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α- AND γ-MELANOCYTE STIMULATING HORMONES IN OBESITY

A study of the key central areas of metabolic regulation

Kim Eerola

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”We know what we are, but know not what we may be”
William Shakespeare

ABSTRACT

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α- and γ-melanocyte stimulating hormones in obesity: A study of the key central areas of metabolic regulation

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The melanocortin system is an important regulator of feeding, energy metabolism, and cardiovascular function and it consists of the pro-opiomelanocortin (POMC) derived melanocyte stimulating hormones (α-, β- and γ-MSH) and their endogenous melanocortin receptors, MC1R to MC5R. In the hypothalamus, α-MSH reduces food intake, and increases energy expenditure and sympathetic tone by binding to MC4R. Mutations affecting the MC4R gene lead to obesity in mammals. On the other hand, the metabolic effects of MC3R stimulation using agonists such as the endogenously expressed γ-MSH have been less extensively explored. The main objective of this study was to investigate the long-term effects of increased melanocortin tone in key areas of metabolic regulation in the central nervous system (CNS) in order to investigate the site-specific roles of both α-MSH and γ-MSH.

The aim was to stereotaxically induce local overexpression of single melanocortin peptides using lentiviral vectors expressing α-MSH (LVi-α-MSH-EGFP) and γ-MSH (LVi-γ-MSH-EGFP). The lentiviral vectors were shown to produce a long-term overexpression and biologically active peptides in cell-based assays. The LVi-α-MSH-EGFP was targeted to the *arcuate nucleus* in the hypothalamus of diet induced obese mice where it reduced weight gain and adiposity independently of food intake. When the *nucleus tractus solitarus* in the brainstem was targeted, the LVi-α-MSH-EGFP treatment was shown to cause a small decrease in adiposity, which did not impact weight development. However, the α-MSH treatment increased heart rate, which was attenuated by adrenergic receptor blockade indicative of increased sympathetic activity. The LVi-γ-MSH-EGFP was targeted to the hypothalamus where it decreased fat mass in mice eating the standard diet, but the effect was abated if animals consumed a high-fat Western type diet. When the diet induced obese mice were subjected again to the standard diet, the LVi-γ-MSH-EGFP treated animals displayed increased weight loss and reduced adiposity.

These results indicate that the long-term central anti-obesity effects of α-MSH are independent of food intake. In addition, overexpression of α-MSH in the brain stem efficiently blocked the development of adiposity, but increased sympathetic tone. The evidence presented in this thesis also indicates that selective MC3R agonists such as γ-MSH could be potential therapeutics in combination with low fat diets.

Key words: melanocortins, central nervous system, obesity, gene therapy, diet induced obesity

TIIVISTELMÄ

Kim Eerola

α- ja γ-melanosyyttejä stimuloivien hormonien sentraaliset vaikutukset lihavuudessa; aineenvaihdunnallisesti keskeiset aivoalueet hypotalamuksessa ja aivorungossa

Biolääketieteen laitos, Farmakologia, Lääkekehitys ja Lääkehoito sekä Lääketutkimuksen tohtoriohjelma Turun Yliopisto, sekä Sydänkeskus, Turun yliopistollinen keskussairaala ja Turun yliopisto

Pro-opiomelanokortiini (POMC) on keskushermoston melanokortiinijärjestemän keskeisin tekijä toimien esiasteena α-, β-, ja γ-melanosyyttejä stimuloiville hormoneille (MSH), jotka säätelevät ruokahalua, energiatasapainoa sekä sydän- ja verenkiertojärjestelmää melanokortiinireseptorien (MC1-5R) välityksellä. α-MSH:n sitoutuminen MC4-reseptoriin hypotalamuksessa vähentää syömistä, lisää energiankulutusta ja sympaattista tonusta akuutisti. MC4-reseptorin mutaatioiden tiedetään aiheuttavan lihavuutta sekä koe-eläimillä että ihmisillä, mutta keskushermostossa esiintyvän MC3-reseptorin ja tämän agonistin, γ-MSH:n roolista lihavuuden synnyssä ja aineenvaihdunnan säätelyssä tiedetään edelleen varsin vähän. Tämän väitöskirjan tavoitteena on ollut selvittää lentivirus-geeniterapian avulla α-MSH:n ja γ-MSH:n pitkäkestoisia vaikutuksia keskushermoston metabolian säätelyn kannalta keskeisillä aivoalueilla.

Osana väitöskirjatutkimusta tuotimme α-MSH (LVi-α-MSH-EGFP) ja γ-MSH (LVi-γ-MSH-EGFP) lentivirukset, joiden funktionaalisuus varmistettiin solupohjaisilla menetelmillä. Virusten todettiin yli-ilmentävän pitkäkestoisesti kutakin MSH peptidiä ja tuottavan biologisesti aktiivista proteiinia. α-MSH:n yli-ilmentäminen hypotalamuksen *arcuatus nucleus* (ARC)-tumakkeessa esti lihavuuden kehittymistä vähentäen hiirten painonkasvua vaikuttamatta ruuan kulutukseen. Vaikutus havaittiin myös aivorungon *nucleus tractus solitarii* (NTS)-tumakkeen alueella. Tällöin vaikutus näkyi lähinnä alentuneena rasvamassana. Tämän lisäksi α-MSH-käsittelyn saaneilla hiirillä todettiin kohonnut sydämensyke, joka oli estettävissä adrenergisten reseptorien salpauksella viitaten kohonneeseen sympaattiseen tonukseen. γ-MSH yli-ilmennys ARC-tumakkeessa vähensi rasvamassan kehittymistä standardirehulla, mutta ruokittaessa koe-eläimiä runsasrasvaisella rehulla tämä ero rasvamassassa hävisi. Lihavuuden kehityttyä runsasrasvaisen rehun vaihtaminen takaisin standardirehuun vähensi γ-MSH käsittelyn saaneiden hiirten rasvamassaa merkittävästi lisäämättä epäsuotuista sympaattista vaikutusta.

Väitöskirjatutkimus osoittaa, että α-MSH aivojen ARC ja NTS tumakkeissa estää lihavuutta muilla kuin suoraan syömistä säätelevillä mekanismeilla ja että aivorungossa α-MSH lisää sympaattista aktiivisuutta. γ-MSH:n myönteiset vaikutukset rasvamassaan olivat riippuvaisia dieetin koostumuksesta. Tämän väitöskirjan löydökset laajentavat ymmärrystä MSH-peptidien sentraalisista vaikutuksista ja näiden peptidien soveltamisesta lihavuuden hoidossa.

Avainsanat: melanokortiinit, keskushermosto, lihavuus, sympaattinen hermosto, ravitsemus

TABLE OF CONTENTS

ABBREVIATIONS

5-HT	serotonin
aa	amino acid
ACTH	adrenocorticotropic hormone
AgRP	agouti related peptide
ANOVA	analysis of variance
ARC	arcuate nucleus of the hypothalamus
BAT	brown adipose tissue
BMI	body mass index
BDNF	brain derived neurotrophic factor
CMV	cytomegalo virus
CNS	central nervous system
DIO	diet induced obesity
DMEM	Dubelcco's modified Eagles medium
EGFP	enhanced green fluorescent protein
eNOS	endothelial nitric oxide synthase
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FFA	free fatty acid
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GPCR	G protein-coupled receptor
HDL	high density lipoprotein
HEK293T	human embryonic kidney cells 293T
HR	heart rate
i.p.	intraperitoneal
IGTT	intraperitoneal glucose tolerance test
iNOS	inducible nitric oxide synthase
IRES	internal ribosomal entry site
LDL	low density lipoprotein
LPL	lipoprotein lipase
LVi	lentiviral vectors with IRES
mRNA	messenger ribonucleic acid
NA	noradrenaline
NO	nitric oxide

NOS	nitric oxide synthase
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
RER	respiratory exchange ratio
PBS	phosphate-buffered saline
PFA	paraformaldehyde
POMC	pro-opiomelanocortin
PRCP	prolylcarboxypeptidase
PVN	paraventricular nucleus
SEM	standard error of mean
SNS	sympathetic nervous system
sBP	systolic blood pressure
T2D	type 2 diabetes
TEE	total energy expenditure
TRH	thyrotropin releasing hormone
UPC1	uncoupling protein 1
VLDL	very-low density lipoprotein
VSMC	vascular smooth muscle cell
WAT	white adipose tissue
WHO	World Health Organization
α-MSH	α-melanocyte stimulating hormone
β-MSH	β-melanocyte stimulating hormone
γ-MSH	γ-melanocyte stimulating hormone
QUICKI	quantitative insulin sensitivity check index

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III:

I Eerola K, Nordlund W, Virtanen S, Dickens AM, Mattila M, Ruohonen ST, Chua SC Jr, Wardlaw SL, Savontaus M, Savontaus E (2013). Lentivirus-Mediated α-Melanocyte-Stimulating Hormone Overexpression in the Hypothalamus Decreases Diet Induced Obesity in Mice. *Journal of Neuroendocrinology* **25**(12):1298-307

II Eerola K, Rinne P, Penttinen AM, Vähätalo L, Savontaus M, Savontaus E (2014). α-MSH Overexpression in the Nucleus Tractus Solitarius Decreases Fat Mass and Elevates Heart Rate. *Journal of Endocrinology*. In press

III Eerola K, Virtanen S, Vähätalo L, Ailanen L, Cai M, Hruby V, Savontaus M, Savontaus E. γ-melanocyte stimulating hormone overexpression in the hypothalamus augments anti-obesity effects in diet induced obese mice. *Manuscript in preparation*

In addition, the thesis also presents some unpublished data.

1. INTRODUCTION

Obesity is a major threat to human health; according to the WHO in 2008, affecting nearly 500 million men and women worldwide. The total costs of obesity to society approaches 6 % of the gross domestic product (GDP) in Western countries (Williams & Frubeck. 2009). The prevalence of obesity has more than doubled since 1980 and there is now growing concern that in 2011 nearly 43 million children under the age of five were obese (WHO). Obesity predisposes to the development of the metabolic syndrome, which is characterized by impaired glucose metabolism accompanied with at least two of the following parameters; elevated blood pressure, dyslipidemia, central obesity and microalbuminuria (WHO).

Environmental factors are undoubtedly a major contributor to the prevalence of obesity in the Western world, however, it fails to explain why people living in the same environment have varying susceptibilities to develop a high body mass index (BMI). WHO defines obesity by the BMI that is a person's weight in kilograms divided by the square of his/her height in meters (kg/m^2). BMI over or equal to 25 is overweight, whereas BMI over 30 defines obesity. The heritability of obesity has been proposed to account for 40-77% of the variation in BMI (Allison*, et al.* 1996, Haworth*, et al.* 2008). In view of the major heritability factor, the study of obesity genetics has been advantageous in clarifying the regulation of energy metabolism and in explaining the development of human obesity (Ramachandrappa & Farooqi. 2011). Moreover, transgenic animal models have nonetheless proven to be useful in understanding the molecular and physiological basis of obesity and human disease. The considerable number and complex regulation of the genes involved in obesity has naturally raised more questions than solutions about metabolic regulation.

The pro-opiomelanocortin system (POMC) is part of a complex network of neurons governing the physiological and behavioral responses involved in obesity (Garfield*, et al.* 2009). POMC derived melanocortin peptides are downstream mediators of leptin, a hormone that closely correlates with body adiposity and the long-term regulation of food intake and energy expenditure. The POMC derived peptides, particularly the melanocortins have been shown to exert multiple physiological effects both centrally and in the periphery.

In order to investigate the central role of neuropeptides in obesity, lentiviral gene delivery vectors expressing biologically active melanocortin peptides were produced and their site-specific role investigated in key central areas of metabolic regulation in mice in this thesis work.

2. REVIEW OF LITERATURE

2.1 GENERAL BACKGROUND

2.1.1 Regulation of energy balance

The central nervous system is a key regulator of energy homeostasis by responding to anorexigenic (factors that reduce hunger) and orexigenic (factors that increase hunger) signals both centrally and peripherally. It is essential that there is a balance between energy intake and energy expenditure in order to maintain normal body weight. The regulatory pathways governing body energy homeostasis are complex and despite the prevalence of obesity today, many individuals do seem to be able to adapt to the energy rich environment.

The prevailing knowledge about basic physiology has layed the foundation for the theories about the regulation of energy homeostasis. One of the first of these was the "glucostatic theory" formally proposed by John Mayer, which postulated that the rise and fall in blood glucose levels were regulating feeding (Mayer. 1953). As the glucostatic theory focused on short-term regulation, the concept adipostat theory introduced long-term regulation of energy balance (Mayer. 1955). The theory of regulation of body homeostasis by negative-feedback loops proposed by Cannon in 1932 introduced the concept where an organism employed a reference signal or a "set-point" to a homeostatic signal, which was defended by compensatory mechanisms (Cannon. 1932). Richter introduced the aspect of instinctive behavior as ways to defend these homeostatic "set-points" (Richter. 1942). Early on, the CNS was seen as the target of these signals and obesity was contributed to anatomical abnormalities associated with this organ. Although damage inflicted on the basomedial hypothalamus was known to induce obesity in humans as early as in 1840 (Mohr. 1993), it was abnormalities in the pituitary gland that were initially thought to be the cause of the endocrine imbalance (Babinski. 1900, Frohlich. 1902). Indeed, the neuronal populations of POMC and AGRP/NPY in the basomedial hypothalamus that were later found to govern the regulation of feeding and body energy homeostasis.

2.1.1.1 LONG TERM SIGNALS OF ENERGY STATE

In mammals, adipose tissue serves the primary site for long-term store of energy. Adipocytes secrete leptin, a hormone that correlates with total fat mass and mediate information about energy storages to the central nervous system (CNS). The optimal function of adipose tissue as an endocrine tissue is fundamental for health and deficiency in leptin or leptin receptor signaling causes severe obesity (Campfield*, et al.* 1995, Halaas*, et al.* 1995, Stephens*, et al.* 1995, Zhang*, et al.* 1994). Several areas of the CNS essential for the regulation of body metabolism harbor leptin receptors, and the activation of these impact energy metabolism in addition to the gonadal, thyroid and adrenal axes (Ahima*, et al.* 1996). Starvation and the subsequent decreases in fat reserves decrease the level of

circulating leptin and activate orexigenic pathways (Frederich*, et al.* 1995). In obesity, prolonged exposure to elevated levels of leptin has been associated with the increase in sympathetic nervous system activity; these nerves are also involved in the mobilization of lipids (Bartness & Song. 2007). The elevation of sympathetic tone also affects many other organs such as blood vessels, kidney, and heart since these are all innervated by postganglionic sympathetic nerves.

2.1.1.2 GLUCOSE HOMEOSTASIS

The supply of nutrients is essential to all living creatures. Despite an adaptation to varying environments and food sources, the brain and muscle cells of higher mammals prefer carbohydrates as their source of energy. Carbohydrates are stored as glycogen throughout the body, especially in muscle cells and the liver. Moreover, the level of glucose in the bloodstream is tightly regulated in order to maintain a steady supply of energy to maintain a conscious state without it reaching toxic concentrations. Glucagon and insulin secreted by the pancreatic islets of α- and β-cells respectively, share the main responsibility for regulating blood glucose levels (Unger & Cherrington. 2012). Insulin decreases blood glucose levels by increasing the uptake of glucose by cells by inducing recruitment of glucose transporters to the cell membrane in the liver and other peripheral tissues and by decreasing glucogenesis in the liver. On the other hand, glucagon increases gluconeogenesis and in the liver and the release of stored glycogen as glucose primarily from the liver. Glycogen can be synthesized by the liver in active process called glycogenesis, converting metabolites from both protein and fat into glycogen. In obesity, the excess supply of nutrients increases the amounts of fatty acids and glucose to be removed from the bloodstream and this requires the synthesis of increased amounts of insulin. Elevated and prolonged exposures to circulating insulin levels lead to the desensitization of the insulin response, which further increases the synthesis of insulin and causes more stress on the pancreas. Ultimately, prolonged inflammation and stress leads to the fibrosis and destruction of the insulin producing cells of the pancreas, resulting in the development of diabetes.

2.1.1.3 ENERGY INTAKE

Energy intake is regulated by a plethora of factors governing meal initiation, termination, and frequency and in humans, also social factors. Moreover, the energy requirements modulate the balance between orexigenic and anorexigenic signals and this impacts on both energy expenditure and meal frequency.

There are both hormonal and neural factors governing meal initiation. Positive associations to a particular food source are mediated through cues from both visual and olfactory sensory neurons that help to alert that it is mealtime. In the CNS, the secretion of orexigenic peptides increase hunger and food intake, whereas the effects of anorexigenic peptides are inhibitory. Ghrelin, a gut-derived hormone stimulates feeding and is secreted during meal intervals and peak its levels before meal initiation. The effects of ghrelin are mediated via afferent neurons to the CNS. The gut plays a role also in meal termination through the secretion of cholecystokinin (CCK), which is a gut-

derived hormone secreted during mealtime. Together with the stimulation of mechanical stretch receptors in the gut wall, the effects of CCK on satiety are mediated by receptors on vagal afferent neurons projecting to the brain stem. In humans, social factors can greatly influence meal size. The term "social facilitation of eating behavior", describes how the number in the company during a mealtime increases the amount of calories ingested (De Castro. 1995).

2.1.1.4 ENERGY EXPENDITURE

Under normal circumstances, total energy requirements exist in a balance with total energy expenditure. However, changes in energy intake can influence energy expenditure to some extent. The total expenditure of energy (TEE) is composed of resting energy expenditure (REE), active thermogenesis, the thermic effect of food and physical activity, which all show significant individual variances. REE accounts for almost two thirds of the daily energy requirement. Many different aspects of normal body function require energy. For instance, the brain and smooth muscle cells of the heart, thorax, gut and intestines require a constant supply of energy in the form of glucose. Furthermore, energy intake requires a large input of energy from the smooth muscle cells surrounding the gut walls, in order to mechanically process food. Thermogenesis is essentially a byproduct of cellular work, however, many mammals are able to produce heat in metabolically active fat tissues, named brown adipose tissue (BAT). In rodents, BAT is activated foremost by cold exposure, generating heat in the vicinity of vital organs such as kidneys, heart as well as around major blood vessels. Food ingestion of high fat diets has also been shown to activate BAT and in recent human studies active BAT has been detected in healthy adults after cold exposure (Virtanen*, et al.* 2009). Thermogenesis by BAT is actively regulated in the CNS by several factors such as insulin, leptin, CRF and serotonin and inhibited by at least two neuropeptide Y (NPY) and galanin (Morrison SF 2014). In rodents, sympathetic outflow neurons from the hypothalamus release noreadrenaline that activates β3-adrenoceptors in BAT. The stimulation of β3- adrenoceptors activates intracellular signaling pathways that promote the expression of uncoupling protein 1 (UCP-1), which drives thermogenesis.

2.1.1.5 CENTRAL REGULATION OF ENERGY BALANCE

The CNS receives its metabolic input both through humoral factors and direct signals from vagal afferent neurons. The hypothalamus is considered as the main central origin of energy control, being affected mainly by humoral effector molecules such as leptin, and insulin. The brain stem additionally receives humoral signals from glucose, ghrelin and neuronal signals from vagal afferent neurons. The vagal afferent signals to the brain stem originate from the mouth, esophagus, stomach, intestine and liver. Moreover, the forebrain also has a role in the control of eating through the reward pathway. Energy expenditure through thermogenesis is a centrally controlled process where the stimulation of SNS outflow neurons activates BAT to produce heat. The forebrain, caudal brain stem and the hypothalamus are linked together through several different and complex neural pathways. Furthermore, most of these neuronal pathways are regulated through negative feedback and there are several overlapping functions.

The most extensively studied feeding center, the hypothalamus is a prime example of the complexity of neuronal pathways. The arcuate nucleus (ARC) of the hypothalamus is composed of the orexigenic NPY/AGRP- and anorexigenic POMC-neurons. Both of these neuronal populations express receptors for serotonin, leptin and insulin and stimulation of POMC neurons release alpha-MSH (α-MSH) and gamma-MSH (γ-MSH) while NPY/AGRP neurons release neuropeptide Y (NPY), AGRP and GABA. Some of these targets of these products are second-order neurons of the paraventricular nucleus (PVN). The ARC AGRP/NPY neurons project to the orexigenic centers of the lateral hypothalamic area (LHA) and the ventromedial nucleus (VMH). Moreover, the AGRP/NPY neurons of the dorsomedial nucleus of the hypothalamus (DMH) also innervate to the PVN to inhibit food intake. The hypothalamus also harbors other neuronal networks involved in energy metabolism. For instance, the lateral hypothalamic area receives orexigenic signals from the nucleus accumbens (NAc) in the forebrain and in response it releases orexin and melanin-concentrating hormone (MCH). The hypothalamus is also a key regulator SNS activity *i.e.* both the VMH and the PVN stimulate whereas the LHA outflow inhibits SNS neurons in the spinal cord. The SNS innervates many important organs and tissues such as heart, blood vessels, kidney and adrenal glands that are responsible for hemodynamics. Moreover, SNS innervates also important metabolic tissues such as the pancreas, WAT and BAT where increased SNS activity induces thermogenesis. (Williams & Frubeck. 2009)

The caudal hindbrain is also an important regulator of metabolism. The area postrema (AP), a target of circulating humoral factors, mediates information through its connections to the nucleus tractus solitarus (NTS). Moreover, the NTS collates a large input of information related to feeding from the periphery through neuronal signals from the afferent neurons. The dorsal motor nucleus of the vagus (DMV) communicates with the NTS and affects peripheral organs via the efferent neurons. Moreover, the NTS is also connected to the oral motor system to affect meal initiation and termination. The hypothalamus and the brain stem are interconnected through several distinct neural projections. For instance, orexinergic neuronal projections from the NTS to the hypothalamus are linked via the parabrachial nucleus (PBN) and the POMC projections from the PVN and the ARC are directly connected with the NTS. The brain stem also largely influences basolateral amygdala (BLA) and the nucleus accumbens (NAc) of the forebrain, which are key regulators of reward. (Williams & Frubeck. 2009)

In obesity, the forebrain is mostly recognized for its involvement in the reward pathway, mediating orosensory pleasure and regulating food intake. It is significant that the reward pathways are able to impact normal body homeostasis by regulating the hedonic energy intake. The neurotransmitters dopamine, GABA, acetylcholine and the endocannabinoids are central mediators of the effects in the forebrain. The endocannabinoids control food preference through endocannabinoid receptors in NAc and in the hypothalamus. The NAc together with the central nucleus of the amygdala (CeA) are involved in opioid mediated eating behavior, while the BLA and higher cortical regions controls food palatability (Balleine*, et al.* 2003). The neural pathways involved in the regulation of body metabolism in the rodent brain are visualized in Figure 1.

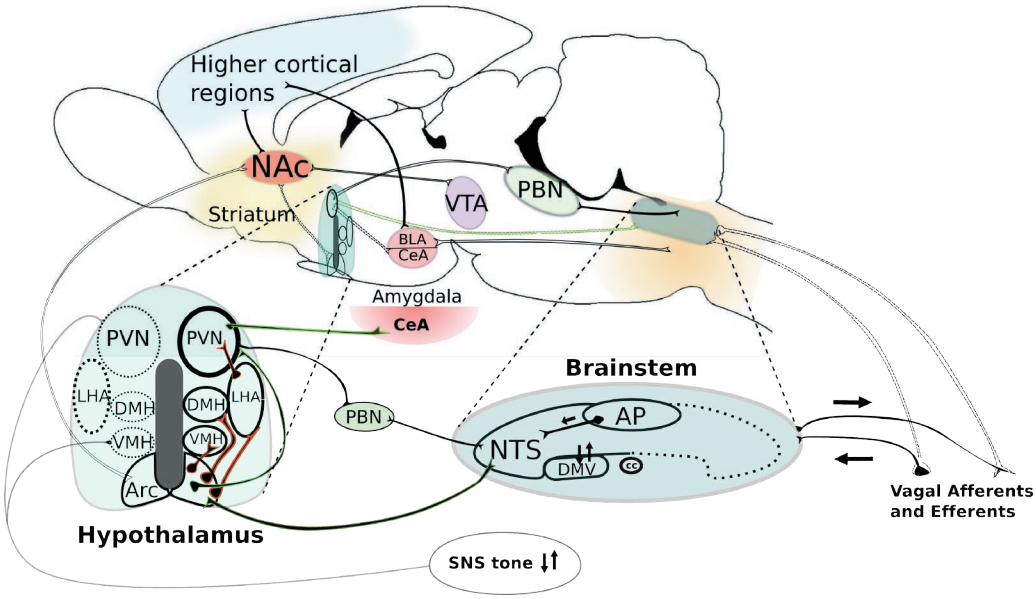

Figure 1. Neural pathways in body metabolism in rodents. Orexigenic (red) and anorexigenic (green) projections. Hypothalamic regions; Arcuate nucleus (ARC), Ventromedial hypothalamus (VMH), Dorsomedial hypothalamus (DMH), Lateral Hypothalamus (LHA), Paraventricular nucleus (PVN). Structures of the Brain stem; Area postrema (AP), Nucleus tractus solitarus (NTS), Dorsomotor nucleus of vagus (DMV), Striatum; Nucleus accumbens (NAc). Mecencephalon; Ventral tegmental area (VTA), Parabranchial Nucleus. Amygdala; Basolateral complex (BLA) and Central nucleus (CeA), Sympathetic nervous system (SNS). Background information for the generation of figure 1 was obtained from (Williams & Frubeck. 2009).

2.1.2 Genetics of obesity

Genes, environmental and social factors contribute to the prevalence of obesity today. It has been proposed that obesity is largely genetically determined, and that changes in the expression of genes and genetic mutations are responsible for as much as 77% of the prevalence of obesity (Haworth*, et al.* 2008). The environmental factor is major contributor to the prevalence of obesity, but it fails to explain why people living in the same environment have varying susceptibilities to develop severe obesity. The progress of finding susceptibility genes and factors that contribute to the development of obesity has been slow and has mostly been determined in monogenic diseases and in genetic syndromes. There are many mutations that have been identified in obesity affecting the CNS and the function of adipose tissue. Animal models of obesity have been useful for investigating metabolic disease and many of the diseases affecting these have proved to be translational to humans. Environmental factors, such as exposure to energy rich diets are often required for the development of obesity. Moreover, it has been demonstrated that in certain populations, there will always be subjects of low- and high-gainers complicating the interpretation of the true effects of genes (Bouchard. 1991). The existence of high and low-gainers may be due to epigenetic factors since mice of

the same genetic strain show significant alterations in the expression of genes related to adipose tissue function (Koza, *et al.* 2006). Secondly, social and environmental stressors have an important impact on feeding behavior and also affect the regulation of energy balance (De Castro. 1995). A variety of stress-evaluation tests have been developed for animals in order to understand the impact of behavior and social factors on genes and diets or obesity treatments, however, many of these have proved inadequate since they cannot be translational to the human disease. The reward pathways in the CNS are especially important in human obesity and recently there have been advances in measuring palatability and food preference also in animals. The genes that cause obesity in humans mostly involve mutations in adipose tissue function and regulatory pathways in the CNS. The genes of modern humans seem to be maladapted to cope with today's energy rich environment. Mutations which affect a combination of genes are part of a larger set of metabolic diseases known as polygenic or common disorders, whereas the most severe cases of obesity involve monogenic mutations.

The monogenic forms of human obesity are severe cases of early onset obesity and involve mutations in single genes such as leptin. In humans, a leptin mutation can cause severe obesity from a young age although this kind of obesity is treatable by daily injections with a recombinant protein. Up to 3 % of patients with severe obesity have been identified as carriers of a dysfunctional leptin gene (Farooqi. 2007). There is evidence that the downstream signaling pathways of leptin are equally important. For instance POMC and its derivatives are also important for normal body homeostasis *i.e.* some subjects with null mutations in the POMC gene are hyperphagic and obese from an early childhood. Characteristic features of individuals with a dysfunctional POMC gene is that they are ACTH deficient and show hypopigmentation of the hair and skin (Krude, *et al.* 1998). There are also well-described point mutations in the POMC gene leading to the loss of parts of the pro-peptide encoding for β- and α-MSH (described in detail in later chapters) (Biebermann et al., 2006; Y. S. Lee et al., 2006). Therefore, mutations in the enzymes involved in the processing of POMC, the prohormone convertase 1 (PCSK1) and carboxypeptidase E (CPE) genes can cause obesity in humans. Mutations in the receptors of the melanocortin peptides such as the MC4R are thought to be responsible for 5-6 % of all early onset obese patients (Farooqi, *et al.* 2003). There are also several identified mutations in the molecules downstream of MC4R such as in the brain-derived neurotrophic factor (BDNF), which is a part of a larger set of growth factors that activate tropomycin-related kinase B (TrkB) (Xu, *et al.* 2003). Mutations and changes in the BDNF loci are also associated with early-onset obesity (Han, *et al.* 2008). Mutations and translocations in the hypothalamic transcription factors such as the single minded 1 (SIM1) thought to be located downstream of MC4R, evoke severe obesity in a manner similar to MC4R mutations (Hung, *et al.* 2007). The FTO gene mutation has been identified in genome wide association studies (GWAS) and is associated in homozygotes with a 1,67-fold increase in obesity probability and a 3-4kg increase in body weight (Frayling, *et al.* 2007). FTO is expressed in the hypothalamus and is linked to energy intake rather than energy expenditure (Cecil, *et al.* 2008). In experimental animals, the overexpression of the FTO gene in mice causes obesity while mice with a non-functional gene are small and exhibits increases in energy expenditure (Church, *et al.* 2010).

2.2 MELANOCORTIN SYSTEM

The melanocortin system consists of the pro-peptide POMC and several downstream cleavage products, which mediate their actions through melanocortin receptors in key neuronal populations in the CNS. Two different 1st order neuronal populations, the POMC expressing neurons and AGRP/NPY expressing neurons receive information about nutritional status from the periphery and affect behavior and metabolism depending on which population dominates. Circulating humoral and nutritional factors such as leptin, insulin and glucose affect the plasticity of POMC neurons. The central melanocortin system regulates peripheral lipid and glucose metabolism independently from energy intake and energy expenditure (Nogueiras*, et al.* 2007) and a significant involvement in hemodynamic processes and in inflammation (Humphreys*, et al.* 2011).

2.2.1 POMC

Pro-opiomelanocortin (POMC) is a 31 kDa pro-peptide which is expressed in the pituitary, medulla and in distinct areas of the CNS and in the periphery. All POMC-derived peptides present in the bloodstream are synthesized from the anterior pituitary with the only exception being the placenta. The distribution, synthesis and composition of these peptide fragments are tissue specific and the processing mechanisms of POMC permit the synthesis of a variety of different regulatory peptides such as ACTH, β-endorphin and the three melanin stimulating hormones alpha, beta and gamma (α-, β-, γ-MSH). Other less prominent peptides derived from POMC are the Corticotrophin-Like Intermediate Protein (CLIP) and beta-lipotropin (β-LPH) (Figure 2).

In the CNS, POMC derived melanocortin peptides regulate energy balance mainly through the activation of MC4R (Butler*, et al.* 2000, Elmquist*, et al.* 1999, Huszar*, et al.* 1997). The natural antagonist of melanocortin receptors, the agouti related peptide (AGRP), exerts an orexigenic effect on energy metabolism. The melanocortins, particularly α-MSH, have also been shown to harbor anti-inflammatory properties and to modulate hemodynamics and sympathetic tone (Baltatzi*, et al.* 2008, Catania*, et al.* 2010, Humphreys*, et al.* 2011). The POMC derived endogenous opioid β-endorphin has also been shown to influence energy balance and β-lipotropin (β-LPH), the precursor of both β-endorphin and γ-LPH is known to be a strong inducer of lipolysis (Bodnar & Klein. 2005, Eichhorn*, et al.* 1995, Richter*, et al.* 1990). Both β-endorphin and its precursor β-LPH are jointly released in physiologically stressful situations such as in pain and during physiological exercise (Petraglia*, et al.* 1990, Salar*, et al.* 1991).

The expression of the POMC gene in the anterior pituitary is increased by cortictropin-releasing hormone (CRH) and vasopressin and inhibited by glucocorticoids. The stress induced increase in POMC gene-expression in the pituitary also increases ACTH secretion. ACTH, a known ligand of MC2R, increases the production of corticosteroids from adrenal membranes. The CLIP peptide has been shown to trigger and prolong the duration of paradoxical sleep in the dorsal raphe nucleus (Cespuglio*, et al.* 1995, Wetzel*, et al.* 1994).

2.2.1.1 POMC PROCESSING

The processing mechanisms of the precursor POMC allow for tissue specific synthesis of different peptides and peptide compositions. The molecular basis for the processing of POMC occurs by the recognition of eight different basic amino acid pairs and one quadruplet in the pro-peptide sequence, which is cleaved by prohormone convertases (PC) PC1/3 and PC2 (Figure 2). PCs belong to the subtilisin family of Ca^{2+}dependent serine proteinases (Seidah*, et al.* 1990, Seidah*, et al.* 1991) and the cleavage of POMC can be regulated by the availability of PC enzymes. The activity of PC1/3 is inhibited by the neuroendocrine-specific-inhibitor proSAAS and PC2 by 7B2 (Fricker*, et al.* 2000, Lindberg*, et al.* 1995).

Processing of POMC in the pituitary is a classical example of the differences in the pro-hormone modification since in this organ, POMC is more extensively cleaved in the intermediate than in the anterior lobe (Figure 2). The corticotropic cells are responsible for the synthesis of POMC in the anterior lobe and processing by PC1/3 results in the generation of pro-γ-MSH, pro-ACTH and β-LPH (Eipper & Mains. 1980). Pro-ACTH can be further cleaved by PC1/3 to form N-terminal pro-opiocortin (N-POC), joining peptide and ACTH (Figure 2). In the melanotropes of the intermediate lobe of the pituitary gland, in the skin and in hypothalamus, $ACTH_{1\text{-}39}$ is cleaved by PC2 to form CLIP, $ACTH_{1\text{-}17}$ and β-LPH. β-LPH is further cleaved by PC2 to form β-$endorphin_{1\text{-}31}$ and γ-LPH (Mains & Eipper. 1981). A small portion of β-LPH is metabolized to β-endorphin in the anterior pituitary. β-$endorphin_{1\text{-}31}$ can be further targeted in the C-terminus to yield β-$endorphin_{1\text{-}26}$, β-$endorphin_{1\text{-}27}$ and a dipeptide glycylglutamine (Boileau*, et al.* 1982, Mains & Eipper. 1981, Smyth & Zakarian. 1980, Smyth*, et al.* 1988). The N-terminal fragment of pro-γ-MSH is partially cleaved at an arginine-lysine site to produce both N-$POMC_{1\text{-}49}$ and Lys-γ_3-MSH (Bennett. 1986). In humans, N-POMC is processed to γ-LPH and β-MSH. Moreover, in the adrenal cortex, the adrenal secretory protease (AsP) can act upon pro-γ-MSH two residues downstream from the dibasic site to produce a mitogen, N-$POMC_{1\text{-}52}$. In addition, the synthesis of α-MSH requires the activity of both carboxypeptidase (CPE) and peptidyl α-amidating monooxygenase (PAM) which first produce desacetyl-α-MSH (da-α-MSH) and further by N-acetyltransferase (N-AT) to form the mature α-MSH. N-AT also diminishes the opiate activity of β-endorphin (Figure 2). The availability of α-MSH in active sites is regulated by a degrading enzyme called propylcarboxypeptidase (PRCP) (Wallingford*, et al.* 2009), which targets the active α-$MSH_{1\text{-}13}$ in the C-terminus producing the inactive α-$MSH_{1\text{-}12}$-form. Excess pro-POMC is produced in order to ensure the availability of the precursor for the rapid synthesis of ligands to ensure receptor activation (Pritchard & White. 2007).

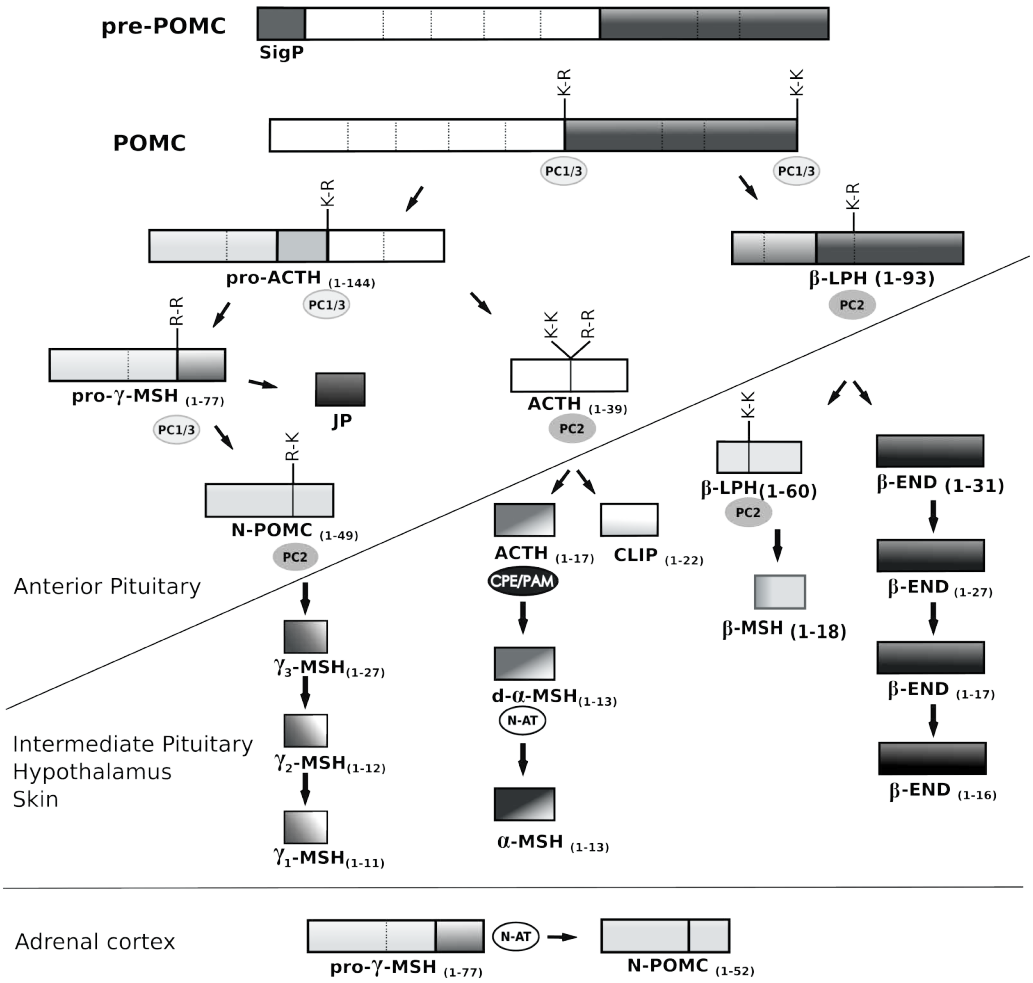

Figure 2. The processing of POMC by PC1/3 and PC2 enzymes, which are expressed in a tissue dependent manner. Figure modified from (Mountjoy. 2010)

2.2.2 Melanocortins

The melanocortins are a well-studied group of neuropeptides. In 1919 Atwell and coworkers isolated a hormone that later would be called melanocyte stimulating hormone due to the way it stimulated pigment cells of both mammalian and amphibian origin to produce melanin (Fitzpatric & Lerner. 1953, Gordon. 1953). The POMC derived peptides, particularly ACTH and the melanocortins are structurally similar and share a conserved core region (Met-Glu-His-Phe-Arg-Trp) where Glu is substituted for a Gly in the γ-MSH peptides (Table 1). The MSH peptides and their receptors are all part of the melanocortin system, which is centrally part of a larger neuronal network regulating and sensing the energy state.

