechanisms are responsible for these changes in both rodents and humans (Yi et al., 2012b).

Collectively, the role of microglia, astrocytes and endothelial cells is pivotal during the initiation and manifestation of diet-induced obesity and has to be considered in order to understand the interplay with the neuronal populations that reside in regions responsible for energy homeostasis regulation.

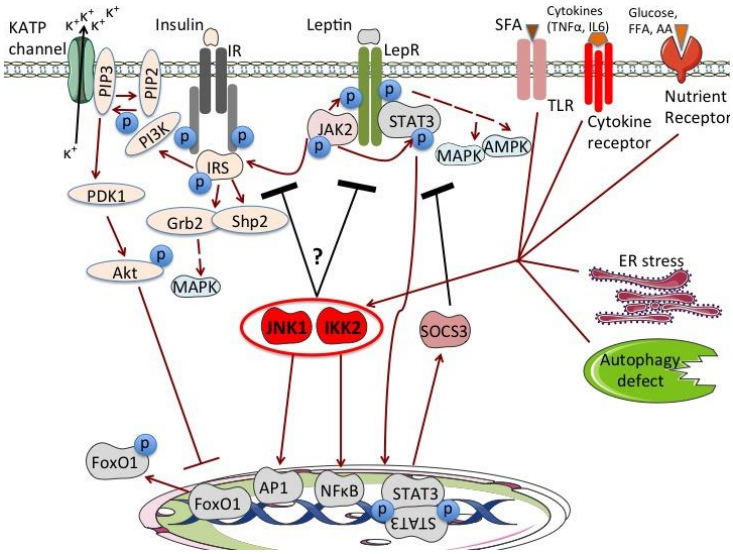

Figure 1. Activation of inflammatory pathways results in insulin and leptin resistance. Binding of insulin leads to a conformational change of the IR, resulting in activation of the endogenous kinase activity, in turn IRS proteins bind to the phosphorylated residues, and are phosphorylated by the IR. Shp2 and Grb2 are activated and Grb2 triggers the MAPK signalling pathway. Phosphorylation of IRS proteins allows for activation of PI3K, which subsequently phosphorylates the membrane lipid PIP2 to generate PIP3. PIP3 binds to KATP channels, leaving them open and resulting in hyperpolarization of the neurons. PIP3 accumulation recruits and allows binding of both PDK1 and AKT. PDK1 phosphorylates and thereby activates AKT, which mediates most of insulin´s effect on glucose and glycogen metabolism, as well as activating protein translation and gene transcription. Binding of leptin leads to recruitment of JAK2, autophosphorylation and phosphorylation of LepR. After Jak2-mediated phosphorylation of STAT3, pSTAT3 dimers activate transcription of target genes. One of these genes is *SOCS3*, and the SOCS3 protein in a feedback loop binds to JAK2 and thereby inhibits STAT3 phosphorylation. Additionally, JAK2 is able to directly activate IRS/PI3K signalling, leading to AKT activation. Increased SFAs, FFAs, glucose, cytokines in the circulation during obesity can signal through TLR, cytokine and nutrient receptors and result in the activation of inflammatory pathways, such as IKK2 and JNK1. Furthermore, ER stress and autophagy defect can also result in IKK2 and JNK1 activation. Both JNK1 and IKK2 have been shown to inducing insulin resistance and activation leptin resistance. It still remains elusive whether JNK1 and/or IKK2 activation *per se*, without any environmental trigger such as high-fat feeding, could still have the same effect in insulin and leptin signalling.

1.6 Ageing

Ageing is a process during which deteriorative changes decrease an organism's ability to survive. These deteriorative changes might occur due to molecular damage, for example caused by oxidative stress, but can also be influenced by genetic variation (Kenyon, 2005). Ageing is associated with pathologies as neoplastic, neurodegenerative and immune diseases (Niccoli and Partridge, 2012) and very importantly the metabolic syndrome (Ford et al., 2010; Hildrum et al., 2007), termed as the metabolic dysfunctions including obesity, insulin resistance and type 2 diabetes.

Improvements in sanitation and medicine keep increasing the average human lifespan since the 1800's (Oeppen and Vaupel, 2002) but there is still a lot to answer concerning the biology of ageing and the determinants of maximum lifespan. On one hand, scientific proof exists that decreasing the caloric intake accompanied by increased insulin sensitivity, termed as caloric restriction, can extend the maximum lifespan (Masoro, 2005). Amelioration of hypothalamic inflammation has also been connected to lifespan extension (Zhang et al., 2013) and resembles some effects of caloric restriction, such as increased insulin sensitivity and reduced growth hormone (Belgardt et al., 2010). On the other hand the disruption of insulin/insulin-like growth factor 1 (IGF1) signalling also extends lifespan, creating a controversy with caloric restriction (Katic and Kahn, 2005). The concept of caloric restriction and the importance of insulin/IGF1 signalling in ageing will be introduced further in detail.

1.6.1 Caloric restriction

The only dietary intervention able to increase maximum lifespan is caloric restriction and was described for the first time almost a century ago (Osborne et al., 1917). Caloric restriction (CR) delays the onset of ageing-associated pathologies (Bronson and Lipman, 1991; Maeda et al., 1985; Roe et al., 1995) and is accompanied by increased insulin sensitivity (Gresl et al., 2003). Other common features of CR include decreased levels of growth hormone (GH), thus retardation of growth, and suppressed IGF1 signalling (Anderson et al., 2009).

CR, the reduction of caloric intake without malnutrition (Masoro, 2005), has been applied successfully in many model organisms including yeast, *C. elegans*, fruit fly, rat, mouse and non-human primates (Taormina and Mirisola, 2014; Colman et al.,

2009; Weindruch and Walford, 1982), although the primate studies still present controversial results (Mattison et al., 2012).

It has been proposed that not only the reduction in caloric intake is essential, varying from 20-40% in the studies using mammals, but also the diet composition is of great significance for the lifespan extension (Taormina and Mirisola, 2014). Indeed, protein, methionine and tryptophan restriction were also used as diets that mimic the effects of caloric restriction and were demonstrated to also result in extended lifespan (Malloy et al., 2006; De Marte and Enesco, 1986; Parrella et al., 2013).

The free radical theory provides an attractive explanation for CR's longevity promoting effects, suggesting that free radical reactivity results in cumulative damage to lipids, proteins and DNA and eventually, senescence. Reduced energy intake, decreases mitochondrial respiration and eventually reactive oxygen species (ROS) production leading to less chronic inflammation resulting in lifespan extension (López-Torres et al., 2002; Sohal et al., 1994).

1.6.2 Insulin/IGF signalling in ageing

Ageing appears to be stochastic and the mechanisms underlying it remain poorly understood but growing evidence supports the important role of insulin/IGF signalling in the ageing process [reviewed in (Katic and Kahn, 2005)].

Although severe reduction in insulin/IGF1 signalling can result in perinatal lethality, insulin resistance and type 2 diabetes (Accili et al., 1996; Brüning et al., 2000; Joshi et al., 1996; Liu et al., 1993), moderate alterations in insulin/IGF1 signalling can extend lifespan in *C. elegans* (Kenyon et al., 1993; Wolkow et al., 2000) and *Drosophila melanogaster* (Clancy et al., 2001; Tatar et al., 2001) and in mice (Blüher et al., 2003; Selman et al., 2008). Specifically, deletion of the insulin receptor in the adipose tissue (Blüher et al., 2003) results in lifespan extension although the mechanism behind this remains elusive and disruption of IRS1 in the brain only results in lifespan extension in female mice (Selman et al., 2008). Furthermore, low plasma levels of IGF1 and growth hormone, as found in the Dwarf mice, are related to life extension (Bartke et al., 2001; Brown-Borg et al., 1996; Flurkey et al., 2001).

1.7 Scientific objectives

In this study we investigate the role of hypothalamic inflammation in the fine balances that regulate energy and glucose homeostasis, as a first aim, and ageing, as a second aim. Specifically, while both inhibition of JNK1 and IKK2, two major inflammatory mediators, in the hypothalamus protects from high-fat diet-associated pathologies, it has not yet been demonstrated in which hypothalamic neuronal population JNK1 and/or IKK2 action deregulates energy and/or glucose homeostasis, and whether neuron-restricted JNK1 and/or IKK2 activation are sufficient to alter energy and glucose homeostasis. Here, we use two models of targeted mouse mutagenesis to constitutively activate JNK1 ($AgRP^{JNK1CA}$) or IKK2 ($AgRP^{IKK2CA/CA}$) in the AgRP orexigenic neurons of the arcuate hypothalamic nucleus in order to examine their role in the regulation of energy and glucose homeostasis, examining insulin and leptin signalling in a molecular and systemic level.

The second aim of this study is to reveal whether conditional ablation of JNK1 in the CNS and pituitary by Nestin Cre ($JNK1^{\Delta Nes}$) affects the ageing process in mice. The phenotype of $JNK1^{\Delta Nes}$ mice resembles the effects of caloric restriction, an intervention that delays ageing. Specifically, $JNK1^{\Delta Nes}$ mice show reduced body weight under normal chow and high-fat diet conditions and improved insulin sensitivity, glucose tolerance and impaired somatic growth with decreased circulating levels of IGF1 and decreased expression of growth hormone in the pituitary (Belgardt et al., 2010). Furthermore, $JNK1^{\Delta Nes}$ mice are protected from hepatic steatosis and present with an anti-inflammatory gene expression pattern in the adipose tissue (Belgardt et al., 2010). The fact that $JNK1^{\Delta Nes}$ phenotype characteristics resemble the features of caloric restriction prompted us to further investigate the impact of JNK1 ablation in the central nervous system in a longitudinal study.

Collectively, the following mutant mouse models are used in this study:

-Mice with AgRP neuron-specific JNK1 constitutive activation ($AgRP^{JNK1CA}$)

-and mice with AgRP neuron-specific IKK2 constitutive activation ($AgRP^{IKK2CA/CA}$)

to investigate the effects of inflammation in energy and glucose homeostasis.

-Mice with Nestin-specific JNK1 ablation ($JNK1^{\Delta Nes}$)

to investigate the effects of hypothalamic inflammation in ageing.

2 Materials & Methods

2.1 Animal care and generation of mice

All animal procedures were conducted in compliance with protocols approved by local government authorities (Bezirksregierung Köln, Cologne, Germany) and were in accordance with NIH guidelines. Mice were housed in groups of 3–5 at 22–24°C using a 12h light/12h dark cycle. Animals were fed NCD (Teklad Global Rodent 2018; Harlan) containing 53.5 % carbohydrates, 18.5 % protein, and 5.5 % fat (12 % of calories from fat) or HFD (HFD; C1057; Altromin) containing 32.7 % carbohydrates, 20 % protein, and 35.5 % fat (55.2 % of calories from fat). Animals had ad libitum access to water at all times, and food was only withdrawn if required for an experiment.

Only male mice were used in these studies to avoid the effect of different stages of estrous cycle on glucose homeostasis. NPY^{GFP}, $R26Stop^{FL}JNK1CA$, $R26Stop^{FL}IKK2CA$, Z/EG, LacZ, $AgRP^{Cre}$, $Nestin^{Cre}$ and $JNK1^{fl/fl}$ mice have been described previously (Könner et al., 2007; Sasaki et al., 2006; Novak et al., 2000; Tong et al., 2008; Belgardt et al., 2010).

2.2 Genotyping

2.2.1 Isolation of genomic DNA

Tail biopsies were obtained at postnatal day 19-21. The samples were incubated for 5h in 500µl Tail Lysis Buffer (100mM Tris pH 8.5, 5mM EDTA, 0.2 % (w/v) SDS, 0.2M NaCl) containing 1 % ProteinaseK (Roche,Germany) at 56°C. DNA was precipitated by adding an equal volume of isopropanol, mixed and pelleted by centrifugation, was washed with 70 % (v/v) Ethanol, dried at RT and redissolved in 50µl ddH2O.

2.2.2 Polymerase chain reaction

For genotypic analysis, polymerase chain reaction (PCR) was performed on tail DNA using the primers given in Table 1. For PCR DreamTaq PCR MasterMix and DNA polymerase (Thermo Scientific, Walldorf, Germany) was used. Standard PCR

contained approx. 50 ng DNA, 25 pMol of each primer, 25 mM dNTP mix and 1 unit DNA polymerase in a 25 ml reaction mix.

Table 1. Genotyping Primers

$AgRP^{Cre}$	GGG CCC TAA GTT GAG TTT TCC T sense
	GAT TAC CCA ACC TGG GCA GAA C antisense
	GGG TCG CTA CAG ACG TTG TTT G antisense (mutant)
$Nestin^{Cre}$	CGC TTC CGC TGG GTC ACT GTC G sense
	TCG TTG CAT CGA CCG GTA ATG CAG GC antisense
LacZ	ATC CTC TGC ATG GTC AGG TC sense
	CGT GGC CTG ATT CAT TCC antisense
GFP	CTG GTC GAG CTG GAC GGC GAC G sense
	CAG GAA CTC CAG CAG GAC CAT G antisense
$JNK1^{fl/fl}$	ACA TGT ACC ATG TAC TGA CCT AAG sense
	CAT TAC TCT ACT CAC TAT AGT AAC A antisense
	GAT ATC AGT ATA TGT CCT TAT AG antisense (deletion)

2.3 Phenotyping

2.3.1 Insulin tolerance test

Insulin tolerance tests were always performed in the morning to avoid deviations of the blood glucose concentration. The mice were injected with 0.75 U of recombinant insulin (Novo Nordisk, Basvaerd, Denmark) per kg of body weight (diluted in saline) and their blood glucose concentration was measured at 0, 15, 30 and 60 minutes post-injection by an automatic glucose monitor (Contour, Bayer, Germany).

2.3.2 Glucose tolerance test

Glucose tolerance tests were performed after a 6h fasting period. 20 % glucose (DeltaSelect, Germany) (10ml/kg body weight) were injected intraperitoneally and blood glucose levels were determined at 0, 15, 30, 60 and 120 minutes post-injection using an automatic glucose monitor (Contour, Bayer, Germany).

2.3.4 Behavioural analysis for learning and memory

Morris Water Maze analysis was performed in order to assess learning and memory in JNK1 $^{\Delta Nes}$ mice. JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ control mice were trained daily for 9 days to memorize the location of a submerged platform in a water pool using spatial recognition markers, and the time taken by each mouse to find the platform was measured by an automatic, software based system, termed acquisition time. Each mouse performed 4 trials per day, entering the pool from different positions and given 60 seconds to find the submerged platform. In case the mouse did not find the platform it was placed on it until it stayed in place for 10 seconds. On the 4th, 7th and 10th day of the test, the platform was removed from the pool, and the amount of time the mouse swam in the quadrant in which the platform had been was automatically measured by a software based system, termed the retention time. In order to assess learning plasticity, the ability to learn a new task, the platform was moved to the opposite quadrant the 11th day of the experiment and the acquisition time was measured for another 3 days.

2.3.5 Indirect calorimetry, physical activity and food intake

All measurements for indirect calorimetry were performed in a PhenoMaster System (TSE systems, Bad Homburg, Germany), which allows measurement of metabolic performance and activity monitoring by an infrared light-beam frame. Mice were placed at room temperature (22 °C–24 °C) in 7.1-l chambers of the PhenoMaster open circuit calorimetry. Mice were allowed to acclimatize in the chambers for at least 24h. Food and water were provided ad libitum in the appropriate devices and measured by the build-in automated instruments. Locomotor activity and parameters of indirect calorimetry were measured for at least the following 48h. Presented data are average values obtained in these recordings. Food intake was measured manually the mice were single-caged at least 4 days prior to the experiment in order to acclimatize to the special food racks.

2.3.6 Body composition and Bone mineral Density

Nuclear magnetic resonance was employed to determine body composition using the NMR Analyzer minispeq mq7.5 (Bruker Optik, Germany). Bone mineral density was measured by computed tomography in anaesthetised mice using the LaTheta micro

CT scanner (Aloka, Tokyo, Japan). The sections were set to 0.6 mm and analysis was performed using the LaTheta software.

2.4 Electrophysiology

2.4.1 Animals and brain slice preparation for electrophysiological experiments

Electrophysiological experiments were performed by Lars Paeger (Kloppenburg laboratory) on brain slices from 35- to 42-day male mice. The animals were anesthetized with halothane (B4388; Sigma-Aldrich, Taufkirchen, Germany) and subsequently decapitated. The brain was rapidly removed and a block of tissue containing the ARC was immediately cut out. Coronal slices (260 µM) containing the ARC were cut with a vibration microtome (HM-650 V; Thermo Scientific, Walldorf, Germany) under cold (4 °C), carbonated (95 % O_2 and 5 % CO_2), glycerol-based modified artificial cerebrospinal fluid (GaCSF) to enhance the viability of neurons. GaCSF contained (in mM): 250 Glycerol, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 10 HEPES, 21 $NaHCO_3$, 5 Glucose adjusted to pH 7.2 with NaOH resulting in an osmolarity of ~310 mOsm. Brain slices were transferred into carbogenated artificial cerebrospinal fluid (aCSF). First, they were kept for 20 min in a 35 °C 'recovery bath' and then stored at room temperature (24 °C) for at least 30 min prior to recording. aCSF 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 resulting in an osmolarity of ~310 mOsm.

2.4.2 Perforated patch recordings

Slices were transferred to a recording chamber (~3 ml volume) and continuously superfused with carbogenated aCSF at a flow rate of ~2 $ml \cdot min^{-1}$. Neurons in ARC were visualized with a fixed-stage upright microscope (BX51WI; Olympus, Hamburg, Germany), using a 60x water immersion objective (LUMplan FI/IR; 60×; 0.9 numerical aperture; 2 mm working distance; Olympus) with infrared-differential interference contrast and fluorescence optics. In the ARC NPY/AgRP neurons were identified by their specific GFP fluorescence (Chroma 41001 filter set; EX: HQ480/40x, BS: Q505LP, EM: HQ535/50m, Chroma, Rockingham, VT, USA).

Current-clamp recordings in the perforated patch configuration were performed with an EPC10 patch-clamp amplifier (HEKA, Lambrecht, Germany) controlled by the PatchMaster software (version 2.32; HEKA) running under Windows. Data were sampled at a rate of 10 kHz and low-pass filtered at 2 kHz with a four-pole Bessel filter. The liquid junction potential between intracellular and extracellular solution was compensated (14.6 mV for normal aCSF; calculated with Patcher's Power Tools plug-in downloaded from http://www3.mpibpc.mpg.de/groups/neher/index.php?page=software for Igor Pro 6 [Wavemetrics, Lake Oswego, OR, USA]).

