UNIVERSITY OF TURKU

THE ROLE OF NEUROPEPTIDE Y IN THE PATHOGENESIS OF THE METABOLIC SYNDROME

A study of liver metabolism in transgenic mice overexpressing neuropeptide Y in noradrenergic neurons

Liisa Ailanen

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To my family

ABSTRACT

Liisa Ailanen

The role of neuropeptide Y in the pathogenesis of the metabolic syndrome: A study of liver metabolism in transgenic mice overexpressing neuropeptide Y in noradrenergic neurons

University of Turku, Faculty of Medicine, Institute of Biomedicine, Pharmacology, Drug Development and Therapeutics, Drug Research Doctoral Programme, Turku, Finland

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The metabolic syndrome (MetS) is a set of symptoms related to obesity, predisposing patients to cardiovascular diseases, and recently hepatosteatosis has been shown to play a major role in its pathogenesis. Neuropeptide Y (NPY) is a neurotransmitter found abundantly in the brain, promoting food intake and energy storage, but also the increased NPY levels in the periphery cause fat accumulation and are associated with impaired glucose tolerance. The main aim of this study was to evaluate the influence of excess NPY on the hepatic metabolism of fatty acids, glucose and cholesterol as a cause of the MetS.

The study was performed with genetically obese mice overexpressing NPY in central noradrenergic neurons and the peripheral sympathetic nervous system (OE-NPY$^{D\beta H}$), which display obesity, impaired glucose tolerance and insulin resistance as they age. This study revealed that obese OE-NPY$^{D\beta H}$ mice exhibited also hepatic accumulation of triglycerides and glycogen, as well as hypercholesterolemia preceded by decreased hepatic fatty acid oxidation and increased synthesis of glycogen and cholesterol. Due to this phenotype, these mice are more prone to type 2 diabetes. Furthermore, hepatic glycogen metabolism could be inhibited by the anti-hyperglycemic agent, metformin, which suggests that changes in glycogen metabolism may associate with the prediabetes encountered in obese OE-NPY$^{D\beta H}$ mice.

The mechanism of action of NPY inducing the hepatic pathologies seems to involve decreased sympathetic activity in the liver of OE-NPY$^{D\beta H}$ mice, similar to that previously detected in adipose tissue. However, there seems to be less involvement of NPY directly on the liver via peripheral Y-receptors. Instead, Y_2-receptors appear to mediate the obesogenic effects of NPY, and they could be a potential drug target for obesity induced by consuming an unhealthy diet combined with excess NPY.

Keywords: neuropeptide Y, the metabolic syndrome, type 2 diabetes, hepatic fatty acid metabolism, glucose metabolism, cholesterol metabolism, Y-receptors, sympathetic nervous system

TIIVISTELMÄ

Liisa Ailanen

Neuropeptidi Y:n merkitys metabolisen oireyhtymän kehittymisessä: Tutkimus maksan aineenvaihdunnasta siirtogeenisellä NPY:tä yli-ilmentävällä hiirimallilla

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Farmakologia, lääkekehitys ja lääkehoito, Lääketutkimuksen tohtoriohjelma, Turku

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Metabolinen oireyhtymä (MBO) on elimistön aineenvaihdunnan häiriötila, joka altistaa sydän- ja vesisuonisairauksille. Sen tekijöitä ovat keskivartalolihavuus, korkea verenpaine sekä glukoosi- ja rasva-aineenvaihdunnan häiriöt. Viime aikoina MBO:ään liittyvä rasvamaksa on kuitenkin nostettu tärkeimmäksi sydäntapahtumien riskille. Neuropeptidi Y (NPY) on yleinen keskushermoston välittäjäaine, joka lisää ruokahalua ja energian varastointia, mutta myös perifeerisen NPY:n lisääntyminen on liitetty elimistön rasvan kerääntymiseen ja glukoosiaineenvaihdunnan heikentymiseen. Tämän tutkimuksen päätarkoitus oli selvittää lisääntyneen NPY:n merkitystä maksan rasvahappo-, glukoosi- ja kolesteroliaineenvaihduntaan sekä MBO:n syntyyn.

Työ toteutettiin tutkimalla NPY:tä noradrenergisessä ja sympaattisessa hermostossa yli-ilmentävää, siirtogeenistä hiirimallia (OE-NPY$^{D\beta H}$). Nämä hiiret lihovat ikääntyessään ja niiden glukoosiaineenvaihdunta heikkenee. Tässä tutkimuksessa todettiin, että OE-NPY$^{D\beta H}$-hiirien maksaan myös kerääntyy rasvaa ja glykogeenia, ja veren kolesterolitasot kohoavat. Nämä johtuvat maksan vähentyneestä rasvahappojen hapettumisesta, sekä lisääntyneestä glykogeeniaineenvaihdunnasta ja kolesterolin synteesistä. Muutokset lisäävät riskiä sairastua tyypin 2 diabetekseen. Diabeteslääke metformiini vähensi maksan glykogeeniaineenvaihduntaa, mistä pääteltiin, että kiihtynyt glykogeeniaineenvaihdunta näyttäisi liittyvän OE-NPY$^{D\beta H}$-hiirien prediabeteksen kehittymiseen.

OE-NPY$^{D\beta H}$-hiirien maksan aineenvaihdunnan häiriöt näyttäisivät johtuvan ylimääräisen NPY:n aiheuttamasta maksan sympaattisen aktiivisuuden vähenemisestä, samoin kuin on aiemmin todettu tapahtuvan rasvakudoksessa. Sen sijaan suorat NPY:n Y-reseptorivälitteiset vaikutukset maksaan eivät ole todennäköisiä. Perifeeriset Y_2-reseptorit välittävät kuitenkin NPY:n lihavuutta aiheuttavia vaikutuksia, ja ne ovat mahdollisia lääkekehityskohteita erityisesti epäterveellisen ruokavalion ja stressistä tai perimästä johtuvan NPY:n ylimäärän aiheuttamassa lihavuudessa.

Avainsanat: neuropeptidi Y, metabolinen oireyhtymä, tyypin 2 diabetes, maksan rasvahappoaineenvaihdunta, glukoosiaineenvaihdunta, kolesteroliaineenvaihdunta, Y-reseptorit, sympaattinen hermosto

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ABBREVIATIONS

ACC	Acetyl-coenzyme A carboxylase
Acox1	Acyl-coenzyme A oxidase 1
Acsl4	Acyl-coenzyme A synthetase long-chain family member 4
Adrb1	Adrenoceptor beta 1
Adrb2	Adrenoceptor beta 2
AgRP	Agouti-related peptide
AMPK	AMP activated protein kinase
α-MSH	α-melanocyte stimulating hormone
Arc	Arcuatus
AUC	Area under curve
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
Dhcr7	7-dehydrocholesterol reductase
CNS	Central nervous system
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase1
Crat	Carnitine acetyltransferase
Cyp7a1	Cytochrome P450 7a1
DβH	Dopamine-beta-hydroxylase
DPP-4	Dipeptidyl peptidase 4
DIO	Diet-induced obesity
DMH	Dorsomedial hypothalamic nucleus
EE	Energy expenditure
Fas	Fatty acid synthase
Fdft1	Farnesyl diphosphate farnesyl transferase1
FDR	False discovery rate
FFA	Free fatty acids
GABA	γ-Aminobutyric acid

GLP-1	Glucagon-like-peptide 1
GLUT	Glucose transporter
GSH	Glutathione
GSSG	Glutathione disulfide
Gys2	Glycogen synthase 2
G6P	Glucose-6-phosphate
G6pc	Glucose-6-phosphatase
GTT	Glucose tolerance test
HDL	High-density lipoprotein
Hmgcr	3-Hydroxy-3-methylglutaryl-CoA reductase
H&E	Hematoxylin and eosin staining
i.c.v.	Intracerebroventricular
IGT	Impaired glucose tolerance
IL1β	Interleukin 1 beta
IL6	Interleukin 6
IL10	Interleukin 10
InsR	Insulin receptor
i.p.	Intraperitoneal
IR	Insulin resistance
ITT	Insulin tolerance test
KD	Knock-down
KO	Knock-out
LF	Labile structure of glycogen
LCFA	Long-chain fatty acid
LDL	Low-density-lipoprotein
Ldlr	LDL receptor
LHA	Lateral hypothalamic area
LC	Locus coeruleus
MC4	Melanocortin 4 receptor
MCH	Melanin concentrating hormone
MetS	The metabolic syndrome
MPO	Medial optic area
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y