Table 1. Comparison of POMC derived ACTH, melanocortins and their analogues. Modified from (Tatro. 1996).

D-Trp-γ-MSH	Tyr-Val-	**Met-Gly-His-Phe-Arg-D-Trp -**	Asp-Arg-Phe-Gly
ACTH	Ser-Tyr-Ser -	**Met-Glu-His-Phe-Arg-Trp -**	Gly-Lys-Pro-Val- + 26 Amino acids
α-MSH	Ac-Ser-Tyr-Ser -	**Met-Glu-His-Phe-Arg-Trp -**	Gly-Lys-Pro-Val
γ2-MSH	Tyr-Val -	**Met-Gly-His-Phe-Arg-Trp -**	Asp-Arg-Phe-Gly
(Lys)-γ1-MSH	(Lys)-Tyr-Val -	**Met-Gly-His-Phe-Arg-Trp -**	Asp-Arg-Phe-NH2
(Lys)-γ3-MSH	(Lys)-Tyr-Val -	**Met-Gly-His-Phe-Arg-Trp -**	Asp-Arg-Phe-Gly- + 14 Amino acids
β-MSH *	Asp-Ser-Gly-Pro-Tyr-Ly:	**Met-Glu-His-Phe-Arg-Trp -**	Gly-Ser-Pro-Lys-Asp
NDP-MSH	Ac-Ser-Tyr-Ser -	Nle-**Glu-His-*D*-Phe-Arg-Trp -**	Gly-Lys-Pro-Val-NH2
Melanotan		Nle-Asp-**His-*D*-Phe-Arg-Trp -**	Lys
SHU9119		Nle-Asp-**His-*D*-Nal-Arg-Trp -**	Lys

*Conserved amino acids shown in **bold** (Lys) = may or may not be present*
*Ac = N-acyl * = Bovine*
NDP-MSH and melanotan are synthetic analogues of α-MSH
SHU9119 is an antagonist of both MC4R and MC3R

2.2.2.1 α-MSH

The most widely studied and best known POMC derived melanocortin, α-MSH, has been shown to possess several different functions both peripherally and centrally. Among the central properties of α-MSH is to mediate the anorexigenic effects of leptin, which is produced by the fat storing cells or adipocytes in the periphery. POMC neurons play a key role in mediating the effects of leptin are in hypothalamus (Cowley*, et al.* 1999, Elmquist*, et al.* 1999). Here, α-MSH is released by POMC neurons and it acts on downstream neurons in the paraventricular nucleus known to express melanocortin receptors, MC4R, and also to a smaller degree MC3R (Adan*, et al.* 1997, Chen*, et al.* 2000). POMC neuronal projections are also able to affect other brain regions such as the NTS of the brain stem where α-MSH can influence meal size after binding to local melanocortin receptors (Zheng*, et al.* 2010). Short-term intracerebroventricular administration of α-MSH or synthetic analogues have been shown to decrease feeding, reduce adiposity and improve glucose metabolism (McMinn*, et al.* 2000, Murphy*, et al.* 1998, Nogueiras*, et al.* 2007).

There is significant overlap in the central effects of α-MSH. Skibicka and Grill compared the effects of a melanocortin agonist in different brain regions which were known to be linked to sympathetic outflow with respect to feeding, locomotor activity and core body temperature. α-MSH exerted an anorexic response in the PVN and the retrochiasmatic area (RCh) of the hypothamus and the NTS and pontine parabranchial nucleus (PBN) of the brain stem, whereas core body temperature was increased by its ablility to stimulate the rostral ventrolateral medulla (RVLM) of the brain stem. Interestingly, only the administrations of α-MSH to the PVN and the PBN were able to stimulate spontaneous locomotor activity (Skibicka & Grill. 2009). In addition, α-MSH has been able to correct hyperphagia in leptin and POMC deficient animals when the peptide was administered peripherally (Cettour-Rose & Rohner-Jeanrenaud. 2002, Yaswen*, et al.* 1999).

There is emerging evidence demonstrating the link between the melanocortins and the HPA axis. For instance, neurons expressing α-MSH and AGRP located in the ARC innervate the PVN and the medial amygdala, which are brain regions known to regulate the HPA-axis. In line with this hypothesis, stress induced anorexia in rodents can be reversed following intracerebroventricular (ICV) injections of melanocortin antagonists (Liu, *et al.* 2007, Vergoni, *et al.* 1999). Moreover, the melanocortin system is associated with adrenal function, as the expression levels of MC4R are not elevated in adrenalectomized rats during re-feeding (Germano et al 2008). Centrally, α-MSH also affects blood pressure through the sympathetic system due to the activation of the MC4R (Butler & Cone. 2002, Hill & Dunbar. 2002, Humphreys, *et al.* 2011, Matsumura, *et al.* 2002, Ni, *et al.* 2006).

The effects of α-MSH as an anti-inflammatory mediator have been well studied both *in vitro* and *in vivo*. A central mechanism for these effects is the capability of α-MSH to inhibit the activation of the nuclear transcription factor NF-κB, which controls the expression of many of the factors involved in inflammation *e.g.* cytokines and their receptors, growth factors, chemokines and adhesion molecules (Catania, *et al.* 2010). Moreover, α-MSH has been shown to induce the expression of a known anti-inflammatory mediator, IL-10 (Bhardwaj et al., 1996). In addition, α-MSH has protective effects on the vasculature by inhibiting the expression of vascular E-selectin and vascular cell adhesion molecule (VCAM)-1, two compounds capable of triggering deleterious vascular damage (Scholzen et al., 2003). There is also recent data showing that α-MSH is able to protect against vascular damage by regulating the availability of vascular nitric oxide (NO) (Rinne, *et al.* 2013).

PRCP governs the availability of active α-MSH in some nerve terminals. In line with this proposal, PRCP null mice have elevated levels of α-MSH and display a characteristic lean phenotype (Wallingford, *et al.* 2009). PRCP is widely distributed in the nervous system and the periphery. Centrally, PCRP is found abundantly expressed in the lateral hypothalamic perifornical region, an area known to trigger orexinergic signaling circuits and the paraventricular and in the ARC of the hypothalamus (Abbott, *et al.* 2000, Bittencourt, *et al.* 1992, de Lecea, *et al.* 1998, Horvath, *et al.* 1999, Sakurai, *et al.* 1998, Skofitsch, *et al.* 1985, Trivedi, *et al.* 1998, Wallingford, *et al.* 2009).

2.2.2.2 β-MSH

β-MSH is the least studied melanocortin peptide because it is not supported by the mouse genome. This is because in mice there is the lack of the proximal di-basic site necessary for the proteolytic cleavage that produces the active peptide. Assumptions have therefore been made that α-MSH, which is expressed in many organisms, is the main central agonist of MCR. Conversely, β-MSH has been implicated in human obesity and mutational studies have shown that the POMC gene displays heterozygous mutations close to the β-MSH peptide sequence (Challis, *et al.* 2002, Hinney, *et al.* 1998, Hinney, *et al.* 2006, Lee, *et al.* 2006). One of these mutations causes a fusion peptide to be formed with β-endorphin, which has been postulated to have a dominant-negative effect (Challis, *et al.* 2002). Loss of function mutations in the β-MSH gene could hypothetically produce an obese phenotype with normal cortisol levels

as compared to the loss of function mutations in the ACTH coding region (Harrold & Williams. 2006). β-MSH and the truncated form (β-MSH_{5-22}) found in human hypothalamus are both capable of inhibiting feeding in a similar manner to α-MSH (Abbott, *et al.* 2000, Bertagna, *et al.* 1986). Additionally in rats, a fragment of β-MSH (β-MSH_{4-7}) has been shown to influence long-term satiety (Kotov, Martynov, Tolypgo, Kelesheva, & Sorochinskaia, 1984). A comparison of melanocortin peptides and their binding affinity for their receptors in transfected cell lines and in brain homogenates isolated from the rat hypothalamus has shown that β-MSH has higher affinity for the MC4R than α-MSH (Harrold, *et al.* 2003, Schioth, *et al.* 1996). A histological analysis of the expression of ACTH, α- and β-MSH in human post-mortem ARC samples revealed that β-MSH was expressed in human POMC neurons (Biebermann, *et al.* 2006). Food restriction is able to affect the level of β-MSH whereas the concentration of α-MSH remains unchanged (Harrold, *et al.* 1999, Harrold & Williams. 2006). Furthermore, hypothalamic levels of β-MSH have been shown to negatively correlate with the plasma insulin concentration, whereas that of β-MSH is essentially unaffected by changes in levels of leptin (Cheung, *et al.* 1997). Similarly to other agonists of MCR, β-MSH has also been shown to inhibit NF-κB and is therefore a potent anti-inflammatory peptide (Muceniece, *et al.* 2005).

2.2.2.3 γ-MSH

γ-MSH exists in three different isoforms with varying affinities for the MC3R but its affinity for the MC4R is lower (Lindblom, *et al.* 1998, Roselli-Rehfuss, *et al.* 1993, Schioth, *et al.* 1996). γ-MSH is produced in the intermediate lobe of the pituitary where it potentiates ACTH-induced steroidogenesis (Farese, *et al.* 1983, Pedersen, *et al.* 1980, Seger & Bennett. 1986). γ-MSH is able to influence hemodynamics by controlling the sympathetic outflow and baroreflex function (Humphreys, *et al.* 2011). Furthermore, γ-MSH is natriuretic by directly affecting renal sodium exertion (Lymangrover, Buckalew, Harris, Klein, & Gruber, 1985). A few studies have also indicated that γ-MSH may be involved in the activation of the vasopressin pathway in the anteroventral region of the third ventricle (Callahan, *et al.* 1988, Gruber & Eskridge. 1986). Similarly to other melanocortins, γ-MSH has been shown to be anti-inflammatory and has been implicated in sexual behavior (Cragnolini, *et al.* 2000, Getting, *et al.* 2001, Xia, *et al.* 2001).

The N-terminal fragment of POMC is cleaved by PC2 and produces a 25 amino acid long peptide, γ_3-MSH, which can be further N-glycosylated (Oki, *et al.* 1982, van Strien, *et al.* 1995). In pituitary cells, γ_3-MSH is able to elevate intracellular Ca^+ levels in growth hormone and prolactin secreting cell lines. Interestingly this activation can be blocked only to 50 % by the use of a melanocortin receptors antagonist (Denef, *et al.* 2003). In comparison to the shorter γ-MSH peptides, γ_3-MSH includes a long C-terminal fragment. The peptide sequence of both γ_2-MSH and γ_1-MSH are similar and have an 11 amino acid long core sequence with either a glycine or an amide group in the C-terminal end, respectively. γ_2-MSH is also selective for the MC3R in preference over both MC4R and MC5R, however, γ_2-MSH shows no selectivity between the MC3R and MC5R in mice (Joseph, *et al.* 2010). Moreover, γ_2-MSH has

about a 3-fold lower affinity for MC receptors compared with γ_1-MSH (Schioth, *et al.* 1996). In addition, both γ_1 and γ_2-MSH have an additional lysine in the N-terminus, which appears to be essential for their full biological activity, at least in the adrenal gland (Pedersen & Brownie. 1983). In behavioral studies, intra-VTA administration of γ_2-MSH induced central analgesia and catalepsy, whereas γ_1-MSH administration stimulated grooming behavior and increased vertical activity (Klusa, *et al.* 1999). These behavioral responses have been speculated to involve the dopaminergic mesolimbic system where (similarly to α-MSH) γ_1-MSH causes activation whereas γ_2-MSH inhibition of $GABA_A$ receptors (Jansone, *et al.* 2004). This conclusion was based on studies where α-MSH mediated analgesia in the VTA could not be blocked by a melanocortin antagonist (Klusa, *et al.* 1998).

The cardiovascular effects of γ-MSH have been well characterized. For instance, intravenous or intracarotid injection of γ_2-MSH has pressor and cardioaccelerator effects which are mediated by the activation of sympathetic outflow (Gruber, *et al.* 1989). Interestingly the sympathetic effects of intravenous γ_2-MSH were independent of both MC3R and MC4R activation pointing to the activation of an entirely different receptor, the FMRFamide gated sodium channel (Humphreys, *et al.* 2011, Li, *et al.* 1996, Ni, *et al.* 2006, Wikberg, *et al.* 2000). The FMRFamide-gated sodium channel (FMRFamide) has important cardioexcitatory and sympathoexcitatory properties and it has been implicated in the development of salt-sensitive hypertension in rats (Huang & Leenen. 2002, Nishimura, *et al.* 2000). Although γ-MSH is able to increase sympathetic activity by activating the FMRFamide at high doses, low doses of γ-MSH have been shown to increase natriuresis and to decrease salt induced hypertension (Humphreys, *et al.* 2011, Lymangrover, *et al.* 1985). Furthermore, central microinjections directed to the NTS could reduce blood pressure due to a reduction in SNS activity (De Wildt, *et al.* 1994, Li, *et al.* 1996). γ-MSH has therefore been postulated to be involved in baroreflex function in the NTS while the natriuretic capacity is mediated by renal MC3R (Humphreys, *et al.* 2011). In line with this theory, elevated dietary sodium dramatically increases the expression of MC3R in the renal inner medullary duct and the level of γ-MSH in plasma and specifically in the neurointermediate lobe of the pituitary (Mayan, *et al.* 1996, Ni, *et al.* 2006). Furthermore, PC2 deficient mice that lack the correct processing of POMC to produce γ-MSH develop salt-sensitive hypertension, which is corrected by infusing γ-MSH peptide, not α-MSH (Ni, *et al.* 2003, Ni, *et al.* 2006). In addition, infusion of γ-MSH in MC3R-deficent mice has no effect on arterial pressure indicating that γ-MSH mediated signaling of MC3R is required to excrete sodium when consuming a high sodium diet (Ni, *et al.* 2003).

MC3R deficient mice have also revealed a link between hypertension and glucose metabolism (Ni & Humphreys. 2008). Salt-sensitive hypertension can be corrected by infusion of γ_2-MSH or blockade of adrenergic receptors and both treatments also normalize fasting glucose levels (Mayan, *et al.* 2003). In this study, treating the salt-induced hypertension with the peripherally acting vasodilator compound, hydralazine, did not alter glucose homeostasis (Mayan, *et al.* 2003). In addition, the salt-induced hypertension elevated noradrenaline levels, but were corrected by infusion of γ_2-MSH. Bromocriptine blockade of pituitary activity in these mice increased plasma noradrenaline levels, but this could be restored by γ_2-MSH administration. In summary, these effects

indicate that it is the increased sympathetic activity that is responsible for the abnormal glucose metabolism. (Ni, *et al.* 2009)

2.2.3 Antagonists of melanocortin action

2.2.3.1 AGOUTI SIGNALING PEPTIDE

The agouti gene encodes for a peripherally expressed 131-amino-acid protein, the agouti signaling peptide (ASIP), that controls the relative amounts of eumelanin (black-brown) and phaeomelanin (red-yellow) produced by hair follicles (Lu, *et al.* 1994). The agouti protein inhibits melanocortin receptor-mediated production of eumelanin by directly blocking MC1R in melanocytes. Additionally, the agouti protein is an antagonist of the MC4R (Gantz, *et al.* 1993, Lu, *et al.* 1994) and it directly modulated feeding in lethal yellow A^Y mice (Dickerson & Gowen. 1947), which ubiquitously overexpress agouti and have an obese phenotype.

2.2.3.2 AGRP

In 1997 Shutter and colleagues described a novel centrally expressed 132-amino acid protein named AGRP that showed 25% homology with agouti. AGRP is expressed only in the ARC of the hypothalamus (Ollmann, *et al.* 1997, Shutter, *et al.* 1997) and is 10-fold upregulated in genetically obese $Lepr^{db}/Lepr^{db}$ and Lep^{ob}/Lep^{ob} mice with leptin signaling deficiency (Ebihara, *et al.* 1999, Ollmann, *et al.* 1997, Shutter, *et al.* 1997). AGRP is a competitive antagonist of both MC3R and MC4R and it co-localizes to neurons that express NPY (Buch, *et al.* 2009, Chen, *et al.* 1999). More recent data suggests that AGRP actually functions as a biased agonist on the MCRs, since it selectively couples to $G_{i/o}$, which decreases cAMP levels (Buch, *et al.* 2009). In addition, both AGRP and α-MSH promote β-arrestin recruitment upon binding to MCRs (Breit, *et al.* 2006). The hypothalamuc NPY/AGRP expressing neurons are distinct from neighboring POMC expressing neurons (Shutter, *et al.* 1997) and project to the adjacent hypothalamic areas in the PVN, DMN and LHA (Broberger, *et al.* 1998, Hahn, *et al.* 1998). The AGRP/NPY neurons increase food intake both by antagonizing melanocortin signaling and by increasing the expression of the fast acting orexigenic peptide NPY and GABA (Krashes, *et al.* 2013, Morton & Schwartz. 2001). Both AGRP and NPY negatively correlate with leptin and the expression of both peptides is reduced following leptin treatment (Mizuno, *et al.* 1998). The increase in AGRP in response to fasting and in genetic leptin deficiency is considerably greater in comparison to that of NPY, which is indicative of a distinct mechanism for AGRP and NPY (Morton & Schwartz. 2001). Furthermore, the orexigenic effects of central AGRP administration have been shown to endure up to a week in contrast to the relatively acute feeding response following NPY delivery which lasts for only hours (Hagan, *et al.* 2000, Rossi, *et al.* 1998).

There is recent data suggesting that AGRP also promotes changes in nutrient partitioning and lipid metabolism in efferent tissues through the reduction of sympathetic activity, which increases adiposity (Dietrich, *et al.* 2010, Joly-Amado, *et al.* 2012). As a result, ablation of AGRP neurons at the neonatal stage leads to a phenotype with

increased adiposity and with an increased lipogenesis and triglyceride content in the liver on carbohydrate rich diets (Joly-Amado, *et al.* 2012). These AGRP deficient animals use lipids as their source of energy making them indistinguishable from WT-controls on a HFD (Joly-Amado, *et al.* 2012). AGRP ablation in adult mice is survivable when the animal has developed adequate fat reserves allowing time for compensatory routes to be activated (Wu, *et al.* 2012).

Although the general view of the orexigenic action of AGRP is that it is mediated by antagonism (or rather a kind of competitive/biased agonism) of the melanocortin receptors, there are studies demonstrating alternative routes for AGRP signaling. Administration of a GABA-agonist to the PBN rescues the phenotype in AGRP ablated mice (Wu, *et al.* 2009). Krashes et al demonstrated that GABA and NPY were responsible for the acute feeding effect, whereas AGRP increased food intake in the long-term after fasting (Krashes, *et al.* 2013). These observations suggest that AGRP/NPY neurons also release GABA, as both are able to induce an orexigenic response in a complementary fashion (Krashes, *et al.* 2013).

2.2.4 NPY

NPY was one of the first peptides to be recognized as a potent orexigenic compound; it was initially isolated from porcine brain in 1982 (Tatemoto, *et al.* 1982). The 36-amino acid peptide NPY has been highly conserved in invertebrate and vertebrate species (Larhammar. 1996) and it shows homology with the other members of the NPY peptide family, peptide YY (PYY) and pancreatic peptide (PP) (Tatemoto, *et al.* 1982). Both NPY and PYY are able to activate the same receptors, Y_1, Y_2, Y_4, Y_5 and Y_6, all belonging to the same family of G_i-coupled receptors (Larhammar. 1996). NPY is found in the nervous system both centrally and in the periphery and is able to interact with other classical stress mediators such as sympathetic neurotransmitters (*e.g.* catecholamines and purines) as well as hormones in the HPA-axis. The general view is that one of the main functions of NPY is to act as a mediator of stress since it is known to be involved in physiological processes linked to stress behavior including feeding (Tatemoto, *et al.* 1982), cardiovascular function (Jacques, *et al.* 1996), anxiety (Giesbrecht, *et al.* 2010), learning and memory (Flood & Morley. 1989), and circadian rhythm (Sindelar, *et al.* 2005).

2.2.4.1 NPY AND THE REGULATION OF FOOD INTAKE

Although NPY has many different effects in the central nervous system, the effect on feeding seems to be most prominent. ICV administration of NPY increases food intake in a dose dependent manner (Clark, *et al.* 1985, Levine & Morley. 1984) and chronic administration evokes a sustained hyperphagia and increased body weight resulting in obesity (Beck, *et al.* 1992, Zarjevski, *et al.* 1993). Increased NPY expression has been demonstrated in DIO animal models and in genetically obese rodent models such as MC4R-deficent mice (Kesterson, *et al.* 1997), agouti lethal yellow (A^y/a) mice (Kesterson, *et al.* 1997), leptin signaling deficient mice and rat (Kesterson, *et al.* 1997, Sanacora, *et al.* 1990, Wilding, *et al.* 1993) and in brown-adipose deficient mice (Tritos, *et al.* 1998). Investigations into

the action of NPY in specific locations in the hypothalamus have identified a feeding response in the PVN, LHA, VMN and the perifornical area (Stanley, *et al.* 1985). LHA is an important activator of the NPY system in the ARC and NPY in turn is able to suppress LHA neurons (Della-Zuana, *et al.* 2002, Fu, *et al.* 2004, Mercer, *et al.* 2011). There is a subpopulation of neurons in the DMN that transiently expresses NPY, whereas the primary input of NPY is from neuronal ARC projections (Bai, *et al.* 1985, Bouret, *et al.* 2004, Thompson & Swanson. 1998). AAV-mediated overexpression of NPY in the LHA and DMN causes hyperphagia and weight gain (Tiesjema, *et al.* 2007, Yang, *et al.* 2009) in lean rats whereas the knock-down of NPY results in a prolonged anorexigenic response in genetically obese OLETF rats (Yang, *et al.* 2009). NPY and AGRP expression co-localize to the same neurons in the hypothalamus and these inhibit POMC neurons by a dual mechanism. NPY binds to Y_1 and Y_2 receptors on downstream POMC neurons inhibiting their firing rate (Acuna-Goycolea, *et al.* 2005, Cowley, *et al.* 2001, Ghamari-Langroudi, *et al.* 2005, Roseberry, *et al.* 2004) and NPY has been implicated in the direct release of α-MSH from POMC neurons by binding to Y_5-receptors (Galas, *et al.* 2002). Moreover, NPY regulation is mediated by an inhibitory mechanism of GABA release in parvocellular neurons of the PVN in post-synaptic fashion (Cowley, *et al.* 1999, Melnick, *et al.* 2007). This effect is most prominent in neuronal populations expressing MC4R (Ghamari-Langroudi, *et al.* 2011). Although NPY/AGRP and POMC neurons co-regulate each other through a similar mechanism, the NPY-mediated orexigenic action in the hypothalamic neurons is not dependent on melanocortin action, evidence for an independent regulatory mechanism for NPY (Aponte, *et al.* 2011).

With respect to the potency of NPY to act as an acute stimulator of feeding in the CNS, models with both germline genetic NPY ablation and overexpression have not produced any profound metabolic phenotype in either mouse or rat backgrounds. The reason is thought to be due to compensatory mechanisms and to the plasticity of the CNS. Thus, postnatal ablation of NPY/AGRP expressing neurons causes lethal starvation (Luquet, *et al.* 2005), whereas it is survivable in neonates neonates (Luquet, *et al.* 2005) and if the animal has attained sufficient fat reserves (Wu & Palmiter. 2011). The general effects of NPY are clear if deficient animals have been crossed with pre-existing obese genetic animal models. For instance, when NPY-deficient mice are crossed with leptin deficient Lep^{ob}/Lep^{ob} mice they produce a phenotype with a reduced food intake and increased energy expenditure (Erickson, *et al.* 1996). On the other hand, if transgenic animals overexpressing NPY (NPY-OE) are presented with a palatable diet where 50% of energy source originates from sucrose, the mice show a rapid development of obesity and increased food intake as compared to WT-control animals (Kaga, *et al.* 2001). Moreover, NPY-OE mice, which overexpress NPY in their noradrenergic neurons, develop increased adiposity and impaired glucose tolerance without affecting the total body weight (Ruohonen, *et al.* 2008).

2.3 MELANOCORTIN RECEPTORS

The POMC derived melanocortin peptides (α-, β- and γ-MSH) and ACTH are endogenous agonists, whereas agouti signaling peptide (ASIP) and AGRP are endogenous antagonists of melanocortin receptors (MCR) (Table 2). There are five known MCR subtypes all

belonging to the family of seven-transmembrane guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCR) (Cone. 2000). The MCR agonists bind with high affinity to their endogenous receptors and this causes a conformation change, which activates an intracellular cyclic adenosine monophosphate (cAMP) signaling pathway. The GPCRs all share high structural homology, although the MCRs are the smallest known type of GPCRs and show high subtype-specific sequence homologies. According to sequence alignment studies, the MC4R and MC5R showing 60% homology, the MC3R and MC1R and the MC2R and MC3R show both 45% and the MC2R and MC4R show 38% homology. In structural terms, the MCRs are composed of seven α-helical transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. In contrast to other GPCRs, the MCRs have short amino- and carboxy-terminal ends and an exceptionally short second extracellular loop (Yang. 2011). The MCRs possess an orthosteric binding-site for the endogenous agonist in the transmembrane region, whereas the endogenous antagonist may also bind to an allosteric site located on the extracellular loop (Oosterom*, et al.* 1999, Yang*, et al.* 1999, Yang & Harmon. 2003).

Translocation to the cell surface and ability of MCRs to induce cAMP activation are modified by Melanocortin 2 receptor accessory proteins 1 and 2 (MRAP1 and MRAP2). (Cooray & Clark. 2011, Hay*, et al.* 2006). MRAP2 is primarly expressed in the CNS, with distinct expression in the cerebellum, pons and parts of the hypothalamus (Asai*, et al.* 2013, Chan*, et al.* 2011). Interestingly, MRAP2 and MC4R mRNA co-localize in the paraventricular nucleus and MRAP2-deficient mice have been shown to display similar characteristics to that of MC4R-deficient mice (Asai*, et al.* 2013). In addition, *in vitro* data shows that MRAP2 enhances the effects of α-MSH to induce cAMP release in MC4R and MC3R suggesting that MRAP2 is also able to regulate the other MCRs (Asai*, et al.* 2013).

There is increasing evidence indicating that some MCRs are also able to activate other signaling pathways such as calcium- and MAPK-mediated signaling (Chai*, et al.* 2007, Chai*, et al.* 2009). Furthermore, mutational studies have shown that specific amino acid residues in the MCRs are required to induce these different signaling pathways (Yang. 2011). In summary, the different binding potencies of the endogenous agonists, different signaling routes and the fact that MCRs have a distinctive tissue distributions result in a diverse set of different physiological functions.

Table 2. Agonist profile, tissue distribution and function of melancortin receptors. Adapted from (Getting. 2006)

Target	Binding capacity	Tissue distribution (mRNA)	Function
MC1-R	α-MSH > ACTH >> γ-MSH	Melanocytes, Keratiocytes, Endothelial cells, Fibroblast, Immune cells, Astrocytes, Neurons	Pigmentary effects, Antipyretic/anti-inflammatory
MC2-R	ACTH	Adrenal cortex	Steroidogenesis
MC3-R	γ-MSH = ACTH > α-MSH	Brain, Heart, Kidney	Autonomic functions, Regulation of energy energy homeostasis
MC4-R	α-MSH > ACTH >> γ-MSH	Brain	Control of feeding and energy homeostasis, Erectile activity
MC5-R	α-MSH > ACTH > γ-MSH	Lymphocytes, Exocrine glands	Regulaion of exocrine secretions, Immunoregulatory functions

2.3.1 MC1R

The MC1R is a 317-amino acid receptor protein and it was the first human MCR to be cloned (Chhajlani & Wikberg. 1992, Mountjoy*, et al.* 1992). The most prominent effect of MC1R activation is the stimulatory effect on the production of melanin, particularly eumelanin (brown-black pigment). α-MSH is the main activator of melanogenesis as it has the highest binding affinity for the MC1R, this being closely followed by $ACTH_{1-39}$. The relative binding affinity of β-MSH is lower than $ACTH_{1-39}$, whereas the affinity of γ-MSH is considered weak and supposedly lacking in melanogenic activity (Table 2) (Abdel-Malek. 2001, Chhajlani & Wikberg. 1992, Suzuki*, et al.* 1996). Interestingly, the truncated peptides $ACTH_{4-10}$ and $ACTH_{1-10}$ do not activate the receptor, which highlights the requirement of the amino and carboxy-terminal ends of the biologically active α-MSH ($ACTH_{1-13}$) (Gantz*, et al.* 1993). Binding of α-MSH to the MC1R in the melanocytes of the skin activates an intracellular signaling cascade, which in turn elevates intracellular cAMP levels further activating the rate limiting enzyme in the process of melanogenesis, tyrosinase (Abdel-Malek*, et al.* 1995, Hunt*, et al.* 1994). The mRNA expression of MC1R is upregulated by melanocortins, endothelin-1 and the microphthalamia-associated transcription factor (MITF) (Abdel-Malek. 2001, Aoki & Moro. 2002).

In addition to its crucial role in the protection of the skin from UV rays, MC1R activation is essential in other physiological functions. MC1R have been described to be involved in the regulation of the immune system where it has been shown to reduce the activation and translocation of the pro-inflammatory cytokine NF-κβ (Manna & Aggarwal. 1998). Furthermore, activation of MC1R in inflammatory cells has been shown to increase the levels of the anti-inflammatory cytokine IL-10 (Bhardwaj*, et al.* 1996). To date, mRNA expression of the MC1R has been described in various immune cells; macrophages (Star*, et al.* 1995), antigen presenting and cytotoxic lymphocytes (Neumann Andersen*, et al.* 2001), astrocytes (Wong*, et al.* 1997), (Bohm*, et al.* 1999) dendritic cells in the circulation (Becher*, et al.* 1999, Salazar-Onfray*, et al.* 2002) and in fibroblasts (Bohm*, et al.* 1999). MC1R is also expressed in endothelial cells (Hartmeyer*, et al.* 1997) and in certain specific neurons of the periaqueductal gray substance in both humans and rats (Xia*, et al.* 1995).

Interestingly, ligand affinity to the MC1R on immunological and endothelial cells appears to be greater than on melanocytes and the effects of MC1R activation in the former cell types produce a physiological response at a picomolar concentration level rather than at the nanomolar level in the latter (Suzuki*, et al.* 1996). Furthermore, the mRNA expression of MC1R is considerably lower in endothelial and immunological cells as compared to that in melanocytes (Catania*, et al.* 2004).

2.3.2 MC2R

The MC2R is known as the ACTH receptor and it is structurally different from the other MCR *e.g.* it shares only 38% sequence identity with the MC4R. The MC2R receptor sequence encodes for a 297-amino acid G-protein coupled receptor (GPCR), which is expressed in the adrenal gland. Here, $ACTH_{1-39}$ is the only known ligand to display affinity

for the MC2R and it influences the production and release of steroids via PKA activation from the adrenal cortex in accordance with the circadian rhythm and in reaction to stress (Buckley & Ramachandran. 1981, Lefkowitz, *et al.* 1970, Mountjoy, *et al.* 1992). $ACTH_{1-39}$ is known to increase the expression of its endogenous receptor in the adrenal gland (Mountjoy, *et al.* 1994). MC2R mRNA has been found in the *zona glomerulosa*, where MC2R is responsible for the production of mineralocorticoids and in the *zona fasciculate*, where it controls the production of glucocorticoids (Mountjoy, *et al.* 1992). MC2R is less prominently present in the *zona reticularis* and only in scattered areas of the adrenal medulla (Xia & Wikberg. 1996). Although some studies have indicated the presence of MC2R on murine adipocytes (Boston & Cone. 1996), the human adipocytes have been consistently shown to lack such expression and therefore ACTH does not appear to regulate lipolysis in humans (Chhajlani. 1996). Moreover, only the adrenal cells and cell lines with endogenous expression of MCRs are able to express the MC2R since it requires the presence of a small membrane protein, the MCR accessory protein (MRAP) (Metherell, *et al.* 2005, Noon, *et al.* 2002). The MC2R has specialized MRAP domains, which are involved in the transport of the receptor (Sebag & Hinkle. 2007, Webb, *et al.* 2009).

2.3.3 MC3R

The MC3R is a 361 amino acid receptor protein which has been considered as the main receptor of γ-MSH, although it shows similar potency to that of $ACTH_{1-39}$ and α-MSH (Table 2). The MC3R is fully activated by the truncated peptides $ACTH_{4-10}$ and $ACTH_{1-10}$, which suggests that the HFRW-core region is required for this activation (Gantz, *et al.* 1993). The MC3R belongs to the GPCR-family, but is coupled to both cAMP- and inositol phospholipid-Ca^{2+} signaling systems (Kim, *et al.* 2002). The MC3R was the third MCR to be cloned and it is expressed in several peripheral tissues such as placenta, gut, kidney, heart, in human monocytes and in mouse peritoneal macrophages (Gantz, *et al.* 1993, Getting, *et al.* 1999, Taherzadeh, *et al.* 1999). In contrast to the MC4R, the central distribution of MC3R is more distinct with abundant expression in the hypothalamic ARC, VMH and in the DMH and in the limbic system (Jegou, *et al.* 2000). The MC3R has also been detected in the hippocampus, septum, thalamus and the midbrain (Roselli-Rehfuss, *et al.* 1993).

The MC3R is involved in the regulation of energy metabolism, inflammatory, autonomic and hemodynamic functions (Butler, *et al.* 2000, Catania, *et al.* 2004, Catania, *et al.* 2004, Chen, *et al.* 2000, Humphreys, *et al.* 2011, Humphreys, *et al.* 2011). The MC3R possesses an auto-inhibitory function on POMC neurons, however MC3Rs are expressed on both the orexigenic AGRP/NPY and on POMC neurons (Berthoud, *et al.* 2006, Li, *et al.* 1996, Skibicka & Grill. 2009). In addition, the MC3R has been linked to the regulation of lipolysis in WAT, which is directly innervated by corticotrophin-releasing hormone (CRH) neurons from the PVN (Bowers, *et al.* 2004, Renquist, *et al.* 2012, Shi & Bartness. 2001). The MC3R has been postulated to increase renal natriuresis in salt-induced hypertension (Humphreys, *et al.* 2011). There are clear indications that the MC3R has anti-inflammatory potential (Getting, *et al.* 1999, Getting & Perretti. 2000, Getting, *et al.* 2002, Getting, *et al.* 2003) and the receptor has been shown to

exert protective effects in situations of myocardial ischemia or in subsequent reperfusion induced injuries in rats (Guarini, *et al.* 2002). More details about the function of MC3R are reviewed in chapter 2.4.2.3, which describes the MC3R-KO mouse.

2.3.4 MC4R

The MC4R gene encodes for a 332-amino acid protein that covers a single exon and it was the second neural MCR to be cloned. It shows high homology with the MC1R and it also shows similar activation potency with respect to its agonists; α-MSH and $ACTH_{1-39}$ have the highest affinity followed by β-MSH, whereas γ-MSH and the truncated peptide, $ACTH_{4-10}$ activate the receptor to a lesser extent (Roselli-Rehfuss, *et al.* 1993). The presence of PRO^{12} in $ACTH_{1-39}$ is required for the activation of the receptor (Gantz, Konda, et al., 1993). The MC4R is highly conserved and shows a remarkable 93% homology between the human and the rat genomes (Alvaro et al., 1996). The MC4R is only found centrally where it exhibits a widespread expression profile in comparison to that of the MC3R, *i.e.* MC4R is expressed in the cortex, hypothalamus, thalamus, brain stem and the spinal cord (Mountjoy, *et al.* 1994)

Evidence gathered from genetic screening studies have revealed that mutations affecting the MC4R gene are involved in the development of obesity in approximately 5 % of all obese subjects. The human data is supported by evidence from animal studies where MC4R-deficient mice develop hyperphagia, hyperinsulinemia and hyperglycemia (Huszar et al., 1997). These animals show enhanced caloric efficiency similar to MC3R-deficient and agouti lethal yellow mice (Ste Marie, *et al.* 2000). Since a double knockout of both MC3R and MC4R leads to increased obesity as compared to MC4R knockout alone, both of these receptors appear to have major roles in the development of obesity (Chen, *et al.* 2000). The MC4R is also believed to be involved in the activation of SNS. Indeed, there is recent evidence from MC4R-deficient mice with selective rescue of the receptor on cholinergic neurons in the brain stem and in the spinal cord clearly demonstrating that the MC4R is responsible for both a decrease in parasympathetic tone and an increase in SNS activity (Sohn, *et al.* 2013). Furthermore, the cholinergic MC4Rs are also responsible for the regulation of insulin release since rescue of the receptor in these neuronal populations in MC4R-deficient mice were able to restore hyperinsulinemia (Scott, *et al.* 2011). The MC4R increases SNS driven activity to both BAT and WAT although these actions involve differential routes (Brito, *et al.* 2007, Song, *et al.* 2005, Song, *et al.* 2008). In addition to the metabolic effects, the MC4R was also reported to modulate erectile function and sexual behavior (Van der Ploeg, *et al.* 2002, Wessells, *et al.* 1998).

2.3.5 MC5R

The MC5R gene was the last MCR to be cloned and identified in humans (Gantz, *et al.* 1994) and in mouse (Labbe, *et al.* 1994); it encodes for a 325-amino acid protein. The MC5R and MC4R show the highest homology among the MCRs and resemble each other with respect to their capability to bind the different agonists. The highest potency

is exhibited by α-MSH followed by $ACTH_{1-10}$ and $ACTH_{4-13}$, whereas the activity of $ACTH_{4-10}$ is substantially weaker as compared to that found for MC3R (Labbe*, et al.* 1994). The MC5R is only expressed in the peripheral tissues *e.g.* liver, lung adipose tissue, skin, skeletal muscle, thymus, immune cells, testis, ovary, mammary gland, stomach and in the duodenum (Catania*, et al.* 2004). The MC5R are expressed on both T-lymphocytes (Taylor & Namba. 2001) and in B lymphocytes (Buggy. 1998) and these findings point to a role for the MC5R in immune regulation. Indeed, α-MSH has been shown to activate the JAK/STAT phosphorylation pathway by binding to the MC5R (Buggy. 1998). Furthermore, the $CD25^+$ $CD4^+$ regulatory T-cells are induced by α-MSH via the MC5R (Taylor & Namba. 2001). MC5R-deficent mice show decreased production of sebaceous lipids in the hair follicles of the skin, which leads to a severe defect in water repulsion and subsequently to defective thermoregulation (Chen*, et al.* 1997). This receptor is required for the production of porphyrins in the Harderian gland as well as for both tear and protein secretion from the lacrimal gland (Chen*, et al.* 1997, Entwistle*, et al.* 1990).

2.4 GENE MODIFIED ANIMAL MODELS IN OBESITY RESEARCH

The purpose of describing the animal models in the following chapter is to highlight the differences and similarities of the models used in metabolic research. The variability between dominant changes and loss-of-function mutations will be explained although many show overlapping or opposing characteristics. The spontaneous mutations in the early laboratory animals lay the foundation for the development of the ever-growing number of animal models of present time. The involvement of genetic mapping has later revealed that many of the metabolic phenotypes of the early genetic models are the consequences of mutations related genes and their signaling cascades. A comparison of the characteristics of these animal models is compiled in Table 3.

2.4.1 Leptin signaling deficiency

Leptin signaling deficiency involves either a loss-of-function mutation in the gene encoding leptin (Lep^{ob}/Lep^{ob}) or in the leptin receptor gene ($Lepr^{db}/Lepr^{db}$) in mice and $Lepr^{fa}/Lepr^{fa}$ in rat). Both mutation types cause a similar phenotype characterized by severe obesity with insulin resistance.