Perforated patch recordings were performed using protocols modified from Horn and Marty (Horn and Marty, 1988) and Akaike and Harata (Akaike and Harata, 1994). Electrodes with tip resistances between 3 and 5 MΩ were fashioned from borosilicate glass (0.86 mm inner diameter; 1.5 mm outer diameter; GB150-8P; Science Products, Hofheim, Germany) with a vertical pipette puller (PP-830; Narishige, London, UK). Perforated patch recordings were performed with ATP and GTP free pipette solution containing (in mM): 128 K-gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 $MgCl_2$ adjusted to pH 7.3 with KOH resulting in an osmolarity of ~300 mOsm. ATP and GTP were omitted from the intracellular solution to prevent uncontrolled permeabilization of the cell membrane (Lindau and Fernandez, 1986). The patch pipette was tip filled with internal solution and back filled with tetraethylrhodamine-dextran (D3308, Invitrogen, Eugene, OR, USA) and amphotericin B containing internal solution (100-250 $\mu g \cdot ml^{-1}$; A4888; Sigma) to achieve perforated patch recordings. Amphotericin B was dissolved in dimethyl sulfoxide (final concentration: 0.2 - 0.5%; DMSO; D8418, Sigma) as described in (Rae et al., 1991) and was added to the modified pipette solution shortly before use. The used DMSO concentration had no obvious effect on the investigated neurons. Experiments were carried out at ~32°C using an inline solution heater (SH27B; Warner Instruments, Hamden, CT, USA) operated by a temperature controller (TC-324B; Warner Instruments). During the perforation process access resistance (R_a) was constantly monitored and experiments were started after R_a and the action potential (AP) amplitude were stable (~10–30 min). A change to the whole-cell configuration was indicated by diffusion of tetraethylrhodamine-dextran into the neuron. Such experiments were rejected. For each neuron, the firing rate and the membrane potential was determined by averaging periods of 5 minutes using Spike2.

2.5 Immunohistochemistry

GFP/phospho-c-Jun and PIP3/LacZ double stainings were performed similarly to previously reported protocols (Belgardt et al., 2008; Plum et al., 2006). The following antibodies were used, α-GFP antibody (Abcam, #13970, dilution 1: 1000), α-p-c-Jun antibody (Cell Signalling, #3270, dilution 1: 800), α-pSTAT3 antibody (Cell Signalling, #9145, dilution 1:100), α-pIκBα antibody (Cell Signalling, #2859, dilution 1:100). After overnight incubation at 4°C, we used goat α-chicken (Jackson, #103-095-155, FITC-coupled, dilution 1:500) and goat α-rabbit (Molecular Probes, #A11012, Alexa 594-coupled, dilution 1:500). Images were captured using a Leica S5 confocal microscope and analyzed, as described below, using ImageJ software (NIH). In the Figures 4B, 4D, 6B, 6D, 9B, cells with immunoreactivity above threshold were counted manually as positive and marked digitally to prevent multiple counts, for each cell immunoreactivity was assessed in every channel (at least 2 sections/animal were used for quantification).

Co-staining for both pSTAT3 and LacZ immunoreactivity was quantified in 10 confocal slices, at 1μm intervals per animal from three animals per group (at least 2 hemisections/animal). In detail, maximum intensity projections were prepared for each series of confocal images, separately for pSTAT3 and LacZ immunoreactivity. Each image was binarized to isolate immunoreactive positive cells from the background and compensate for differences in fluorescence intensity. To determine co-localization, the integrated density of overlapping pixels was measured from the pSTAT3 and LacZ binarized images (Figure 2). The integrated density, depicted in Figure 6C, is therefore proportional to the intensity of pSTAT3 and LacZ immunoreactivity co-localization. To quantify the intensity of pSTAT3 immunoreactivity in non-LacZ cells ($LacZ^-$), the same binarized images of pSTAT3 and LacZ immunoreactivity were used for analysis as for Figure 6E. Specifically, the binarized image of LacZ was subtracted from the binarized image of pSTAT3 to define the pSTAT3 immunoreactivity in $LacZ^-$ cells (non-AgRP neurons), and the integrated density of the resulting image defined the intensity depicted in Figure 6E.

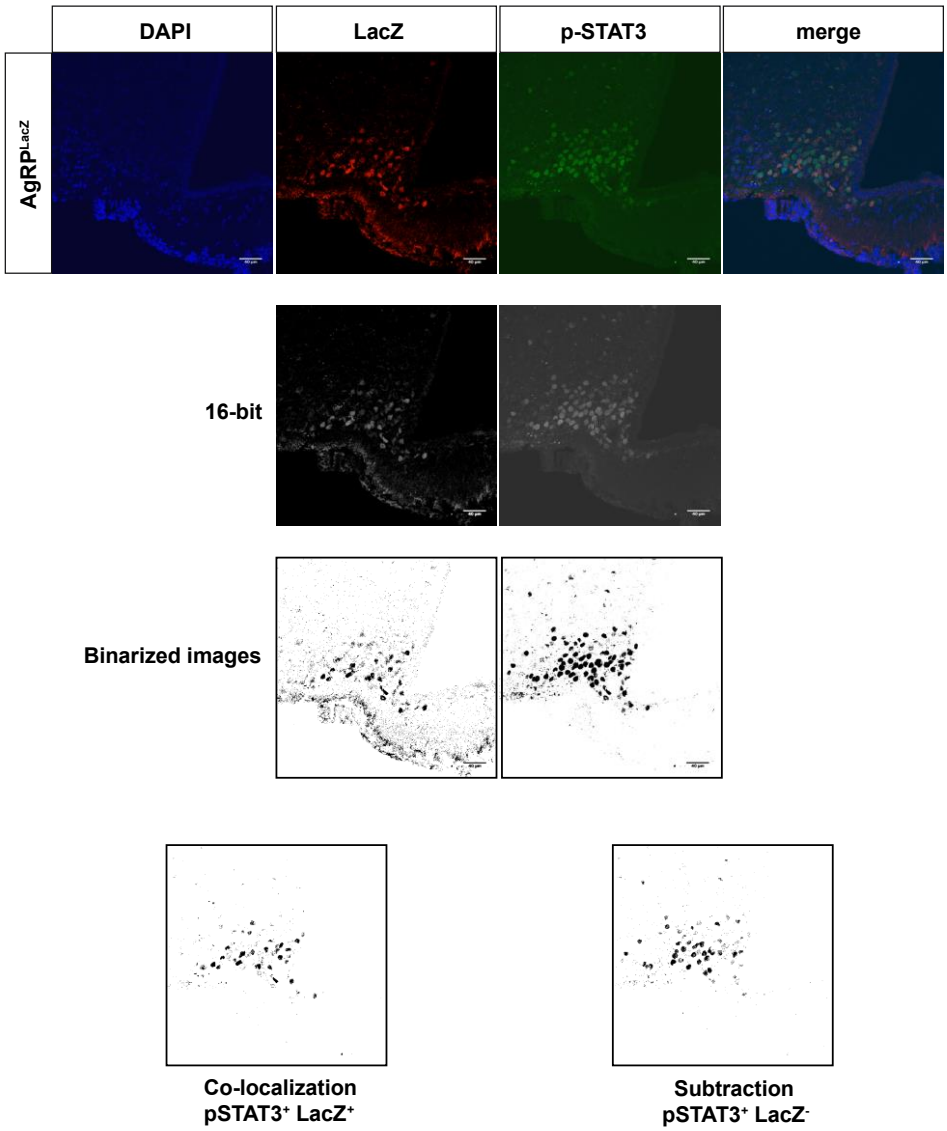

Figure 2. Immunoreactivity quantification. To quantify the immunoreactivity, each image was converted to 16-bit format and then binarized to isolate immunoreactive positive cells from the background and compensate for differences in fluorescence intensity. To determine co-localization, the integrated density of overlapping pixels was measured, from the pSTAT3 and LacZ binarized images. The integrated density is proportional to the intensity of pSTAT3 and LacZ immunoreactivity co-localization. To quantify the intensity of pSTAT3 immunoreactivity in non-LacZ cells ($LacZ^-$), the binarized image of LacZ was subtracted from the binarized image of pSTAT3 to define the pSTAT3 immunoreactivity in $LacZ^-$ cells (non-AgRP neurons), and the integrated density of the resulting image defined the intensity.

For semi-quantitative analysis of PIP3 levels in AgRP neurons, β-galactosidase positive neurons were assessed in ARC slices of control $AgRP^{LacZ}$, $AgRP^{JNK1CA;LacZ}$ and $AgRP^{IKK2CA;LacZ}$ mice, which had been injected either with saline or insulin as previously described (Plum et al., 2006). In detail, the amount of PIP3

was classified as low (immunoreactive cytoplasmic dots/sprinkles in the proximity of the nucleus at background levels, i.e. 6 or fewer dots, no cloudy aspect, no confluent areas), moderate (dots/sprinkles at levels above background, i.e. more than 6 dots, cloudy aspect), or high (more than 20 dots/sprinkles, cloudy with confluent areas) (Figure 3).

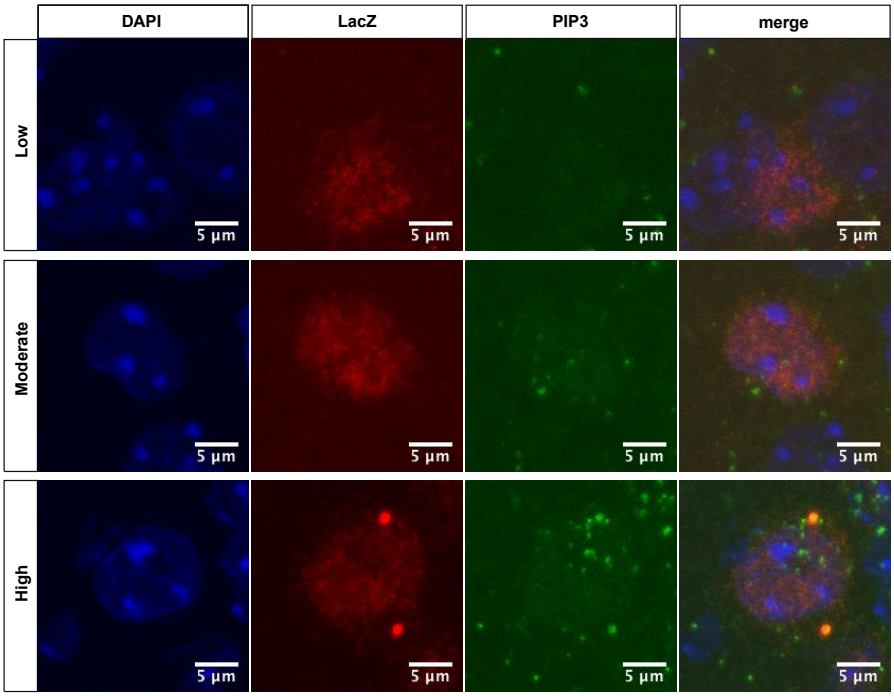

Figure 3. PIP3 quantification. The amount of PIP3 was classified as low (immunoreactive cytoplasmic dots/sprinkles in the proximity of the nucleus at background levels, i.e. 6 or fewer dots, no cloudy aspect, no confluent areas), moderate (dots/sprinkles at levels above background, i.e. more than 6 dots, cloudy aspect), or high (more than 20 dots/sprinkles, cloudy with confluent areas.

Determination of adipocyte surface

Haematoxylin and eosin staining was performed in epididymal/parametrial adipose tissue. In order to quantify the adipocyte surface in epididymal/parametrial adipose tissue, pictures were taken with a bright-field Leica microscope and the surface size was determined using the provided Leica software.

2.6 Data analysis and statistical methods

Data analysis was performed with Igor Pro 6 (Wavemetrics) and Graphpad Prism (version 5.0b; Graphpad Software Inc., La Jolla, CA, USA). Numerical values are given as mean ± standard error. Boxplots are generated according to Tukey, means

are reflected by "+" and medians by the dash, respectively. To determine differences in means *t* tests and 2-way ANOVA analyses were performed. A significance level of 0.05 was accepted for all tests. *$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$ *versus* controls.

2.7 Chemicals and Enzymes

Table 2. Chemicals and enzymes

0.9% saline, sterile	Delta Select, Pfullingen, Germany
2,2,2-Tribromethanol (Avertin)	Sigma-Aldrich, Seelze, Germany
Agarose (Ultra Pure)	Invitrogen, Karlsruhe, Germany
Aprotinin	Sigma-Aldrich, Seelze, Germany
Bacillol	Bode Chemie, Hamburg, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Seelze, Germany
Bromphenol blue	Marck, Darmstadt, Germany
Calcium chloride	Merck, Darmstadt, Germany
Diaminobenzidin (DAB)	Dako, Denmark
Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany
di-Natriumhydrogenphosphat	Merck, Darmstadt, Germany
Ethanol, absolute	Applichem, Darmstadt, Germany
Ethidium bromide	Sigma-Aldrich, Seelze, Germany
Ethylendiamide tatraacetate (EDTA)	Applichem, Darmstadt, Germany
Glucose 20%, sterile	DeltaSelect, Pfullingen, Germany
Glycerol	Serva, Heidelberg, Germany
HEPES	Applichem, Darmstadt, Germany
Hydrogen peroxide	Sigma-Aldrich, Seelze, Germany
Insulin (human)	Novo Nordisk, Basvaerd, Denmark
Isopropanol	Roth, Karlsruhe, Germany
Ketamine hydrochloride	Sigma-Aldrich, Seelze, Germany
Leptin	NHPP, USA
Magnesium chloride	Merck, Darmstadt, Germany
Methanol	Roth, Karlsruhe, Germany
Nitrogen (liquid)	Linde, Pullach, Germany
Paraformaldehyde (PFA)	Fluka, Sigma-Aldrich, Seelze, Germany
Phosphate buffered saline (PBS)	Gibco, Eggenstein, Germany
Potassium hydroxide	Merck, Darmstadt, Germany

Rompun (Xylazine)	Bayer, Germany
Sodium chloride	Applichem, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Applichem, Darmstadt, Germany
Sodium fluoride	Merck, Darmstadt, Germany
Sodium hydroxide	Applichem, Darmstadt, Germany
Sodium orthovanadate	Sigma-Aldrich, Seelze, Germany
Sodium pyruvate	Sigma-Aldrich, Seelze, Germany
Tissue freezing medium	Jung, Heidelberg, Germany
Tris	AppliChem, Darmstadt, Germany
Vectashield (with DAPI)	Vector, Burlingame, USA
Proteinase K	Roche, Basel, Switzerland
DreamTaq DNA Polymerase	Thermo Scientific, Walldorf, Germany

3 Results

3.1 Hypothalamic inflammation in obesity and insulin resistance

3.1.1 Activation of JNK signalling in AgRP neurons occurs in obesity and increases firing of these cells

Hypothalamic inflammation, and particularly activation of JNK signalling, is observed after HFD consumption (Belgardt et al., 2010) but the relative contribution of the different hypothalamic cell types remains elusive. Therefore, to assess whether JNK-activation occurs in AgRP neurons during obesity development, we employed a reporter mouse strain that allows for genetic identification of AgRP/NPY-co-expressing neurons via the expression of humanized *Renilla* Green Fluorescent Protein (hrGFP) under the control of the mouse *Npy* promoter (NPY^{GFP}) and fed 8-week-old mice with NCD (NCD:NPY^{GFP}) or HFD for ten days (HFD:NPY^{GFP}). Immunostaining for phosphorylated c-Jun -as a read-out for JNK-activation and a crucial regulator of hypothalamic inflammation (De Souza et al., 2005; Belgardt et al., 2010; Sabio et al., 2010; Unger et al., 2010)- in combination with GFP performed revealed a ~3-fold increase of p-c-Jun immunoreactivity in GFP positive neurons in NPY^{GFP} mice after HFD consumption compared to the NCD fed NPY^{GFP} mice (Figure 4A, B). These data indicate that indeed early during the course of HFD-feeding JNK is activated in AgRP neurons.

To address whether JNK-activation *per se* in AgRP neurons, as observed upon obesity development and HFD consumption (Figure 4A, B), is sufficient to induce metabolic abnormalities we sought to generate a genetic mouse model for cell type-specific JNK-activation. Constitutive activation of JNK has previously been achieved by expression of a constitutively active version of the upstream kinase JNKK2/MKK7 (MKK7D) (Wang et al., 1998) or a fusion protein consisting of JNKK2/MKK7 and JNK1 (JNKK2-JNK1) (Zheng et al., 1999). Thus, we generated mice allowing for the conditional expression of a MKK7D-JNK1 fusion protein upon Cre-recombinase-induced excision of a transcriptional Stop cassette ($R26Stop^{FL}$JNK1CA mice) from the ROSA26-locus (Pal et al., 2013). In order to activate JNK signalling in AgRP neurons, we crossed $R26Stop^{FL}$JNK1CA mice with mice expressing the Cre-recombinase in these cells ($AgRPCre^{+/-}$) (Tong et al., 2008).

To investigate the efficiency of this transgenic approach to activate JNK, we generated reporter mice expressing GFP in AgRP neurons, by crossing the $AgRP^{Cre}$ with the Z/EG mice, in the presence or absence of JNK1CA expression. Immunostaining for phosphorylated c-Jun -as a read-out for JNK-activation- in combination with GFP revealed that less than 20% of GFP^{+}-AgRP neurons exhibited p-c-Jun immunoreactivity in lean control animals, whereas in $AgRP^{JNK1CA;GFP}$ mice more than 70% of GFP^{+}-AgRP neurons showed detectable phosphorylation of c-Jun (Figure 4C, D), in fact to similar degree as observed upon HFD-feeding. Importantly, constitutive JNK activation in AgRP neurons did not affect the total number of GFP^{+}-AgRP cells, although JNK1 had been implicated in a cell-type specific regulation of cell death and survival (Bogoyevitch, 2006), (Figure 4E). Taken together, these results indicate that the $AgRP^{JNK1CA}$ mice are a suitable model to investigate the physiological consequences of chronically activated JNK1 signalling in AgRP neurons without altering AgRP-neuron viability.

To determine the effect of activated JNK-signalling on the cellular properties of AgRP neurons, we first investigated the electrophysiological parameters of AgRP neurons. Here, we employed the NPY^{GFP} reporter mouse strain and crossed it to $AgRP^{JNK1CA}$ and the respective control mice. Perforated patch-clamp recordings of genetically identified AgRP/NPY-neurons revealed that activation of JNK-signalling in AgRP neurons of NPY^{GFP};$AgRP^{JNK1CA}$ mice increased the spontaneous firing frequency of these cells and depolarized the membrane potential compared to AgRP/NPY-neurons of lean control littermates (NPY^{GFP};JNK1CA;$AgRP^{Cre-/-}$) (Figure 4F-H). We did not observe any differences in other basic electrophysiological properties, such as input resistance, cell capacitance and spike frequency adaptation (Figure 5A-C).