NTS	Nucleus of the solitary tract
OE-NPY$^{D\beta H}$	Mouse model overexpressing NPY under DβH promoter
ORO	Oil red O staining
PAS	Periodic acid-Schiff staining
Pgc1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PCK1	Phosphoenolpyruvate carboxykinase 1
p.o.	Per os
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PTT	Pyruvate tolerance test
Pygl	Liver glycogen phosphorylase
PYY	Peptide YY
PVN	Paraventricular nucleus
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
s.c.	Subcutaneus
SCN	Suprachiasmatic nucleus
SEM	Standard error of mean
SF	Stable structure of glycogen
SGLT2	Sodium-glucose co-transporter 2
SNS	Sympathetic nervous system
Srb1	Scavenger receptor, class B, type 1
Srebp1c	Sterol regulatory element binding transcription factor 1
STZ	Streptozotocin
TG	Triglyceride
TGC	Total glycogen content
Tgfb1	Transforming growth factor beta 1
Th	Tyrosine hydroxylase
TNFα	Tumor necrosis factor alpha
TRAP	Total peroxyl radical trapping antioxidant potential
T2D	Type 2 diabetes
VLDL	Very-low-density lipoprotein
VMH	Ventromedial nucleus
WAT	White adipose tissue

WD	Western type diet
WT	Wildtype
Y1R	Neuropeptide Y Y_1-receptor
Y2R	Neuropeptide Y Y_2-receptor
Y5R	Neuropeptide Y Y_5-receptor

LIST OF ORIGINAL PUBLICATIONS

I Ailanen L, Ruohonen ST, Vähätalo LH, Tuomainen K, Eerola K, Salomäki-Myftari H, Röyttä M, Laiho A, Ahotupa M, Gylling H & Savontaus E. The metabolic syndrome in mice overexpressing neuropeptide Y in noradrenergic neurons. Journal of Endocrinology 2017; 234(1): 57-72; doi: 10.1530/JOE-16-0223.

II Ailanen L, Bezborodkina NN, Virtanen L, Ruohonen ST, Malova AV, Okovityi SV, Chistyakova EY & Savontaus E. Metformin normalizes the structural changes in glycogen preceding prediabetes in mice overexpressing neuropeptide Y in noradrenergic neurons. Pharmacology Research and Perspectives 2018; 6(2):e00389; doi: 10.1002/prp2.389.

III Ailanen L*, Vähätalo LH*, Salomäki-Myftari H, Mäkelä S, Orpana W, Ruohonen ST & Savontaus E. Peripherally administered Y_2-receptor antagonist BIIE0246 prevents diet-induced obesity in mice with excess neuropeptide Y, but enhances obesity in control mice. Frontiers in Pharmacology 2018; doi: 10.3389/fphar. 2018.00319

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1 INTRODUCTION

The prevalence of obesity and related diseases has increased enormously over the past few decades. Today, about every other Finnish adult is overweight (body mass index (BMI) > 25 g/m^2) and one in five is obese (BMI > 30 g/m^2). Overweight and obesity are more common in men (66%) than in women (44%) (National Institute of Health and Welfare 2017). Similar statistics of obesity are present in other Western countries, but also developing countries suffer from the same phenomenon (World Health Organization (WHO) 2018). Obesity predisposes to a wide range of diseases. It increases the risk for metabolic disorders (i.e. the Metabolic Syndrome (MetS) and type 2 diabetes (T2D)), and obese middle aged people have been shown to have as much as a ten times higher risk to develop T2D as compared to healthy ones (Mustajoki 2017). In addition, cardiovascular diseases, many cancers, osteoarthritis, asthma, depression and dementia are more common among obese patients.

The MetS is a cluster of metabolic disorders combined with visceral obesity and disruptions in lipid and glucose metabolism, which predispose patients to cardiovascular diseases. Accumulation of lipids into the internal organs, e.g. into the liver, induce non-alcoholic fatty liver disease (NAFLD) and insulin resistance (IR). NAFLD is the most prevalent liver disease in the world today, concerning 25% of the adult population worldwide. In addition to obesity, NAFLD is associated with other metabolic comorbidities for example, almost 25% of subjects with NAFLD suffer from T2D and over 40% are diagnosed with the MetS, which when present together, further increase the risk for liver fibrosis and cardiovascular diseases (Younossi*, et al.* 2016). IR induces hyperglycemia and hyperinsulinemia, which further enhance lipid accumulation into the tissues, and leads to a vicious cycle of imbalanced energy metabolism. Today, there are effective drugs to treat different symptoms of the MetS, but no drugs are available to treat NAFLD. Therefore, adopting a healthier lifestyle and weight loss are the only effective way to treat NAFLD.

Obesity and its related metabolic disorders are mostly caused by consumption of an energy-rich diet and a sedentary lifestyle, but also the genetic background and stress may impair energy homeostasis. Neuropeptide Y (NPY) is a powerful stimulant of feeding and the storage of energy (Levine & Morley. 1984, Zarjevski, *et al.* 1993), but it is also involved in stress-induced obesity (Kuo, *et al.* 2007, Zhang, *et al.* 2014). Human gain-of-

function polymorphism in *NPY* gene, rs16139, increases NPY levels during sympathetic activity (Kallio, *et al.* 2001), and predisposes to weight gain and metabolic disorders (Jaakkola, *et al.* 2006, Karvonen, *et al.* 1998, Nordman, *et al.* 2005, Ukkola & Kesaniemi. 2007). In order to clarify the role of NPY in the catecholaminergic system, Ruohonen and colleagues (Ruohonen, *et al.* 2008) created a mouse model overexpressing NPY in noradrenergic neurons and peripheral sympathetic nervous system (SNS) (OE-NPY$^{D\beta H}$). Heterozygous OE-NPY$^{D\beta H}$ mice display obesity, and late-onset impairment of glucose tolerance (IGT) and hepatic triglyceride (TG) accumulation (Ruohonen, *et al.* 2008), and the body adiposity and IGT are even more obvious with an earlier onset in a homozygous OE-NPY$^{D\beta H}$ model (Vähätalo, *et al.* 2015). Paradoxically, sympathetic activity is increased in heterozygous OE-NPY$^{D\beta H}$ mice, but decreased in homozygous OE-NPY$^{D\beta H}$ mice, which illustrates the dual role of NPY on the catecholaminergic system depending on the level of NPY (Ruohonen, *et al.* 2009, Vähätalo, *et al.* 2015).

This study focuses on characterizing the hepatic phenotype of OE-NPY$^{D\beta H}$ mice, and identifying the mechanisms by which NPY influences the metabolism of hepatic fatty acids, cholesterol and glucose. The aim is to find novel drug targets to combat NAFLD-induced metabolic disturbances.

2 REVIEW OF THE LITERATURE

2.1 Physiological control of energy homeostasis

The body's energy balance consists of food intake, basal energy expenditure (EE), physical activity and thermogenesis. Food intake is responsible for energy intake, whereas total EE consists of the other three components. In order to maintain a constant body weight and adiposity, energy intake should be in balance with the expenditure.

The control of food intake and EE involves a complex interaction between central and peripheral hormonal and neural signaling, influenced by external or internal stimuli, such as fasting or food intake, physical activity, cold, stress, or body adiposity. Peripheral hormones, which originate from the gut, pancreas or adipose tissue, regulate appetite by signaling about hunger or satiety, or providing information about the long-term energy storage in the body. They activate the central areas of energy balance in the hypothalamus and the brainstem via vagal signals or directly via circulation. The hypothalamic arcuate nucleus (Arc) is "the metabolic center", which contains both anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) expressing neurons (POMC/CART), and orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) expressing neurons (NPY/AgRP) or only NPY expressing neurons. NPY/AgRP neurons release also γ-aminobutyric acid (GABA). Some of the peripheral signals activate Arc directly, whereas other signals are mediated via the nucleus of the solitary tract (NTS) in the brainstem (Morton, *et al*. 2014, Valassi*, et al*. 2008). An overview of hormonal regulation of the energy intake and expenditure is presented in Fig. 1.