2.4.1.1 THE OBESE (Lep^{ob}/Lep^{ob}) MOUSE

The Lep^{ob}/Lep^{ob} mouse was discovered in an outbred colony in 1949 at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The phenotype is inherited via an autosomal recessive mutation positioned on chromosome 6 (Coleman & Hummel. 1973) and it was transferred to the well-characterized C57Bl background (Ingalls*, et al.* 1950). The mice were severely obese, hyperinsulinemic and hyperglycemic (Garthwaite*, et al.* 1980, Mayer. 1953). The pancreatic islets of these mice are up to ten-fold larger than in wild-type animals and have therefore been used in many studies involving β-cell function (Bray & York. 1979). The development of diabetes in Lep^{ob}/ Lep^{ob} mice has helped in

Table 3. Comparison of the specific metabolic characteristics of obese Lep^{ob}/Lep^{ob}, $Lepr^{db}/Lepr^{db}$, A^{y}/a, MC3R-KO, MC4R-KO, CPE-KO and POMC-KO mice and the genetically lean PRCP-KO and MSH-OE. + = increase, - = decrease, N = normal, ND = not defined, (M) = only in male. Table modified from (Butler & Cone. 2002)

	Leptin and MC-signaling deficiency							**Increased MC-tone**	
Charachteristics	**Lep^{ob}/Lep^{ob}**	**$Lepr^{db}/Lepr^{db}$**	**A^{Y}/a**	**MC3R-KO**	**MC4R-KO**	**CPE-KO**	**POMC-KO**	**PRCP-KO**	**MSH-OE**
Food intake	+ +	+ +	+	N	+	+ +	+	-	N
Weight gain	+ +	+ +	+ +	+/N	+ +	+ + +	+ +	-	-
NAL	-	-	+	-	+	+	+	-	-/N
Fat mass	+ +	+ +	+	+	+ +	+ + +	+ +	-	- (M)
Lean mass	-	-	+	-	+	+	ND	+	N
Glucose	+ +	+	+ (M)	+	+ + +	+ + +	N	N	N
Insulin	+ + +	+ + +	+	+	+ +	N	N	-	-
Leptin	- - -	+ + +	+ + +	+	+ +	+ +	+ +	ND	N
HPA-axis	+	+	+	+	N	N	- -	ND	-
SNS-activity	-	-	-	N	-	N	-	ND	+
Thyroid funtion	-	-	N	N	N	-	ND	-	Increased
Metabolic rate	Decreased	Decreased	Decreased	N	Decreased	Decreased	Decreased	Increased	Increased
Locomotor activity	Decreased	Decreased	Decreased	Decreased	Decreased (M)	N	ND	Increased	Decreased
Thermogenesis	Impaired	Impaired	N	N	Impaired	Impaired	ND	Increased	N
Reproduction	Infertile	Infertile	Functional	Functional	Functional	Infertile	ND	ND	Decreased
Immune function	Impaired	Impaired	Impaired	N	N	Impaired	Impaired	N	ND

the study of diabetes related illnesses such as in cardiomyopathy and in abnormal left ventricular function (Giacomelli & Wiener. 1979). An additional characteristic of these animals is that they are infertile and have impaired immune functions. Over 40 years after the discovery of the Lep^{ob}/Lep^{ob} mouse, extensive work using positional cloning was undertaken to identify the cause of the phenotype and in 1994, Friedman and co-workers identified leptin as a circulating factor produced by adipose tissue (Friedman*, et al.* 1991, Zhang*, et al.* 1994). The phenotype could be almost completely reversed by exogenous leptin therapy or by transfection of the leptin gene (Halaas*, et al.* 1995, Larcher*, et al.* 2001, Pelleymounter*, et al.* 1995).

The mice gain weight rapidly regardless of the initial hypophagia showing both hyperplastic and hypertrophic growth of both subcutaneous and intra-abdominal fat (Alonso & Maren. 1955). The Lep^{ob}/Lep^{ob} mice have impaired longitudinal growth and thus have a shorter anus to nose length as compared to wild-type littermates (Dubuc. 1976). Not only is the fat mass increased, but it is also redistributed to non-adipose tissue such as liver and muscle and the protein deposition is reduced due to the decreases in glucose uptake and glycogen synthesis (Le Marchand-Brustel & Jeanrenaud. 1978). During the progression of obesity, the animals also develop glucose intolerance, fasting hyperglycemia and resistance to exogenous insulin (Bergen*, et al.* 1975, Liu & Yin. 1974, Thurlby & Trayhurn. 1978). At 6 months of age, the weight accumulation attenuates and even starting to decline. There are small variations in the phenotype depending on the background.

2.4.1.2 THE DIABETIC ($Lepr^{db}/Lepr^{db}$) MOUSE

The mutation causing the phenotype in diabetic *$LepR^{db}$* - mice originates in chromosome 4 (Coleman & Hummel. 1973) and encodes for the leptin receptor gene (Chehab*, et al.* 2004). A number of different alleles have been identified and the mutation has been bred into several different backgrounds. The phenotype of these mice is almost identical to that of the Lep^{ob}/Lep^{ob} mice if the mutation is on the same background and includes the db^{3J}-allele (Hummel*, et al.* 1972).

The initial development of the phenotype in $Lepr^{db}/Lepr^{db}$ mice is similar in all backgrounds. In comparison to the phenotype of Lep^{ob}/Lep^{ob} mice, a small increase in insulin levels is observed by day 10-12 (Coleman & Hummel. 1973) accompanied by increased adiposity (Le Marchand-Brustel & Jeanrenaud. 1978). Insulin levels continue to rise modestly until the time of weaning when levels increase rapidly by as much as 10-fold in comparison to wild-type littermates at two to three months of age. On the C57BL/KS background, the hyperinsulinemia is transient and after three months of age the insulin levels suddenly drop due to atrophy of the β-islets and the animals start to lose weight before dying at six to eight months of age (Like & Chick. 1970). Conversely, on the C57BL/6J background, the state of hyperinsulinemia is retained and as a result the mice show a mild hyperglycemia, but display severe obesity similarly to that of Lep^{ob}/Lep^{ob} mice (Coleman & Hummel. 1973, Hummel*, et al.* 1972). The weight of the kidneys is increased with glomerular infiltration approximately at the age of five months (Like*, et al.* 1972). Similarly to the situation in Lep^{ob}/Lep^{ob} mice, fat is deposited in both subcutaneous and intra-abdominal areas with hypertrophic growth on the

C57BL/Ks background, whereas the C57BL/6J show both adipose tissue hypertrophy and hyperplasia (Meade, *et al.* 1979). Hyperphagia is observed after weaning (Le Marchand-Brustel & Jeanrenaud. 1978), however, the obesity is not caused attributable solely to hyperphagia and the reduced locomotor activity (Cox & Powley. 1977, Yen & Acton. 1972).

2.4.1.3 THE FATTY ($Lepr^{fa}/Lepr^{fa}$) RAT

The mutation causing diabetes and obesity in the Fatty $Lepr^{fa}/Lepr^{fa}$ rat was first described by Zucker and Zucker in 1961 (Zucker & Zucker. 1963). Similarly to the $Lepr^{db}/Lepr^{db}$ mice, these rats also lack functional leptin receptors and this is manifested in an obese, hyperinsulinemic and hyperlipemic phenotype. The development of the phenotype is also similar to that of the $Lepr^{db}/Lepr^{db}$ mice. Similarly to the other leptin signaling deficient animal models, the Fatty rat has elevated levels of NPY, low SNS tone and impaired BAT thermogenesis.

2.4.2 MC signaling deficiency

Functional melanocortin signaling is essential for body homeostasis and involves the synthesis, processing and degradation of melanocortin peptides and the functional binding and activation of melanocortin receptors. Mutations in any of these steps can cause changes in body energy homeostasis.

2.4.2.1 YELLOW OBESE (A^y/a) MICE

The yellow obese mouse was one of the earliest obese laboratory animals described by Lataste in 1883 and by Cuneot in 1905 (Cuneot. 1905, Lataste. 1883). However, the gene was not cloned and characterized until 1992 when a mutation was identified on chromosome 2 in the agouti locus which encodes for agouti signaling peptide (ASIP) (Bultman, *et al.* 1992). As described in chapter 2.2.3, ASIP was found to control the relative amounts of eumelanin (black-brown) and phaeomelanin (red-yellow) produced by hair follicles (Lu, *et al.* 1994) by antagonizing the MC1R. In the yellow obese mice, a 120 to 170 kbp deletion positions the ASIP gene under a ubiquitous promoter and this causes the coat to take on a yellow appearance. Normally ASIP is only expressed in the periphery, however, in the Yellow obese mice (Duhl, *et al.* 1994, Michaud, *et al.* 1993) there is impaired melanocortin signaling in both peripheral and central compartment. In addition to the yellow coat color, the yellow mice develop mature-onset obesity, type II-diabetes, hyperleptinemia, increased linear growth (in contrast to Lep^{ob}/Lep^{ob} mice) and infertility. The characteristics of the yellow obese have been confirmed to involve the agouti loci by creating using transgenic mice that have been engineered to ubiquitously overexpress the gene (Klebig, *et al.* 1995).

Heterozygous yellow obese mice have a somewhat milder obesity phenotype but the homozygous A^y/A^y mutation is lethal in the embryonic stage. The body weight increases linearly after puberty (8-12 weeks) and gradually reaches as high as 40 g (Carpenter &

Mayer. 1958, Castle. 1941, Dickie & Woolley. 1946). Increased adiposity is linked to increased fat cell size with only minor hyperplasia (Johnson & Hirsch. 1972). Obesity is significantly increased if the animals are fed obesogenic diets (Carpenter & Mayer. 1958). Plasma insulin is increased as a consequence of the increased obesity and decreased overall metabolic rate, although oxygen consumption is identical to that found in wild-type littermates (Bartke & Gorecki. 1968). The yellow mice have a reduced thermogenic capacity and are unable to maintain core body temperature when exposed to cold environments. Furthermore, locomotor activity is reduced in both young and in old A^Y/a mice (Dickerson & Gowen. 1947).

2.4.2.2 CARBOXYPEPTIDASE E-DEFICIENT MICE

Carboxypeptidase E (CPE) is required to remove the basic residues from the intermediate products of prohormone processing in order to produce a bioactive hormone. CPE is involved in the processing of several different hormones; initially it was described as an encephalin convertase (Fricker & Snyder. 1982, Hook*, et al.* 1982). CPE is required for the biological activity of α-MSH and the CPE-deficient mice resemble obese leptin signaling deficient animals, being both hyperphagic and infertile. This animal model has revealed CPE's role in bone modeling, neuroprotection and in emotional responding. The spontaneous mutation of the fat/fat mouse (Coleman & Eicher. 1990) is in fact located in the near proximity of the CPE locus on chromosome 8, hence the name $CPE^{fat/fat}$. Later, a KO mouse lacking both exons 4 and 5 of the CPE gene was created in order to elucidate that the genetic traits of the $CPE^{fat/fat}$ –mouse were a consequence of a non-functional CPE gene (Cawley*, et al.* 2004).

The CPE deficiency manifests as severe obesity in all backgrounds. Although, CPE-KO mice are born small as compared to their wild-type littermates, by week eight they are already significantly heavier (Cawley*, et al.* 2004, Leiter*, et al.* 1999). Interestingly, the mice continue to gain weight up to one year of age when both males and females can be as large as two or even three times the size of heterozygous or wild type littermates. Weight gain is dependent on the increase in fat mass alone and contributes to approximately 50% of body weight in CPE-KO of both genders, although female mice are slightly leaner. The substantial increase in weight has been attributed to hyperphagia in the adult mice; these can reach up to 70-80 grams of weight (Leiter*, et al.* 1999, Yuan*, et al.* 2004). The CPE-deficient animals also show a decreased metabolic rate and reduced spontaneous locomotor activity (Leiter*, et al.* 1999). The CPE-deficient mice develop diabetes with a slight variation in the progression depending on background (Cawley*, et al.* 2004, Leiter*, et al.* 1999, Naggert*, et al.* 1995).

The CPE-KO females develop both severe hyperglycemia, glucose intolerance and later also insulin-resistance whereas the male mice are less severely affected (Cawley*, et al.* 2004). The $CPE^{fat/fat}$ females do not develop the hyperglycemia encountered in the male mice (Leiter*, et al.* 1999). One interesting feature is that proinsulin levels are exceptionally high in both models, which is due to a secretory and processing defect in the β-islets of the pancreas (Irminger*, et al.* 1997, Leiter*, et al.* 1999).

2.4.2.3 MC3R-KO

The mutant mice which are deficient in functional MC3Rs present a unique metabolic phenotype. At first glance, the obesity observed in MC3R-KO animals fed on a standard diet is only modest, with no apparent differences in total body weight compared to wild-type animals (Butler*, et al.* 2000, Chen*, et al.* 2000) and with no published evidence for hyperphagia in the backgrounds in mice consuming either in normal or high fat diets (Butler. 2006). Although the contribution of the MC3R in metabolic regulation has been unclear, recent data obtained using the MC3R-KO mice has indicated a critical role of MC3R in the adaptation to food intake and in the maintenance of glucose homeostasis during periods of restricted feeding (Sutton*, et al.* 2010). Furthermore, there is also a report that the MC3R displayed significant importance in salt-sensitive hypertension, as functional MC3R are important mediators of salt-induced natriuresis (Humphreys*, et al.* 2011).

When consuming a standard diet, the MC3R-KO mice are indistinguishable from WT and heterozygous animals. However, if the animals are weaned to a low fat chow and then presented with a high fat chow diet for six weeks they exhibit a modest weight gain (Butler*, et al.* 2000). Interestingly, the acquired weight is attributable entirely to an increase in fat mass (40%) and a modest decrease in fat free mass when fed regular chow (Butler*, et al.* 2000, Butler. 2006). Although the increase in adiposity might be dependent on a decreased spontaneous physical activity measured only during nighttime in wheel cages or using the photobeam system, it is more probably explained by an overall increase in obesity (Butler*, et al.* 2000, Butler. 2006, Thorburn & Proietto. 2000). More significantly, the increase in adiposity has been explained as being due to a disproportion in substrate utilization. In fact, the respiratory exchange ratio (RER) of MC3R-KO on the B6 background is significantly increased reflecting a difference of 12-14% in substrate utilization (Butler*, et al.* 2000, Butler. 2006, Elia & Livesey. 1988). Although there is no difference in the carbohydrate balance, there is an estimated 7 kJ/day imbalance in fat intake and fat oxidation also seen in MC3R-KO female mice (Butler. 2006). A sexual dimorphism has been observed since only the female MC3R-KO mice are prone to gain weight on a chow with a modest fat content (25% kJ/fat) and have a increased weight gain even in comparison to their male counterparts fed a high fat diet (60%kJ/fat) (Chen*, et al.* 2000). Recent data has shown that MC3Rs' are important in the adaptation to metabolic conditions. If feeding is restricted only to the daytime, the MC3R-KO mice are unable to efficiently produce and use glucose as reflected in decreased activity of gluconeogenic genes and increased lipogenic enzymes in the liver (Sutton*, et al.* 2010). In fact, the increased levels of lipogenic enzymes during restricted feeding suggest that MC3R-KO mice might convert carbohydrates into fatty acids and further into ketone bodies in the liver pointing to an important role of MC3R in regulating the rhythmicity of liver metabolism (Sutton*, et al.* 2010). Furthermore, the MC3R deficient mice show significantly reduced activity and wakefulness around food presentation and decreased expression in the genes involved in the regulation of rhythmic clock genes (Sutton*, et al.* 2008). Recent data has shown that MC3R-deficient mice have decreased lipolysis in WAT and decreased central mRNA levels of AGRP and NPY during fast and impaired fasting induced re-feeding (Renquist*, et al.* 2012). In addition to the regulation of excess sodium excretion and to the maintenance of normal blood pressure under high salt conditions,

the MC3R is also involved in regulating mechanisms governing glucose homeostasis under exceptional conditions such as in salt-induced hypertension (Humphreys, *et al.* 2011). The MC3R-KO mouse exhibit mild insulin resistance on a HFD, however, they develop fasting hyperglycemia and hyperinsulemia associated with hypertension if consuming high sodium diets (Butler. 2006, Chen, *et al.* 2000, Ni & Humphreys. 2008). The abnormal glucose homeostasis and insulin resistance seen in the MC3R-KO mice has been assumed to involve increased sympathetic outflow and a decreased blood flow to skeletal muscle (Humphreys, *et al.* 2011). Interestingly, the phenotype of MC3R-deficient mice closely resembles the symptoms associated with Cushing's syndrome as also corticosterone levels are elevated in this mouse model (Renquist, *et al.* 2012).

2.4.2.4 MC4R-KO

The development of the MC4R-KO mice represented a turning point in melanocortin research. These mice displayed many of the same characteristics as encountered in the leptin signaling deficient mouse models (Lep^{ob}/Lep^{ob} and $Lepr^{db}/Lepr^{db}$) and the agouti yellow mouse (A^{Y}/a). Similarly to the $Lepr^{db}/Lepr^{db}$ mouse lacking functional leptin receptors, the MC4R-KO mice were unresponsive to the effects of both peripheral and central administrations of leptin and thus they demonstrated the importance of melanocortin receptors as key mediators of leptin signaling in the central nervous system (Marsh, *et al.* 1999). The MC4R deficient mice are grossly obese and hyperphagic with a significantly increased lean mass and nose to anus length when fed energy rich diets. Furthermore, both insulin and leptin levels are increased and glucose levels are elevated only in male homozygous mice, whereas levels remained unchanged in female mice (Huszar, *et al.* 1997).

Although the MC4R-KO is maintained on different backgrounds, the phenotype of these is almost identical. On the standard rodent chow, hyperphagia is observed in the homozygous MC4R-KO mice as early as 3-5 weeks of age. Both lean mass (20%) and longitudinal length (10-12%) are increased, which is also observed in humans carrying MC4R mutations (Albarado, *et al.* 2004, Coll, *et al.* 2004, Hinney, *et al.* 2013, MacKenzie. 2006). The introduction of high fat or more palatable diets markedly augments hyperphagia in MC4R-KO mice similarly to the agouti yellow A^{Y}/a mice (Butler, *et al.* 2000, Xu, *et al.* 2003). The sensitivity of both the A^{Y}/a and the MC4R-KO to palatable or high fat conteining diets has been linked to the centrally expressed brain-derived neurotrophic factor (BDNF), which is decreased in the ventromedial hypothalamus (VMH) in both models and in food deprived mice (Xu, *et al.* 2003). The effect of BDNF is particularly noticeable in the consumption of high fat diets as administration of BDNF to A^{Y}/a mice reduces food consumption only when the animals are fed a high fat diet (Xu, *et al.* 2003). Peripheral or central administration of the non-selective melanocortin agonists does not reduce food intake in the MC4R-KO mice suggesting that the MC4R is the main regulator of feeding rather than the MC3R (Chen, *et al.* 2000, Kumar, *et al.* 2009, Marsh, *et al.* 1999). Interestingly, a reduction in food intake can be observed if young lean or pair-fed MC4R-KO mice are treated with leptin pointing to the involvement of alternative routes for leptin signaling besides the MC4R (Butler. 2006, Marsh, *et al.* 1999). The phenotype of MC4R-KO mice on the B6 background, which

is a known model used for the development of diet-induced obesity in mice, reveals a sexual dimorphism in hyperphagia. Female MC4R-KO on the B6 background are more sensitive towards hyperphagia when fed a high fat diet and they also display a higher caloric intake than either wild-type or Lep^{ob}/Lep^{ob} mice (Albarado, *et al.* 2004). On the other hand male mice on the B6 background are not hyperphagic when presented low fat chow (Fan, *et al.* 2004). MC4R-KO mice on the B6 background are also insensitive to the administration of cholecystokinin (CCK), which suggests that functional MC4R are required for the effects of the gut derived CCK to mediate satiety (Butler. 2006, Fan, *et al.* 2004). Pair-feeding MC4R-KO mice according to the food consumed by wild-type littermates reduces weight gain and adiposity, however, it does not completely prevent it (Marsh, *et al.* 1999, Ste Marie, *et al.* 2000). The MC4R-KO mice display reduced energy expenditure due to a reduction in the sympathetic activity (Rahmouni, *et al.* 2003, Sohn, *et al.* 2013) and physical activity (Butler. 2006, Chen, *et al.* 2000, Ste Marie, *et al.* 2000). Diet induced thermogenesis (DIT) normally associated with an increase of 10-20% in oxygen consumption is not present in MC4R deficient mice, evidence of a maladaptation to dietary changes and an increase in feeding efficiency (Butler. 2006). Furthermore, the reduced fatty acid oxidation together with a decreased DIT in the MC4R-KO mice is indicative of an increase in the respiratory exchange ratio (RER) (Albarado, *et al.* 2004). Indeed, higher RER values have been observed in both MC4R-KO males on standard low fat chow and in females on a purified high fat diet on the B6 background (Albarado, *et al.* 2004, Butler. 2006).

2.4.2.5 POMC-KO

The POMC-KO mouse was created by deletion and replacement of exon 3 of the murine Pomc1 gene with a neomycin cassette (Yaswen, *et al.* 1999). The POMC-deficient mice share many of the same characteristics of mice with dysfunctional melanocortin signaling, although a distinct feature of the model is a total adrenal deficiency due to the lack of ACTH (Yaswen, *et al.* 1999).

The POMC-KO mice are hyperphagic, have an increased weight gain both when fed a normal or a high fat diet and have increased longitudinal growth similarly to MC4R-KO and A^{Y}/a mice (Butler, *et al.* 2000, Yaswen, *et al.* 1999). The weight of POMC-KO mice is increased after weaning and continues to accumulate until the age of four months, when they can be twice the weight of wild-type animals (Yaswen, *et al.* 1999). Leptin levels remain high and glucose and insulin levels remain normal (Yaswen, *et al.* 1999). POMC-KO animals show a maladaptation to energy rich diets because of a higher caloric intake and decreased energy expenditure, which exaggerates their obesity in comparison to wild-type littermates (Yaswen, *et al.* 1999). In line with the red hair color presents in human subjects with POMC deficiency, the POMC-KO mice have a slightly yellow coat color, which becomes more prominent in older animals (Yaswen, *et al.* 1999). Similarly to MC5R-KO mice, the POMC-KO mice have a decreased ability to shed water from their fur and are therefore unable to normally regulate their body temperature (Chen, *et al.* 1997, Yaswen, *et al.* 1999). The adrenal insufficiency affects both corticosterone and aldosterone concentrations which fall below detectable level (Yaswen, *et al.* 1999). Plasma catecholamine levels are less affected since only adrenaline levels show a small decrease, and dopamine levels may show a marginal increase due to a compensatory effect (Yaswen, *et al.* 1999). The deficiencies of POMC also lead to

a severe underdevelopment of adrenal glands although these are not macroscopically detectable (Yaswen, *et al.* 1999).

2.4.3 Elevation of melanocortin tone

Increasing the melanocortin tone in transgenic animals has been implemented in only a handful of studies. Ubiquitous overexpression of α- and γ_3-MSH in both lean and obese mice has resulted in a weight reduction without changes in food intake. However, the results show gender differences between the different backgrounds (Savontaus, *et al.* 2004). Inactivation of the α-MSH degrading enzyme, PRCP, produces a lean phenotype with a shorter nose-to-anus length and resistance to high-fat diet (HFD)-induced obesity (Wallingford, *et al.* 2009).

2.4.3.1 MSH-OE

The MSH-OE mouse has been produced in three different genetic backgrounds; lean (C57BL/6J.129-A^{wJ} db^{3J}), in db^{3J}/ db^{3J} and in A^{Y}/a mice. Although there were some gender differences in all backgrounds, the transgene overexpression caused significant reductions in weight development in mice in the obese $Lepr^{db}$/$Lepr^{db}$ background (Savontaus, *et al.* 2004). In addition, transgenic mice on the C57BL/6J.129-A^{wJ} db^{3J} background have attenuated weight gain in the absence of feeding effects and display higher energy consumtion and nutrient partitioning when feeding on a high fat diet (Lee, *et al.* 2007).

The MSH-OE mouse was created by insertion of a gene segment including both α- and γ_3-MSH under the general CMV-promoter into fertilized C57BL/6xCBA F2 oocytes. The founders were then mated with agouti-colored mice from a B6 strain (Chua, *et al.* 2002, Kowalski, *et al.* 2001) in order to facilitate the visualization and selection of transgene expression. The selected transgenic mice were mated with both db^{3J}/ db^{3J} and A^{Y}/a mice (Savontaus, *et al.* 2004). Transgene expression was detected centrally in the hypothalamus, brain stem and cerebellum, whereas peripheral expression was detected in the adrenal glands, skin, muscle, liver, kidney, heart, stomach and intestine. The levels of α- and γ_3-MSH in brain areas important for metabolic regulation were increased 1.5- to 2-fold in the mediobasal hypothalamus (MBH) and by 2- to 4-fold in the brain stem. Lean MSH-OE mouse are readily distinguishable from the agouti-colored WT mice by their black coat-color, which is a result of a stimulatory effect of melanocortins on the melanin production in the skin. The weight gain is significantly reduced in both male and female mice on the db^{3J}/ db^{3J}-background, however, adiposity was only decreased in male mice. There was no difference in nose-to-anus length (NAL), food intake or in glucose metabolism in these mice. Adiposity and food intake were similar, however, there were significant differences in glucose metabolism. Some gender-related differences were observed in the lean mice and on the A^{y}-background. Weight gain was significantly reduced in obese lean male mice starting from 7-weeks of age, whereas this effect is only apparent in female mice on the A^{y}-background starting from the same age. Only the lean male mice displayed shorter NAL and exhibited reduced adiposity in comparison to

control. Conversely, only female mice on the A^y-background were shorter at three months of age. Both fed and fasted plasma insulin levels were reduced in both male and female lean mice, however, there was no difference in plasma glucose levels. However, the fasted and stimulated insulin levels were significantly decreased only in female mice on the A^y-background. When the effects of dietary fat content was analysed in transgenic mice on the C57BL/6J.129-A^{wJ} db^{3J} background, they displayed higher oxygen consumption and respitatory quotient only on a high fat diet (Lee*, et al.* 2007). These findings were consistent with the displayed modest reduction in weight gain in the absence of a feeding effect (Lee*, et al.* 2007). In addition, the HFD attenuated the increase in corticosterone levels in the transgenic mice (Lee*, et al.* 2007). The hemodynamic characteristics of lean MSH-OE mice have been evaluated revealing an improved baroreflex control of heart rate (Rinne*, et al.* 2008, Rinne*, et al.* 2013).

The effects of central overexpression of the complete precursor of MSH peptides have previously been studied using POMC-adeno associated virus (POMC-AAV) in rats. POMC-overexpression in the ARC of the hypothalamus has been associated with decreased hyperphagia in both leptin deficient and in aged obese rats (Li*, et al.* 2003, Li*, et al.* 2005), but failed to reduce the weight gain if animals were fed a high fat diet (Zhang*, et al.* 2010). In addition, a parallel study showed that the treatment could also increase weight gain and total caloric intake if treated animals first received standard rodent chow, which was later changed to a high-fat diet (Li*, et al.* 2007). The effects of POMC-overexpression in the NTS of the brain stem and the VTA in the tegmentum have also shown to reduce weight gain in diet-induced obesity (Andino*, et al.* 2011, Zhang*, et al.* 2010).

2.4.3.2 PRCP-KO

Extracellular α-MSH is regulated by a specific degrading enzyme, PRCP, which degrades the active form of α-MSH. The PRCP-KO mouse lacks functional PRCP and has almost 60% higher hypothalamic levels of the active α-MSH_{1-13} (as opposed to the inactive α-MSH_{1-12}) (Wallingford*, et al.* 2009). The mice lacking functional PRCP are significantly leaner and have decreased food intake, adiposity and NAL length (Wallingford*, et al.* 2009). Expression analysis of PRCP showed abundant localization centrally in the cortex, brain stem, hippocampus and the hypothalamus. High levels of PRCP were found in the lateral-perifornical area, *zona incerta* and the dorsomedial nucleus of the hypothalamus, whereas in the ARC, the expression was significantly lower (Jeong & Diano. 2014). PRCP was found mostly opposite to or in the close proximity of neuronal POMC axon terminals, suggesting that PRCP is responsible for inactivating α-MSH at the release sites (Wallingford*, et al.* 2009). Obesity is also attenuated in PCRP-KO mice when the animals were exposed to a high fat diet (45% fat). The observed decrease in weight was significant starting from the first week and lasten to the end of the 18-week experiment. It is also kown that PRCP also regulates the thyroid axis as PRCP-deficient mice have higher levels of thyrotropin-releasing hormone (TRH) levels and administration of triiodothyronine (T_3) can normalize the abnormal TRH levels (Jeong*, et al.* 2012).

2.5 THE CNS AS A TARGET FOR PEPTIDE AND GENE DELIVERY

The CNS is regulated by a multitude of different factors. The general conductors of the CNS are the major neurological circuits composed of neurotransmitters and a multitude of different neuropeptides. A considerable amount of effort has been expended in attempts to understand the way the CNS is regulated, however, much still remains unknown. Electrophysiological measurements are conducted on *ex vivo* tissue slices in order to determine neuronal connections. One way to study the different physiological functions of neuropeptides has been to deliver them or their analogues to the anatomically distinct areas or nuclei of the CNS in order to clarify site-specific effects or into the ventricular spaces in order to examine the general central effect of neuropeptides.

The function of ventricles in the CNS is to sustain buoyancy, protection, and chemical stability. The ventricles are filled with cerebrospinal fluid (CSF), which is produced by the choroid plexus and epidymal cells. Many neuropeptides are found in CSF and the ventricular space can therefore be considered as one of the natural delivery pathways of neuropeptides. Although the ventricular space cavity connects the CNS and the spinal chord, it can also be divided anatomically to distinct parts. The two lateral ventricles occupy the cerebral hemisphere and a third ventricle is connected to the lateral ventricles by a passage (*interventricular foramina*). The fourth ventricle is connected to the third ventricle by a narrow passage (cerebral aqueduct) and by the preceding central canal that continues down to the spinal chord. Additionally, CSF fluid passes to the subarachnoid space via openings in the fourth ventricle. The choroid plexus is a bundle of small capillaries surrounding epidymal cells, which forms the blood-brain barrier (BBB) by allowing only the passage of blood plasma into the CSF. Moreover, the BBB is considered to be semipermeable particularly at the ARC of the hypothalamus and where it permits the controlled, selective transport of various nutrients and energy signals such as leptin (Banks. 2006). CSF circulates from the lateral to the third ventricle continuing to the fourth ventricle and further to the spinal chord. In the arachnoid space, CSF returns to the blood through villi located in the dural sinuses of the meninges (Kandel*, et al.* 2000).

The anatomy of the CNS has been outlined and each divided into functionally distinct parts. Previously, the specific nuclei and existing neuronal projections have been characterized using mechanical disruption and direct delivery of neuropeptides and analogues, whereas the techniques used today involve knocking out distinct populations of neurons and receptors or by genetic rescue or overexpression of neuropeptides or their receptors in specific loci using gene delivery vectors. The information gathered in these studies together with illustrative histological data have been combined into anatomical maps. The specific locations of the CNS in these maps are defined by the anatomy of the skull bone, which is used in the stereotaxic delivery of neuropeptides and genes.

Stereotaxis is used in single or continuous delivery to the CNS. With both approaches, the anatomy of the skull bone is used in order to target specific areas or ventricles of the CNS. The parietal bone structures of the skull are divided into several different anatomical parts by a connective tissue joints (sutures). The meeting points of the sagittal and the coronal sutures are termed bregma (and additionally an interaural line in rodents), which is defined in coronal brain anatomy as the middle or zero point. This bregma-related line

is by definition invariable between vertebrates of the same genetic strain and is therefore useful in defining the anatomy of the CNS.

2.6 GENE DELIVERY VECTORS

Gene delivery is useful in the study of the acute and long-term effects of genes *in vitro* and *in vivo* and in the targeting and destruction of unwanted cells, such as cancer cells (Vile & Russell. 1994). Viral gene delivery vectors that produce stable over-expression have been proven to be effective in the characterization of neuronal networks and for analyzing the physiological effects of therapeutics and endogenous peptides in different anatomical locations of the CNS and for treating human diseases (Ridet & Privat. 1995). A gene delivery vector artificially carries foreign genetic material in the form of DNA or RNA into a cell, where it can be either be integrated into the host genome allowing long-term expression or transiently expressed. Viral gene delivery vectors exploit the specialized molecular mechanisms of naturally occurring viruses to efficiently transport their target genomes inside the cells that they infect. The subsequent infection and delivery of the genetic material is termed transduction and the infected cells are defined as having been transduced. Viral vectors can be divided into integrating or non-integrating classes. Furthermore, the non-integrating viruses may also replicate. The main classes of viral vectors are different retroviruses such as the integrating and non-replicating lentiviruses, the non-integrating and replicating adenoviruses and integrating adeno-associated viruses. Lentiviruses use the genes of the human immunodeficiency virus (HIV) to integrate to the genome of many types of cells (Trono. 2000). The lentiviral vectors have been rendered non-replicative by removing the genes responsible for the production of new viral particles from the vectors themselves. The target genes of lentiviruses integrate into the host genome by using retroviral reverse transcriptase and recombination processes. This molecular event is random and previously it has resulted in the activation of oncogenes in human trials using lentiviruses. Moreover, non-integrating adenoviruses are used to eliminate specific cells (such as cancer cells), since infection by adenoviruses results in the production of new viral particles and ultimately results in the bursting and destruction of the infected cells (Barry*, et al.* 2012). The characteristics of adenoviruses can be modified and targeted to specific cells by changing the characteristics of their core proteins. There are several different strains of adenoviruses and these can be combined and redesigned in order to match the specific target cells. Adeno-associated viruses (AAV) are viruses that resemble adenoviruses, however, they function more like retroviruses. The AAV does not evoke an immunological response and in that respect it differs from the adenoviruses and there are no known adverse effects in terms of integration of genes into the host genome, which is one reason why they have proven useful in human gene therapy (Grieger & Samulski. 2012).

3. AIMS OF THE STUDY

The current study is an investigation of the central effects of POMC-derived melanocortin peptide overexpression in obesity. There is compelling evidence indicating that the melanocortin system is essential for the regulation of energy metabolism. α-MSH decreases obesity effects, but elevates sympathetic nervous system activity in the short term and the specific role of γ-MSH is still unclear. Transgenic mice overexpressing α- and γ-MSH show attenuated weight gain, however, no conclusion about the specific effects of each MSH peptides or the target area could be made. In addition, rats with targeted central overexpression of the propeptide, POMC show similar effects. The main objectives of this study were to determine the site-specific long-term effects of α-MSH or γ-MSH overexpression only in key areas of metabolic regulation in the CNS. The specific tasks investigated in this thesis were:

1. To develop lentiviral gene delivery vectors overexpressing γ-and α-MSH and to demonstrate that the gene delivery vectors integrate and produce stable expression and biologically active peptides *in vitro* and *in vivo*

2. To determine the long-term anti-obesity effects of α-MSH-OE in the ARC of the hypothalamus in diet induced obese mice.

3. To determine the anti-obesity effects of long-term α-MSH-OE in the NTS of the brain stem in diet induced obese mice and to investigate if the treatment would cause any elevation in SNS tone.

4. To elucidate the effects of γ-MSH-OE in hypothalamus in the regulation of feeding and body metabolism in mice feeding on different diets and in the treatment of diet induced obesity.

4. MATERIALS AND METHODS

4.1 LENTIVIRUSES

4.1.1 DNA constructs

The α-MSH gene construct used the signal- and sorting peptides of the mouse POMC pro-peptide followed by the α-MSH sequence starting with the endogenous endoprotease cleavage site and ending in the C-terminal glycine allowing for amidation and an inserted stop codon (Schafer*, et al.* 1992) (Figure 3). The construct was generated by amplifying two separate fragments from the MSH-L construct described earlier (Savontaus*, et al.* 2004) and included a part of the 5' untranslated region, the signal sequence, the sorting sequence, the joining peptide and α-MSH. The codon for the C-terminal glycine is immediately followed by a stop codon. The first fragment encodes the signal sequence and the sorting sequence and second part of the joining peptide sequence and α-MSH (Figure 3). The fragments are amplified with primer-pairs rMSH1 and rMSH1-Sac, and rMSH3-Sac and rMSH2 (Table 4). Both amplified products include a Sac II restriction site aiding in the ligation of fragments to produce the DNA construct, which is identical to both rat and mouse α-MSH (Figure 3).

Table 4. Primers used for the generation of constructs. Restriction sites (underlined) Sac II used for the generation and ligation of α-MSH construct and Mlu I and Sal I for γ-MSH. Mutations (bold) were used to generate stop codon in γ-MSH template.

α-MSH			
Template DNA	Origin: MSH-L plasmid (Savontaus E. et al 2004)		Restriction site
Primers	rMSH1	5'-GTC CTC AGA AAG CTT CCT TTC CGC-3	-
	rMSH1-Sac	5'-CGT TGC CTG GAA ACC GCG GCG TCT C-3'	Sac II
Primers	rMSH3-Sac	5'-AGC CAA GTC CGC GGA GGG CAA GC-3'	Sac II
	rMSH2	5'-CAC AGG GCG AAG CTT CTA GCC CAC CGG-3'	-
γ-MSH			
Template cDNA	Origin: Mouse hypothalamic mRNA		Restriction site
Primers	gMSH for-1	5'-TGC CTT TAC GCGTCA GGG GT-3'	MluI
	gMSH rev-2	5'-**cta t**GT CGA CTC TTC CTC CGC ACG C**TA** CTG C-3'	SalI

Mouse hypothalamic cDNA was used as a template to create the γ-MSH construct. The construct includes the signal- and sorting-sequences of POMC and the endogenous cleavage sites for appropriate intracellular processing (Figure 3). The γ_3-MSH sequence was amplified using primers (forward and reverse, respectively) gMSH for-1 and gMSH rev-2 (Table 4). The forward primer includes an MluI restriction (underlined) site achieved by changing two bases (bold) and the reverse primer had a SalI restriction site and extra codons for aiding restriction in the 5´-end (underlined and bold) and a stop codon (bold) in 3'-end (Table 4).

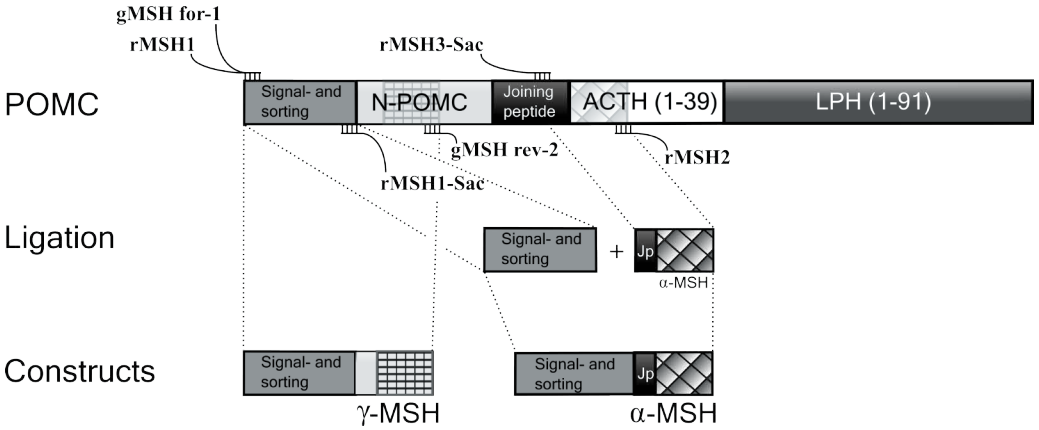

Figure 3. Generation of MSH constructs from POMC sequence. α-MSH sequence was amplified from a DNA plasmid including the N-terminal POMC and ATCH sequences together with the signal and sorting sequences, which were ligated together. The γ-MSH sequence was amplified from mouse hypothalamic mRNA.