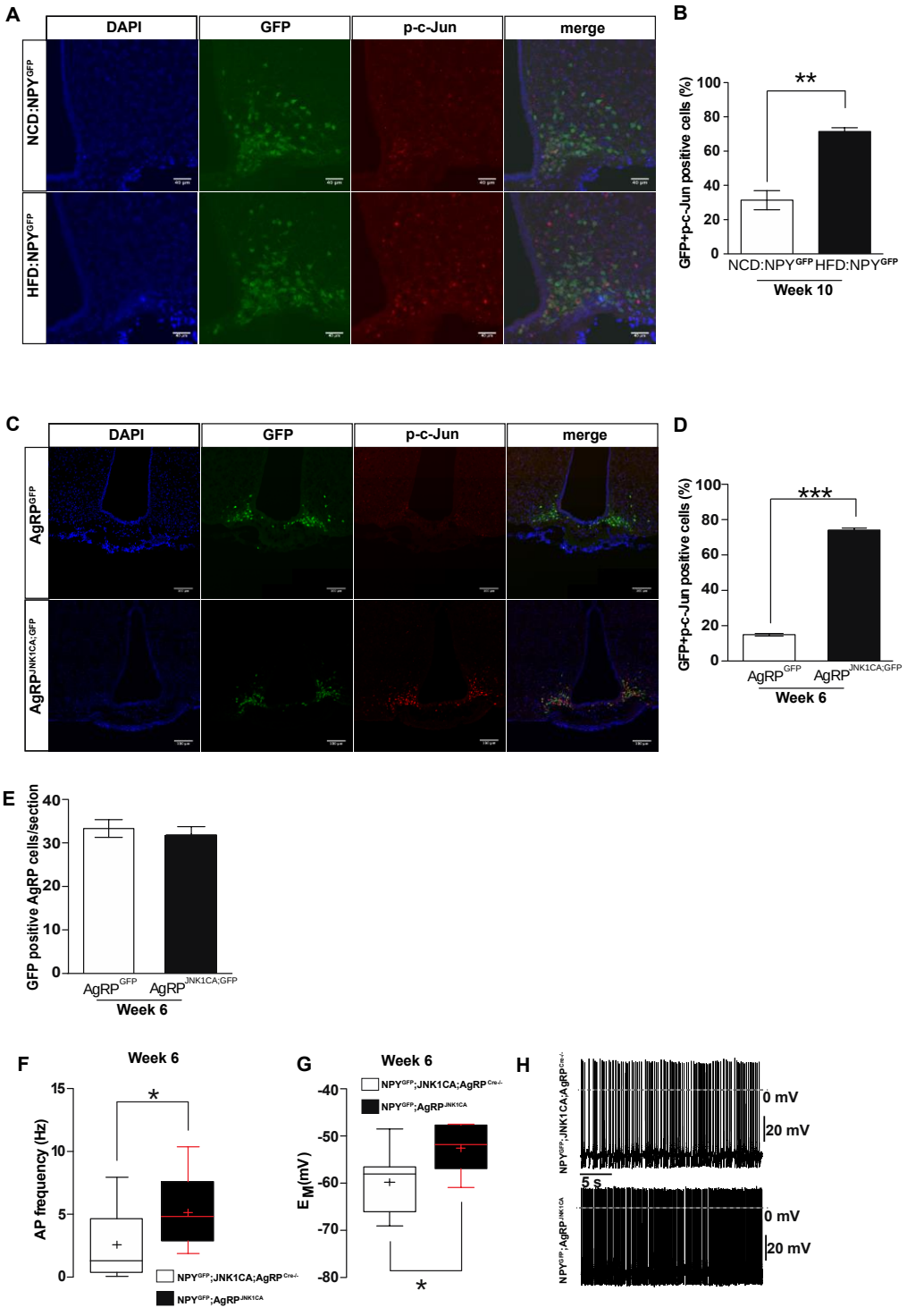

Figure 4. Activation of JNK signalling in AgRP neurons occurs in obesity and increases firing of these cells. A, B) High-fat diet consumption for ten days is sufficient to trigger c-Jun phosphorylation in AgRP neurons of NPY^{GFP} reporter mice. Immunostaining for phospho-c-Jun and GFP performed in arcuate nuclei of NPY^{GFP} reporter mice at the age of 10 weeks fed with normal chow diets (NCD) or high-fat diet for ten days (HFD). The number of p-c-Jun and GFP double positive neurons was significant increased in NPY^{GFP} mice fed with HFD (HFD:NPY^{GFP}; *n*=3) compared to NPY^{GFP} mice

fed with normal chow diet (NCD:NPY^{GFP}; *n*=3). Scale bar, 40 µm. C, D) Expression of the JNK1CA protein in AgRP neurons leads to cell-specific phosphorylation of the JNK-target c-Jun *in vivo*. AgRP neurons were visualized by immunostaining for GFP in AgRP^{GFP} and AgRP$^{JNK1CA;GFP}$ reporter animals. Immunostaining for phospho-c-Jun revealed few AgRP neurons positive for p-c-Jun in control reporter animals, whereas the majority of AgRP neurons in AgRP$^{JNK1CA;GFP}$ mice showed clear immunoreactivity for p-c-Jun. Quantification of p-c-Jun and GFP positive AgRP neurons is depicted in D in control AgRP^{GFP} (*n*=3) and AgRP$^{JNK1CA;GFP}$ (*n*=3) reporter mice. Scale bar, 100 µm.

E) Chronic JNK1 activation does not affect AgRP neuron numbers. All GFP^{+} AgRP neurons were counted in control AgRP^{GFP} (*n*=3) and AgRP$^{JNK1CA;GFP}$ (*n*=3) reporter mice. No difference in neuron counts per slide was detected between genotypes.

F) Spontaneous action potential frequencies of NPY^{GFP};JNK1CA;AgRP$^{Cre-/-}$ (*N*=3 mice; *n*=13 AgRP neurons) and NPY^{GFP};ARP^{JNK1CA} (*N*=3 mice; *n*=9 AgRP neurons).

G) Membrane potentials of NPY^{GFP};JNK1CA;AgRP$^{Cre-/-}$ (*N*=3 mice; *n*=13 AgRP neurons) and NPY^{GFP}/ARP^{JNK1CA} (*N*=3 mice; *n*=8 AgRP neurons).

H) Representative recordings of spontaneous action potential frequencies of NPY^{GFP};JNK1CA;AgRP$^{Cre-/-}$ and NPY^{GFP}/ARP^{JNK1CA} mice at the age of 6 weeks.

Electrophysiological experiments were performed by Lars Paeger. Displayed values are means +/- SEM. ***p<0.001.

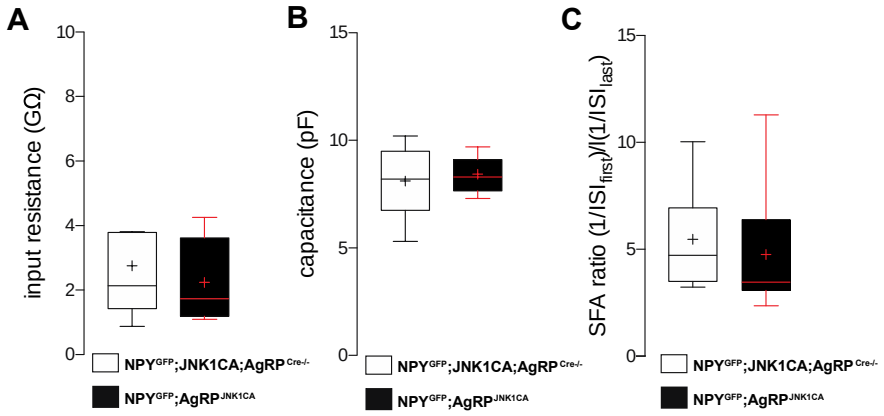

Figure 5. JNK1 activation does not affect input resistance, capacitance and spike frequency adaptation ratio in AgRP neurons. Input resistances A, cell capacitances B and spike frequency adaptation (SFA) ratios C show no differences between control (NPY^{GFP};JNK1CA;AgRP$^{Cre-/-}$) and NPY^{GFP};AgRP^{JNK1CA} mice. Electrophysiological experiments were performed by Lars Paeger.

3.1.2 JNK activation in AgRP neurons causes cellular and systemic leptin resistance

Apart from regulating neuronal firing of AgRP neurons, leptin exerts its regulatory role on feeding behaviour through STAT3-dependent regulation of neuropeptide expression, including that of AgRP and NPY (Bates et al., 2003). Thus, we directly assessed leptin's ability to activate STAT3-phosphorylation in AgRP neurons of $AgRP^{JNK1CA}$ and control mice. To this end, we employed mice, which allow for Cre-dependent expression of ß-galactosidase (Plum et al., 2006) and crossed these animals with $AgRP^{JNK1CA}$ mice, yielding both, control mice with genetically marked AgRP neurons ($AgRP^{LacZ}$ mice) as well as $AgRP^{JNK1CA;LacZ}$ mice. Whereas *in vivo* leptin stimulation markedly induced Stat3 phosphorylation in AgRP neurons of 10-week-old control mice, leptin's ability to induce pSTAT3-immunoreactvity in AgRP neurons of $AgRP^{JNK1CA;LacZ}$ mice was largely reduced, demonstrated both as the percentage of pSTAT3 immunoreactive LacZ positive cells per section (Figure 6A, B) and as intensity of the pSTAT3 immunoreactivity in LacZ positive cells (Figure 6C). Thus, activating JNK-signalling in AgRP neurons induces leptin resistance in these cells. This effect is AgRP cell-specific, since pSTAT3 immunoreactivity after leptin stimulation is not affected in non-AgRP cells (Figure 6D and Figure 6E).

To investigate systemic leptin sensitivity, control and $AgRP^{JNK1CA}$ mice were injected twice daily for 3 consecutive days with saline followed by twice-daily injections for 3 consecutive days with 2mg/kg leptin. Leptin treatment significantly reduced food intake and body weight in both, control and $AgRP^{JNK1CA}$ mice at 8 weeks of age (Figure 6F, G). Importantly, at the age of 15 weeks, leptin treatment failed to suppress food intake and to reduce body weight in $AgRP^{JNK1CA}$ mice, whereas control mice still responded to the food intake- and body weight-reducing action of leptin (Figure 6H, I). Of note, leptin resistance at this age occurred in weight-matched animals, to rule out a potential confounding effect of increased body weight between the two genotypes. Collectively, these experiments reveal that chronic activation of JNK-signalling in AgRP neurons is sufficient to initially cause cell-autonomous and subsequently systemic leptin resistance.

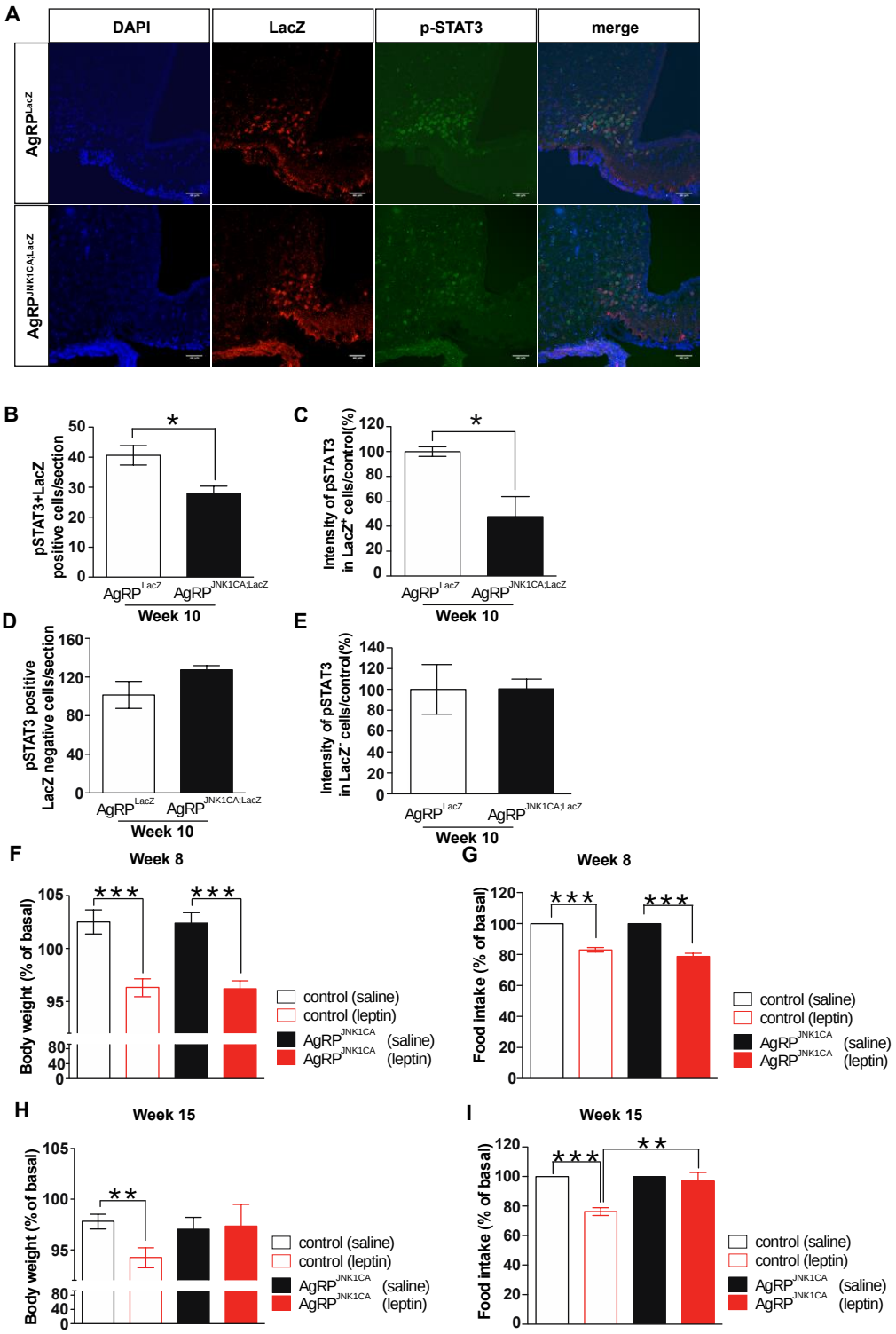

Figure 6. JNK1 activation causes AgRP neuron-specific and subsequently systemic leptin resistance. A) Representative pSTAT3 immunostaining of AgRP^{LacZ} and AgRP$^{JNK1CA;LacZ}$ after fasting for 16h and intraperitoneal leptin stimulation (1mg/kg) in 10 week-old animals. Scale bar, 40 µm. B) Average number of pSTAT3 and LacZ immunoreactive cells per section of control AgRP^{LacZ} (*n*=3) and AgRP$^{JNK1CA;LacZ}$ (*n*=3) mice. C) Intensity of pSTAT3 immunoreactivity in AgRP neurons of control AgRP^{LacZ} (*n*=3) and AgRP$^{JNK1CA;LacZ}$ (*n*=3) mice depicted as the percentage of the control. D) Average number pSTAT3 immunoreactive non-AgRP (LacZ negative) cells after leptin stimulation is

indistinguishable between $AgRP^{LacZ}$ (*n*=3) and $AgRP^{JNK1CA;LacZ}$ (*n*=3) mice. E) The intensity of pSTAT3 immunoreactivity in non-AgRP ($LacZ^-$) cells after leptin stimulation is indistinguishable between $AgRP^{LacZ}$ (*n*=3) and $AgRP^{JNK1CA;LacZ}$ (*n*=3) mice. F) Changes in body weight after intraperitoneal leptin treatment in control (*n*=7) and $AgRP^{JNK1CA}$ (*n*=7) mice at 8 weeks of age. Data represent percentage of basal body weight (the body weight in the beginning of each treatment) after a 3-day (72h) treatment with twice-daily injections (12h apart) of saline followed by a 3-day treatment with twice-daily injections of 2mg/kg leptin. G) Changes in food intake after intraperitoneal leptin treatment in control (*n*=7) and $AgRP^{JNK1CA}$ (*n*=7) mice at 8 weeks of age. Data represent daily food intake after a 3-day (72h) treatment with twice-daily injections (12h apart) of saline followed by a 3-day treatment with twice-daily injections of 2mg/kg leptin. H) As in F but at 15 weeks of age (*n*=10; 4). I) As in G but at 15 weeks of age (*n*=10; 4). Displayed values are means +/- SEM. *p<0.05; **p<0.01; ***p<0.001.

3.1.3 Activation of JNK signalling in AgRP neurons causes obesity

To address the effect of activating JNK signalling in AgRP neurons on energy homeostasis, we monitored the body weight of male control and $AgRP^{JNK1CA}$ mice. Whereas body weight did not differ between genotypes at the beginning of our analysis, $AgRP^{JNK1CA}$ mice displayed progressively increased body weight starting at the age of 5 weeks, which continued until the end of our study at 28 weeks of age (Figure 7A). Moreover, analysis of body composition revealed increased adipose tissue mass in $AgRP^{JNK1CA}$ compared to control mice (Figure 7B), which was associated with adipocyte hyperplasia (Figure 7C, D).

In light of the increased body weight and adiposity of $AgRP^{JNK1CA}$ mice, we aimed to identify the mechanism of how AgRP-neuron-specific activation of JNK-signalling caused a positive energy balance. These analyses revealed that daily food intake was significantly increased upon activation of JNK-signalling in AgRP-cells (Figure 7E), whereas locomotor activity and energy expenditure remained unaltered in $AgRP^{JNK1CA}$ mice compared to control mice (Figure 7F,G). Thus, activation of JNK signalling in AgRP neurons is sufficient to promote hyperphagia and obesity development in mice.

