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NATURAL BORN WEIGHT GAINERS

The Mechanisms of Obesity in Transgenic Mice Overexpressing Neuropeptide Y

Laura Vähätalo

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To the wonderful people I have met during this journey

ABSTRACT

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Natural born weight gainers: The mechanisms of obesity in transgenic mice overexpressing neuropeptide Y

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Neuropeptide Y (NPY) is a neurotransmitter promoting energy storage by activating Y-receptors and thus affecting food intake, thermogenesis and adipose tissue metabolism. NPY is expressed both in the central and sympathetic nervous system. Hypothalamic NPY is known to stimulate feeding, but the effects of noradrenergic neuron NPY are more ambiguous. Chronic stress stimulates fat accumulation via NPY release from noradrenergic neurons. Furthermore, polymorphism in the human *Npy* gene has been associated with metabolic disturbances and increased NPY secretion after sympathetic stimulation. The main objective of this study was to clarify the mechanisms of noradrenergic neuron NPY in the development of obesity.

The metabolic phenotype of a homozygous mouse overexpressing NPY in the brain noradrenergic neurons and sympathetic nervous system (OE-NPY$^{D\beta H}$ mouse) was characterized. OE-NPY$^{D\beta H}$ mice had an increased fat mass and body weight, which caused impairments of glucose metabolism and hyperinsulinaemia with age. There were no differences in energy intake or expenditure, but the sympathetic tone was down-regulated and the endocannabinoid system activated. Furthermore, peripheral Y2-receptors in energy-rich conditions played an important role in mediating the fat-accumulating effect of NPY.

These results indicate that noradrenergic neuron NPY promotes obesity via direct effects in the periphery and by modulating the sympatho-adrenal and endocannabinoid systems. Additionally, NPY in the central noradrenergic neurons is believed to possess many important roles. The phenotype of the OE-NPY$^{D\beta H}$ mouse resembles the situations of chronic stress and *Npy* gene polymorphism and thus these mice may be exploited in testing novel drug candidates for the treatment of obesity.

Keywords: neuropeptide Y, obesity, Y-receptors, endocannabinoid system, sympathetic nervous system

TIIVISTELMÄ

Laura Vähätalo

Luontaiset painon kerryttäjät: Lihavuuden mekanismit siirtogeenisella NPY:tä yli-ilmentävällä hiirellä

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Neuropeptidi Y (NPY) on hermovälittäjäaine, joka Y-reseptorien välityksellä edistää energian varastointia. NPY vaikuttaa useisiin energiatasapainon osa-alueisiin kuten syömiseen, lämmöntuottoon ja rasva-aineenvaihduntaan. NPY:tä ilmennetään sekä keskus- että sympaattisessa ääreishermostossa. Hypotalaamisen NPY:n tiedetään olevan voimakkain keskushermoston syömistä lisäävä hermovälittäjäaine. Sen sijaan noradrenergisissa hermoissa ilmennettävän NPY:n tehtävät ovat vielä tuntemattomampia. Krooninen stressi vapauttaa NPY:tä noradrenergisista hermoista ja näin lisää rasvan kertymistä. Lisäksi ihmisellä on havaittu metabolisiin häiriöihin assosioituva *Npy*-geenin polymorfia, jossa NPY:n vapautuminen on lisääntynyt sympaattisen aktivaation aikana. Tämän tutkimuksen tavoitteena oli tutkia tarkemmin mekanismeja, joilla noradrenergisten hermojen NPY osallistuu lihavuuden kehittymiseen.

Ensin selvitettiin siirtogeenisen homotsygootisti NPY:tä aivojen noradrenergisissa hermoissa ja sympaattisessa hermostossa yli-ilmentävän hiirimallin (OE-NPY$^{D\beta H}$ hiiri) metabolinen ilmiasu. OE-NPY$^{D\beta H}$-hiirten ruumiin paino ja rasvamassan määrä olivat lisääntyneet, mikä myöhemmällä iällä johti huonontuneeseen glukoosiaineenvaihduntaan ja kohonneeseen veren insuliinipitoisuuteen. Syömisessä ja energiankulutuksessa ei havaittu eroja. Sen sijaan sympaattinen aktiivisuus oli madaltunut ja endokannabinoidijärjestelmä aktivoitunut OE-NPY$^{D\beta H}$-hiirellä. Lisäksi perifeerisilla Y2-reseptoreilla on energiapitoisen ruokavalion yhteydessä tärkeä rooli NPY:n aiheuttamassa lisääntyneessä rasvamassan määrässä.

Tämä tutkimus osoittaa, että noradrenergisten hermojen NPY edistää lihavuuden syntyä suorien periferisten vaikutusten kautta sekä muokkaamalla sympaattisen hermoston ja endokannabinoidijärjestelmän toimintaa. Näiden lisäksi aivojen noradrenergisella NPY:llä on osuutensa lihavuuden kehittymisessä. OE-NPY$^{D\beta H}$-hiirten ilmiasu muistuttaa kroonisen stressin sekä *Npy*-geenin polymorfian aiheuttamaa metabolista tilaa ja OE-NPY$^{D\beta H}$-hiirtä voidaankin hyödyntää testattaessa uusia lääkekandidaatteja lihavuuden ja metabolisen oireyhtymän hoitoon.

Avainsanat: neuropeptidi Y, lihavuus, Y-reseptorit, endokannabinoidijärjestelmä, sympaattinen hermosto

TABLE OF CONTENTS

ABBREVIATIONS

2-AG	2-arachidonoylglycerol
α-MSH	α-melanocyte stimulating hormone
AAV	adeno-associated virus
Adrb3	β3-adrenoceptor
AEA	anandamide
Agrp	agouti related peptide
Arc	arcuate nucleus
BAT	brown adipose tissue
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CART	cocaine and amphetamine-regulated transcript
CB1R	cannabinoid receptor type-1
CB2R	cannabinoid receptor type-2
CCK	cholecystokinin
CNS	central nervous system
CRH	corticotropin-releasing hormone
DMH	dorsomedial hypothalamic nucleus
DβH	dopamine-beta-hydroxylase
GI	gastrointestinal
GLP-1	glucagon like peptide-1
HE	hematoxylin and eosin
HPA	hypothalamic-pituitary-adrenal
HSL	hormone sensitive lipase
ICV	intracerebroventicular
i.p.	intraperitoneal
KD	knock-down
KO	knock-out
LacZ	β-galactosidase
LC	locus coeruleus

LHA	lateral hypothalamus
LPL	lipoprotein lipase
MCH	melanin-concentrating hormone
MS	metabolic syndrome
NA	noradrenaline
NEFA	non-esterified fatty acids
NPY	neuropeptide Y
NTS	nucleus tractus solitaries
OE-NPY$^{D\beta H}$	mouse model overexpressing NPY under DβH promoter
p.L7P	leucine-to-proline polymorphism
PFA	perifornical area
PVN	paraventricular nucleus
POMC	pro-opiomelanocortin
PP	pancreatic polypeptide
Prm1	protamine-1
PYY	peptide YY
qPCR	quantitative PCR
RER	respiratory exchange ratio
s.c.	subcutaneous
SEM	standard error of mean
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
TH	tyrosine hydroxylase
TRH	thyrotropin-releasing hormone
TTM	total tissue mass
UCP1	uncoupling protein1
VMH	ventromedial hypothalamus
WAT	white adipose tissue
WT	wild-type
YRs	Y-receptors

LIST OF ORIGINAL PUBLICATIONS

I Ruohonen ST, Vähätalo LH & Savontaus E. Diet-induced obesity in mice overexpressing neuropeptide Y in noradrenergic neurons. International Journal of Peptides 2012; 2012: 452424.

II Vähätalo LH, Ruohonen ST, Mäkelä S, Kovalainen M, Huotari A, Mäkelä KA, Määttä JA, Miinalainen I, Gilsbach R, Hein L, Ailanen L, Mattila M, Eerola K, Röyttä M, Ruohonen S, Herzig KH & Savontaus E. Neuropeptide Y in the noradrenergic neurones induces obesity and inhibits sympathetic tone in mice. ACTA Physiologica 2015; 213(4): 902-19.

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1. INTRODUCTION

Obesity is defined as an excessive fat accumulation, which may impair health (WHO 2015b). The World Health Organization (WHO) has defined obesity according to the body mass index (BMI), which refers to a person's weight in kilograms divided by the square of the height in meters (kg/m^2). BMI ≥25 kg/m^2 represents overweight and BMI ≥30 kg/m^2 obesity (WHO 2015b). In 2014, 39 % of adults (age ≥ 18) were overweight and 13 % obese. The constantly rising prevalence of obesity is clearly associated with the economical status of a population and thus the highest prevalence is found in the westernized (developed) countries and lowest in some African (malnourished) countries. The United States is one of the countries with very high prevalences, in 2014 67 % of its adult population was overweight and 34 % were obese. In Finland, the situation is almost as alarming as in USA, since respective percentages in 2014 were 55 % and 21 % (WHO 2015a).

It has been debated whether or not overweight or obesity can be considered as a disease. Weight gain alone associates with more serious diseases such as osteoarthritis and other musculoskeletal disorders related to carrying an excess weight (Rigby et al. 2009, WHO 2015b). Furthermore, the increased fat mass predisposes the individual to type 2 diabetes, cardiovascular diseases, non-alcoholic fatty liver disease (NAFLD), gallstones, cancer and metabolic syndrome (MS), which is a term for combined symptoms of impaired glucose tolerance, insulin resistance, dyslipidemias, hypertension and coronary heart disease (Bray 2004, Despres & Lemieux 2006). Since obesity can increase the prevalence of other serious diseases, it represents a major financial burden on the health care system.

Body weight is normally maintained at a rather constant level since there are many efficient energy metabolism regulating systems in the human body. However, environmental factors such as the availability of energy dense food and lack of physical activity disturb the energy homeostasis and promote the development of obesity. In addition to environmental inputs, genetic factors are important in determining the susceptibility to gain weight. The role of genes can be studied with gene-modified animal models and energy-rich diets can be exploited to mimick the environmental factors in the development of obesity.

Neuropeptide Y (NPY) is a neurotransmitter involved in several physiological and pathophysiological functions such as the cardiovascular system, anxiety, alcohol consumption and epilepsy. In energy metabolism, NPY promotes positive energy balance by stimulating food intake, promoting fat storage and inhibiting thermogenesis (Billington et al. 1991, Sainsbury et al. 1997, Zarjevski et al. 1993). NPY is expressed in the brain and in the peripheral sympathetic neurons. Hypothalamic NPY stimulates food intake but the role of noradrenergic neuron NPY is less clear. Chronic stress is known to release NPY from the noradrenergic sympathetic nerves and thus induces abdominal obesity (Kuo et al. 2007). Similarly, a single nucleotide Leucine-to-Proline (p.L7P) polymorphism in the human *Npy* gene has been shown to increase NPY secretion during

sympathetic stimulation and this polymorphism is associated with traits of metabolic disorders (Kallio et al. 2001, Pesonen 2008). Furthermore, our research group has previously revealed the importance of noradrenergic neuron NPY in the development of mild adiposity, disturbances in glucose metabolism and cardiovascular function (Ruohonen et al. 2008, 2009a & 2009b). Recently it was shown that noradrenergic neuron NPY was also responsible for the stress-induced weight gain (Zhang et al. 2014). Thus, noradrenergic neuron NPY clearly plays an important role in energy metabolism but the actual mechanisms have remained unknown. The focus of this thesis was to clarify the role and the mechanisms of noradrenergic neuron NPY in the development of obesity by exploiting a transgenic mouse model overexpressing NPY in the noradrenergic neurons. Furthermore, the interplay was studied between NPY excess and energy-rich diet or the other energy metabolism regulating systems, i.e. endocannabinoids and the sympathetic nervous system.

2. REVIEW OF THE LITERATURE

2.1 Regulation of energy metabolism

2.1.1 Food intake

Feeding is a complex process, which not only aims at satisfying the energy demands of physiological processes, but is also affected by emotional, cognitive and cultural factors. The direct neuroendocrine modulators of food intake are either orexigenic (feeding stimulative) or anorexigenic (feeding suppressive) and the net effect of these signals eventually decides whether eating will be started and when it is terminated. Summary of food intake regulation is presented in figure 1 and explained in more detail in the main text.

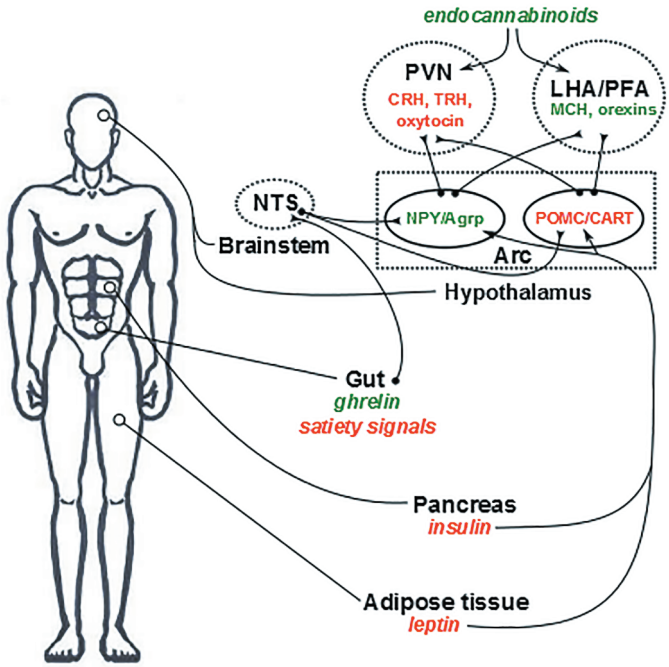

Figure 1. Food intake regulation. Orexigenic NPY/Agrp neurons and anorexigenic POMC/CART neurons are located in the ascuate nucleus (Arc) of hypothalamus and project to other central areas involved in the regulation of eating behavior. Additionally central endocannabinoids, satiety signals from gut and peripheral adiposity signals insulin and leptin take part in the food intake regulation. Orexigenic pathways are represented in green. They stimulate each other and inhibit anorexigenic pathways represented in red. Agrp=Agouti related peptide, Arc=arcuate nucleus, CART=cocaine and amphetamine-regulated transcript, CRH=corticotropin-releasing hormone, LHA=lateral hypothalamus, NPY=neuropeptide Y, NTS=nucleus tractus solitarius, PFA=perifornical area, PVN=paraventricular nucleus, POMC=pro-opiomelanocortin, TRH=thyrotropin-releasing hormone. Figure modified from Schwartz et al. (2000).

Feeding regulative signals form a complicated network in the central nervous system (CNS) receiving adiposity and satiety signals from many peripheral tissues such as the

gastrointestinal (GI) tract and adipose tissue. The main site of regulation is located in the hypothalamic Arcuate nucleus (Arc). Both orexigenic neurons releasing neuropeptide Y (NPY) and Agouti related peptide (Agrp) (Hahn et al. 1998) as well as anorexigenic neurons releasing pro-opiomelanocortin (POMC)-derived α-melanocyte stimulating hormone (α-MSH) and cocaine and amphetamine-regulated transcript (CART) peptide (Elias et al. 1998) are expressed in the Arc. These neurons project from the Arc to the paraventricular nucleus (PVN), the lateral hypothalamus (LHA) and the perifornical area (PFA), which secrete second-order regulators of feeding behavior. The PVN expresses several neuropeptides that reduce food intake i.e. corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and oxytocin, whereas the LHA and the adjacent PFA secrete neuropeptides, which stimulate food intake i.e. melanin-concentrating hormone (MCH) and orexins. The importance of these neuropeptides in the particular hypothalamic areas has been highlighted by conducting lesion studies which have revealed that a lesion in the PVN evoked increased food intake and elevated body weight (Leibowitz et al. 1981). In contrast, a lesion in the LHA has reduced these parameters (Hernandez & Hoebel 1989). Endocannabinoids discussed in more detail in section 2.1.5 'Endocannabinoid system in the regulation of energy metabolism' also modulate the release of neuropeptides in PVN and LHA (Quarta et al. 2011).

The central network receives satiety signals from the GI-tract via the vagus nerve, which projects to the hindbrain in the nucleus tractus solitarius (NTS). The NTS gathers peripheral signals and interconnects with the forebrain areas such as the PVN (Ter Horst et al. 1989). Furthermore, the NTS expresses leptin and melanocortin 4 (MC4) receptors (Mercer et al. 1998, Mountjoy et al. 1994) as well as POMC (Bronstein et al. 1992). Thus, the NTS is an important nucleus in the food intake regulating network.

There are several neurotransmitters regulating the food intake. NPY is the most potent feeding stimulative factor of the hypothalamus and its properties will be discussed in more detail in sections 2.2 'Neuropeptide Y (NPY)' and 2.3 'Physiological functions of NPY'. Another orexigenic peptide, Agrp, co-localizes in the same neurons with NPY. Both transgenic (Ollmann et al. 1997) and intracerebroventicular (ICV) (Rossi et al. 1998) administration of Agrp cause intensive hyperphagia, but it is not known whether Agrp functions as a competitive antagonist or as an inverse agonist for the central melanocortin receptors (Low 2011). Furthermore, orexin and opioid receptor related mechanisms appear to be involved in the action of Agrp (Hagan et al. 2001). Anorexigenic POMC is cleaved into smaller peptides called melanocortins. α-MSH is very important in the feeding behavior; this peptide inhibits food intake via melanocortin receptors MC3R and MC4R (Fan et al. 1997). Mice lacking MC4R are hyperphagic and obese (Huszar et al. 1997), and mutations in the *MC4R* gene have been linked with approximately 5 % of the early-onset morbid obesity in human populations (Farooqi et al. 2003) thus being the most common monogenic cause of obesity (Langhans et al. 2009).

Peripheral adiposity signals, i.e. leptin and insulin, modulate orexigenic and anorexigenic neurons in the Arc. Leptin and insulin are peripheral hormones produced in adipose tissue

and pancreatic beta-cells, respectively. They are called adiposity signals because their concentration in the blood is proportional to the body fat content. Despite their different origins, both hormones reduce food intake by stimulating the POMC/CART neurons and inhibiting NPY/Agrp neurons in the Arc in response to negative energy balance (Langhans et al. 2009). The crucial role of leptin in enrgy homeostasis is evidenced in leptin-deficient *ob/ob* mice and leptin receptor-deficient *db/db* mice, which are both hyperphagic and morbidly obese (Boozer & Mayer 1976, Cox & Powley 1977, Dubuc 1976). Moreover, obesity is commonly associated with increased leptin levels indicating that leptin resistance occurs both in humans (Considine et al. 1996) and in obese animal models (Mantzoros et al. 1998, Marsh et al. 1999). Insulin resistance is also a common symptom in obese subjects. The main function of insulin is to regulate the blood glucose level but it also inhibits food intake via hypothalamic insulin receptors. Both central (McGowan et al. 1990) and peripheral (VanderWeele et al. 1982) insulin administrations have been demonstrated to reduce food intake by inhibiting NPY neurons (Williams 2009). Furthermore, insulin deficiency such as diabetes mellitus is commonly associated with hyperphagia (Leedom & Meehan 1989).

Food intake, especially the feeding duration and meal size, is also regulated by short-term satiety signals secreted from the GI tract as a response to gastric filling or distension. The most important satiety peptides are cholecystokinin (CCK), glucagon like peptide-1 (GLP-1), ghrelin and peptide YY (PYY), which all, with the exception of ghrelin, reduce food intake (Valassi et al. 2008). CCK is a satiety hormone delivering the sense of fullness from the vagal sensory terminals of the upper gut to the NTS. GLP-1 promotes satiety, stimulates insulin secretion as a response to increased glucose levels, suppresses glucagon secretion and delays gastric emptying. Ghrelin, as an orexigenic peptide, induces feeding via stimulation of the NPY/Agrp pathway and inhibition of the POMC pathway. Additionally, it has a multifunctional role in the control of glucose and lipid metabolism, gastric mobility and acid secretion. PYY in turn reduces food intake by inhibiting NPY signaling in the hypothalamus (Batterham et al. 2002). PYY binds to the same receptors as NPY and its properties will be discussed in more detail later. Additionally, PYY is able to inhibit gastric motility and accelerate nutrient absorption (Holzer et al. 2012).

2.1.2 Energy expenditure

Energy acquired from nutrient intake is consumed in several processes of the body. Energy expenditure can be crudely divided into obligatory energy utilization in the physiological processes of the body, adaptive thermogenesis and movement-related energy expenditure (Tseng et al. 2010). Although one should not overlook the importance of physical activity in the regulation of body weight, the majority of the energy is used in the obligatory and adaptive thermogenesis. Obligatory thermogenesis includes the standard metabolic rate, i.e. energy used for cellular function and the thermic effect of food. Adaptive thermogenesis refers to those processes which are activated in order

to generate heat to stay warm. Additionally, extra energy is needed during periods of growth and reproduction.

Adaptive thermogenesis can be further divided into cold-induced shivering thermogenesis, non-shivering thermogenesis and diet-induced thermogenesis. In cold-induced shivering thermogenesis, the skeletal muscles shiver in order to generate heat as a response to an acute cold exposure. Shivering and non-shivering cold-induced thermogenesis are independent processes, activated separately. Non-shivering thermogenesis occurs in brown adipose tissue (BAT) and is an adaptative reaction to prolonged cold exposure. BAT is also responsible for diet-induced thermogenesis, which aims at dissipating the excess energy gained from food with a high energy content. Both non-shivering and diet-induced thermogenesis are dependent on the presence of a 32 kDa protein called uncoupling protein 1 (UCP1), which is located in the inner membrane of mitochondria; it allows the uncoupling of the electrons to move back along the proton gradient, and thus releasing energy as heat instead of using it for the production ATP (Klingenberg & Huang 1999). UCP1 is expressed only in brown adipocytes and it is necessary for normal thermoregulation; this has been demonstrated on UCP1-deficient mice, which are sensitive to cold and compensate for the impairment in BAT thermogenesis by prolonged shivering (Golozoubova et al. 2001). However, UCP1 ablated mice are not obese or hyperphagic when housed in a normal laboratory environment (Enerbäck et al. 1997). The importance of UCP1 activity in the regulation of energy metabolism became evident when UCP1-deficient mice were housed in thermoneutral conditions (~30° C); the animals did not consume energy in heat production but developed obesity both when fed normal chow or high-fat diet (Feldmann et al. 2009).

BAT is extensively innervated by sympathetic nerves (Cottle & Cottle 1970) and BAT thermogenesis is stringently regulated via sympathetic β-adrenoceptors (Landsberg et al. 1984). When the body temperature drops below thermoneutrality, cold-sensitive neurons in the skin and in the CNS are activated, the sympathetic nervous system (SNS) becomes stimulated, noradrenaline (NA) is released and the amount of cyclic AMP is increased via β_3-adrenoceptor activation. Cyclic AMP further activates lipolysis and the resulting free fatty acids stimulate *Ucp1* expression (Lowell & Spiegelman 2000). The importance of SNS regulation is evidenced by the NA-induced enhancement in thermogenesis (Landsberg et al. 1984). Furthermore, mice with ablated NA and adrenaline are cold intolerant not only because of the resulting impairment in BAT function but also because of failure in peripheral vasoconstriction during cold (Thomas & Palmiter 1997). Thyroid hormones also have an important role in the adaptive thermogenesis (Silva 2006). During cold exposure, SNS stimulation of BAT activates type II iodothyronine 5'-deiodinase (D2) enzyme, which is responsible for the conversion of the prohormone thyroxine (T4) into the active form of the hormone, triiodothyronine (T3). The increase in BAT T3 levels further amplifies the SNS tone, activating lipolysis and UCP1 stimulation. On the other hand, catecholamines increase D2 activity, thus illustrating the interaction between thyroid hormones and catecholamines in the regulation of BAT thermogenesis.

Since the mechanisms regulating adaptive thermogenesis and food intake are intimately intertwined, many key neurotransmitters affecting food intake are important also in the regulation of thermogenesis. For example, several hormones such as insulin, glucagon, glucocorticoids and leptin regulate thermogenesis, but the mechanisms are indirect and ultimately feed back to the regulation of SNS and thyroid hormone pathway.

2.1.3 White adipose tissue as energy storage

White adipose tissue (WAT) is destined to store excess energy in the form of triglycerides. WAT consists of white adipocytes and stromal vascular cells including fat precursor cells, fibroblasts, immune cells and blood vessel cells. The latter cells are responsible for the appropriate angiogenesis and immune cell infiltration, which are needed not only for the vitality of the adipose tissue but also for its expansion capability (Sun et al. 2011). One microscopic characteristic of the white adipocyte is the large lipid droplet separating the triglyceride reservoir from the cell cytoplasm and a nucleus flattened into the edges of adipocytes. Since adipocytes can store such a large lipid load, their size can vary enormously (diameter 20-200 μm). Mature adipocytes are not able to differentiate, and thus when existing adipocytes have reached their maximum volume, then new adipocytes are formed from the pre-existing preadipocytes which originally differentiate from mesenchymal stem cells in the process of adipogenesis. (Langin et al. 2009, Tortora & Grabowski 2004) Adipose tissue mass can be increased either via hyperplasia, i.e. increased number of adipocytes or via hypertrophy, i.e. enlarged size of adipocytes.

Triglycerides are formed from one glycerol molecule and three fatty acid molecules. During the process called lipogenesis, lipids are synthesized from their precursors, which are acquired mostly from digested macronutrients, and stored in WAT (Fig. 2, Tortora & Grabowski 2004, Vazquez-Vela et al. 2008). Lipid accumulation in the adipocytes depends on the uptake rate of circulating fatty acids, which is regulated by lipoprotein lipase (LPL), an enzyme which hydrolyses triglycerides into free fatty acids in the chylomicrons (Zechner et al. 2000). Once fatty acids and glycerol are ingested inside the adipocyte, they are converted to triglycerides via a pathway of anabolic reactions. Triglycerides can be further modified to other lipids such as lipoproteins according to the body's requirements. Lipogenesis is stimulated by insulin, which increases the activity and gene expression of enzymes involved in lipogenesis i.e. fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) (Sul & Wang 1998).

In the case of energy deficiency, triglycerides in WAT are hydrolyzed back to fatty acids and glycerol in the process called lipolysis (Langin 2006, Tortora & Grabowski 2004, Vazquez-Vela et al. 2008). The hydrolysis of triglycerides is mediated via several enzymes, i.e. adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoglyceride lipase (MGL). The hydrolysis products are then exported and catabolized in order to produce energy in the form of ATP. Glycerol is converted to glyceraldehyde 3-phosphate and metabolized further either to glucose or pyruvic acid depending on the ATP supply of the cell. Fatty acids are catabolized in the mitochondria in a process of beta oxidation,

which serially removes two carbon fragments from the fatty acid side chain and produces acetyl coenzyme A, which is further degraded to ATP in the Krebs cycle. Lipolysis is most effectively stimulated by catecholamines and natriuretic peptides i.e. atrial and brain natriuretic peptides, which stimulate adenylyl and guanylyl cyclase to produce cyclic AMP and GMP, respectively. These second messengers further activate HSL and thus assist in the hydrolysis of triglycerides. There are also other lipolysis stimulators e.g. growth hormone (GH), glucocorticoids, thyroxine and tumour necrosis factor-α (TNF-α). Lipolysis is inhibited by insulin which inhibits the actions of several lipases.