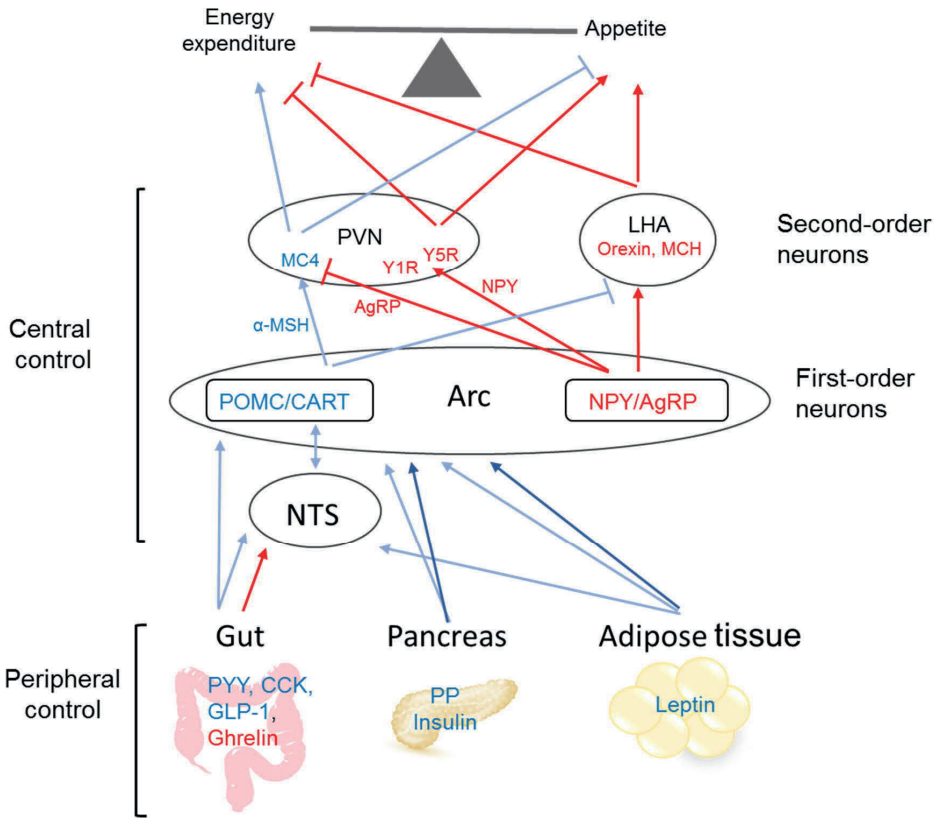

Figure 1 The control of energy intake and expenditure by interaction of peripheral and central signaling

Peripheral short-term signals of satiety (light blue) and hunger (red) mostly originate from the gut, but also from pancreas (pancreatic peptide, PP). In addition, long-term signaling related to the mass of adipose tissue and energy storage are mediated by leptin and insulin (dark blue). Vagal nerve mediates the stimulus of gut hormones and PP to the nucleus of the solitary tract (NTS) in the brainstem, from where the afferent fibres project to the arcuatus nucleus (Arc), to the so-called first-order neurons. Arc contains both anorexigenic POMC/CART neurons, orexigenic NPY/AgRP neurons, which also contain GABA, and NPY neurons. NPY/AgRP and NPY neurons inhibit POMC/CART neurons directly by releasing GABA and NPY, respectively. Activation of the POMC/CART neurons stimulate the expression of α-melanocyte stimulating hormone (α-MSH), its binding to melanocortin 4 receptor (MC4) in paraventricular nucleus (PVN) mediates the appetite suppressing and energy expenditure stimulating action. In lateral hypothalamic area (LHA), POMC/CART neurons suppress the secretion of orexigenic orexin and melanin concentrating hormone (MCH) with the same outcome for the energy balance. Activation of NPY/AgRP neurons stimulate the expression of neuropeptide Y (NPY). The orexigenic action of NPY is mediated via NPY Y_1- and Y_5-receptors (Y1R and Y5R, respectively) in PVN, and it stimulates the secretion of orexin and MCH in LHA. Agouti-related peptide (AgRP) inhibits the anorexigenic stimulus of α-MSH by antagonizing MC4-receptors in PVN. In addition, the release of oxytocin in PVN, which is projected to NTS, increases satiety (not shown). The balance of anorexigenic and orexigenic signals in the body is prerequisite to constant energy homeostasis. CART = cocaine- and amphetamine-regulated transcript, CCK = cholecystokinin, GABA = γ-aminobutyric acid, GLP-1 = glucagon-like-peptide 1, POMC = pro-opiomelanocortin, PYY = peptide YY, blue arrows and text = anorexigenic signals, red arrows and text = orexigenic signals. (Modified from Morton, *et al.* 2014, Valassi, *et al.* 2008 and Woods. 2004)

There are many important endogenous, peripheral satiety signals e.g. gut derived peptide YY (PYY), cholecystokinin (CCK) and glucagon-like-peptides 1 and 2 (GLP-1 and GLP-2, respectively), and pancreatic polypeptide (PP), originating from pancreas and the gut. They are all secreted in postprandial state, and they activate the sympathetic and vagal pathways to stimulate NTS in the brainstem (Fig. 1) (Morton, *et al.* 2014, Valassi*, et al.* 2008, Woods. 2004).

Ghrelin is the only known circulating hunger hormone, which is secreted by the stomach (Morton, *et al.* 2014). It stimulates its receptors in both the brainstem and the hypothalamic NPY/AgRP neurons, which increases the secretion of NPY and AgRP, leading to increased appetite and storing of fat (Fig. 1) (Tschop*, et al.* 2000). Ghrelin also stimulates the secretion of growth hormone in the pituitary gland (Kojima*, et al.* 1999, Nakazato*, et al.* 2001).

Circulating leptin and insulin emit signals to the hypothalamus about the adiposity and the energy stored in the body, and thus they mostly function as long-term signals of stored energy in the body (Fig. 1). Both of these hormones decrease NPY secretion, which suppresses food intake and increases EE (Schwartz*, et al.* 1991). In addition, they decrease blood glucose (Schwartz*, et al.* 1996).

Leptin is the most critical peripheral hormone involved in energy homeostasis, as its deficiency leads to morbid obesity (Münzberg & Morrison. 2015). Leptin is released by white adipose tissue (WAT) during feeding or activation of the SNS, and its circulating levels are highly dependent on the adipose mass in the body (Münzberg & Morrison. 2015, Caron, *et al.* 2018). Its influence is mediated by leptin receptors in the hypothalamus and in NTS (Münzberg & Morrison. 2015). Paradoxically, high leptin levels are also detected in obese subjects due to the expansion of adipose tissue, leading to central leptin resistance (Münzberg & Morrison. 2015, Erickson*, et al.* 1996). Leptin resistance has been suggested to result from the attenuated transport of leptin crossing the blood brain barrier (BBB), and inhibition of leptin activated cell signaling. Specifically, leptin receptors in the Arc, which detect circulating leptin levels, are particularly sensitive to leptin resistance (Barateiro, *et al.* 2017, Münzberg & Morrison, 2015). Leptin resistance-induced hyperleptinemia leads to overactivation of SNS, which causes disturbances in energy metabolism (Guarino, *et al.* 2017, Kalil & Haynes. 2012, Schlaich, *et al.* 2015). For example, activation of SNS stimulates gluconeogenesis (i.e. glucose

production) in the liver and lipolysis (i.e. degradation of serum TGs into free fatty acids (FFA)) in adipose tissue, inhibits insulin production in pancreas, and attenuates glucose uptake in muscle tissue by constricting the arterioles (Schlaich, *et al.* 2015). Due to the fundamental role of leptin in energy homeostasis, leptin deficient *ob/ob* mice and leptin receptor deficient *db/db* mice have become commonly used models in research into obesity and T2D (Schwartz*, et al.* 1996).

Insulin is secreted by the pancreatic beta cells in the Islets of Langerhans in postprandial state. Similarly to leptin, it suppresses the appetite by decreasing NPY secretion from the Arc (Fig. 1), but its effect on inhibiting food intake is not comparable to leptin (Morton, *et al.* 2014, Schwartz*, et al.* 1991). Insulin deficiency in various diabetes models has been shown to increase NPY levels in the hypothalamus, at least in Arc and paraventricular nucleus (PVN) (Abe*, et al.* 1991). Leptin and insulin are interconnected, as insulin decreases leptin secretion, whereas leptin decreases insulin secretion (MacDougald*, et al.* 1995, Schwartz*, et al.* 1996).

In the brain, the hypothalamic neurons controlling energy balance can be divided into the first and second-order neurons (Fig. 1). The Arc contains the first-order neurons, i.e. the anorexigenic POMC/CART and the orexigenic NPY/AgRP neurons. As discussed above, these neurons can be activated by direct signals either from the periphery or from neuronal signals via NTS. POMC is the full-length gene, from which the anorexigenic melanocortins, e.g. α-melanocyte stimulating hormone (α-MSH), are originated. CART similarly to α-MSH suppresses food intake and increases EE. Instead, NPY/AgRP neurons are activated during food deprivation and decreased fat storage, or independently by the release of ghrelin from the stomach (Morton, *et al.* 2014, Nakazato*, et al.* 2001). Inhibition of POMC/CART neurons in the Arc after the release of GABA from NPY/AgRP neurons and the release of NPY from NPY neurons enhances the hunger response (Loh, *et al.* 2015).