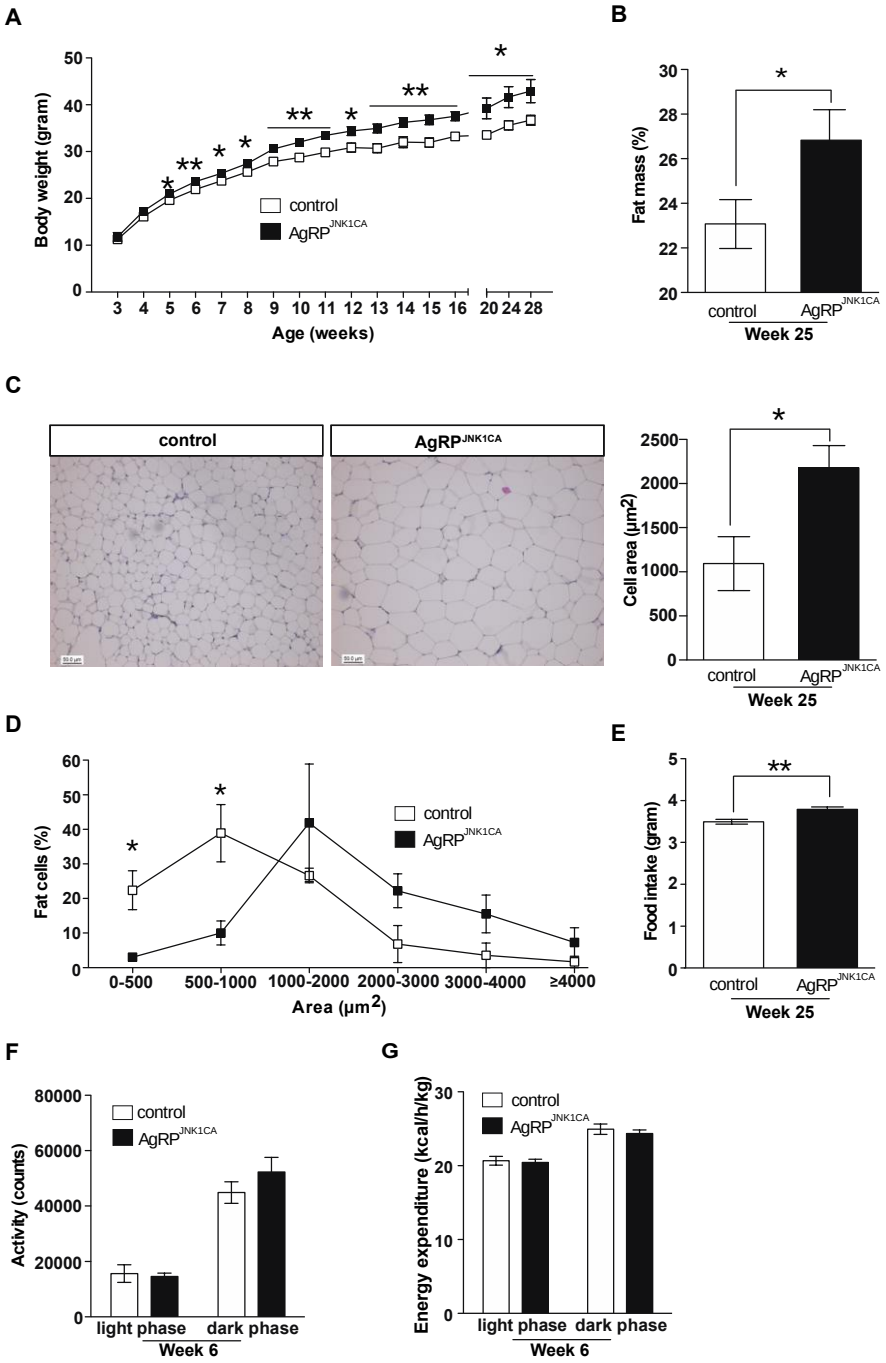

Figure 7. **Chronic JNK1 signalling in AgRP neurons results in increased body weight and adiposity.** A) Average body weight of control (*n*= 20) and AgRP^{JNK1CA} mice (*n*= 20) on normal diet. B) Body fat as measured by nuclear magnetic resonance analysis of control (*n*= 12) and AgRP^{JNK1CA} mice (*n*= 14) on normal diet at the age of 25 weeks. C) Representative pictures of epididymal adipose tissue paraffin sections. Scale bar, 50µm. Average fat cell area of control (*n*= 3) and AgRP^{JNK1CA} mice (*n*= 4) at the age of 25 weeks. D) Fat cell area distribution of control (*n*= 3) and AgRP^{JNK1CA} mice (*n*= 4) at the age of 25 weeks. E) Average ad libitum food intake at the age of 6 weeks of control (*n*= 12) and AgRP^{JNK1CA} mice (*n*= 12). F) Locomotor activity at the age of 6 weeks of control (*n*= 7) and AgRP^{JNK1CA} mice (*n*= 7). G) Energy expenditure at the age of 6 weeks of control (*n*= 7) and AgRP^{JNK1CA} mice (*n*= 7). Displayed values are means +/- SEM. *p<0.05; **p<0.01.

3.1.4 Unaltered glucose homeostasis in AgRP^{JNK1CA} mice

Insulin action in AgRP neurons is critical for the control of hepatic glucose output (Könner et al., 2007; Lin et al., 2010). Insulin activates the PI3 kinase cascade in AgRP neurons, which catalyses the generation of phosphatidylinositol 3,4,5 triphosphate (PIP3) from phosphatidylinositol 4,5 diphosphate (PIP2). To examine whether JNK activation leads to the development of insulin resistance, we investigated the effect of constitutive JNK activation in AgRP neurons on insulin's ability to activate PI3-kinase signalling in these cells by immunostaining for PIP3 in genetically marked β-galactosidase-positive AgRP neurons of mice expressing LacZ in AgRP neurons in the presence or absence of JNK1CA expression (AgRP$^{JNK1CA;LacZ}$ and AgRP^{LacZ}, respectively). In both, AgRP^{JNK1CA} and control mice, insulin treatment resulted in comparable PIP3 formation in AgRP neurons, indicating that activation of JNK-signalling did not cause neuronal insulin resistance in this cell type (Figure 8A, B). Basal levels of immunoreactive PIP3 were also indistinguishable between AgRP^{LacZ} and AgRP$^{JNK1CA;LacZ}$ mice (Figure 8C). Consistent with unaltered insulin action in AgRP neurons, systemic glucose homeostasis remained unaffected in AgRP^{JNK1CA} compared to control mice as assessed by insulin and glucose tolerance tests (Figure 8D, E). Thus, these experiments revealed the development of leptin but not insulin resistance as a consequence of JNK activation in AgRP neurons.

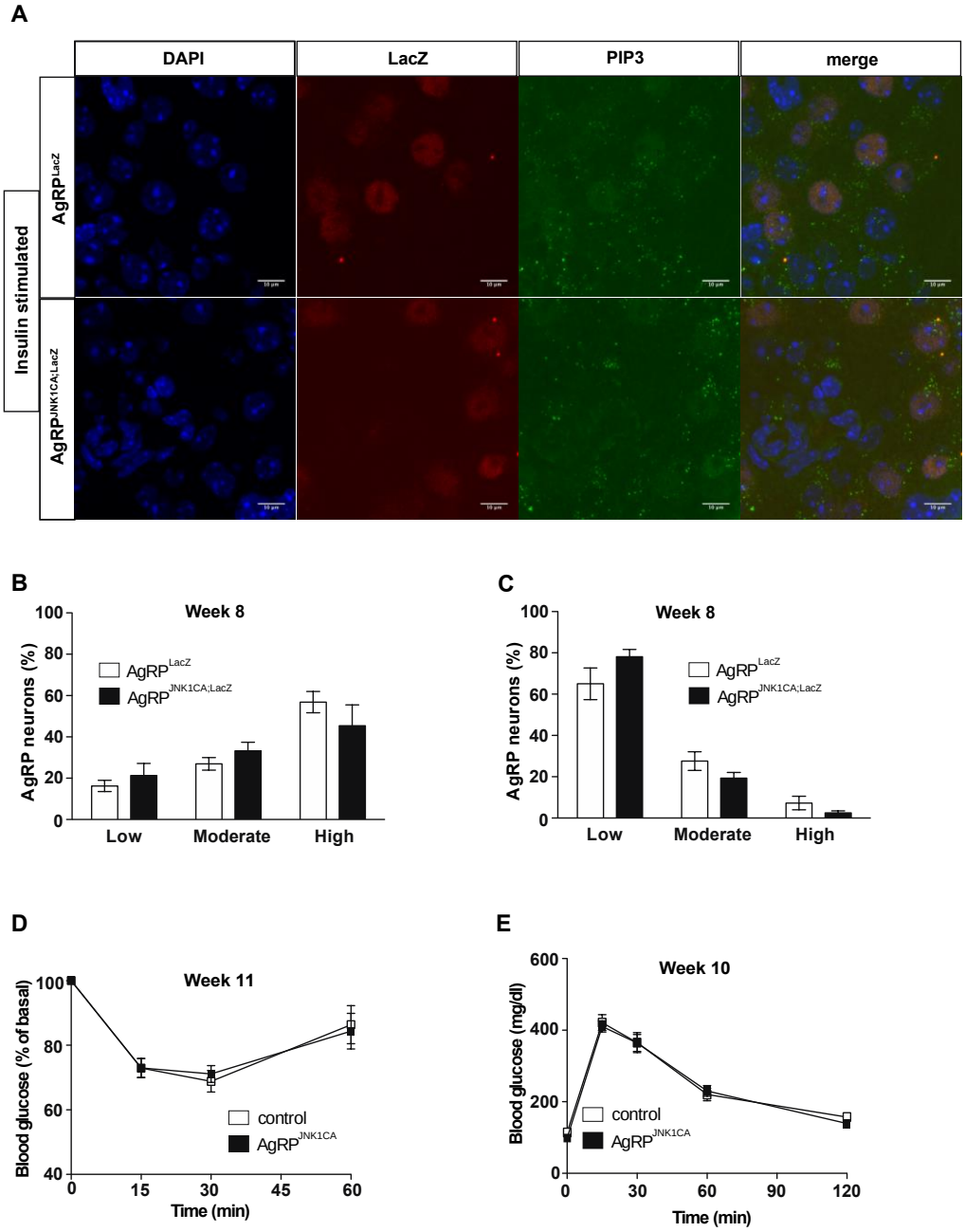

Figure 8. Unaltered AgRP-neuronal insulin sensitivity and glucose homeostasis in AgRP^{JNK1CA} mice. A) Representative PIP3 immunostaining of AgRP^{LacZ} and AgRP$^{JNK1CA;LacZ}$ after intravenous insulin stimulation with 5U of insulin. Scale bar, 10 µm. B) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (*N*=5 mice; *n*=460 AgRP neurons) and AgRP$^{JNK1CA;LacZ}$ (*N*=3 mice; *n*=148 AgRP neurons) mice after fasting for 16h and insulin stimulation (in the vena cava inferior) with 5U of insulin for 10 minutes. C) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (*N*=3 mice; *n*=234 AgRP neurons) and AgRP$^{JNK1CA;LacZ}$ (*N*=3 mice; *n*=346 AgRP neurons) mice in basal state (saline injection in the vena cava inferior) after fasting for 16h. D) Blood glucose levels as percentage of the initial blood glucose during an insulin tolerance test of control (*n*=14) and AgRP^{JNK1CA} mice (*n*=18). E) Blood glucose levels during glucose tolerance test of control (*n*=9) and AgRPJ^{NK1CA} (*n*=11). The mice were fasted for 6h before the injection with 20% glucose (10 ml/kg). Displayed values are means +/- SEM. *p<0.05; **p<0.01; ***p<0.001.

3.1.5 Activation of IKK2 signalling increases the firing rate of AgRP neurons

Since activation of JNK signalling in AgRP neurons was sufficient to induce obesity and leptin resistance, but neither caused neuronal insulin resistance nor impaired systemic glucose metabolism, we sought to investigate whether chronic activation of IKK2 signalling in AgRP neurons would impact on the regulation of energy homeostasis and/or glucose homeostasis. Therefore, we generated mice expressing a constitutive active IKK2 mutant (IKK2CA) (Sasaki et al., 2006) specifically in AgRP neurons ($AgRP^{Cre}R26Stop^{fl/wt}IKK2EE$, referred to as $AgRP^{IKK2CA}$ mice). Given that in this mouse model IKK2EE-expression is driven by the endogenous ROSA-26-promoter, which exhibits lower expression than the construct employed for expressing the JNK1CA variant, we generated homozygous mice expressing two copies of IKK2CA ($AgRP^{IKK2CA/CA}$) to achieve a more robust expression of the IKK2CA-variant.

To test the functionality of this approach we employed pIκBα immunostaining as a read-out for IKK2 activation in AgRP neurons of reporter mice expressing ß-galactosidase in AgRP neurons upon Cre-mediated expression of LacZ from the ROSA26-locus. Since both, the ß-galactosidase reporter, as well as the IKK2CA-allele were expressed from the ROSA-allele, we compared the degree of pIκBα immunoreactivity in control $AgRP^{LacZ}$ mice and $AgRP^{IKK2CA;LacZ}$ mice, which express only one copy of the IKK2CA-allele. Whereas approximately 45% of β-galactosidase-positive neurons displayed pIκBα immunoreactivity in control $AgRP^{LacZ}$ mice, AgRP-specific constitutive activation of IKK2 resulted in pIκBα immunoreactivity in approximately 70% of β-galactosidase-positive neurons in $AgRP^{IKK2CA;LacZ}$ mice (Figure 9A, B). Constitutive IKK2 activation in AgRP neurons did not affect the total number of $LacZ^{+}$-AgRP cells (Figure 9C). Collectively, these experiments reveal successful activation of IKK2-signalling in AgRP neurons in mice, already in those that express only one copy of the IKK2CA-allele.

Next we performed electrophysiological recordings of genetically marked NPY^{GFP}-neurons of control NPY^{GFP};IKK2CA/CA;$AgRP^{Cre-/-}$ or NPY^{GFP};$AgRP^{IKK2CA/CA}$ mice. Similar to what we had observed in NPY^{GFP};$AgRP^{JNK1CA}$ mice, activation of IKK2-signalling in AgRP neurons increased their firing rate compared to AgRP neurons of control mice (Figure 9D-F). Again, other basic electrophysiological properties remained unaltered (Figure 10A-C). These

experiments indicate that activation of both major inflammatory mediators JNK1 and IKK2 propagate increased activity of orexigenic AgRP/NPY-neurons in the ARC in a cell-autonomous manner.

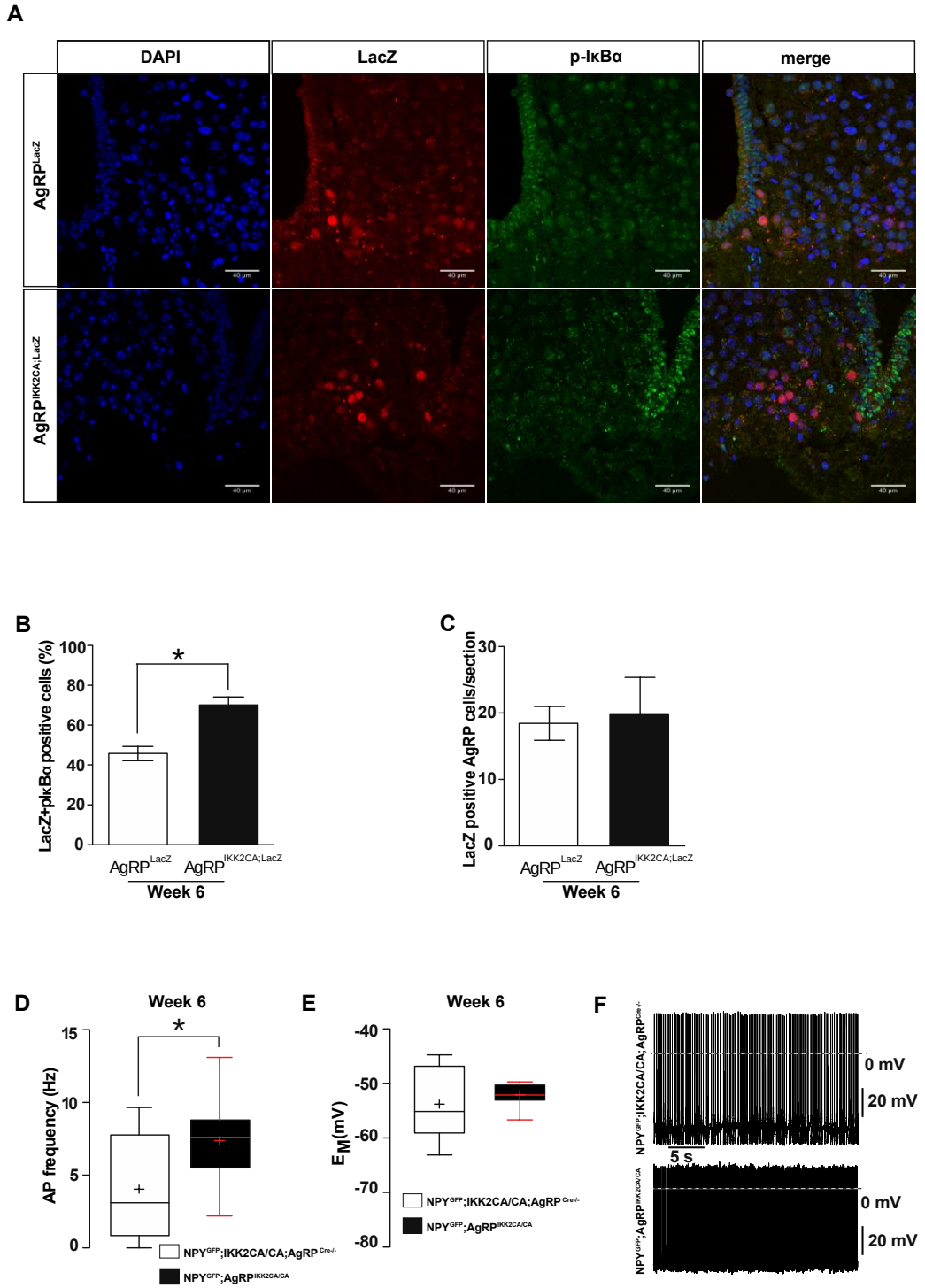

Figure 9. Activation of IKK2 signalling increases the firing rate of AgRP neurons. A, B) Expression of the IKK2CA protein in AgRP neurons leads to cell-specific phosphorylation of the IKK2 target IκBα *in vivo*. AgRP neurons were visualized by immunostaining for β-galactosidase in AgRP^{LacZ} and AgRP$^{IKK2CA;LacZ}$ reporter animals. Immunostaining for phospho-IκBα revealed 45% of the AgRP neurons positive in control reporter animals, whereas 70% of AgRP neurons in AgRP$^{IKK2CA;LacZ}$ mice

showed clear immunoreactivity for p-IκBα. Quantification of p-IκBα positive and β-galactosidase positive AgRP neurons is depicted in B in control AgRP^{LacZ} (*n*=3) and AgRP$^{IKK2CA;LacZ}$ (*n*=3) reporter mice. Scale bar, 50 µm. C) Chronic IKK2 activation does not affect AgRP neuron numbers. β-galactosidase positive AgRP neurons were counted in control AgRP^{LacZ} (*n*=3) and AgRP$^{IKK2CA;LacZ}$ (*n*=3) reporter mice. No difference in neuron counts per slide was detected between genotypes. Displayed values are means +/- SEM. *p<0.05. D) Spontaneous action potential frequencies of NPY^{GFP};IKK2CA/CA;AgRP$^{Cre-/-}$ (*N*=3 mice; *n*=17 AgRP neurons) and NPY^{GFP};ARP$^{IKK2CA/CA}$ (*N*=3 mice; *n*=11 AgRP neurons). E) Membrane potentials of NPY^{GFP};IKK2CA/CA;AgRP$^{Cre-/-}$ (*N*=3 mice; *n*=17 AgRP neurons) and NPY^{GFP};ARP$^{IKK2CA/CA}$ (*N*=3 mice; *n*=11 AgRP neurons). F) Representative recordings of spontaneous action potential frequencies of NPY^{GFP};IKK2CA/CA;AgRP$^{Cre-/-}$ (*N*=3 mice; *n*=17 AgRP neurons) and NPY^{GFP};ARP$^{IKK2CA/CA}$ (*N*=3 mice; *n*=11 AgRP neurons) mice at the age of 6 weeks. Electrophysiological experiments were performed by Lars Paeger.