4.1.2 Lentiviral constructs

In the production of the second-generation lentiviruses, the α-MSH and γ-MSH constructs were ligated to the lentiviral vector plasmid pWPI-EGFP (LVi-EGFP) to produce pWPI-α-MSH-EGFP and pWPI-γ-MSH-EGFP plasmids, respectively (Figure 4). In the bicistronic pWPI vector, the gene construct precedes the Encephalomyocarditis virus internal ribosomal entry site (EMCV IRES) and EGFP, which allows for the simultaneous expression of the transgene and the reporter gene (Jang*, et al.* 1988, Pelletier & Sonenberg. 1988). The pWPI vector plasmids use the EF1-α promoter, which is a general promoter (Lee*, et al.* 1992). In addition, the plasmids also include the WPRE (woodchuck hepatitis post-transcriptional regulatory element), which has been shown to enhance the expression of genes in different cell lines (Klein*, et al.* 2006).

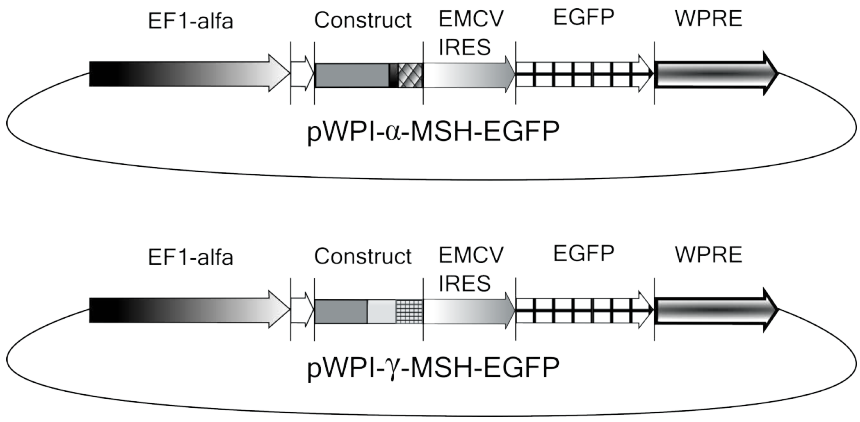

Figure 4. The α-MSH and γ-MSH gene constructs were subcloned into pWPI plasmid to produce pWPI-α-MSH-EGFP and pWPI-γ-MSH-EGFP.

4.1.3 Generation of lentiviruses

Lentiviruses were produced in human embryonic kidney cells (HEK293T) that were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (LVi-α-MSH-EGFP) or advanced DMEM (LVi-γ-MSH-EGFP) with 2% fetal bovine serum supplemented with 2 mM L-glutamine (1.5 g/l sodium bicarbonate and 4.5 g/l glucose), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich Co.,St. Louis, MO, USA) and with the LVi-γ-MSH-EGFP also 0.03 mM cholesterol (Sigma-Aldrich Co.,St. Louis, MO, USA). Addition of cholesterol to the growth medium of transfected cells aids the budding of cells (Mitta*, et al.* 2005). Normal cell culture conditions in cell culture incubators (37° C and 5% CO_2) were used for the production of all viruses. First, 2.5 million low passage HEK293T cells (passage < 7) were plated on 10 cm cell culture plates and grown until 50 % confluency. Viral particles were produced by calcium phosphate transient co-transfection of the HEK293T cells with the lentiviral vector plasmid (pWPI-α-MSH-EGFP or pWPI-γ-MSH-EGFP and pWPI-EGFP) and two helper plasmids: pCMV-pR8.91 (a packaging plasmid) and pMD2G (an VSV-G envelope plasmid) (Figure 5). The viruses were collected and filtered (0.45 µm) and then concentrated by ultracentrifugation in 50000 x G (LVi-α-MSH-EGFP) or 20000 x G (LVi-γ-MSH-EGFP) in 33 ml conical ultracentrifuge tubes (Beckman Coulter, Inc., CA, USA). A lower concentration speed has been shown to improve titers (al Yacoub*, et al.* 2007). Viral pellets were resuspended in sterile PBS solution (Sigma-Aldrich Co.,St. Louis, MO, USA).

4.1.4 Analysis of lentiviruses

Titers (transducing units/ml (TU/ml)) were determined by fluorescence based flow cytometry (Dittrich & Gohde. 1969, Göhde. 1968) (Figure 5). First, thawed viral suspension was added in a 1:5 dilution series to a known number of cells on a 24-well plate. Since the viruses induce EGFP expression, the relative amounts of EGFP-positive (EGFP+) cells were assessed in each well by fluorescence based flow cytometry. Titers were calculated from wells with dilution series showing 10-20 % EGFP+ cells by the equation: **TU = N x (EGFP+) x DF** (N = cells at infection, EGFP+ = percent EGFP+ cell in well, DF = dilution factor):

The ability of the LVi-α-MSH-EGFP virus to produce biologically active α-MSH was tested in a modified melanin in vitro assay by measuring melanin production in B16F10 melanocytes that express MC1-R (Siegrist & Eberle. 1986). The lentivirus vectors were analyzed by first plating 5000 B16F10 on 96-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine (1.5 g/l sodium bicarbonate and 4.5 g/l glucose), penicillin (100 U/ml), and streptomycin (100 µg/ml). On the following day, the cells were infected with a MOI 100 (Multiplication of infectivity x 100) of the LVi-α-MSH-EGFP or LVi-EGFP virus and compared with untreated control cells. The concentration of melanin in the growth medium was determined by absorbance measurement at 405 nm 72 hours after the infection (Figure 5).

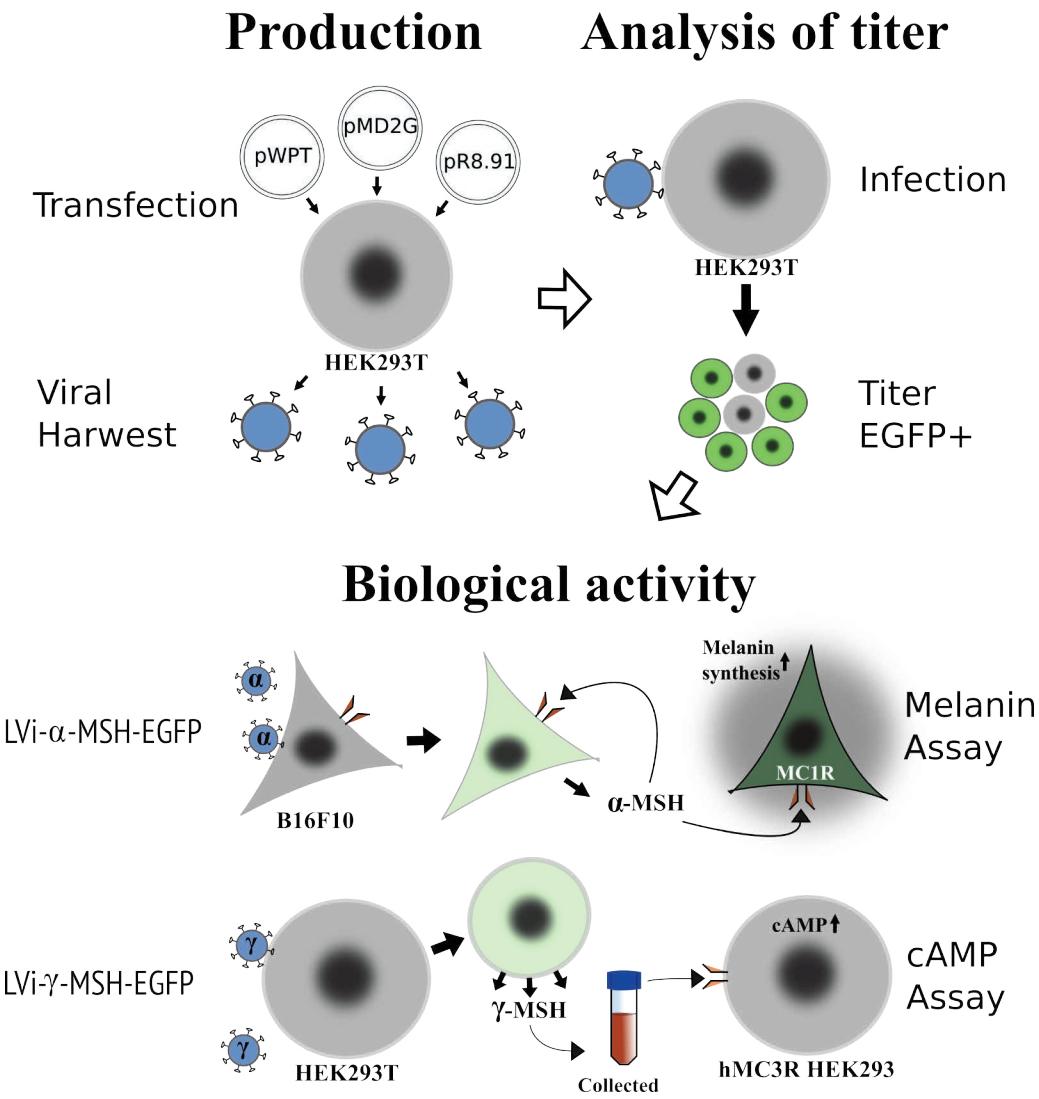

Figure 5. Schematic presentation of the LVi-EGFP, LVi-α-MSH-EGFP and LVi-γ-MSH-EGFP lentivirus, titer determination and biological activity analysis protocol.

The ability of the LVi-γ-MSH-EGFP virus to produce biologically active γ-MSH was tested by stimulation of a HEK293 cell line expressing human MC3R (hMC3R-cells) (Grieco*, et al.* 2002) and by measuring the elevation in intracellular cAMP levels using a commercially available kit (Perkin Elmer) according to manufacturer's instructions. The main goal of this method was to infect a separate cell line (HEK293T) using the LVi-γ-MSH-EGFP in order to produce γ-MSH peptide in the cell growth medium, which was used to stimulate the hMC3R cell line in a cAMP assay (Figure 5). First, a HEK293T cell line was infected with a MOI 100 of the LVi-γ-MSH-EGFP or LVi-EGFP. After this, 100 000 infected and non-infected cells were plated on 24-well plates and grown until confluency. Subsequently, the growth mediums of the cells were collected, pooled and stored in -80 C until later use. For the cAMP measurements, 10000 hMC3R cells grown

in Minumum essential media (Gibco® MEM, Thermo Fisher Scientific Inc, MA, USA), 10% fetal bovine serum, 4 mM L-glutamine (1.5 g/l sodium bicarbonate and 4.5 g/l glucose), penicillin (100 U/ml), streptomycin (100 µg/ml) and 50 µM of G418 (Sigma-Aldrich, MI, USA) were added to the wells of a 96-well plate and incubated with an equal amount of growth medium from non-infected HEK 293T cells (control medium), different concentrations of synthetic γ-MSH peptide (Tocris Biosciences Cat. No. 4272, Bristol, United Kingdom) diluted in control medium or the growth medium from LVi-EGFP and LVi-γ-MSH-EGFP infected cells. The assay measured the amount of bound intracellular cAMP, which negatively correlated with unbound cAMP using Eu-labeled antibodies.

4.2 ANIMALS (I-III)

Two-month-old C57BL/6N male mice were obtained either from the local laboratory animal facilities (University of Turku) (I) or from Harlan Laboratories B.V. (Venray, The Netherlands) (I-III). The mice were kept in an animal room maintained at 21 ± 1°C with a fixed 12-h light/12-h dark cycle. Food and water were available *ad libitum*, except when experiments with pair-feeding were being conducted (I).

4.2.1 Anesthesia and analgesia

In the stereotactic operations for analgesia either ketamine (75mg/kg) (Pfizer Inc, NY, NY, USA) (I-II) or buprenorphine (0.1 mg/kg) (Temgesic® Schering-Plough, New Jersey, USA) (III) was administered intraperitoneally 20 min prior to the injections and the animals were anesthetized using isoflurane. In addition, intraperitoneal or subcutaneous buprenorphine was administered if animals showed any signs of pain (immobilization etc.). At the end of the experiments, the mice were sacrificed in deep terminal sodium-pentobarbital (60 mg/kg) Mebunat Vet® (Orion Co. Espoo, Finland) (I, II) or ketamine (Ketalar®, Pfizer Inc. New York, USA) plus medetomidine (Domitor®, Orion Co. Espoo, Finland) (III) anesthesia in combination with intracardiac puncture.

4.2.2 Ethical aspects

Animal care was in accordance with the guidelines of the International Council of Laboratory Animal Science (ICLAS) and with the European Community Council Directive 2010/63/EU, and all experimental procedures were approved by the national animal care and use committee. Experiments were designed in order to minimize the number of animals used.

4.2.3 Diets

Standard rodent chow (SDS, Essex, UK) was used unless stated otherwise. Special diets obtained from Research Diets (New Brunswick, NJ) were used to induce obesity and are listed in detail in Table 5. A high-fat Western type diet (D12451) was used in study I and in the pair-feeding study. A Western diet including a moderate level of fat (D05022804) was used in study II and normal Western diet (D12079B) was used in study III. Standard rodent chow was used mainly in study III.

Table 5. Information about the main components of the diets used in each study.

Study (I-III)	Standard		I		II		III	
Product	Chow		D12451		D05022804		D12079B	
gm%	g%	kcal%	g%	kcal%	g%	kcal%	g%	kcal%
Protein	21	17.5	24	20	18	17	20	17
Carbohydrate	90	75.1	41	35	62	58	50	43
Fat	3.7	7.4	24	45	12	25	21	40
Total		100		100		100		100
kcal/g	3.52		4.73		4.25		4.68	
Cholesterol	-		-		1.5		1.5	

4.2.4 Study design

In studies I-II, control groups were included for both the stereotaxic operation (non-treated control) and the lentivirus vector (saline) (Table 6). Since both saline and non-treated controls did not show significant differences in both studies I-II, they were excluded from the last experiment in order to reduce the numbers of animals needed.

Table 6. Information about the number of treated animals in addition to the number of animals left at the end and the number used for statistics in each study. In study I; One mouse in the LVi-EGFP died from operative trauma and five mice in the LVi-EGFP and three mice in the LVi-α-MSH-EGFP were euthanized before the end of the experiment due to other complications. Study I was divided into two separate experiments of which the other was a pair-feeding study where LVi-EGFP PF were fed similarly to matched pair in the LVi-α-MSH-EGFP group. Due to an unexplained cause in the pair-feeding study, there was an outbreak of dermatitis. Ultimately, two mice in LVi-α-MSH-EGFP and three mice in the LVi-EGFP PF groups had to be euthanized because of severe complications related to the dermatitis, although all treatment groups were severely compromised. In study II, one animal in the LVi-EGFP died from operative trauma and one LVi-α-MSH-EGFP treated animal suffered from a hematoma. In addition, one LVi-α-MSH-EGFP died after telemetric implantation. In study III, one animal in the LVi-EGFP group died from operative trauma and one animal in each treatment group died of other causes.

Experimental setups		**I**			
Treatment Group		**LVi-EGFP**	**LVi-α-MSH-EGFP**	**Saline**	**Non-treated**
Use of Animals	**Start**	**12**	**12**	**6**	**6**
	End	**7**	**9**	**6**	**6**
Animals included in statistics		**4**	**4**	**5**	**6**
I (pair-feeding)					
Treatment Group		**LVi-EGFP**	**LVi-α-MSH-EGFP**	**LVi-EGFP PF**	
Use of Animals	**Start**	**13**	**13**	**14**	
	End	**13**	**11**	**11**	
Animals included in statistics		**7**	**7**	**7**	
II					
Treatment Group		**LVi-EGFP**	**LVi-α-MSH-EGFP**	**Saline**	**Non-treated**
Use of Animals	**Start**	**16**	**16**	**6**	**6**
	End	**15**	**16**	**6**	**6**
Animals included in statistics		**10**	**9**	**5**	**6**
Telemetric Implantation		**4**	**5**	**-**	**-**
III					
Treatment Group		**LVi-EGFP**	**LVi-γ-MSH-EGFP**		
Use of Animals	**Start**	**20**	**20**		
	End	**18**	**19**		
Animals included in statistics		**17/15**	**16/13**		

4.3 EXPERIMENTAL PROTOCOLS

4.3.1 Stereotaxis (I-III)

Analgesia was administered 20 min prior to each operation intraperitoneally and the animals were anesthetized with isoflurane. Saline, $2.1*10^5$ TU (I-II) or $6*10^5$ TU (III) of LVi-EGFP, and LVi-α-MSH-EGFP (I-II) or LVi-γ-MSH-EGFP (III) in similar volume was delivered bilaterally either to the ARC (I, III) or NTS (II) by stereotaxis. Before each injection, the animals were carefully positioned to a mouse adaptor in a stereotaxic frame from Stoelting (Stoelting Co., Wood Dale, IL, USA). A Stoelting QSI injector was

used to deliver a total volume of 1 µl of the viral suspension at 0.1µl /min. The needle was withdrawn slowly 5 (I-II), 10 (I PF, III) after infusion.

The stereotaxic coordinates or the point of the injection according to the Bregma for the ARC were Anterior-Posterior (AP) 1.70 mm, Dorsal-Ventral (DV) -6.00 mm and Medial-Lateral (ML) ± 0.05 mm (I) and AP 1.70 mm, DV -5.90 mm and ML ± 0.13 mm at a 10° angle towards the midline (III) The stereotaxic coordinates or the point of the injection according to the Interaural structure for NTS injections were Anterior-Posterior (AP) - 3.50 mm, Dorsal-Ventral (DV) - 4.50 mm and Medial-Lateral (ML) ± 0.05 mm (II). Stereotaxic coordinated were obtained from Franklin & Paxinos. 1997 "The Mouse Brain: In Stereotaxic Coordinates"-atlas and refined using methylene blue test injections (Franklin & Paxinos. 1997).

4.3.2 Exclusion criteria (I-III)

At the end of first two studies, the coronal brain sections of both LVi-α-MSH-EGFP and LVi-EGFP injected animals were analyzed for EGFP expression to define the site of injection. Mice with detectable EGFP in the ARC inside the region of Bregma AP -1.45 to -2 mm according to Franklin and Paxinos at least unilaterally were included in the final results. EGFP expression in the ventricular space was an exclusion criterion, since this was considered as evidence of a ventricular administration or leakage of the viral suspension. In study I, four mice out of a total of seven LVi-EGFP injected and four out of nine LVi-α-MSH-EGFP injected mice showed EGFP in the ARC including one bilaterally (Table 6). In the pair-feeding experiment of study I, seven mice in each treatment group displayed EGFP in the ARC with two LVi-α-MSH-EGFP and one pair-fed LVi-EGFP treated mice bilaterally (Table 6). In study II, mice with injections that entered the fourth ventricle or that were located outside the NTS region of Interaural AP -3.20 to -3.80 mm (Franklin & Paxinos. 1997) were excluded from the final results (n = 5 in the LVi-α-MSH-EGFP and n = 3 in the LVi-EGFP group). Overall ten mice in the LVi-EGFP group and nine in the LVi-α-MSH-EGFP group were included in the final results (Table 6). Exclusion criteria in study III were based on unresponsiveness to diets and thus low weight gain in DIO sensitive mice consuming the Western diet for 13-weeks (one LVi-EGFP and three LVi-γ-MSH-EGFP) and weight loss resistant mice (two LVi-EGFP and three LVi-γ-MSH-EGFP) were excluded from experiment (Table 6).

4.3.3 Body weight and food intake assessments (I-III)

Body and food weights were measured biweekly (I, II) or weekly (I, III) in the first experiment, and daily food intake was calculated from the change in food weight divided by the number of days. In the pair-feeding experiment, the mice and food were weighed daily, and mice in the pair-fed LVi-EGFP treated group were given the amount of food consumed by a weight matched pair in the LVi-α-MSH-EGFP treated group during the corresponding experimental day. Food spillage was accounted for in all measurements by carefully checking the bedding (Tapvei aspen) for fallen red (I), blue (II), yellow (III) or brown-colored (chow) food crumbs and including the crumbs into the remaining food. In study III, fasting and re-feeding were also assessed. The experiments include measurements

of *ad libitum* feeding during the first two hours of the dark period 24 hours before the re-feeding analysis. Animals were fasted during the light period (12 hours) prior to re-feeding. Food was weighed manually two-, twelve- and 24-hours from the beginning of re-feeding.

4.3.4 Basal glucose and glucose tolerance test (I-III)

For basal blood glucose level and the glucose tolerance test (GTT), the mice were administered intraperitoneally with glucose (5% [wt/vol], 1 g/kg body weight) after a four-hour fast. Tail vein blood samples (5 μL) were withdrawn by incision at 0, 20, 40, 60, and 90 minutes from mice restrained shortly. Glucose concentration was measured with the Precision Xtra Glucose Monitoring Device (Abbott Diabetes Care, Abbott Park, IL).

4.3.5 Estimation of energy expenditure (II)

The assessment of Total energy expenditure (TEE_{bal}) was calculated according to the energy obtained from consumed food together with the relative gain or loss of energy from lean and fat mass change over time according to the following equation: $\mathbf{TEE_{bal}}$ = **EI - (Δsomatic fat energy + Δsomatic fat-free energy)**, where EI = total energy intake (kcal), Δsomatic fat energy = change in fat mass (g) x 9.0 kcal / g for negative change or 12.3 kcal/g for positive change, Δsomatic fat-free energy = change in lean mass (g) x 1.0 kcal / g for negative change or 2.2 kcal / g for positive change. The estimation technique has been validated and shows a high correlation with total energy expenditure as measured with indirect calorimetry (Ravussin*, et al.* 2013).

4.3.6 Hemodynamic parameters (II)

4.3.6.1 BLOOD PRESSURE BY TAIL-CUFF

Systolic blood pressure (sBP) and heart rate (HR) were measured in conscious restrained animals optically using the tail-cuff system (TSE System International Group, Bad Homburg, Germany). The sBP and HR were assessed on day three after an acclimation for two consecutive days. The mean of 6-10 measurements from each mouse was recorded.

4.3.6.2 BLOOD PRESSURE TELEMETRY AND PHARMACOLOGICAL TESTING

sBP and HR were measured in conscious, unrestrained mice using a radiotelemetry system (TA11PA-C10 and Dataquest software, Data Sciences International) as previously described (Rinne*, et al.* 2008, Rinne*, et al.* 2013). Baseline values for hemodynamic parameters during the first recovery week were recorded for three consecutive days and the results are given as 12 h means over non-active light and active dark periods. At the end, autonomic control of BP and HR was assessed pharmacologically using muscarinic blockade by atropine (2 mg/kg), β1-adrenergic blockade by metoprolol (4 mg/kg) and α_1-adrenergic blockade with prazosin (1 mg/kg) comparing to saline (10 mL/kg) as described earlier (Rinne*, et al.* 2013).

4.3.7 Body composition (II-III)

Body composition (fat tissue and lean tissue in grams) was assessed utilizing an EchoMRI-700 quantitative nuclear magnetic resonance whole body composition analyzer (Echo Medical Systems, Houston, TX). Awake mice were placed in the holder for a duration of 1-3 minutes in this measurement.

4.3.8 Metabolic cage and home cage assessments (II-III)

Mice were weighed and placed into metabolic cages (Harlan Laboratories Inc, Indianapolis, Ind, USA) allowing the collection of urine and feces for over a period of 24 hours. Water and food consumption was measured and the fecal stool and total urine were weighed and collected for further analysis. In the home cage water intake assessment, water bottles were first filled and weighed and then added to each cage carefully. The water intake was measured for 72-hours and 24-hour water intake was calculated as a three-day average of the total amount of water consumed by the animal.

4.3.9 Voluntary running wheel activity (III)

Running wheel activity was measured prior to and three weeks after the treatments and three weeks after the beginning of the Western diet. Low-Profile wireless running wheels for mice (Med Associates, Inc. Vermont, USA) were used to measure 24-hour running wheel activity in the home cage. Weight change and food intake during the 24-hour analysis were also measured.

4.3.10 Tissue collection and preparation (I-III)

At the end of experiments, the mice were sacrificed after a four-hour fast. Blood samples were collected with terminal intracardiac puncture under deep pentobarbital sodium (70 mg/kg) anesthesia (I) or by decapitation (I PF). A total of 500μL blood per mice was collected in serum separation tubes (CAPIJECT® T-MG) (Terumo Medical Corporation, Elkton, MD, USA).

Whole brain samples were collected, rinsed in ice-cold PBS solution (pH 7.4), dried and frozen with isopentane in O.C.T solution (Sakura Finetek, Torrance, CA, USA). Brain sections were collected with a Leica CM-3050S cryostat in 10 μm sections (Leica Microsystems GmbH, Wetzlar, Germany) and samples were stored isolated from air at -80°C (I-III).

In study I; subcutaneous, epididymal, mesenteric, and retroperitoneal white adipose tissue (WAT), and interscapular brown adipose tissue (BAT) depots were collected and weighed at sacrifice. In addition liver, heart and kidney were collected and weighed. In study II; mesenteric and retroperitoneal WAT and interscapular BAT depots, liver, heart and kidney were collected and weighed. In study III; a single lobe of intrascapular BAT and adrenal gland were collected, snap frozen and stored at -80 until analysis of mRNA.

4.4 BIOCHEMICAL ANALYSIS

4.4.1 Immunohistochemistry (I-II)

Immunohistochemistry (IHC) was performed using the avidin–biotin–peroxidase method. Briefly, samples were post-fixed in 4 % paraformaldehyde-PBS solution for 5 minutes and incubated for 20 minutes in 3% H_2O_2 in methanol in order to inactivate endogenous peroxidase. 10% normal horse serum in PBST 0.1 % solution was used to block Fc receptors. The α-MSH antibody (Phoenix Pharmaceuticals, Inc. catalogue number: H-043-01) was incubated at a concentration of 1/1000 overnight in 4°C in PBST 0.1 % solution. Subsequently, the sections were incubated with a biotinylated horse anti-rabbit IgG antibody (1/300) (Vector Laboratories, CA, USA) 1 h at room temperature in PBS solution. VectorElite ABC (Vector Laboratories, CA, USA) was used to amplify the signal and visualized using 3,3-diaminobenzidine (DAB). The tissue was then counter-stained using cresyl violet in order to visualize cell nuclei. The antibody used in IHC was specific to α-MSH as incubation of primary antibody solution and 100 mM of synthetic α-MSH (Tocris Bioscience, Bristol, United Kingdom Cat. No. 3013) abolished all immunoreactivity.

4.4.2 Image analysis and quantification (I-II)

Brain samples were examined with a Leica DM R microscope (Leica Microsystems GmbH, Wetzlar, Germany) under fluorescence filters or visible light fluorescent images were acquired from samples post-fixed in 4% PFA-PBS solution, dried and mounted with a fluorescent preserving hardening mounting medium with DAPI (Vector Laboratories Inc, Burlingame, CA, USA) using an Olympus DP70 camera and DP software (Olympus Co., Japan) (I) or a Zeiss Axioimager M1 and ZEN 2012 software (Carl Zeiss Microscopy LLC, New York, USA) (II). DAB stained sections were visualized using a BestScope BUC4-500C camera (BestScope International Limited, Beijing, China) and IScapture 2.6 software (Fuzhou Xintu Photonics Co., Fuijan, China)**.** The image series were then merged together using Windows Ice 1.3.3.0 software (Microsoft Co., USA). Pictures were analyzed using Adobe Photoshop 8.0 (Adobe Systems Inc., USA).

Coronal sections covering the ARC (I) or NTS (II) in 100 μm increments were immunostained for α-MSH and positive DAB staining was quantified manually (I) or by using automated DAB calculations (II). The ARC and NTS regions were cropped from the images according to Franklin and (Franklin & Paxinos. 1997). In study I, quantification was carried out by counting the number of neurons with detectable levels of α-MSH in the ARC region. The researcher was blinded to the treatment group, the mean of three counts per sample was used with an n of 6 in each group. In study II, cropped images were analyzed with ImageJ 1.47 software (Rasband. 1997-2012), brown DAB staining was isolated and the pixel count of specific DAB staining calculated automatically using the color_deconvolution/H&E DAB-program (Ruifrok*, et al.* 2003). Pixel counts were acquired from both hemispheres separately with four mice per group and from one hemisphere only in three additional mice in the LVi-α-MSH-EGFP group. The results include 3-8 samples per rostrocaudal segment in each group. Areas under the

curve (AUC) for the mean pixel count curves in Figure 9 were calculated and used to estimate the relative α-MSH levels in the whole NTS area.

4.4.3 Liver and serum lipids (I-III)

Liver lipid contents were isolated and purified with the Folch method (Folch*, et al.* 1957) (I). Serum and liver triglycerides were quantified with Free Glycerol Reagent (F6428) and Triglyceride Reagent (T2449; Sigma Diagnostics). The non-esterified free fatty acids (NEFA) were determined with NEFA-C Reagent set (Wako Chemicals GmbH, Neuss, Germany).

4.4.4 Cholesterol levels and non-esterified fatty acids (I-III)

Serum total cholesterol levels were measured with Cayman Cholesterol Assay Kit (Cayman Chemical Company, MI, USA) (I) and the levels of non-esterified free fatty acids (NEFA) were determined from serum with NEFA-C Reagent set (Wako Chemicals GmbH, Neuss, Germany) (I-III).

4.4.5 Serum insulin and glucose (I-II)

The serum insulin concentration was measured using Mercodia Ultrasensitive Mouse Insulin ELISA Kit (Mercodia AB, Uppsala, Sweden). Serum glucose levels were determined using Cayman Glucose Colorimetric Assay Kit (Cayman Chemical Company, MI, USA).

4.4.6 Urinary creatinine (II)

24-h urine samples were collected in metabolic cages and the specimens were stored at −80 °C until assayed with a creatinine assay (Cayman Chemical Company, Ann Arbor, MI) relying on the Jaffé reaction to estimate the creatinine concentration which was then multiplied with the 24-hour urine volume.

4.5 GENE EXPRESSION (II-III)

4.5.1 Quantitative polymerase chain reaction

The mRNA levels of uncoupling protein 1 (UCP1) in BAT and tyrosine hydroxylase from adrenal gland were analyzed using quantitative real-time PCR (qPCR). Briefly, the mRNA was first extracted and converted to cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA) according to the manufacturer's instructions on a GeneAmp PCR System 9600 (Perkin Elmer). BAT mRNA was isolated using Qiazol (Chomczynski & Sacchi. 2006) and adrenal gland mRNA using a RNeasy Micro Kit

according to the manufacturer's instructions (Qiagen N.V. Venlo, Netherlands). Target genes uncoupling protein 1 (UCP1) and tyrosine hydroxylase (TH) were quantitated with 7300 Real-Time PCR System (Applied Biosystems) relative to the housekeeping genes: Ribosomal protein s29 (Rps29) (II) or β-actin (Mouse ACTB) (III) using the SYBR-Green method (Kapa Biosystems, Woburn, MA). The primer pairs are presented in Table 7.

Table 7. Primer-pairs used in assessment of mRNA levels of genes. All results were correlated according to known and commonly used house-keeping genes.

Houskeeping genes:	
β-actin	Sequence
Upstream	**5'-GGC CTC TAC GAC TCA GTC CA-3**
Downstream	**5'-TAA GCC GGC TGA GAT CTT GT-3'**
Rps29	Sequence
Upstream	**5'-ATG GGT CAC CAG CAG CTC TA-3'**
Downstream	**5'-AGC CTA TGT CCT TCG CGT ACT-3'**
Target genes	
UCP1	Sequence
Upstream	**5'-ACT GCC ACA CCT CCA GTC ATT-3'**
Downstream	**5'-CTT TGC CTC ACT CAG GAT TGG-3'**
TH	Sequence
Upstream	**5'-CCC AAG GGC TTC AGA AGA G-3'**
Downstream	**5'-GGG CAT CCT CGA TGA GAC T-3**

4.6 STATISTICAL METHODS

Weight development, food intake, body composition, telemetric measurements over time, glucose tolerance test and voluntary running wheel activity were analyzed using two-way ANOVA. Whenever the interaction was significant, the treatment effects were compared at different time points with Bonferroni post-hoc analysis. The parametric unpaired t-test was used for comparing normally distributed single-parameters and Mann–Whitney test for parameters with small numerical nomenclature between LVi-α-MSH-EGFP or LVi-γ-MSH-EGFP and LVi-EGFP control treatments. Areas under the curves were calculated with the trapezoidal method. Statistical analyses were carried out with GraphPad Prism 6.0. Data are presented as means ± SEM. The results were considered statistically significant at $P < 0.05$.

5. RESULTS

5.1 LENTIVIRAL VECTORS

5.1.1 Biological activity of viruses

The ability of the LVi-α-MSH-EGFP to generate biologically active α-MSH was tested in B16F10 melanocyte cells, which are known to express MC1R. As a result of MC1-receptor activation, the LVi-α-MSH-EGFP infected melanocytes produced significantly more melanin than cells infected with the LVi-EGFP control virus and uninfected control cells indicating that the virally produced α-MSH is biologically active (Figure 6 A). Furthermore, the lentiviruses induced 71.7 ± 1.4 % infectivity in the B16F10 cells with a MOI100, which demonstrated the capability of the lentiviral vectors to induce viral transduction.

The ability of the LVi-γ-MSH-EGFP to produce biologically active peptides was analyzed by using the growth medium of HEK293T cells infected with the LVi-γ-MSH-EGFP. The cell growth medium of LVi-γ-MSH-EGFP infected HEK293T cells resulted in significantly smaller levels of bound cAMP as compared with cells infected with the LVi-EGFP control virus (Figure 6 B).

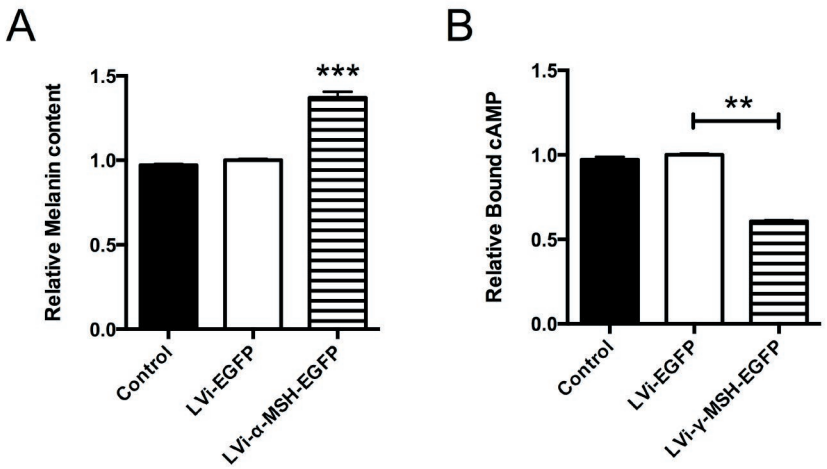

Figure 6. *In vitro* biological activity of LVi-α-MSH-EGFP and LVi-γ-MSH-EGFP lentiviruses. A: The relative amount of melanin produced by the LVi-α-MSH-EGFP (n = 11) lentiviral vector compared to LVi-EGFP (n = 12) and non-infected control (n = 6) in B16F10 cells. B: The relative stimulatory capacity of the growth medium of non-infected control (n = 5) and LVi-EGFP (n = 5) or LVi-γ-MSH-EGFP infected (n = 6) HEK293T cells to induce the release of bound cAMP in hMC3R cells ***P < 0.001, **P < 0.01, one-way ANOVA followed by Kruskal-Wallis post-hoc test

5.2 IN VIVO GENE EXPRESSION ANALYSIS

5.2.1 EGFP expression

Coronal brain sections of animals injected with LVi-EGFP and LVi-α-MSH-EGFP lentiviruses in studies I-II were analyzed for the exact position of EGFP expression visually in DAPI stained sections in a fluorescent microscope. EGFP expression was found in the medial and basal region of the hypothalamic ARC (Figure 7 A) and in the medial and dorsal regions of the NTS in the brain stem (Figure 7 B).

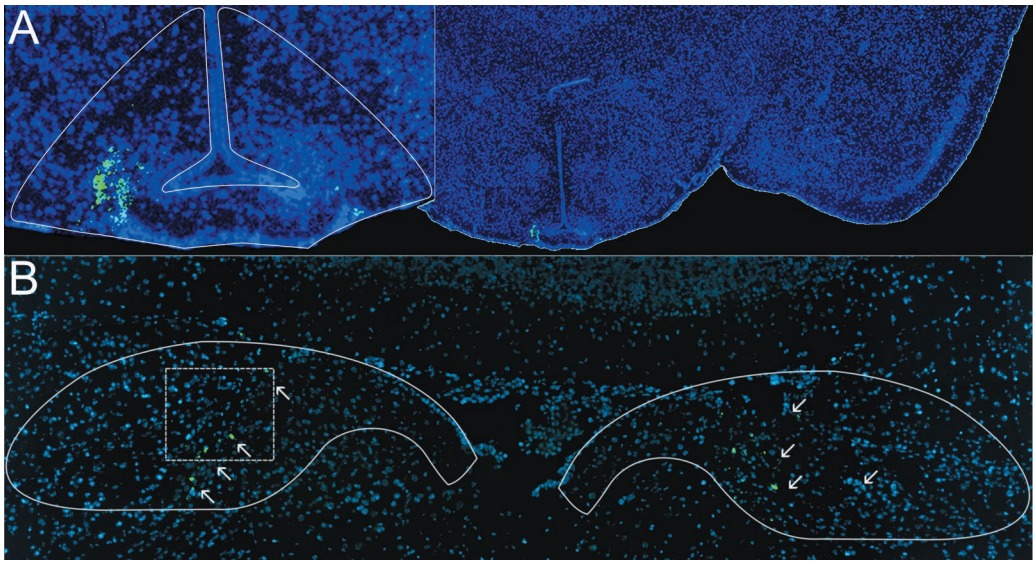

Figure 7. Location of lentivirally induced EGFP expression (green) in A: ARC injections and in B: NTS injections. Figure on the left in A is a magnification of the background coronal section. Marked area represents the corresponding target brain area and the white square in B indicated the location of DAB staining of α-MSH immunoreactivity (Figure 9 D). DAPI (blue) is a nuclear stain. Arrows indicate location of EGFP transgene expression in the NTS.

5.2.2 Transgene and α-MSH immunostaining

Hypothalamus

The level of α-MSH overexpression in the ARC was semiquantified using immunohistochemistry. There was a 62 % increase in the number of α-MSH stained neurons in the ARC in LVi-α-MSH-EGFP compared with LVi-EGFP injected mice (42 ± 6.0 vs. 26 ± 2.3, *P < 0.05) (Figure 8).