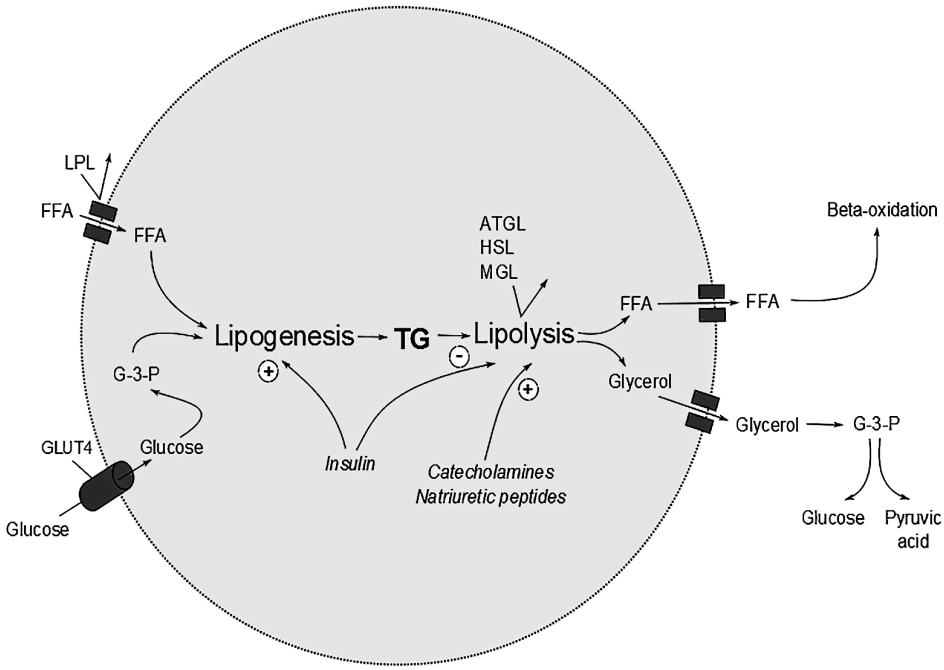

Figure 2. Lipogenesis and lipolysis in white adipocyte. Lipogenesis synthesizes triglycerides from one molecule of glycerol-3-phosphate (G-3-P) and three molecules of free fatty acids (FFA). Glycerol-3-phosphate is formed in the cell via phosphorylation from glucose, which is imported inside the cell via GLUT4-transporter. Lipoprotein lipase (LPL) enzyme regulates FFA intake. In lipolysis triglycerides are hydrolyzed back to FFAs and glycerol via lipolysis stimulating enzymes adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoglyceride lipase (MGL). The resulting glycerol and FFA are exported outside from the adipocyte and processed further in order to produce energy. Figure modified from Langin et al. (2009).

2.1.4 Sympathetic nervous system in the regulation of energy metabolism

SNS is a part of the autonomic nervous system and it is responsible for the "fight-or-flight" response in the body by releasing NA from post-ganglionic neurons. NA activates G protein-coupled adrenergic receptors (α_1-α_2, β_1-β_3) in the tissues and thus causes a wide spectrum of effects depending on the target tissue and receptor subtype. In 1990's, obesity was linked to decreased sympathetic tone, which has led to the so-

called “Mona Lisa” hypothesis standing for the phrase “Most Obesities kNown Are Low In Sympathetic Activity” (Bray 1991). It has been shown in rodents that sympathetic activity is down-regulated in obesity attributable to hyperphagia, hypothalamic lesions or genetic manipulations (Bray 1991, Young & Landsberg 1983). Obese humans also exhibit decreased plasma noradrenaline levels (Peterson et al. 1988) and a higher body fat percent and these values correlate with lower sympathetic nerve activity in muscles (Spraul et al. 1993). However, later this paradigm has been criticized because SNS over-activity is present in patients with many cardiovascular disorders, such as hypertension and congestive heart disease, which are more frequent in obese population (Davy & Orr 2009). The current opinion is that low SNS activity is a risk for weight gain, but SNS over-activity is a characteristic for the metabolic symptoms which accompany obesity.

In order to understand the imbalance in SNS tone in patients with the metabolic syndrome, a compartment model has been developed (Kreier et al. 2003). In this model, the whole body and the different symptoms of metabolic disorder have been divided into distinct compartments, which are regulated differently by the autonomic nervous system. For example, the organs situated in visceral compartment receive a predominant parasympathetic activation, whereas the thoracic and movement compartments are shifted towards SNS over-activity. Reduced sympathetic activity contributes to the pathophysiology of obesity at several levels. As described earlier, sympathetic nervous system activates the adaptive BAT thermogenesis, thus explaining the decreased energy expenditure in obese population. Furthermore, SNS directly stimulates lipolysis in humans via β_1 and β_2-adrenoceptors (Barbe et al. 1996) and in rodents also via β_3-adrenoceptors (Arch & Wilson 1996). Thus, reduced sympathetic activity will decrease the lipolysis rate and promote weight gain directly in the adipose tissue. However, noradrenaline and other catecholamines have a relatively higher affinity for α_2-receptors, which exert an inhibitory effect on lipolysis (Langin 2006). Thus, the ultimate *in vivo* effect of sympathetic activation on fat accumulation will be dependent on both endocrine and paracrine regulation as well as the type and location of fat tissue (Dodt et al. 2003). As mentioned, the metabolic syndrome with hypertension and insulin resistance associates with SNS over-activity (Kreier et al. 2003). The increased sympathetic drive elevates heart rate and vascular resistance, and thus increases blood pressure, which if prolonged, causes hypertension. Furthermore, insulin sensitivity is impaired because of the haemodynamic changes attributable to SNS over-activity. On the other hand, activation of the parasympathetic nervous system increases the accumulation of intra-abdominal fat.

In summary, the role of sympathetic nervous system in the regulation of energy balance can be investigated at the organ level but not at the whole body level. Additionally, many confounding factors such as nutritional and disease states further complicate the clarification about the role of sympathetic tone in the energy metabolism.

2.1.5 Endocannabinoid system in the regulation of energy metabolism

The endocannabinoid system consists of lipid mediators known as endocannabinoids, the metabolic enzymes for these compounds and their molecular targets, i.e. the cannabinoid

receptors type-1 (CB1R) and type-2 (CB2R). The two most studied endocannabinoids are arachidonic acid derived anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) (Pagotto et al. 2006). Several other lipid mediators acting via CB-receptors have also been identified but the physiological role of these compounds is still somewhat unclear (De Petrocellis et al. 2004). The endocannabinoid system participates in several physiological functions such as nociception (Cravatt & Lichtman 2004), neuroprotection (Panikashvili et al. 2001 & 2005) and regulation of cardiovascular (Cravatt & Lichtman 2004, Mendizabal & Adler-Graschinsky 2003), immune and inflammatory systems (Klein et al. 2003, Massa et al. 2004). Importantly, endocannabinoids modulate energy homeostasis, i.e. they regulate both appetite and hedonistic aspects of food intake (Harrold & Williams 2003) and energy expenditure.

AEA and 2-AG can activate both of the G protein-coupled CB-receptors. However, the CB1R is more important in energy metabolism since it is expressed not only in many of the brain areas controlling food intake and reward, such as the hypothalamus and the mesolimbic system, but also in peripheral organs controlling lipid and glucose metabolism, such as the WAT and the liver (Pagotto et al. 2006). CB2Rs are expressed almost exclusively in immune and blood cells and their function is to mediate the immunomodulatory effects of endocannabinoids (Howlett et al. 2002).

Endocannabinoids affect energy homeostasis at several levels (Fig. 3). They modulate the feeding behavior by regulating CRH and TRH releasing neurons in PVN and MCH synthetizing neurons in LHA (Quarta et al. 2011). Central administration of AEA or 2-AG evokes hyperphagia, which can be attenuated with a CB1R-antagonist or an inverse agonist (Kirkham et al. 2002, Williams & Kirkham 1999). Furthermore, CB1R knock-out (KO) mice are lean and have reduced food intake (Cota et al. 2003). However, endocannabinoids exert much wider effects on energy and glucose metabolism at the level of peripheral organs. In adipocytes, endocannabinoid system activation promotes adipogenesis and lipid accumulation (Karaliota et al. 2009, Teixeira et al. 2010) whereas administration of a CB1R-antagonist enhances lipolysis (Jbilo et al. 2005). Treatment with a CB1R-antagonist also stimulates BAT thermogenesis and enhances glucose uptake both in BAT and WAT in diet-induced obese mice independently of its anorectic or weight-reducing effects (Bajzer et al. 2011). Furthermore, CB1R activation has been shown to enhance basal and glucose-induced insulin release from pancreatic β-cells (Malenczyk et al. 2013, Matias et al. 2006) and inhibit insulin signaling and glucose uptake in skeletal muscle myotubes (Cavuoto et al. 2007, Lindborg et al. 2010). In hepatocytes, elevated AEA levels and CB1R overactivity contribute to the hepatic insulin resistance and formation of steatosis (Liu et al. 2012, Malenczyk et al. 2013,), which is prevented in CB1R-deficiency (Osei-Hyiaman et al. 2005). Since the endocannabinoid system modulates a wide variety of target tissues controlling metabolism, it is not surprising that both obese animal models and obese patients have dysregulated CB1R activity with abnormal endocannabinoid levels in both central and peripheral tissues (Engeli et al. 2005, Massa et al. 2010). Thus, the endocannabinoid system is a promising

target for drug development. In fact, the CB1 antagonist, rimonabant, was previously used for the treatment of obesity, but withdrawn from the market due to the increased risk for psychiatric adverse events (Christensen et al. 2007). However, the compounds acting only on the peripheral CB1 receptors are still viewed as attractive therapeutic options for the treatment of metabolic diseases.

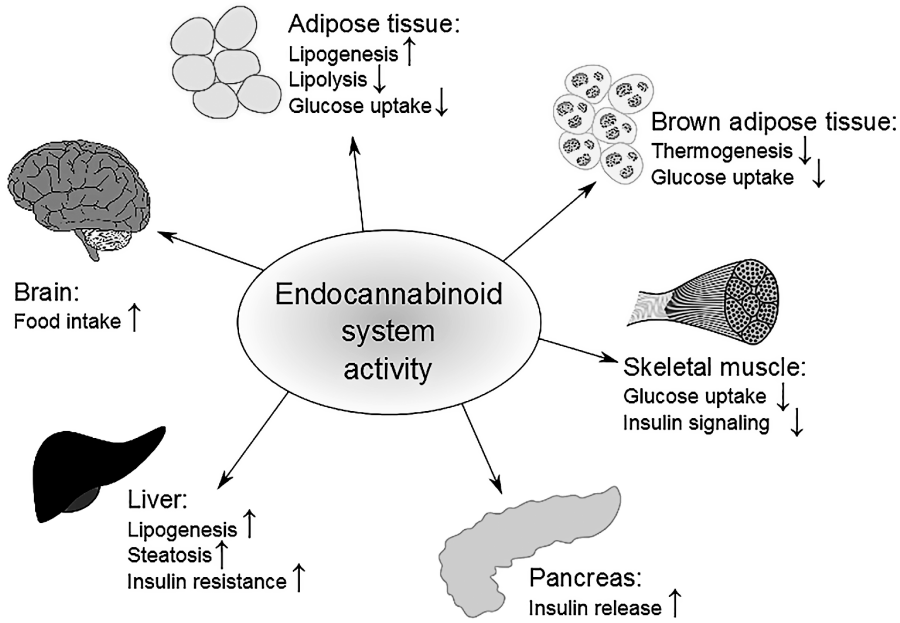

Figure 3. The multiple effects of endocannabinoid system on energy metabolism. Figure modified from Lipina et al. (2012).

The endocannabinoid system undertakes cross-talk with other peptides regulating energy metabolism. Leptin negatively regulates the endocannabinoid system because the elevated hypothalamic 2-AG and AEA levels of *db/db* and *ob/ob* mice can be normalized by acute leptin treatment (Di Marzo et al. 2001). In contrast to leptin, ghrelin increases hypothalamic endocannabinoid levels (Kola et al. 2008). Furthermore, administration of the CB1R antagonist rimonabant acutely modifies the hypothalamic expression of MCH, CART, α-MSH and NPY by activating the neurons co-localizing these neuropeptides (Verty et al. 2009). The cross-talk between endocannabinoid and NPY system will be discussed in more detail in section 2.3.7 'NPY and endocannabinoid system'.

2.2 Neuropeptide Y (NPY)

2.2.1 General

Neuropeptide Y is a 36 amino acid peptide, which was originally isolated from pig brain by Tatemoto and colleagues in 1982 (Tatemoto 1982). Neuropeptide was

named ‘Y’ because of the tyrosine (abbreviation Y) residues found at both ends and in the middle of the peptide. NPY belongs to pancreatic polypeptide family, which includes also pancreatic polypeptide (PP) and PYY. All of these three peptides have many similarities e.g. chemical structure, affinity for Y-receptors (YRs) and ability to modulate food intake. PP and PYY are expressed in endocrine type of cells in pancreas and GI tract, where they regulate pancreatic secretion and gastric motility (Ekblad & Sundler 2002).

NPY can be co-localized in the same neurons with other neuropeptides or neurotransmitters such as Agrp, somatostatin and gamma-aminobutyric acid (GABA) (McDonald & Pearson 1989). In sympathetic nerve endings, NPY is co-localized with noradrenaline, and in adrenal chromaffin cells it is accompanied by both noradrenaline and adrenaline (Cavadas et al. 2011, Everitt et al. 1984). It is stored in large dense-cored vesicles (diameter >80 nm), where it is released together with noradrenaline after prolonged sympathetic stimulus such as physical exercise or cold exposure. Small dense-cored vesicles (diameter ~50 nm) containing only noradrenaline are secreted more rapidly after an acute sympathetic stimulus (Ekblad et al. 1984, Fried et al. 1985, Potter 1988). Thus, the resting plasma levels of NPY in most species, with the exception of rats, are relatively low (Ericsson et al. 1987). Due to the long half-life of NPY (approx. 20 min), the plasma NPY concentration declines relatively slowly after the cessation of a prolonged sympathetic stimulus (Potter 1988).

2.2.2 NPY in the central nervous system

The widespread distribution of NPY throughout the brain e.g. basal ganglia, hippocampus, cortical, limbic, and hypothalamic areas supports the important role of NPY in various physiological systems. The NPY concentration is highest in the hypothalamic nuclei Arc and its projection site, the PVN. The neurons in the Arc and the PVN further project to other parts of the hypothalamus e.g. the medial preoptic area (MPOA), the LHA, the anterior hypothalamic area, the periventricular nucleus, the ventromedial hypothalamus (VMH), the dorsomedial hypothalamic nucleus (DMH) and the posterior hypothalamus (Chronwall et al. 1985). In these neurons, NPY may co-exist with other neurotransmitters (Chronwall et al. 1984, Hahn et al. 1998) in addition to catecholamines (Bai et al. 1985). In the brainstem, NPY has been detected in catecholaminergic neurons that synthesize either adrenaline or noradrenaline (Sawchenko et al. 1985). The ventrolateral medulla oblongata with A1/C1 neurones, the locus coeruleus (LC) and the NTS which sends NPY projections to the PVN are the most important sites for NPY expression in the brainstem (Chronwall et al. 1985, Everitt et al. 1984). NPY immunoreactive cell bodies can be found also in cortex and in several locations included in the limbic system e.g. hippocampus, dentate gyrus, amygdala and nucleus stria terminalis (Chronwall et al. 1985). Furthermore, NPY is detected in spinal cord, more specifically in dorsal horn (Polak & Bloom 1984). Most of the NPY distribution studies have been conducted in rodents but the NPY

expression panel in CNS is very similar between the rodents and humans despite the structural differences of the brain (Caberlotto et al. 2000).

2.2.3 NPY in the peripheral nervous system

In addition to the CNS, NPY is widely expressed in peripheral tissues. NPY-immunoreactive nerve fibers in SNS innervate blood vessels (Ekblad et al. 1984, Sundler et al. 1983), the heart (Gu et al. 1984), respiratory tract (Sheppard et al. 1984), the gut (Sundler et al. 1983), the pancreas (Sundler et al. 1983), the liver (Taborsky et al. 1994), the adipose tissue (Cannon et al. 1986, Kuo et al. 2007), the thyroid gland (Grunditz et al. 1984), the eye (Terenghi et al. 1983) and the genital tract (Adrian et al. 1984). NPY is produced in the chromaffin cells of adrenal medulla and thus particularly high NPY concentrations are found in the adrenal glands (Varndell et al. 1984). NPY in chromaffin cells is mostly co-localized in the same cells with adrenaline (Kuramoto et al. 1986) but also expressed in NA-containing granules of adrenal medulla (Varndell et al. 1984). In non-sympathetic peripheral neurons, NPY is present in the myenteric and submucous ganglia, which are a part of the enteric nervous system innervating the small intestine wall and regulating the function of the circular muscle (Furness et al. 1983).

2.2.4 Y-receptors (YRs)

The effects of NPY are mediated via Gi/Go-coupled receptors named Y1, Y2, "Y3", Y4, Y5 and y6. In addition to NPY, these receptors bind the other members of pancreatic polypeptide family (PYY, PP) and C-terminal fragments of NPY (NPY_{2-36}, NPY_{3-36}, NPY_{13-36}, NPY_{18-36}) with different affinities (Silva et al. 2002). The expression of YRs varies between species and tissues.

Y1R was cloned in 1990 by Eva et al. and it is widely expressed both in rodent and human CNS and peripheral tissues (Eva et al. 1990). Y1R is found for example in the thalamic and hypothalamic regions, visceral adipose tissue, pancreas, adrenal glands and blood vessels (Eva et al. 1990, Kopp et al. 2002, Matsuda et al. 2002, Morgan et al. 1998, Yang et al. 2008). Y1R displays the highest affinities for NPY and PYY (Silva et al. 2002).

Y2R was cloned in 1995 by Rose et al. Y2 is mostly a presynaptic receptor, which suppresses the release of NPY and other transmitters (Colmers et al. 1991). Y2R, like Y1R, is expressed widely in the CNS e.g. in hypothalamus, hippocampus, amygdala and the brainstem (Parker & Herzog 1999, Stanic et al. 2006). In the periphery, Y2R is expressed or linked to the function of many organs including blood vessels, visceral white adipose tissue, intestine as well as being found on sympathetic and parasympathetic nerve endings (Cox & Cuthbert 1990, Grundemar & Hakanson 1990, Kuo et al. 2007, Stjernquist & Owman 1990, Wahlestedt et al. 1986, Zukowska-Grojec et al. 1998). Y2R has good affinity for NPY, PYY and C-terminal fragments of NPY (Silva et al. 2002,

Wahlestedt et al. 1986). However, PP and C-terminally truncated fragments bind to this receptor with only a low affinity.

Y3R has not been cloned but based on pharmacological studies it has been proposed to exist (Grundemar et al. 1991, Lee & Miller 1998). One characteristic of the Y3-receptor is its considerably higher affinity for NPY in comparison with other neurotransmitters such as PYY (Lee & Miller 1998). The expression profile of Y3R has been constructed based on the pharmacological studies. Y3R is believed to regulate the cardiovascular function and vascular permeability in the rat NTS, vascular endothelial cells and lung (Chen et al. 2007, Grundemar et al. 1991, Hirabayashi et al. 1996, Nan et al. 2004). Additionally, Y3R is thought to be expressed in the adrenal capsule and chromaffin cells (Bernet et al. 1994, Norenberg et al. 1995).

Y4R was cloned in 1995 by Bard et al. (1995) and it is occasionally called PP1-receptor because of its markedly higher affinity for PP in comparison with other ligands (Gehlert et al. 1996). The Y4R tissue expression profile is similar in rodents and humans. Since PP is located peripherally, also Y4R is widely expressed in the periphery e.g. pancreas, gut, skeletal muscle, lung and heart (Bard et al. 1995, Yan et al. 1996). Furthermore, Y4R is expressed in the brain, particularly in the hypothalamus but also in the brainstem e.g. the area postrema, the NTS and in the dorsal vagal complex (Bard et al. 1995, Campbell et al. 2003, Larsen & Kristensen 1997, Parker & Herzog 1999).

Y5R was cloned in 1996 by Gerald et al. (Gerald et al. 1996, Weinberg et al. 1996) and it is expressed predominantly in the CNS, i.e. in the hypothalamus, especially in the PVN (Gerald et al. 1996), but also in the cortex, the hippocampus and the brainstem (Grove et al. 2000). In the periphery, Y5R is expressed in the small intestine, colon, pancreas, spleen and testis (Goumain et al. 1998, Statnick et al. 1998, Weinberg et al. 1996). Y5R has the highest affinity for NPY and PYY (Silva et al. 2002).

The y6R was cloned in 1996 by Weinberg et al. but it was originally referred to Y5R and only later designated as y6R (Burkhoff et al. 1998). The y6R is expressed as a functional receptor only in mice and rabbits (Yulyaningsih et al. 2011). It is not expressed at all in rats (Burkhoff et al. 1998) and in some other species, including humans, it is expressed in a truncated form with a premature stop codon (Matsumoto et al. 1996, Nakamura et al. 1997). Despite its inactivation, the gene transcript has been detected for instance in the human heart skeletal muscle and adrenal glands (Gregor et al. 1996, Matsumoto et al. 1996). In mice, y6R has been reported to be expressed centrally in the suprachiasmatic nucleus, the anterior hypothalamus, the bed nucleus of stria terminalis and the ventromedial nucleus (Weinberg et al. 1996), and peripherally in the kidney and testis (Burkhoff et al. 1998). The y6R has similar binding properties as Y4R with its highest affinity for PP and a lower affinity for PYY and NPY (Gregor et al. 1996).

2.3 Physiological functions of NPY

The summary of the effects of NPY on the regulation of energy metabolism is presented in figure 4.

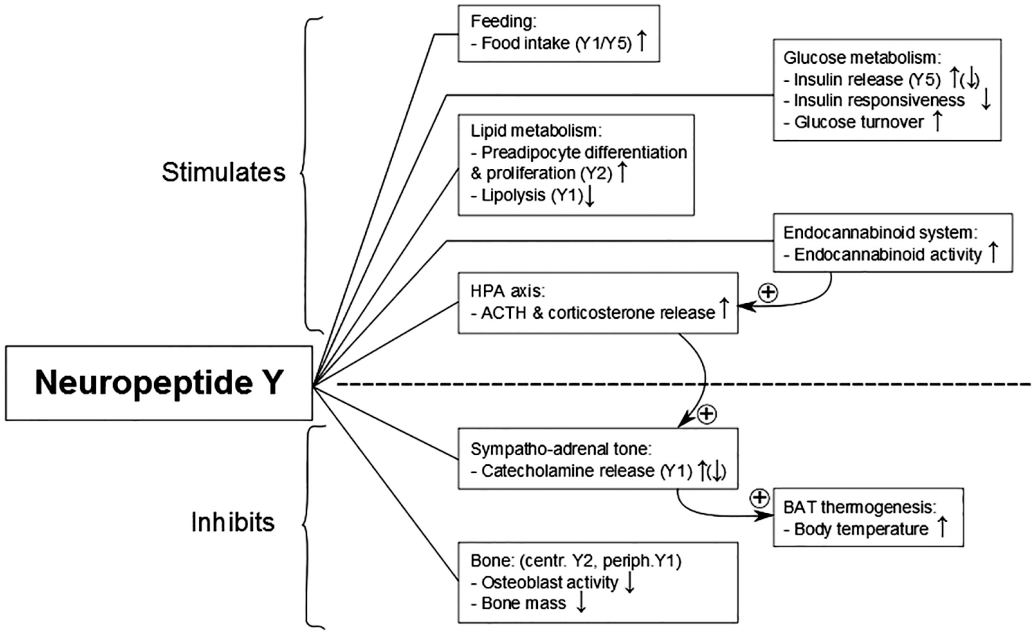

Figure 4. Summary of the NPY's effects on different systems involved in the regulation of energy metabolism. The Y-receptors (YRs) that are known to mediate these effects are marked in the figure. Those systems that are stimulated by NPY are represented above the dashed line and those systems that are inhibited are marked below the line. ↑/+ = stimulatory effect, ↓/- = inhibitory effect, ACTH = adrenocorticotropic hormone, HPA = hypothalamic-pituitary-adrenal.

2.3.1 NPY in the regulation of food intake

It was first shown by Clark et al. (1984) that ICV NPY injection stimulates food intake in a dose-dependent manner in satiated rats, a finding soon confirmed by others (Levine & Morley 1984, Stanley & Leibowitz 1984). Consequently, NPY's role in the regulation of food intake has been an area of active investigation.

More specific ICV injections to the separate hypothalamic nuclei have shown that the PFA within the LHA is the most sensitive hypothalamic area for NPY-induced feeding, but NPY stimulates feeding also in the PVN, the VMH and on some occasions in the DMH (Li et al. 1994, Stanley et al. 1985a & 1993). However, the most dense NPYergic nerve innervation, is present in the PVN (Gray et al. 1986), which thus is the main locus of NPY-mediated feeding. From the PVN, the NPYergic nerves further project to the hindbrain and other areas mediating the feeding response. The NPY concentration in the PVN increases before eating and declines soon after the initiation of eating (Kalra

et al. 1991). Acute and chronic injections of NPY into the PVN induce a strong feeding response without tolerance (Stanley & Leibowitz 1985, Stanley et al. 1986). However, food intake slowly returns to normal after the cessation of NPY administration (Stanley et al. 1986). The importance of NPY in the stimulation of food intake has also been shown in situations of energy deficiency. In fasted animals, NPY release is increased but returns to the normal level after 24-h of feeding (Kalra et al. 1991). Furthermore, NPY expression in the Arc is increased by both food deprivation and food restriction (Brady et al. 1990, Sahu et al. 1988). There is a report that food deprivation increases NPY expression also in the PVN and remains elevated for a day after *ad libitum* eating interpreted to reflect NPY synthesis in the Arc from where it is transported to the PVN (Sahu et al. 1988).

NPY affects food intake by reducing the latency to eat and by delaying satiety (Leibowitz & Alexander 1991, Sindelar et al. 2005). Furthermore, NPY increases preference for carbohydrates (Stanley et al. 1985b), which is also seen as increased respiratory quotient after NPY injection to the PVN (Menendez et al. 1990). These effects are mediated via Y1Rs and Y5Rs (Leibowitz & Alexander 1991, Mashiko et al. 2003). The stimulation of Y2R via PYY, on the other hand, reduces food intake (Batterham et al. 2002, Feletou & Levens 2005). In addition to food intake, NPY in the PVN mildly increases drinking as a direct effect rather than secondary to increased food intake (Stanley et al. 1985a, Stanley & Leibowitz 1985). The feeding response triggered by NPY is known to be different from the orexigenic effects of noradrenaline and adrenaline (Stanley & Leibowitz 1985, Leibowitz et al. 1988). For example, separate silencing of NPYergic and catecholaminergic innervations in the brainstem has no effect on glucoprivic feeding, whereas simultaneous silencing of both systems significantly suppresses glucoprivic feeding (Li et al. 2009), thus suggesting that these independent feeding regulating systems can compensate one another.

2.3.2 NPY in the regulation of energy expenditure and brown adipose tissue thermogenesis

The effect of NPY on energy expenditure at the level of physical activity or basal metabolic rate has not been widely studied. Germline NPY-deficient mice display suppressed locomotor activity in an open field test (Karl et al. 2008), which could be mediated via increased sympathetic tone, but in animal behaviour is associated more with anxiety than with the regulation of energy homeostasis. However, NPY is known to regulate energy expenditure by affecting BAT thermogenesis. This was first found as a decrease in the body temperature induced by central NPY administration in animals at their normal environmental temperature (Esteban et al. 1989, Morioka et al. 1986) or having prostaglandin E_2-induced fever (Inui et al. 1989). Subsequently, it became clarified that the hypothermic effect is mediated via SNS since microinjection of NPY into the PVN suppressed the sympathetic nerve activity to BAT (Egawa et al. 1991) and NPY administration to the lateral ventricle feeding-independently decreased BAT GDP

binding, which is an indicator of BAT thermogenic activity (Billington et al. 1991). Thus, the effects of NPY on energy expenditure and sympathetic activity are very closely interwoven but seem to be one-directional, since sympathetic stimulation of BAT thermogenesis does not affect hypothalamic NPY levels (Bing et al. 1998). However, in one study, acute cold-exposure was shown to increase NPY levels in hypothalamic areas via unknown mechanisms (McCarthy et al. 1993).