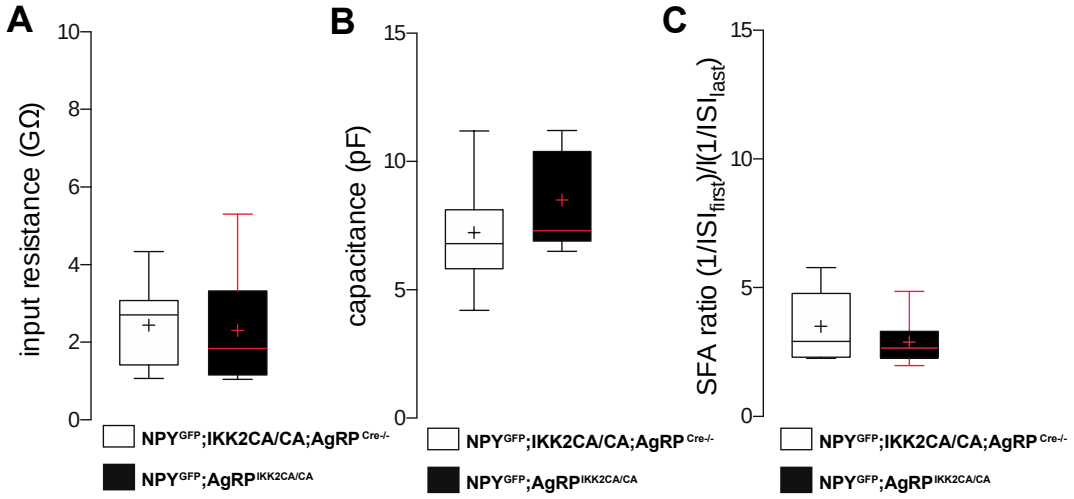

Figure 10. IKK2 activation does not affect input resistance, capacitance and spike frequency adaptation ratio in AgRP neurons. Input resistances A, cell capacitances B and spike frequency adaptation ratios C show no differences between control (NPY^{GFP};IKK2CA/CA;AgRP$^{Cre-/-}$) and NPY^{GFP};AgRP$^{IKK2CA/CA}$ mice. Electrophysiological experiments were performed by Lars Paeger.

3.1.6 Activation of IKK2 signalling in AgRP neurons does not affect leptin sensitivity or body weight

In contrast to what we observed in AgRP^{JNK1CA} mice, leptin-mediated suppression of food intake was retained in AgRP$^{IKK2CA/CA}$ mice to the same degree as in control animals even at the age of 15 weeks, when AgRP^{JNK1CA} mice develop profound systemic leptin resistance (Figure 11A, B). Taken together, activating IKK2-signalling in AgRP neurons does not interfere with body weight maintenance or leptin sensitivity in contrast to what is observed upon activation of JNK signalling in the same neurons.

Upon validation of the functionality of the constitutively active IKK2 variant expressed in AgRP neurons, we performed a metabolic characterization of AgRP$^{IKK2CA/CA}$ and their respective control mice. In contrast to what we had observed in AgRP^{JNK1CA} mice, AgRP$^{IKK2CA/CA}$ mice did not develop increased body weight or adiposity (Figure 11C, D) despite a slightly elevated food intake compared to controls (Figure 11E). Similarly, locomotor activity and energy expenditure remained unaltered between genotypes (Figure 11F, G).

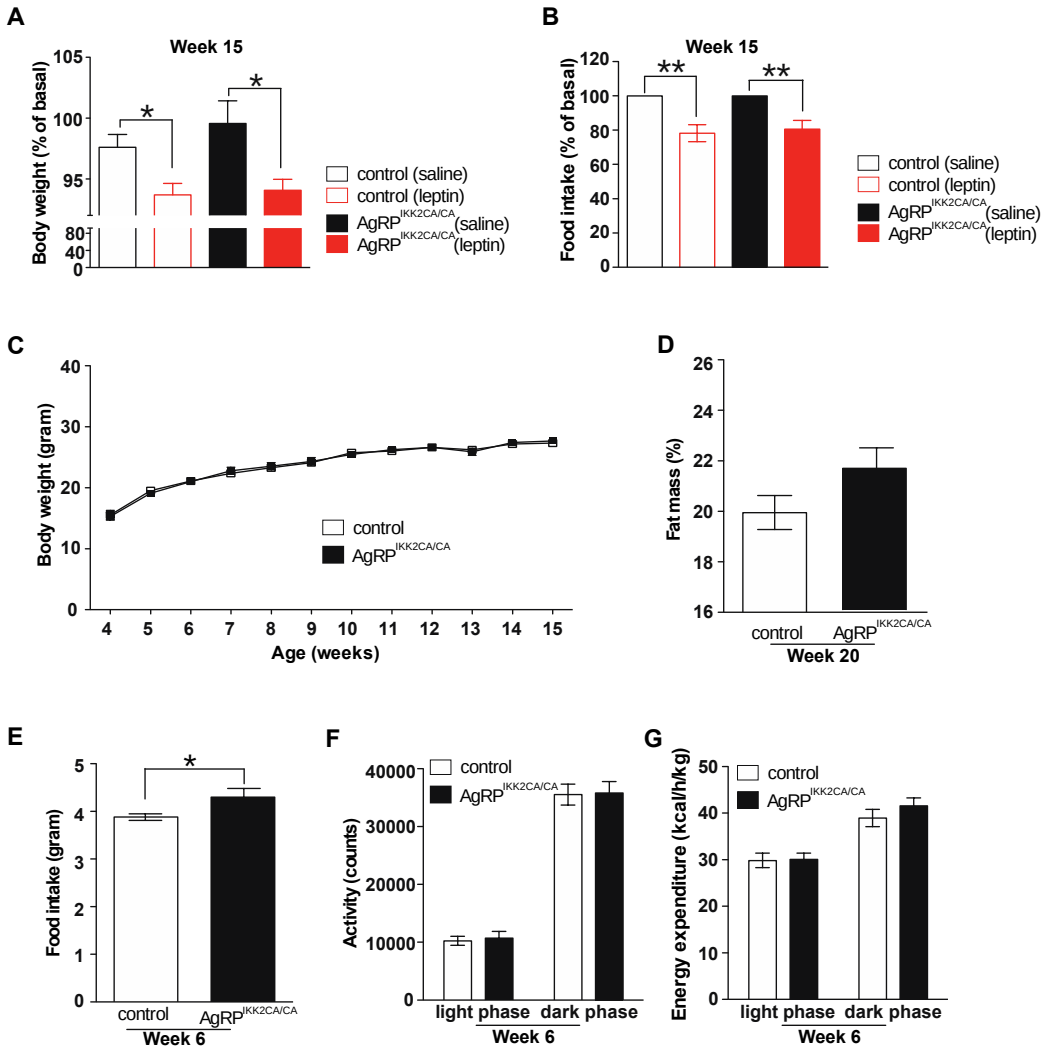

Figure 11. Chronic activation of IKK2 signalling in AgRP neurons does not affect body weight and leptin sensitivity. A) Changes in body weight after intraperitoneal leptin treatment in control (*n*=7) and AgRP^{JNK1CA} (*n*=7) mice at 15 weeks of age. Data represent percentage of basal body weight (body weight in the beginning of each treatment) after a 3-day (72h) treatment with twice-daily injections (12h apart) of saline followed by a 3-day treatment with twice-daily injections of 2mg/kg leptin. B) Changes in food intake after intraperitoneal leptin treatment in control (*n*=5) and AgRP^{JNK1CA} (*n*=5) mice at 15 weeks of age. Data represent daily food intake after a 3-day (72h) treatment with twice-daily injections (12h apart) of saline followed by a 3-day treatment with twice-daily injections of

2mg/kg leptin. C) Average body weight of control (*n*= 20) and AgRP$^{IKK2CA/CA}$ mice (*n*= 20) on normal chow diet. D) Body fat as measured by nuclear magnetic resonance analysis of control mice (*n*= 8) and AgRP$^{IKK2CA/CA}$ mice (*n*= 7) on normal chow diet at the age of 20 weeks. E) Average ad libitum food intake of control (*n*= 10) and AgRP$^{IKK2CA/CA}$ mice (*n*= 7) at the age of 6 weeks. F) Locomotor activity of control (*n*= 20) and AgRP$^{IKK2CA/CA}$ mice (*n*= 19). G) Energy expenditure at the age of 6 weeks of control (*n*= 20) and AgRP$^{IKK2CA/CA}$ mice (*n*= 19) at the age of 6 weeks. Displayed values are means +/- SEM. *p<0.05; **p<0.01.

3.1.7 Impaired glucose homeostasis in AgRP$^{IKK2CA/CA}$ mice

Despite unaltered body weight and adiposity, AgRP$^{IKK2CA/CA}$ mice displayed impaired insulin sensitivity and mild glucose intolerance as compared to control mice (Figure 12A, B). Since glucose homeostasis critically depends on the ability of insulin to activate PI3-kinase in AgRP neurons leading to subsequent membrane hyperpolarization of these neurons, we investigated the ability of insulin to activate PI-3-kinase signalling in AgRP neurons of control AgRP^{LacZ} mice and AgRP$^{IKK2CA;LacZ}$ mice. Whereas intravenous insulin stimulation induced high levels of immunoreactive PIP3 in approximately 60% of AgRP neurons in control mice, this proportion was significantly reduced to 40% in mice expressing only one IKK2CA-allele (Figure 12C, D). Of note, at basal levels the amount of PIP3 formation was also indistinguishable between AgRP$^{IKK2CA;LacZ}$ and control mice (Figure 12E). Thus, activating IKK2-signalling in AgRP neurons causes cell-autonomous insulin resistance and subsequently impairs systemic insulin sensitivity in the absence of altered body weight regulation.

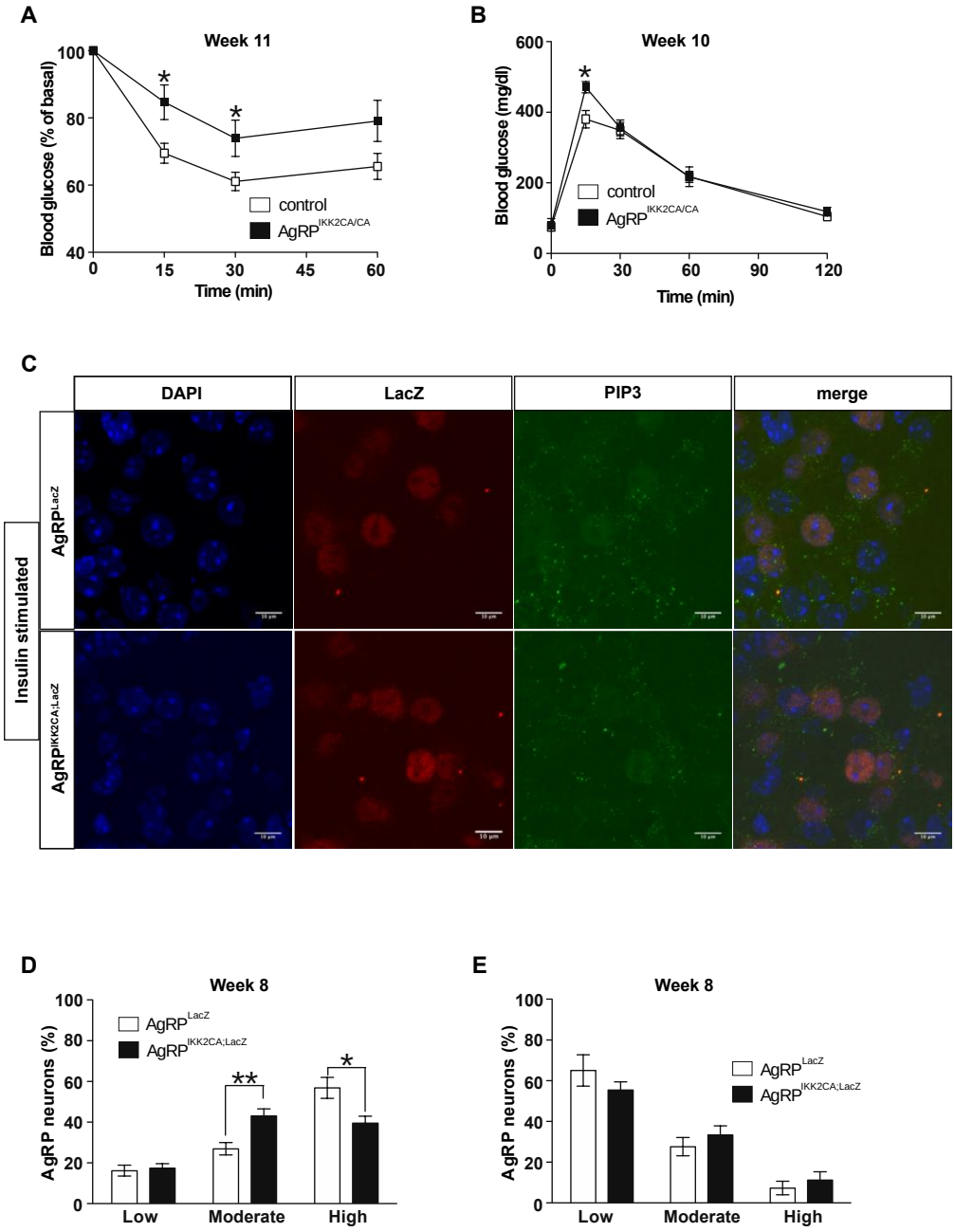

Figure 12. Impaired glucose homeostasis in AgRP$^{IKK2CA/CA}$ mice. A) Blood glucose levels as percentage of the initial blood glucose during an insulin tolerance test of control (*n*=21) and AgRP$^{IKK2CA/CA}$ (*n*=20). B) Blood glucose levels during glucose tolerance test of control (*n*=8) and AgRP$^{IKK2CA/CA}$ (*n*=7). The mice were fasted for 6h before the experiment. C) Representative PIP3 immunostaining of AgRP^{LacZ} (same as depicted in Figure 8A) and AgRP$^{IKK2CA;LacZ}$ mice after intravenous insulin stimulation with 5U of insulin. Scale bar, 10 µm. D) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (*N*=5 mice; *n*=460 AgRP neurons, same as depicted in Figure 8B) and AgRP$^{IKK2CA;LacZ}$ (*N*=5 mice; *n=357* AgRP neurons) mice after fasting for 16h and insulin stimulation (in the vena cava inferior) with 5U of insulin for 10 minutes. E) Quantification of PIP3 levels in AgRP neurons of AgRP^{LacZ} (*N*=3 mice; *n*=234 AgRP neurons, same as depicted in Figure 8C) and AgRP$^{IKK2CA;LacZ}$ (*N*=3 mice; *n*=256 AgRP neurons) mice in basal state (saline injection in the vena cava inferior) after fasting for 16h. Displayed values are means +/- SEM. *p<0.05; **p<0.01.

3.2 Hypothalamic inflammation in ageing

3.2.1 Extended median and maximum lifespan in JNK1$^{\Delta Nes}$ mice

To address the role of hypothalamic inflammation in ageing we performed a longitudinal study using the JNK1$^{\Delta Nes}$ mice. The JNK1$^{\Delta Nes}$ mice have been demonstrated to be protected by HFD-induced insulin resistance, having reduced levels of circulating IGF1 and reduced somatic growth, characteristics that resemble the effects of caloric restriction, a dietary intervention that prolongs lifespan (Belgardt et al., 2010). Log-rank testing was used to evaluate differences between lifespan in male JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ control mice, fed HFD since weaning. Median survival in JNK1$^{\Delta Nes}$ mice was significantly extended by 52 days (from 603 to 655 days, p=0.038) accounting for an 8.6% increase relative to that of JNK1$^{fl/fl}$ mice. Furthermore, maximum lifespan, which was calculated as the mean age of the 20% of the oldest animals of each genotype, was also significantly increased in JNK1$^{\Delta Nes}$ mice by 7.2% (from 762 to 818 days, p=0.007) relative to JNK1$^{fl/fl}$ mice (Figure 13). Mice were monitored daily and examined weekly for macroscopic pathological changes. In order to minimize pain, discomfort and distress the mice were euthanized when they fulfilled the moribund criteria scoring (Burkholder et al., 2012). Furthermore, mice that died during the course of the experiments were eliminated. Of note, there were no significant differences in the proportion of each mode of death between the two groups. Primary causes of death were ulcerative dermatitis (27%) and rectal prolapse (18%), also many mice were found dead without clear indications for the reason of death (28%). We next sought to determine the underlying mechanisms of the lifespan extension by investigating ageing markers and metabolic parameters at different ages.

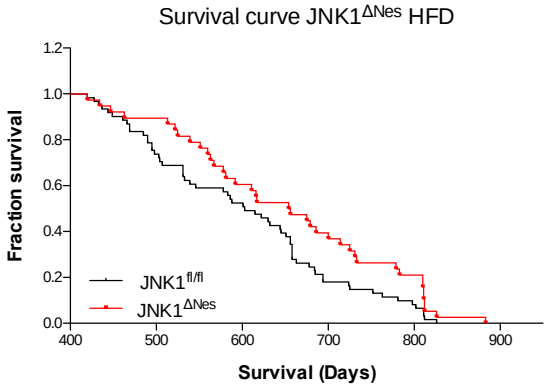

Figure 13. Ablation of JNK1 by Nestin^{Cre} results in extended lifespan in mice. Median and maximum lifespan were significantly increased in JNK1$^{\Delta Nes}$ male mice fed HFD. JNK1$^{fl/fl}$ (*n*= 61), JNK1$^{\Delta Nes}$ (*n*= 38).

3.2.2 Decreased body weight in JNK1$^{\Delta Nes}$ mice up to 1 year of age

It has previously been demonstrated that the JNK1$^{\Delta Nes}$ mice have decreased body weight under high-fat diet conditions compared to the control JNK1$^{fl/fl}$ mice until 16 weeks of age (Belgardt et al., 2010). In this longitudinal study body weight difference persists until 1 year of age but is gradually lost probably due to the age-dependent wasting (sarcopenia) that is observed in both groups of mice (Figure 14). Furthermore, we measured the body weight of Nestin^{Cre} and Bl6 littermate mice in order to assure that the body weight difference between JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ is not due to Nestin^{Cre} activity. Indeed, there was no body weight difference between Nestin^{Cre} and Bl6 mice, with their body weights being similar to those of JNK1$^{fl/fl}$ control mice (Figure 15). Collectively, the decreased body weight of JNK1$^{\Delta Nes}$ mice is not due a side effect of Nestin^{Cre} activity and is not present through the whole study.