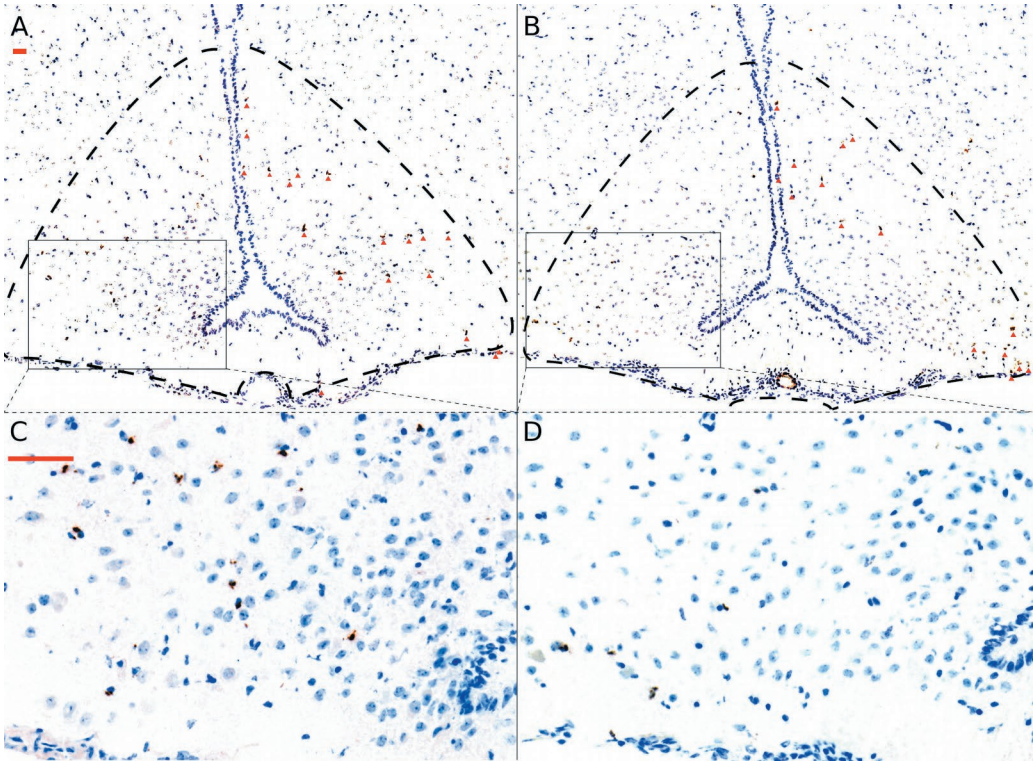

Figure 8. Immunohistochemical staining in study I of α-MSH in the ARC of the hypothalamus of representative samples of A: LVi-α-MSH-EGFP and B: LVi-EGFP treated animals. Positive DAB (brown) staining highlighted in the right hemisphere with red cones (A and B). High-resolution images of staining close to the third ventricle on the left hemisphere of C: LVi-α-MSH-EGFP and D: LVi-EGFP treated animals. Scale bar (red) indicates a distance of 50 µm.

Brain stem

The α-MSH immunoreactivity in NTS injections was determined in coronal sections using immunohistochemistry of LVi-EGFP and LVi-α-MSH-EGFP treatments groups. The α-MSH immunoreactivity in the LVi-EGFP control group was mostly located in the close proximity of the fourth ventricle or the central canal in the commissural NTS with minor positive locations in the dorsal and medial NTS (Figure 9 A-C). In contrast, α-MSH immunoreactivity in the LVi-α-MSH-EGFP samples was evident as a more intense and widespread staining descending from dorsolateral regions and dorsomedial to the medial NTS (Figure 9 D-E) and spreading on average over an area of 255 ± 14 µm in width (n = 5).

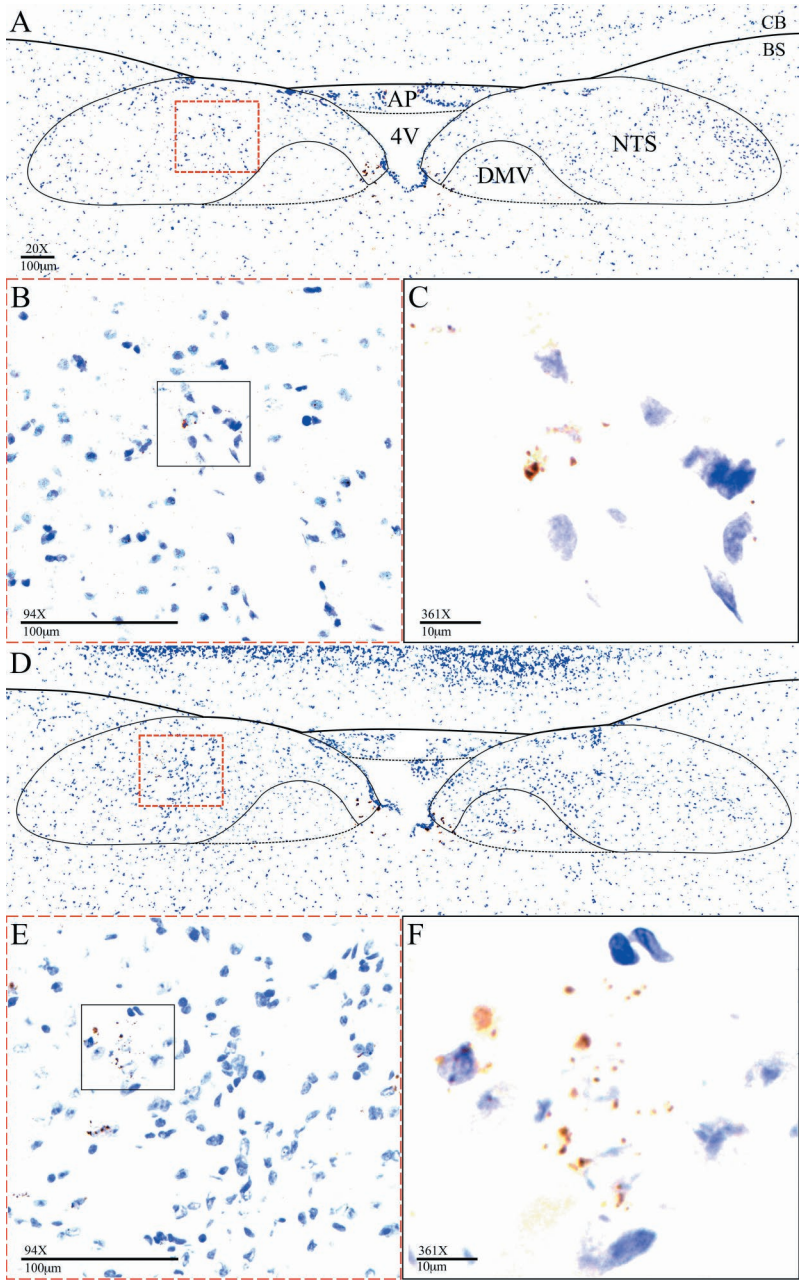

Figure 9. Representative images from study II of α-MSH immunoreactivity in the coronal sections of the brain stem in the interaural -3.5 mm position of LVi-EGFP (A-C) and LVi-α-MSH-EGFP (D-F) injected mouse. A, D: 20x image of the nucleus tractus solitarius (black outline) and infection site (red box). B, E: 40x image of the injection site. C, F: Magnified 63x image of the black box in B and E. Cresyl (blue color) indicates nuclei and DAB (brown color) the location of α-MSH immunoreactivity. Scale bar and magnification factor located in the lower left corner of each image. The white dotted box indicates the injection site and white arrows point to EGFP. AP = area postrema, BS = brain stem, CB = cerebellum, DMV = dorsal motor nucleus of the vagus, NTS = nucleus tractus solitarius, 4V = the fourth ventricle.

The data obtained from IHC-staining analysis was used in order to measure the major location of α-MSH overexpression in the brain stem (Figure 10) and to compare this data to the determined target site. The quantification results indicate a significant 65 % increase in α-MSH immunoreactivity in the interaural -3.5 mm sections in accordance with the coordinates used in the injections and the actual sites of successful injections (Figure 10). The overall α-MSH immunoreactivity in the NTS as estimated by comparing the AUC values of the mean pixel count curves (Figure 10) was 31% more in the LVi-α-MSH-EGFP mice (AUC: 1288 vs. 983 pixels x section). In addition, NTS injections were plotted schematically (Figure 11) according to both EGFP and α-MSH immunereactivity in order to visualize the target sites and confirm the results obtained from semiquantification.

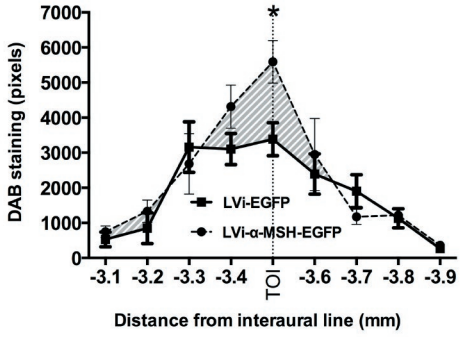

Figure 10. Semiquantification of α-MSH immunoreactivity in study II (DAB stained pixels) in the NTS -3.9 to -3.1 mm from the interaural structure in LVi-EGFP (n = 4) and LVi-α-MSH-EGFP (n = 6) treated animals. TOI = Target of infection. * P < 0.05, Mann-Whitney.

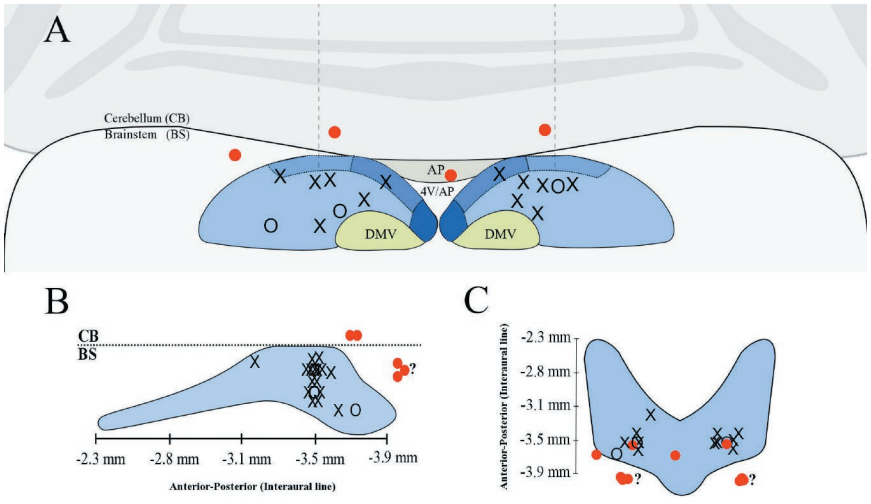

Figure 11. A: Schematic figure of coronal brain sectionin study II including injection sites, X = bilateral injection pair, O = unilateral hit, Red dot = injection missed NTS, AP = area postrema, DMV = dorsal motor nucleus of vagus, 4V = fourth ventricle, CC = central channel, NTS divided into; commissural (closest to 4V/CC), medial (middle of NTS), dorsomedial (next to commissural NTS) and dorsolateral (next to dorsomedial). B: Schematic figure of sagittal view in (A), C: Schematic figure of superior view in (A).

5.3 THE EFFECTS OF α-MSH OR γ-MSH OVEREXPRESSION ON WEIGHT AND FOOD INTAKE

5.3.1 In the hypothalamus

5.3.1.1 α-MSH

The first *in vivo* experiment in study I assessed the metabolic effects of LVi-α-MSH-EGFP compared with LVi-EGFP treated mice over six weeks after the injections. LVi-α-MSH-EGFP treated mice gained significantly less weight (8.4 ± 0.4 g) compared to LVi-EGFP treated mice (12.3 ± 0.6 g, $P < 0.05$) during the experiment. The body weight gain was statistically significantly reduced in the LVi-α-MSH-EGFP group starting from day 7 (Figure 12 A). The LVi-α-MSH-EGFP treated mice consumed less food over the first week than the LVi-EGFP treated mice, but subsequently feeding increased to the same level and remained there until the end of the experiment (Figure 12 C). Viral infection *per se* had no effect on weight gain or food intake, as there were no differences between LVi-EGFP injected and saline injected animals (Figure 12 A, C).

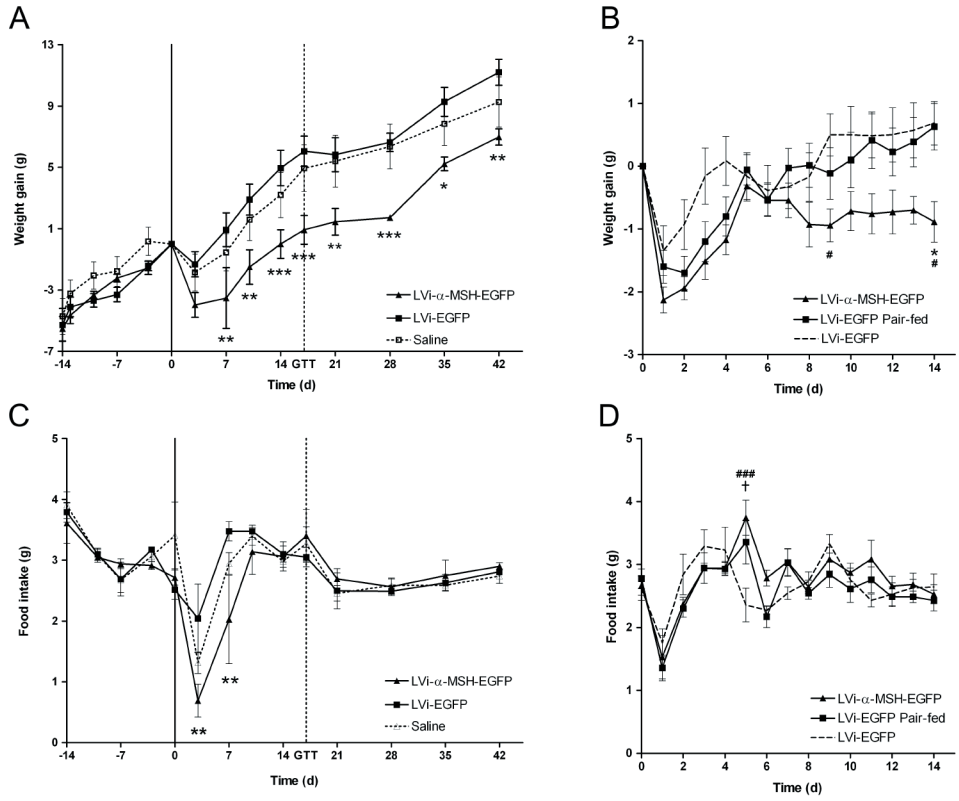

Figure 12. A: Weight change and C: daily food intake of mice injected to the arcuate nucleus with lentiviral vectors LVi-α-MSH-EGFP (n = 4) and LVi-EGFP (n = 4) or saline (n = 5) in study I. B: Weight change and D: daily food intake of weight matched LVi-α-MSH-EGFP treated group (n = 7), pair-fed LVi-EGFP treated group (n = 7) and *ad libitum* fed LVi-EGFP treated control group (n = 7). LVi-α-MSH-EGFP compared to pair-fed LVi-EGFP (*) and LVi-EGFP control (#), pair-fed LVi-EGFP compared to LVi-EGFP (†): ***P < 0.001, **P < 0.01, *P < 0.05, ###P < 0.001, #P < 0.05, †P < 0.05.

The aim of the pair-feeding experiment of study I was to define the role of feeding in the weight development of LVi-α-MSH-EGFP treated mice by pair-feeding a group of LVi-EGFP treated mice to the level of LVi-α-MSH-EGFP treated mice and comparing their weight gain. Although food intake was not significantly decreased in LVi-α-MSH-EGFP mice in this experiment, the weight gains between LVi-α-MSH-EGFP and LVi-EGFP groups were significantly different over the two week period after the injections (interaction of treatment and time: $P < 0.0001$ and $P = 0.0002$, respectively, Figure 12 B, D). Food intake and weight gain tended to be lower on the first days after the injections in LVi-α-MSH-EGFP treated and pair-fed mice compared to LVi-EGFP treated *ad libitum* fed mice (Figure 12 B, D). Subsequently, food intake increased to the level of LVi-EGFP treated mice. Despite the similar food intake in all three groups after day 5, the LVi-α-MSH-EGFP group continued to gain less weight in comparison to both LVi-EGFP treated groups (Figure 12 B, D).

5.3.1.2 γ-MSH

The *in vivo* experiment of study III aimed at defining the metabolic role of the γ-MSH over-expression period of 26 weeks, which was divided into an initial six-week chow diet, followed by a 13-week Western diet and a final seven-week chow diet intervention.

Post-treatment chow diet period

The LVi-γ-MSH-EGFP treatment decreased the cumulatice food intake during the first two weeks after stereotaxic delivery of lentiviral treatments compared to control (55.8 ± 0.9 vs. 58.7 ± 0.9 g *$P < 0.05$), but the weekly measurements were not significantly different (Figure 13 B). There were no differences in the total food intake over the course of the post-treatment chow period (169.5 ± 2.0 vs. 170.1 ± 2.0 g P = NS). Pre-treatment weight was regained within two weeks in both groups and overall weight development was similar in both groups during the six-week chow period (Figure 13 A). The weight reduction during the 12-hour fast prior to re-feeding was similar in both groups (-1.1 ± 0.1 vs. -1.2 ± 0.1 g P = NS). There was no difference in the acute feeding (2h) after fasting (0.77 ± 0.05 vs. 0.75 ± 0.04 g P = NS) or under *ad libitum* conditions after the beginning of the dark period (0.63 ± 0.03 vs. 0.61 ± 0.04 g, P = NS). However, the treatment increased food intake over 24 hours time in LVi-γ-MSH-EGFP animals after reintroduction to food (4.26 ± 0.10 vs. 4.54 ± 0.10 g *$P < 0.05$).

Diet induced obesity period

The relative weight change (Figure 13 C), daily food intake (Figure 13 D) and cumulative food intake during the first two weeks (51.1 ± 1.4 vs. 49.7 ± 0.9 g P = NS) and total food intake during the Western diet period (290.7 ± 5.1 vs. 282.4 ± 4.1 g P = NS) were similar between the LVi-EGFP and LVi-γ-MSH-EGFP treatment groups. The weight tended to decrease more in the LVi-γ-MSH-EGFP group during the 12 hour fast before re-feeding (-1.54 ± 0.09 vs. -1.30 ± 0.08 g $P = 0.06$). Similarly to chow re-feeding assessment, there was no difference in the acute re-feeding (0.60 ± 0.06 vs. 0.50 ± 0.04 g P=NS) or normal feeding (0.49 ± 0.06 vs. 0.48 ± 0.06 g, P = NS). However, in contrast to the results on chow there was no differences in the 24-hour food intake between the treatments after re-access to food (3.55 ± 0.10 vs. 3.36 ± 0.10 g P = NS).

Weight loss with chow diet

After the 13-week Western diet period, the diet was reverted back to standard rodent chow for a duration of seven weeks. The LVi-γ-MSH-EGFP treated mice had a significantly increased relative weight loss compared to control (Figure 13 E). Food intake was similar in both treatment groups during this period (Figure 13 F).

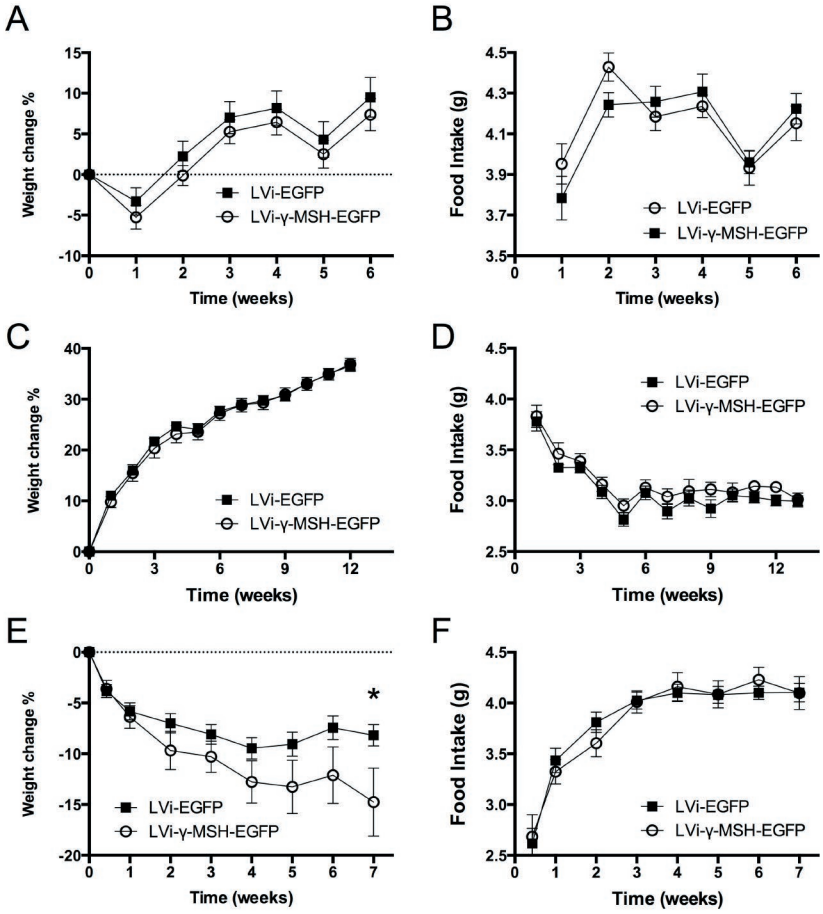

Figure 13. Relative weight change and food intake from all three parts of study III. Data from the initial chow period, A: Relative weight change, B: Food intake and the DIO Western diet period, C: Relative weight change and D: Food intake of LVi-EGFP (n = 17) and LVi-γ-MSH-EGFP (n = 16) injected mice. E: Relative weight change and F: Food intake during the seven-week weight loss with chow diet-period in LVi-EGFP (n = 15) and LVi-γ-MSH-EGFP (n = 13) injected mice *P < 0.05.

5.3.2 In the brain stem

5.3.2.1 α-MSH

There was no difference in relative body weight change between the treatments over time until day 53 (Figure 14 A). In the end, LVi-α-MSH-EGFP treated mice weighted significantly less than the LVi-EGFP mice (35.3 ± 0.9 vs. 38.5 ± 0.9 g *P < 0.05). The operation needed for viral injections caused a decrease in the body weights in both

groups, and the initial weight was achieved in the LVi-EGFP group by day 21 and in the LVi-α-MSH-EGFP group by day 28. On day 39 after three consecutive days of tail-cuff measurements, there was a difference in the feeding relative to baseline food intake and in total food intake (LVi-EGFP: 2.3 ± 0.1 vs. LVi- α-MSH-EGFP: 2.8 ± 0.1 g **P < 0.01). The fluctuation in food intake in both treatment groups might have been related to weekly routines in the animal facilities or to the disturbed weight development after the experimental procedures.

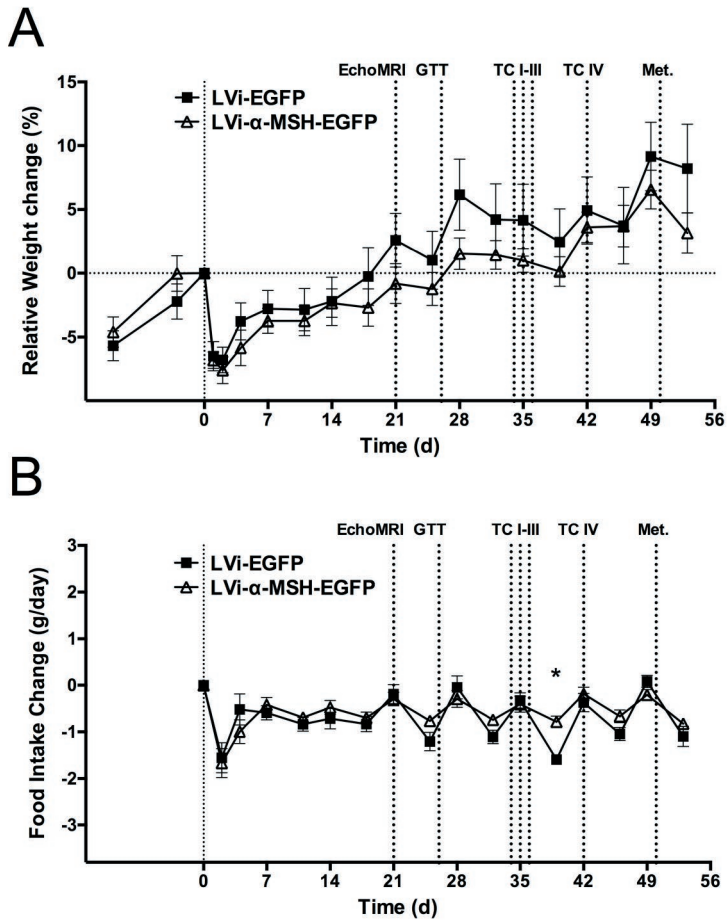

Figure 14. Results from study II. A: Relative weight change, B: food intake change from baseline in food intake of mice injected to the NTS with lentiviral vectors LVi-α-MSH-EGFP (n = 9) or LVi-EGFP (n = 10) *P < 0.05, Two-way-ANOVA followed by Bonferroni post-test. Time points of body composition analysis (EchoMRI), glucose tolerance test (GTT), tail-cuff blood pressure training and measurements (TC) and urine and fecal collections in the metabolic cages (Met.) are indicated as dotted vertical lines.

5.4 THE EFFECTS OF α-MSH OR γ-MSH OVEREXPRESSION ON BODY COMPOSITION, ADIPOSITY AND TISSUE WEIGHTS

5.4.1 In the hypothalamus

5.4.1.1 α-MSH

After sacrifice, liver, heart and the fat depots were individually weighed. The weight of the mesenteric fat depot was significantly decreased and the retroperitoneal fat depot showed a tendency to decrease in LVi-α-MSH-EGFP group compared with the LVi-EGFP group (Table 8), but there were no differences in subcutaneous, epididymal, or interscapular brown fat weights between treatments (Table 8). Moreover, there were no difference in heart weight, but the liver of the LVi-α-MSH-EGFP treated mice was significantly smaller (Table 8).

Table 8. Isolated tissues including fat depots from study I: LVi-EGFP (n = 4) and LVi-α-MSH-EGFP (n = 4), study II: LVi-EGFP (n = 10) and LVi-α-MSH-EGFP (n = 8 and study III: LVi-EGFP (n = 15) and LVi-γ-MSH-EGFP (n = 13). **P > 0.01 *P > 0.05, non-parametric t-test was used in Study I and parametric t-test in Studies II and III.

	Tissue	LVi-EGFP	LVi-α-MSH-EGFP
STUDY I	Subcutaneous fat (mg)	429 ± 27	461 ± 105
	Epigonadal fat (mg)	1343 ± 70	1243 ± 82
	Retroperitoneal fat (mg)	385 ± 21	195 ± 54
	Mesenteric fat (mg)	993 ± 96	729 ± 64 *
	Interscapular BAT (mg)	156 ± 34	143 ± 13
	Sum of fat depots (mg)	**5308 ± 168**	**4525 ± 445**
	Liver (mg)	1942 ± 35	1562 ± 42 *
	Heart (mg)	132 ± 7	146 ± 9
	Tissue	**LVi-EGFP**	**LVi-α-MSH-EGFP**
STUDY II	Retroperitoneal fat (mg)	335 ± 18	256 ± 27 *
	Mesenteric fat (mg)	807 ± 69	552 ± 38 **
	Interscapular BAT (mg)	247 ± 14	188 ± 17 *
	Liver (mg)	2109 ± 116	2075 ± 158
	Heart (mg)	148 ± 3	149 ± 7
	Kidney (mg)	214 ± 5	208 ± 9
	Tissue	**LVi-EGFP**	**LVi-γ-MSH-EGFP**
STUDY III	Subcutaneous fat (mg)	651 ± 43	565 ± 82
	Epigonadal fat (mg)	1259 ± 82	1054 ± 121 (P= 0.09)
	Retroperitoneal fat (mg)	600 ± 47	502 ± 70
	Mesenteric fat (mg)	868 ± 46	741 ± 88 (P= 0.10)
	Inguinal fat (mg)	620 ± 40	491 ± 73 *
	Interscapular BAT (mg)	290 ± 17	269 ± 27
	Sum of fat depots (mg)	**5681 ± 295**	**4525 ± 445 (P= 0.08)**
	Liver (mg)	1851 ± 110	1764 ± 110
	Heart (mg)	142 ± 4	151 ± 5
	Kidney (mg)	205 ± 7	213 ± 7

5.4.1.2 γ-MSH

Body composition was analyzed before treatments and at three-week intervals from the beginning of each diet period. During the post-treatment chow diet period the increase in fat mass to lean mass ratio was attenuated significantly at week six in the LVi-γ-MSH-EGFP group (25 ± 12 vs. 70 ± 18 % *P < 0.05). There was no significant difference in fat mass (Figure 15 A), but lean mass tended to be increased compared to control (Figure 15 B). During the diet induced obesity period, the increases in both fat and lean mass were similar (Figure 15 C, D) nor were there any differences in total fat mass (19.4 ± 0.8 vs. 19.5 ± 0.9 g P = NS) or total lean mass (22.2 ± 0.3 vs. 22.6 ± 0.4 g P = NS) at the end of the Western diet in LVi-EGFP and LVi-γ-MSH-EGFP treated mice respectively. However, during the weight loss with the chow diet-period the LVi-γ-MSH-EGFP treatment significantly decreased fat mass at week six (Figure 15 E), however, lean mass was similar between the treatments during the period (Figure 15 F).

Liver, heart, kidney and adipose tissue depots were weighed at the end of study III and the inguinal fat depot was significantly decreased and epigonadal, mesenteric and total fat depot weights tended to be decreased in the LVi-γ-MSH-EGFP treated mice compared to LVi-EGFP control (Table 8). There was no difference in the weight of subcutaneous, retroperitoneal or BAT between the treatments. In addition, the weights of liver, heart and kidney were similar between the treatments (Table 8).

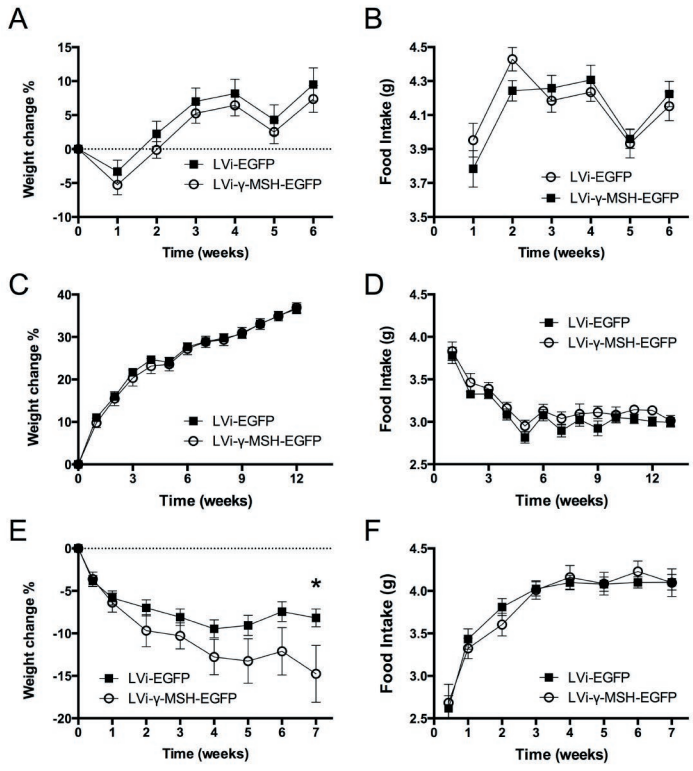

Figure 15. Body composition measurements from Study III. A: Fat mass and B: lean mass from post-treatment chow diet period in LVi-EGFP (n = 17) and LVi-γ-MSH-EGFP (n = 16) injected mice. C: Fat mass and D: lean mass during the diet induced obesity period. E: Fat mass and F: lean mass on the weight loss with chow diet-period in LVi-EGFP (n = 15) and LVi-γ-MSH-EGFP (n = 13) treated mice. *P < 0.05 post-hoc test.

5.4.2 In the brain stem

5.4.2.1 α-MSH

The LVi-α-MSH-EGFP treated animals showed a significantly lower absolute fat mass (7.3 ± 0.6 vs 9.8 ± 0.6 g ***P < 0.001) and change in fat mass 56 days after the injection (Figure 16 A). There were no significant differences in lean mass (24.2 ± 0.4 vs. 24.3 ± 0.4 P = NS) or lean mass change (Figure 16 B) between the groups over time.

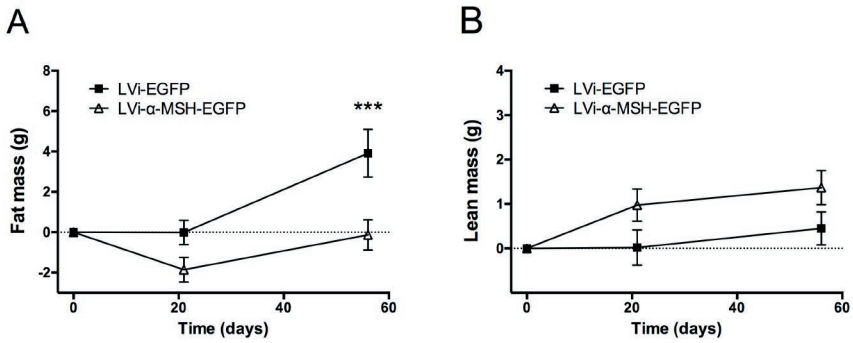

Figure 16. Body composition measurement in LVi-EGFP (n = 10) and LVi-α-MSH-EGFP (n = 9) treated mice in study II. A: Fat mass change and B: lean mass change ***P < 0.001 two-way anova post-hoc test.

The weights of mesenteric and retroperitoneal WAT, and interscapular BAT depots were significantly smaller in the LVi-α-MSH-EGFP injected group as compared to the LVi-EGFP group at the end of study II (Table 8).

5.5 THE EFFECTS OF α-MSH OR γ-MSH OVEREXPRESSION ON GLUCOSE METABOLISM

5.5.1 In the hypothalamus

5.5.1.1 α-MSH

Intraperitoneal glucose tolerance test performed three weeks after the injections in study I showed that LVi-α-MSH-EGFP treated mice cleared blood glucose faster than to the LVi-EGFP group (Figure 17 A, B), but there was no difference in fasted glucose concentrations. Viral infection had no effect on glucose tolerance, as there were no differences between LVi-EGFP and saline treated groups.

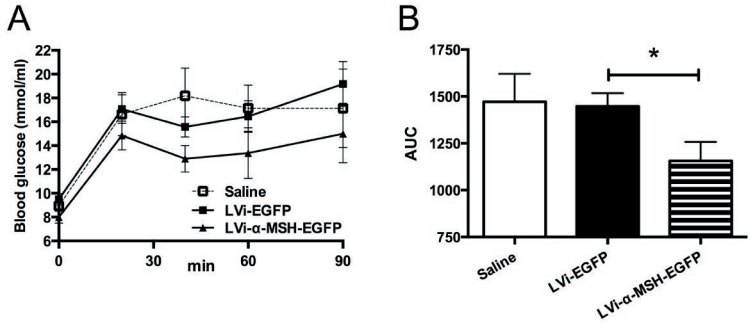

Figure 17. Blood glucose data in A: GTT and B: are under the curve (AUC) of (A) in LVi-EGFP (n = 4) and LVi-α-MSH-EGFP (n = 4) treated mice of study I. *P > 0.05 t-test

5.5.1.2 γ-MSH

Basal glucose levels assessed week three after injections on the standard chow diet and were similar in both groups (8.1 ± 0.3 vs. 7.8 ± 0.3 mmol/L, P = NS). The levels were also similar between the groups during the Western diet-period and during the weight loss with the chow diet-period (data not shown). GTT was assessed six weeks after the last diet change on the weight loss period, but there were no differences between the treatment groups (data not show).

5.5.2 In the brain stem

5.5.2.1 α-MSH

In study II, there was no significant difference in serum glucose levels after a four hour fasting period or after glucose load in GTT analyzed over time (data not shown) or in the area under the curve (LVi-EGFP: 627 ± 48 vs. LVi-α-MSH-EGFP: 638 ± 31 minutes x mmol/L). There were no differences in serum insulin levels at the end between the treatments (Table 10).

5.6 THE CARDIOVASCULAR EFFECTS OF α-MSH OVEREXPRESSION

5.6.1 In the brain stem

Study II assessed systolic blood pressure (sBP) and heart rate (HR) using the tail-cuff method, which showed no significant differences in HR and sBP in the LVi-α-MSH-EGFP (701 ± 21 b.p.m. and 130 ± 4 mmHg) compared with the LVi-EGFP group (712 ± 15 b.p.m. and 119 ± 5 mmHg, P = NS and P = 0.15, respectively). In the radiotelemetric assessments, the baseline HR was consistently higher in the LVi-α-MSH-EGFP group during the active dark cycle, but no changes were noted in MAP (Figure 18 A, B). To study autonomic control of cardiac function, acute cardiovascular responses to autonomic

blocking agents were monitored. The HR response to a control injection with saline was enhanced in the LVi-α-MSH-EGFP group without any significant effect on MAP (Figure 18 C, D). This difference was abolished when the mice were treated with metoprolol (Figure 18 E, F) evidence of increased sympathetic activity in the LVi-α-MSH-EGFP-treated mice. Furthermore, HR responses to muscarinic blockade with atropine were attenuated in the LVi-α-MSH-EGFP group (Figure 18 G), indicating lower cardiac vagal activity. Interestingly, the compensatory increase in HR as a response to vasodilatation induced by the α_1-adrenergic receptor antagonist prazosin was enhanced in the LVi-α-MSH-EGFP group (Figure 18 G). These altered HR responses to autonomic blocking agents occurred without there being any significant differences in MAP responses between the groups (Figure 18 H).

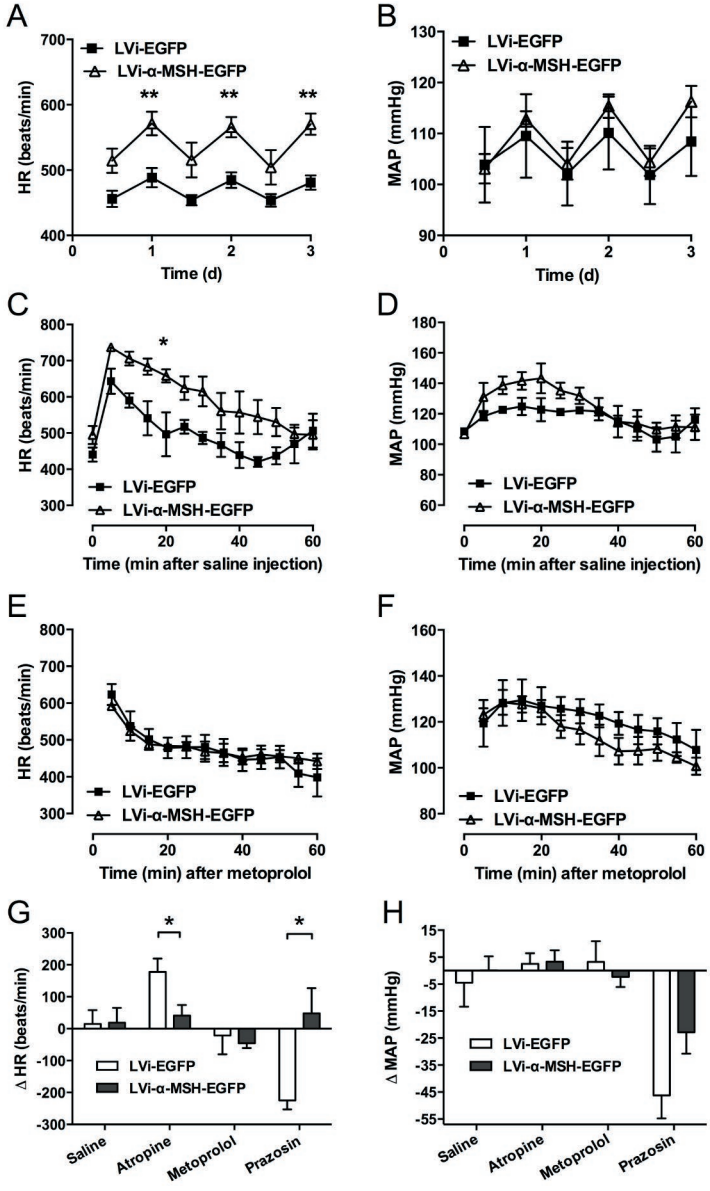

Figure 18. Telemetric cardiovascular measurements of LVi-EGFP (n = 4) and LVi-α-MSH-EGFP (n = 4) treated mice in study II. A: Mean arterial pressure (MAP) and B: Heart rate (HR). Effects of saline injection on C: MAP and D: HR. Effects of metoprolol injection on E: HR and F: MAP. *P < 0.05, ** < 0.05; two-way ANOVA followed by Bonferroni posttest. The change from baseline in G. MAP and H. HR 45-60 minutes after the administration of saline or autonomic nervous system blocking agents, atropine, metoprolol or prazosin. * P < 0.05; Mann-Whitney.