Several hypothalamic brain areas are involved in the regulation of sympathetic outflow to the BAT (Holt et al. 1987). In addition to the previously mentioned PVN, NPY knock-down (KD) after transduction by adeno-associated virus (AAV), in the DMH potentiate BAT thermogenesis as evidenced with increased UCP1 protein expression in BAT (Chao et al. 2011). Furthermore, UCP1 positive "brite" cells are found in the subcutaneous inguinal WAT of NPY-KD rats as a result of potentiated sympathetic activity. Moreover, in the Arc, NPY inhibits BAT thermogenesis by decreasing the sympathetic activity, which is postulated to be mediated via Y1Rs (Shi et al. 2013). Extra-hypothalamic NPY can also affect thermogenesis since an NPY injection into the sulcal prefrontal cortex (SPC) has inhibited energy expenditure but did not reduce locomotor activity (McGregor et al. 1990).

2.3.3 NPY in the regulation of white adipose tissue and lipid metabolism

Adipocytes express NPY, Y1Rs and Y2Rs with some species-dependent variation (Kuo et al. 2007, Serradeil-Le Gal et al. 2000, Yang et al. 2008). Since adipose tissue is a very heterogenic tissue containing different cell types, NPY may affect adipose tissue also via indirect ways, for example via the inflammatory system (Zhang et al. 2014). Furthermore, adipose tissue is an endocrine organ producing a variety of hormones and adipokines which may change the expression of NPY or vice versa. For example, NPY expression in WAT can be stimulated by corticosterone or insulin treatment, which is in concordance with the increased levels of blood corticosterone, insulin and WAT NPY concentrations of obese animal models (Yang et al. 2008). However, it is challenging to determine the causality of these endocrine factors and because of the complexity of the neuroendocrine circuits, this review concentrates mainly on the direct endocrine and the neural effects of NPY.

Kuo et al. (2007) were the first researchers to show that peripheral NPY could have a direct role in lipid metabolism. They reported that stress-induced NPY release from sympathetic nerves upregulates NPY expression in WAT and facilitates the fat growth by increasing both proliferation and differentiation of preadipocytes via Y2Rs. Subsequently, Yang et al. (2008) confirmed that NPY is synthesized in visceral WAT and it stimulates proliferation of preadipocytes. However, they postulated that these effects are mediated via Y1Rs, which was in disagreement with other studies (Rosmaninho-Salgado et al. 2012). Today, the general consensus is that the promotion of adipogenesis is mediated primarily via Y2Rs.

In addition to the stimulation of adipogenesis, NPY inhibits lipolysis in both isolated adipocytes and fully differentiated 3T3-L1 adipocytes, and this is mediated via

Y1Rs (Bradley et al. 2005, Labelle et al. 1997, Valet et al. 1990). Consequently, an NPY antagonist (S.A.0204) stimulates lipolysis (Margareto et al. 2000). Furthermore, sympathetic nerve-secreted NPY has been shown to down-regulate lipolysis in an *ex vivo* model co-culturing 3T3-L1 adipocytes and sympathetic neurons, where adipocyte-derived soluble factors further up-regulate the secretion of NPY, thus revealing a feedback loop between WAT and SNS-derived NPY (Turtzo et al. 2001). Indirectly, NPY facilitates adipose tissue growth by stimulating the angiogenic activities in WAT via Y2Rs (Ekstrand et al. 2003, Kuo et al. 2007).

In addition to peripheral NPY, central NPY participates in the regulation of lipid metabolism. Central NPY administration increases very low density lipoprotein (VLDL) triglyceride secretion to the bloodstream (Stafford et al. 2008). Furthermore, both acute and chronic ICV NPY administrations increase WAT LPL activity independent of food intake (Billington et al. 1991 & 1994, Stanley et al. 1986, Zarjevski et al. 1993). Lipogenesis is also mediated via increases in glucose uptake and hepatic *de novo* lipogenesis (Zarjevski et al. 1993).

2.3.4 NPY in the regulation of glucose metabolism

Chronic NPY infusion to the brain (Vettor et al. 1994) or acute microinjections into the specific hypothalamic nuclei or the brainstem (Dunbar et al. 1992) increase blood insulin levels. NPY can elevate insulin levels food intake dependently via Y5Rs and food intake independently via Y1Rs (Gao et al. 2004). Additionally, central NPY changes the peripheral tissue responsiveness to insulin by increasing glucose uptake into WAT and impairing glucose utilization in the muscle, the latter phenomenon being associated with hyperphagia (Vettor et al. 1994). On the other hand, NPY has been shown to decrease insulin sensitivity by stimulating the glucose production, but not the glucose disposal (Marks & Waite 1997).

Since NPY nerve fibers are found in the pancreas (Sundler et al. 1983), NPY presumably exerts direct effects on insulin release from pancreatic beta cells. Intravenous administration of high-doses of NPY increase basal insulin release but inhibits glucose-stimulated insulin secretion in rats (Pettersson et al. 1987). In isolated rat pancreatic islets, NPY decreases both basal and glucose-stimulated insulin release (Moltz & McDonald 1985). However, NPY overexpression in pancreatic islets has no effect on insulin secretion *in vivo* while pancreatic islets isolated from these transgenic mice show reduced glucose-stimulated insulin secretion (Machida et al. 2014). On the other hand, NPY antiserum increases insulin release (Wang et al. 1994) and pancreatic islets of NPY deficient mice show increased basal and glucose induced insulin secretion (Imai et al. 2007). Inhibition of insulin secretion is mediated via pancreatic Y1Rs (Morgan et al. 1998).

NPY and insulin have bidirectional interaction. Both central and subcutaneous insulin administration abolished the elevated NPY levels in the hypothalamic Arc-PVN axis of

fasted rats (Malabu et al. 1992, Schwartz et al. 1992). Furthermore, hypoinsulinemic conditions, i.e. food deprivation, diabetes and intensive exercise are associated with increased NPY levels (Lewis et al. 1993, Williams et al. 1989). However, the role of insulin in the regulation of hypothalamic NPY expression applies only to the situations with normal insulin sensitivity. For example, in obese Zucker rats, ICV insulin administration is not able to reduce the fasting-induced rise in the hypothalamic NPY mRNA levels (Schwartz et al. 1991).

2.3.5 NPY, sympathoadrenal tone and stress

As described in previous sections, catecholamines in the sympathetic nervous system are interconnected with NPY on several levels of energy metabolism, i.e. the regulation of food intake, BAT thermogenesis and adipose tissue metabolism. Centrally administered NPY down-regulates the sympathoadrenal tone in specific tissues (Egawa et al. 1991, Matsumura et al. 2000), and widespread NPY overexpression exerts a sympatholytic effect (Michalkiewicz et al. 2003). On the other hand, hypothalamic NPY-KD stimulates the SNS activity and thus prevents diet-induced obesity (Chao et al. 2011). Furthermore, NPY directly inhibits noradrenaline release from the sympathetic nerves of mesenteric (Westfall et al. 1987) and renal arteries (Malmstrom et al. 2002). However, there are some contradictory findings, where NPY has been shown to stimulate catecholamine release from mouse adrenal chromaffin cells in a Y1R-dependent manner (Cavadas et al. 2001 & 2006). On the other hand, Hexum et al. have shown with bovine chromaffin cells that NPY reduces tyrosine hydroxylase (TH) and adenylyl cyclase activity (Zheng et al. 1997, Zhu et al. 1992). This hypothesis has been supported by one recent study (Wang et al. 2013), where after an acute stress NPY suppresses TH activity and inhibits the stress-related increase in circulating catecholamines via Y1Rs. Additionally, loss of NPY leads to elevated TH-immunoreactivity in murine chromaffin cells.

Stress is an intense stimulus activating the sympathoadrenal system and promoting the release of NPY, adrenaline and noradrenaline in proportions depending on the type of the stress (Zukowska-Grojec 1995). Severe or prolonged stress has been demonstrated to stimulate the release of NPY and lead to abdominal obesity as described above (Kuo et al. 2007). Mild and short-term stress favors the release of noradrenaline, which can cause a weight loss through β-adrenoceptor-mediated lipolysis. Thus, stress can either induce or reduce the weight gain depending on the intensity of the stressor and pathway being activated. Alternatively, stress can promote weight gain via stress hormones, glucocorticoids, released from the adrenal cortex as a response to the activation of hypothalamic-pituitary-adrenal (HPA) axis. Interestingly however, chronic stress is rarely associated with elevated cortisol levels in humans (Dallman et al. 2003) and hypercorticosteronemia in chronically stressed mice becomes normalized already after 2 weeks of daily stress although the adipogenic effect is still evident with continuous stress (Kuo et al. 2007). It has been shown *in vitro* that when sympathetic neurons are stressed by the glucocorticoid, dexamethasone, they release more NPY in comparison

with catecholamines (Kuo et al. 2007). Thus, glucocorticoids via up-regulation of NPY can shift the balance between catecholamines and NPY towards the adipogenic NPY. Furthermore, release of NPY during stress acutely activates a negative feedback loop, which via Y2Rs inhibits its own adrenal expression and thus hinders the pathologically overactive stress response (Wang & Whim 2013). At the same time, central NPY infusion increases plasma adrenocorticotropic hormone (ACTH) and corticosterone levels (Sainsbury et al. 1997).

2.3.6 NPY and bone

Bone metabolism is a strictly regulated process of bone resorption and bone formation. The net effect of catabolic and anabolic signals determines whether bone mass is increased or lost. In bone, osteoblasts produce NPY and express Y1Rs but not Y2Rs (Baldock et al. 2007, Lundberg et al. 2007). NPYergic fibres in bone are mostly located in the blood vessels but also around bone lining and marrow cells (Ahmed et al. 1993, Bjurholm et al. 1988). A central infusion of NPY reduces bone volume and hypothalamic NPY overexpression reduces osteoblast activity both in cortical and cancellous bone (Baldock et al. 2009, Ducy et al. 2000). *In vivo* NPY inhibits the differentiation of bone marrow stromal cells (BMSC) to osteoblast-like cells (Amano et al. 2007). The loss of NPY increases osteoblast activity and thus is associated with greater bone mass (Baldock et al. 2009). NPY regulates bone via both NPYergic and sympathetic neurons (Shi et al. 2010).

The catabolic effect of NPY on bone formation is mediated via central Y2Rs and peripheral osteoblastic Y1Rs. An Arc-specific Y2R-deletion increases bone volume as a result of enhanced osteoblast activity and bone formation without a change in bone resorption (Baldock et al. 2002 & 2009). Additionally, BMSCs extracted from $Y2R^{-/-}$ mice show increased mineralization and elevated numbers of osteoprogenitor cells (Lundberg et al. 2007). The phenotype of hypothalamic $Y2R^{-/-}$ mice is identical to the phenotype of mice with germline Y2R deletion suggesting that the central Y2Rs are responsible for the regulation of bone (Baldock et al. 2002). Similar to the situation in $Y2R^{-/-}$ mice, germline $Y1R^{-/-}$ mice exhibit an increase in bone formation but bone resorption as evidenced by the increased osteoclast surface is also altered. However, the hypothalamic loss of Y1Rs does not alter bone mass or bone formation and thus osteoblastic Y1Rs are solely responsible for the changes in bone homeostatis. (Baldock et al. 2007) Furthermore, BMSCs isolated from mice lacking Y1Rs in mature osteoblasts display increased mineralization (Lee et al. 2010).

2.3.7 NPY and the endocannabinoid system

Similarities in the regulation of energy metabolism and their localization in the same brain areas have led to speculation that there is a possible interaction between NPY and the endocannabinoid systems. The exact mechanism is not completely clear but undeniably there is an interplay between these systems. It has been shown that AEA

augments resting and KCl-evoked NPY release in the rat hypothalamic explant model and this effect can be blocked by treatment with a CB1R-antagonist (Gamber et al. 2005). Furthermore, intraperitoneal (i.p.) administration of the CB1R-antagonist rimonabant (also known as SR141716) decreases hypothalamic NPY mRNA and protein levels and abolishes the orexigenic effect of NPY (Poncelet et al. 2003, Verty et al. 2009). However, inhibition of NPY is not solely the reason for decreased food intake after CB1R-blockade since rimonabant has been shown to reduce food intake also in NPY-deficient mice (Di Marzo et al. 2001). Thus, NPY and the endocannabinoid systems act both independently and synergistically in the regulation of food intake. However, body weight is regulated synergistically by both systems since dual blockade of CB1 and NPY signaling in rimonabant-treated $NPY^{-/-}$ mice induces a greater weight loss than blockade of either system alone (Zhang et al. 2010a). The reduced body weight and adiposity are mediated via fat oxidation and HPA axis (Zhang et al. 2010a). Fat oxidation is promoted independently by NPY and endocannabinoids, while the regulation of HPA axis requires both of the systems as evidenced by the inability of rimonabant to increase serum corticosterone levels in $NPY^{-/-}$ mice.

2.4 Gene modified animal models of NPY

A summary of the metabolic phenotypes of NPY knock-out and overexpression animal models is presented in table 1.

2.4.1 NPY knock-out models

There are several NPY-deficient mouse models from different backgrounds (Erickson et al. 1996a, Karl et al. 2008, Patel et al. 2006) but none of these animals shows a lean phenotype or exhibits any other significant changes in energy metabolism under normal conditions. However, metabolic challenges, such as fasting, can unmask the alterations in the regulation of energy metabolism. First NPY-deficient ($NPY^{-/-}$) mouse model in C57BL x 129/Sv background was constructed by Erickson et al. (1996a). These $NPY^{-/-}$ mice grow and eat normally under basal conditions, but show a reduced hyperphagic response to 48-h food deprivation and a more intensive hypophagic response to leptin administration (Bannon et al. 2000, Erickson et al. 1996a). Obesity develops normally after chemical lesions of the hypothalamus or when they are fed high-fat diet (Hollopeter et al. 1998). Furthermore, the endocrine function involving the gonadotropic, thyrotropic and corticotropic axes is normal in $NPY^{-/-}$ mice in a C57BL x 129/Sv background (Erickson et al. 1997). $NPY^{-/-}$ mice with an obesity-prone C57BL background paradoxically gain weight and adipose tissue when they achieve adulthood without alterations in food intake (Segal-Lieberman et al. 2003). In these $NPY^{-/-}$ mice, the weight gain may result from hyperinsulinaemia and increased pancreatic islet mass with higher basal and glucose-stimulated insulin secretion (Imai et al. 2007). After 24-h fasting, $NPY^{-/-}$ mice eat less and compensate less efficiently for the fasting-induced

weight loss (Segal-Lieberman et al. 2003). Another study, although unable to report the obesogenic phenotype of the NPY$^{-/-}$ mice with the C57BL background, suggested that the blunted hyperphagia and body weight detected after fasting were attributable to the increased energy expenditure as evidenced by increased oxygen consumption and BAT UCP1 expression during the period of fasting (Patel et al. 2006). Moreover, the fasting-induced increase in hypothalamic Agrp expression was absent in NPY$^{-/-}$ mice and they were less susceptible to diet-induced obesity.

As a summary of studies with NPY$^{-/-}$ mice, NPY is not essential for maintaining the body weight. An NPY deficiency may even induce the development of late-onset obesity, since it may trigger the activation of other compensatory mechanisms. Additionally, food intake is not affected by germline NPY loss but fasting unmasks the predictable changes in food intake with decreased re-feeding and a poor ability to maintain the body weight in NPY$^{-/-}$ mice. A similar effect has also been seen in *ob/ob* mice crossed with NPY$^{-/-}$ mice, which show decreased food intake, increased energy expenditure and thus attenuated obesity (Erickson et al. 1996b). However, the phenotype of *ob/ob* mice is not totally normalized by NPY deficiency and thus NPY is not the only central factor mediating the effects of leptin deficiency.

2.4.2 Receptor-specific knock-out models

The phenotype of Y1R-deficient mice supports the hypothesis that the Y1R is involved in NPY-induced feeding since Y1R$^{-/-}$ mice show significantly reduced NPY-induced food intake (Kanatani et al. 2000). However, Y1Rs can mediate obesogenic effects also via mechanisms other than simply feeding. Y1R-deficient mice paradoxically develop mild late-onset obesity and hyperinsulinaemia without any increase in their food intake (Kushi et al. 1998). Since these mice are not hyperphagic, the authors claimed that obesity had developed due to the impaired control of insulin secretion or lower energy expenditure as evidenced by alterations in adipose tissue UCP1 levels. Another Y1R$^{-/-}$ mouse model devised by Pedrazzini et al. (1998) shows a similar obesogenic phenotype, with elevated blood insulin levels and reduced glucose-induced insulin release. These mice display mild declines in their basal and NPY-induced food intake but markedly reduced fasting-induced feeding. Consequently, obesity in Y1R$^{-/-}$ mice is not explained by increased food intake but rather by decreased energy expenditure evidenced as reduced dark-phase locomotor activity, and dysfunction in the regulation of insulin signaling (Pedrazzini et al. 1998). Experiments conducted by Zhang et al. (2010b) using Y1R$^{-/-}$ mice further support the role of Y1R in the regulation of energy expenditure. These investigators demonstrated that Y1R$^{-/-}$ mice prefer lipids as the oxidative fuel source but are physically more active and thus do not experience any difference in the net energy expenditure. One explanation for obese phenotype of Y1R$^{-/-}$ mice is that the lack of functional Y1Rs is compensated by more intense signaling via other YRs such as Y5R.

Y2R$^{-/-}$ mice on 129/Sv x Balb/c background are hyperphagic and mildly obese but physically more active than Y2R$^{+/+}$ mice (Naveilhan et al. 1999). Furthermore, Y2R-

deficient mice have normal NPY- and fasting-induced feeding responses but have substantially attenuated leptin-induced hypophagia. These findings support an inhibitory role of Y2R in the regulation of food intake and body weight. However, another germline, the $Y2R^{-/-}$ mice with a C57BL/6 x 129/SvJ background, show decreased body weight and adiposity with mild hypophagia in male and hyperphagia in female mice (Sainsbury et al. 2002a). The different mouse strains and gene deletion techniques may explain the discrepancies between these $Y2R^{-/-}$ phenotypes.

$Y4R^{-/-}$ mice display a lean phenotype with reduced body weight and adiposity (Sainsbury et al. 2002b). Since $Y4R^{-/-}$ mice have increased plasma PP levels and the phenotype is similar to that encountered in the PP overexpression model (Ueno et al. 1999), PP has been postulated to be responsible for the lean phenotype of $Y4R^{-/-}$ mice, for example by reducing nutrient uptake. Interestingly, Y2R and Y4R double knock-out mice have a lean phenotype with a more intense decrease in body weight and adiposity than deletion of either receptor alone, thus suggestive of a synergistic role of Y2Rs and Y4Rs (Sainsbury et al. 2003, Zhang et al. 2010c). $Y2R^{-/-}/Y4R^{-/-}$ double knock-out mice have decreased blood leptin and insulin levels, increased bone mass and they are mildly hyperphagic despite the slightly reduced food intake found in the single receptor KO-mice. The synergistic effect seems to be associated with differences in mitochondrial oxidative capacity (Zhang et al. 2010c). However, physical activity is increased and the respiratory exchange ratio decreased equally in double and single knock-outs pointing to an independent role of Y2Rs and Y4Rs in the regulation of locomotor activity and substrate utilization.

Young $Y5R^{-/-}$ mice eat and grow normally but develop late-onset obesity accompanied by fatty liver and hyperphagia (Marsh et al. 1998). However, neither fasting-induced nor NPY-induced feeding are increased in $Y5R^{-/-}$ mice (Kanatani et al. 2000). This rather surprising phenotype may result from compensation of Y5Rs with Y1R-mediated signaling since it has been reported that treatment with a Y1R-antagonist is able to abolish the NPY-induced feeding response in $Y5R^{-/-}$ mice (Marsh et al. 1998). This has been interpreted as support for a role of Y1Rs and Y5Rs in feeding behavior although it has been debated, whether both of these receptors are needed for the NPY-mediated control of food intake (Nguyen et al. 2012, Raposinho et al. 2004).

In general it can be stated, that deletion of one receptor is not the optimum way to study the function of specific receptors. The problem with a constitutive receptor knock-out method is that receptors may have different functions during the periods of embryonic and postnatal development than they have in adulthood. Thus, the phenotype of an adult animal may reflect more the consequences of perturbation in normal development than the functional significance of that receptor in the adult animal. Adult-onset receptor knock-out studies describe more reliably the role of specific YR in the regulation of energy metabolism. However, compensatory mechanisms may evolve to replace the loss of one receptor e.g. other receptors may become more efficient, and this reservation seems to apply especially to the NPY system where several YRs are able to influence the same physiological processes.

Table 1. Summary of the metabolic phenotypes of NPY knock-out and overexpression animal models.

Knock-out models								
Species/strain	**Modif. Meth.**	**Body weight**	**Food intake**	**EE**	**Lipid metab.**	**Gluc metab.**	**Other**	**Reference**
Mouse C57BL x 129Sv	Neomycin resistance cassette	↔ fast-induced weight loss ↔ leptin-induced weight loss ↑	↔ fast-induced ↔↓ leptin-induced hypophagia ↑		adiposity ↔ leptin ↔	gluc ↔ ins ↔	seizures ↑ anxiogenic hyperanalgesia	Erickson et al. 1996 Erickson et al. 1997 Bannon et al. 2000
Mouse C57BL	Neomycin resistance cassette	↔ ↑ ↓(HFD) fast-induced weight loss ↑	↔ fast-induced ↓	RER ↔ loc.activity ↔ fasting EE ↑ fasting O2 ↑	adiposity ↔ ↑ adiposity ↓ (HFD) leptin ↔ trigly ↔	gluc ↔ gluc ↔ (HFD) ins ↑ ins ↔ (HFD)		Patel et al. 2006 Segal-Lieberman et al. 2003 Imai et al. 2007
Mouse C57BL	NPY-Cre construct	↔		loc. activity ↓	adiposity ↓		anxiogenic bone mass ↑	Karl et al. 2008
Overexpression models								
Species/strain	**Modif. Meth.**	**Body weight**	**Food intake**	**EE**	**Lipid metab.**	**Gluc metab.**	**Other**	**Reference**
Mouse C57BL/6	Microinjection	↔			adiposity ↓ leptin ↔	gluc ↔ ins ↔		Ste Marie et al. 2005
Rat Sprague- Dawley	Microinjection OE in NPYergic neurons	↔					BP responses ↑ anti-stress behaviour	Michalkiewicz et al. 2001 Thorsell et al. 2000 Michalkiewicz et al. 2000
Mouse Bdf1	Microinjection OE in CNS	↑(HSD) fast-induced weight loss↓ (HSD)	↑(HSD)	EE ↔ (HSD)	trigly ↔ (HSD) chol ↔ (HSD)	gluc ↑ (HSD) ins ↑ (HSD) OGTT↔ (HSD)		Kaga et al. 2001 Inui et al. 1998
Rat Wistar	AAV OE in Arc	↑	↑ (light)		adiposity ↑, chol ↑ leptin ↑, trigly↑	gluc ↑ ins ↑		Sousa-Ferreira et al. 2011
Rat Wistar	AAV OE in PVN	↑	meals ↑	loc. activity ↓ temp ↓ (dark)	adiposity ↑ leptin ↑	gluc ↑ ins ↑		Tiesjema et al. 2007 Tiesjema et al. 2009
Rat / Sprague- Dawley	AAV OE in DMH	↑	meal size ↑ (dark)	metabolic efficiency ↓	adiposity ↑ leptin ↔	gluc ↔ ins ↑		Zheng et al. 2013
Rat Wistar	AAV OE in LH	↑	meal size ↑	loc. activity ↓ temp ↓ (dark)	adiposity ↑ leptin ↑	gluc ↔ ins ↑		Tiesjema et al. 2007

AAV = adeno-associated virus, BP = blood pressure, chol = cholesterol, dark = dark-time, EE = energy expenditure, gluc = glucose, HFD = high-fat diet, HSD = high-sucrose diet, ins = insulin, light = light-time, loc. activity = locomotor activity, OE = overexpression, OGTT = oral glucose tolerance test, RER = respiratory exchange ratio,temp = temperature, trigly = triglycerides

2.4.3 NPY overexpression models

NPY overexpression models have been less studied in the field of obesity and energy metabolism but widely used in the area of epileptogenesis and anxiety (Christiansen et al. 2014, Noe et al. 2009). Kaga et al. (2001) were the first to reveal the obesogenic effects in gene modified NPY-overexpressing mice. Overexpression was localized in the CNS with Thy-1 promoter and the homozygous mice developed obesity when fed a sucrose-loaded diet. The gain in body weight was due to an increase in Y1R-mediated food intake without any change in oxygen consumption. During the same year, a rat model overexpressing NPY widely in the central and peripheral nervous systems was devised (Michalkiewicz et al. 2001). However, these transgenic rats surprisingly had no differences in their body weights, but showed increased blood pressure responses. A mouse model overexpressing NPY also failed to display any significant changes in body weight or feeding behavior (Ste Marie et al. 2005).

Conventional transgene insertion methods with oocyte microinjections have now been replaced by more advanced methods such as targeted gene insertion via AAV-vectors. This kind of inducible transgene overexpression has been particularly exploited in studies clarifying the role of NPY in different brain nuclei. AAV-induced NPY overexpression in several hypothalamic nuclei cause an increase in food intake and body weight or alters the diurnal rhythm of feeding. In the PVN, NPY evokes a temporary weight gain, which is due to the increased number of meals but this can be prevented by pair-feeding (Tiesjema et al. 2007 & 2009). Additionally the HPA-axis activity is increased whereas body temperature together with locomotor activity is reduced independently of food intake in rats overexpressing NPY in the PVN. In LH, NPY increases meal size but also affects the diurnal patterns, equalizing light- and dark-phase feeding and body temperature (Tiesjema et al. 2007). In the DMH, NPY overexpression induces hyperphagia also by increasing the size of the meals during the dark-phase, but this is evident only after feeding with a high-fat diet (Zheng et al. 2013). Additionally, these rats show increased body weight, decreased metabolic efficacy and insulin insensitivity. NPY overexpression in the Arc causes hyperphagia during light-phase and body weight gain with dysfunctions in adipocytes, such as increased adipocyte cell size and an unexpected decline in the protein level of the adipogenic marker, peroxisome proliferator-activated receptor γ2 (PPARγ-2) (Sousa-Ferreira et al. 2011).

In conclusion, NPY overexpression models have been used only occasionally in the field of obesity and energy metabolism research. The role of NPY overexpression in the different loci of the hypothalamus is well documented. Hypothalamic NPY seems to disrupt the diurnal rhythms of feeding behavior and thus to cause weight gain and obesity.