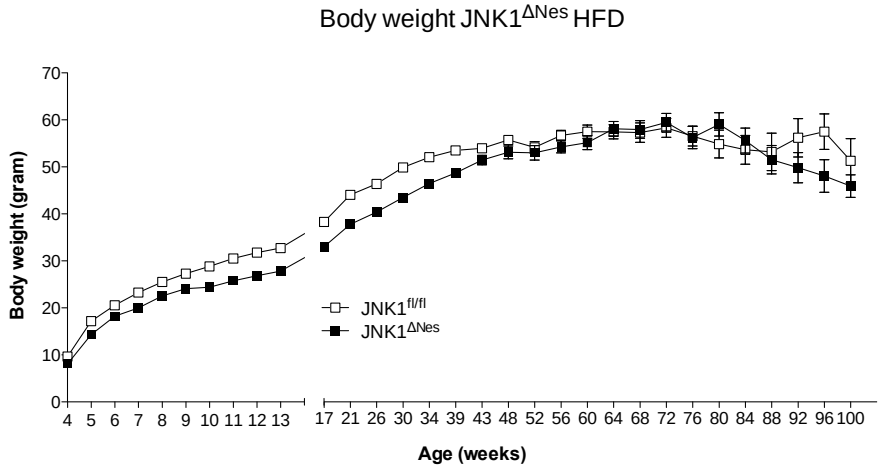

Figure 14. JNK1$^{\Delta Nes}$ mice have decreased body weight up to one year of age. The body weight of male mice upon HFD was measured weekly until week 13 and monthly until week 100. The JNK1$^{\Delta Nes}$ mice weighed less up to the age of one year ($p<0.0001$) compared to the JNK1$^{fl/fl}$ control mice. JNK1$^{fl/fl}$ (*n*= 6-57), JNK1$^{\Delta Nes}$ (*n*= 7-44).

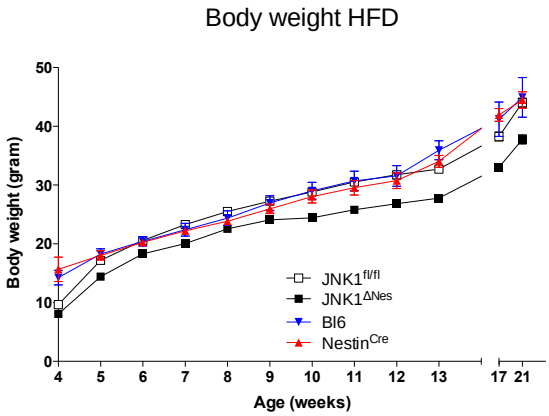

Figure 15. Nestin^{Cre} activity does not affect body weight regulation. JNK1$^{fl/fl}$ (*n*= 27), JNK1$^{\Delta Nes}$ (*n*= 33), Bl6 (*n*= 5), Nestin^{Cre} (*n*= 10).

3.2.3 Enhanced insulin sensitivity in JNK1$^{\Delta Nes}$ mice

During caloric restriction lowered fasting insulin and glucose levels combined with increased insulin sensitivity have been reported. Similarly, the JNK1$^{\Delta Nes}$ mice remain insulin sensitive after short-term high-fat feeding and also have decreased blood glucose and serum insulin (Belgardt et al., 2010). Here, we investigated insulin sensitivity longitudinally and could demonstrate that JNK1$^{\Delta Nes}$ mice are insulin sensitive also at 3, 6, 9, 12 and 15 months of age, under high-fat feeding, compared to the insulin resistant JNK1$^{fl/fl}$ mice (Figure 16). This enhanced insulin sensitivity, sustained also after prolonged high-fat feeding, resembles the positive effects of caloric restriction and is potentially contributing to the extended median and maximum lifespan of JNK1$^{\Delta Nes}$ mice.

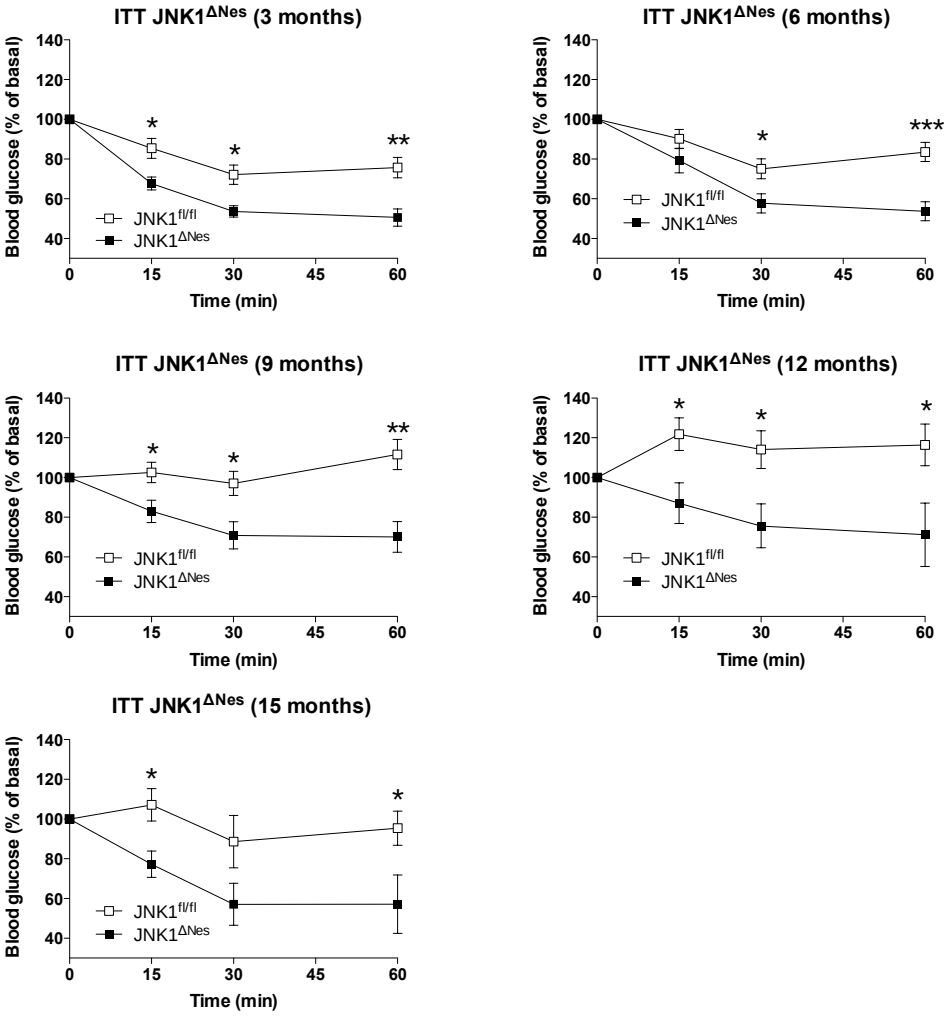

Figure 16. The JNK1$^{\Delta Nes}$ mice remain insulin sensitive upon HFD consumption until the age of 15 months. Blood glucose levels as percentage of the initial blood glucose during insulin tolerance tests at the age of 3 (*n*= 23; 13), 6 (*n*= 20; 13), 9 (*n*= 17; 11), 12 (*n*= 13; 7) and 15 (*n*= 7; 5) months of age of JNK1$^{fl/fl}$ and JNK1$^{\Delta Nes}$ mice. Displayed values are means +/- SEM. *p<0.05; **p<0.01; ***p<0.001.

3.2.4 Increased adiposity in JNK1$^{\Delta Nes}$ mice

Calorically restricted mice remain lean throughout life (Anderson et al., 2009), in contrast other longevity models present with increased adiposity (Flurkey et al., 2001). We measured fat content in JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ mice at the ages of 7 and 12 months. At 7 months of age there was a significant increase in adiposity in JNK1$^{\Delta Nes}$ compared to JNK1$^{fl/fl}$ mice, which persisted at 12 months of age (Figure 17). The increase in adiposity in JNK1$^{\Delta Nes}$ mice might be due to the reduced lipolytic effects of growth hormone (Davidson, 1987; Salomon et al., 1989). It is evident that although the JNK1$^{\Delta Nes}$ mice resemble the phenotype of calorically restricted mice, not all the characteristics are similar in these two models.

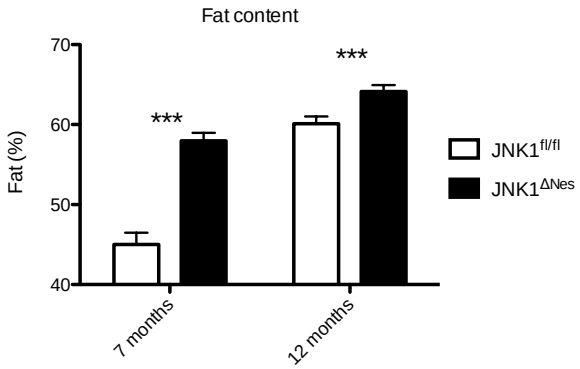

Figure 17. JNK1$^{\Delta Nes}$ mice have increased adiposity. Body fat as measured by magnetic tomography analysis of JNK1$^{fl/fl}$ mice (*n*= 11) and JNK1$^{\Delta Nes}$ mice (*n*= 30) on HFD at the age of 7 months and 12 months (*n*= 7; 17). Displayed values are means +/- SEM. ***p<0.001.

3.2.5 Increased energy expenditure in JNK1$^{\Delta Nes}$ mice

We next performed calorimetric analyses in JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ mice at 7 and 14 months of age. Oxygen consumption, CO_2 production, energy expenditure are increased and respiratory quotient decreased in JNK1$^{\Delta Nes}$ mice compared to JNK1$^{fl/fl}$ control mice at 7 months of age (Figure 18A-D). At 14 months of age the JNK1$^{\Delta Nes}$ mice still present a tendency (p= 0.12) for increased energy expenditure compared to the JNK1$^{fl/fl}$ control mice but the difference is not significant (Figure 18G-J). Of note, no differences in locomotor activity were observed between JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ mice in 7 or 14 months of age (Figure 18F, K). Collectively, the JNK1$^{\Delta Nes}$ mice have increased metabolic rate at 7 months of age but due to the age-related decline of metabolic rate in JNK1$^{fl/fl}$ mice there is no difference at 14 months of age between the two groups.

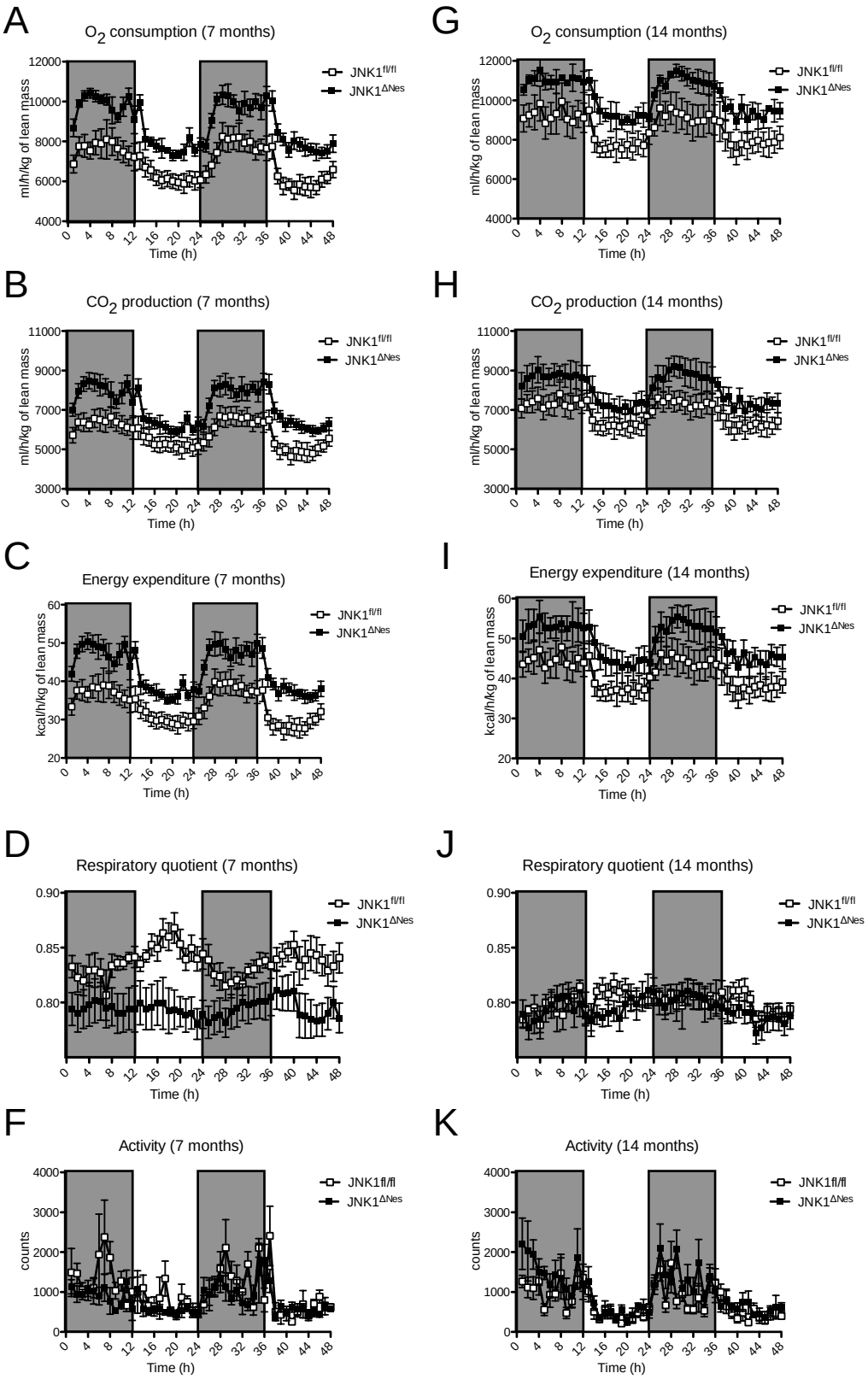

Figure 18. JNK1$^{\Delta Nes}$ mice have increased energy expenditure at the age of 7 months. A) O_2 consumption (p= 0.005; *n*= 6; 5), B) CO_2 production (p= 0.018; *n*= 6; 5), C) Energy expenditure (p= 0.007; *n*= 6; 5), D) Respiratory quotient (p= 0.04; *n*= 6; 6) and F) Activity (*n*= 6; 6) in JNK1$^{fl/fl}$ and JNK1$^{\Delta Nes}$ mice at the age of 7 months. G) O_2 consumption (*n*= 6; 5), H) CO_2 production (*n*= 6; 5), I) Energy expenditure (*n*= 6; 5), J) Respiratory quotient (*n*= 7; 7) and K) Activity (*n*= 7; 7) in JNK1$^{fl/fl}$

and JNK1$^{\Delta Nes}$ mice at the age of 14 months. The grey background represents the night phase and the white background the light phase.

3.2.6 Declined Bone Mineral Density in JNK1$^{\Delta Nes}$ mice

The effects of caloric restriction are not always beneficial and the decreased circulating levels of growth hormone and IGF1 have been correlated to decreased bone mineral density (BMD) (Devlin et al., 2010). BMD-decline is an important age-related health problem and ageing marker. Therefore, we measured the BMD of JNK1$^{\Delta Nes}$, which also present with reduced growth hormone and IGF1 levels (Belgardt et al., 2010), and JNK1$^{fl/fl}$ mice at 6 and 10 months of age. Using computed tomography scan we measured cortical, cancellous and whole BMD of JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ control mice setting as region of interest (ROI) the bones of the for- and hind limbs. Already from 6 months of age the JNK1$^{\Delta Nes}$ mice show reduced cortical BMD compared to the JNK1$^{fl/fl}$ control mice (Figure 19). At 10 months of age the JNK1$^{\Delta Nes}$ mice show reduced cortical, cancellous and whole BMD compared to the JNK1$^{fl/fl}$ control mice (Figure 19). Collectively, it is evident that the increased lifespan in JNK1$^{\Delta Nes}$ mice is accompanied by negative side effects, similar to those found in calorically restricted mice, as the reduced BMD (Devlin et al., 2010).

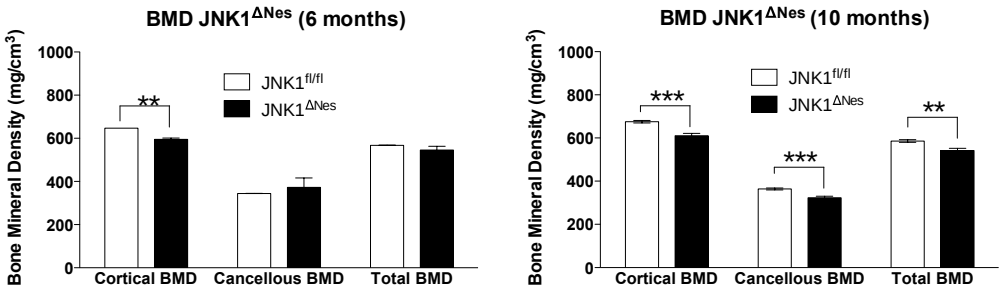

Figure 19. JNK1$^{\Delta Nes}$ mice have decreased bone mineral density. Cortical, cancellous and total BMD as measured by computed tomography scan in JNK1$^{fl/fl}$ (*n*= 4) and JNK1$^{\Delta Nes}$ (*n*= 3) at the age of 6 months and JNK1$^{fl/fl}$ (*n*= 8) and JNK1$^{\Delta Nes}$ (*n*= 6) at the age of 10 months.

3.2.7 Improved learning plasticity in JNK1$^{\Delta Nes}$ mice

There are three JNK isoforms with JNK3 being more prevalent in the brain (Weston and Davis, 2007). Brain-specific deletion of JNK3 has previously been demonstrated to protect from Aβ formation in an Alzheimer's disease model (Yoon et al., 2012). Furthermore, JNK has been indicated to be a negative regulator of associative learning (Sherrin et al., 2010). Therefore, in the JNK1$^{\Delta Nes}$ mice we investigated

spatial memory and learning abilities hypothesizing that they could also be protected from the detrimental effects of ageing in Morris Water Maze performance. JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ control mice were trained daily for 9 days to memorize the location of a submerged platform in a water pool using spatial recognition markers, and the time taken by each mouse to find the platform was measured by an automatic, software based system, termed acquisition time (Figure 20A). Each mouse performed 4 trials per day, entering the pool from different positions and given 60 seconds to find the submerged platform. On the 4th, 7th and 10th day of the test (Probe day 1, 2 and 3, respectively), the platform was removed from the pool, and the amount of time the mouse swam in the quadrant in which the platform had been, was automatically measured by a software based system and was termed the retention time (Figure 20B). There were no differences observed between JNK1$^{\Delta Nes}$ and JNK1$^{fl/fl}$ control mice in acquisition and retention time at 6 and 15 months of age (Figure 20A, B), but JNK1$^{\Delta Nes}$ mice were significantly faster when reverse acquisition time was assessed at 6 months of age. More specifically, after the 10-day experiment in 6-month-old mice the position of the platform was changed, in order to assess their ability to learn a new task, and the experiment was continued for another 3 days, with the JNK1$^{\Delta Nes}$ mice being significantly faster already from the first day (Figure 20C). The lower reverse acquisition time in the JNK1$^{\Delta Nes}$ mice proposes an increased learning plasticity, the ability to learn new tasks, which deserves further investigation in the future.