POMC/CART and NPY/AgRP neurons mediate the anorexigenic or orexigenic signals to the second-order hypothalamic neuropeptide neurons which are located mostly in PVN and lateral hypothalamic area (LHA) (Fig. 1). In PVN, α-MSH mediates the anorexigenic signal via the melanocortin 4 receptor (MC4), whereas AgRP and NPY mediate the orexigenic signal by antagonising MC4-receptors and by activating NPY Y_1- and Y_5-receptors, respectively. Projections from PVN to NTS also enhance satiety signals. In

LHA, POMC/CART neurons suppress the secretion of orexigenic orexin and melanin concentrating hormone (MCH), which leads to a positive energy balance. In contrast, NPY/AgRP neurons stimulate the release of both orexin and MCH, which triggers increased food intake and decreased EE (Loh, *et al.* 2015, Morton, *et al.* 2014, Valassi, *et al.* 2008). LHA also integrates the reward system, closely related to feeding, with information concerning energy homeostasis (Loh, *et al.* 2015, Morton, *et al.* 2014).

While the endocrine control has the main responsibility for energy homeostasis, the neural component is also important. However, there seem to be some discrepancies in the literature (Guarino, *et al.* 2017, Tentolouris, *et al*. 2006). Many studies support the concept that low sympathetic activity, i.e. low levels of noradrenaline and adrenaline, is a risk for obesity (Tentolouris, *et al*. 2006). Administration of several orexigenic peptides or neurotransmitters (e.g. NPY, β-endorphin and galanin) have been shown to reduce sympathetic activity and to inactivate thermogenesis in brown adipose tissue (BAT) (Bray. 2000, Egawa, *et al.* 1991). Similarly, agonists of β_2 and β_3 adrenoceptors have been shown to reduce food intake (Bray. 2000). In line with this, anorexigenic components (e.g. leptin and CCK), cold and food intake seem to activate the SNS, which in turn stimulates BAT and increases EE (Guarino, *et al.* 2017). Therefore, almost 30 years ago Bray (1991) proposed his famous Mona Lisa hypothesis of obesity, "**M**ost **o**besities k**n**own **a**re **l**ow **i**n **s**ympathetic **a**ctivity". However, as also overactivity of the SNS has been linked to metabolic disturbances, and furthermore both stress-induced weight gain and loss have been recognized, the effect of the SNS on energy metabolism still seems somewhat unclear (Kuo, *et al.* 2007, Michopoulos. 2016, Morton *et al.* 2014). It has been calculated that the contribution of the SNS accounts for only about 5% of the total daily EE (Tentolouris, *et al*. 2006).

There is extensive genetic variation in the components of energy homeostasis, which may cause an imbalance in weight control and predispose subjects to obesity and obesity related disorders. In humans, leptin deficiency is one of the most powerful (but rare) inducers of morbid obesity, which means that it can be treated by administration of leptin (Licinio*, et al.* 2004). However, the increased energy intake in relation to decreased expenditure, i.e. consumption of an unhealthy and energy rich diet combined with a sedentary lifestyle, is the most common reason for weight gain and obesity in the modern world. The prolonged excessive intake of fat and sugars disturbs the sensitive control of energy homeostasis, which originally was developed to protect the body from a shortage

of the nutrients, instead of overfeeding. Once this disturbance occurs, it may be hard to maintain a constant body weight even if energy intake recovers to the normal level.

2.2 Obesity related metabolic disorders

2.2.1 The metabolic syndrome

Obesity predisposes to a wide range of disease, most importantly to metabolic disorders, the MetS and T2D, which increase the risk for cardiovascular events. The MetS is a set of metabolic disorders, characterized by the co-existence of obesity, dyslipidemia, impaired glucose metabolism and hypertension. The Finnish national criteria of the MetS is based on the definition created by harmonizing the definitions of the MetS prepared by different organizations (National Cholesterol Education Panel (NCEP) Adult Treatment Panel III (ATP III), WHO, International Diabetes Federation (IDF), National Heart, Lung and Blood Institute (NHBLI), American Heart Association (AHA), World Heart Federation and World Obesity) in 2009 (Lam & LeRoith. 2015, Mustajoki, 2017). The Harmonization defines that MetS exists, when three out of five of the following symptoms have been diagnosed: central obesity (>90 cm in women and >100 cm in men (in Finland)), dyslipidemia (TG >1.7 mmol/l, and high-density (HDL) cholesterol <1.0 mmol/l in women and <1.3 mmol/l in men), impaired glucose metabolism (fasting blood glucose >5.6 mmol/l) or previously diagnosed T2D, and hypertension (blood pressure >130/85 mm Hg (systolic / diastolic)) (Huang, 2009, Lam & LeRoith. 2015, Mustajoki, 2017). However, this definition includes ethnic-specific waist circumference cut-off values. For example, South Asian populations, which have more visceral adiposity for given waist circumference values and are therefore more prone to hepatosteatosis compared to Europeans and they have lower cut-off values for central obesity (Lam & LeRoith. 2015).

The definition of the MetS has been developed to provide clinicians with a simple tool to identify a subpopulation at a greater risk of developing cardiovascular diseases (Huang 2009). High low-density (LDL) cholesterol and TG levels, and low HDL cholesterol levels increase the risk for atherosclerosis and stroke, hypertension increases the burden on the heart, whereas in addition to these macrovascular complications, hyperglycemia predisposes to microvascular complications, e.g. neuropathy, nephropathy and

retinopathy. Although hepatic steatosis is not officially counted as a trait of the MetS, increasing evidence shows that it is the hepatic manifestation of the MetS and an independent cause for cardiovascular events or liver-related death induced by increased secretion of very-low-density lipoproteins (VLDL), hepatokines and procoagulant factors into the circulation (Adams, *et al.* 2005, Lu, *et al.* 2013, Marchesini, *et al.* 2003, Sunny, *et al.* 2017).

2.2.2 Hepatosteatosis

Abdominal obesity is a sign of accumulation of visceral fat, i.e. fat which surrounds the internal organs and accumulates into the tissues, such as liver (Jeong, *et al.* 2008, Koo, 2013). Obesity is diagnosed as a comorbidity in half of all subjects with NAFLD. NAFLD refers to hepatosteatosis with no association with excessive alcohol consumption (<21 (men) or <14 (women) drinks of alcohol per week) or other diseases causing a secondary accumulation of TGs into the liver (Chalasani, *et al.* 2012). The histological criterion for hepatosteatosis is that lipid droplets are detected in half of the hepatocytes (Kleiner, *et al.* 2005). The clinical criterion for hepatosteatosis is that the content of TGs exceeds 5% of the total weight of the liver (Kleiner, *et al.* 2005). However, accurate clinical diagnosis demands liver biopsy, which is too invasive and an excessively expensive method that it could be used in the basic healthcare. Therefore, in the case of the diagnosis of simple steatosis, liver biopsy is usually replaced by other (non-invasive, but less accurate) techniques (imaging or biochemistry). As NAFLD strongly co-exists with the MetS, abnormal liver biomarkers in patients with the symptoms of the MetS rather well predict the presence of steatosis (Chalasani, *et al.* 2012).

There are several ways in which TGs can accumulate into the liver (Fig. 2). The increased input consists of fatty acid uptake and hepatic *de novo* lipogenesis. Fatty acids, which are transported to the liver, originate from nutrition or WAT. Naturally, fat-rich nutrition increases fatty acid uptake. However, increased fatty acid uptake also takes place, when expanding adipose tissue increases lipolysis in WAT and FFAs are transported into the liver. Hepatic lipogenesis may be stimulated e.g. in the case of accumulation of glycogen storage (5% of liver weight), when the excess glucose taken up by the liver is used for producing TGs (Koo, 2013, Postic & Girard. 2008). In addition, other external stimuli, e.g. centrally administered NPY, may stimulate lipogenesis (Zarjevski, *et al.* 1993).

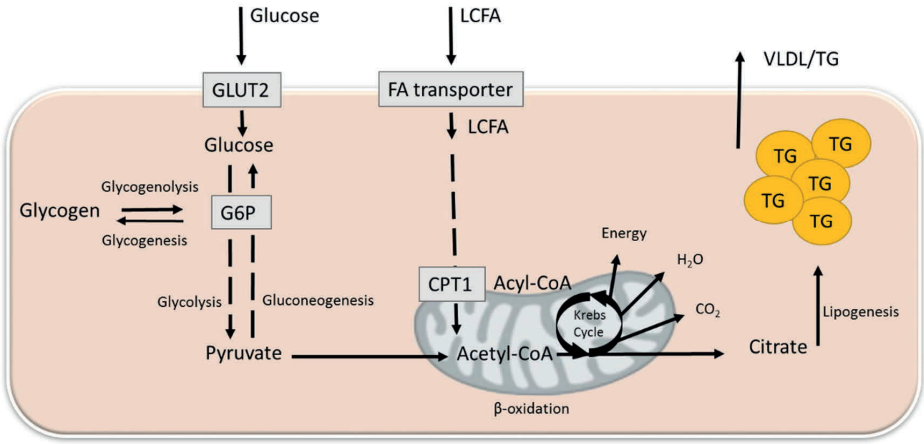

Figure 2 Liver glucose and fatty acid metabolism.