5.7 THE EFFECTS ON LOCOMOTION

5.7.1 In the hypothalamus

5.7.1.1 γ-MSH

Voluntary running wheel activity in study III was assessed a week before and four weeks after the treatments on the standard rodent chow and four weeks after the shift to the Western diet. The pre-treatment assessment served as an introduction to the running wheels and was characterized by several peak activity points (revolutions/hour) during the dark period (Figure 19 A). The animals were divided into treatment groups based on total running wheel activity such that the mean activity during the dark period was similar (Figure 19 B). There was no difference in the running wheel activity between the groups during the post-treatment chow diet period assessment (Figure 19 C) and the mean running wheel activity during the dark period was also similar between the treatment groups (Figure 19 D). Consumption of the Western diet markedly reduced running wheel activity in both treatment groups but the activity of LVi-γ-MSH-EGFP was slightly higher as compared to control at the beginning and during the last hour of the dark period (Figure 19 E). Interestingly, the running wheel activity was completely abolished in both groups after the dark period (Figure 19 E). The LVi-γ-MSH-EGFP treated mice displayed a significantly higher mean running wheel activity during the dark period (Figure 19 F).

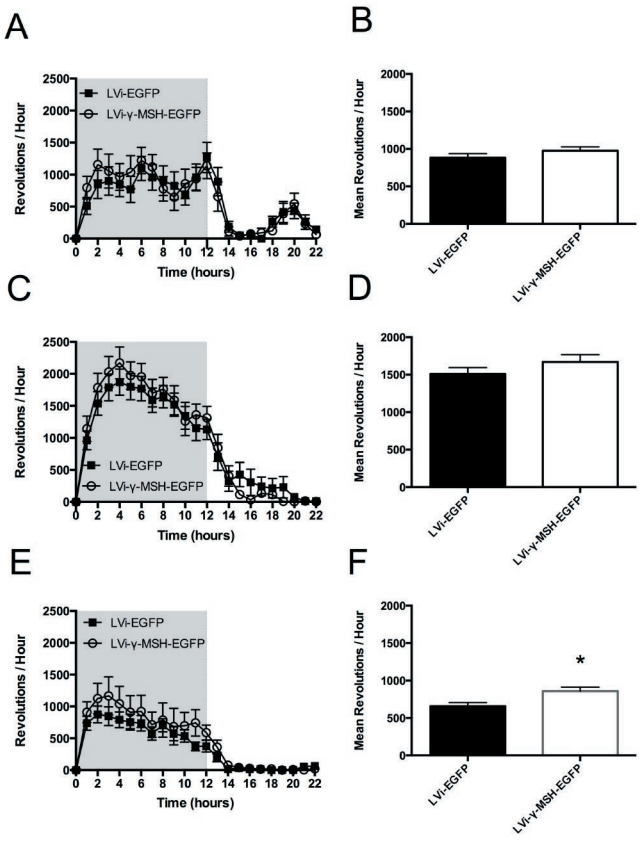

Figure 19. A: Running wheel activity prior to injections, B: Pre-treatment-mean running wheel activity per hour during dark cycle, C: Post-treatment chow diet-running wheel activity four weeks after injections, D: Post-treatment chow diet period-mean running wheel activity during dark period, E: Western diet-running wheel activity, F: Western diet-mean running wheel activity during dark period in LVi-EGFP (n = 17) and LVi-γ-MSH-EGFP (n = 16) injected mice *P < 0.05.

5.7.2 In the brain stem

5.7.2.1 α-MSH

Locomotion was assessed in mice implanted with radiotelemetric devices in study II, but there were no differences between the treatment groups during the assessment period (Figure 20).

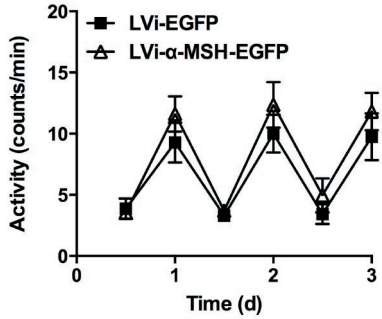

Figure 20. Physical activity showing mean values for 12-hour light and dark periods over a three-day baseline measurements in LVi-EGFP (n = 4) and LVi-α-MSH-EGFP (n = 4) treated mice.

5.8 METABOLIC EFFECTS

5.8.1 In the hypothalamus

5.8.1.1 γ-MSH

In study III, the 24-hour food intake, water intake, urine volume and stool weights were assessed in metabolic cages. The 24-hour water intake was significantly smaller in the LVi-γ-MSH-EGFP (Table 9). The 24-hour food intake, weight loss, total urine volume and total feces weight were similar in both groups (Table 9). The average 24-hour home cage water intake assessed over 72-hours was similar in the two groups (3.30 ± 0.25 vs. 3.20 ± 0.28 mL P = NS).

5.8.2 In the brain stem

5.8.2.1 α-MSH

In study II, the LVi-α-MSH-EGFP treated animals had lower 24-hour urine volume as compared to the LVi-EGFP treated group (Table 9). The levels of 24-hour creatinine excretion did not differ, indicating that there was no difference in their renal function (Table 9). There were no significant differences in water and food intake or the weight of the stool (Table 9).

Table 9. Parameters from the 24-hour metabolic cage assessment in both study II and III. In study II: LVi-EGFP (n = 10) was compared to LVi-α-MSH-EGFP (n = 9) and in study III: LVi-EGFP (n = 15) was compared to LVi-γ-MSH-EGFP (n = 13) *P < 0.05 non-parametric in study II and parametric in study III.

		LVi-EGFP	LVi-γ-MSH-EGFP
Study III	Food (g/24h)	3.32 ± 0.11	3.26 ± 0.14
	Feces (g/24h)	0.30 ± 0.01	0.28 ± 0.02
	Water (mL/24h)	4.24 ± 0.56	2.92 ± 0.29 *
	Urine (mL/24h)	0.26 ± 0.04	0.28 ± 0.04
		LVi-EGFP	LVi-α-MSH-EGFP
Study II	Food (g/24h)	3.85 ± 0.32	3.65 ± 0.27
	Feces (g/24h)	0.36 ± 0.03	0.37 ± 0.03
	Water (mL/24h)	3.03 ± 0.38	2.36 ± 0.36
	Urine (mL/24h)	0.97 ± 0.14	0.47 ± 0.11 *
	Creatinine (µg/24h)	391 ± 36	388 ± 148

5.9 THE EFFECTS ON ENERGY EXPENDITURE

5.9.1 In the hypothalamus

5.9.1.1 γ-MSH

Energy expenditure was calculated by assessing the total energy gained from the change in lean and fat mass and reducing it from total energy intake. Energy expenditure was similar in both LVi-EGFP and LVi-γ-MSH-EGFP treated mice over the three diet periods in study III (Figure 21).

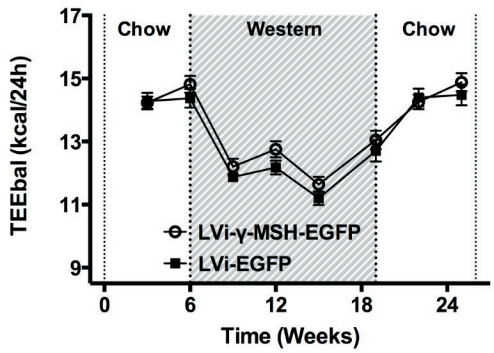

Figure 21. Energy expenditure estimate (TEE_{bal}) over time in mice treated with LVi-EGFP (n = 17/15) and LVi-γ-MSH-EGFP (n = 16/13) in study III.

5.9.2 In the brain stem

5.9.2.1 α-MSH

In study II, there were no differences in the calculated energy expenditure (TEE_{bal}) between the treatment groups from day 21 to 56 (LVi-EGFP: 11.8 ± 0.2 kcal/24h vs. LVi-α-MSH-EGFP: 12.1 ± 0.4 kcal/24h).

5.10 GENE EXPRESSION AND BIOCHEMICAL ASSAYS

5.10.1 In the hypothalamus

5.10.1.1 α-MSH

At the end of study I, it was decided to assess liver triglyceride content, serum levels of total cholesterol and triglycerides, but there were no differences detected between the LVi-EGFP and LVi-α-MSH-EGFP treatments (Table 10).

5.10.1.2 γ-MSH

In study III, quantitative assessment of UCP1 mRNA levels in BAT and adrenal TH did not reveal any differences between the LVi-EGFP and LVi-γ-MSH-EGFP treatments (Table 10). Moreover, the non-esterified fatty acids (NEFA) in the serum of LVi-γ-MSH-EGFP tended to be lower than the corresponding value in the LVi-EGFP mice, but there were no differences in the serum triglyceride content (Table 10).

5.10.2 In the brain stem

5.10.2.1 α-MSH

Study II assessed serum levels of NEFA, cholesterol and insulin but there were no differences observed between the LVi-EGFP and LVi-α-MSH-EGFP treatments. Despite the lower BAT weight (Table 8), the thermogenic capacity assessed as UCP1 mRNA level was also similar (Table 10).

Table 10. Results from biochemical assays and mRNA quantifications in Study I-III. Study I: LVi-EGFP (n = 4) was compared to LVi-α-MSH-EGFP (n = 4). Study II: LVi-EGFP (n = 10) was compared to LVi-α-MSH-EGFP (n = 9). Study III: LVi-EGFP (n = 15) was compared to LVi-γ-MSH-EGFP (n = 13). NEFA, non-esterified fatty acids; BAT, brown adipose tissue; BAT UCP1, Brown adipose tissue uncoupling protein 1, TH, tyrosine hydrolase.

	Sample	LVi-EGFP	LVi-α-MSH-EGFP
I	Serum NFFA mmol/l	0.26 ± 0.03	0.25 ± 0.04
	Serum Cholesterol (mg/µl)	2.87 ± 0.17	2.10 ± 0.43
	Liver Triglycerides mmol/l	5.54 ± 0.97	5.18 ± 0.32
	Sample	**LVi-EGFP**	**LVi-α-MSH-EGFP**
II	Serum NEFA (mmol/L)	0.37 ± 0.03	0.35 ± 0.04
	Serum Cholesterol (mg/mL)	2.63 ± 0.17	2.97 ± 0.29
	Serum Insulin (µg/L)	0.77 ± 0.22	1.02 ± 0.24
	BAT UCP1 mRNA	1.06 ± 0.13	1.01 ± 0.16
	Sample	**LVi-EGFP**	**LVi-γ-MSH-EGFP**
III	Serum NEFA (mmol/L)	0.41 ± 0.03	0.35 ± 0.02 (P = 0.08)
	Serum Triglycerides (mmol/L)	1.33 ± 0.09	1.29 ± 0.12
	BAT UCP1 mRNA	1.00 ± 0.08	0.93 ± 0.06
	adrenal TH mRNA	1.00 ± 0.16	1.07 ± 0.14

6. DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Gene therapy

The introduction of genes to cells using gene delivery vectors is a well-established method and gene delivery has been attemped in humans to cure rare genetic disorders with some success. In contrast to pharmacological approaches, viral gene delivery provides a benefit of anatomically targeted treatment and long-term expression when used in animal studies to investigate the role of genes in the CNS. Melanocortin peptides are locally expressed and since these neuropeptides have a short half-life, central infusion was not considered as an alternative in this study. Therefore lentiviral vectors were chosen since these have been shown to induce long-term site-specific overexpression in the CNS. Genetically engineered transgene animal models are a well-characterized and reliable way to study the impact of genes. The insertion or removal of genes using recombination with the CRE-LOX system (Sauer. 1987, Sauer & Henderson. 1988) has been useful for the development of transgene or knock-out animals with region or tissue specific genetic modifications. Knock-out or transgenic animals are extremely useful when characterizing the general phenotype of abolishment or over-expression of a target gene, respectively. However, the development and characterization of TG-animals are often laborious, time-consuming and expensive.

6.1.2 Animal models

The study of obesity in laboratory rodents extends back to the very beginning of the development and characterization of animal models intended for research. The spontaneous mutations that generated obesity in these early animal models have later been established and many have also been recognized in humans. Although laboratory rodents such as mice have been proven useful models in obesity research and since the genes that govern energy intake and energy metabolism are highly conserved between species, there are still questions that need to be considered. Mice are exceptionally resistant to the effects of obesity and the emotional elements, which are highly influential in human obesity, are different and challenging to interpret in mice. Another disadvantage in metabolic research is that within a population of genetically identical animals and presumably in humans, there will be a sub-population of low- and high-gainers making interpretation of results challenging (Bouchard. 1991). Closer examination of these subpopulations has revealed a variation in the genetic expression profiles, which has been linked to epigenetic and prenatal remodeling (Koza*, et al.* 2006). The C57BL/6N mice used in all of these studies are one of the most commonly used mouse strains and develop obesity when fed *ad libitum* with both normal and especially on high-fat diets (Collins*, et al.* 2004). In addition, the C57BL/6N strain also develops atherosclerosis when fed a high fat diet (Schreyer*, et al.* 1998). In summary, the C57BL/6N strain is highly suitable for obesity related studies.

In order to accelerate obesity in mouse strains, special rodent diets were used. In comparison to the standard rodent chow diet, these obesogenic diets mimic the highly processed Western type foods including a high animal based fat and sugar content and relatively small amount of dietary fiber. These diets rapidly induce obesity in both mice and rats; however, some individuals still remain resistant to obesity and this is a factor which must be taken into account (Bouchard. 1991). Pair-feeding of a control group with the group receiving the treatment may help elucidate food intake dependence. When the effects of α-MSH overexpression were investigated in the hypothalamus, pair-feeding of the treatment groups was conducted in order to investigate the acute effects of feeding on weight development.

6.1.3 Lentiviral constructs

The basis for this thesis was to utilize the potential of lentiviral gene delivery vectors for the generation of a local overexpression of MSH peptides in the CNS. Lentiviral vectors are easily adaptable to suit different target tissues due to the use of different promoters, but have shown exceptional potential in numerous studies involving the CNS. Therefore, we chose a second-generation lentiviral construct including a general neuronal EF1-α promoter and VSV-G core protein was chosen since this has been shown to be transduced almost exclusively neurons in the long-term (Jakobsson*, et al.* 2003). The genome of the second-generation of lentiviral vectors is divided into three different plasmids in the production phase in order to eliminate the possibility of a theoretical multi-recombination event resulting in a self-replicative vector. The production and measurement of lentiviral concentration were determined using validated methods (Trono. 2000, Wiznerowicz & Trono. 2005). A clear disadvantage with the method used in this thesis is that over-expression is not confined to specifc neuronal populations in the target area. In addition, we are not able to exclude the possibility of off-target effects related to neuronal projections to other brain regions. The combination of genetically modified mice with Cre-recombinase confined to certain neuronal populations such as POMC neurons with gene delivery vectors including LoxP-flanked gene expression cassettes would offer superior specificity over the use of “normal” overexpression vectors.

It is highly relevant to show that gene delivery vectors expressing peptides are able to produce biologically active compounds. Defective peptides may bind receptors without causing activation and thus act as antagonists of endogenous receptors or affect the binding of endogenous compounds. Therefore, it was considered important to decisively demonstrate the biological activity of each viral vector before *in vivo* studies. Both the α-MSH (LVi-α-MSH-EGFP) and γ-MSH lentiviral vector (LVi-γ-MSH-EGFP) were shown to efficiently activate melanocortin receptors *in vitro*. Moreover, the *in vivo* studies showed that the lentiviruses induced long-term moderate overexpression of the MSH peptides.

The use of fluorescent reporter genes such as the GFP and EGFP have proven useful in the investigation of gene expression (Chalfie. 1995, Inouye & Tsuji. 1994) and also in the validation of the location of gene expression by lentiviral transduction in our studies.

Although GFP and EGFP are readily used in research, there is a disadvantage when using green fluorephores. In fact, GFP and EGFP have fairly low penetration in tissue due to a moderately high energy and shorter wavelength compared to fluorophores of the far-red spectrum. Another disadvantage in the use of fluorophores is the bleaching factor or destruction of the peptide responsible for emitting the light. In order to minimize the effects of bleaching, analysis of EGFP expression in the *in vivo* studies was conducted immediately following sectioning and the effects of light and oxygen exposure were avoided.

6.1.4 Stereotaxic delivery

In order to determine the effects of neuropeptides in specific locations of the CNS, stereotaxis was used to deliver the MSH overexpressing lentiviral vectors in mice. Although stereotaxis is a well-utilized method in research, the literature associated with methodology is scarce. Target area coordinates are predominantly obtained from brain maps and optimized by using dyes (Franklin & Paxinos. 1997).

Delivery to specific nuclei in the CNS of small rodents such as mice represents a major challenge. Firstly, the target nuclei in the mouse CNS are tiny and are very challenging to target. Secondly, the accuracy of the method is highly dependent on the anatomy and size of the scull, which vary significantly even between mice of the same species, size and gender. Correct positioning of an animal in the frame is essential and ultimately relies on visual cues, which are analyzed subjectively by the operator. The margin of error is significant if positioning is not corrected between each animal and this variance between individuals increased the number of animals needed for each study. A laser-guide was developed in order to correct the inexact method of visual analysis of positioning the animal onto the stereotaxic frame. The laser is attached to a specialized holder and is aimed onto the skull bone. The method involves positioning of the animal according to the laser, which is reflected from skull bone onto a set target. The hypothesis was that this surface would be a more homologous target and would provide an accurate determination of head position as opposed to the fastening points of the ear cavities and incisors of the upper jaw. Indeed, it was possible to increase the success rate of the injections with this system.

The methodology involved in stereotaxic drug aministration is rather invasive and ultimately includes the penetration through brain tissue in order to reach the target areas. For instance, in intrahypothalamic injections it was necessary to penetrate the hippocampus, thalamus and the medial hypothalamus and in intrabrainstem injection also the cerebellum. In fact, mechanical disruption of brain tissue was previously used to analyze the physiological function brain areas. The needle tract of the injection represents a relatively small disturbances of the brain tissue reaching to target sites, but it was not possible to determine whether the injections caused any disruption of specific neuronal projections. Animals were excluded from experiments if the injections penetrated into the ventricles or produced hematomas in the CNS. In intrahypothalamic injections, there was also a comparison made of the weight and food intake of non-treated versus saline injections. The saline injected animals had a similar weight development as compared

to non-treated control after recovery from operations and showed that the injection did not cause significant damage in the treated animals. Although the protrusion of CNS did not significantly impact the development of the studied animals, all results represent the difference between animals treated similarly with a lentivirus and the only variable in the final results was the presence or lack of the target gene.

6.1.5 Characterization of obesity and hemodynamic changes

The development of obesity involves many different parameters and the timescale in which these occur varies. For instance, the accumulation of fat mass is an essential parameter in obesity for instance compared to weight gain or food intake. Increased fat mass in metabolically active peritoneal fat deposits is the initial sign in the development of obesity. After there was an increase in fat mass, then other factors such as disturbed glucose metabolism could be detected.

The development of obesity was assessed with a battery of different methods. Weight and food intake measurements were the primary parameters in the characterization of changes in body metabolism. Since both parameters were manually assessed on normal laboratory scales, there will automatically be a small variance in the results in addition to the daily fluctuations in the normal weight of mice. However, these variations are corrected over time and by increasing the number of treatment subjects. Body composition including fat mass, fat-free mass and free water were analyzed with commercially available and standardized quantitative nuclear magnetic resonance (NMR). The assessment of body composition using NMR is highly dependent on the level of immobilization in the target subject and small movements can cause large variations in the results. The variation eventually leads to repetition of the assessments. Analysis of glucose metabolism (glucose and insulin tolerance) was an important face of the study, however, the method is rather stressful for the animals and may interfere with weight and fat development. End-point data include a large subset of both commercially available and in house methods. Many of the methods involved in the assessment of serum and tissue parameters are now more sensitive than before and require smaller sample volumes. In the case of small rodents such as mice, small sample volumes are critical in order to reduce the requirements of animals for each experiment. Different metabolic parameters are obtained from the blood, tissues, urine and feces. Serum levels of glucose, insulin, cholesterol, triglycerides and non-esterified free fatty acids and triglycerides from liver in addition to urine creatinine and catecholamine levels. BAT activity and adrenal gland activity were determined by analyzing mRNA levels and information about lipid content different tissues homogenates and in feces were obtained using commercial kits. However, there is a certain degree of variance in the results obtained from these measurements, which often leads to non-significance in the statistical evaluation of the final results. Information about locomotor activity and voluntary running wheel activity were measured using commercially available light beam cages and running wheels. However, some animals did not acclimatize to the presence of running wheel devices in the home-cage and exhibited low running activity. Blood pressure was assessed using the tail-cuff method. However, this method has some limitations since the animals may be subjected to slight stress. Therefore, the less stressful method of radiotelemetry was

also included, as this has been assessed as being the benchmark method for the study of hemodynamic parameters in mice (Rinne, *et al.* 2008).

6.2 THE EFFECTS OF LONG-TERM CENTRAL α- AND γ-MSH OVEREXPRESSION

Table 11. Summary of the main findings in studies I-III

Study	Viral vector	CNS Target Area	Diet	Main outcomes				
				Food intake	*Body composition (Adiposity)*	*Hemodynamic and SNS effects*	*Glucose metabolism*	*Locomotion*
I	α-MSH	ARC	45% Fat	NS	na	na	↑	na
II	α-MSH	NTS	10% Fat	NS	↓	↑	NS	NS
III	γ-MSH	ARC	Chow / 40% Fat	↑ (Fasting)	↓ (Chow)	NS	NS	↑ (Chow)

↑ = Increase ↓ = Decrease NS = non significant NA = Not analyzed

6.2.1 Food intake and weight development

The present studies showed that chronic α-MSH overexpression in the hypothalamus attenuated weight development in mice on a high-fat diet without affecting food intake. The α-MSH treatment in the brain stem was unable to significantly impact on weight or food intake during experiment. On the other hand, γ-MSH overexpression in the hypothalamus decreased the weight of obese mice when they were returned to the low-fat chow diet without affecting food intake. In addition, fasting for 12 hours increased the 24-hour feeding in the γ-MSH treatment group.

The acute and chronic effects of melanocortins are distinct and the reduction of certain effects over time is the result of desensitization caused by receptor internalization and other compensatory mechanisms (Shinyama, *et al.* 2003). Acutely, α-MSH administration to the ventricular cavity and directly to the paraventricular nucleus of the hypothalamus has decreased food intake (Kask, *et al.* 2000, McMinn, *et al.* 2000, Soos, *et al.* 2010, Wirth, *et al.* 2001, Zhang, *et al.* 2010). α-MSH is synthesized in first-order neurons in the ARC, which project to second-order neurons in PVN, LHA and also to the NTS of the brain stem (Berthoud, *et al.* 2006, Elmquist, *et al.* 1999, Williams, *et al.* 2000, Zheng, *et al.* 2005) (Figure 22). The primary target of second-order neurons in the PVN is MC4R and animals deficient of MC4R do not respond to the anorexigenic effects of melanocortin agonists (Chen, *et al.* 2000, Marsh, *et al.* 1999). Hypophagia is among the effects that is diminished after prolonged exposure of MC3R/MC4R agonist, in genetic targeting of POMC and in the present studies (Kievit, *et al.* 2013, Li, *et al.* 2003, Li, *et al.* 2005, Li, *et al.* 2007, Zhang, *et al.* 2010).

The roles of γ-MSH and the activation of MC3R in food intake and weight development have remained unclear. As mentioned in previous sections, the MC3R-deficient mice represent an interesting metabolic phenotype with increased fat to lean mass ratio, impaired lipolysis under fast, decreased fasting induced re-feeding and defective circadian rhythm in food expectancy (see Butler. 2006). As compared to

α-MSH, there are very few studies characterizing the metabolic effects of γ-MSH. Infusion of a MC3R agonist directly to the hypothalamus and to the periphery was shown to increase food intake (Lee*, et al.* 2008, Marks*, et al.* 2006). Moreover, Cowley and colleagues (Cowley*, et al.* 2001) showed that γ-MSH could directly inhibit the electrophysiological activity in POMC neurons (Figure 22). By using lentiviral gene therapy, it was hoped to investigate the chronic effects of γ-MSH in the hypothalamus. During re-feeding measurements on the chow diet after a 12-hour fast, a small increase in food intake was also observed, which should de facto be related to an inhibition of anorexigenic activity after the acute re-feeding phase. Thus, the small increase in fasting induced re-feeding, which may in fact represent POMC auto-inhibition. Evidence seems to suggest that the mechanisms of γ-MSH in the hypothalamus are more complicated concerning food intake and weight development than has previously been though since the treatment caused significant weight loss in the DIO mice that had been reintroduced to the standard low-fat diet.

6.2.2 Body composition

The present studies demonstrated that α-MSH overexpression in the hypothalamus decreased obesity and the size of metabolically active fat depots, whereas the treatment attenuated body adiposity without significantly affecting weight development and food intake in the brain stem. γ-MSH was demonstrated to exert a positive modulatory effect on body composition, which was dependent on the ingestion of the low fat standard chow diet.

The central melanocortin system has been directly linked to peripheral lipid metabolism (Nogueiras*, et al.* 2007) and the long-term anti-obesity effects of melanocortins have been associated with increased SNS activity, which drives lipolysis and energy expenditure. The increase in energy expenditure by melanocortins is at least partly due to direct activation of SNS-mediated BAT thermogenesis. Recent data have directly linked the melanocortin system to peripheral lipid metabolism and one of the target sites of melanocortins, the PVN has been shown to directly innervate WAT (Bowers*, et al.* 2004, Nogueiras*, et al.* 2007, Shi & Bartness. 2001) (Figure 22). The MC4R has been shown to activate distinct SNS innervation to ‘BAT and WAT (Brito*, et al.* 2007, Song*, et al.* 2005, Song*, et al.* 2008)and regulate both sympathetic and parasympathetic preganglionic neurons (Sohn*, et al.* 2013).

With respects to the brain stem, it was concluded that α-MSH overexpression reduced fat mass without impacting on the weight under the observation period. Moreover, although food intake was transiently decreased over the first few days, α-MSH overexpression in the NTS also increased SNS-activity without affecting BAT thermogenesis at in the end of the experiment (Figure 22).

γ-MSH overexpression in the ARC attenuated the increase in fat to lean mass ratio, but surprisingly only on the standard rodent chow diet. The modulation of body composition did not result in significant changes in weight or total food intake. Interestingly, when the diet was changed to a high-fat Western diet, the positive effects

in body composition became attenuated. In an effort to clarify the effects of the diet in the γ-MSH treated animals, the high fat western type diet was changed to standard low-fat chow. The γ-MSH treatment decreased weight more than occurred in the controls and this reduction was dependent on a reduction in fat mass. These results indicate that central MC3Rs could indeed be involved in the lipolysis of WAT in the periphery (Figure 22). Moreover, the MC3R-deficient mice have increased respiratory exchange ratio (RER) and thus prefer carbohydrates as the main energy source (Butler & Cone. 2003). Overexpression of γ-MSH in the hypothalamus might shift the preference towards fat utilization.

6.2.3 Metabolic, hemodynamic and other effects

Glucose metabolism was assessed by GTT and analysis of serum glucose and insulin levels from the collected blood of sacrificed animals. Other parameters included analysis of serum lipids and activity assessment of BAT and adrenal gland. Urine was collected in metabolic cages to permit the measurement of excreted creatinine. Locomotor activity was assessed in voluntary running wheels and in telemetric cages. Hemodynamic parameters were analyzed using telemetric devices and tail-cuff measurements.

Hypothalamic α-MSH overexpression improved glucose clearance, however, the other measured parameters were similar. Improved glucose clearance in the α-MSH treated mice correlated with a decreased overall obesity. Assessment of hemodynamic parameters and SNS activity was not implemented because of the existing consensus about the effects of α-MSH and analogues on hemodynamic regulation in the hypothalamus.

Mice treated with the LVi-α-MSH-EGFP virus displayed a significantly decreased urine secretion without affecting their drinking behavior. It appeared that renal function had been affected and it was hypothesized that in the brain stem, α-MSH was either affecting renal SNS activity or decreasing blood pressure. It was possible to detect a tendency towards an increased blood pressure in the α-MSH treated mice using the tail-cuff method, which eventually was the reason for resorting to telemetric implantation and a more accurate assessment of the hemodynamic effects using radiotelemetry. The α-MSH treated mice exhibited increased heart rate during the recovery phase and acutely when mice were administered saline. Furthermore, the α-MSH treated animals had attenuated tachycardic response when administered with both adrenergic and parasympathetic blockers suggesting increased sympathetic activity and decreased vagal tone in cardiac control. The expression of α-MSH was localized in the brain stem, indicating an involvement of α-MSH in this brain region in the increase in SNS activity and vagal tone (Figure 22). There were no differences in glucose metabolism, which was reflected in feeding behavior and in weight development.

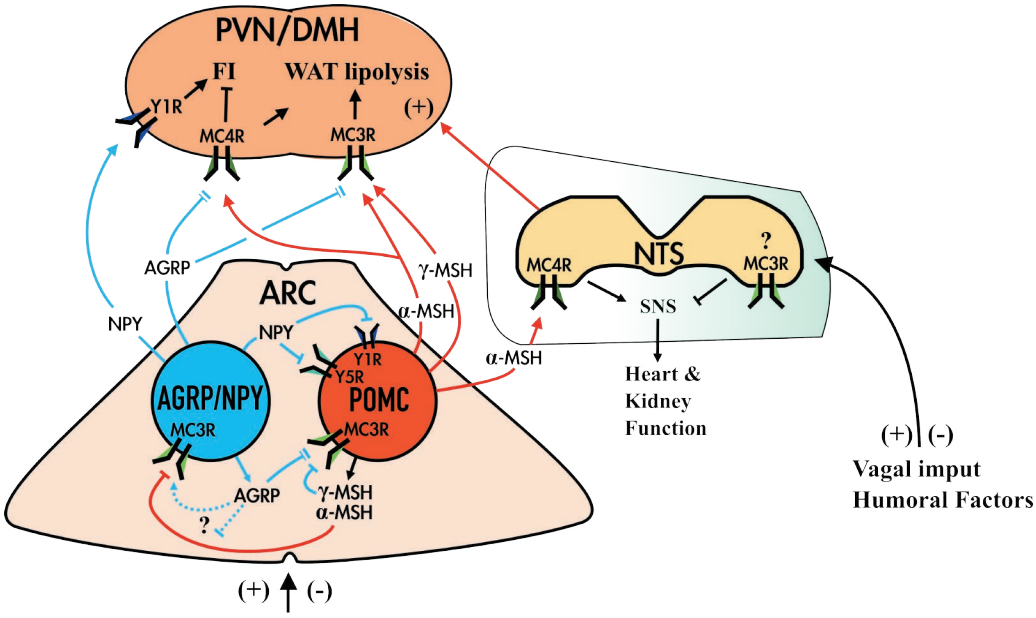

Figure 22. Schematic presentation of the chronic effects of melanocortins in the regulation of body metabolism in the CNS. Orexigenic (blue), anorexigenic (red) projections and factors with positive (+) and negative role (-) involved in the regulation of food intake, energy expenditure, thermogenesis, body composition and SNS-activity. AGRP is an inverse agonist of MC-receptors, whereas NPY acts on Y-receptors to increase feeding and decrease energy expenditure. γ-MSH seems to act as an auto-receptor on POMC neurons in certain situations but the effect on AGRP/NPY neurons is unclear. α-MSH modulates body composition by reducing fat mass, but have cardioaccelerator and renal effects related to increased SNS-activity modulated by the NTS.

We assessed the locomotion of mice treated with LVi-γ-MSH-EGFP using voluntary running wheel activity in addition to basal glucose, glucose tolerance, BAT UCP1 mRNA and tyrosine hydroxylase mRNA levels in the adrenal gland in order to assess the level of SNS activity. As mentioned in section (6.2.1.2), the γ-MSH treatment increased voluntary running wheel activity on the high-fat Western-type diet. The contributing factor for this effect is not known, but it might relate to food seeking behavior, which has been speculated in some studies (Butler. 2006, Herwig, *et al.* 2013). γ-MSH treatment did not have any direct impact on glucose metabolism, although the mice displayed lower fat mass. Moreover, the UCP1 and TH levels were similar, suggesting that the overall SNS activity was unchanged.

We were unable to conduct hemodynamic analysis of LVi-γ-MSH-EGFP mice. Nevertheless, the MC3R is involved in hemodynamic regulation both centrally and peripherally. MC3R-deficent mice have been reported to be more prone to salt-induced hypertension that is attributed to a decreased natriuretic action (Humphreys, *et al.* 2011). Furthermore, γ-MSH has been shown to both decrease and increase blood pressure (De Wildt, *et al.* 1994, Huang & Leenen. 2002, Li, *et al.* 1996, Nishimura, *et al.* 2000). The pressor effects associated with γ-MSH infusion centrally seem to be dependent on

the activation of FMRFamide-gated sodium channels, which causes sympathoexcitation (Ni, *et al.* 2006). Microinjection of γ-MSH to the NTS has been shown to decrease blood pressure, which suggest that γ-MSH may be involved in the regulation of baroreflex function (Humphreys, *et al.* 2011) (Figure 22). The chronic effects of γ-MSH on hemodynamic function centrally in the hypothalamus and brain stem are not known and would therefore be interesting targets of investigation with the LVi-γ-MSH-EGFP lentivirus vector.

6.3 COMPLEXITY OF THE CNS

The melanocortin system indisputably plays an important role in the regulation of body metabolism and energy intake, but is also involved in an array of other functions both centrally and peripherally. Furthermore, the POMC mediated neuropeptides are accompanied by several other elements in the CNS, which include different neuronal subtypes and a multitude of different receptors and their effector molecules (for more information 2.1.1.5). Not only is the specific location of where the neuropeptides are released important, but also several different modulators can impact on the outcome. Moreover, both receptor expression and effector availability are regulated and in most cases by several non-related mechanisms. In addition, many functions in the CNS are tightly interconnected. When assessing body metabolism and food intake, the emotional and behavioral aspects must also be contemplated. Assimilation of this level of complexity is extremely demanding and contradicts the desire of researchers to simplify the mechanisms involved. At present, scientists are still only appreciating the level of overlap in the CNS and there needs to be an overwhelming consensus about each independent mechanism before they can be convincingly inter-related.

6.4 THERAPEUTIC ASPECT OF MELANOCORTINS

While melanocortins do seem to possess the therapeutic potential, there are several hurdles which need to be overcome. Firstly, the anti-obesity effects of melanocortins are centrally mediated and thus synthetic agonists of melanocortin receptors are required to penetrate through the BBB. Secondly, MC4R agonists increase sympathetic nervous activity, which leads to hemodynamic changes and this is a major cause for concern. Thirdly, the anti-obesity effects of MC4R agonists attenuate over time, due to receptor internalization and elevations in the levels of the degrading enzymes and in other opposing factors (Kumar, *et al.* 2009, Shinyama, *et al.* 2003, Wallingford, *et al.* 2009).

In 2009, Kumar and coworkers tested several newly developed melanocortin agonists in MC3R- and MC4R-deficient animals and revealed that their anti-obesity effects, including the reduction in food intake, were MC4R-dependent (Kumar, *et al.* 2009). The agonist that caused sustained anti-obesity effects, BIM-22493, was then taken to a pre-clinical trial in Rhesus macaques where it also decreased obesity, but also decreased blood pressure. BIM-22493 caused a transient decrease in food intake, which suggests that other factors contributed to the anti-obesity effects. These results also indicated that BIM-22493 did not cause receptor internalization and most importantly, it did not cause

adverse SNS-related effects. Since melanocortins are able to cause activation of several different intracellular signaling pathways, there is the possibility that BIM-22493 was able to cause activation in one or several cascades that were responsible for the anti-obesity effects, but not in those that cause SNS-activation. RM-493 (BIM-22493) is currently undergoing phase II trials.

The therapeutic potential of MC3R agonists have not been thoroughly investigated and the present studies revealed that an MC3R agonist could prove useful in diet intervention since the γ-MSH overexpressing lentiviral vector targeted to the hypothalamus decreased fat mass even when mice were consuming low-fat. Although the MC3R-deficient mice have helped to clarify many of the effects associated with MC3R action, there are only a few studies that have investigated the effects of exclusive stimulation of this single receptor subtype (Lee M. et al 2008; Marks DL et al 2006). Studies of chronic stimulation of MC3R/MC4R with γ-MSH have not been conducted and the results presented in this thesis are therefore novel. Previous studies in laboratory rodents have indicated MC3R agonists to be responsible for hyperphagia and inhibition of the anorexigenic POMC neurons (Cowley*, et al.* 2001, Lee*, et al.* 2008, Marks*, et al.* 2006) and thus have resulted in the conclusion that MC3R lacks therapeutic potential in combatting obesity. It was found that γ-MSH over-expression in the hypothalamus caused hyperphagia after fasting, but not under normal circumstances. Moreover, the treatment had no impact on weight gain or body composition when animals consumed the high-fat diet, but it did reduce fat mass with the standard low-fat diets. Since the phenotype of MC3R-deficient mice shares similar characteristics with the symptoms of patients suffering from Cushing's syndrome *e.g.* increased adiposity, decreased lean mass, decreased immune function, elevated corticosterone, defective fasting induced HPA-axis activation and increased sensitivity to salt-induced hypertension (Butler*, et al.* 2000, Chen*, et al.* 2000, Ellacott*, et al.* 2007, Ni*, et al.* 2003, Renquist*, et al.* 2012), it would be interesting to investigate the therapeutic potential of MC3R-agonists in the treatment of this syndrome.

In conclusion, although the therapeutic benefits of melanocortin receptor agonists have been questioned due to adverse effects, the true potential of the activation of both central and peripheral melanocortin agonist are still emerging. Recently, a selective MC4R agonist has been shown to lack adverse SNS effects and the present investigations of long-term central γ-MSH over-expression have shown that this compound can cause a decrease in adiposity without elevating SNS-activity. In summary, the melanocortins still hold potential as anti-obesity therapeutics.

7. SUMMARY AND CONCLUSIONS

Although α-MSH is anorexigenic with acute administrations, the long-term effect on obesity was independent of food intake in the hypothalamus and brain stem. Treatment with α-MSH decreased the fat mass and elevated SNS-activity in the brain stem. On the other hand, hypothalamic γ-MSH treatment reduced adiposity, but only when animals were fed the standard low-fat diet and in that situation it did not affect overall SNS activity. The main findings and conclusions of the thesis are presented below:

1. High titer lentiviral gene delivery vectors overexpressing γ-and α-MSH were generated and shown to induce long-term expression of biologically active peptides in vitro and in vivo.