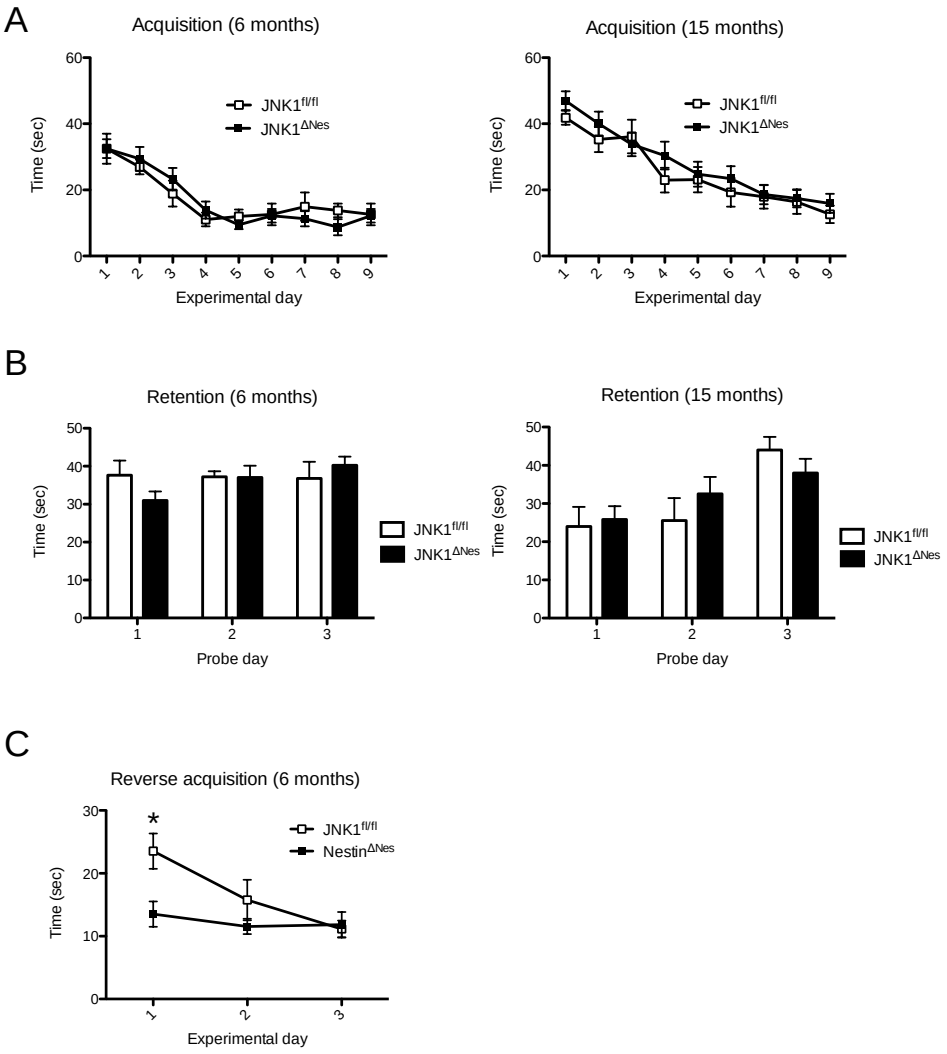

Figure 20. JNK1$^{\Delta Nes}$ mice have increased learning plasticity. Learning and memory were assessed by the Morris Water Maze test. A) There were no differences in acquisition time, the time needed to find the submerged platform, between the JNK1$^{fl/fl}$ (*n*= 8) and JNK1$^{\Delta Nes}$ (*n*= 11) at 6 months of age and JNK1$^{fl/fl}$ (*n*= 11) and JNK1$^{\Delta Nes}$ (*n*= 14) at 15 months of age. B) There were no differences in retention time, the time a mouse stays at the quadrant when the platform is removed, between the JNK1$^{fl/fl}$ (*n*= 8) and JNK1$^{\Delta Nes}$ (*n*= 11) at 6 months of age and JNK1$^{fl/fl}$ (*n*= 11) and JNK1$^{\Delta Nes}$ (*n*= 14) at 15 months of age.

4 Discussion

4.1 Hypothalamic inflammation in obesity and insulin resistance

Among the various challenges facing mankind in the 21st century, the obesity epidemic is an immense socioeconomic and health threat. In our evolutionary history we were never exposed to such an oversupply of calories before. Food was scarce, survival and reproduction were ever-present struggles, and fat, a rare energy-dense source with extremely high relative value for our reward system. Therefore, our hypothalamic neuronal circuits that regulate energy homeostasis are primarily programmed to drive food consumption to ensure survival. Importantly, we are also supplied with mechanisms to counteract excess energy intake during the times of abundance; peripheral hormones such as insulin and leptin signal to the hypothalamus to limit food intake. Prolonged and excessive fat consumption, however, results in resistance to the anorexigenic effect of insulin and leptin. Furthermore, the modern sedentary lifestyle is responsible for a major decrease in energy expenditure, making the positive energy balance even more pronounced. In addition, genetic and epigenetic parameters are added to the equation making the search for a therapy against obesity a never-ending scientific battle.

Taking advantage of our already existing anorexigenic mechanisms might prove to be a successful therapeutic strategy to counteract obesity. More specifically, if we ensure that insulin and leptin persistently mediate their effects even after prolonged fat consumption, persons may remain lean by reducing their caloric intake whenever necessary. Therefore, the mechanisms that are involved in insulin and leptin resistance initiation and manifestation, such as hypothalamic inflammation, require thorough investigation. While hypothalamic activation of inflammatory pathways including that of JNK- and IKK2-mediated signalling had been reported early upon high-fat feeding of mice (De Souza et al., 2005; Olofsson et al., 2013; Thaler et al., 2012), the functional significance of this phenomenon remained unclear. Recent experiments demonstrating that attenuation of neuronal IKK2-, JNK1-, TLR- and ER-stress-signalling protect from the development of diet-induced obesity (Zhang et al., 2008; Kleinridders et al., 2009; Belgardt et al., 2010) indicate, that hypothalamic inflammation, indeed contributes to the manifestation of the metabolic syndrome.

However, the contribution of different cell types that reside in the hypothalamus, such as astrocytes, microglia, stem cells, endothelial cells and neurons, to the initiation and manifestation of hypothalamic inflammation, is only partially understood (García-Cáceres et al., 2013; Gao et al., 2014; Purkayastha and Cai, 2013; Gosselin and Rivest, 2008). Moreover, due to the diversity and partial functional antagonism of distinct hypothalamic neurons, we sought to clarify the role of inflammatory signalling in the orexigenic AgRP neurons of the arcuate nucleus in the manifestation of the metabolic syndrome. To this end, the present study clearly reveals that activation of JNK1 and IKK2 in these cells is sufficient to initiate key features of the metabolic syndrome, even in the absence of environmental triggers, such as high-fat feeding. At the same time, our results demonstrate that activation of inflammatory signalling pathways in neurons is a crucial determinant in the pathogenesis of obesity and impaired insulin sensitivity.

4.1.1 JNK1 activation in AgRP neurons results in leptin resistance and obesity

JNK activation has been reported in the hypothalamus after HFD consumption (Belgardt et al., 2010; De Souza et al., 2005). Here we used a mouse model of targeted mutagenesis to constitutively activate JNK1 specifically in AgRP neurons, to investigate its effects on energy and glucose homeostasis in mice fed NCD. In detail, JNK1 was fused to its upstream kinase MKK7, which was rendered constitutively active through two point mutations, and this fusion protein was expressed in the ROSA26 locus after a CAGS promoter, for robust expression (Pal et al., 2013). In AgRP neurons, the levels of p-c-Jun, the downstream target of JNK1, were similar in mice expressing JNK1 in a constitutively active form and mice fed HFD for a short time, demonstrating that our model for hypothalamic inflammation mimics the effects of HFD consumption. Importantly, we were able to show that JNK1 activation does not affect the viability of the neurons, consistent with previous studies reporting the role of JNK3 in mediating the apoptotic response of neurons to stress, and not JNK1 or JNK2 (Kuan et al., 2003; Yang et al., 1997).

We next investigated the molecular effects of JNK1 activation in AgRP neurons, which revealed that it initially promotes neuronal leptin resistance and subsequently resistance to leptin's anorexigenic effects on food consumption and body weight reduction. These findings are consistent with reports that mice with

targeted disruption of LepR signalling in the AgRP neurons develop hyperphagia and obesity (van de Wall et al., 2008). Moreover, AgRP neurons have been reported to be one of the first hypothalamic neuronal populations to sense changes in plasma metabolic signals and develop cellular leptin resistance (Olofsson et al., 2013), possibly as a consequence of activating inflammatory signalling cascades, i.e. JNK. Indeed, it has been demonstrated that JNK-activation can promote SOCS3 expression, a well characterized negative regulator of leptin signalling (Qin et al., 2007; Bjørbaek et al., 1999; Howard et al., 2004). In agreement with our observations, JNK inhibition in the ARC of DIO leptin resistant mice reinstates the anorexigenic effects of leptin (Koch et al., 2014). Interestingly, in this study it was also shown that even leptin deficient mice acquire leptin resistance upon HFD consumption pointing to the role of activated inflammatory pathways, independent of hyperleptinemia to be able to cause leptin resistance (Koch et al., 2014).

On the other hand, JNK-activation in AgRP neurons fails to promote cell-autonomous and systemic insulin resistance, despite the fact that JNK-dependent serine phosphorylation of IRS-1 has been proposed to cause insulin resistance, at least *in vitro*, in myeloid progenitor cells (Aguirre et al., 2002). However, mice with a mutation of IRS-1 serine 307 to alanine, that prevents this phosphorylation, are surprisingly more insulin resistant under high-fat diet conditions than their control littermates (Copps et al., 2010). These results indicate that JNK activation does not necessarily result in attenuation of insulin signalling via IRS-1 ser307-phosphorylation *in vivo*, consistent with what we observe in AgRP neurons. Furthermore, it is possible that the fusion protein MKK7-JNK1, as it was previously reported (Zheng et al., 1999), is primarily located in the nucleus and therefore does not directly access the IRS to phosphorylate it and trigger insulin resistance, yet is still able to trigger leptin resistance indirectly by regulating SOCS3 expression. Interestingly, translocation of c-Jun to the nucleus does not require JNK signalling but binding of c-Jun enhances nuclear accumulation of JNK (Schreck et al., 2011).

4.1.2 IKK2 activation in AgRP neurons triggers insulin resistance

In order to investigate the effects of IKK2 activation in AgRP neurons we generated mice that express a constitutively active form of IKK2 (IKK2EE) (Sasaki et al., 2006) specifically in AgRP neurons. IKK2EE harbours mutations that result in two

aminoacid substitutions (S177E and S181E) that mimic the charge of the phosphorylated and activated form of IKK2. Using this approach, IKK2 activation resulted in a significant increase in pIκBα in AgRP neurons, yet a more moderate one compared to the robust increase seen in p-c-Jun of the $AgRP^{JNK1CA}$ mice. This is likely due to lack of the CAGS promoter. The heterozygous mice ($AgRP^{IKK2CA}$) did not exhibit a significant alteration in glucose homeostasis, therefore we used homozygous mice ($AgRP^{IKK2CA/CA}$) that are expected to closer mimic the 2-fold increase in IKK2 activation observed after HFD consumption (Zhang et al., 2008). Of note, previous studies using the same transgenic mice also reported higher levels of IKK2 activation and stronger effects in homozygous mice (Vlantis et al., 2011).

IKK2 activation in AgRP neurons causes cell-autonomous and systemic insulin resistance in the absence of leptin resistance and obesity. In fact this observation is consistent with the notion that disruption of insulin signalling in the AgRP neurons impairs systemic insulin sensitivity through impairing insulin's ability to suppress hepatic glucose production but without affecting body weight regulation (Könner et al., 2007). IKK2 has been shown to trigger insulin resistance (Cai et al., 2005; Yuan et al., 2001) by directly interacting with IRS (Gao et al., 2002) and indirectly by inducing TNF expression, which is also known to inhibit insulin signalling (de Alvaro et al., 2004; Gao et al., 2003). In contrast to our observations, previous studies have shown that ablation of IKK2 specifically from the AgRP neurons was sufficient to largely prevent obesity and leptin resistance upon HFD feeding (Zhang et al., 2008). This points to the possibility that IKK2 in AgRP neurons might act synergistically with other activated inflammatory pathways, and is necessary to promote weight gain and leptin resistance upon HFD consumption, but its activation *per se* is not sufficient to initiate all the aspects of the metabolic syndrome, under NCD conditions.

4.1.3 JNK1 or IKK2 activation result in increased firing of AgRP neurons

Activation of either inflammatory signalling branch, JNK1 or IKK2, results in increased basal firing of AgRP neurons. In fact, both JNK and IKK2 have been demonstrated to regulate glutamatergic signalling and synapse maturation, raising the possibility that both inflammatory kinases promote AgRP-neuron excitability via similar or distinct pathways (Thomas et al., 2008; Bockhart et al., 2009; Ahn and

Choe, 2010; Schmeisser et al., 2012). Importantly, inflammatory signalling has been demonstrated by multiple studies to influence neuronal excitability by regulating sodium, potassium and calcium channels (Amaya et al., 2006; Beyak and Vanner, 2005; Flake, 2004; Malykhina et al., 2004; Malykhina et al., 2006; Peng et al., 2011; Riazi et al., 2008; Rodgers et al., 2009; Rus et al., 2005; Yoshimura and de Groat, 1999).

Interestingly, despite the fact that in both models AgRP firing is increased by ~2-fold, only the AgRP^{JNK1CA} mice develop obesity. While optogenetic stimulation has clearly defined that robustly increasing AgRP-neuron firing evokes voracious feeding (Aponte et al., 2011), our experiments reveal, that increases in AgRP firing *per se*, does result in increased food intake, but does not necessarily cause obesity. Despite increased firing at young age, as detected in our animals, compensatory mechanisms might normalize electrical activity of these cells in the long-term despite increased JNK and IKK2-activity. For example, leptin action has been demonstrated to modulate the intrinsic excitability of AgRP neurons (Baver et al., 2014) and subsequent leptin resistance in the AgRP^{JNK1CA} mice might therefore affect their excitability differentiating them from the leptin sensitive AgRP$^{IKK2CA/CA}$ mice. Similarly, insulin resistance blunts signalling via PIP3 that has been shown to bind to KATP channels, resulting in hyperpolarisation and thus silencing of the neurons (MacGregor et al., 2002; Plum et al., 2006). Additionally, AgRP neurons receive excitatory and inhibitory input (Bouret et al., 2012; Pinto et al., 2004; Shanley et al., 2001; Villanueva and Myers, 2008), which could be affected by JNK1 and IKK2 in distinct ways, due to their differential effects in leptin and insulin signalling. Ultimately we cannot rule out the possibility of unidentified cellular heterogeneity in AgRP neurons -as observed for POMC neurons- that may contribute to these differential outcomes (Williams et al., 2010).

4.2 Hypothalamic inflammation in ageing

In order to investigate the role of hypothalamic inflammation in ageing we performed a longitudinal study with the JNK1$^{\Delta Nes}$ mice. JNK1$^{\Delta Nes}$ mice have been demonstrated to resemble the effects of caloric restriction, an intervention that delays ageing. Specifically, JNK1$^{\Delta Nes}$ mice show reduced body weight under normal chow and high-fat diet conditions and improved insulin sensitivity, glucose tolerance and impaired

somatic growth with decreased circulating levels of IGF1 and decreased expression of growth hormone in the pituitary (Belgardt et al., 2010). Here, ablation of JNK1 by $Nestin^{Cre}$ resulted in a significant extension of 8.6 % in median lifespan and 7.2 % in maximum lifespan. Similarly, hypothalamic inflammation has been reported to influence ageing as CNS ablation of IKK2 by $Nestin^{Cre}$ can also extend median lifespan by 23 % and maximum lifespan by 20%, and activation of IKK2 accelerates the ageing process (Zhang et al., 2013).

The $JNK1^{\Delta Nes}$ mice remain insulin sensitive, even after prolonged HFD consumption, a characteristic that resembles the effects of caloric restriction (Masoro, 2005; Gresl et al., 2003) compared to the $JNK1^{fl/fl}$ mice that develop insulin resistance already at 3 months of age. The effect of CR to reduce blood glucose levels and enhance insulin sensitivity has not only been reported for rodents (Masoro et al., 1992) but also for monkeys, and is proposed to be one of the mechanisms that contribute to lifespan extension (Bodkin et al., 1995; Cefalu et al., 1997; Kemnitz et al., 1994).

Although the $JNK1^{\Delta Nes}$ mice upon HFD consumption weigh less up to the age of 1 year, compared to the $JNK1^{fl/fl}$ mice, they develop diet-induced obesity. The $JNK1^{\Delta Nes}$ have increased adiposity, already at 7 motnhs of age, compared to the $JNK1^{fl/fl}$ mice and are not protected from age-related sarcopenia, characteristics that distinguish them from the caloric restriction phenotype (Colman et al., 2008). Interestingly, increased adiposity has also been reported for the long-lived pituitary dwarf mice (Flurkey et al., 2001) and for insulin/IGF *C. elegans* mutants with extended lifespan (Ashrafi et al., 2003; Kenyon et al., 1993; Kimura et al., 1997; Wolkow et al., 2000). Moreover, adiposity can be uncoupled from longevity in the case of leptin deficient mice as they retain increased fat content (48 %) compared to control mice (14 % body fat) when subjected to CR yet both groups show the same lifespan extension (Harrison et al., 1984). Collectively, our results, in agreement with other studies, suggest that longevity is regulated by alterations in the metabolic function rather than absolute fat mass.