2.4.4 Animal models of peripheral NPY

There are a few mouse models available which highlight the importance of peripheral NPY and YRs in the regulation of energy homeostasis. Kuo et al. (2007) have reported

that NPY released from sympathetic neurons as a response to mild chronic stress is able to facilitate diet-induced obesity in mice. A gain of WAT mass without any difference in food intake or body weight is the primary response to the stress, but after a prolonged chronic exposure to a stressor, metabolic syndrome-like symptoms such as impaired glucose tolerance, hyperinsulinaemia, liver steatosis and hypertension start to develop. Additionally, NPY administration directly into the subcutaneous fat increase the adipose tissue weight and volume by approximately 50 %. Administration of NPY causes an adipogenic effect locally in adipose tissue by stimulating macrophage infiltration, fat angiogenesis and adipogenesis via Y2Rs as evidenced by the decreased vascularity and increased apoptosis after fat-targeted Y2R-KD.

This primary phenotype of mice which display increases in stress-induced NPY release is very similar to the phenotype of transgenic mouse model studied by our research group. In order to study the effects of extra-hypothalamic NPY, we have created a mouse model (OE-NPY$^{D\beta H}$) overexpressing NPY in the brain noradrenergic neurons and the peripheral sympathetic nerves via the *dopamine-beta-hydroxylase* (*DβH*) gene promoter (Ruohonen et al. 2008). The heterozygous OE-NPY$^{D\beta H}$ mice show an approximately 1.8-fold NPY overexpression in adrenal glands and a 1.3-fold overexpression in the brainstem without displaying any significant changes in the hypothalamic NPY levels. This mild overexpression in the SNS leads to increased adiposity, liver triglycerinemia, late-onset impairment in glucose tolerance together with increased blood insulin, leptin and ghrelin levels. However, neither their body weight nor their food intake is significantly increased. Furthermore, OE-NPY$^{D\beta H}$ mice are susceptible to vascular neointima formation (Ruohonen et al. 2009a) and their sympathoadrenal activity is increased since blood pressure during surgical stress, basal blood adrenaline levels and sympathetically regulated BAT activity are all elevated (Ruohonen et al. 2009b). Despite the clear metabolic phenotype in OE-NPY$^{D\beta H}$ mice, it cannot be concluded whether the phenotype is a primary response to the elevated NPY levels in noradrenergic neurons or whether NPY overexpression modulates the endogenous NPY levels in non-noradrenergic cells, such as in the hypothalamus, and thus secondarily induces the phenotype. To address this problem, Zhang et al. (2014) have re-introduced NPY expression only in the noradrenergic neurons by crossing the OE-NPY$^{D\beta H}$ mouse with NPY$^{-/-}$ mouse (catNPY). Studies with these catNPY mice have indicated that noradrenergic neuron NPY is responsible for promoting the weight gain when exposed to a high-fat diet by inhibiting the diet-induced thermogenesis, and under chronic stress by increasing corticosterone levels and lipogenic activity.

Further support for the importance of peripheral NPYergic signaling emerges from experiments in the mice with adult-onset peripheral Y1R or Y2R-KD which display disruption of energy homeostasis (Shi et al. 2011, Zhang et al. 2014). Since the total ablation of one player, such as one receptor type, in the complex NPY signaling system can often trigger activation of compensatory mechanisms and thus mask the true function of that receptor, the conditional KD of one receptor in one individual tissue may be a

better strategy for revealing the true function of each receptor. Peripheral $Y1R^{-/-}$ mice have normal body weight and food intake but their respiratory exchange ratio (RER) during the light-phase is decreased and there are increases in the activities of liver enzymes related to fatty acid oxidation (Zhang et al. 2014). This suggests that peripheral Y1R is important in the regulation of lipid oxidation. Furthermore, peripheral Y1R-KD prevents diet-induced obesity probably due to the mildly increased energy expenditure. Adult-onset peripheral Y2R-KD causes similar resistance to diet-induced obesity, but the reduced weight gain is accompanied by improved glucose tolerance, increased energy expenditure, physical activity, RER and paradoxically increased food intake (Shi et al. 2011). When fed a normal chow diet, peripheral $Y2R^{-/-}$ mice do not show differences in body composition but have increased activity, RER and decreased energy expenditure. Thus, peripheral Y2Rs seem to be important in mediating the effects of NPY in the regulation of fuel selection, physical activity and glucose metabolism.

2.5 Leucine-Proline (p.L7P) polymorphism in human *Npy* gene

In humans, there is a single nucleotide polymorphism (SNP) named p.L7P (rs16139, 1128 T>C) in the exon 2 of the *Npy* gene. In the polymorphic form, amino acid 7 in the *Npy* signal peptide is changed from leucine to proline because of a single nucleotide mutation (T→C) in the amino acid sequence. Although the signal peptide is cut off from the final protein structure, its presence is important for the proper folding and packaging of the peptide. In the case of L7P polymorphism, the exact difference in molecular mechanisms of action between alleles is not known (Ding et al. 2005). In *in vitro* experiments, endocrine cells transfected with the L7P form have shown that the prohormone is synthesized normally, but the secretory capacity of the mutated cells is elevated (Mitchell et al. 2008). In human endothelial cells with the p.P7 allele, NPY-immunoreactivity appeared to be increased in comparison with the normal allele cells (Kallio et al. 2001). Furthermore, p.P7 subjects have higher plasma NPY levels during strong sympathetic stimulation (Kallio et al. 2001) although their basal NPY secretion is reduced (Kallio et al. 2003). Thus, the presence of p.L7P in the signal peptide of *Npy* gene seems to cause enhanced processing of preproNPY in the cells and to accelerate the release of the NPY peptide.

The carrier frequency for the p.P7 variant is 6-15% in a Caucasian population with significantly lower frequencies found in other ethnicities (Pesonen 2008, Yeung et al. 2011). The prevalence of the p.P7 variant increases towards Northern Europe and again from east to west. Hence, Finland is one of the countries with the highest prevalence of the p.L7P substitution.

L7P polymorphism associates with many aspects of the metabolic syndrome such as lipid metabolism, blood glucose levels and cardiovascular function (Pesonen 2008). Birth weight and BMI of normal and overweight p.P7 individuals are increased (Ding et al. 2005, Karvonen et al. 2000). Furthermore, p.L7P associates with increased serum

triglyceride levels in young boys and elevated total and low density lipoprotein (LDL) cholesterol levels in obese and normal-weight men independently from body weight or food intake (Karvonen et al. 1998 & 2006). Most of the association studies have only been conducted in one gender. Interestingly however, atherosclerosis related inflammation and increased plasma NPY levels in diabetic p.P7 subjects have been shown to be more pronounced in males than in females (Jaakkola et al. 2012). Although many of the significant associations in p.P7 variants seem to be dependent on both the study population and on environmental risk factors, increased food intake has not been observed in the mutation carriers in any of the epidemiological studies (Karvonen et al. 1998, Yeung et al. 2011). There may be reasons why p.L7P does not seem to affect feeding behavior; people under-report their food intake or p.L7P may affect only episodic-like feeding, which is difficult to monitor in follow-up studies (Yeung et al. 2011). The L7P polymorphism associates also with higher basal blood glucose, reduced insulin levels at rest and in response to an oral glucose tolerance test, as well as to an earlier onset of type 2 diabetes (Jaakkola et al. 2005 & 2006, Kallio et al. 2003). Additionally, the fasting plasma glucose level is higher in the carriers of p.P7 variant and consequently the frequency of p.P7 variant is higher in men with impaired glucose tolerance or type 2 diabetes than in their non-diabetic male counterparts (Nordman et al. 2005). Thus, p.L7P seems to predispose to certain metabolic disturbances such as type 2 diabetes.

One important fact related to p.L7P is the relatively recent finding about its linkage disequilibrium with another more frequent SNP in the *Npy* gene promoter region (rs16147, NPY-399C>T) (Zhou et al. 2008). Both rs16139 and rs16147 have been reported to associate with elevated NPY secretion, but rs16147 has been studied more extensively in the field of stress response and emotion rather than in energy metabolism (Zhou et al. 2008). In fact, these SNPs have been mostly investigated separately and there are only a few publications which have investigated both of these SNPs (Jaakkola et al. 2012, Laddha et al. 2014, Zhou et al. 2008). However, Jaakkola et al. (2012) have shown that the NPY-399T allele without the p.P7 allele is associated with an increased risk for asthma and thus these SNPs do appear to exert some individual effects even though they are in linkage disequilibrium.

As a conclusion, the differences in the vesicular processing of NPY and the association studies in the carriers of p.L7P highlight the importance of elevated SNS-derived NPY levels in the development of metabolic consequences in humans. However, the mechanisms governing these metabolic alterations are still far from clear at the tissue level. It was the working hypothesis of this thesis project that the transgenic mouse model overexpressing NPY in the brain noradrenergic neurons and SNS (OE-NPY$^{D\beta H}$) would be useful in clarifying these mechanisms.

3. AIMS OF THE STUDY

The aim of the current study was to investigate the role of noradrenergic neuron NPY in the development of obesity and metabolic disorders. This study continues previous studies with heterozygous transgenic OE-NPY$^{D\beta H}$ mice, which have concluded that NPY overexpression in noradrenergic neurons has an important role in the pathogenesis of metabolic disorders. The goal here was to elucidiate in more detail the mechanisms responsible for the metabolic impairments seen in OE-NPY$^{D\beta H}$ mice. In this way, it was hoped that this model could be utilized in identifying novel drug targets for the treatment of the metabolic syndrome, especially in the cases with altered NPY levels, such as in subjects carrying the p.L7P mutation or in disturbances secondary to chronic stress.

The specific aims of this study were:

1) To intensify the metabolic phenotype of heterozygous OE-NPY$^{D\beta H}$ mice for the purpose of being able to conduct subsequent mechanistic studies and drug interventions

   a) by investigating the effect of a high-calorie diet on the metabolic phenotype of heterozygous OE-NPY$^{D\beta H}$ mice.

   b) by increasing the transgene copy number and studying the metabolic phenotype of homozygous OE-NPY$^{D\beta H}$ mice.

2) To evaluate the role of the catecholamine system in the obesity phenotype.

3) To study the role of endocannabinoid system in the metabolic phenotype of homozygous OE-NPY$^{D\beta H}$ mice in a CB1R-antagonism intervention study.

4) To determine the role of peripheral Y1Rs and Y2Rs in the metabolic phenotype of OE-NPY$^{D\beta H}$ mice via an Y1R- and Y2R-antagonism intervention study.

4. MATERIALS AND METHODS

4.1 In vivo experiments

4.1.1 Animals

Homozygous OE-NPY$^{D\beta H}$ (studies I-IV), heterozygous OE-NPY$^{D\beta H}$ (study I) mice and wild-type (WT) mice originating from the same C57BL/6N strain were used. Animals were housed in the facilities of Central Animal Laboratory (University of Turku) and they had fixed environmental conditions with 21±3 °C temperature and 12-h light/12-h dark cycle. Mice were fed *ad libitum* with standard rodent chow (SDS, Essex, U.K.; Harlan Laboratories, Indianapolis, IN, USA) and tap water was available, unless stated otherwise.

The gender and age of the animals varied between the cohorts and thus are described more specifically in the results section. In general, in study I, the effect of western diet was studied in heterozygous male and female mice and in homozygous females at the age of 3-4 months. In study II, the metabolic phenotype of homozygous OE-NPY$^{D\beta H}$ mice was studied at the age of 2, 4 and 7 months in female and male mice, and further mechanistic studies were performed with male mice at 4-5 months, unless stated otherwise. In drug intervention studies (study III & IV), 4-month-old homozygous OE-NPY$^{D\beta H}$ males were used.

OE-NPY$^{D\beta H}$ mice have been shown to be more responsive to the effects of stress (Ruohonen et al. 2009b), which was taken into consideration in the experimental procedures. Mice were group-housed unless single-housing was justified for the experiment. After changes in the environmental conditions, there was a one week habituation period without any experimental procedures in order to minimize the effect of stress on the results. For the same reason, saline injections were performed before the drug administration in the drug intervention studies (study III & IV).

4.1.2 Ethical aspects

Animal care and all the experimental procedures were approved by the Regional State Administrative Agency for Southern Finland as the presenting officers of national Animal Experiment Board (ELLA), and carried out in accordance with the guidelines of the International Council of Laboratory Animal Science (ICLAS). Ethical 3R's principle (Reduction, Replacement, Refinement) was followed. The number of animals used was reduced to the minimum within the limits of detecting statistically significant and physiologically relevant differences. Mostly sample sizes were kept to a minimum (n=8-12 mice/group), but exact n-values are reported in the results section. During tissue collection, all the metabolically interesting tissues were dissected and stored for future use in order to avoid the need for extra animals as a source of tissue samples. The replacement of *in vivo* experiments with *in vitro* methods was in this case impossible since

we were interested in the physiological mechanisms at the whole body level. Refinement was acknowledged by using only representative individuals in the experiments. For this purpose, mice were constantly monitored by animal facility employees and in the case of aggressive behavior or illness, mice were separated and removed from the experiment (<5% of total animal number). The abnormalities irrelevant for the phenotype or treatment are not reported. Pain caused in the invasive experiments was minimized with proper analgesia (buprenorfine) and anesthesia with medetomidine as described later in section 4.1.10 'Anesthesia and tissue collection'.

4.1.3 Generation of the homozygous OE-NPY$^{D\beta H}$ mouse line (studies I & II)

Genomic DNA was isolated from the tail samples with Gentra Puregene Mouse Tail Kit (Qiagen, Hilden, Germany) and the genotype determined with quantitative PCR (qPCR). The transgene was targeted to the noradrenergic neurons via the human *DβH* gene promoter as described previously (Ruohonen et al. 2008). Specific TaqMan primers and probe (Table 2) were designed for β-galactosidase (LacZ) reporter gene sequence of the transgene with the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). LacZ expression level was quantified with the ΔΔCT-method and esimated relatively to the housekeeping gene β-actin (*Actb*) (Mouse ACTB, VIC/MGB Probe, Primer Limited, Applied Biosystems). The resulting gene expression of 0, 1 or 2 was equivalent to the copy number of the transgene in WT, heterozygous and homozygous mice, respectively. The correct genotypes were verified from the first litters of each mouse line.

The transgene insertion site was located with the inverse PCR technique in order to exclude the possibility for gene disruption with the homozygous transgene insertion (Fig. 5). Genomic DNA extracted from the liver of homozygous OE-NPY$^{D\beta H}$ mice was digested in 37°C for 16 hours with the restriction enzyme RsaI (Fermentas, Vilnius, Lithuania), which has a restriction site in the LacZ sequence of the transgene 3'-end. Restriction was verified with gel electrophoresis and restricted DNA purified with PCR Purification kit (Qiagen). DNA was then self-ligated in 21°C for 16 hours with T4 ligase (Fermentas) and purified. An inverse 'RsaI forward' and 'RsaI reverse' primer pair (Table 2) was designed within the LacZ end from the RsaI restriction site onwards. Pfu DNA polymerase (Fermentas) amplified the flanking sequence and the resulting amplicon was detected from the gel and purified prior to sequencing. The flanking sequence was sequenced with a 'flanking LacZ forward' primer at The Finnish Microarray and Sequencing Centre, Turku, Finland with 3130xl Genetic Analyzer (Applied Biosystems). The sequencing result was aligned with mouse genome with NCBI BLAST tool. Transgene insertion site was located in the antisense orientation in chromosome 16, approximately at site 10796500 of NC_000082.6 genomic sequence. Inverse PCR and sequencing designed for the DβH-end of the transgene confirmed that the insertion site was in chromosome 16. For DβH-end AciI restriction enzyme (New England Biolabs, Ipswich, MA, USA), PCR primers 'AciI forward', 'AciI reverse' and 'flanking DBH reverse' sequencing primer were used. The location of the transgene was further verified

by amplifying DNA with PCR across the junction site at LacZ-end of the transgene. The 'Chr16 forward' primer in the chromosome 16 and 'LacZ junction reverse' primer in the LacZ sequence were able to amplify 206 bp amplicon in heterozygous and homozygous OE-NPY$^{D\beta H}$ mice but not in WT mice.

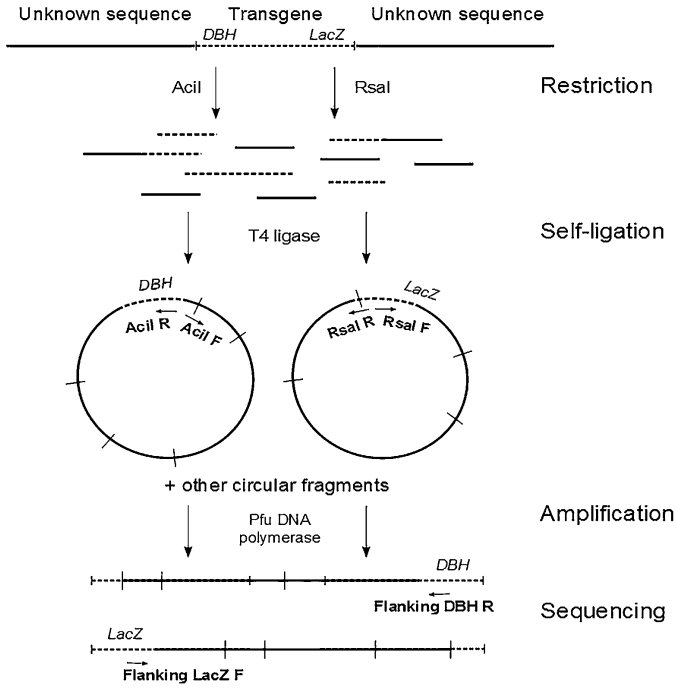

Figure 5. Inverse PCR in determining the transgene insertion site in the genome. Genomic DNA was first restricted into small nucleotide fragments with a restriction enzyme cutting DNA sequence from the transgene sequence. Then small DNA fragments were self-ligated to circular DNAs. The transgene end and following flanking sequence were amplified with inverse PCR primers and sequenced. DBH = dopamine-beta-hydroxylase, LacZ = β-galactosidase, F = forward, R = reverse.

Table 2. PCR primers and probe used in the genotyping and inverse PCR

Primer/probe name	Sequence
AciI forward	5'-TGGATCTCTGAGGTGACCAAGC-3'
AciI reverse	5'-GGCCGATGCAGTGAGGGCTA-3'
Chr16 forward	5'-GCTCCTGTTTTTCATCGGACG-3'
Flanking DBH reverse	5'-CGATGCAGTGAGGGCTAGCGAGACA-3'
Flanking LacZ forward	5'-CTTTCTGACAAACTCGGCCTCGACTCTA-3'
LacZ forward	5'-TGGCTGGAGTGCGATCTTC-3'
LacZ junction reverse	5'-GCCGCTATTTCTCTGTTCTCG-3'
LacZ probe	6FAM-CCGATACTGTCGTCGTC-MGB
LacZ reverse	5'-GAGTTTGACCGTCTACGTGC-3'
RsaI forward	5'-CTTTCTGACAAACTCGGCCT-3'
RsaI reverse	5'-GTCATGTAGCCAAATCGGGA-3'

4.1.4 Body composition

Mice were weighed always before tissue collection and weekly during detailed monitoring i.e. active growth at the age of 1-3 months (study II), diet (study I & IV) or drug interventions (study III & IV).

Body composition was measured either with DEXA scan (Lunar PIXImus2, GE Medical Systems, Madison, WT, USA) in study II or with EchoMRI-700 (Echo Medical Systems LLC, Houston, TX, USA) in studies I, III & IV. For the DEXA scanning, mice were lightly anaesthesized with ketamine (45 mg/kg) and medetomidine (0.8 mg/kg) as described in section 4.1.10 'Anesthesia and tissue collection'. Atipamezole (Antisedan, Orion Oyj, Espoo, Finland) 2 mg/kg i.p. was used for the reversal of the anaesthesia. DEXA measures fat-%, bone mineral density (BMD), bone mineral content (BMC) and total tissue mass (TTM), and estimates for lean-% (100% – fat-%), fat mass (TTM x (fat-%/100)) and lean mass (TTM x (lean-%/100)) can be calculated. EchoMRI does not require anaesthesia but each animal was scanned twice in order to minimize the error caused by the minor movements of the animal. Average values for body fat mass and lean tissue mass were calculated from two separate scans.

4.1.5 Food intake

Food intake per cage was measured weekly from group-housed mice at the age of 1–4 months (n = 10–13 cages/genotype, study II), during diet (study I & IV) or drug interventions (study III & IV). Individual food intake was measured from chow-fed males 5–6 months of age for 3 days (study II). Additionally, food intake was measured simultaneously with different experimental set-ups i.e. physical activity (study I), metabolic cages (study II) and automated monitoring system of energy expenditure (study II).

The challenge of a 16-h overnight fast followed by 24-h re-feeding period was performed with an automated monitoring system LabMaster (section 4.1.6 'Energy expenditure', Study II). Fasting was repeated in a group of female mice in order to obtain tissue samples from fasted animals. Fasted mice were compared with a set of control females fed *ad libitum*.

4.1.6 Energy expenditure (studies I & II)

Locomotor activity and metabolic performance i.e. heat production/energy expenditure (kcal/h) and RER were measured in individually housed male mice with an automated monitoring system (LabMaster®, TSE Systems GmbH, Bad Homburg, Germany, study II). Alternatively, spontaneous locomotor activity was measured with a photo-beam recording system (San Diego Instruments, San Diego, CA, USA) in 10 minute intervals for 24 hours (study I).

In order to investigate the energy output, faeces were collected from single-housed mice over 24 hours and faecal lipid content was quantified (study II). Briefly, faecal mass was dried under air flow in 60 °C for 24 hours, faecal lipids were extracted from the dry faecal mass with Folch method (Folch et al. 1957), and the weight of the lipid content was determined. Urine was collected in metabolic cages with a fractional urine collector (Techniplast Gazzada, Buguggiate, Italy) which had a freezing unit storing urine samples at +4 °C (study II). Urine was collected for 48 hours in 6-h fractions (from 06:00, 12:00, 18:00 and 24:00 onwards). Acetic acid (1% v/v) and sodium disulphide (0.1% w/v) were used as catecholamine preservatives, and mineral oil was included to prevent evaporation.

4.1.7 Body temperature (studies I & II)

Intraperitoneal body temperature was measured with telemetry transmitters (TA-F10, DSI, New Brighton, MN, USA, study II). Transmitters were inserted into the abdominal cavity under isoflurane anaesthesia, and mice were treated subcutaneously (s.c.) with 0.1 mg/kg buprenorphine (Temgesic, Schering-Plough, Brussels, Belgium) once before and once after the surgery. During the telemetry, mice were single-housed in their home cages, which were placed on the telemetry receivers and the core body temperature was recorded for 5 seconds every 5 minutes. The 24-h recovery and 48-h basal body temperature were measured after 24-h and a one week recovery period from the surgery, respectively. Alternatively, rectal temperature was measured at sacrifice with a thermometer (Ellab, Roedovre, Denmark, study I).

Challenge of cold exposure in +5…+9 °C was performed in order to study the BAT thermogenesis (study II). The cohorts of 4-h cold with intraperitoneal telemetry transmitters, 4-h cold in automated monitoring system LabMaster, 2-h cold in home cages and room temperature controls were examined.

4.1.8 Glucose and insulin tolerance tests (studies I, II & III)

Glucose (5% or 10% wt/vol, 1 g/kg) and insulin (0,5 IU or 1 IU/kg, Protaphane FlexPen, Novo Nordisk, Baggvaerd, Denmark) were administered i.p. (per os in 7-month-old males to avoid over 1 ml i.p. dosing volumes) after 4- and 1-h fasting periods respectively. Tail vein glucose was measured at 0, 20, 40, 60 and 90 min in GTT and 0, 20, 40 and 60 min in ITT with the Precision Xtra Glucose Monitoring Device (Abbott Diabetes Care, Abbott Park, IL, USA).

4.1.9 Diet and drug interventions (studies I, III & IV)

Diet intervention was used in study I and diet together with drug intervention in study IV. The drug administered in study III was a CB1R inverse agonist and in study IV it was an Y1R- or Y2R-antagonist. The time-lines of intervention studies are presented in figure 6.

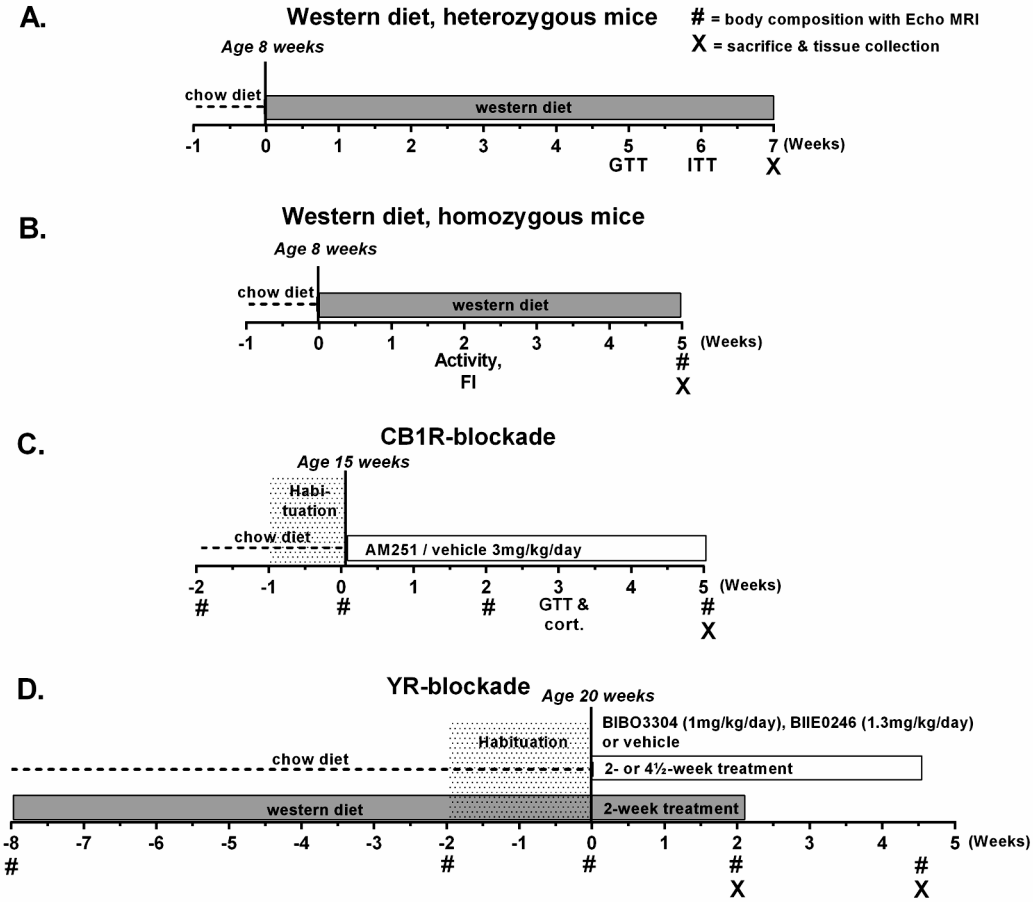

Figure 6. The timelines of diet and drug intervention studies. Western type diet (grey box) intervention was used for (A) heterozygous and (B) homozygous OE-NPY$^{D\beta H}$ and WT mice. (C) CB1R-blockade with inverse agonist AM251 (3mg/kg/day) and (D) Y1R- or Y2R-blockade with BIBO3304 (1mg/kg/day) or BIIE0246 (1.3mg/kg/day), respectively, were performed for homozygous OE-NPY$^{D\beta H}$ and WT mice after habituation period (dotted area). # = EchoMRI body composition measurement, X = sacrifice and tissue collection.