The liver takes up glucose from diet and fatty acids from diet and adipose tissue. Glucose is stored into the liver as glycogen via glycogenesis or used for energy i.e. lipogenesis via glycolysis. Fatty acids are first oxidized to short-chain acyl-coenzyme A (Acyl-CoA) in peroxisomes, and then oxidized to energy in mitochondria. In a pathological condition (e.g. in insulin resistance), these mechanisms are unbalanced, and the liver produces glucose by gluconeogenesis and triglycerides via lipogenesis, but reduces fatty acid oxidation due to a dysfunction of mitochondria. This leads to elevated blood glucose and hepatosteatosis. CPT1=carnitine-acetyl-transferase 1, FA = fatty acid, GLUT2 = Glucose transporter 2, G6P=Glucose-6-phosphate, LCFA = long-chain fatty acid, TG=triglyceride. (Modified from Postic & Girard. 2008).

A decreased fatty acid output is another mechanism by which TGs can accumulate in the liver. It includes decreased fatty acid oxidation and secretion of TGs to other tissues via VLDL (Postic & Girard. 2008). β-oxidation is the most common route by which fatty acids are oxidized. Long-chain, very-long-chain (> C_{20}) and branched-chain fatty acids are first oxidized in peroxisomes to shorter fatty acids and H_2O_2. In mitochondrial β-oxidation, short- and middle-chain (< C_{20}) fatty acids are oxidized to acetyl-coenzyme A (acetyl-CoA) and further in the Krebs cycle to produce energy, H_2O and CO_2. Microsomal ω-oxidation is minor in normal conditions, and increased only in pathological conditions (e.g. in diabetes), or when β-oxidation routes are saturated.

There is a controversy about whether the accumulation of TGs into the liver decreases or increases fatty acid oxidation (Kohjima*, et al.* 2007, Koliaki, *et al.* 2015, Mansouri, *et al.* 2018). This discrepancy has been explained by different states of the disease. In the short-term, the accumulation of TGs compensatorily increases both fatty acid oxidation and VLDL secretion. However as steatosis progresses, the constant overload of FFAs into the mitochondria will lead to their dysfunction and decreased β-oxidation (Koliaki, *et al.* 2015, Mansouri, *et al.* 2018).

Mitochondrial dysfunction together with ω-oxidation produces reactive oxygen species (ROS), which in turn stimulate the production of proinflammatory cytokines, and are involved in inflammation and the progression of NASH (Kohjima, *et al.* 2007, Reddy. 2001) (discussed in more detail in chapter 2.2.3). While simple steatosis is a reversible state and may be cured by adopting a healthier life style, 5-10% of NAFLD cases progress to NASH, which is an irreversible state and may progress further to cirrhosis or hepatocarcinoma. There are several ways to classify the state of steatosis. Classification, where histopathologic subtypes correlate with clinical outcomes is presented in Table 1.

Table 1 Classification of non-alcoholic fatty liver disease

Subtype	*Pathology*	*Clinicopathologic correlation*
Type 1	Simple steatosis alone	No NASH
Type 2	Steatosis + lobular inflammation only	No NASH
Type 3	Steatosis + hepatocellular ballooning	NASH without fibrosis
Type 4	Steatosis, ballooning, Mallory bodies or fibrosis	NASH with fibrosis

NASH = Non-alcoholic steatohepatitis. The table is modified from Cabezas 2012.

2.2.3 Insulin resistance and inflammation

NAFLD is closely related to IR and prediabetes, which may progress to T2D over time. IR develops with obesity, as lipids accumulate in the liver, muscle and adipose tissue. In addition to the insulin independent glucose receptor GLUT2, glucose can enter the target cell via the insulin sensitive glucose transporter GLUT4. In IR, insulin signaling fails to phosphorylate the tyrosine residues on the insulin receptor, and glucose cannot be transported into the cell. In muscles, IR decreases glucose disposal and in adipose tissue, it increases lipolysis, which leads to increased secretion of FFAs to the other tissues. However when one considers whole body glucose metabolism, hepatic IR is suggested to be of paramount importance, as the liver is the only tissue capable of producing glucose via gluconeogenesis. Inadequate insulin signaling leads to activation of gluconeogenic enzymes (e.g. phosphoenolpyruvate carboxykinase 1 (PCK1), glucose-6-phosphatase (G6PC)) and increased hepatic glucose production. The combination of decreased glucose transport into the tissues and increased glucose production lead to hyperglycemia and hyperinsulinemia, a state called prediabetes. Eventually the continuous

hyperinsulinemia leads to a failure of pancreatic beta cells and the onset of T2D (Smith & Kahn. 2016, Postic & Girard. 2008).

Obesity is associated with a low grade inflammation due to pro-inflammatory responses as well as FFA-induced lipotoxicity, which may enhance the development of IR. Furthermore, IR and inflammation are all closely linked with steatosis. Unlike glucose uptake, hepatic lipogenesis responds to circulating insulin in IR, which enhances the accumulation of TGs during hyperinsulinemia (Postic & Girard. 2008). Thus, steatosis, IR and inflammation stimulate each other, but there is no clear understanding about which one is the cause and which is the consequence (Fig. 3). Obesity related inflammation is a complex combination of metabolic disruptions with the production of both pro- and anti-inflammatory mediators e.g. by adipocytes (adipokines) or immune cells (e.g. interleukines), in which an excessive intake of dietary sugars and fat seems to play a significant role. In particular, the accumulation of FFAs instead of TGs is considered to be harmful for liver metabolism because of their reactive nature, but also excess cholesterol has been shown to cause similar signs of lipotoxicity in the liver (Wouters, *et al.* 2008, Yamaguchi, *et al.* 2007).

The expanding adipose tissue in obesity seems to be the most likely source of the activation of pro-inflammatory responses. As discussed above, in addition to nutritional FFAs, accelerated lipolysis in the expanding WAT is a major origin of these signals, i.e. high levels of FFAs cause lipotoxicity (Fig. 3). Furthermore, activation of macrophages in the WAT increases these cells' production of cytokines and adipokines, which may also stimulate the inflammatory responses. Resistin, an inflammatory adipokine formed by the macrophages in WAT, has been shown to associate with obesity, inflammation and T2D (Fu, *et al.* 2006, McTernan, *et al.* 2003, Shetty, *et al.* 2004), and independently to induce IGT and IR (Rajala, *et al.* 2003, Steppan, *et al.* 2001). Instead, the levels of an anti-inflammatory adipokine, adiponectin, are decreased in obesity (Arita, *et al.* 1999, Hu, *et al.* 1996), and low levels of adiponectin may predispose to IR, steatosis and T2D (Hotta, *et al.* 2001, Targher, *et al.* 2004, Yamauchi, *et al.* 2001).

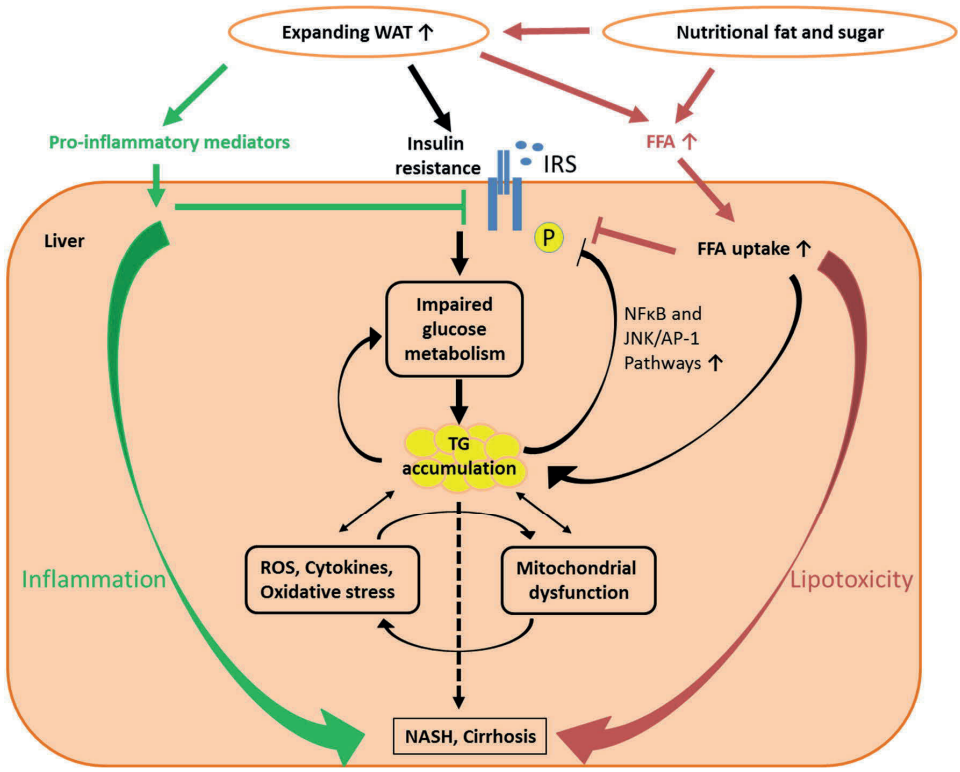

Figure 3 The association of steatosis, insulin resistance and inflammation in the liver.