The metabolic rate of $JNK1^{\Delta Nes}$ mice is increased at 7 months of age, as they consume more oxygen and have increased energy expenditure normalized to their lean body mass, in agreement with previous studies investigating the effects of JNK1 ablation in the CNS (Sabio et al., 2010), although their activity levels are similar to those of $JNK1^{fl/fl}$ mice. In contrast, multiple studies in the past have shown the

relationship between CR and reduced metabolic rate. In some cases only a transient reduction of metabolic rate occurred after the initiation of CR in rats and monkeys which was normalised in the long term (Lane et al., 1996; McCarter and Palmer, 1992; Ramsey et al., 2000). Other studies, have shown that CR results in a sustained reduction in metabolic rate in rhesus monkeys (DeLany et al., 1999). The theory that reduced metabolic rate might contribute to the lifespan extension was proposed for the first time by Sacher in 1977 but it has been challenged, as there have been reports showing a positive correlation between metabolic rate and longevity in mice (Speakman et al., 2004). Although it has been proposed that oxygen consumption positively correlates with oxidative DNA damage, and therefore senescence, (Adelman et al., 1988; Loft et al., 1994) there are multiple studies showing that the opposite occurs in many situations and the factors that determine the mitochondrial ROS generation are more complex than simply increases in oxygen consumption [reviewed in (Barja, 2007)]. Therefore, it cannot be concluded whether the increased metabolic rate seen in the $JNK1^{\Delta Nes}$ mice present at 7 months of age, is causative to any differences in lifespan.

The $JNK1^{\Delta Nes}$ mice displayed reduced BMD compared to $JNK1^{fl/fl}$ mice. BMD decline is an ageing marker that is also a consequence of caloric restriction (Devlin et al., 2010; Villareal, 2006). BMD decline is likely to occur due to the reduced levels of IGF1 and growth hormone in $JNK1^{\Delta Nes}$ mice (Belgardt et al., 2010). More specifically, growth hormone and IGF1 are important for bone formation as they facilitate the proliferation and differentiation of chondrocytes, the cells responsible for bone growth (Olney, 2003; Mohan et al., 2003). Another possibility is that the increased levels of T3 and TSHβ, as reported for $JNK1^{\Delta Nes}$ mice (Belgardt et al., 2010), affect BMD. Interestingly, T3 exerts anabolic actions during growth but catabolic actions in adult skeleton [reviewed in (Bassett and Williams, 2009)]. Moreover, hyperthyroidism, even in a subclinical level, has been associated with increased fracture risk and decreased BMD in postmenopausal women (Morris, 2007). Collectively, $JNK1^{\Delta Nes}$ mice present with reduced BMD, which might be due to their affected HPA axis and resembles the negative effect of caloric restriction on BMD.

Lastly, while brain-specific deletion of JNK3 has previously been demonstrated to protect from Aβ formation in an Alzheimer's disease model (Yoon et al., 2012) and JNK has been indicated to be a negative regulator of associative

learning (Sherrin et al., 2010), learning *per se* was not affected in JNK1$^{\Delta Nes}$ mice, compared to JNK1$^{fl/fl}$ mice. Interestingly, while the Morris Water Maze test determined no differences in learning, when the submerged platform was moved, in order to assess the ability of the mice to learn a new task, the JNK1$^{\Delta Nes}$ mice learnt the new task significantly faster. Therefore we concluded that JNK1 affects learning plasticity. This hypothesis warrants further investigation using immunohistochemical analyses to reveal the impact of JNK1 ablation on, for example, synapse formation and maturation.

4.3 Targeting inflammation, is it the solution?

Our study clearly demonstrated the detrimental effects of JNK1 and IKK2 activation in AgRP neurons in the initiation of insulin and leptin resistance, respectively. Furthermore, ablation of JNK1 by Nestin^{Cre} resulted in lifespan extension and enhanced insulin sensitivity upon HFD consumption. As a future perspective, the use of anti-inflammatory therapeutics to target hypothalamic inflammation should be considered. While significant research efforts have been made, more thorough investigations regarding the specificity and duration of inflammation amelioration is required to determine the therapeutic potential of reducing hypothalamic inflammation in metabolic disease.

4.3.1 Strategies and positive effects of hypothalamic inflammation amelioration

Multiple studies have examined the role of anti-inflammatory and anti-oxidant substances/strategies to ameliorate inflammation and its detrimental effects in the brain during obesity and/or HFD consumption. Natural foods, containing polyunsaturated fatty acids, chemical compounds, dietary interventions and exercise can that have anti-inflammatory actions in the hypothalamus will be discussed further.

Dietary unsaturated fatty acids such as C18:3 and C18:1 have been demonstrated to correct hypothalamic inflammation, revert insulin resistance, reduce adiposity and inhibit the AMPK/ACC pathway in the hypothalamus (Cintra et al., 2012). Pimentel *et al.* demonstrated the benefits of omega 3 PUFAs in improving central and peripheral inflammatory profiles via the reduction of intracellular inflammatory mediators (Pimentel et al., 2013). Moreover, utilizing immortalized

hypothalamic neurons Wellhauser *et al.* determined that omega 3 fatty acids act through the receptor GPR120, to mediate their anti-inflammatory effects (Wellhauser and Belsham, 2014). Furthermore, it has been recently demonstrated that fatty acid esters of hydroxyl fatty acids correlate with insulin sensitivity, improve insulin secretion and reduce inflammation (Yore et al., 2014).

Multiple natural plant-derived substances have been demonstrated to exert anti-inflammatory effects in the hypothalamus, restoring insulin and leptin signalling. Ursolic acid, found in many fruits, has been shown to inhibit NFκB and ER stress restoring insulin signalling and improving impaired cognition induced by HFD consumption (Lu et al., 2011). Hydroalcoholic extract of *Solidago chilensis* also improves hypothalamic insulin sensitivity after HFD consumption reducing NFκB, p-IκB and pJNK levels in mice (Melo et al., 2011). *Momordica charantia* (bitter melon) ameliorates HFD-associated changes in the blood brain barrier permeability and reduces glial cell activation, oxidative stress and pro-inflammatory markers in the brain (Nerurkar et al., 2011). The plant terpenoid compound ginsenoside Rb1, when administered intraperitoneally, can decrease the expression of inflammatory markers and negative regulators of leptin signalling, restoring its anorexigenic effect in the hypothalamus of HFD-fed mice (Wu et al., 2014). Also substances in the grape skin and seeds (Charradi et al., 2012) and green tea (Okuda et al., 2014) have been demonstrated to reduce brain lipotoxicity, and HFD-induced hypothalamic inflammation, respectively. Collectively, these studies advocate that dietary supplementation with natural substances can exert potent anti-inflammatory actions in the hypothalamus and potentially enhance insulin and leptin sensitivity.

Interestingly, Milanski et al. demonstrated that ICV administration of immunoneutralizing antibodies against TLR4 and TNFα results in reduced hypothalamic inflammation accompanied by the improved hypothalamic leptin sensitivity and insulin signal transduction in the liver, reduced hepatic steatosis and reduced hepatic glucose production mediated by parasympathetic signals (Milanski et al., 2012). Although, this approach could not be directly translated to human application, it indicates the potential for novel therapeutics.

Ageing also causes hypothalamic insulin resistance and increased levels of NFκB, p38 and PTPs (García-San Frutos et al., 2012). Ageing-associated hypothalamic inflammation can be ameliorated by dietary interventions such as caloric restriction (García-San Frutos et al., 2012). Exercise as an intervention has

also been proven to protect against HFD-induced hypothalamic inflammation, Yi et al. demonstrated that treadmill running reduces hypothalamic inflammation, microglial activation and improves glucose tolerance in HFD-fed $LDLR^{-/-}$ mice (Yi et al., 2012a). Similarly, Ropelle et al. demonstrated that exercise-induced IL6 and IL10 synergistically act to suppress hyperphagia-related obesity by blocking IKK2 and ER stress, reconstituting leptin and insulin sensitivity and reducing hypothalamic inflammation (Ropelle et al., 2010).

Taken together, these studies highlight the diverse successful approaches to revert hypothalamic inflammation, by dietary supplementation, lifestyle interventions and development of potential therapeutics with significant results in rodents.

4.3.2 Negative effects of inflammation amelioration

Anti-inflammatory therapeutics have been employed against obesity and insulin resistance however many of them did not yield the desired effects [reviewed in (Gao and Ye, 2012; Ye and McGuinness, 2013)]. Inflammatory pathways are highly conserved through evolution and although they appear to have detrimental effects during the course of obesity multiple studies using targeted mouse mutagenesis have demonstrated their importance and even their protective role in energy homeostasis. Cytokines and signalling pathways that are increased in obesity, such as NFκB, TNFα and pro-inflammatory interleukins, have been demonstrated to be detrimental when ablated or down-regulated.

The role of NFκB in metabolism has been extensively studied but it remains elusive whether and how it can be targeted to improve metabolic abnormalities. Unexpectedly, constitutive activation of IKK2 in adipose tissue prevents diet-induced obesity in mice (Jiao et al., 2012) and the conditional disruption of IKK2 fails to prevent obesity and insulin resistance (Röhl et al., 2004). NFκB can induce energy expenditure and its deficiency can stimulate the progression of non-alcoholic steatohepatitis in mice by promoting NKT-cell-mediated responses (Locatelli et al., 2013). Recently a study has provided evidence that overexpression of p65 in macrophages ameliorates atherosclerosis in $ApoE^{-/-}$ mice (Ye et al., 2013). Furthermore, IKK2 and JNK are required for activating the innate immune response to viral infection (Chu et al., 1999).

JNK inhibition has been proven beneficial in diseases such as inflammatory bowel disease (Mitsuyama et al., 2006) and asthma (Chialda et al., 2005). Interestingly, JNK1 deficiency increases skin tumour incidence, induced by a tumour promoter (She et al., 2002), while JNK2 deficiency suppresses it (Chen et al., 2001). Furthermore, although our results indicate that ablation of JNK1 in the CNS increases lifespan in mice there have been studies in flies where JNK1 activation also increased lifespan as much as 80% by activating hormesis (Wang et al., 2003). Specifically, JNK1 activation is believed to activate a stress-induced longevity pathway by conferring tolerance to oxidative stress.

The effects of TNF, the most investigated cytokine family, in energy homeostasis and metabolic diseases are also controversial. In the absence of TNFα/β, mice have increased body weight compared to control mice and develop hepatic steatosis in a high cholesterol diet (Schnyder-Candrian et al., 2005). Furthermore, HFD-fed TNFR1 and 2 deficient mice are insulin resistant (Pamir et al., 2009) and develop obesity and diabetes (Schreyer et al., 1998).

Recent evidence supports the beneficial role of IL6 to alternatively activate macrophages to limit endotoxemia and insulin resistance (Mauer et al., 2014). Furthermore, mice lacking IL6 develop hepatic inflammation and hepatic insulin resistance (Matthews et al., 2010) and Wunderlich et al. demonstrated that IL6 signalling in liver parenchymal cells suppresses hepatic inflammation and improves insulin action (Wunderlich et al., 2010). Even in the CNS human IL6 has been shown to enhance leptin's action to reduce food intake (Sadagurski et al., 2010). IL1 and IL6 mediate GLP1R effects to suppress food intake (Shirazi et al., 2013) and combined deficiency of those interleukins can cause obesity in young mice (Chida et al., 2006).

The pro-inflammatory IL1 was also demonstrated to have a protective role in multiple studies. IL1 mediates glucose-induced food intake suppression (Mizuno et al., 2013) and in absence of IL1 signalling in IL1R deficient mice the suppression of feeding by the gut hormone xenin, was impaired (Kim et al., 2014a). Lastly, long-term exposure to HFD results in the exacerbated development of glucose intolerance and insulin resistance in IL1βR1 deficient mice compared to control mice (García et al., 2006).

Further studies have been conducted proving the beneficial role of the pro-inflammatory cytokine IL18. IL18 can control energy homeostasis by suppressing appetite and weight regain in food-deprived mice, although its levels are generally

elevated in obese individuals (Zorrilla et al., 2007). IL18$^{-/-}$ mice are hyperphagic and become overweight (Zorrilla et al., 2007) and IL18 null mutation not only increases food intake and weight but also reduces energy expenditure and lipid substrate utilization in HFD-fed mice (Zorrilla and Conti, 2014). Furthermore, IL18 activates skeletal muscle AMPK and reduces weight gain and insulin resistance (Lindegaard et al., 2013).

Collectively, there is evidence that disrupting pro-inflammatory signalling by NFκB, TNF and interleukins is not always beneficial and could potentially present undesired effects. In order to develop therapies against obesity and insulin resistance the role of the molecules participating in the inflammatory responses has to be clearly defined and there should be specific tissue or even cell-specific targeting.

4.4 Conclusions and Future perspectives

In this study we demonstrate the importance of hypothalamic inflammation in obesity, insulin resistance initiation and manifestation, and ageing regulation. We used two genetic mouse models to trigger inflammation in the AgRP neurons and could show that JNK1 activation results in obesity and leptin resistance whereas IKK2 activation results in insulin resistance, even in the absence of an environmental trigger, such as high-fat feeding. Furthermore, we demonstrated that JNK1 ablation in the CNS results in lifespan extension and protection from HFD-induced insulin resistance but on the other hand increased adiposity and reduced bone mineral density.

In the future, hypothalamic JNK1 and IKK2 could be used as potential therapeutic targets to counteract obesity- and ageing-associated pathologies. It is of great importance to investigate the timeframe of diet-induced hypothalamic inflammation initiation, the dynamic regulation of these kinases and their role in multiple other processes, apart from insulin and leptin signalling, and also regard the negative effects of inflammation inhibition. Furthermore, the molecular effects of JNK1 ablation in the CNS should be thoroughly studied in order to reveal the mechanism that results in this lifespan increase. Moreover, the role of different cell types residing in the hypothalamus in hypothalamic inflammation is an important area for further investigation. Are subpopulations of AgRP neurons responsible for different processes? How are the hypothalamic neuronal circuits affected by

inflammation? Future studies will further define the role of hypothalamic inflammatory pathways in obesity and ageing.

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Erklärung

Die vorliegende Arbeit wurde in der Zeit von September 2011 bis Januar 2015 am Institut für Genetik, Arbeitsgruppe Modern Mouse Genetics and Metabolism, Universität zu Köln und im Max-Planck-Institut für Stoffwechselforschung Köln, Forschungsgruppe Neuronal Control of Metabolism unter Anleitung von Herrn Prof. Dr. Jens C. Brüning angefertigt.

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig 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 dieser Promotionsordnung sind mir bekannt.

Köln, Februar 2015

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(Eva Tsaousidou)

Teilpublikation

Tsaousidou E, Paeger L, Belgardt BF, Pal M, Wunderlich CM, Brönneke H, Collienne U, Hampel B, Wunderlich FT, Schmidt-Supprian M, Kloppenburg P, and Brüning JC. Distinct roles for JNK- and IKK-activation in Agouti-Related Peptide neurons in the development of obesity and insulin resistance. *Cell Rep*. 2014 Nov 20; 9; 1-12.

Eva Tsaousidou, M.Sc.

Geburtsort	Thessaloniki
Geburtstag	20. März 1989
Nationalität	griechisch
Adresse	Vor den Siebenburgen 74, Köln, Deutschland, DE-50676
Email	eva.tsaousidou@sf.mpg.de
Telefonnummer	+49 177 509 54 18

STUDIUM

MSc Oktober 2010-Dezember 2012

Universität zu Köln, Deutschland

Master of Science in Biological Sciences, Spezialisierung: Genetik

'The role of constitutive c-Jun N-terminal kinase 1 (JNK1) activation in AgRP neurons'

Supervisor: Prof. Dr. Jens C. Brüning

BSc September 2006-Juli 2010

Aristotle Universität, Thessaloniki, Griechenland

Bachelor of Science in Biologie, Richtung: Molekulare Biologie, Genetik und Biotechnologie

'Cloning in *E. coli*, purification and functional analysis of mTRAF6'

Supervisor: Prof. Dr. George Mosialos

Gymnasium (Apolytirio Lykeiou) September 2000-Juni 2006

24. Gymnasium, Thessaloniki

VERÖFFENTLICHUNGEN

Tsaousidou E, Paeger L, Belgardt BF, Pal M, Wunderlich CM, Brönneke H, Collienne U, Hampel B, Wunderlich FT, Schmidt-Supprian M, Kloppenburg P, and Brüning JC. Distinct roles for JNK- and IKK-activation in Agouti-Related Peptide neurons in the development of obesity and insulin resistance. *Cell Rep*. 2014 Nov 20; 9; 1-12.

Rother E, Belgardt BF, **Tsaousidou E**, Hampel B, Waisman A, Myers MG Jr, Bruening JC. Acute selective ablation of rat insulin promoter-expressing (RIPHER) neurons defines their orexigenic nature. *PNAS*. 2012 Oct 30; 109(44): 18132-7.

KONFERENZ TEILNAHMEN

Vortrag Mai 2014

EMBO/EMBL Symposium, Translating Diabetes

Heidelberg, Deutschland

Poster Januar 2014

Keystone Meeting, Obesity: A Multisystems Perspective

Vancouver, British Columbia, Kanada. Poster No: J2 3044.

Danksagung

I would sincerely like to thank:

Prof. Dr. Brüning for allowing me to be a part of his excellent laboratory, trusting me with amazing projects, inspiring and motivating me all these years.

Prof. Dr. Kloppenburg, Prof. Dr. Korsching and Dr. Lichtenberg for agreeing to form my thesis committee.

Dr. Bengt Belgardt for being a true mentor and for trusting me with the continuation of his projects.

Prof Dr. Kloppenburg and Dr. Paeger for the electrophysiological experiments' collaboration.

Prof. Dr. Mosialos for the mentoring, support and also for believing in me!

Hayley (for all the hugs), Martin, Katarina, Ismene, Nasim and Derk for reading and correcting parts of this Thesis and giving me great ideas, you guys rock!

Jens Alber und Kerstin Marohl nicht nur für die Unterstützung und die perfekte Mausarbeit aber auch für die Freundschaft!

Brigitte Hampel für die wunderschönen Färbungen und Sigrid Irlenbusch die beste LaborMama!

The International Graduate School in Development Health and Disease and Graduate School for Biological Sciences for trusting me with a fellowship and their support through the years. Especially, I would like to thank Dr. Witt who taught me that science goes beyond any borders!

All the current and past members of the Brüning, Wunderlich and Kornfeld lab, particularly Hayley, Katarina, Martin, Tim, Sebastian, Elaine, Sophie, Jan Wilhelm, Thomas, Claudia, Hella for their scientific and mental support, it has been a privilege to meet these amazing scientists/people.

My lovely friends who are close and far away, I know that without them I would never make it! Special thanks to Babis, for the countless scientific/philosophical discussions, his music and friendship since a decade!

I would like to thank my parents and my brother, for teaching me the true values in life and for their endless love, ευχαριστώ μαμά, μπαμπά και Χρήστο, σας αγαπώ πολύ! Lastly, I would like to thank Derk for always being there, making me smile and tolerating my craziness!