2. The anti-obesity effects of long-term α-MSH overexpression in the arcuate nucleus of the hypothalamus in diet induced obese mice were shown to be independent of any reduction in food intake.

3. The long-term effects of α-MSH-OE in the nucleus tractus solitarus of the brain stem in diet induced obese mice were decreased adiposity without significant effect on food intake during the course of the study. Radiotelemetric assessment showed that the treatment increased heart rate, which was attenuated by adrenergic blockade. In addition, the treatment decreased urine secretion, which together with hemodynamic assessment pointed towards SNS-activation.

4. The presence of γ-MSH-OE in arcuate nucleus of the hypothalamus improved adiposity without affecting weight development on the standard diet. However, feeding the high fat diet abolished this effect. The treatment decreased weight and fat mass in obese mice when the diet was changed back to the standard diet. In addition, the treatment also increased voluntary running wheel activity and re-feeding after fasting, two observations which are opposite to the findings observed in MC3R-deficient mice.

In conclusion, the outcome of the investigations conducted in this thesis indicates that central α-MSH mediated food independent anti-obesity effects over the long-term. Furthermore, the results demonstrated a potential therapeutic role for γ-MSH in the obesity when combined with dietary intervention.

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REFERENCES

Abbott CR, Rossi M, Kim M, AlAhmed SH, Taylor GM, Ghatei MA, Smith DM & Bloom SR 2000 Investigation of the melanocyte stimulating hormones on food intake. lack of evidence to support a role for the melanocortin-3-receptor. *Brain research* **869** 203-210.

Abdel-Malek Z, Swope VB, Suzuki I, Akcali C, Harriger MD, Boyce ST, Urabe K & Hearing VJ 1995 Mitogenic and melanogenic stimulation of normal human melanocytes by melanotropic peptides. *Proceedings of the National Academy of Sciences of the United States of America* **92** 1789-1793.

Abdel-Malek ZA 2001 Melanocortin receptors: Their functions and regulation by physiological agonists and antagonists. *Cellular and molecular life sciences : CMLS* **58** 434-441.

Acuna-Goycolea C, Tamamaki N, Yanagawa Y, Obata K & van den Pol AN 2005 Mechanisms of neuropeptide Y, peptide YY, and pancreatic polypeptide inhibition of identified green fluorescent protein-expressing GABA neurons in the hypothalamic neuroendocrine arcuate nucleus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25** 7406-7419.

Adan RA, Oosterom J, Toonen RF, Kraan MV, Burbach JP & Gispen WH 1997 Molecular pharmacology of neural melanocortin receptors. *Receptors & channels* **5** 215-223.

Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E & Flier JS 1996 Role of leptin in the neuroendocrine response to fasting. *Nature* **382** 250-252.

al Yacoub N, Romanowska M, Haritonova N & Foerster J 2007 Optimized production and concentration of lentiviral vectors containing large inserts. *The journal of gene medicine* **9** 579-584.

Albarado DC, McClaine J, Stephens JM, Mynatt RL, Ye J, Bannon AW, Richards WG & Butler AA 2004 Impaired coordination of nutrient intake and substrate oxidation in melanocortin-4 receptor knockout mice. *Endocrinology* **145** 243-252.

Allison DB, Kaprio J, Korkeila M, Koskenvuo M, Neale MC & Hayakawa K 1996 The heritability of body mass index among an international sample of monozygotic twins reared apart. *International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity* **20** 501-506.

Alonso LG & Maren TH 1955 Effect of food restriction on body composition of hereditary obese mice. *The American Journal of Physiology* **183** 284-290.

Andino LM, Ryder DJ, Shapiro A, Matheny MK, Zhang Y, Judge MK, Cheng KY, Tumer N & Scarpace PJ 2011 POMC overexpression in the ventral tegmental area ameliorates dietary obesity. *The Journal of endocrinology* **210** 199-207.

Aoki H & Moro O 2002 Involvement of microphthalmia-associated transcription factor (MITF) in expression of human melanocortin-1 receptor (MC1R). *Life Sciences* **71** 2171-2179.

Aponte Y, Atasoy D & Sternson SM 2011 AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nature neuroscience* **14** 351-355.

Asai M, Ramachandrappa S, Joachim M, Shen Y, Zhang R, Nuthalapati N, Ramanathan V, Strochlic DE, Ferket P, Linhart K *et al* 2013 Loss of function of the melanocortin 2 receptor accessory protein 2 is associated with mammalian obesity. *Science (New York, N.Y.)* **341** 275-278.

Babinski MJ 1900 Tumeur du corps pituitaire san acromegalie et avec arret de developpement des organes genitaux. . *Rev. Neurol* **8** 531–533.

Bai FL, Yamano M, Shiotani Y, Emson PC, Smith AD, Powell JF & Tohyama M 1985 An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. *Brain research* **331** 172-175.

Balleine BW, Killcross AS & Dickinson A 2003 The effect of lesions of the basolateral amygdala on instrumental conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23** 666-675.

Baltatzi M, Hatzitolios A, Tziomalos K, Iliadis F & Zamboulis C 2008 Neuropeptide Y and alpha-melanocyte-stimulating hormone: Interaction in obesity and possible role in the development of hypertension. *International journal of clinical practice* **62** 1432-1440.

Banks WA 2006 Blood-brain barrier and energy balance. *Obesity (Silver Spring, Md.)* **14 Suppl 5** 234S-237S.

Barry MA, Weaver EA & Chen CY 2012 Mining the adenovirus "virome" for systemic oncolytics. *Current Pharmaceutical Biotechnology* **13** 1804-1808.

Bartke A & Gorecki A 1968 Oxygen consumption by obese yellow mice and their normal littermates. *The American Journal of Physiology* **214** 1250-1252.

Bartness TJ & Song CK 2007 Thematic review series: Adipocyte biology. sympathetic and sensory innervation of white adipose tissue. *Journal of lipid research* **48** 1655-1672.

Becher E, Mahnke K, Brzoska T, Kalden DH, Grabbe S & Luger TA 1999 Human peripheral blood-derived dendritic cells express functional melanocortin receptor MC-1R. *Annals of the New York Academy of Sciences* **885** 188-195.

Beck B, Stricker-Krongrad A, Nicolas JP & Burlet C 1992 Chronic and continuous intracerebroventricular infusion of neuropeptide Y in long-evans rats mimics the feeding behaviour of obese zucker rats. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* **16** 295-302.

Bennett HP 1986 Biosynthetic fate of the amino-terminal fragment of pro-opiomelanocortin within the intermediate lobe of the mouse pituitary. *Peptides* **7** 615-622.

Bergen WG, Kaplan ML, Merkel RA & Leveille GA 1975 Growth of adipose and lean tissue mass in hindlimbs of genetically obese mice during preobese and obese phases of development. *The American Journal of Clinical Nutrition* **28** 157-161.

Bertagna X, Lenne F, Comar D, Massias JF, Wajcman H, Baudin V, Luton JP & Girard F 1986 Human beta-melanocyte-stimulating hormone revisited. *Proceedings of the National Academy of Sciences of the United States of America* **83** 9719-9723.

Berthoud HR, Sutton GM, Townsend RL, Patterson LM & Zheng H 2006 Brainstem mechanisms integrating gut-derived satiety signals and descending forebrain information in the control of meal size. *Physiology & Behavior* **89** 517-524.

Bhardwaj RS, Schwarz A, Becher E, Mahnke K, Aragane Y, Schwarz T & Luger TA 1996 Pro-opiomelanocortin-derived peptides induce IL-10 production in human monocytes. *Journal of immunology (Baltimore, Md.: 1950)* **156** 2517-2521.

Biebermann H, Castaneda TR, van Landeghem F, von Deimling A, Escher F, Brabant G, Hebebrand J, Hinney A, Tschop MH, Gruters A *et al* 2006 A role for beta-melanocyte-stimulating hormone in human body-weight regulation. *Cell metabolism* **3** 141-146.

Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, Vale W & Sawchenko PE 1992 The melanin-concentrating hormone system of the rat brain: An immuno- and hybridization histochemical characterization. *The Journal of comparative neurology* **319** 218-245.

Bodnar RJ & Klein GE 2005 Endogenous opiates and behavior: 2004. *Peptides* **26** 2629-2711.

Bohm M, Schulte U, Kalden H & Luger TA 1999 Alpha-melanocyte-stimulating hormone modulates activation of NF-kappa B and AP-1 and secretion of interleukin-8 in human dermal fibroblasts. *Annals of the New York Academy of Sciences* **885** 277-286.

Boileau G, Lariviere N, Hsi KL, Seidah NG & Chretien M 1982 Characterization of multiple forms of porcine anterior pituitary proopiomelanocortin amino-terminal glycopeptide. *Biochemistry* **21** 5341-5346.

Boston BA & Cone RD 1996 Characterization of melanocortin receptor subtype expression in murine adipose tissues and in the 3T3-L1 cell line. *Endocrinology* **137** 2043-2050.

Bouchard C 1991 Heredity and the path to overweight and obesity. *Medicine and science in sports and exercise* **23** 285-291.

Bouret SG, Draper SJ & Simerly RB 2004 Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24** 2797-2805.

Bowers RR, Festuccia WT, Song CK, Shi H, Migliorini RH & Bartness TJ 2004 Sympathetic innervation of white adipose tissue and its regulation of fat cell number. *American journal of physiology.Regulatory, integrative and comparative physiology* **286** R1167-75.

Bray GA & York DA 1979 Hypothalamic and genetic obesity in experimental animals: An autonomic and endocrine hypothesis. *Physiological Reviews* **59** 719-809.

Breit A, Wolff K, Kalwa H, Jarry H, Buch T & Gudermann T 2006 The natural inverse agonist agouti-related protein induces arrestin-mediated endocytosis of melanocortin-3 and -4 receptors. *The Journal of biological chemistry* **281** 37447-37456.

Brito MN, Brito NA, Baro DJ, Song CK & Bartness TJ 2007 Differential activation of the sympathetic innervation of adipose tissues by melanocortin receptor stimulation. *Endocrinology* **148** 5339-5347.

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

Buch TR, Heling D, Damm E, Gudermann T & Breit A 2009 Pertussis toxin-sensitive signaling of melanocortin-4 receptors in hypothalamic GT1-7 cells defines agouti-related protein as a biased

agonist. *The Journal of biological chemistry* **284** 26411-26420.

Buckley DI & Ramachandran J 1981 Characterization of corticotropin receptors on adrenocortical cells. *Proceedings of the National Academy of Sciences of the United States of America* **78** 7431-7435.

Buggy JJ 1998 Binding of alpha-melanocyte-stimulating hormone to its G-protein-coupled receptor on B-lymphocytes activates the jak/STAT pathway. *The Biochemical journal* **331 (Pt 1)** 211-216.

Bultman SJ, Michaud EJ & Woychik RP 1992 Molecular characterization of the mouse agouti locus. *Cell* **71** 1195-1204.

Butler AA 2006 The melanocortin system and energy balance. *Peptides* **27** 281-290.

Butler AA & Cone RD 2003 Knockout studies defining different roles for melanocortin receptors in energy homeostasis. *Annals of the New York Academy of Sciences* **994** 240-245.

Butler AA & Cone RD 2002 The melanocortin receptors: Lessons from knockout models. *Neuropeptides* **36** 77-84.

Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M & Cone RD 2000 A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* **141** 3518-3521.

Callahan MF, Kirby RF, Johnson AK & Gruber KA 1988 Sympathetic terminal mediation of the acute cardiovascular response of gamma 2-MSH. *Journal of the Autonomic Nervous System* **24** 179-182.

Campfield LA, Smith FJ, Guisez Y, Devos R & Burn P 1995 Recombinant mouse OB protein: Evidence for a peripheral signal linking adiposity and central neural networks. *Science (New York, N.Y.)* **269** 546-549.

Cannon WB 1932 *The wisdom of the body*, p., 1 l., 19-312Anonymous New York: W. W. Norton & company, inc.

Carpenter KJ & Mayer J 1958 Physiologic observations on yellow obesity in the mouse. *The American Journal of Physiology* **193** 499-504.

Castle WE 1941 Influence of certain color mutations on body size in mice, rats, and rabbits. *Genetics* **26** 177-191.

Catania A, Gatti S, Colombo G & Lipton JM 2004 Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacological reviews* **56** 1-29.

Catania A, Lonati C, Sordi A, Carlin A, Leonardi P & Gatti S 2010 The melanocortin system in control of inflammation. *TheScientificWorldJournal* **10** 1840-1853.

Catania A, Lonati C, Sordi A, Carlin A, Leonardi P & Gatti S 2010 The melanocortin system in control of inflammation. *TheScientificWorldJournal* **10** 1840-1853.

Cawley NX, Zhou J, Hill JM, Abebe D, Romboz S, Yanik T, Rodriguiz RM, Wetsel WC & Loh YP 2004 The carboxypeptidase E knockout mouse exhibits endocrinological and behavioral deficits. *Endocrinology* **145** 5807-5819.

Cecil JE, Tavendale R, Watt P, Hetherington MM & Palmer CN 2008 An obesity-associated FTO gene variant and increased energy intake in children. *The New England journal of medicine* **359** 2558-2566.

Cespuglio R, Marinesco S, Baubet V, Bonnet C & el Kafi B 1995 Evidence for a sleep-promoting influence of stress. *Advances in Neuroimmunology* **5** 145-154.

Cettour-Rose P & Rohner-Jeanrenaud F 2002 The leptin-like effects of 3-d peripheral administration of a melanocortin agonist are more marked in genetically obese zucker (fa/fa) than in lean rats. *Endocrinology* **143** 2277-2283.

Chai B, Li JY, Zhang W, Wang H & Mulholland MW 2009 Melanocortin-4 receptor activation inhibits c-jun N-terminal kinase activity and promotes insulin signaling. *Peptides* **30** 1098-1104.

Chai B, Li JY, Zhang W, Ammori JB & Mulholland MW 2007 Melanocortin-3 receptor activates MAP kinase via PI3 kinase. *Regulatory peptides* **139** 115-121.

Chalfie M 1995 Green fluorescent protein. *Photochemistry and photobiology* **62** 651-656.

Challis BG, Pritchard LE, Creemers JW, Delplanque J, Keogh JM, Luan J, Wareham NJ, Yeo GS, Bhattacharyya S, Froguel P *et al* 2002 A missense mutation disrupting a dibasic prohormone processing site in pro-opiomelanocortin (POMC) increases susceptibility to early-onset obesity through a novel molecular mechanism. *Human molecular genetics* **11** 1997-2004.

Chan LF, Metherell LA & Clark AJ 2011 Effects of melanocortins on adrenal gland physiology. *European journal of pharmacology* **660** 171-180.

Chehab FF, Qiu J & Ogus S 2004 The use of animal models to dissect the biology of leptin. *Recent progress in hormone research* **59** 245-266.

Chen AS, Metzger JM, Trumbauer ME, Guan XM, Yu H, Frazier EG, Marsh DJ, Forrest MJ, Gopal-Truter S, Fisher J *et al* 2000 Role of the melanocortin-4 receptor in metabolic rate and food intake in mice. *Transgenic research* **9** 145-154.

Chen AS, Metzger JM, Trumbauer ME, Guan XM, Yu H, Frazier EG, Marsh DJ, Forrest MJ, Gopal-Truter S, Fisher J *et al* 2000 Role of the melanocortin-4 receptor in metabolic rate and food intake in mice. *Transgenic research* **9** 145-154.

Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L *et al* 2000 Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nature genetics* **26** 97-102.

Chen P, Li C, Haskell-Luevano C, Cone RD & Smith MS 1999 Altered expression of agouti-related protein and its colocalization with neuropeptide Y in the arcuate nucleus of the hypothalamus during lactation. *Endocrinology* **140** 2645-2650.

Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ & Cone RD 1997 Exocrine gland dysfunction in MC5-R-deficient mice: Evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* **91** 789-798.

Chen XW, Ying WZ, Valentin JP, Ling KT, Lin SY, Wiedemann E & Humphreys MH 1997 Mechanism of the natriuretic action of gamma-melanocyte-stimulating hormone. *The American Journal of Physiology* **272** R1946-53.

Cheung CC, Clifton DK & Steiner RA 1997 Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* **138** 4489-4492.

Chhajlani V 1996 Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochemistry and molecular biology international* **38** 73-80.

Chhajlani V & Wikberg JE 1992 Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS letters* **309** 417-420.

Chomczynski P & Sacchi N 2006 The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. *Nature protocols* **1** 581-585.

Chua S,Jr, Liu SM, Li Q, Yang L, Thassanapaff VT & Fisher P 2002 Differential beta cell responses to hyperglycaemia and insulin resistance in two novel congenic strains of diabetes (FVB- lepr (db)) and obese (DBA- lep (ob)) mice. *Diabetologia* **45** 976-990.

Church C, Moir L, McMurray F, Girard C, Banks GT, Teboul L, Wells S, Bruning JC, Nolan PM, Ashcroft FM *et al* 2010 Overexpression of fto leads to increased food intake and results in obesity. *Nature genetics* **42** 1086-1092.

Clark JT, Kalra PS & Kalra SP 1985 Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. *Endocrinology* **117** 2435-2442.

Coleman DL & Eicher EM 1990 Fat (fat) and tubby (tub): Two autosomal recessive mutations causing obesity syndromes in the mouse. *The Journal of heredity* **81** 424-427.

Coleman DL & Hummel KP 1973 The influence of genetic background on the expression of the obese (ob) gene in the mouse. *Diabetologia* **9** 287-293.

Coll AP, Farooqi IS, Challis BG, Yeo GS & O'Rahilly S 2004 Proopiomelanocortin and energy balance: Insights from human and murine genetics. *The Journal of clinical endocrinology and metabolism* **89** 2557-2562.

Collins S, Martin TL, Surwit RS & Robidoux J 2004 Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: Physiological and molecular characteristics. *Physiology & Behavior* **81** 243-248.

Cone RD 2000 Haploinsufficiency of the melanocortin-4 receptor: Part of a thrifty genotype? *The Journal of clinical investigation* **106** 185-187.

Cooray SN & Clark AJ 2011 Melanocortin receptors and their accessory proteins. *Molecular and cellular endocrinology* **331** 215-221.

Cowley MA, Pronchuk N, Fan W, Dinulescu DM, Colmers WF & Cone RD 1999 Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: Evidence of a cellular basis for the adipostat. *Neuron* **24** 155-163.

Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD & Low MJ 2001 Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* **411** 480-484.

Cox JE & Powley TL 1977 Development of obesity in diabetic mice pair-fed with lean siblings. *Journal of comparative and physiological psychology* **91** 347-358.

Cragnolini A, Scimonelli T, Celis ME & Schioth HB 2000 The role of melanocortin receptors in sexual behavior in female rats. *Neuropeptides* **34** 211-215.

Cuneot L 1905 Les races pures et leurs combinaisons chez les souris. *Arch Xool Exp Gen* **3** 123-132.

De Castro JM 1995 Social facilitation of food intake in humans. *Appetite* **24** 260.

de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS,2nd *et al* 1998 The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. *Proceedings of the National Academy of Sciences of the United States of America* **95** 322-327.

De Wildt DJ, Van der Ven JC, Van Bergen P, De Lang H & Versteeg DH 1994 A hypotensive and bradycardic action of gamma 2-melanocyte-stimulating hormone

(gamma 2-MSH) microinjected into the nucleus tractus solitarii of the rat. *Naunyn-Schmiedeberg's archives of pharmacology* **349** 50-56.

Della-Zuana O, Presse F, Ortola C, Duhault J, Nahon JL & Levens N 2002 Acute and chronic administration of melanin-concentrating hormone enhances food intake and body weight in wistar and sprague-dawley rats. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* **26** 1289-1295.

Denef C, Lu J & Swinnen E 2003 Gamma-MSH peptides in the pituitary: Effects, target cells, and receptors. *Annals of the New York Academy of Sciences* **994** 123-132.

Dickerson GE & Gowen JW 1947 Hereditary obesity and efficient food utilization in mice. *Science (New York, N.Y.)* **105** 496-498.

Dickie MM & Woolley GW 1946 The age factor in weight of yellow mice; weight reduction of ageing yellows and "thin-yellows" revealed in littermate comparisons. *The Journal of heredity* **37** 365-368.

Dietrich MO, Antunes C, Geliang G, Liu ZW, Borok E, Nie Y, Xu AW, Souza DO, Gao Q, Diano S *et al* 2010 Agrp neurons mediate Sirt1's action on the melanocortin system and energy balance: Roles for Sirt1 in neuronal firing and synaptic plasticity. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30** 11815-11825.

Dittrich W & Gohde W 1969 Impulse fluorometry of single cells in suspension. *Zeitschrift fur Naturforschung.Teil B: Chemie, Biochemie, Biophysik, Biologie* **24** 360-361.

Dubuc PU 1976 The development of obesity, hyperinsulinemia, and hyperglycemia in ob/ob mice. *Metabolism: clinical and experimental* **25** 1567-1574.

Duhl DM, Stevens ME, Vrieling H, Saxon PJ, Miller MW, Epstein CJ & Barsh GS 1994 Pleiotropic effects of the mouse lethal yellow (ay) mutation explained by deletion of a maternally expressed gene and the simultaneous production of agouti fusion RNAs. *Development (Cambridge, England)* **120** 1695-1708.

Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, Tamaki M, Yoshioka T, Hayase M, Matsuoka N *et al* 1999 Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* **48** 2028-2033.

Eerola K, Rinne P, Penttinen AM, Vahatalo L, Savontaus M & Savontaus E 2014 Alpha-MSH overexpression in the nucleus tractus solitarius decreases fat mass and elevates heart rate. *The Journal of endocrinology* .

Eerola K, Nordlund W, Virtanen S, Dickens AM, Mattila M, Ruohonen ST, Chua SC,Jr, Wardlaw SL, Savontaus M & Savontaus E 2013 Lentivirus-mediated alpha-melanocyte-stimulating hormone overexpression in the hypothalamus decreases diet induced obesity in mice. *Journal of neuroendocrinology* **25** 1298-1307.

Eichhorn P, Schwandt P & Richter WO 1995 Proopiomelanocorticotropin (POMC) peptides and lipoprotein lipase activity in vitro. *Peptides* **16** 665-671.

Eipper BA & Mains RE 1980 Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocrine reviews* **1** 1-27.

Elia M & Livesey G 1988 Theory and validity of indirect calorimetry during net lipid synthesis. *The American Journal of Clinical Nutrition* **47** 591-607.

Ellacott KL, Murphy JG, Marks DL & Cone RD 2007 Obesity-induced inflammation in white adipose tissue is attenuated by loss of melanocortin-3 receptor signaling. *Endocrinology* **148** 6186-6194.

Elmquist JK 2001 Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* **25 Suppl 5** S78-82.

Elmquist JK, Elias CF & Saper CB 1999 From lesions to leptin: Hypothalamic control of food intake and body weight. *Neuron* **22** 221-232.

Entwistle ML, Hann LE, Sullivan DA & Tatro JB 1990 Characterization of functional melanotropin receptors in lacrimal glands of the rat. *Peptides* **11** 477-483.

Erickson JC, Hollopeter G & Palmiter RD 1996 Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science (New York, N.Y.)* **274** 1704-1707.

Fan W, Ellacott KL, Halatchev IG, Takahashi K, Yu P & Cone RD 2004 Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nature neuroscience* **7** 335-336.

Farese RV, Ling NC, Sabir MA, Larson RE & Trudeau WL,3rd 1983 Comparison of effects of adrenocorticotropin and lys-gamma 3-melanocyte-stimulating hormone on steroidogenesis, adenosine 3',5'-monophosphate production, and phospholipid metabolism in rat adrenal fasciculata-reticularis cells in vitro. *Endocrinology* **112** 129-132.

Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T & O'Rahilly S 2003 Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *The New England journal of medicine* **348** 1085-1095.

Farooqi S 2007 Insights from the genetics of severe childhood obesity. *Hormone research* **68 Suppl 5** 5-7.

Fitzpatric TB & Lerner AB 1953 Terminology of pigment cells. *Science (New York, N.Y.)* **117** 640.

Flood JF & Morley JE 1989 Dissociation of the effects of neuropeptide Y on feeding and memory: Evidence for pre- and postsynaptic mediation. *Peptides* **10** 963-966.

Folch J, Lees M & Sloane Stanley GH 1957 A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry* **226** 497-509.

Franklin KBJ & Paxinos G Eds. 1997 *The mouse brain in stereotaxic
 coordinates*, 1st edn. San Diego: Academic Press.

Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, Perry JR, Elliott KS, Lango H, Rayner NW *et al* 2007 A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science (New York, N.Y.)* **316** 889-894.

Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB & Flier JS 1995 Leptin levels reflect body lipid content in mice: Evidence for diet-induced resistance to leptin action. *Nature medicine* **1** 1311-1314.

Fricker LD & Snyder SH 1982 Enkephalin convertase: Purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules. *Proceedings of the National Academy of Sciences of the United States of America* **79** 3886-3890.

Fricker LD, McKinzie AA, Sun J, Curran E, Qian Y, Yan L, Patterson SD, Courchesne PL, Richards B, Levin N *et al* 2000 Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20** 639-648.

Friedman JM, Leibel RL, Siegel DS, Walsh J & Bahary N 1991 Molecular mapping of the mouse ob mutation. *Genomics* **11** 1054-1062.

Frohlich A 1902 Path of impulses for inhibition under decerebrate rigidity. *The Journal of physiology* **28** 14-19.

Fu LY, Acuna-Goycolea C & van den Pol AN 2004 Neuropeptide Y inhibits hypocretin/orexin neurons by multiple presynaptic and postsynaptic mechanisms: Tonic depression of the hypothalamic arousal system. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24** 8741-8751.

Galas L, Tonon MC, Beaujean D, Fredriksson R, Larhammar D, Lihrmann I, Jegou S, Fournier A, Chartrel N & Vaudry H 2002 Neuropeptide Y inhibits spontaneous alpha-melanocyte-stimulating hormone (alpha-MSH) release via a Y(5) receptor and suppresses thyrotropin-releasing hormone-induced alpha-MSH secretion via a Y(1) receptor in frog melanotrope cells. *Endocrinology* **143** 1686-1694.

Gantz I, Shimoto Y, Konda Y, Miwa H, Dickinson CJ & Yamada T 1994 Molecular cloning, expression, and characterization of a fifth melanocortin receptor. *Biochemical and biophysical research communications* **200** 1214-1220.

Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J & Yamada T 1993 Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *The Journal of biological chemistry* **268** 15174-15179.

Gantz I, Konda Y, Tashiro T, Shimoto Y, Miwa H, Munzert G, Watson SJ, DelValle J & Yamada T 1993 Molecular cloning of a novel melanocortin receptor. *The Journal of biological chemistry* **268** 8246-8250.

Garfield AS, Lam DD, Marston OJ, Przydzial MJ & Heisler LK 2009 Role of central melanocortin pathways in energy homeostasis. *Trends in endocrinology and metabolism: TEM* **20** 203-215.

Garthwaite TL, Martinson DR, Tseng LF, Hagen TC & Menahan LA 1980 A longitudinal hormonal profile of the genetically obese mouse. *Endocrinology* **107** 671-676.

Getting SJ 2006 Targeting melanocortin receptors as potential novel therapeutics. *Pharmacology & therapeutics* **111** 1-15.

Getting SJ & Perretti M 2000 MC3-R as a novel target for antiinflammatory therapy. *Drug news & perspectives* **13** 19-27.

Getting SJ, Christian HC, Flower RJ & Perretti M 2002 Activation of melanocortin type 3 receptor as a molecular mechanism for adrenocorticotropic hormone efficacy in gouty arthritis. *Arthritis and Rheumatism* **46** 2765-2775.

Getting SJ, Allcock GH, Flower R & Perretti M 2001 Natural and synthetic agonists of the melanocortin receptor type 3 possess anti-inflammatory properties. *Journal of leukocyte biology* **69** 98-104.

Getting SJ, Gibbs L, Clark AJ, Flower RJ & Perretti M 1999 POMC gene-derived peptides activate melanocortin type 3 receptor on murine macrophages, suppress cytokine release, and inhibit neutrophil migration in acute experimental inflammation. *Journal of immunology (Baltimore, Md.: 1950)* **162** 7446-7453.

Getting SJ, Christian HC, Lam CW, Gavins FN, Flower RJ, Schioth HB & Perretti M 2003 Redundancy of

a functional melanocortin 1 receptor in the anti-inflammatory actions of melanocortin peptides: Studies in the recessive yellow (e/e) mouse suggest an important role for melanocortin 3 receptor. *Journal of immunology (Baltimore, Md.: 1950)* **170** 3323-3330.

Ghamari-Langroudi M, Srisai D & Cone RD 2011 Multinodal regulation of the arcuate/paraventricular nucleus circuit by leptin. *Proceedings of the National Academy of Sciences of the United States of America* **108** 355-360.

Ghamari-Langroudi M, Colmers WF & Cone RD 2005 PYY3-36 inhibits the action potential firing activity of POMC neurons of arcuate nucleus through postsynaptic Y2 receptors. *Cell metabolism* **2** 191-199.

Giacomelli F & Wiener J 1979 Primary myocardial disease in the diabetic mouse. an ultrastructural study. *Laboratory investigation; a journal of technical methods and pathology* **40** 460-473.

Giesbrecht CJ, Mackay JP, Silveira HB, Urban JH & Colmers WF 2010 Countervailing modulation of ih by neuropeptide Y and corticotrophin-releasing factor in basolateral amygdala as a possible mechanism for their effects on stress-related behaviors. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30** 16970-16982.

Göhde W 1968 Automatisches meß- und zählgerät für die teilchen einer dispersion. In . Germany: .

Gordon M 1953 The influence of the mating system upon normal and atypical pigment cell growth. *Acta - Unio Internationalis Contra Cancrum* **9** 787-793.

Grieco P, Balse-Srinivasan P, Han G, Weinberg D, MacNeil T, Van der Ploeg LH & Hruby VJ 2002 Synthesis and biological evaluation on hMC3, hMC4 and hMC5 receptors of gamma-MSH analogs substituted with L-alanine. *The journal of peptide research : official journal of the American Peptide Society* **59** 203-210.

Grieger JC & Samulski RJ 2012 Adeno-associated virus vectorology, manufacturing, and clinical applications. *Methods in enzymology* **507** 229-254.

Gruber KA & Eskridge SL 1986 Central vasopressin system mediation of acute pressor effect of gamma-MSH. *The American Journal of Physiology* **251** E134-7.

Gruber KA, Eskridge-Sloop SL, Eldridge JC & Callahan MF 1989 ACTH-induced hypertension in rats: Fact or artifact? *The American Journal of Physiology* **256** R1308-12.

Guarini S, Schioth HB, Mioni C, Cainazzo M, Ferrazza G, Giuliani D, Wikberg JE, Bertolini A & Bazzani C 2002 MC(3) receptors are involved in the protective effect of melanocortins in myocardial ischemia/reperfusion-induced arrhythmias. *Naunyn-Schmiedeberg's archives of pharmacology* **366** 177-182.

Hagan MM, Rushing PA, Pritchard LM, Schwartz MW, Strack AM, Van Der Ploeg LH, Woods SC & Seeley RJ 2000 Long-term orexigenic effects of AgRP-(83--132) involve mechanisms other than melanocortin receptor blockade. *American journal of physiology. Regulatory, integrative and comparative physiology* **279** R47-52.

Hahn TM, Breininger JF, Baskin DG & Schwartz MW 1998 Coexpression of agrp and NPY in fasting-activated hypothalamic neurons. *Nature neuroscience* **1** 271-272.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK & Friedman JM 1995 Weight-reducing effects of the plasma protein encoded by the obese gene. *Science (New York, N.Y.)* **269** 543-546.

Han JC, Liu QR, Jones M, Levinn RL, Menzie CM, Jefferson-George KS, Adler-Wailes DC, Sanford EL, Lacbawan FL, Uhl GR *et al* 2008 Brain-derived neurotrophic factor and obesity in the WAGR syndrome. *The New England journal of medicine* **359** 918-927.

Harrold JA & Williams G 2006 Melanocortin-4 receptors, beta-MSH and leptin: Key elements in the satiety pathway. *Peptides* **27** 365-371.

Harrold JA, Widdowson PS & Williams G 2003 Beta-MSH: A functional ligand that regulated energy homeostasis via hypothalamic MC4-R? *Peptides* **24** 397-405.

Harrold JA, Widdowson PS & Williams G 1999 Altered energy balance causes selective changes in melanocortin-4(MC4-R), but not melanocortin-3 (MC3-R), receptors in specific hypothalamic regions: Further evidence that activation of MC4-R is a physiological inhibitor of feeding. *Diabetes* **48** 267-271.

Hartmeyer M, Scholzen T, Becher E, Bhardwaj RS, Schwarz T & Luger TA 1997 Human dermal microvascular endothelial cells express the melanocortin receptor type 1 and produce increased levels of IL-8 upon stimulation with alpha-melanocyte-stimulating hormone. *Journal of immunology (Baltimore, Md.: 1950)* **159** 1930-1937.

Haworth CM, Plomin R, Carnell S & Wardle J 2008 Childhood obesity: Genetic and environmental overlap with normal-range BMI. *Obesity (Silver Spring, Md.)* **16** 1585-1590.

Hay DL, Poyner DR & Sexton PM 2006 GPCR modulation by RAMPs. *Pharmacology & therapeutics* **109** 173-197.

Herwig A, de Vries EM, Bolborea M, Wilson D, Mercer JG, Ebling FJ, Morgan PJ & Barrett P

2013 Hypothalamic ventricular ependymal thyroid hormone deiodinases are an important element of circannual timing in the siberian hamster (phodopus sungorus). *PloS one* **8** e62003.

Hill C & Dunbar JC 2002 The effects of acute and chronic alpha melanocyte stimulating hormone (alphaMSH) on cardiovascular dynamics in conscious rats. *Peptides* **23** 1625-1630.

Hinney A, Volckmar AL & Knoll N 2013 Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis. *Progress in molecular biology and translational science* **114** 147-191.

Hinney A, Becker I, Heibult O, Nottebom K, Schmidt A, Ziegler A, Mayer H, Siegfried W, Blum WF, Remschmidt H *et al* 1998 Systematic mutation screening of the pro-opiomelanocortin gene: Identification of several genetic variants including three different insertions, one nonsense and two missense point mutations in probands of different weight extremes. *The Journal of clinical endocrinology and metabolism* **83** 3737-3741.

Hinney A, Bettecken T, Tarnow P, Brumm H, Reichwald K, Lichtner P, Scherag A, Nguyen TT, Schlumberger P, Rief W *et al* 2006 Prevalence, spectrum, and functional characterization of melanocortin-4 receptor gene mutations in a representative population-based sample and obese adults from germany. *The Journal of clinical endocrinology and metabolism* **91** 1761-1769.

Hook VY, Eiden LE & Brownstein MJ 1982 A carboxypeptidase processing enzyme for enkephalin precursors. *Nature* **295** 341-342.

Horvath TL, Diano S & van den Pol AN 1999 Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: A novel circuit implicated in metabolic and endocrine regulations. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19** 1072-1087.

Huang BS & Leenen FH 2002 Brain amiloride-sensitive phe-met-arg-phe-NH(2)--gated na(+) channels and na(+)-induced sympathoexcitation and hypertension. *Hypertension* **39** 557-561.

Hummel KP, Coleman DL & Lane PW 1972 The influence of genetic background on expression of mutations at the diabetes locus in the mouse. I. C57BL-KsJ and C57BL-6J strains. *Biochemical genetics* **7** 1-13.

Hummel KP, Coleman DL & Lane PW 1972 The influence of genetic background on expression of mutations at the diabetes locus in the mouse. I. C57BL-KsJ and C57BL-6J strains. *Biochemical genetics* **7** 1-13.

Humphreys MH, Ni XP & Pearce D 2011 Cardiovascular effects of melanocortins. *European journal of pharmacology* .

Hung CC, Luan J, Sims M, Keogh JM, Hall C, Wareham NJ, O'Rahilly S & Farooqi IS 2007 Studies of the SIM1 gene in relation to human obesity and obesity-related traits. *International journal of obesity (2005)* **31** 429-434.

Hunt G, Todd C, Cresswell JE & Thody AJ 1994 Alpha-melanocyte stimulating hormone and its analogue Nle4DPhe7 alpha-MSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes. *Journal of cell science* **107 (Pt 1)** 205-211.

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD *et al* 1997 Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* **88** 131-141.

Ingalls AM, Dickie MM & Snell GD 1950 Obese, a new mutation in the house mouse. *The Journal of heredity* **41** 317-318.

Inouye S & Tsuji FI 1994 Evidence for redox forms of the aequorea green fluorescent protein. *FEBS letters* **351** 211-214.

Irminger JC, Verchere CB, Meyer K & Halban PA 1997 Proinsulin targeting to the regulated pathway is not impaired in carboxypeptidase E-deficient cpefat/cpefat mice. *The Journal of biological chemistry* **272** 27532-27534.

Jacques D, Tong Y, Dumont Y, Shen SH & Quirion R 1996 Expression of the neuropeptide Y Y1 receptor mRNA in the human brain: An in situ hybridization study. *Neuroreport* **7** 1053-1056.

Jakobsson J, Ericson C, Jansson M, Bjork E & Lundberg C 2003 Targeted transgene expression in rat brain using lentiviral vectors. *Journal of neuroscience research* **73** 876-885.

Jang SK, Krausslich HG, Nicklin MJ, Duke GM, Palmenberg AC & Wimmer E 1988 A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *Journal of virology* **62** 2636-2643.

Jansone B, Bergstrom L, Svirskis S, Lindblom J, Klusa V & Wikberg JE 2004 Opposite effects of gamma(1)- and gamma(2)-melanocyte stimulating hormone on regulation of the dopaminergic mesolimbic system in rats. *Neuroscience letters* **361** 68-71.

Jegou S, Boutelet I & Vaudry H 2000 Melanocortin-3 receptor mRNA expression in pro-opiomelanocortin neurones of the rat arcuate nucleus. *Journal of neuroendocrinology* **12** 501-505.

Jeong JK & Diano S 2014 Prolyl carboxypeptidase mRNA expression in the mouse brain. *Brain research* **1542** 85-92.

Jeong JK, Szabo G, Kelly K & Diano S 2012 Prolyl carboxypeptidase regulates energy expenditure and the thyroid axis. *Endocrinology* **153** 683-689.

Johnson PR & Hirsch J 1972 Cellularity of adipose depots in six strains of genetically obese mice. *Journal of lipid research* **13** 2-11.

Joly-Amado A, Denis RG, Castel J, Lacombe A, Cansell C, Rouch C, Kassis N, Dairou J, Cani PD, Ventura-Clapier R *et al* 2012 Hypothalamic AgRP-neurons control peripheral substrate utilization and nutrient partitioning. *The EMBO journal* **31** 4276-4288.

Joseph CG, Yao H, Scott JW, Sorensen NB, Marnane RN, Mountjoy KG & Haskell-Luevano C 2010 Gamma(2)-melanocyte stimulation hormone (gamma(2)-MSH) truncation studies results in the cautionary note that gamma(2)-MSH is not selective for the mouse MC3R over the mouse MC5R. *Peptides* **31** 2304-2313.

Kaga T, Inui A, Okita M, Asakawa A, Ueno N, Kasuga M, Fujimiya M, Nishimura N, Dobashi R, Morimoto Y *et al* 2001 Modest overexpression of neuropeptide Y in the brain leads to obesity after high-sucrose feeding. *Diabetes* **50** 1206-1210.

Kandel ER, Schwartz JH & Jassell TM 2000 *Principles of neural science*, p.1414, 4th edn. Anonymous New York: McGraw-Hill, Health Professions Division.