Insulin resistance (IR) is associated with increased adiposity, inflammation and mitochondrial dysfunction. IR is caused by WAT-derived pro-inflammatory responses (green lines and arrows) and increased free fatty acid (FFA) levels (red lines and arrows), but also hepatic steatosis can activate inflammatory responses (black lines and arrows), which lead to IR. IR, on the other hand, impairs glucose metabolism and enhances steatosis. The same inflammatory responses also trigger tissue damage and the progression of simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. FFA = Free fatty acid, IRS = Insulin receptor substrate, JNK/AP-1 = c-Jun N-terminal kinase and activator protein-1, NF-κB = Nuclear factor κB, ROS = Reactive oxygen species, TG = Triglyceride, WAT = White adipose tissue. (Modified from Rinella 2015)

In addition to the WAT-derived inflammatory responses, increased hepatic FFAs induce the accumulation of TGs, leading to mitochondrial dysfunction and the production of ROS and cytokines (e.g. tumor necrosis factor α (TNFα)), as well as endoplasmic reticulum stress, all of which activate the pro-inflammatory pathways (Fig. 3) (Reddy, 2001, de Luca & Olefsky. 2008, Mansouri, *et al.* 2018). Activation of hepatic inflammatory pathways, (e.g. nuclear factor κB (NF-κB), and c-Jun N-terminal kinase and activator protein-1 (JNK/AP-1) pathways) and their downstream cytokine signaling

(e.g. interleukins 6 (IL-6) and 1β (IL-1β)) may, in turn, enhance the development of IR (Fig. 3) (Cai, *et al.* 2005, Hirosumi, *et al.* 2002). These factors impair insulin signaling by inducing the phosphorylation of serine 307 in the insulin receptor instead of phosphorylating tyrosine residues (Aguirre, *et al.* 2000). Hepatic inflammatory responses are believed to originate from both the hepatocytes and the liver-specific macrophages, Kupffer cells. The NF-κB pathway has been shown to activate the pro-inflammatory actions of Kupffer cells, but not to induce their proliferation (Cai, *et al.* 2005). TNFα is believed to play a major role in IR, as it has been shown to attenuate mitochondrial function in the hepatocytes and to associate with increased FFAs (Uysal, *et al.* 1997). Mice deficient in TNFα and its receptor do not develop IR despite the presence of diet-induced (DIO) or genetic obesity (Uysal, *et al.* 1997). In addition to IR, all these hepatic inflammatory responses may also induce the progression of NASH.

Obesity, hepatosteatosis, IR and inflammation thus represent a complex set of metabolic disturbances in the body, and some individuals seem to be more prone to these disturbances than others. Although many mechanisms of inflammatory responses are known, there is still no clear understanding of the actual cause underpinning the inflammation or the critical point when NAFLD actually progresses to NASH. Furthermore, there is still an evident need to find novel drug targets in this area, especially drugs capable of specifically targetting the disease.

2.3 Neuropeptide Y (NPY)

2.3.1 General

NPY is a 36-amino-acid long neurotransmitter. It belongs to the NPY family together with PYY and PP, which are all processed from 94-95 amino-acid long pro-hormones. NPY was first isolated by Tatemoto and colleagues in the early 1980s from porcine brain (Tatemoto. 1982), and it was named after the tyrosine (Y) residues present at both ends of the amino acid chain. The amino acid sequence of NPY has been highly conserved across species throughout evolution, e.g. there is as much as 92% sequence identity between mammals and cartilaginous fishes (Larhammar, *et al.* 1993).

NPY, PYY and PP share a similar chemical structure, and they are recognized by six NPY Y receptors (Y_1-Y_5 and y_6) with different affinities. All of these receptors also modulate food intake, but in contrast to the orexigenic NPY, PYY and PP are satiety hormones and, thus inhibit food intake. PYY is produced by the gut, where it is released from L cells in the postprandial state. Although full-length PYY binds to all Y-receptors, the most abundant form of PYY in the circulation, $PYY_{3–36}$, binds to the hypothalamic Y_2-autoreceptor, through which it inhibits NPY secretion and decreases food intake (Batterham*, et al.* 2002, Ekblad & Sundler. 2002, Murphy & Bloom. 2006). However, $PYY_{3–36}$ has also been shown, at least partly, to inhibit food intake triggered by vagal stimulation of NTS (Koda*, et al.* 2005). PP is released by the endocrine pancreas and the gut in postprandial state and has the highest binding affinity to Y_4- and Y_5-receptors (Adrian*, et al.* 1976, Batterham*, et al.* 2003). PP is a peripheral hormone, as it is unable to cross the BBB (Sainsbury*, et al.* 2002).

NPY is expressed in the central nervous system (CNS) and in the peripheral SNS. In the brain, NPY is one of the most abundant peptides, and it is widely expressed throughout the brain, with the exception of the cerebellum. The highest NPY immunoreactivity is detected in the cortical, hypothalamic and limbic regions. In the hypothalamus, *NPY* is expressed in the Arc and its projection sites, e.g. the PVN, the dorsomedial hypothalamic nucleus (DMH) and the ventromedial nucleus (VMH), which are the main sites controlling appetite (Allen*, et al.* 1983, Beck*, et al.* 1992, Chronwall*, et al.* 1985). There are other NPY immunoreactive locations in the limbic system e.g. hippocampus, amygdala and dentate gyrus. In the brainstem, *NPY* is expressed in the main noradrenergic sites in the CNS, in locus coeruleus (LC) in pons, and in A1/C1 neurons and NTS in the medulla oblongata, from where the noradrenergic neurons project to the PVN (Allen*, et al.* 1983, Chronwall*, et al.* 1985). Furthermore, NPY is expressed in the dorsal horn of the spinal cord (Chronwall*, et al.* 1985).

In the periphery, the main site of *NPY* expression is the chromaffin cells of adrenal medulla, where it is co-localized and co-released with catecholamines (i.e. adrenaline and noradrenaline) (Varndell*, et al.* 1984, Whim. 2006). In addition, NPY is co-released with noradrenaline in the large dense-core vesicles of the sympathetic nerve endings innervating not only vascular smooth muscle cells but also a wide range of different tissues (gut, pancreas, heart, liver, BAT, WAT, respiratory tract, thyroid gland and eyes) (Cannon*, et al.* 1986, Ekblad*, et al.* 1984, Fried*, et al.* 1985, Grunditz*, et al.* 1984, Gu*, et*

al. 1984, Kuo, *et al.* 2007, Sheppard, *et al.* 1984, Sundler, *et al.* 1983, Taborsky, *et al.* 1994, Terenghi, *et al.* 1983). NPY is released during conditions of intense sympathetic activation, e.g. in stress (Ekblad, *et al.* 1984), and it potentiates and prolongs the vasoconstrictor effect of noradrenaline and adenosine triphosphate (ATP) (Lundberg, *et al.* 1990, Westfall, *et al.* 1995). However, NPY can also act as a vasoconstrictor on its own, and it is able to reverse noradrenaline-induced adrenergic desensitization of nerves (Wahlestedt, *et al.* 1990b).

2.3.2 Y-receptors

Similar to the neurohormone NPY, its receptors are expressed all over the body. Y-receptors are structurally a heterogeneous group of $G_{i/o}$-protein coupled receptors, but they all recognize NPY. Binding of the ligand to the Y-receptor reduces the intracellular production of cyclic adenosine monophosphate (cAMP) from ATP by inhibiting adenylate cyclase, and it reduces Ca^{2+} channel current (Fig. 4) (Gerald, *et al.* 1995, Herzog, *et al.* 1992, Reynolds & Yokota. 1988).