In study I, heterozygous OE-NPY$^{D\beta H}$ and WT mice were fed *ad libitum* with a western-type diet (42 kcal% fat, 15kcal% protein, 43% carbohydrates, code 829100, Special Diets Services, Essex, UK) for 7 weeks at the age of 8-15 weeks. GTT and ITT were performed after 5 and 6 weeks of diet, respectively. After consuming the western diet for 7 weeks, mice were sacrificed and tissues collected. Additionally, a 5-week western diet experiment was performed in age-matched homozygous OE-NPY$^{D\beta H}$ and WT females. Physical activity and individual food intake were studied after 2 weeks of consumption of the diet. Body composition was measured at the age of 13 weeks before sacrifice and tissue collection.

In study III, CB1Rs were blocked for 5 weeks with the specific CB1R inverse agonist, AM251 (Tocris Bioscience, Bristol, UK). Male homozygous OE-NPY$^{D\beta H}$ or WT mice at

the age of 15 weeks received daily i.p. injections of 3mg/kg AM251 or vehicle containing DMSO, Tween® 80 (Fisher Scientific, Fair Lawn, NJ, USA) and 0.9 % NaCl (1:1:18). The dose of AM251 was selected based on a previous study showing a moderate but significant effect on food intake and fat mass (Hildebrandt et al. 2003). Before the drug treatment mice had a one week habituation period with daily saline injections. They were divided into the treatment groups based on the baseline body weights on the week 0. Group-housed mice were weighed daily and food intake per cage was measured weekly. Body composition was measured at 1½ week before the drug treatment, at the initiation of the drug treatment and after 2 and 5 weeks of treatment (referred to as weeks -1½, 0, 2 and 5). A glucose tolerance test (GTT) was performed and plasma corticosterone levels measured after 3-weeks' drug treatment. After the 5-weeks' treatment period, at the age of 20 weeks, mice were sacrificed and tissues collected.

In study IV, Y1Rs or Y2Rs were blocked with specific Y1R-antagonist (1 mg/kg, BIBO3304 trifluoroacetate, Tocris Bioscience, Bristol, UK), or Y2R-antagonist (1.3 mg/kg, BIIE0246, Tocris Bioscience). The dose for BIBO3304 was selected based on a previous publication showing a significant effect in neural tube formation (Yuzuriha et al. 2007) and the dose for BIIE0246 was calculated based on the amount of substance of the BIBO3304 since there were no reference studies available during the initiation of the drug treatment. Male homozygous OE-NPY$^{D\beta H}$ or WT mice at the age of 20 weeks received daily i.p. injections of antagonist or vehicle (containing the same components as in study III). Mice had a 2-weeks' habituation period with daily saline injections before the drug treatment. Half of the chow-fed mice were treated with YR-antagonists for the whole 4½ weeks and half were sacrificed and tissue collected already after 2-weeks' treatment. Additionally, antagonist treatment was administered for two weeks to the mice fed on a western diet (RD Western Diet, 41 kcal% fat, 17 kcal% protein, 43 kcal% carbohydrates, Research Diets, New Brunswick, NJ, USA) for 8 weeks before and 2 weeks during drug treatment. Mice were divided into the treatment groups based on their body weight on week 0. Group-housed mice were weighed twice per week and food intake per cage was measured weekly. Body composition was measured at the initiation of western diet, habituation and drug treatment period, and after 2-weeks' drug treatment and at the sacrifice (referred as weeks -8, -2, 0, 2 and 4½).

4.1.10 Anesthesia and tissue collection

Prior to tissue collection, mice were anesthetized with ketamine (75 mg/kg, i.p., Ketalar, Pfizer Oy, Finland, or Ketaminol, Intervet Boxmeer, Netherlands) and medetomidine (1 mg/kg, i.p., Domitor, Orion Oyj, Finland or Cepetor, CP-Pharma Burgdorf, Germany). A terminal blood sample was collected by heart puncture or from the inferior vena cava (study I) after a 4-h fast (in study IV 3-h fast). Serum or plasma (study I) was separated by centrifugation at 4000 rpm for 10 minutes. Subcutaneous, epididymal/gonadal, retroperitoneal and mesenteric WAT pads, BAT and liver were collected and weighed. Additionally, adrenal glands (study II), the brain (study II, III & IV), pancreas (study

III) and soleus muscle (study III) were collected. The medial basal hypothalamus was isolated with a mouse brain block using a 3-mm section caudal to the optic nerve chiasma, excluding hippocampal and cortical areas. The 3-mm brainstem section extended 2-mm caudal from the hypothalamic section excluding the cerebellum and cerebral cortex. Tissue samples were snap frozen in liquid nitrogen and stored with blood samples at −70°C.

4.2 Ex vivo experiments

4.2.1 White adipose tissue structure (study II)

Retroperitoneal WAT pads were embedded in paraffin and sectioned on microscopic slides. The morphology was visualized with hematoxylin and eosin (HE) staining and macrophages identified with Mac-3 staining. Mac-3 staining was carried out as previously described (Haukkala et al. 2009) with some modifications (Mac-3 rat anti-mouse monoclonal antibody, catalogue #550292 (BD Pharmingen, San Diego, CA, USA), dilution 1:500, endogenous peroxidase blocked in 3% w/v H_2O_2).

In order to determine the size of the adipocytes and extract the adipocytes prior to lipolysis assay, the adipocytes were isolated as previously described (Di Girolamo et al. 1971). Isolated adipocytes were imaged under a light microscope and diameters were calculated using Cell A imaging software (Soft Imaging System GmbH, Münster, Germany). Adipocyte sizes were determined from gonadal fat pads of female mice and mesenteric adipocytes of male mice were isolated for lipolysis assay. The lipolysis assay was modified from previously described protocols (Berger & Barnard 1999, Chapados et al. 2008). The average volumes of spherical freely floating adipocytes were calculated based on the average diameters of the cells ($V = \pi \times d^3/6$) and the number of adipocytes in each solute was calculated based on the average volume of a single adipocyte. Adipocytes (3000 cells) were then incubated with concentration series of isoprenaline for 1 hour and the reaction stopped by incubating the samples on ice. Glycerol concentration was measured from the cell medium with Free Glycerol Assay Kit (Biovision, Mountain View, CA, USA).

4.2.2 Brown adipose tissue structure (study II)

Interscapular BAT was fixed in formaldehyde, embedded in paraffin and sectioned on microscope slides. Basic morphology was visualized with HE-staining and a more specific analysis of the size and appearance of BAT mitochondria with electron microscopy, which was performed on samples which had been fixed in 4% paraformaldehyde with 2% glutaraldehyde.

4.2.3 Bone structure (study II)

The bone structure of female mice was studied with Micro-CT bone using Skyscan 1072 scanner (Skyscan, Belgium) as described previously (Määttä et al. 2012). Fixed tibiae

and femora were analyzed and crossectional images were reconstituted with NRecon v 1.4 and analysis was conducted with CTan v. 1.12 software (Skyscan, Kontich, Belgium). Defined density standards were used to calibrate the device for BMD analysis. Lengths of tibiae were measured with a caliper. Ash weight was measured after burning away all organic material for 20 hours at 600°C in a Thermolyne 62700 furnace (Thermolyne, Cole-Parmer, Vernon Hills, IL, USA).

4.3 Biochemical analyses

4.3.1 Transgene and NPY expression (study II)

In order to locate the transgene expression site in the brainstem and the hypothalamus of homozygous OE-NPY$^{D\beta H}$ mice, frozen coronary brain sections (40 µm) of respective sites were stained with LacZ as previously described (Ruohonen et al. 2008). NPY protein concentrations were quantified from the homogenates of hypothalamus, brainstem and adrenal gland with EURIA-NPY radioimmunoassay (RIA) kit (Euro-Diagnostics, Malmö, Sweden) as described before (Ruohonen et al. 2008). *Npy* mRNA levels were quantified with real-time qPCR as described in section 4.3.5 'Gene expression analyses with real-time qPCR'.

4.3.2 Biochemical markers in the blood

Serum insulin levels were measured with ELISA kit (Mercodia AB, Uppsala, Sweden). Serum triglycerides were quantified with serum triglyceride determination kit (TR0100 Sigma, St. Louis, MO, USA) and non-esterified fatty acids (NEFA) with NEFA-HR(2) kit (Wako Diagnostics, Richmond, VA, USA). Serum ghrelin levels and corticosterone levels were determined with RIA kits (Linco Research, St. Charles, MO, USA; ImmunoChemTM Double Antibody Corticosterone, MP Biomedicals, LLC, Orangeburg, NY, USA) and serum leptin with MILLIPLEX Mouse Adipokine Multiplex Assay (cat. no. MADKMAG-71K, Millipore Corporationa, Billerica, MA, USA). Serum total thyroxine levels were quantified with Total T4 determination kit (NovaTec Immundiagnostica GmbH, Dietzenbach, Germany). All the commercial measurements were performed as duplicates and according to the manufacturers' instructions.

4.3.3 Catecholamine concentration in the urine

Urine noradrenaline and adrenaline concentrations were quantified by HPLC as previously described (Gilsbach et al. 2007).

4.3.4 Endocannabinoid level in the tissues

For tissue endocannabinoid measurements, lipids were extracted from the hypothalamus, epididymal WAT, pancreas, liver and soleus muscle. Then, the two endocannabinoids

AEA and 2-AG were purified from the lipid extracts and quantitated with isotope dilution liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-MS) as described previously (Bartelt et al. 2011).

4.3.5 Gene expression analyses with real-time qPCR

For gene expression analyses, total RNA was isolated from tissue samples. RNAs from BAT and WAT samples were extracted with Trizol Reagent (Invitrogen, Carlsbad, CA, USA), from the adrenal glands with RNeasy Micro Kit (Qiagen) or with Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems) and from the brain sections with RNeasy Lipid Tissue Mini Kit (Qiagen). DNase treatment was performed with TURBO DNA-free™ Kit (Ambion Inc., Austin, TX, USA). RNA was converted to cDNA with High Capacity RNA-to-cDNA Kit (Applied Biosystems) and pre-designed TaqMan® Gene Expression assays (Applied Biosystems) or SYBR Green (KAPA SYBR® FAST ABI Prism®, Kapa Biosystems, Woburn, MA, USA) technique with separate primers were used for quantification. A list of the primers and pre-designed assays used is presented in the Table 3. Target genes were quantified with 7300 Real-Time PCR System (Applied Biosystems) relative to the housekeeping gene *Actb*, β-2 microglobulin (*B2m*), ribosomal protein S29 (*Rps29*) or eukaryotic 18S rRNA (TaqMan® Endogenous Control, Primer Limited, Applied Biosystems). Formula $2^{-\Delta\Delta CT}$ was used to calculate the gene expression relative to the expression level of WT mice.

4.4 Statistical analyses

The differences between the genotypes were compared with unpaired Student's test. Logarithmic transformations or nonparametric Mann–Whitney's U-test were used if data were not normally distributed with D'Agostino and Pearson omnibus normality test. Comparisons between the two genotypes and both sex or different challenges were analyzed with 2-way ANOVA and Bonferroni's or Sidak's post hoc test. Parameters including the time effect (weight gain, food intake, GTT, ITT, energy expenditure, RER, locomotor activity and temperature) were analyzed with repeated measures 2-way ANOVA and Bonferroni's or Sidak's post-hoc tests. The comparison of energy expenditure and body temperature was carried out by analysis of covariance (ANCOVA) with body weight and total WAT weight as covariates respectively. In study III, body weight development between treatments in each genotype was determined using the Generalized Linear Mixed Model and fitted for body weight accounting for individual mice with Toeplitz covariance structure. P-values for multiple comparisons were adjusted using simulation based method. Correlation analyses were performed by computing Pearson's correlation coefficients. Statistical analyses were carried out with Graphpad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) with the exception of ANCOVA analyses, which were calculated with IBM SPSS statistics version 21 (IBM,

Armonk, NY, USA). Data are presented as means ± the standard error of the mean (SEM), and the results were considered statistically significant if $P < 0.05$.

Table 3. Primers and probes used in qPCR gene expression studies.

Gene name	Sequence / TaqMan Assay ID	Method	Tissue	Endog. control	Study
Agouti-related peptide (*Agrp*)	F: 5'-CTTTCGCGGAGGTGCTAGAT-3' R: 5'-AGGACTCGTGCAGCCTTACAC-3'	SYBR Green	HT	*Actb*	II
β-galactosidase (*LacZ*)	F: 5'-TGGCTGGAGTGCGATCTTC-3' R:5'-GAGTTTGACCGTCTACGTGC-3' probe: 6FAM-CCGATACTGTCGTCGTC-MGB	TaqMan	gDNA	*Actb*	I, II, III
B-2 microglobulin (*B2m*)	Mm 00437762_m1	TaqMan	WAT/e	-	II
B-actin (*Actb*)	F: 5'-TCCATCATGAAGTGTGACGT-3' R: 5'- GAGCAATGATCTTGATCTTCA-3'	SYBR Green	HT BS AG	- - -	II, IV IV IV
B-actin (Mouse ACTB, VIC/MGB probe)	pre-designed	TaqMan	several	-	I, II, III
Carboxylesterase 3 (*Ces3*)	F: 5'-TGGTATTTGGTGTCCCATCA-3' R: 5'-GCTTGGGCGATACTCAAACT-3'	SYBR Green	WAT/r	*Rps29*	IV
Corticotropin-releasing hormone (*Crh*)	F: 5'-AAGAATACTTCCTCCGCCTGG-3' R: 5'-GTTGCTGTGAGCTTGCTGAGC-3'	SYBR Green	HT	*Actb*	II
Dopamine beta hydroxylase (*Dbh*)	Mm00460472_m1	TaqMan	BS AG	*Actb* *Actb*	II II
Fatty acid binding protein 4 (*Fabp4*)	F: 5'-GGATGGAAAGTCGACCACAA-3' R: 5'-AGTGTTCGTTCCTCGGAGTG-3'	SYBR Green	WAT/r	*Rps29*	IV
Hormone sensitive lipase (*Lipe*)	Mm00495359_m1 F: 5'-GCGCTGGAGGAGTGTTTTT-3' R: 5'-CGCTCTCCAGTTGAACCAAG-3'	TaqMan SYBR Green	WAT/e WAT/r	*18S* *Rps29*	II IV
Lipoprotein lipase (*Lpl*)	Mm00434770_m1 F: 5'-CTCGCTCTCAGATGCCCTAC-3' R: 5'-AGGCCTGGTTGTGTTGCTT-3'	TaqMan SYBR Green	WAT/e WAT/r	*18S* *Rps29*	II IV
Matrix metallopeptidase (*Mmp3*)	F: 5'-TTGTTCTTTGATGCAGTCAGC-3' R: 5'-GATTTGCGCCAAAAGTGC-3'	SYBR Green	WAT/r	*Rps29*	IV
Neuropeptide Y (*Npy*)	Mm03048253_m1 F: 5'-CTCCGCTCTGCGACACTAC-3' R: 5'-GGAAGGGTCTTCAAGCCTTGT-3' F: 5'-CCGCTCTGCGACACTACAT-3' R: 5'-TGTCTCAGGGCTGGATCTCT-3'	TaqMan SYBR Green SYBR Green	AG AG BS HT	*Actb* *Actb* *Actb* *Actb*	II, IV IV IV IV
Pro-opiomelanocortin (*Pomc*)	F: 5'-CAAGCCGGTGGGCAACAAACG-3' R: 5'-CTAATGGCCGCTCGCCTTCAAG-3'	SYBR Green	HT BS	*Actb* *Actb*	II, IV II
Ribosomal protein S29 (Rps29)	F: 5'-ATGGGTCACCAGCAGCTCTA-3' R: 5'-AGCCTATGTCCTTCGCGTACT-3'	SYBR Green	WAT/r	-	IV
Tyrosine hydroxylase (*Th*)	Mm00447557_m1 F: 5'-CCCAAGGGCTTCAGAAGAG-3' R: 5'-GGGCATCCTCGATGAGACT-3'	TaqMan SYBR Green	AG BS HT	*Actb* *Actb* *Actb*	II II, IV II, IV
Uncoupling protein 1 (*Ucp1*)	Mm01244861_m1 F: 5'-ACTGCCACACCTCCAGTCATT-3' R: 5'-CTTTGCCTCACTCAGGATTGG-3'	TaqMan SYBR Green	BAT BAT	*Actb* *Actb*	I, II IV
Uncoupling protein 2 (*Ucp2*)	Mm00495907_g1	TaqMan	WAT/e	*B2m*	II

AG = adrenal gland, BS = brainstem, gDNA = genomic DNA, HT =hypothalamus, WAT/e = epididymal white adipose tissue, WAT/r = retroperitoneal white adipose tissue

5. RESULTS AND DISCUSSION

5.1 Western diet in heterozygous OE-NPY$^{D\beta H}$ mice (study I)

The first approach adopted to intensify the phenotype of heterozygous OE-NPY$^{D\beta H}$ mice was the high-calorie western type diet. It was hypothesized that OE-NPY$^{D\beta H}$ mice would be more susceptible to diet-induced obesity in comparison with WT mice. This was true for female OE-NPY$^{D\beta H}$ mice, which gained significantly more weight during the 7-weeks' diet in comparison with WT mice (Fig. 7A). However, there was no difference between the genotypes in the males. Consistently, WAT and BAT weights were significantly increased in OE-NPY$^{D\beta H}$ females but not in males (Fig. 7B-C). Weight gain was accompanied by impaired glucose tolerance and insulin sensitivity in female OE-NPY$^{D\beta H}$ mice (Original publication I).

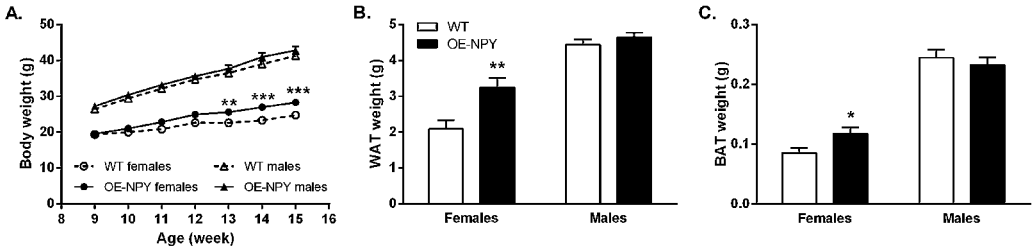

Figure 7. Weight gain and adiposity in heterozygous OE-NPY$^{D\beta H}$ and WT mice after 7-week western diet. (A) Body weight gain during consumption of the western diet. (B) Total weight of different WAT pads and (C) BAT weight after the western diet. Values are expressed as means ± SEM (n=9-16/genotype). * = P<0.05, ** = P<0.01, *** = P<0.001 with repeated measures ANOVA and Bonferroni's post-hoc test (A) or Student's t-test (B-C).

HE-staining revealed lipid accumulation to BAT in all groups except WT female mice (Original publication I). However, the BAT *Ucp1* mRNA expression level (females: WT 1.09±0.13, OE-NPY$^{D\beta H}$ 1.24±0.16, P=NS; males: WT 1.13±0.18, OE-NPY$^{D\beta H}$ 1.16±0.15, P=NS) or rectal body temperature (females: WT 35.9±0.4°C, OE-NPY^{DBH} 35.7±0.4°C, P=NS; males: WT 35.4±0.2°C, OE-NPY^{DBH} 35.7±0.3°C, P=NS) did not differ between the genotypes. Additionally, physical activity and food intake measured in western diet-fed homozygous OE-NPY$^{D\beta H}$ and WT females were similar in both genotypes (Original publication I), and thus the obesogenic phenotype of female OE-NPY$^{D\beta H}$ mice could not be explained by increased energy intake or decreased energy consumption. Plasma insulin or cholesterol levels were not changed between the genotypes in either sex.

In conclusion, NPY overexpression in noradrenergic neurons was able to reverse the resistance of C57BL female mice to diet-induced obesity (Novak et al. 2006). However, in males, the susceptibility to diet-induced obesity overrode the effect of NPY and OE-NPY$^{D\beta H}$ males gained weight similarly to WT males (55% of initial body weight). Since consumption of the western diet did intensify the metabolic phenotype of heterozygous

OE-NPY$^{D\beta H}$ female mice, it could have been utilized in the following mechanistic studies. However, NPY has been shown to participate in the estrogen-dependent anorectic effects (Baskin et al. 1995, Bonavera et al. 1994), which may complicate the interpretation of the results. Thus, male mice were preferred and it was decided to investigate the effects of increasing the transgene copy number as one way to intensify the phenotype of OE-NPY$^{D\beta H}$ mice.

5.2 Generation of the homozygous OE-NPY$^{D\beta H}$ mouse line

5.2.1 Transgene insertion site and NPY expression (study II)

A homozygous OE-NPY$^{D\beta H}$ mouse line was constructed by breeding the heterozygous OE-NPY$^{D\beta H}$ mice and genotyping the offspring with real-time qPCR. NPY overexpression in the noradrenergic areas i.e. the brainstem and adrenal glands showed evidence of significantly increased NPY protein and/or mRNA levels (Table 4). Surprisingly, hypothalamic *Npy* mRNA expression was also increased in OE-NPY$^{D\beta H}$ mice. The transgene expression in the brain was studied more closely with LacZ staining, which revealed β-galactosidase expression not only in the LC nucleus of the brainstem, an area densely innervated with noradrenergic nerves, but also in the hypothalamic nuclei such as the PVN and the DMH (Original publication II). The hypothalamic transgene was minor in comparison with the staining in the brainstem, but in agreement with a previous report which detected ectopic transgene expression in the hypothalamus with the promoter construct used in OE-NPY$^{D\beta H}$ mice (Hoyle et al. 1994).

Table 4. NPY mRNA and protein expression levels in 4-month-old OE-NPY$^{D\beta H}$ and WT male mice.

	mRNA (relative quantity)			protein (nmol/g protein)		
	WT	OE-NPY$^{D\beta H}$	P	WT	OE-NPY$^{D\beta H}$	P
Brainstem	1.00 ± 0.04	1.97 ± 0.12	<0.001	1.6 ± 0.1	3.4 ± 0.9	<0.05
Hypothalamus	1.00 ± 0.12 #	1.96 ± 0.18 #	<0.001	34.6 ± 8.4	50.7 ± 10.3	NS
Adrenal gland	1.00 ± 0.02	1.18 ± 0.21	<0.05	165.9 ± 17.4	183.3 ± 26.0	NS

Values are expressed as means ± SEM (n=6-11/group). # = measured from females, NS=non significant. Statistical comparisons are conducted with Student's t-test or Mann-Whitney test.

In order to exclude the possibility that homozygous transgene insertion would have deleted a known gene affecting energy metabolism, the transgene insertion site in the genome was determined. The transgene insertion site was located in chromosome 16 approximately at site 10796500 of NC_000082.6 genomic sequence within the second exon of protamine-1 (*Prm1)* gene. *Prm1* is expressed only in the spermatids and is responsible for the nuclear reorganization of spermatids during the spermatogenesis (Balhorn 2007). *Prm1* is necessary for the fertility (Lee et al. 1995) but there is no evidence that it would participate in other physiological functions (Balhorn 2007). Thus, it

is unlikely that the interruption of *Prm1* gene would affect the phenotype of homozygous OE-NPY$^{D\beta H}$ mice, which do not show considerable impairment in reproduction (study I).

5.3 Metabolic phenotype of the homozygous OE-NPY$^{D\beta H}$ mouse

5.3.1 Body composition (study II)

Body weight was not significantly increased in homozygous OE-NPY$^{D\beta H}$ female or male mice during weekly monitoring between the ages of 1-3 months (Original publication II). However, a difference in weight started to emerge around 3 months and at the age of 4 months, OE-NPY$^{D\beta H}$ mice were significantly heavier (Fig. 8A-B) in comparison with their WT counterparts. The total weight of different WAT depots was increased in OE-NPY$^{D\beta H}$ males already at the age of 2 months (Fig. 8C-D).

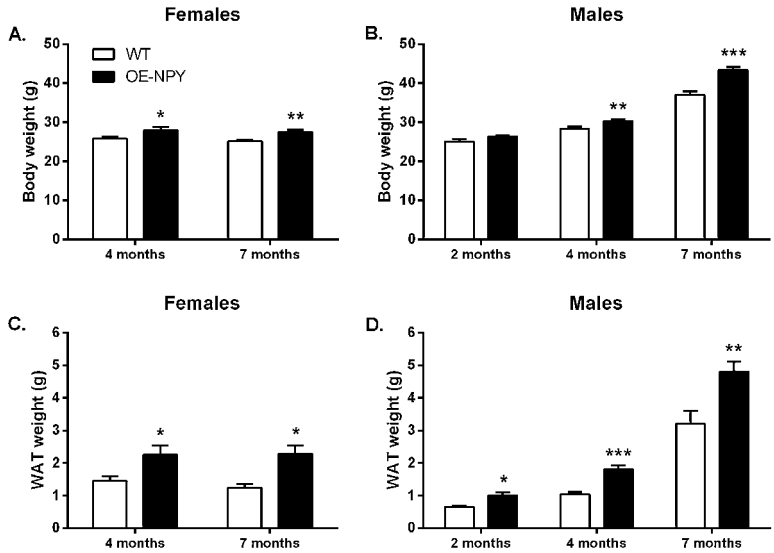

Figure 8. Body weight (A-B) and total weight of different WAT pads (C-D) at the ages of 2, 4 and 7 months in homozygous OE-NPY$^{D\beta H}$ and WT mice. Values are expressed as means ± SEM (n=6-12). * = P<0.05, ** = P<0.01, *** = P<0.001 with Student's t-test or Mann-Whitney test.

The difference in body adiposity was evident also in the DEXA scan and EchoMRI. The DEXA scan revealed increased fat-% in OE-NPY$^{D\beta H}$ mice (Table 5) and EchoMRI increased fat mass (Original publication IV) and fat mass gain during the drug interventions (Original publications III & IV). The DEXA scan also noted the tendency of female OE-NPY$^{D\beta H}$ mice towards decreased lean mass, whereas in male OE-NPY$^{D\beta H}$ mice, lean mass tended to be increased. However, the same male individuals were slightly longer (WT 9.6±0.2 cm, OE-NPY$^{D\beta H}$ 9.9±0.2 cm; P < 0.05), which probably explains the

opposite finding in lean mass of male mice since EchoMRI during the drug interventions detected a similarly decreased absolute lean mass (Original publication IV) and lean mass gain (Original publications III & IV). The increased body length was not evident in any other male or female mouse groups at different ages. DEXA scan further revealed decreased values of BMD and BMC in OE-NPY$^{D\beta H}$ females, while males showed no difference between the genotypes (Table 5). Thus, bone was studied more closely in female mice. There were no differences in the length or ash weight of tibiae. However, volumetric analysis revealed a slight decline in the tissue volume of the trabecular and cortical bone in OE-NPY$^{D\beta H}$ mice which could be a reflection of weakened bone structure. Furthermore, cortical mean total cross-sectional tissue area, perimeter and mean polar moment of inertia were reduced in OE-NPY$^{D\beta H}$ mice. Similarly, a decline in cortical bone tissue volume has been reported in mice with osteoblast and osteocyte-specific NPY overexpression (Matic et al. 2012).

Table 5. The body composition measured with DEXA scan and bone micro-Ct analysis of 5-month-old homozygous OE-NPY$^{D\beta H}$ and WT mice.