Kask A, Rago L, Wikberg JE & Schioth HB 2000 Differential effects of melanocortin peptides on ingestive behaviour in rats: Evidence against the involvement of MC(3) receptor in the regulation of food intake. *Neuroscience letters* **283** 1-4.

Kesterson RA, Huszar D, Lynch CA, Simerly RB & Cone RD 1997 Induction of neuropeptide Y gene expression in the dorsal medial hypothalamic nucleus in two models of the agouti obesity syndrome. *Molecular endocrinology (Baltimore, Md.)* **11** 630-637.

Kievit P, Halem H, Marks DL, Dong JZ, Glavas MM, Sinnayah P, Pranger L, Cowley MA, Grove KL & Culler MD 2013 Chronic treatment with a melanocortin-4 receptor agonist causes weight loss, reduces insulin resistance, and improves cardiovascular function in diet-induced obese rhesus macaques. *Diabetes* **62** 490-497.

Kim CS, Lee SH, Kim RY, Kim BJ, Li SZ, Lee IH, Lee EJ, Lim SK, Bae YS, Lee W *et al* 2002 Identification of domains directing specificity of coupling to G-proteins for the melanocortin MC3 and MC4 receptors. *The Journal of biological chemistry* **277** 31310-31317.

Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, Edwards CM, Heath MM, Stanley SA, Seal LJ *et al* 2000 Hypothalamic localization of the feeding effect of agouti-related peptide and alpha-melanocyte-stimulating hormone. *Diabetes* **49** 177-182.

Klebig ML, Wilkinson JE, Geisler JG & Woychik RP 1995 Ectopic expression of the agouti gene in transgenic mice causes obesity, features of type II diabetes, and yellow fur. *Proceedings of the National Academy of Sciences of the United States of America* **92** 4728-4732.

Klein R, Ruttkowski B, Knapp E, Salmons B, Gunzburg WH & Hohenadl C 2006 WPRE-mediated enhancement of gene expression is promoter and cell line specific. *Gene* **372** 153-161.

Klusa V, Svirskis S, Opmane B, Muceniece R & Wikberg JE 1999 Behavioural responses of gamma-MSH peptides administered into the rat ventral tegmental area. *Acta Physiologica Scandinavica* **167** 99-104.

Klusa V, Svirskis S, Opmane B, Muceniece R, Skujins A, Mutulis F, Wikberg JE & Schioth HB 1998 Evaluation of behavioural effects of neural melanocortin receptor antagonists injected ICV and in VTA in rats. *Neuropeptides* **32** 573-580.

Kowalski TJ, Liu SM, Leibel RL & Chua SC,Jr 2001 Transgenic complementation of leptin-receptor deficiency. I. rescue of the obesity/diabetes phenotype of LEPR-null mice expressing a LEPR-B transgene. *Diabetes* **50** 425-435.

Koza RA, Nikonova L, Hogan J, Rim JS, Mendoza T, Faulk C, Skaf J & Kozak LP 2006 Changes in gene expression foreshadow diet-induced obesity in genetically identical mice. *PLoS genetics* **2** e81.

Krashes MJ, Shah BP, Koda S & Lowell BB 2013 Rapid versus delayed stimulation of feeding by the endogenously released AgRP neuron mediators GABA, NPY, and AgRP. *Cell metabolism* **18** 588-595.

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

Kumar KG, Sutton GM, Dong JZ, Roubert P, Plas P, Halem HA, Culler MD, Yang H, Dixit VD & Butler AA 2009 Analysis of the therapeutic functions of novel melanocortin receptor agonists in MC3R- and MC4R-deficient C57BL/6J mice. *Peptides* **30** 1892-1900.

Labbe O, Desarnaud F, Eggerickx D, Vassart G & Parmentier M 1994 Molecular cloning of a mouse melanocortin 5 receptor gene widely expressed in peripheral tissues. *Biochemistry* **33** 4543-4549.

Larcher F, Del Rio M, Serrano F, Segovia JC, Ramirez A, Meana A, Page A, Abad JL, Gonzalez MA, Bueren J *et al* 2001 A cutaneous gene therapy approach to human leptin deficiencies: Correction of the murine ob/ob phenotype using leptin-targeted keratinocyte grafts. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **15** 1529-1538.

Larhammar D 1996 Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. *Regulatory peptides* **62** 1-11.

Larhammar D 1996 Structural diversity of receptors for neuropeptide Y, peptide YY and pancreatic polypeptide. *Regulatory peptides* **65** 165-174.

Lataste F 1883 Trois questions: Naturaliste. *Bull. Sci. du dip. du Nord* **8** 364.

Le Marchand-Brustel Y & Jeanrenaud B 1978 Pre- and postweaning studies on development of obesity in mdb/mdb mice. *The American Journal of Physiology* **234** E568-74.

Le Marchand-Brustel Y & Jeanrenaud B 1978 Pre- and postweaning studies on development of obesity in mdb/mdb mice. *The American Journal of Physiology* **234** E568-74.

Lee M, Kim A, Chua SC,Jr, Obici S & Wardlaw SL 2007 Transgenic MSH overexpression attenuates the metabolic effects of a high-fat diet. *American journal of physiology.Endocrinology and metabolism* **293** E121-31.

Lee M, Kim A, Conwell IM, Hruby V, Mayorov A, Cai M & Wardlaw SL 2008 Effects of selective modulation of the central melanocortin-3-receptor on food intake and hypothalamic POMC expression. *Peptides* **29** 440-447.

Lee S, Francoeur AM, Liu S & Wang E 1992 Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family. *The Journal of biological chemistry* **267** 24064-24068.

Lee YS, Challis BG, Thompson DA, Yeo GS, Keogh JM, Madonna ME, Wraight V, Sims M, Vatin V, Meyre D *et al* 2006 A POMC variant implicates beta-melanocyte-stimulating hormone in the control of human energy balance. *Cell metabolism* **3** 135-140.

Lefkowitz RJ, Roth J, Pricer W & Pastan I 1970 ACTH receptors in the adrenal: Specific binding of ACTH-125I and its relation to adenyl cyclase. *Proceedings of the National Academy of Sciences of the United States of America* **65** 745-752.

Leiter EH, Kintner J, Flurkey K, Beamer WG & Naggert JK 1999 Physiologic and endocrinologic characterization of male sex-biased diabetes in C57BLKS/J mice congenic for the fat mutation at the carboxypeptidease E locus. *Endocrine* **10** 57-66.

Leiter EH, Kintner J, Flurkey K, Beamer WG & Naggert JK 1999 Physiologic and endocrinologic characterization of male sex-biased diabetes in C57BLKS/J mice congenic for the fat mutation at the carboxypeptidease E locus. *Endocrine* **10** 57-66.

Levine AS & Morley JE 1984 Neuropeptide Y: A potent inducer of consummatory behavior in rats. *Peptides* **5** 1025-1029.

Li G, Mobbs CV & Scarpace PJ 2003 Central pro-opiomelanocortin gene delivery results in hypophagia, reduced visceral adiposity, and improved insulin sensitivity in genetically obese zucker rats. *Diabetes* **52** 1951-1957.

Li G, Zhang Y, Cheng KY & Scarpace PJ 2007 Lean rats with hypothalamic pro-opiomelanocortin overexpression exhibit greater diet-induced obesity and impaired central melanocortin responsiveness. *Diabetologia* **50** 1490-1499.

Li G, Zhang Y, Wilsey JT & Scarpace PJ 2005 Hypothalamic pro-opiomelanocortin gene delivery ameliorates obesity and glucose intolerance in aged rats. *Diabetologia* **48** 2376-2385.

Li SJ, Varga K, Archer P, Hruby VJ, Sharma SD, Kesterson RA, Cone RD & Kunos G 1996 Melanocortin antagonists define two distinct pathways of cardiovascular control by alpha- and gamma-melanocyte-stimulating hormones. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **16** 5182-5188.

Like AA & Chick WL 1970 Studies in the diabetic mutant mouse. I. light microscopy and radioautography of pancreatic islets. *Diabetologia* **6** 207-215.

Like AA & Chick WL 1970 Studies in the diabetic mutant mouse. II. electron microscopy of pancreatic islets. *Diabetologia* **6** 216-242.

Like AA, Lavine RL, Poffenbarger PL & Chick WL 1972 Studies in the diabetic mutant mouse. VI. evolution of glomerular lesions and associated proteinuria. *The American journal of pathology* **66** 193-224.

Lindberg I, van den Hurk WH, Bui C & Batie CJ 1995 Enzymatic characterization of immunopurified prohormone convertase 2: Potent inhibition by a 7B2 peptide fragment. *Biochemistry* **34** 5486-5493.

Lindblom J, Schioth HB, Larsson A, Wikberg JE & Bergstrom L 1998 Autoradiographic discrimination of melanocortin receptors indicates that the MC3 subtype dominates in the medial rat brain. *Brain research* **810** 161-171.

Liu CM & Yin TH 1974 Caloric compensation to gastric loads in rats with hypothalamic hyperphagia. *Physiology & Behavior* **13** 231-238.

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

Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP & Wilkison WO 1994 Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* **371** 799-802.

Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP & Wilkison WO 1994 Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* **371** 799-802.

Luquet S, Perez FA, Hnasko TS & Palmiter RD 2005 NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science (New York, N.Y.)* **310** 683-685.

Lymangrover JR, Buckalew VM, Harris J, Klein MC & Gruber KA 1985 Gamma-2MSH is natriuretic in the rat. *Endocrinology* **116** 1227-1229.

MacKenzie RG 2006 Obesity-associated mutations in the human melanocortin-4 receptor gene. *Peptides* **27** 395-403.

Mains RE & Eipper BA 1981 Synthesis and secretion of ACTH, beta-endorphin, and related peptides. *Advances in Biochemical Psychopharmacology* **28** 35-47.

Manna SK & Aggarwal BB 1998 Alpha-melanocyte-stimulating hormone inhibits the nuclear transcription factor NF-kappa B activation induced by various inflammatory agents. *Journal of immunology (Baltimore, Md.: 1950)* **161** 2873-2880.

Marks DL, Hruby V, Brookhart G & Cone RD 2006 The regulation of food intake by selective stimulation of the type 3 melanocortin receptor (MC3R). *Peptides* **27** 259-264.

Marsh DJ, Hollopeter G, Huszar D, Laufer R, Yagaloff KA, Fisher SL, Burn P & Palmiter RD 1999 Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. *Nature genetics* **21** 119-122.

Matsumura K, Tsuchihashi T, Abe I & Iida M 2002 Central alpha-melanocyte-stimulating hormone acts at melanocortin-4 receptor to activate sympathetic nervous system in conscious rabbits. *Brain research* **948** 145-148.

Mayan H, Ni XP, Almog S & Humphreys MH 2003 Suppression of gamma-melanocyte-stimulating hormone secretion is accompanied by salt-sensitive hypertension in the rat. *Hypertension* **42** 962-967.

Mayan H, Ling KT, Lee EY, Wiedemann E, Kalinyak JE & Humphreys MH 1996 Dietary sodium intake modulates pituitary proopiomelanocortin mRNA abundance. *Hypertension* **28** 244-249.

Mayer J 1955 The physiological basis of obesity and leanness. part II. *Nutrition abstracts and reviews. Series A: Human and experimental* **25** 871-883.

Mayer J 1953 Glucostatic mechanism of regulation of food intake. *The New England journal of medicine* **249** 13-16.

McMinn JE, Wilkinson CW, Havel PJ, Woods SC & Schwartz MW 2000 Effect of intracerebroventricular alpha-MSH on food intake, adiposity, c-fos induction, and neuropeptide expression. *American journal of physiology.Regulatory, integrative and comparative physiology* **279** R695-703.

Meade CJ, Ashwell M & Sowter C 1979 Is genetically transmitted obesity due to an adipose tissue defect? *Proceedings of the Royal Society of London.Series B, Containing papers of a Biological character. Royal Society (Great Britain)* **205** 395-410.

Melnick I, Pronchuk N, Cowley MA, Grove KL & Colmers WF 2007 Developmental switch in neuropeptide Y and melanocortin effects in the paraventricular nucleus of the hypothalamus. *Neuron* **56** 1103-1115.

Mercer RE, Chee MJ & Colmers WF 2011 The role of NPY in hypothalamic mediated food intake. *Frontiers in neuroendocrinology* **32** 398-415.

Metherell LA, Chapple JP, Cooray S, David A, Becker C, Ruschendorf F, Naville D, Begeot M, Khoo B, Nurnberg P *et al* 2005 Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nature genetics* **37** 166-170.

Michaud EJ, Bultman SJ, Stubbs LJ & Woychik RP 1993 The embryonic lethality of homozygous lethal yellow mice (ay/ay) is associated with the disruption of a novel RNA-binding protein. *Genes & development* **7** 1203-1213.

Mitta B, Rimann M & Fussenegger M 2005 Detailed design and comparative analysis of protocols for optimized production of high-performance HIV-1-derived lentiviral particles. *Metabolic engineering* **7** 426-436.

Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA & Mobbs CV 1998 Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes* **47** 294-297.

Mohr B 1993 Neuropathology communication from dr. mohr, privat docent in wurzburg. 1840. *Obesity research* **1** 334-335.

Morton GJ & Schwartz MW 2001 The NPY/AgRP neuron and energy homeostasis. *International journal of obesity and related metabolic disorders*

: journal of the International Association for the Study of Obesity **25 Suppl 5** S56-62.

Mountjoy KG 2010 Functions for pro-opiomelanocortin-derived peptides in obesity and diabetes. *The Biochemical journal* **428** 305-324.

Mountjoy KG, Bird IM, Rainey WE & Cone RD 1994 ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. *Molecular and cellular endocrinology* **99** R17-20.

Mountjoy KG, Robbins LS, Mortrud MT & Cone RD 1992 The cloning of a family of genes that encode the melanocortin receptors. *Science (New York, N.Y.)* **257** 1248-1251.

Mountjoy KG, Robbins LS, Mortrud MT & Cone RD 1992 The cloning of a family of genes that encode the melanocortin receptors. *Science (New York, N.Y.)* **257** 1248-1251.

Mountjoy KG, Mortrud MT, Low MJ, Simerly RB & Cone RD 1994 Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Molecular endocrinology (Baltimore, Md.)* **8** 1298-1308.

Muceniece R, Zvejniece L, Kirjanova O, Liepinsh E, Krigere L, Vilskersts R, Baumane L, Gordjusina V, Kalvinsh I, Wikberg JE *et al* 2005 Beta-MSH inhibits brain inflammation via MC(3)/(4) receptors and impaired NF-kappaB signaling. *Journal of neuroimmunology* **169** 13-19.

Murphy B, Nunes CN, Ronan JJ, Harper CM, Beall MJ, Hanaway M, Fairhurst AM, Van der Ploeg LH, MacIntyre DE & Mellin TN 1998 Melanocortin mediated inhibition of feeding behavior in rats. *Neuropeptides* **32** 491-497.

Naggert JK, Fricker LD, Varlamov O, Nishina PM, Rouille Y, Steiner DF, Carroll RJ, Paigen BJ & Leiter EH 1995 Hyperproinsulinaemia in obese fat/fat mice associated with a carboxypeptidase E mutation which reduces enzyme activity. *Nature genetics* **10** 135-142.

Neumann Andersen G, Nagaeva O, Mandrika I, Petrovska R, Muceniece R, Mincheva-Nilsson L & Wikberg JE 2001 MC(1) receptors are constitutively expressed on leucocyte subpopulations with antigen presenting and cytotoxic functions. *Clinical and experimental immunology* **126** 441-446.

Ni XP & Humphreys MH 2008 Abnormal glucose metabolism in hypertensive mice with genetically interrupted gamma-melanocyte stimulating hormone signaling fed a high-sodium diet. *American journal of hypertension* **21** 1284-1287.

Ni XP, Butler AA, Cone RD & Humphreys MH 2006 Central receptors mediating the cardiovascular actions of melanocyte stimulating hormones. *Journal of hypertension* **24** 2239-2246.

Ni XP, Pearce D, Butler AA, Cone RD & Humphreys MH 2003 Genetic disruption of gamma-melanocyte-stimulating hormone signaling leads to salt-sensitive hypertension in the mouse. *The Journal of clinical investigation* **111** 1251-1258.

Nishimura M, Ohtsuka K, Takahashi H & Yoshimura M 2000 Role of FMRFamide-activated brain sodium channel in salt-sensitive hypertension. *Hypertension* **35** 443-450.

Nogueiras R, Wiedmer P, Perez-Tilve D, Veyrat-Durebex C, Keogh JM, Sutton GM, Pfluger PT, Castaneda TR, Neschen S, Hofmann SM *et al* 2007 The central melanocortin system directly controls peripheral lipid metabolism. *The Journal of clinical investigation* **117** 3475-3488.

Nogueiras R, Wiedmer P, Perez-Tilve D, Veyrat-Durebex C, Keogh JM, Sutton GM, Pfluger PT, Castaneda TR, Neschen S, Hofmann SM *et al* 2007 The central melanocortin system directly controls peripheral lipid metabolism. *The Journal of clinical investigation* **117** 3475-3488.

Noon LA, Franklin JM, King PJ, Goulding NJ, Hunyady L & Clark AJ 2002 Failed export of the adrenocorticotrophin receptor from the endoplasmic reticulum in non-adrenal cells: Evidence in support of a requirement for a specific adrenal accessory factor. *The Journal of endocrinology* **174** 17-25.

Oki S, Nakao K, Tanaka I, Kinoshita F, Naki Y & Imura H 1982 Characterization of gamma-melanotropin-like immunoreactivity and its secretion in an adrenocorticotropin-producing mouse pituitary tumor cell line. *Endocrinology* **111** 418-424.

Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I & Barsh GS 1997 Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science (New York, N.Y.)* **278** 135-138.

Oosterom J, Nijenhuis WA, Schaaper WM, Slootstra J, Meloen RH, Gispen WH, Burbach JP & Adan RA 1999 Conformation of the core sequence in melanocortin peptides directs selectivity for the melanocortin MC3 and MC4 receptors. *The Journal of biological chemistry* **274** 16853-16860.

Pedersen RC & Brownie AC 1983 Lys-gamma 3-melanotropin binds with high affinity to the rat adrenal cortex. *Endocrinology* **112** 1279-1287.

Pedersen RC, Brownie AC & Ling N 1980 Pro-adrenocorticotropin/endorphin-derived peptides: Coordinate action on adrenal steroidogenesis. *Science (New York, N.Y.)* **208** 1044-1046.

Pelletier J & Sonenberg N 1988 Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334** 320-325.

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T & Collins F 1995 Effects of the obese gene product on body weight regulation in ob/ob mice. *Science (New York, N.Y.)* **269** 540-543.

Petraglia F, Bacchi Modena A, Comitini G, Scazzina D, Facchinetti F, Fiaschetti D, Genazzani AD, Barletta C, Scavo D & Genazzani AR 1990 Plasma beta-endorphin and beta-lipotropin levels increase in well trained athletes after competition and non competitive exercise. *Journal of endocrinological investigation* **13** 19-23.

Pritchard LE & White A 2007 Neuropeptide processing and its impact on melanocortin pathways. *Endocrinology* **148** 4201-4207.

Ramachandrappa S & Farooqi IS 2011 Genetic approaches to understanding human obesity. *The Journal of clinical investigation* **121** 2080-2086.

Rasband WS 1997-2012 ImageJ (http://Imagej.nih.gov/ij/). In . Bethesda, Maryland, USA,: National Institute of Health.

Ravussin Y, Gutman R, LeDuc CA & Leibel RL 2013 Estimating energy expenditure in mice using an energy balance technique. *International journal of obesity (2005)* **37** 399-403.

Renquist BJ, Murphy JG, Larson EA, Olsen D, Klein RF, Ellacott KL & Cone RD 2012 Melanocortin-3 receptor regulates the normal fasting response. *Proceedings of the National Academy of Sciences of the United States of America* **109** E1489-98.

Richter CP 1942 Total self regulatory functions in animals and human beings
. In , p. 63-103.

Richter WO, Jacob BG & Schwandt P 1990 Processing of the lipid-mobilizing peptide beta-lipotropin in rabbit adipose tissue. *Molecular and cellular endocrinology* **71** 229-238.

Ridet JL & Privat A 1995 Gene therapy in the central nervous system: Direct versus indirect gene delivery. *Journal of neuroscience research* **42** 287-293.

Rinne P, Harjunpaa J, Makela S & Savontaus E 2013 Genetic and pharmacological mouse models of chronic melanocortin activation show enhanced baroreflex control of heart rate. *Regulatory peptides* **182** 19-27.

Rinne P, Harjunpaa J, Scheinin M & Savontaus E 2008 Blood pressure regulation and cardiac autonomic control in mice overexpressing alpha- and gamma-melanocyte stimulating hormone. *Peptides* **29** 1943-1952.

Rinne P, Nordlund W, Heinonen I, Penttinen AM, Saraste A, Ruohonen ST, Makela S, Vahatalo L, Kaipio K, Cai M *et al* 2013 Alpha-melanocyte-stimulating hormone regulates vascular NO availability and protects against endothelial dysfunction. *Cardiovascular research* **97** 360-368.

Roseberry AG, Liu H, Jackson AC, Cai X & Friedman JM 2004 Neuropeptide Y-mediated inhibition of proopiomelanocortin neurons in the arcuate nucleus shows enhanced desensitization in ob/ob mice. *Neuron* **41** 711-722.

Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, Entwistle ML, Simerly RB & Cone RD 1993 Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proceedings of the National Academy of Sciences of the United States of America* **90** 8856-8860.

Rossi M, Kim MS, Morgan DG, Small CJ, Edwards CM, Sunter D, Abusnana S, Goldstone AP, Russell SH, Stanley SA *et al* 1998 A C-terminal fragment of agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. *Endocrinology* **139** 4428-4431.

Ruifrok AC, Katz RL & Johnston DA 2003 Comparison of quantification of histochemical staining by hue-saturation-intensity (HSI) transformation and color-deconvolution. *Applied Immunohistochemistry & Molecular Morphology : AIMM / Official Publication of the Society for Applied Immunohistochemistry* **11** 85-91.

Ruohonen ST, Pesonen U, Moritz N, Kaipio K, Roytta M, Koulu M & Savontaus E 2008 Transgenic mice overexpressing neuropeptide Y in noradrenergic neurons: A novel model of increased adiposity and impaired glucose tolerance. *Diabetes* **57** 1517-1525.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S *et al* 1998 Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* **92** 573-585.

Salar G, Alessio L, Govoni S, Battaini F, Iob I, Goss I & Trabucchi M 1991 Evaluation of beta-endorphin/beta-lipotropin immunoreactivity content in the CSF of patients affected by deafferentation pain syndromes. *Pharmacological research : the official journal of the Italian Pharmacological Society* **23** 181-186.

Salazar-Onfray F, Lopez M, Lundqvist A, Aguirre A, Escobar A, Serrano A, Korenblit C, Petersson M, Chhajlani V, Larsson O *et al* 2002 Tissue distribution and differential expression of melanocortin 1 receptor, a malignant melanoma marker. *British journal of cancer* **87** 414-422.

Sanacora G, Kershaw M, Finkelstein JA & White JD 1990 Increased hypothalamic content of preproneuropeptide Y messenger ribonucleic acid in genetically obese zucker rats and its regulation by food deprivation. *Endocrinology* **127** 730-737.

Sauer B 1987 Functional expression of the cre-lox site-specific recombination system in the yeast saccharomyces cerevisiae. *Molecular and cellular biology* **7** 2087-2096.

Sauer B & Henderson N 1988 Site-specific DNA recombination in mammalian cells by the cre recombinase of bacteriophage P1. *Proceedings of the National Academy of Sciences of the United States of America* **85** 5166-5170.

Savontaus E, Breen TL, Kim A, Yang LM, Chua SC,Jr & Wardlaw SL 2004 Metabolic effects of transgenic melanocyte-stimulating hormone overexpression in lean and obese mice. *Endocrinology* **145** 3881-3891.

Schafer MK, Stoffers DA, Eipper BA & Watson SJ 1992 Expression of peptidylglycine alpha-amidating monooxygenase (EC 1.14.17.3) in the rat central nervous system. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **12** 222-234.

Schioth HB, Muceniece R & Wikberg JE 1996 Characterisation of the melanocortin 4 receptor by radioligand binding. *Pharmacology & toxicology* **79** 161-165.

Schreyer SA, Wilson DL & LeBoeuf RC 1998 C57BL/6 mice fed high fat diets as models for diabetes-accelerated atherosclerosis. *Atherosclerosis* **136** 17-24.

Scott MM, Williams KW, Rossi J, Lee CE & Elmquist JK 2011 Leptin receptor expression in hindbrain glp-1 neurons regulates food intake and energy balance in mice. *The Journal of clinical investigation* **121** 2413-2421.

Sebag JA & Hinkle PM 2007 Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. *Proceedings of the National Academy of Sciences of the United States of America* **104** 20244-20249.

Seger MA & Bennett HP 1986 Structure and bioactivity of the amino-terminal fragment of pro-opiomelanocortin. *Journal of steroid biochemistry* **25** 703-710.

Seidah NG, Day R, Marcinkiewicz M, Benjannet S & Chretien M 1991 Mammalian neural and endocrine pro-protein and pro-hormone convertases belonging to the subtilisin family of serine proteinases. *Enzyme* **45** 271-284.

Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M & Chretien M 1990 cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: Tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA and cell biology* **9** 415-424.

Shi H & Bartness TJ 2001 Neurochemical phenotype of sympathetic nervous system outflow from brain to white fat. *Brain research bulletin* **54** 375-385.

Shinyama H, Masuzaki H, Fang H & Flier JS 2003 Regulation of melanocortin-4 receptor signaling: Agonist-mediated desensitization and internalization. *Endocrinology* **144** 1301-1314.

Shinyama H, Masuzaki H, Fang H & Flier JS 2003 Regulation of melanocortin-4 receptor signaling: Agonist-mediated desensitization and internalization. *Endocrinology* **144** 1301-1314.

Shutter JR, Graham M, Kinsey AC, Scully S, Luthy R & Stark KL 1997 Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. *Genes & development* **11** 593-602.

Siegrist W & Eberle AN 1986 In situ melanin assay for MSH using mouse B16 melanoma cells in culture. *Analytical Biochemistry* **159** 191-197.

Sindelar DK, Palmiter RD, Woods SC & Schwartz MW 2005 Attenuated feeding responses to circadian and palatability cues in mice lacking neuropeptide Y. *Peptides* **26** 2597-2602.

Skibicka KP & Grill HJ 2009 Hypothalamic and hindbrain melanocortin receptors contribute to the feeding, thermogenic, and cardiovascular action of melanocortins. *Endocrinology* **150** 5351-5361.

Skibicka KP & Grill HJ 2009 Hypothalamic and hindbrain melanocortin receptors contribute to the feeding, thermogenic, and cardiovascular action of melanocortins. *Endocrinology* **150** 5351-5361.

Skofitsch G, Jacobowitz DM & Zamir N 1985 Immunohistochemical localization of a melanin concentrating hormone-like peptide in the rat brain. *Brain research bulletin* **15** 635-649.

Smyth DG & Zakarian S 1980 Selective processing of beta-endorphin in regions of porcine pituitary. *Nature* **288** 613-615.

Smyth DG, Darby NJ & Maruthainar K 1988 Sequential formation of beta-endorphin-related peptides in porcine pituitary. *Neuroendocrinology* **47** 317-322.

Sohn JW, Harris LE, Berglund ED, Liu T, Vong L, Lowell BB, Balthasar N, Williams KW & Elmquist JK 2013 Melanocortin 4 receptors reciprocally regulate sympathetic and parasympathetic preganglionic neurons. *Cell* **152** 612-619.

Sohn JW, Harris LE, Berglund ED, Liu T, Vong L, Lowell BB, Balthasar N, Williams KW & Elmquist JK 2013 Melanocortin 4 receptors reciprocally regulate sympathetic and parasympathetic preganglionic neurons. *Cell* **152** 612-619.

Song CK, Jackson RM, Harris RB, Richard D & Bartness TJ 2005 Melanocortin-4 receptor mRNA is expressed in sympathetic nervous system outflow neurons to white adipose tissue. *American journal of*

physiology.Regulatory, integrative and comparative physiology **289** R1467-76.

Song CK, Vaughan CH, Keen-Rhinehart E, Harris RB, Richard D & Bartness TJ 2008 Melanocortin-4 receptor mRNA expressed in sympathetic outflow neurons to brown adipose tissue: Neuroanatomical and functional evidence. *American journal of physiology.Regulatory, integrative and comparative physiology* **295** R417-28.

Soos S, Petervari E, Szekely M, Jech-Mihalffy A & Balasko M 2010 Complex catabolic effects of central alpha-MSH infusion in rats of altered nutritional states: Differences from leptin. *Journal of molecular neuroscience : MN* .

Stanley BG, Chin AS & Leibowitz SF 1985 Feeding and drinking elicited by central injection of neuropeptide Y: Evidence for a hypothalamic site(s) of action. *Brain research bulletin* **14** 521-524.

Star RA, Rajora N, Huang J, Stock RC, Catania A & Lipton JM 1995 Evidence of autocrine modulation of macrophage nitric oxide synthase by alpha-melanocyte-stimulating hormone. *Proceedings of the National Academy of Sciences of the United States of America* **92** 8016-8020.

Ste Marie L, Miura GI, Marsh DJ, Yagaloff K & Palmiter RD 2000 A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors. *Proceedings of the National Academy of Sciences of the United States of America* **97** 12339-12344.

Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM & Kriauciunas A 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* **377** 530-532.

Sutton GM, Josephine Babin M, Gu X, Hruby VJ & Butler AA 2008 A derivative of the melanocortin receptor antagonist SHU9119 (PG932) increases food intake when administered peripherally. *Peptides* **29** 104-111.

Sutton GM, Begriche K, Kumar KG, Gimble JM, Perez-Tilve D, Nogueiras R, McMillan RP, Hulver MW, Tschop MH & Butler AA 2010 Central nervous system melanocortin-3 receptors are required for synchronizing metabolism during entrainment to restricted feeding during the light cycle. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* **24** 862-872.

Suzuki I, Cone RD, Im S, Nordlund J & Abdel-Malek ZA 1996 Binding of melanotropic hormones to the melanocortin receptor MC1R on human melanocytes stimulates proliferation and melanogenesis. *Endocrinology* **137** 1627-1633.

Suzuki I, Cone RD, Im S, Nordlund J & Abdel-Malek ZA 1996 Binding of melanotropic hormones to the melanocortin receptor MC1R on human melanocytes stimulates proliferation and melanogenesis. *Endocrinology* **137** 1627-1633.

Rahmouni K, Haynes WG, Morgan DA & Mark AL 2003 Role of melanocortin-4 receptors in mediating renal sympathoactivation to leptin and insulin. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23** 5998-6004.

Taherzadeh S, Sharma S, Chhajlani V, Gantz I, Rajora N, Demitri MT, Kelly L, Zhao H, Ichiyama T, Catania A *et al* 1999 Alpha-MSH and its receptors in regulation of tumor necrosis factor-alpha production by human monocyte/macrophages. *The American Journal of Physiology* **276** R1289-94.

Tatemoto K, Carlquist M & Mutt V 1982 Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* **296** 659-660.

Tatro JB 1996 Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. *Neuroimmunomodulation* **3** 259-284.

Taylor A & Namba K 2001 In vitro induction of CD25+ CD4+ regulatory T cells by the neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH). *Immunology and cell biology* **79** 358-367.

Thompson RH & Swanson LW 1998 Organization of inputs to the dorsomedial nucleus of the hypothalamus: A reexamination with fluorogold and PHAL in the rat. *Brain research.Brain research reviews* **27** 89-118.

Thorburn AW & Proietto J 2000 Biological determinants of spontaneous physical activity. *Obesity reviews : an official journal of the International Association for the Study of Obesity* **1** 87-94.

Thurlby PL & Trayhurn P 1978 The development of obesity in preweaning obob mice. *The British journal of nutrition* **39** 397-402.

Tritos NA, Elmquist JK, Mastaitis JW, Flier JS & Maratos-Flier E 1998 Characterization of expression of hypothalamic appetite-regulating peptides in obese hyperleptinemic brown adipose tissue-deficient (uncoupling protein-promoter-driven diphtheria toxin A) mice. *Endocrinology* **139** 4634-4641.

Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH & Guan XM 1998 Distribution of orexin receptor mRNA in the rat brain. *FEBS letters* **438** 71-75.

Trono D 2000 Lentiviral vectors: Turning a deadly foe into a therapeutic agent. *Gene therapy* **7** 20-23.

Unger RH & Cherrington AD 2012 Glucagonocentric restructuring of diabetes: A pathophysiologic and therapeutic makeover. *The Journal of clinical investigation* **122** 4-12.

Van der Ploeg LH, Martin WJ, Howard AD, Nargund RP, Austin CP, Guan X, Drisko J, Cashen D, Sebhat I, Patchett AA *et al* 2002 A role for the melanocortin 4 receptor in sexual function. *Proceedings of the National Academy of Sciences of the United States of America* **99** 11381-11386.

van Strien FJ, Devreese B, Van Beeumen J, Roubos EW & Jenks BG 1995 Biosynthesis and processing of the N-terminal part of proopiomelanocortin in xenopus laevis: Characterization of gamma-MSH peptides. *Journal of neuroendocrinology* **7** 807-815.

Vergoni AV, Bertolini A, Wikberg JE & Schioth HB 1999 Selective melanocortin MC4 receptor blockage reduces immobilization stress-induced anorexia in rats. *European journal of pharmacology* **369** 11-15.

Vile R & Russell SJ 1994 Gene transfer technologies for the gene therapy of cancer. *Gene therapy* **1** 88-98.

Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S *et al* 2009 Functional brown adipose tissue in healthy adults. *The New England journal of medicine* **360** 1518-1525.

Wallingford N, Perroud B, Gao Q, Coppola A, Gyengesi E, Liu ZW, Gao XB, Diament A, Haus KA, Shariat-Madar Z *et al* 2009 Prolylcarboxypeptidase regulates food intake by inactivating alpha-MSH in rodents. *The Journal of clinical investigation* **119** 2291-2303.

Webb TR, Chan L, Cooray SN, Cheetham ME, Chapple JP & Clark AJ 2009 Distinct melanocortin 2 receptor accessory protein domains are required for melanocortin 2 receptor interaction and promotion of receptor trafficking. *Endocrinology* **150** 720-726.

Wessells H, Fuciarelli K, Hansen J, Hadley ME, Hruby VJ, Dorr R & Levine N 1998 Synthetic melanotropic peptide initiates erections in men with psychogenic erectile dysfunction: Double-blind, placebo controlled crossover study. *The Journal of urology* **160** 389-393.

Wetzel W, Balschun D, Janke S, Vogel D & Wagner T 1994 Effects of CLIP (corticotropin-like intermediate lobe peptide) and CLIP fragments on paradoxical sleep in rats. *Peptides* **15** 237-241.

Wikberg JE, Muceniece R, Mandrika I, Prusis P, Lindblom J, Post C & Skottner A 2000 New aspects on the melanocortins and their receptors. *Pharmacological research : the official journal of the Italian Pharmacological Society* **42** 393-420.

Wilding JP, Gilbey SG, Bailey CJ, Batt RA, Williams G, Ghatei MA & Bloom SR 1993 Increased neuropeptide-Y messenger ribonucleic acid (mRNA) and decreased neurotensin mRNA in the hypothalamus of the obese (ob/ob) mouse. *Endocrinology* **132** 1939-1944.

Williams DL, Kaplan JM & Grill HJ 2000 The role of the dorsal vagal complex and the vagus nerve in feeding effects of melanocortin-3/4 receptor stimulation. *Endocrinology* **141** 1332-1337.

Williams G & Frubeck G Eds. 2009 *Obesity science to practice*, p.585UK: John Wiley & Sons, Ltd.

Wirth MM, Olszewski PK, Yu C, Levine AS & Giraudo SQ 2001 Paraventricular hypothalamic alpha-melanocyte-stimulating hormone and MTII reduce feeding without causing aversive effects. *Peptides* **22** 129-134.

Wiznerowicz M & Trono D 2005 Harnessing HIV for therapy, basic research and biotechnology. *Trends in biotechnology* **23** 42-47.

Wong KY, Rajora N, Boccoli G, Catania A & Lipton JM 1997 A potential mechanism of local anti-inflammatory action of alpha-melanocyte-stimulating hormone within the brain: Modulation of tumor necrosis factor-alpha production by human astrocytic cells. *Neuroimmunomodulation* **4** 37-41.

Wu Q & Palmiter RD 2011 GABAergic signaling by AgRP neurons prevents anorexia via a melanocortin-independent mechanism. *European journal of pharmacology* **660** 21-27.

Wu Q, Whiddon BB & Palmiter RD 2012 Ablation of neurons expressing agouti-related protein, but not melanin concentrating hormone, in leptin-deficient mice restores metabolic functions and fertility. *Proceedings of the National Academy of Sciences of the United States of America* **109** 3155-3160.

Wu Q, Boyle MP & Palmiter RD 2009 Loss of GABAergic signaling by AgRP neurons to the parabrachial nucleus leads to starvation. *Cell* **137** 1225-1234.

Xia Y & Wikberg JE 1996 Localization of ACTH receptor mRNA by in situ hybridization in mouse adrenal gland. *Cell and tissue research* **286** 63-68.

Xia Y, Wikberg JE & Krukoff TL 2001 Gamma(2)-melanocyte-stimulating hormone suppression of systemic inflammatory responses to endotoxin is associated with modulation of central autonomic and neuroendocrine activities. *Journal of neuroimmunology* **120** 67-77.

Xia Y, Wikberg JE & Chhajlani V 1995 Expression of melanocortin 1 receptor in periaqueductal gray matter. *Neuroreport* **6** 2193-2196.

Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, Tecott LH & Reichardt LF 2003 Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. *Nature neuroscience* **6** 736-742.

Yang L, Scott KA, Hyun J, Tamashiro KL, Tray N, Moran TH & Bi S 2009 Role of dorsomedial hypothalamic neuropeptide Y in modulating

food intake and energy balance. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29** 179-190.

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

Yang YK & Harmon CM 2003 Recent developments in our understanding of melanocortin system in the regulation of food intake. *Obesity reviews : an official journal of the International Association for the Study of Obesity* **4** 239-248.

Yang YK, Thompson DA, Dickinson CJ, Wilken J, Barsh GS, Kent SB & Gantz I 1999 Characterization of agouti-related protein binding to melanocortin receptors. *Molecular endocrinology (Baltimore, Md.)* **13** 148-155.

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

Yen TT & Acton JM 1972 Locomotor activity of various types of genetically obese mice. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* **140** 647-650.

Yuan Q, Fontenele-Neto JD & Fricker LD 2004 Effect of voluntary exercise on genetically obese cpefat/fat mice: Quantitative proteomics of serum. *Obesity research* **12** 1179-1188.

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

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

Zhang Y, Rodrigues E, Gao YX, King M, Cheng KY, Erdos B, Tumer N, Carter C & Scarpace PJ 2010 Pro-opiomelanocortin gene transfer to the nucleus of the solitary track but not arcuate nucleus ameliorates chronic diet-induced obesity. *Neuroscience* **169** 1662-1671.

Zheng H, Patterson LM, Phifer CB & Berthoud HR 2005 Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. *American journal of physiology. Regulatory, integrative and comparative physiology* **289** R247-58.

Zheng H, Patterson LM, Rhodes CJ, Louis GW, Skibicka KP, Grill HJ, Myers MG,Jr & Berthoud HR 2010 A potential role for hypothalamomedullary POMC projections in leptin-induced suppression of food intake. *American journal of physiology. Regulatory, integrative and comparative physiology* **298** R720-8.

Zucker TF & Zucker LM 1963 Fat accretion and growth in the rat. *The Journal of nutrition* **80** 6-19.