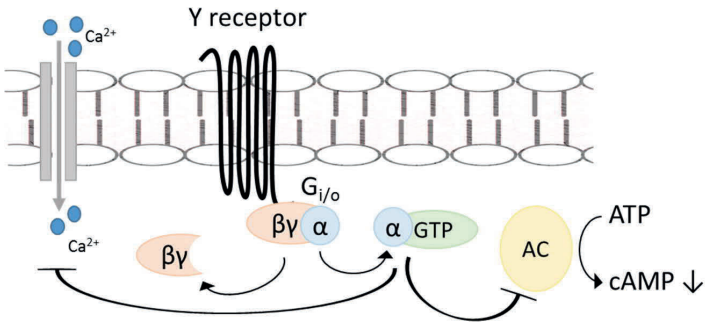

Figure 4 Intracellular signaling of Y-receptors

Y-receptors are $G_{i/o}$-protein coupled receptors. Activation of the Y-receptor inhibits adenylate cyclase (AC), which reduces the production of cyclic adenosine monophosphate (cAMP) from ATP. Activation of the Y-receptor can also induce hyperpolarization through G_i, which reduces the Ca^{2+} channel current. Different Y-receptors regulate different types of Ca^{2+} channels. (Modified from Brumovsky, *et al.* 2007)

Y_1-receptors are stimulated by NPY and PYY (Lundell, *et al.* 1997); they are the most abundant Y-receptors in the brain, and are mostly located post-synaptically. Their distribution in the brain ranges from different nuclei of the hypothalamus and the brainstem to pituitary gland, hippocampus and amygdala (Kishi, *et al.* 2005, Naveilhan,

et al. 1998, Wolak, *et al.* 2003). A widespread distribution is detected also in periphery: in the vascular smooth muscle cells in different organs (kidney, spleen and mesenteric WAT) (Malmström & Lundberg. 1997, Malmström. 2000, Malmström. 2001, Phillips, *et al.* 1998, Reynolds & Yokota. 1988), in the cardiomyocytes (McDermott, *et al.* 1997), in adrenal chromaffin cells (Cavadas, *et al.* 2001), in the liver (Zhang, *et al.* 2010), in the gut (Cox, *et al.* 2001, Goumain, *et al.* 1998) and in the immune system (dendritic cells, natural killer cells, mast cells, T and B cells, and macrophages) (Bedoui, *et al.* 2003, Wheway, *et al.* 2005). In the vasculature, activation of Y_1-receptors causes vasoconstriction (Malmström & Lundberg. 1997, Malmström. 2000, Malmström. 2001, Phillips, *et al.* 1998, Reynolds & Yokota. 1988).

Y_1-receptors play a significant role in the control of energy homeostasis. Their activation in PVN triggers a feeding response and a preference for a high carbohydrate diet (Kalra, *et al.* 1991a, Leibowitz & Alexander. 1991, Lopez Valpuesta, *et al.* 1996). Furthermore, activation of the Y_1-receptor inhibits EE and fat utilization as a source of energy, which independent of food intake, increases weight gain and adiposity (Henry, *et al.* 2005). Peripheral Y_1-receptors are reported to be expressed in preadipocytes and suggested to mediate NPY-induced adipogenesis (Yang, *et al.* 2008, Zhang, *et al.* 2015). In the liver, Y_1-receptors have been shown to be involved in fatty acid oxidation and lipogenesis (Zhang, *et al.* 2010).

Y_2-receptors are stimulated by the amidated carboxy-terminal of NPY fragments (e.g. NPY_{13-36}) and $PYY_{3–36}$. These receptors are mostly located pre-synaptically and act as auto-receptors in the CNS and SNS. Thus, their activation decreases NPY secretion (Wahlestedt, *et al.* 1990a). Y_2-receptors are expressed extensively in the brain, e.g. in the hypothalamus and the brainstem (Stanić, *et al.* 2006). In addition, Y_2-receptors are localized post-synaptically on the cell membranes of the peripheral tissues e.g. they have been detected in the epithelial cells in vasculature along with Y_1-receptors (Zukowska Grojec, *et al.* 1998), in cardiomyocytes (McDermott, *et al.* 1997), in adrenal chromaffin cells (Cavadas, *et al.* 2001), in the enteric neurons of the gut (Cox, *et al.* 2001, Goumain, *et al.* 1998, Voisin, *et al.* 1990) and in WAT (Kuo, *et al.* 2007). Depending on the localization (pre- or post-synaptically), activation of Y_2-receptors can evoke apparently opposite responses. The activation of central Y_2-auto-receptors by a Y_2-agonist, $PYY_{3–36}$, decreases food intake, improves glucose tolerance and stimulates insulin secretion by increasing plasma GLP-1 levels (Batterham, *et al.* 2002, Chandarana, *et al.* 2013). In

adipose tissue, Y_2-receptors have been shown to mediate the adipogenic effect of NPY (Zukowska Grojec, *et al.* 1998), as well as the stress-induced proliferation and differentiation of adipocytes (Kuo, *et al.* 2007, Rosmaninho-Salgado, *et al.* 2012).

The knowledge of the Y_3-receptor is based only on pharmacological studies, as it is the only Y-receptor that has not been cloned. Y_3-receptors are activated by NPY, but not by PYY or PP (Glaum, *et al.* 1997, Lee & Miller. 1998). They are known to be located at least in the brainstem in NTS and area postrema (Glaum, *et al.* 1997, Lee & Miller. 1998). However, there is also evidence for their presence in cardiomyocytes (Balasubramaniam, *et al.* 1990) and endothelial cells (Chen, *et al.* 2007, Movafagh, *et al.* 2006), as well as in adrenal chromaffin cells (Cavadas, *et al.* 2001). The central Y_3-receptors mediate the cardiovascular effects of NPY (Grundemar, *et al.* 1991), whereas the binding of NPY to Y_3-receptors in heart and in vasculature induces the proliferation of the cells (Chen, *et al.* 2007, Movafagh, *et al.* 2006).

Y_4-receptors are mostly expressed in the peripheral tissues: in the pancreas, in the endothelial cells of the gut (both in small and large intestine) (Bard, *et al.* 1995, Cox, *et al.* 2001, Goumain, *et al.* 1998), in the heart (Bard, *et al.* 1995), and in the adrenal chromaffin cells (Cavadas, *et al.* 2001). In the CNS, Y_4-receptors have been detected in the brainstem (in NTS and area postrema), as well as in the hypothalamus and amygdala (Tasan, *et al.* 2009). The Y_4-receptor has the greatest affinity for PP, but less for NPY and PYY (Lundell, *et al.* 1997). As PP is expressed only peripherally, NPY is suggested to mediate the central actions of the Y_4-receptor. However, should PP be administered centrally, then the Y_4-receptor mediates its effect of negative energy balance and reduced food intake (Asakawa, *et al.* 1999). Central Y_4-receptors are involved also in fertility, and they may evoke a reduction in aggressive behavior (Sainsbury, *et al.* 2002c). Our knowledge about the physiological role of Y_4-receptors has been mainly based on experiments conducted with Y_4-receptor knock-out (KO) mouse model, as potential specific agonists for Y_4-receptor have been discovered only recently (Schubert, *et al.* 2017, Sliwoski, *et al.* 2016).

Y_5-receptors have the highest affinity for NPY, NPY_{2-36} and NPY_{3-36} (Lundell, *et al.* 1997, Statnick. 1998). There is convincing evidence that Y_5- and Y_1-receptors are interconnected, and activation of the transcription of one receptor compensatorily affects its counterpart. The genes encoding Y_1- and Y_5-receptors exist in close proximity, and

they have been suggested to derive from the duplication of the original gene (Herzog, *et al.* 1997). Y_5-receptors are mostly expressed in the CNS, but the distribution is limited only to those neurons that express also Y_1-receptors, and the expression level is quite low as compared to Y_1- and Y_2-receptors (Naveilhan, *et al.* 1998). Y_5-receptors are expressed in the hypothalamus (PVN, Arc and supraoptic nucleus), where similarly to Y_1-receptors, they mediate the feeding response of NPY (Gerald, *et al.* 1996). In addition, they have been detected in hippocampus, amygdala and in the brainstem (LC, A1 and C1 nuclei and NTS), lateral septum and cerebellum (Gerald, *et al.* 1995, Morin & Gehlert. 2006, Silva, *et al.* 2003, Wolak, *et al.* 2003). Y_5-receptors are expressed also in the periphery, although to a lesser extent. They have been detected at least in the adrenal chromaffin cells (Cavadas, *et al.* 2001), testis, spleen and pancreas (Statnick. 1998) but their existence in the gut is controversial (Cox, *et al.* 2001, Goumain, *et al.* 1998).

Activation of Y_5-receptors in PVN leads to a feeding response (Leibowitz & Alexander. 1991). However, a deficiency of Y_5-receptors or their antagonism is not solely able to inhibit the feeding response to NPY because of the compensatory effects mediated by Y_1-receptors. Only simultaneous antagonism or double-KO of both receptors induces hypophagia (Mashiko, *et al.* 2009, Nguyen, *et al.* 2012). In addition to a feeding response, the Y_5-receptor has also been shown to mediate NPY's ability to control hormone release (i.e. negative control of gonadotropic axis) (Raposinho, *et al.* 1999).