	Females			Males		
	WT	**OE-NPY$^{D\beta H}$**	**P**	**WT**	**OE-NPY$^{D\beta H}$**	**P**
Fat-% (%)	17.6 ± 6.1	30.0 ± 5.2	<0.001	23.3 ± 7.3	31.2 ± 6.4	<0.05
Lean mass (g)	20.9 ± 1.5	20.5 ± 1.4	0.05	26.2 ± 2.0	27.9 ± 1.8	0.08
BMD (mg/cm^3)	57.1 ± 0.6	54.7 ± 0.9	<0.05	55.0 ± 0.7	53.6 ± 0.7	NS
BMC (g)	0.52 ± 0.01	0.45 ± 0.01	<0.001	0.46 ± 0.01	0.45 ± 0.02	NS
Trabecular TV (mm^3)	2.32 ± 0.05	2.16 ± 0.04	<0.05	ND	ND	-
Cortical TV (mm^3)	0.24 ± 0.003	0.23 ± 0.003	<0.05	ND	ND	-
Cortical tissue area (mm^2)	1.50 ± 0.02	1.42 ± 0.02	<0.05	ND	ND	-
Bone perimeter (mm)	10.33 ± 0.10	9.92 ± 0.12	<0.05	ND	ND	-
Mean polar moment of inertia (mm^4)	0.46 ± 0.01	0.41 ± 0.01	<0.05	ND	ND	-

Values are expressed as means ± SEM (n=8-13/group). BMD = bone mineral density, BMC = bone mineral content, NS = non significant, TV = tissue volume. Statistical comparisons are conducted with Student's t-test or Mann-Whitney test.

5.3.2 Energy intake and expenditure (study II)

Food intake did not differ between the genotypes, when measured either with group-housed mice at the age of active growth (Original publication II) or with individually housed 5–6-month-old male mice (WT 4.8±0.3 g/day, OE-NPY$^{D\beta H}$ 4.6±0.2 g/day, P=NS). Furthermore, total and diurnal patterns of eating, energy expenditure and RER measured with automated monitoring system LabMaster were similar in both genotypes (Fig. 9A-C). The total 24-h locomotor activity did not differ between the genotypes, but activity during light-time was significantly reduced in OE-NPY$^{D\beta H}$ mice (Fig. 9D). However, this phenomenon alone cannot explain the weight gain, i.e. the mechanism is neither exclusively associated with increased energy intake nor decreased energy expenditure. Energy intake can be altered also at the level of nutrient absorption. However, wet or dry faecal mass, lipid content of the faeces, water intake or urine volume did not differ between the genotypes and thus do not imply that changes had occurred in nutrient

absorption in OE-NPY$^{D\beta H}$ mice, although NPY may participate in the regulation of gastric motility (Fujimiya & Inui 2000) and intestinal absorption (Cox 2007).

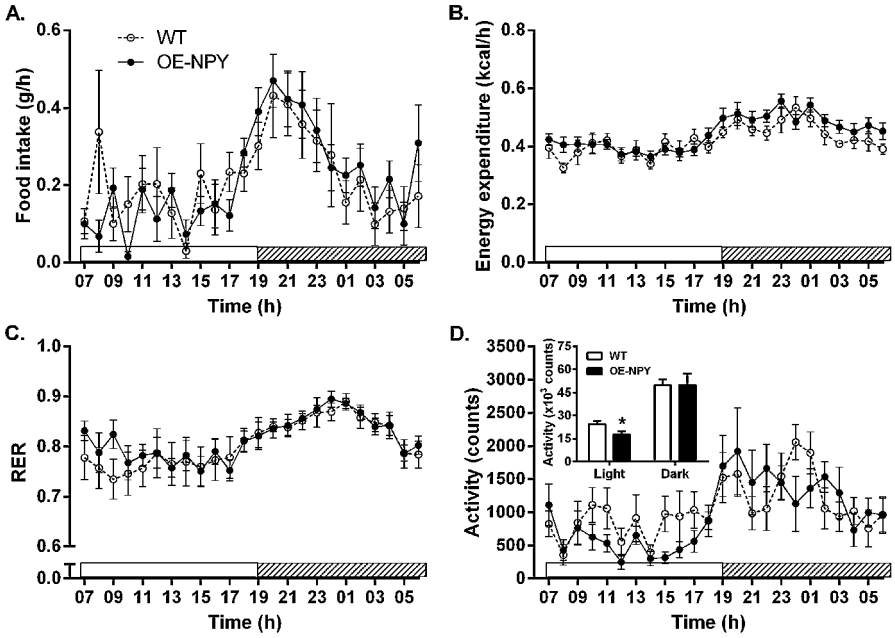

Figure 9. Diurnal energy intake and expenditure of 4-month-old homozygous OE-NPY$^{D\beta H}$ and WT male mice. (A) Food intake, (B) energy expenditure, (C) respiratory exchange ratio (RER) and (D) locomotor activity during 24-h period from the first full day and night of 4-day LabMaster monitoring. Total light- and dark-time locomotor activity during the 4-day measurement period (D). Values are expressed as means ± SEM (n=8/genotype). White box = light-time, lined box = dark-time. * = $P < 0.05$ with Student's t-test. Energy expenditure is adjusted for body weight by ANCOVA (B). Adjusted energy expenditure is presented at common body weight of 29.825 g. Other statistical comparisons (A, C-D) are conducted with repeated measures ANOVA.

Biomarkers involved in the appetite regulation were quantified from the blood and the hypothalamus of 4-month-old female mice (section 5.3.7 'Response to overnight fasting challenge'). Serum ghrelin levels or hypothalamic *Agrp* mRNA expression did not differ between the genotypes under basal conditions (Fig. 16A-B). However, the level of the anorexigenic *Pomc* mRNA expression in the hypothalamus was increased in OE-NPY$^{D\beta H}$ mice, probably as a response to weight gain (Fig. 16C).

5.3.3 Lipid metabolism (study II)

One potential mechanism to explain the weight gain observed in OE-NPY$^{D\beta H}$ mice could be at the level of WAT. The cell size of white adipocytes was increased in OE-NPY$^{D\beta H}$ mice as revealed with HE-staining (Original publication II) and in isolated gonadal adipocytes (Fig. 10A). Serum leptin levels were significantly increased in 7-month-old female and

male OE-NPY$^{D\beta H}$ mice due to their greater adipose tissue mass (Table 6). Furthermore, serum triglycerides and NEFA levels were reduced in OE-NPY$^{D\beta H}$ mice with some variation between the sexes and the age groups. These findings are believed to reflect increased lipid uptake and decreased lipolysis in OE-NPY$^{D\beta H}$ mice. Both adipogenic and antilipolytic actions of NPY have been reported before (Valet et al. 1990, Kuo et al. 2008).

Table 6. Serum leptin and lipid levels in fasted homozygous OE-NPY$^{D\beta H}$ and WT mice.

	4 months			7 months		
Females	**WT**	**OE-NPY$^{D\beta H}$**	**P**	**WT**	**OE-NPY$^{D\beta H}$**	**P**
Leptin (ng/ml)	ND	ND	-	3.0 ± 0.8	8.6 ± 1.3	<0.01
Triglycerides (mg/ml)	0.21 ± 0.06	0.25 ± 0.07	NS	0.39 ± 0.03	0.32 ± 0.04	<0.05
NEFAs (mmol/l)	0.40 ± 0.05	0.27 ± 0.02	0.06	0.38 ± 0.02	0.37 ± 0.02	NS

	4 months			7 months		
Males	**WT**	**OE-NPY$^{D\beta H}$**	**P**	**WT**	**OE-NPY$^{D\beta H}$**	**P**
Leptin (ng/ml)	ND	ND	-	7.7 ± 1.4	13.5 ± 1.6	<0.05
Triglycerides (mg/ml)	0.29 ± 0.02	0.24 ± 0.03	NS	0.40 ± 0.04	0.30 ± 0.04	0.05
NEFAs (mmol/l)	0.24 ± 0.01	0.21 ± 0.03	NS	0.33 ± 0.01	0.28 ± 0.02	<0.05

Values are expressed as means ± SEM (n=6-12/group). NS = non significant, NEFAs = non-esterified fatty acids. Statistical comparisons are conducted with Student's t-test or Mann-Whitney test.

In the isolated mesenteric adipocytes, the β-agonist, isoprenaline, stimulated lipolysis significantly more in OE-NPY$^{D\beta H}$ adipocytes (Fig. 10B), which may be explained by a reduction in catecholaminergic tone (section 5.3.6 'Catecholaminergic system') sensitizing the β-adrenoceptors to a sympathetic stimulus. However, the mRNA expression of lipolysis regulating enzymes HSL (*Lipe*) and LPL (*Lpl*), or fat oxidation promoting uncoupling protein 2 (*Ucp2*) in epididymal WAT did not differ between the genotypes (Original publication II). Even though there was increased adiposity, there were no signs of inflammation in OE-NPY$^{D\beta H}$ mice, since the number of macrophages remained the same in retroperitoneal WAT of both genotypes (data not shown).

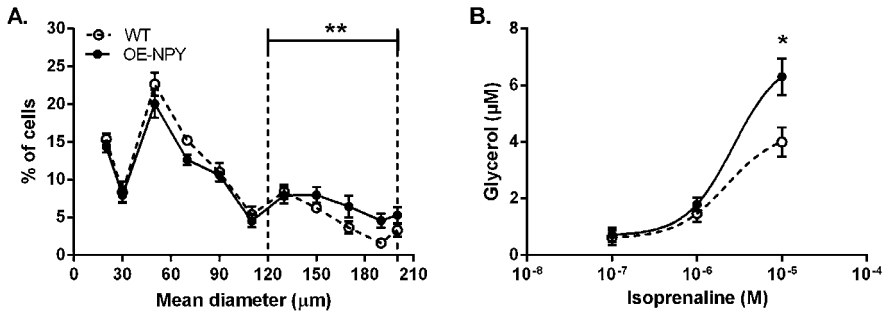

Figure 10. Adipocyte cell size and lipolysis activity in 4-month-old homozygous OE-NPY$^{D\beta H}$ and WT mice. (A) Size of isolated gonadal adipocytes of female mice (n=6/genotype). (B) Isoprenaline-stimulated glycerol release in mesenteric adipocytes isolated from male mice (n=4-5/genotype). Values are expressed as means ± SEM. * = P<0.05, ** = P<0.01 with repeated measures ANOVA (A) or 2-way ANOVA (B) and Bonferroni's post-hoc test.

5.3.4 BAT and thermogenesis (study II)

Homozygous OE-NPY$^{D\beta H}$ mice had increased BAT weight already at the age of 2 months (males: WT 81.4±6.41 mg, OE-NPY$^{D\beta H}$ 122.0±10.3 mg, $P < 0.05$) and the morphology in HE-staining resembled WAT with lipid droplets inside the brown adipocytes (Fig. 11A). Electron microscopy revealed that the BAT of OE-NPY$^{D\beta H}$ mice had an increased fat content but the size and structure of their mitochondria were normal (data not shown). There was no difference in basal core body temperature between the genotypes (Fig. 11B). However, the levels of the thermogenesis marker *Ucp1* mRNA expression in BAT were significantly decreased in OE-NPY$^{D\beta H}$ mice (Fig. 12A). When this result is supplemented with the evidence of increased mass and fat content of the BAT, it is hypothesized that there is impairment of BAT function in OE-NPY$^{D\beta H}$ mice, which likely contributes to the weight gain as has been shown with other obese rodent models (Arbeeny et al. 1995, Sell et al. 2004).

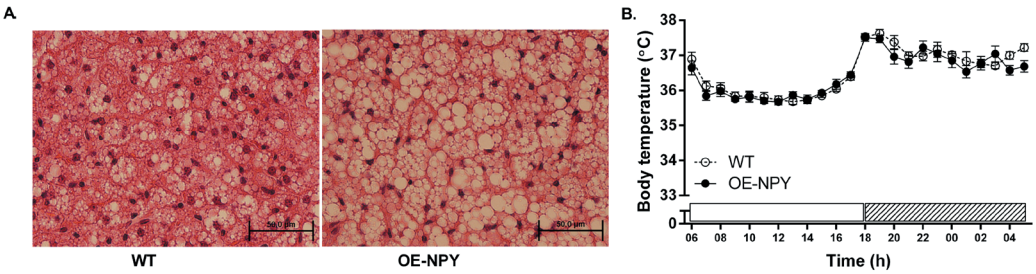

Figure 11. BAT morphology and body temperature of OE-NPY$^{D\beta H}$ and WT mice. (A) BAT morphology of 2-month-old male mice in HE-staining (scale bar = 50 µm). (B) Intraperitoneal body temperature of 5–6 month-old male mice (n= 7-9/genotype). Values are expressed as means ± SEM. Statistical comparisons are conducted with repeated measures ANOVA (B).

BAT thermogenesis was further studied during 2- and 4-h cold exposures. Cold significantly increased *Ucp1* mRNA expression in both genotypes in comparison with room temperature (Fig. 12A). However, the reduced *Ucp1* expression of OE-NPY$^{D\beta H}$ mice in comparison with WT mice was evident also after the cold exposures. Cold exposure reduced the intraperitoneal body temperature in both genotypes but the shapes of body temperature curves were significantly different (interaction $P<0.01$) in OE-NPY$^{D\beta H}$ and WT mice (Fig. 12B). However, when only the individuals matched for WAT mass (n=4/genotype) were compared, the shapes of body temperature curves were identical and OE-NPY$^{D\beta H}$ mice had significantly higher body temperature (Fig. 12C) suggesting improved cold tolerance. Similarly to the results obtained in the lipolysis assay, low basal sympathetic activity hypersensitizing β-adrenoceptors in BAT may explain this increased thermogenic activity after an intense sympathetic stimulus such as cold exposure (section 5.3.6 'Catecholaminergic system'). RER or energy expenditure did not differ between the genotypes during cold challenges (data not shown). However, locomotor activity, measured with the automated monitoring system LabMaster, was

reduced in OE-NPY$^{D\beta H}$ mice during the 4-h cold exposure (Fig. 12D). Serum NEFA levels increased significantly in both genotypes after 2-h cold exposure as an indication of cold-stimulated lipolysis but this parameter did not differ between the genotypes (Original publication II).

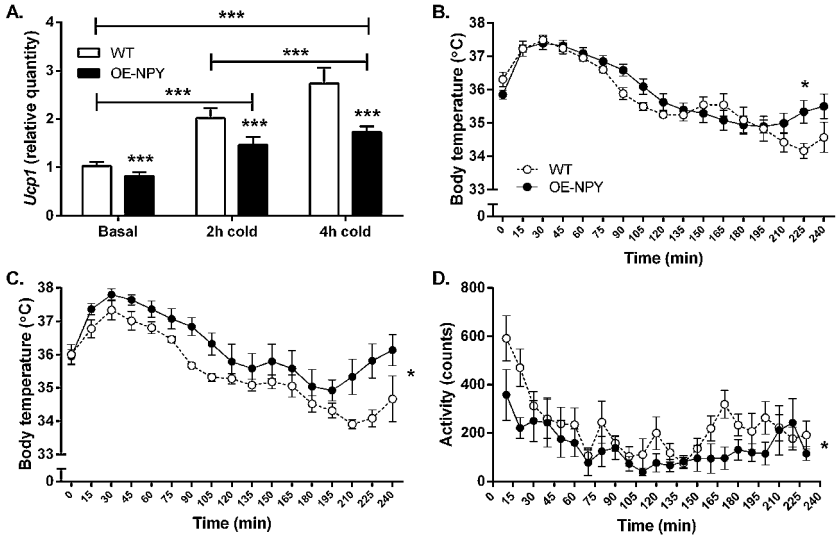

Figure 12. Response to cold challenges in male OE-NPY$^{D\beta H}$ and WT mice at the age of 4-5 months. (A) Uncoupling protein 1 (*Ucp1*) mRNA expression in room temperature and after 2- or 4-h cold-exposed mice. Intraperitoneal body temperature during 4-h cold exposure (B) in the whole cohort or (C) in WAT mass-matched mice (n=4/genotype). (D) Locomotor activity during 4-h cold exposure. Values are expressed as means ± SEM (n=7-10/group). * = P<0.05, *** = P<0.001 with 2-way ANOVA (A) or repeated measures ANOVA and Sidak's post-hoc test (B-D).

5.3.5 Glucose metabolism (study II)

Fasting blood glucose levels were increased in OE-NPY$^{D\beta H}$ males at the age of 3 and 6 months but not in female mice (Table 7). Glucose clearance after an i.p. glucose load was impaired at the 20 min time-point in 3-month-old OE-NPY$^{D\beta H}$ female mice (Fig. 13A). In 6-month-old OE-NPY$^{D\beta H}$ females, glucose tolerance only tended to be impaired. In OE-NPY$^{D\beta H}$ males, glucose clearance was significantly impaired at the ages of 3 and 6 months (Fig. 13B). A delay in glucose clearance was seen also in the AUC values in both females and males (Table 7). The glucose response to insulin was impaired in female OE-NPY$^{D\beta H}$ only at the age of 6 months (Fig. 13C). Male OE-NPY$^{D\beta H}$ mice showed a tendency towards impaired insulin sensitivity at the age of 3 months, which developed to a statistically significant level by the age of 6 months (Fig. 13D). However, AUC values of the glucose response to insulin were significantly increased in OE-NPY$^{D\beta H}$ mice already at the age of 3 months in both female and male mice. The impairment in glucose metabolism developed to evoke significant hyperinsulinaemia by the age of 6 months in OE-NPY$^{D\beta H}$ males (Table 7).

Table 7. Fasting blood glucose and insulin levels, and area under the curve (AUC) values of glucose (GTT) and insulin tolerance (ITT) tests in homozygous OE-NPY$^{D\beta H}$ and WT mice.

	3 months			6 months		
Females	**WT**	**OE-NPY$^{D\beta H}$**	**P**	**WT**	**OE-NPY$^{D\beta H}$**	**P**
Fasting glucose (mmol/l)	8.3 ± 0.6	8.4 ± 0.4	NS	8.0 ± 0.3	8.3 ± 0.3	NS
GTT AUC (mmol/l x min)	925 ± 22	1042 ± 65	0.06	911 ± 25	982 ± 31	0.09
ITT AUC (mmol/l x min)	377 ± 26	405 ± 11	<0.05	381 ± 17	430 ± 14	<0.05
Fasting insulin (µg/l) #	0.21 ± 0.05	0.27 ± 0.04	NS	0.24 ± 0.01	0.25 ± 0.02	NS

	3 months			6 months		
Males	**WT**	**OE-NPY$^{D\beta H}$**	**P**	**WT**	**OE-NPY$^{D\beta H}$**	**P**
Fasting glucose (mmol/l)	9.6 ± 0.3	10.9 ± 0.3	<0.01	9.0 ± 0.4	11.0 ± 0.5	<0.01
GTT AUC (mmol/l x min)	1040 ± 32	1223 ± 35	<0.01	997 ± 78	1622 ± 111	<0.001
ITT AUC (mmol/l x min)	434 ± 35	516 ± 23	<0.01	388 ± 21	503 ± 27	<0.05
Fasting insulin (µg/l) #	0.33 ± 0.03	0.58 ± 0.14	NS	0.36 ± 0.05	1.09 ± 0.18	<0.001

Values are expressed as means ± SEM (n=7-12/group). # = measured at the age of 4 and 7 months, NS = non significant. Statistical comparisons are conducted with Student's t-test or Mann-Whitney test.

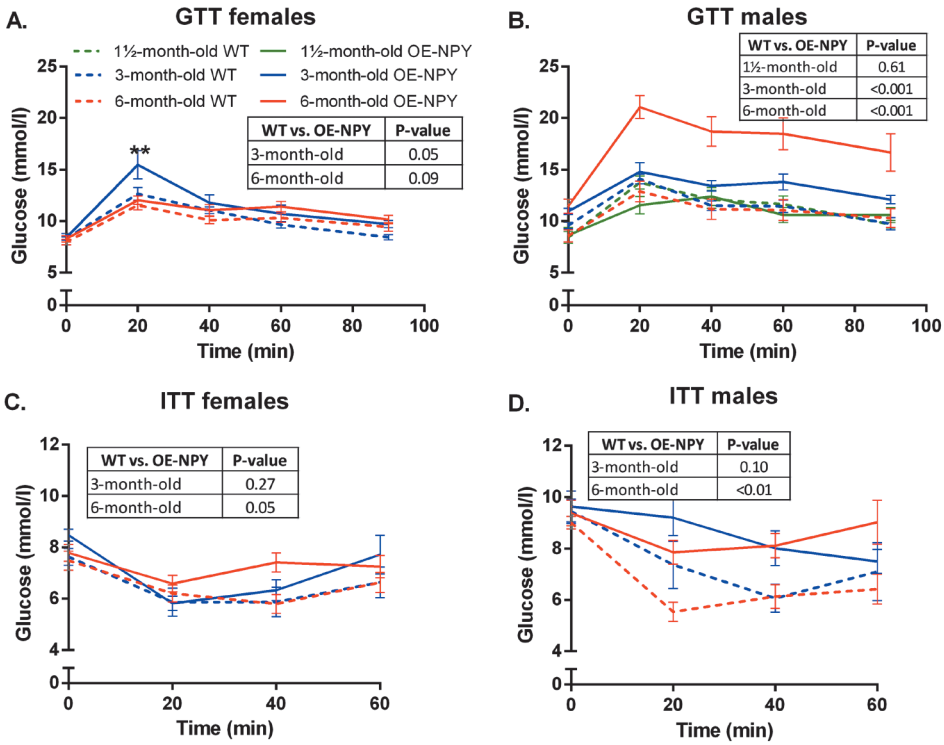

Figure 13. Glucose tolerance and insulin sensitivity in OE-NPY$^{D\beta H}$ and WT mice. Blood glucose response for a dose of (A-B) glucose (5% wt/vol, 1g/kg) and (C-D) insulin (1 IU/kg) at the age of 1½, 3 and 6 months. Values are expressed as means ± SEM (n=7-12/group). Statistical comparisons between age-matched WT and OE-NPY$^{D\beta H}$ mice analyzed with repeated measures ANOVA are marked in the tables. ** = P<0.01 with Sidak's post hoc test at 20 min (A).

NPY is known to affect insulin secretion not only at the central level but also directly at the level of the pancreatic β-cells (Gao et al. 2004, Moltz & McDonald 1985). However, in OE-NPY$^{D\beta H}$ mice, the impairment in the glucose tolerance developed age-dependently after the increase in body adiposity, which suggests that the changes in glucose tolerance were a secondary response to fat accumulation rather than a direct effect of NPY.

5.3.6 Catecholaminergic system (study II)

As mentioned in previous sections, down-regulated basal sympatho-adrenal tone was postulated to promote fat gain in OE-NPY$^{D\beta H}$ mice. This was evidenced with significantly decreased urine adrenaline levels in OE-NPY$^{D\beta H}$ mice in both of the 6-h dark period fractions, while noradrenaline levels displayed no differences between the genotypes (Fig. 14A-B). Furthermore, the expression levels of the catecholamine synthesizing enzymes *Th* (WT 1.01±0.06, OE-NPY$^{D\beta H}$ 0.81±0.04, P<0.01) and *Dbh* (WT 1.01±0.08, OE-NPY$^{D\beta H}$ 0.75±0.09, P<0.05) were decreased in the brainstem of OE-NPY$^{D\beta H}$ mice but there were no differences detected in the adrenal gland or the hypothalamus (Original publication II).

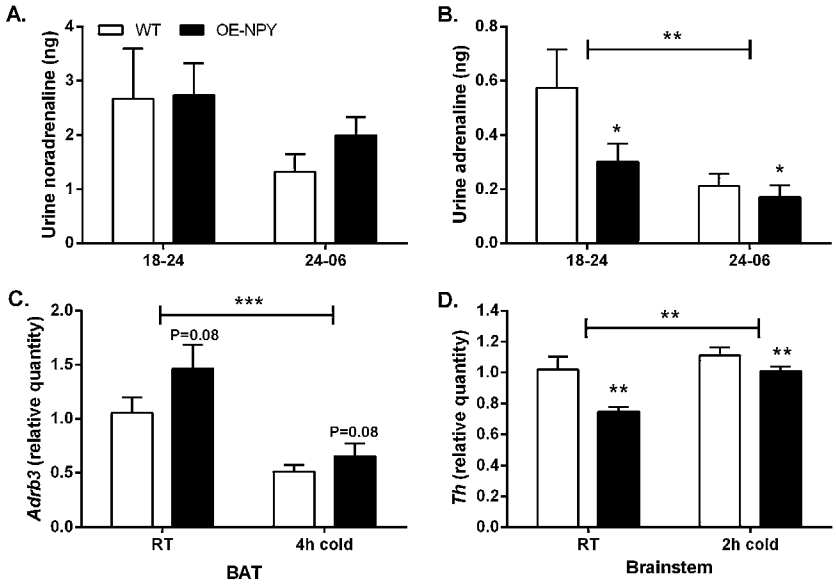

Figure 14. Catecholaminergic system in 4-5-month-old male OE-NPY$^{D\beta H}$ and WT mice. Urine (A) noradrenaline and (B) adrenaline levels in 6-h night fractions. (C) Relative β3-adrenoceptor (*Adrb3*) mRNA expression in BAT and (D) tyrosine hydroxylase (*Th*) mRNA expression in the brainstem under room temperature (RT) conditions and after 2-h or 4-h cold exposure. Values are expressed as means ± SEM (n=4-9/group). * = P<0.05, ** = P<0.01, *** = P<0.001 with 2-way ANOVA.

Furthermore, BAT *Adrb3* mRNA expression under basal conditions tended to be increased in OE-NPY$^{D\beta H}$ mice when compared with WT mice (Fig. 14C). Cold challenges

significantly decreased BAT *Adrb3* expression as a response to an intense sympathetic stimulus and increased the brainstem *Th* in both genotypes, but the genotype differences were maintained (Fig. 14C-D). However, WAT *Adrb3* mRNA expression was not altered in OE-NPY$^{D\beta H}$ mice under basal conditions or after the cold exposure in spite of the increased lipolytic response to isoprenaline in WAT lipolysis assay (section 5.3.3 'Lipid metabolism').

These findings in OE-NPY$^{D\beta H}$ mice are in agreement with the known inhibitory effect of NPY on tyrosine hydroxylase promoter activity (Cavadas et al. 2006). Furthermore, rats overexpressing NPY have exhibited reduced urine catecholamine levels (Michalkiewicz et al. 2001). The reduced sympathetic tone may also explain the relatively minor bone phenotype in OE-NPY$^{D\beta H}$ mice. Low sympathetic activity has been shown to stimulate bone formation (Takeda et al. 2003), and thus the mild bone resorptive phenotype in OE-NPY$^{D\beta H}$ mice may result from a subtle imbalance between bone resorption stimulated by excess NPY and bone formation stimulated by the low sympathetic activity. However, it remains unclear to what extent and on what level NPY excess inhibits catecholaminergic tone. Since *Th* is downregulated in the brainstem of OE-NPY$^{D\beta H}$ mice, it seems that NPY inhibits the sympathetic efferent neurons at least at the central level. Additionally, it is not possible to distinguish which metabolic consequences in OE-NPY$^{D\beta H}$ mice result from downregulated sympathetic tone. However, it is interesting to note that OE-NPY$^{D\beta H}$ mice are still able to respond normally or even more intensively to acute sympathetic stimulus.

5.3.7 Response to overnight fasting challenge (study II)

An overnight 16-h fasting challenge decreased body weight to a similar extent in both genotypes (Fig. 15A) but induced a more intensive re-feeding response in OE-NPY$^{D\beta H}$ mice (Fig. 15B). Locomotor activity or energy expenditure did not differ between the genotypes during the fasting or the following 24-h re-feeding period (data not shown), but RER was increased in OE-NPY$^{D\beta H}$ mice due to accelerated re-feeding (Fig. 15C).

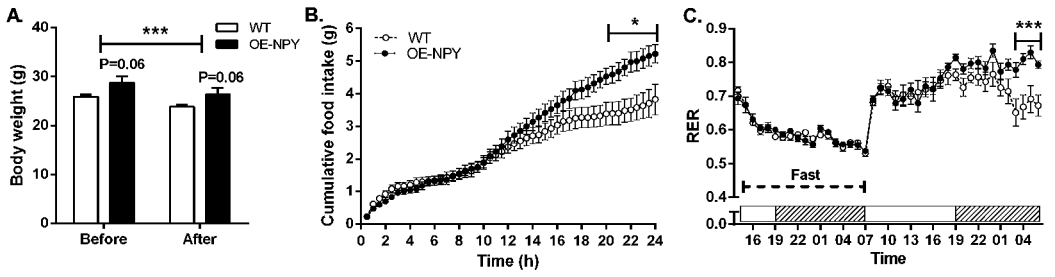

Figure 15. Response to overnight fasting in OE-NPY$^{D\beta H}$ and WT mice. (A) Body weight before and after the 16-h fasting in 5-month-old female mice (n=10-11/genotype). (B) Cumulative food intake after the fasting and (C) respiratory exchange ratio (RER) during the fasting and the following re-feeding period in 5-month-old male mice (n=8/genotype). Values are expressed as means ± SEM. * = P<0.05, *** = P<0.001 with 2-way ANOVA (A) or repeated measures ANOVA and Sidak's post-hoc test (B-C).

Fasting significantly increased serum ghrelin levels and hypothalamic *Agrp* mRNA expression in both genotypes when compared with the fed mice (Fig. 16A-B). However, there was no difference between the genotypes. Fasting did not affect hypothalamic *Pomc* mRNA levels (Fig. 16C) but elevated the hypothalamic *Npy* mRNA expression in both genotypes (Fig. 16D). OE-NPY$^{D\beta H}$ mice retained their significantly increased *Pomc* and *Npy* levels in comparison with WT mice also in the fasted state.

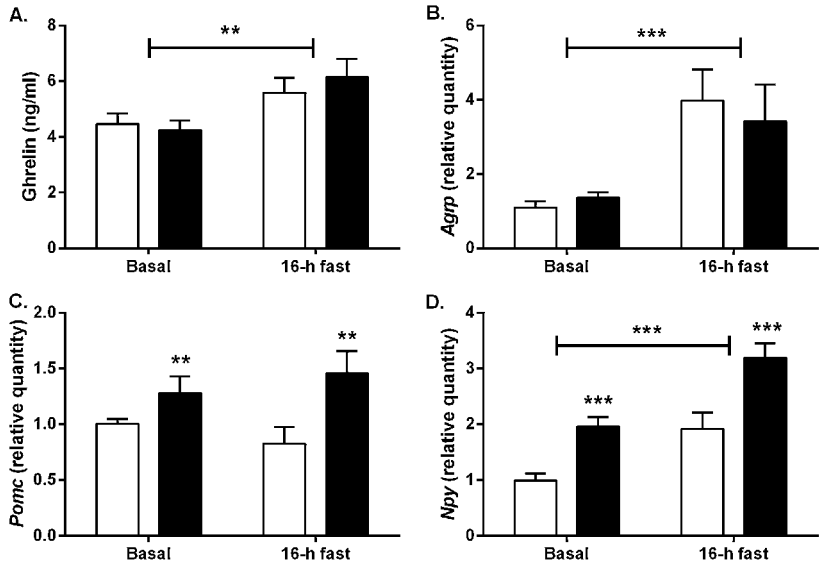

Figure 16. Appetite biomarkers of 5-month-old fed and fasted OE-NPY$^{D\beta H}$ and WT female mice. (A) Serum ghrelin levels, hypothalamic (B) *Agrp*, (C) *Pomc* and (D) *Npy* mRNA expressions in *ad libitum* fed and 16-h fasted mice. Values are expressed as means ± SEM (n=6-10/group). ** = P<0.01, *** = P<0.001 with 2-way ANOVA.

Taken together, OE-NPY$^{D\beta H}$ mice showed no difference in basal food intake (section 5.3.2 'Energy intake and expenditure') but exhibited increased fasting-induced re-feeding thought to be due to the significantly higher hypothalamic *Npy* expression in comparison with fasted WT mice. An opposite effect, i.e. attenuated re-feeding after fasting, has been reported in rats with NPY overexpression in the Arc (Sousa-Ferreira et al. 2011). Thus, it is proposed that hypothalamic NPY is not responsible for the metabolic phenotype of OE-NPY$^{D\beta H}$ mice, but extra-hypothalamic effects of NPY exert an independent role in the pathogenesis of obesity.

5.4 Homozygous OE-NPY$^{D\beta H}$ mice and endocannabinoid system (study III)

As presented in the review of literature, endocannabinoids are important in the regulation of energy homeostasis and these transmitters are known to interact with NPY. Therefore, it was hypothesized that endocannabinoids could mediate some of the effects of NPY

on the development of obesity and metabolic disturbances. The tissue levels of two endogenous endocannabinoids, i.e. AEA and 2-AG, levels were significantly altered in homozygous OE-NPY$^{D\beta H}$ mice (Table 8). In the hypothalamus, 2-month-old OE-NPY$^{D\beta H}$ mice had reduced AEA concentrations but elevated 2-AG levels. At the age of 4 months, only 2-AG levels were increased in the hypothalamus. The opposite change in endocannabinoid levels at the age of 2 months is not surprising since (i) AEA can inhibit the brain 2-AG biosynthesis and (ii) 2-AG can act as a substrate for the AEA catabolic enzyme and thus to be degraded instead of AEA (Di Marzo & Maccarrone 2008). Moreover, AEA can bind to non-cannabinoid receptors and be regulated differently from 2-AG. Since food intake is not altered in OE-NPY$^{D\beta H}$ mice, the increase in hypothalamic 2-AG concentrations may rather be a secondary effect of leptin resistance caused by WAT accumulation (Di Marzo et al. 2001) than a direct effect of NPY.

Table 8. Tissue endocannabinoid i.e. anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels in male OE-NPY$^{D\beta H}$ and WT mice.

	2 months		4 months		7 months	
AEA (pmol/g)	**WT**	**OE-NPY$^{D\beta H}$**	**WT**	**OE-NPY$^{D\beta H}$**	**WT**	**OE-NPY$^{D\beta H}$**
Hypothalamus	22.3 ± 0.8	12.0 ± 1.0*	14.8 ± 2.5	13.6 ± 3.4	32.4 ± 2.9	25.0 ± 9.9
WAT/epid	40.0 ± 1.2	28.0 ± 1.6*	52.0 ± 2.2	84.0 ± 3.6*	39.9 ± 2.8	46.4 ± 3.6
Pancreas	34.2 ± 0.9	28.1 ± 0.9*	26.8 ± 1.8	35.5 ± 3.0*	32.6 ± 2.8	39.7 ± 9.0
Liver	30.6 ± 3.9	22.3 ± 2.7	44.7 ± 3.9	30.2 ± 2.0	53.0 ± 4.3	101.8 ± 15.4*
Soleus muscle	83.0 ± 10.4	12.6 ± 1.5*	59.3 ± 3.5	80.0 ± 8.5	83.7 ± 5.0	95.2 ± 5.0

	2 months		4 months		7 months	
2-AG (nmol/g)	**WT**	**OE-NPY$^{D\beta H}$**	**WT**	**OE-NPY$^{D\beta H}$**	**WT**	**OE-NPY$^{D\beta H}$**
Hypothalamus	2.9 ± 0.2	4.6 ± 0.2*	1.9 ± 0.1	3.1 ± 0.1*	4.1 ± 0.2	3.9 ± 0.3
WAT/epid	0.77 ± 0.03	1.11 ± 0.09*	0.69 ± 0.05	0.51 ± 0.04	0.63 ± 0.13	0.62 ± 0.04
Pancreas	7.6 ± 0.5	10.7 ± 0.5*	7.9 ± 0.5	6.8 ± 0.7	8.0 ± 0.5	8.0 ± 1.0
Liver	3.8 ± 0.1	4.1 ± 0.3	3.9 ± 0.3	4.4 ± 0.03	4.0 ± 0.2	4.8 ± 0.3
Soleus muscle	1.6 ± 0.2	0.7 ± 0.1*	2.7 ± 0.1	2.5 ± 0.1	3.0 ± 0.3	1.8 ± 0.3

Values are expressed as means ± SEM (n=4/group). * = P<0.05 with ANOVA and Bonferroni's post-hoc test.

The increased adiposity in 2-month-old OE-NPY$^{D\beta H}$ mice was accompanied with an increase of 2-AG and a decrease of AEA in epididymal WAT. The opposite changes in endocannabinoid levels are probably attempting to compensate for each other. At the age of 4 months, AEA levels were elevated, which may reinforce the triglyceride storage in WAT. Adipose tissue endocannabinoid levels have been shown to be elevated in both obese humans and mice (D'Eon et al. 2008, Matias et al. 2006). Similarly to epididymal WAT, pancreatic 2-AG levels were elevated in OE-NPY$^{D\beta H}$ mice at the age of 2 months and AEA levels were reduced at the age of 2 months but increased at 4 months. With respect to the liver endocannabinoids, the only significant difference detected between the genotypes was the increased levels of AEA in 7-month-old OE-NPY$^{D\beta H}$ mice.

The altered endocannabinoid levels may participate in the development of impaired glucose tolerance and hyperinsulinaemia in OE-NPY$^{D\beta H}$ mice since it has been previously

reported that activation of endocannabinoid system enhances both basal and glucose-induced insulin release from the β-cells (Malenczyk et al. 2013, Matias et al. 2006), and contributes to the development of hepatosteatosis and insulin resistance (Liu et al. 2012, Osei-Hyiaman et al. 2005). Additionally, increased hypothalamic 2-AG levels have been reported to participate in the hepatic glucose production and insulin resistance (O'Hare et al. 2011). However, liver AEA levels were increased only in 7-month-old mice with evident liver steatosis (data not shown) and thus alteration in endocannabinoids is postulated to be a consequence of hepatosteatosis rather than a reason for this condition. The endocannabinoids present in muscle tissue may also regulate the glucose metabolism since CB1 activation in myotubes is known to inhibit insulin signaling and glucose uptake (Silvestri & Di Marzo 2012). However, both endocannabinoids were decreased in the soleus muscle of 2-month-old OE-NPY$^{D\beta H}$ mice without significant differences at the older ages. Thus, the reduced endocannabinoid levels in the soleus muscle may rather reflect an adaptive mechanism counteracting the increased skeletal muscle glucose uptake caused by increased WAT accumulation. This would indicate that reduced endocannabinoid levels in skeletal muscle are able to protect mice from glucose intolerance and thus glucose metabolism is not impaired in OE-NPY$^{D\beta H}$ mice until the endocannabinoid levels in skeletal muscle are no longer reduced.

The effect of endocannabinoid system on the development of the metabolic phenotype in OE-NPY$^{D\beta H}$ mice was further studied by blocking CB1Rs with a specific inverse agonist AM251 for 5 weeks. CB1R-blockade significantly reduced body weight gain in both genotypes (Fig. 17A), while OE-NPY$^{D\beta H}$ mice gained significantly more weight in comparison with WT mice in both CB1R inverse agonist and vehicle-treated groups. Furthermore, CB1R-blockade reduced food intake in WT mice but not in OE-NPY$^{D\beta H}$ mice (Fig. 17B), which may suggest that in WT mice, the weight reduction was caused by the hypophagic response. While in OE-NPY$^{D\beta H}$ mice, the blockade of CB1Rs may have prevented the effects of altered peripheral endocannabinoids and thus reduced the body weight gain. CB1R-blockade decreased fat mass and lean mass gain in both genotypes (Fig. 17C-D). The fat mass gain in OE-NPY$^{D\beta H}$ mice was reduced almost to the level present in the vehicle-treated WT mice.

Glucose tolerance tended to be impaired in vehicle-treated OE-NPY$^{D\beta H}$ mice in comparison with WT mice (Fig. 18A). The CB1R inverse agonist treatment for 3 weeks almost significantly ($P=0.06$) improved glucose tolerance in OE-NPY$^{D\beta H}$ mice to the same level with WT mice, but it did not affect the glucose tolerance of WT mice. Furthermore, CB1R-blockade for three weeks tended to increase serum corticosterone levels especially in OE-NPY$^{D\beta H}$ mice although there was no statistical significance between the genotypes (Fig. 18B). This increase in the corticosterones of OE-NPY$^{D\beta H}$ mice is thought to result from the inhibition of the higher hypothalamic 2-AG levels in OE-NPY$^{D\beta H}$ mice, which under normal conditions, may tonically inhibit the HPA axis. There is one previous study highlighting the necessity of both NPY and endocannabinoid signaling in the regulation of corticosterone release, which supports this conclusion (Zhang et al. 2010a).

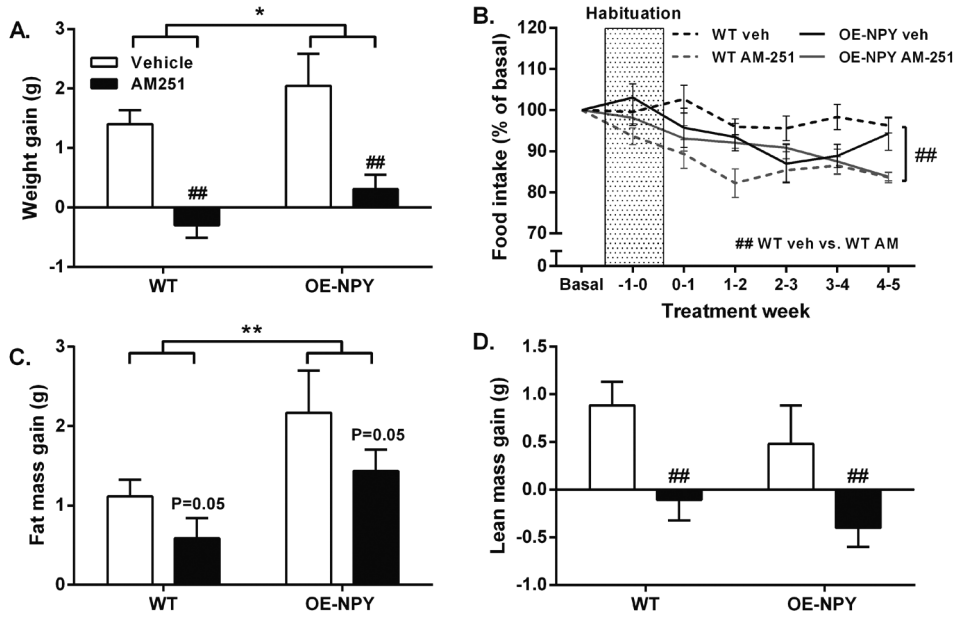

Figure 17. Body composition and food intake of OE-NPY$^{D\beta H}$ and WT male mice in CB1R-blockage intervention study. (A) Body weight gain (B) food intake (n=5-6 cages/genotype), (C) fat mass gain and (D) lean mass gain measured by EchoMRI after 5-week CB1R inverse agonist (AM251, 3mg/kg/day, i.p.) or vehicle treatment. Values are expressed as means ± SEM (n=9-12/group). * = P<0.05, ** = P<0.01 comparing different genotypes within one treatment; ## = P<0.01, ### = P<0.001 comparing different treatments with 2-way ANOVA (A, C-D) or repeated measures ANOVA (B).

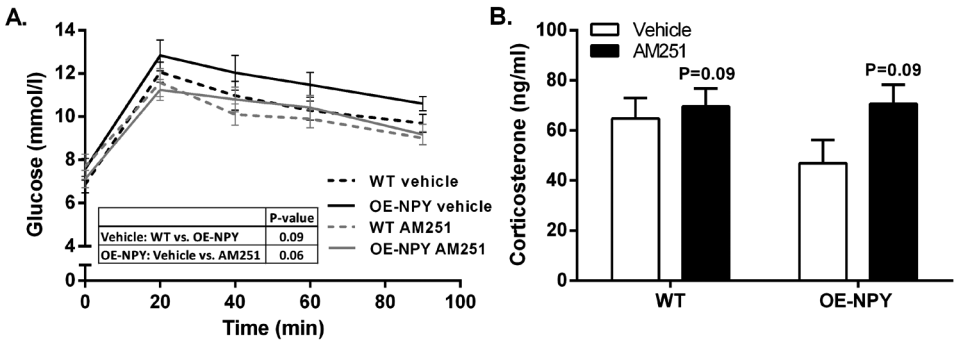

Figure 18. Glucose tolerance and blood corticosterone levels of OE-NPY$^{D\beta H}$ and WT male mice in CB1R-blockade intervention study. (A) Blood glucose response for a glucose dose (5% wt/vol, 1g/kg) and (B) fasting blood corticosterone levels after 3-week inverse agonist (AM251, 3mg/kg/day, i.p.) or vehicle drug treatment. Values are expressed as means ± SEM (n=9-12/group). Statistical comparisons are conducted with repeated measures ANOVA (A) or 2-way ANOVA (B).

In conclusion, alterations in endocannabinoid levels precede and may at least partly be responsible for the metabolic phenotype of OE-NPY$^{D\beta H}$ mice. However, it is not known whether endocannabinoid, NPY and sympathoadrenal systems affect energy metabolism independently or synergistically. It has been shown that NPY's feeding stimulative effect requires the presence of an intact CB1 system (Poncelet et al. 2003), which supports the interwoven system involving both endocannabinoids and NPY. On the other hand, the CB1R inverse agonist reduced body weight and depressed the fat mass gain in both genotypes and thus the beneficial effects of CB1R-blockade in OE-NPY$^{D\beta H}$ mice may represent simply a net effect of a reduced endocannabinoid system and stimulated NPY system. There are previous studies supporting the independent effects of the two systems, i.e. CB1-antagonism and NPY deficiency acted additively on body weight gain and lipid oxidation, and CB1-antagonism reduced food intake independently from NPY (Di Marzo et al. 2001, Zhang et al. 2010a). Furthermore, CB1R-activation has been shown to inhibit noradrenaline release and reduce sympathetic tone (Ishac et al. 1996, Kunos et al. 2008). Thus, CB1R-blockade may decrease fat accumulation via activation of the sympathoadrenal system and BAT thermogenesis (Boon et al. 2014), with these effects being intensified in OE-NPY$^{D\beta H}$ mice with hypersensitized adrenergic receptors. Unfortunately, BAT and catecholaminergic tone were not investigated during CB1R-blockade. Due to the complicated interplay between the endocannabinoid, sympathoadrenal and NPY systems, one can only speculate on the ways that noradrenergic neuron NPY overexpression *in vivo* affected both endocannabinoid and sympathoadrenal systems. However, it is not known which of the metabolic changes in OE-NPY$^{D\beta H}$ mice would be primary effects attributable to the noradrenergic neuron NPY and which would be secondary responses mediated via other energy metabolism regulating systems.

5.5 Homozygous OE-NPY$^{D\beta H}$ mice and direct effects of NPY via peripheral Y1Rs and Y2Rs

Blockade of peripheral Y1Rs with the specific antagonist BIBO3304 exerted no major effects on the phenotype of WT or OE-NPY$^{D\beta H}$ mice fed either with chow diet or with a western type diet. Y1R-antagonism initially increased body weight gain and BAT weight in both genotypes (Original publication IV). Additionally, in OE-NPY$^{D\beta H}$ mice, the gain in lean mass and food intake were increased after the 2-week Y1R-antagonist treatment. However, these differences were not evident after the 4½-week treatment period, suggesting that the effects of peripheral Y1R-blockade become compensated after longer term drug administration. In contrast to these findings, previous publications have shown a mild hypophagic effect during chronic administration of a peripheral Y1R-antagonist (Sousa et al. 2012), and increased lipid oxidation and resistance to diet-induced obesity in mice with a peripheral Y1R-KD (Zhang et al. 2010). However, in the present studies, energy expenditure was not investigated, which might have revealed mild differences in energy utilization not reflected as a significant difference in the body composition.

Unlike the situation with Y1Rs, blockade of peripheral Y2Rs affected the energy homeostasis of WT and OE-NPY$^{D\beta H}$ mice. In mice fed a chow diet, the Y2R-antagonist significantly increased the body weight gain in both genotypes (Fig. 19A-B). However, there were no increases in the gains of fat (Fig. 19C-D) or lean mass (Original publication IV). Thus, it is postulated that Y2R-antagonism can evoke an increase in body weight via mild, but statistically non-significant, anabolic effects in several peripheral tissues. One explanation for increased weight gain in Y2R-antagonist-treated OE-NPY$^{D\beta H}$ mice is increased food intake (Fig. 19E). In contrast, there was no evidence of hyperphagia in the WT mice.

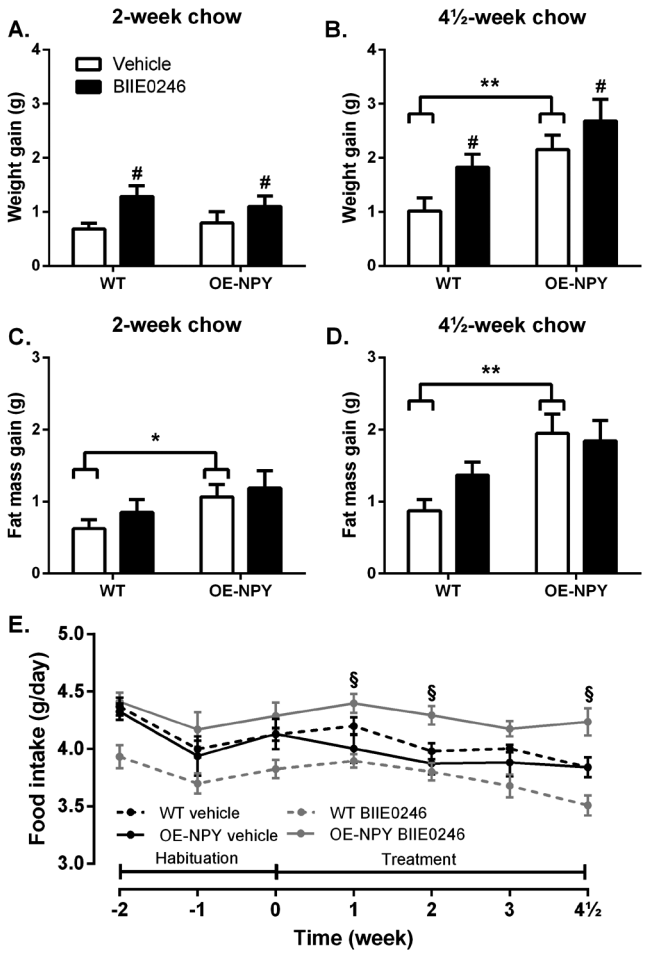

Figure 19. Body composition and food intake in chow-fed Y2R-antagonist-treated OE-NPY$^{D\beta H}$ and WT mice. (A-B) Body weight and (C-D) fat mass gain after 2-week (n=19-25/group) or 4½-week (n=11-12/group) Y2R-antagonist (BIIE0246, 1.3mg/kg/day, i.p.) or vehicle administration. (E) Food intake during habituation and treatment period (n=19-25/group until week 2 and n=10-13/group from 2 to 4½ weeks). Values are expressed as means ± SEM. * = P<0.05, ** = P<0.01 comparing different genotypes in vehicle-treated mice with student's t-test. # = P<0.05 comparing different treatments within one genotype with 2-way ANOVA. § = P<0.05 comparing different treatments in OE-NPY$^{D\beta H}$ mice with repeated measures ANOVA and Sidak'c post-hoc test.

Y2R-antagonist did not significantly affect blood glucose or serum NEFA levels in either genotype (Original publication IV). In WT mice, Y2R-antagonist tended to decrease serum triglycerides and significantly increased serum cholesterol and insulin concentrations to the level of vehicle-treated OE-NPY$^{D\beta H}$ mice (Fig. 20A-D). In OE-NPY$^{D\beta H}$ mice, serum insulin levels were significantly increased after treatment with the Y2R-antagonist.

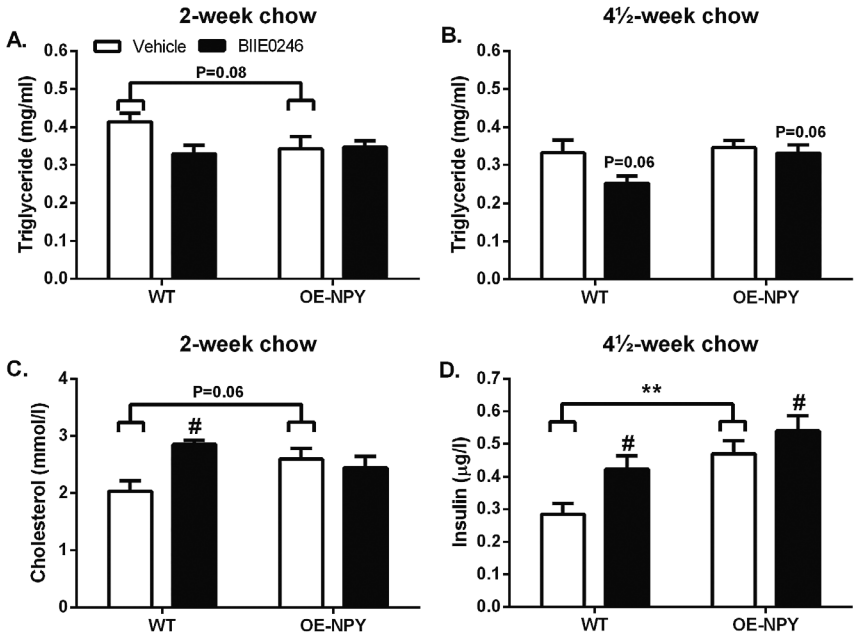

Figure 20. Serum lipid and insulin levels in chow-fed Y2R-antagonist-treated OE-NPY$^{D\beta H}$ and WT mice. Serum triglycerides after (A) 2-week and (B) 4½-week Y2R-antagonist (BIIE0246, 1.3mg/kg/day, i.p.) or vehicle administration. (C) Serum cholesterol levels in the 2-week cohort and (D) insulin levels in the 4½-week cohort. Values are expressed as means ± SEM (n=6-13/group). ** = P<0.01 comparing different genotypes in vehicle-treated mice with student's t-test. # = P<0.05 comparing different treatments within one genotype with 2-way ANOVA.

Taken together, the phenotype of chow-fed Y2R-antagonist-treated WT mice resembled the metabolic phenotype of OE-NPY$^{D\beta H}$ mice, and thus is postulated to result from increased NPY levels caused by blockade of presynaptic Y2-autoreceptors regulating the NPY release (Wang & Whim 2013). However, *Npy* mRNA expression in hypothalamus or adrenal gland did not significantly differ between the treatments in either genotype (Original publication IV). On the other hand, mRNA expression represents the synthesis of the gene and may not be comparable with the amount of active peptide. In OE-NPY$^{D\beta H}$ mice, the mechanism for weight gain may also be hyperphagia induced by increased NPY levels. This is in concordance with the stress-related hyperphagia evidenced

previously as fasting-induced feeding. However, our findings were contradictory to a previous Y2R-study which could detect no difference in body composition in chow-fed mice with adult-onset Y2R-KD in peripheral tissues (Shi et al. 2011). However, the approaches used to block the Y2R-signalling were different these two studies. Shi et al. (2011) ablated Y2Rs in peripheral target tissues without alterations in NPY neurons. However, the peripheral Y2R-antagonism applied in the present study blocked also the pre-synaptic Y2Rs controlling NPY release causing an obesogenic phenotype similar to that seen in the adult-onset hypothalamic $Y2R^{-/-}$ mice (Shi et al. 2013). As a matter of fact, peripherally administered YR-antagonist may have affected YRs in the central nervous system since hypothalamus lacks an effective blood-brain-barrier (Rodriguez et al. 2010).

When combined with a western type diet, treatment with the Y2R-antagonist caused similar but more intense anabolic effects in WT mice when compared with regular chow diet. Body weight and fat mass gain of western diet-fed WT mice were significantly elevated after Y2R-antagonist treatment although there was no difference in food intake (Fig. 21A-C). However, in OE-$NPY^{D\beta H}$ mice, Y2R-antagonist treatment tended to reduce fat mass gain and caused mild hypophagia at the 2-week time point. No differences were detected in blood glucose or lipid levels between the genotypes or the treatments (Original publication IV). However, the provision of an energy-rich diet increased the serum cholesterol levels when compared to chow diet and thus the diet effects may have overwhelmed the effects of Y2R-antagonism.

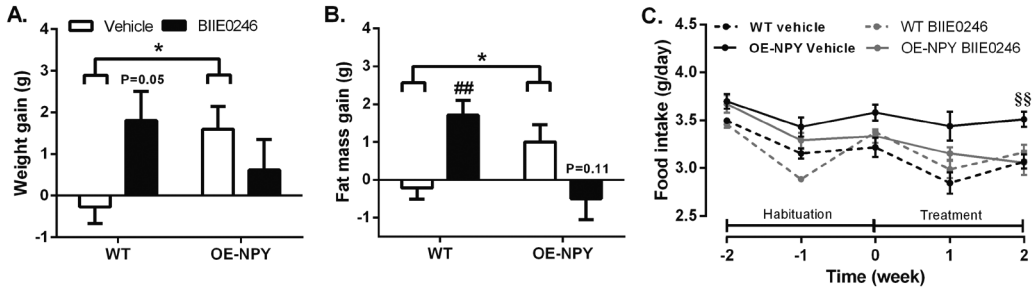

Figure 21. Body composition and food intake in a western diet-fed Y2R-antagonist-treated OE-$NPY^{D\beta H}$ and WT mice. (A) Body weight and (B) fat mass gain after 2-weeks Y2R-antagonist (BIIE0246, 1.3mg/kg/day, i.p.) or vehicle administration. (C) Food intake during habituation and treatment period. Values are expressed as means ± SEM (n=8-12/group). * = $P<0.05$ comparing different genotypes in vehicle-treated mice with student's t-test. ## = $P<0.01$ comparing different treatments within one genotype with 2-way ANOVA and §§ = $P<0.01$ comparing different treatments in OE-$NPY^{D\beta H}$ mice with repeated measures ANOVA and Sidak's post-hoc test.

The tendency of the Y2R-antagonist to reduce the fat mass gain in OE-$NPY^{D\beta H}$ mice consuming the western diet is consistent with a previous study reporting that peripheral

Y2R-KD could attenuate diet-induced obesity (Shi et al. 2011). Furthermore, chronic stress together with an energy-rich diet has been shown to induce NPY release from SNS and to cause fat mass gain via direct Y2R-mediated adipogenic effects in the adipose tissue (Kuo et al. 2007). NPY is thought to regulate adipogenesis in two ways, directly by stimulating the proliferation and differentiation of adipocytes, and indirectly by promoting angiogenesis (Kuo et al. 2007). However, the expressions of adipogenic (*Fabp4*, *Ces3*) and angiogenic (*Mmp3*) genes in the retroperitoneal WAT were not altered after Y2R-antagonism in either genotype (Original publication IV). Treatment with the Y2R-antagonist tended to increase the expression of the enzymes responsible for both triglyceride uptake (*Lpl*) and breakdown (*Lipe*) in both genotypes (Original publication IV), which is suggestive of accelerated lipid turn-over but does not explain different response for Y2R-antagonism in different genotypes. However, it is possible that the distribution of the Y2Rs and thus the effects of peripheral NPY differ between the WAT depots. Thus, the failure to detect any statistically significant difference in the expression levels of adipogenic and angiogenic genes in retroperitoneal WAT does not rule out the possibility that it is the anti-angiogenic and anti-adipogenic effects of Y2R-antagonism that are mediating the reduced fat mass gain in OE-NPY$^{D\beta H}$ mice.

Since food intake was decreased in Y2R-antagonist-treated OE-NPY$^{D\beta H}$ mice but not in WT mice, the expressions of feeding regulative genes were studied in the brainstem and the hypothalamus. Y2R-antagonism significantly increased and tended to increase the *Npy* expression in the brainstem and the hypothalamus of both genotypes, respectively (Fig. 22A-B) but not in adrenal gland (Original publication IV). This supports the conclusion that blockade of presynaptic Y2Rs increases the NPY levels. Since Y2Rs are hypothesized to be upregulated in OE-NPY$^{D\beta H}$ mice, the increase in *Npy* mRNA expression is more prominent in OE-NPY$^{D\beta H}$ mice when compared to WT mice. Furthermore, hypothalamic *Pomc* mRNA expression was reduced in Y2R-antagonist-treated WT mice to the level of OE-NPY$^{D\beta H}$ mice (Fig. 22C), which fits with the obesogenic effects of Y2R-antagonism in WT mice. However, the *Th* expression of either genotype was not changed by the Y2R-antagonist and thus the blockade of peripheral Y2Rs seems to cause the metabolic alterations via mechanisms other than central modulation of the sympathetic tone. Thus, these alterations in the expressions of genes regulating food intake do not explain the weight reduction and mild hypophagia in OE-NPY$^{D\beta H}$ mice. However, similar differences in the hypothalamic gene expressions have been reported with normophagic mice with an NPY neuron-specific Y2R-deletion in the Arc (Shi et al. 2010).

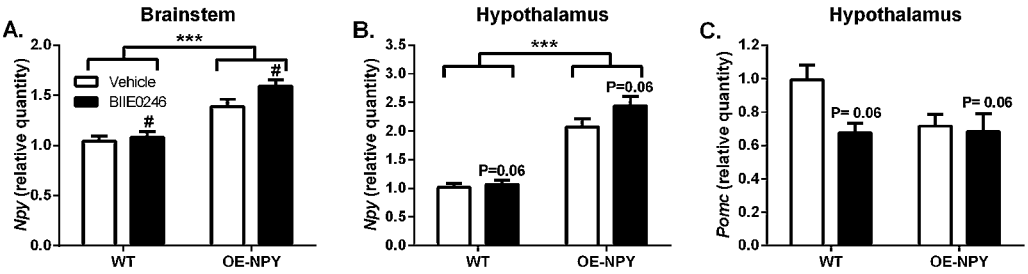

Figure 22. Gene expression of feeding regulatory genes in western diet-fed Y2R-antagonist-treated OE-NPY$^{D\beta H}$ and WT mice. *Npy* mRNA expression in (A) the brainstem and (B) the hypothalamus, and (C) *Pomc* mRNA expression in the hypothalamus after 2-week Y2R-antagonist (BIIE0246, 1.3mg/kg/day, i.p.) or vehicle administration. Values are expressed as means ± SEM (n=7-12/group). *** = P<0.001 comparing different genotypes and # = P<0.05 comparing different treatments within one genotype with 2-way ANOVA.

As a conclusion, peripheral Y2R-antagonism evoked obesogenic effects in chow-fed WT and OE-NPY$^{D\beta H}$ mice as well as in WT mice consuming a western diet most likely by blocking the presynaptic Y2Rs and stimulating NPY release. Thus, it seems that peripheral Y2Rs during a 'healthy' diet do not mediate the energy storing effects of NPY. However, when confronted with an energy-rich diet, peripheral presumably postsynaptic Y2Rs are responsible for the NPY's obesogenic effects in the peripheral target organs such as adipose tissue.

5.6 The mechanisms of noradrenergic neuron NPY causing the development of obesity in OE-NPY$^{D\beta H}$ mice

The hypothesized mechanisms of noradrenergic neuron NPY to cause metabolic alterations in homozygous OE-NPY$^{D\beta H}$ mice are presented in figure 23. Noradrenergic neuron NPY has clear feeding-independent adipogenic effects, which impair glucose metabolism and predispose to the metabolic disorder. These effects are hypothesized to be mediated via several mechanisms. Noradrenergic neuron NPY in the brain and/or in the SNS may promote fat accumulation. Peripheral Y2-receptors seem to have an important role, at least during energy surplus. This effect is hypothesized to take place in WAT, since Kuo et al. (2007) have previously reported that WAT-targeted Y2R-antagonism reduces visceral fat accumulation induced by elevated NPY released from SNS during chronic stress in combination with an energy-rich diet. However, it cannot be ruled out that noradrenergic neuron NPY exerted its observed metabolic changes either via other peripheral tissues or through other YRs. In the future, the role peripheral YRs in different target tissues will need to be clarified before it will be possible to exploit the putative beneficial effects of YR-blockade, such as prevention of fat gain, without the harmful side effects, such as decline in bone mass.

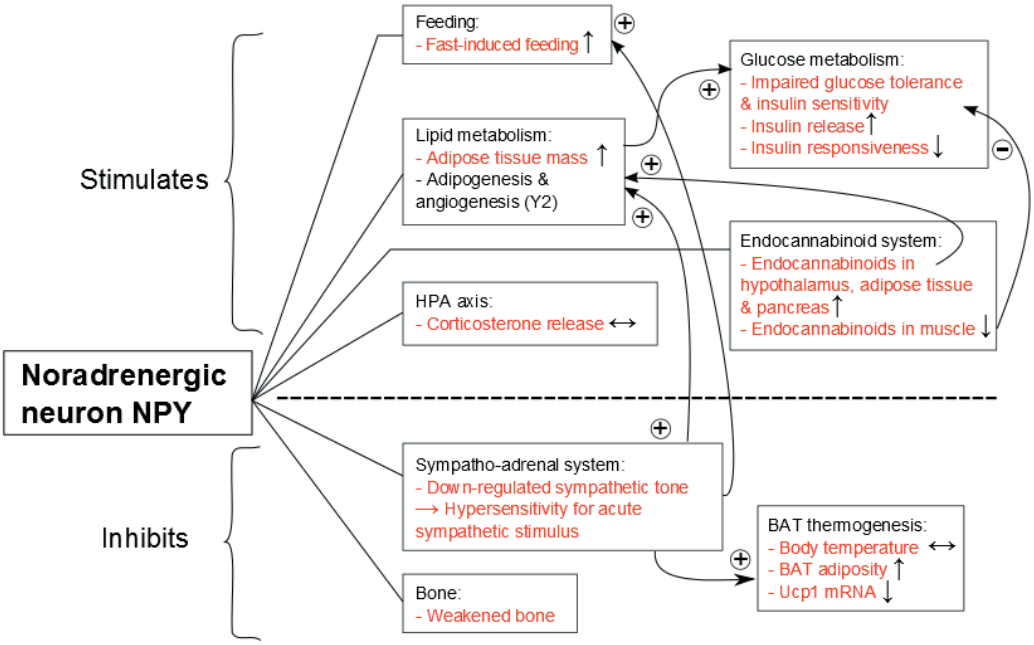

Figure 23. Summary of the effects of noradrenergic neuron NPY on the energy metabolism. The findings evident in OE-NPY$^{D\beta H}$ mice are labeled in red. Noradrenergic neuron NPY stimulates fast-induced feeding and increases adipose tissue mass via down-regulation of sympathetic activity. Adiposity further impairs glucose tolerance and insulin sensitivity. Inhibited sympathetic tone increases BAT adiposity and impairs BAT function. Furthermore, noradrenergic NPY increases endocannabinoid levels in several energy metabolism regulating organs, which may further participate in the fat mass gain and impaired glucose metabolism.The systems that are stimulated by noradrenergic neuron NPY are represented above the dashed line and the systems that are inhibited are marked below the line. ↑ or + = stimulatory effect, ↓ or - = inhibitory effect, ↔ = no change.

Since peripheral blockade of Y1Rs or Y2Rs, in the experimental conditions used, did not attenuate the metabolic phenotype of OE-NPY$^{D\beta H}$ mice, the brain noradrenergic neuron NPY seems to have an important role in mediating the adipogenic effects. Although the original aim was to study the extra-hypothalamic NPY in the brain, minor but evident transgene expression was detected also in the hypothalamus of OE-NPY$^{D\beta H}$ mice, which could have been responsible for the metabolic effects. However, in comparison with other hypothalamic NPY overexpression models, the phenotype of OE-NPY$^{D\beta H}$ mice is very different, which may result from milder NPY overexpression (2-fold increase in OE-NPY$^{D\beta H}$ mice vs. 3.6-fold increase by Sousa-Ferreira et al. 2011). Most importantly, homozygous OE-NPY$^{D\beta H}$ mice were not hyperphagic as is the case in the hypothalamic models (Sainsbury et al. 1997, Shi et al. 2013, Sousa-Ferreira et al. 2011). Furthermore, NPY overexpression in the Arc has been shown to inhibit sympathetic tone of BAT via downregulation of Th activity in the PVN (Shi et al. 2013). However, hypothalamic *Th* was not altered in OE-NPY$^{D\beta H}$ mice. Additionally, NPY overexpression in the Arc is known to impair the ability of the hypothalamic neuronal pathway to respond to changes

in blood leptin levels and this leads to attenuated fasting-induced feeding (Sousa-Ferreira et al. 2011). On the contrary, OE-NPY$^{D\beta H}$ mice showed increased re-feeding. Thus, extra-hypothalamic, i.e. noradrenergic neuron NPY in the brainstem rather than hypothalamic NPY is postulated to be responsible for the metabolic consequences evident in OE-NPY$^{D\beta H}$ mice. Consequently, our research group has already started an experiment to investigate the effects of virus vector-derived noradrenergic neuron NPY only in the brainstem. The aim is to distinguish between the role of brain noradrenergic neuron NPY and that of NPY in SNS.

In addition to direct YRs-mediated effects, noradrenergic neuron NPY down-regulates sympatho-adrenal and stimulates endocannabinoid activity. The complex interplay between NPY, sympatho-adrenal and endocannabinoid system was discussed in sections 5.3.6 'Catecholaminergic system and 5.4 'Homozygous OE-NPY$^{D\beta H}$ mice and endocannabinoid system'. Decreased sympathetic tone is involved in the NPY-induced defects in the functions of WAT and BAT. This was evidenced in OE-NPY$^{D\beta H}$ mice but has been reported also in previous studies demonstrating that NPY excess in CNS inhibits BAT function via decreased sympathetic tone (Shi et al. 2013) whereas in the periphery, it causes fat accumulation associated with decreased plasma and fat tissue catecholamine levels (Kuo et al. 2007). Elevated endocannabinoid levels may also promote fat gain in adipose tissue but are not solely responsible for the metabolic effects of noradrenergic neuron NPY.

5.7 Methodological considerations

There are some methodological aspects of this study which need to be considered. First of all, OE-NPY$^{D\beta H}$ mice are shown to react more intensively to stress (Ruohonen et al. 2009b), which affects the food intake, body composition and behavior in general. Since energy homeostasis was studied, the possibility for stress-induced alterations in the parameters investigated had to be taken into account. Invasive procedures, such as glucose tolerance test and drug administration, and the introduction to a new environment, such as single-housing, affected the body weight more in OE-NPY$^{D\beta H}$ than in their WT counterparts. A habituation period of one week was used to minimize the effect of stress on the results. Despite this precaution, the energy homeostasis parameters of OE-NPY$^{D\beta H}$ mice measured in unusual housing conditions may have been affected by the stress. For example, the prolonged stress reaction during the single-housing in automated monitoring system may have overrated the energy expenditure of OE-NPY$^{D\beta H}$ mice.

The intervention studies (studies III & IV) were performed during the time when the phenotype was still developing, i.e. at the age of 3-4 months. However, the stressful effects of daily injections were underestimated and the 4-month-old OE-NPY$^{D\beta H}$ mice in study III had not yet developed a complete metabolic phenotype with significantly impaired glucose tolerance. Furthermore, the increase in body weight and the gain in fat mass were reduced during the administration period due to the stressful injections.

Subsequently, one could speculate that the pre-existence of a clear metabolic phenotype or longer drug administration periods could have resulted in larger significant differences between the genotypes. On the other hand, in order to affect the mechanisms regulating the metabolic phenotype in OE-NPY$^{D\beta H}$ mice, it was essential to examine the younger animals with their still developing metabolic phenotype. One approach to reduce the decline in body weight gain caused by daily injections could have been automated administration systems such as implanted osmotic minipumps. However, implantation would have needed anesthesia and recovery from the implantation surgery, which again would have evoked different kinds of stress responses in OE-NPY$^{D\beta H}$ and WT mice.

Another aspect to consider in the intervention studies is the pharmacokinetics and dose of the drugs. In study III, the dose of CB1R inverse agonist AM251 was selected based on a previous study showing a moderate but significant effect on food intake and fat mass (Hildebrandt et al. 2003). AM251 is able to cross the blood-brain barrier (Li et al. 2009) and in the current study, it reduced the food intake of WT mice, which supports the assumption that the selected dose was efficient. In comparison with AM251, the Y1R-antagonist BIBO3304 and the Y2R-antagonist BIIE0246 have been less extensively utilized in the energy homeostasis studies. Both antagonists, however, are highly selective for their receptors (Doods et al. 1999, Wieland et al. 1998) and unable to cross the blood-brain-barrier (Brothers et al. 2010, Dozio et al. 2007), which was important since it was desired to investigate the role of specific peripheral receptors. However, hypothalamus as one of the rare brain areas lacking an effective blood-brain-barrier (Rodriguez et al. 2010) and expressing both Y1Rs and Y2Rs may have centrally mediated the effects of YR-antagonists. The dose for BIBO3304 was selected based on a previous publication, which showed a clear effect in neural tube development after chronic administration (Yuzuriha et al. 2007). However, there were no reference studies for chronic Y2R-antagonist treatment at the time when the experiment was planned, and thus the dose for BIIE0246 was calculated based on the amount of substance of the BIBO3304. In a more recent study, acute administration of BIIE0246 at a slightly higher dose caused a clear physiological effect (Forbes et al. 2012), which thus supports the dose selected in study IV. Additionally, one can speculate if the dosing frequency or timing was optimal. The half-life of BIIE0246 in mouse is less than 3 hours and thus not very suitable for chronic drug administration (Brothers et al. 2010). However, the drug's half-life is markedly longer than the other Y2R-antagonists, thus making it the best of the available Y2R-antagonists for use in these kinds of experiments. The half-life of BIBO3304 is not known but the previous chronic studies with peripheral dosing pattern of once or twice per day hint that it has a relatively long half-life (Sousa et al. 2012, Yuzuriha et al. 2007). One possibility is that due to the relatively short half-life of the drugs and administration in the morning, the drugs are no longer effective during the dark when mice are active. After all, there is no reason to assume that the drugs in the intervention studies were ineffective since statistically significant differences were detected between the vehicle and drug-treated animals.

5.8 Comparison with other noradrenergic neuron NPY mouse models

With the OE-NPY$^{D\beta H}$ mouse model, one cannot conclude whether NPY overexpression in noradrenergic neurons is causing the metabolic alterations, or whether NPY overexpression modifies the level of endogenous NPY. However, Zhang et al. (2014) have bred OE-NPY$^{D\beta H}$ mice with NPY$^{-/-}$ mice and created a mouse model expressing NPY only in the noradrenergic neurons without endogenous NPY expression. These catNPY mice show increased body weight and adiposity in comparison with WT and NPY$^{-/-}$ mice only in an obesogenic environment i.e. during HFD or chronic stress. The lack of difference in the chow-fed mice may result from the lower levels of NPY in comparison with the OE-NPY$^{D\beta H}$ mice. When consuming a HFD, the body weight gain in catNPY mice was mediated by reduced diet-induced thermogenesis. In homozygous OE-NPY$^{D\beta H}$ mice, BAT function is reduced due to a lower sympathetic activity, which was not studied in catNPY mice. Furthermore, stress-induced obesity in catNPY mice is known to be dependent on the increased corticosterone levels and decreased locomotor activity. CatNPY mice also show increased RER, which is suggested to result from increased lipogenic activity. Since chronic stress increases NPY secretion (Kuo et al. 2007), the stressed catNPY mice mimic the NPY overexpression of OE-NPY$^{D\beta H}$ mice rather well. OE-NPY$^{D\beta H}$ mice have increased lipogenic activity although blood corticosterone levels are not altered since OE-NPY$^{D\beta H}$ mice are not stressed. However, it has to be noted that diet exerts dramatic effects on the phenotype of OE-NPY$^{D\beta H}$ mice, and thus the results from HFD-fed catNPY mice and chow-fed OE-NPY$^{D\beta H}$ mice cannot be directly compared. Zhang et al. (2014) postulated also that glucose metabolism is mediated by non-catecholaminergic NPY, which supports the proposal here that impaired glucose metabolism in OE-NPY$^{D\beta H}$ mice results from fat accumulation and activation of the endocannabinoid system.

Our research group has previously studied heterozygous OE-NPY$^{D\beta H}$ mice (Ruohonen et al. 2008). Despite the same origin, the homozygous OE-NPY$^{D\beta H}$ mice are not simply a more intensive version of heterozygous mice; there are some mechanistical differences evident in the phenotypes of the heterozygous and homozygous OE-NPY$^{D\beta H}$ mice. Homozygous OE-NPY$^{D\beta H}$ mice have a more pronounced increase in WAT mass in comparison with heterozygous mice (50-84% vs. 20-25% increase in comparison with WT mice, respectively), which contributes to the development of increased body weight and earlier disturbances in glucose metabolism. However, in contrast to homozygous OE-NPY$^{D\beta H}$ mice, their heterozygous counterparts show increased circulating adrenaline levels and a similar trend in noradrenaline levels (Ruohonen et al. 2009b). Furthermore, BAT is more thermogenically active as evidenced with increased BAT mitochondrial GDP binding (Ruohonen et al. 2009b). In addition, plasma ghrelin levels are increased (Ruohonen et al. 2008). These features of heterozygous OE-NPY$^{D\beta H}$ mice can be explained by increased sympathetic tone (Mundinger et al. 2006). It has been shown *in vitro* that NPY stimulates catecholamine release in a dose-dependent manner (Renshaw et al. 2000), and thus the contrasting findings in the catecholaminergic activity

of heterozygous and homozygous mice cannot be explained by the copy number. It does seem that *in vivo* there is a more complicated interplay between NPY and catecholamines. Due to the differences in the fundamental mechanisms regulating energy metabolism, such as catecholamines and thermogenesis, these two mice strains, i.e. heterozygous and homozygous OE-NPY$^{D\beta H}$ mice, should be considered as separate mouse models.

5.9 Comparability with human p.L7P

The original inspiration for creating a mouse model overexpressing NPY in the noradrenergic neurons of the brain and in the sympathetic nervous system came from the L7P polymorphism in humans. The phenotype of the OE-NPY$^{D\beta H}$ mice and its use in mechanistic studies may help to understand the obesity occurring in carriers of the polymorphism. There are several common features evident in homozygous OE-NPY$^{D\beta H}$ mice and human carriers of the p.P7 variant. Epidemiological follow-up studies have shown that the p.L7P moderately increases weight gain but does not affect dietary intake (Ding et al. 2005, Yeung et al. 2011), which fits with the current mouse data. It has been speculated that instead of simply increasing the total food intake, exaggerated episodic binge eating would be more likely the case in the carries of p.P7 (Yeung et al. 2011). In OE-NPY$^{D\beta H}$ mice, hyperphagia was seen in stressful situations such as re-feeding after fasting and during drug intervention studies. Moreover, the healthy carriers of p.P7 variant show an antilipolytic effect when NPY release is stimulated by exercise (Kallio et al. 2001). Similarly, OE-NPY$^{D\beta H}$ mice have reduced blood lipid levels as a marker for reduced lipolysis. Furthermore, human studies have reported lower plasma noradrenaline levels in the carriers of the p.P7 mutation (Kallio et al. 2003) suggesting a downregulated sympathetic tone, which is evident in homozygous OE-NPY$^{D\beta H}$ mice as well.

Consequently, the metabolic phenotype of OE-NPY$^{D\beta H}$ mice correlates rather well with the clinical findings in carriers of the p.P7 variant. Thus, OE-NPY$^{D\beta H}$ mice may not serve just as a model for the human metabolic syndrome in general but also for human L7P polymorphism. The mechanistical findings about noradrenergic neuron NPY in the development of metabolic phenotype in OE-NPY$^{D\beta H}$ mice should be examined in the carriers of the p.P7 variant. For example, the role of endocannabinoid system in the p.L7P should be investigated. Furthermore, blockade of peripheral Y2-receptors could represent an effective form of treatment for obesity in the carriers of L7P polymorphism. The OE-NPY$^{D\beta H}$ mouse model could also be utilized in the development of other drug treatments for metabolic disturbances associated with L7P polymorphism.

6. SUMMARY AND CONCLUSIONS

These studies with homozygous OE-NPY$^{D\beta H}$ mice have shown that increased noradrenergic neuron NPY in the brainstem and peripheral sympathetic nerves significantly affect energy balance. The following conclusions can be drawn from the studies included in this thesis based on the aims set:

1) The metabolic phenotype of heterozygous OE-NPY$^{D\beta H}$ mice was successfully intensified by increasing the transgene copy number.

   a) Consumption of a high-calorie western diet intensified the metabolic phenotype of heterozygous OE-NPY$^{D\beta H}$ females, i.e. increased body weight gain, adiposity and impaired glucose tolerance and insulin sensitivity. However, in heterozygous OE-NPY$^{D\beta H}$ males, susceptibility to diet-induced obesity overrode the effect of excess NPY and no difference was detected in body composition or glucose tolerance between the genotypes.

   b) A homozygous OE-NPY$^{D\beta H}$ mouse line was created by quantifying the transgene copy number with real-time qPCR. Homozygous overexpression of NPY in noradrenergic neurons increased body weight and adiposity, which led to impaired glucose tolerance and insulin resistance. Increased lipogenesis, reduced lipolysis and BAT thermogenic activity were the mechanisms detected in adipose tissue responsible for the obesogenic effects of NPY. Neither energy intake nor expenditure was altered in the homozygous OE-NPY$^{D\beta H}$ mouse in comparison with WT mice.

2) Noradrenergic neuron NPY inhibited catecholaminergic tone, i.e. homozygous OE-NPY$^{D\beta H}$ mice had a decreased urine adrenaline concentration and the expression levels of catecholamine synthetizing enzymes were reduced in the brainstem. The reduced sympathetic tone together with increased NPY inhibited BAT thermogenesis and WAT lipolytic activity.

3) Noradrenergic neuron NPY overexpression affected endocannabinoid levels of several tissues and these alterations preceded the metabolic disturbances in the homozygous OE-NPY$^{D\beta H}$ mice. Chronic CB1R-blockade normalized the metabolic phenotype of OE-NPY$^{D\beta H}$ mice to the level of vehicle-treated WT mice.

4) Peripheral Y1Rs play no major role in the metabolic phenotype of OE-NPY$^{D\beta H}$ mice. Treatment with an Y2R-antagonist increased body weight gain of chow-fed OE-NPY$^{D\beta H}$ mice, but tended to reduce fat mass gain during western diet. Thus peripheral Y2Rs mediate the obesogenic effects of NPY seen with consumption of an energy-rich diet, but this effect is not evident with a healthy diet.

In conclusion, these findings highlight the important role of extra-hypothalamic NPY in the pathogenesis of obesity and metabolic disorders. The feeding-independent mechanisms of noradrenergic neuron NPY inducing weight gain may be exploited when developing treatment for obesity associated with chronic stress or L7P polymorphism in the *Npy* gene.

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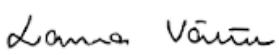

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