Similar to Y_3-receptors, our knowledge of the y_6-receptor is very limited, as mice and rabbits are the only species known to express a functional form of the receptor (Gregor, *et al.* 1996, Matsumoto, *et al.* 1996, Rose, *et al.* 1997). In rats, y_6-receptors are not expressed at all (Burkhoff, *et al.* 1998). In humans, the y_6-receptor is a pseudogene and its transcription does not produce a functional protein due to the deletion of one nucleotide leading to the absence of the seventh transmembrane region of the receptor (Gregor, *et al.* 1996, Rose, *et al.* 1997). However, mRNA of y_6-receptor is expressed in human brain, heart, gut, muscle and adrenal glands (Burkhoff, *et al.* 1998, Gregor, *et al.* 1996, Matsumoto, *et al.* 1996), which suggests that the receptor still may have some function in the human body. In the mouse brain, y_6-receptors are expressed in the suprachiasmatic nucleus (SCN) of hypothalamus (Weinberg, *et al.* 1996). In the periphery, y_6-receptors have been detected at least in testes (Yulyaningsih, *et al.* 2014). y_6–receptors recognize circulating $NPY_{13\text{-}36}$ and PP, but not PYY (Matsumoto, *et al.* 1996, Yulyaningsih, *et al.* 2014). The role of y_6-receptors remained a mystery until a recent experiment conducted

with y_6-receptor KO mice, which claimed that also y_6-receptors are involved in the control of energy homeostasis (Yulyaningsih, *et al.* 2014). That study suggested that PP-induced hypophagia and increased EE could be mediated via y_6–receptors located in SCN because of the ineffective BBB in the hypothalamus (Yulyaningsih, *et al.* 2014).

Table 2 The properties of Y-receptors in energy metabolism

Receptor	Expression sites	The most potent endogenous ligands	Special characteristics
Y_1	- Wide distribution in the brain - Vascular smooth muscle cells - Cardiomyocytes - Adrenal chromaffin cells - Liver - Gut - Immune system	NPY, PYY	- Widely expressed in CNS and peripheral tissues - Mediates the orexigenic and energy storing actions of NPY
Y_2	- Wide distribution in the brain (especially in the hypothalamus and the brainstem) - Vascular epithelial cells - Cardiomyocytes - Adrenal chromaffin cells - Gut - WAT	NPY_{13-36}, PYY_{3-36}	- Involved in energy homeostasis - Opposite effects on energy homeostasis depending on the expression site: - pre-synaptic in CNS and SNS → auto-receptor (NPY ↓) - post-synaptic in peripheral tissues → mediates the actions of NPY → adiposity↑
Y_3	- Brainstem (NTS, area postrema) - Cardiomyocytes - Endothelial cells - Adrenal chromaffin cells	NPY	- Limited knowledge only - Receptor has not been cloned
Y_4	- Pancreas - Gut - Heart - Adrenal chromaffin cells - Limited distribution in the brain (brainstem, hypothalamus, amygdala)	PP	- Mostly expressed in the peripheral tissues
Y_5	- Wide distribution in the brain - Limited distribution in the peripheral tissues (adrenal chromaffin cells, testis, spleen, pancreas)	NPY, NPY_{2-36}, NPY_{3-36}	- Co-existence with Y_1-receptor in CNS - Mediates the feeding response of NPY
y_6	- Humans: brain, heart, gut, muscle and adrenal glands - Mice: hypothalamus and testes	NPY_{13-36}, PP	- Limited knowledge only - Pseudogene in humans - Involved in energy homeostasis in mice

CNS = Central nervous system, NTS = Nucleus of the solitary tract, SNS = Sympathetic nervous system, WAT = White adipose tissue

2.4 Physiological functions of NPY

Because of NPY's widespread expression in the brain and periphery, as well as its high degree of conservation throughout evolution, it has been suggested that NPY must perform a broad range of vital physiological functions (Chronwall, *et al.* 1985, Larhammar, *et al.* 1993). NPY is known to be involved in many activities e.g. in the regulation of energy homeostasis, blood pressure, behavior (anxiolysis, stress), memory, alcohol consumption, circadian rhythms and hormone release (Lin, *et al.* 2004). However, studies with NPY KO mice have been shown that despite the broad range of activities in the body, a deficiency of NPY is not life-threatening (Karl, *et al.* 2008). The multitude of actions of NPY in both the CNS and the periphery, combined with the diversity of the study designs complicate clarification of the role of NPY in different physiological states. Fig. 5 illustrates the contribution of central and peripheral NPY to energy homeostasis, and in the following chapters, they will be discussed in more detail.

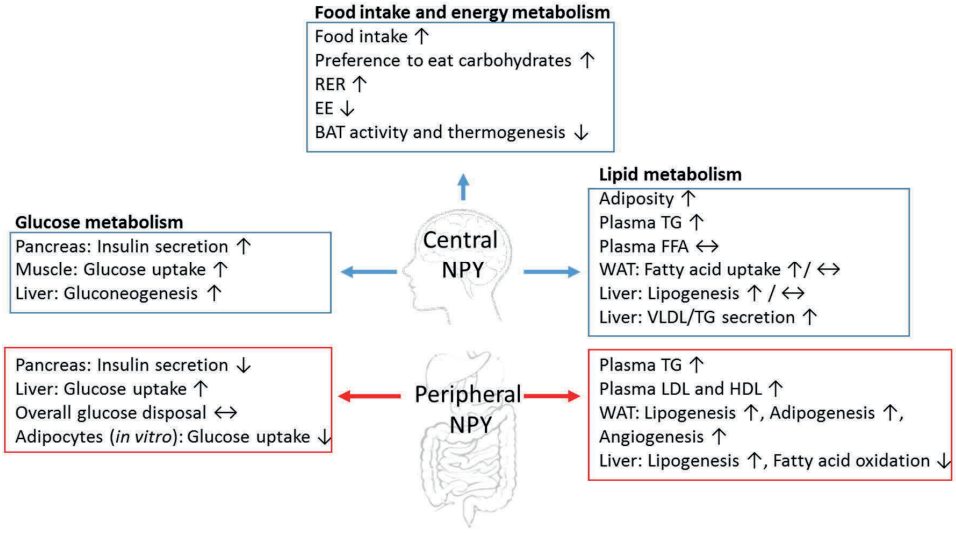

Figure 5 Central and peripheral actions of NPY in energy homeostasis

NPY has a fundamental role in energy homeostasis. Central NPY (blue) induces hyperphagia and decreases energy expenditure (EE), but central and peripheral NPY (red) has somewhat diverse actions on glucose and lipid metabolism. This highlights the complicated set of actions of NPY on glucose and lipid metabolism. BAT = Brown adipose tissue, WAT = White adipose tissue, FFA = Free fatty acid, TG = Triglyceride, HDL = High-density lipoprotein, LDL = Low-density lipoprotein, VLDL = Very low-density lipoprotein, RER = Respiratory exchange ratio, ↑ = Increased, ↓= Decreased, ↔ = No effect.

2.4.1 Food intake and energy expenditure

As discussed previously, the feeding response is influenced by orexigenic and anorexigenic signals from central and peripheral origins, and hypothalamic NPY, is the most powerful stimulator of a feeding response. Fasting or some other stimulus leading to a negative energy balance increases NPY levels, especially in the *central* areas of energy control, i.e. in Arc and PVN (Beck, *et al.* 1990, Brady, *et al.* 1990), as well as in other hypothalamic areas (medial optic area (MPO) and dorsomedial nucleus (DMH)) (Fig. 5) (Lewis, *et al.* 1993). NPY secretion follows a circadian rhythm; in rodents, high levels of NPY are detected in PVN or LHA in early dark hours or prior to the introduction of food, which is followed by clear peaks also in food intake and later there is a significant decrease of NPY concentrations (Kalra, *et al.* 1991b, McKibbin, *et al.* 1991, Stricker-Krongrad, *et al.* 1997).

Intracerebroventricular injection (i.c.v.), i.e central administration of NPY induces an immediate feeding response (Levine & Morley. 1984), and hyperphagia is retained during repeated dosing eventually leading to gain of weight and adiposity (Stanley, *et al.* 1986). However, food intake and body weight are normalized soon after the cessation of NPY administration (Stanley, *et al.* 1986). NPY administration or viral vector induced *Npy* overexpression directly in PVN, VMH, Arc and other hypothalamic nuclei have been shown to induce hyperphagia in rodents, but also a preference for a high-carbohydrate diet in preference over a high-fat or high-protein diet (Fig. 5) (Morley, *et al.* 1987, Shi, *et al.* 2013, Stanley, *et al.* 1986, Stricker-Krongrad, *et al.* 1997). The preference for a high-carbohydrate diet is especially detected in early dark hours, but to a lesser extent during late dark hours (Tempel & Leibowitz. 1990). This high-carbohydrate diet preference may be a consequence of NPY's ability to switch to utilizing carbohydrates as a fuel resource over fat, i.e. increase the respiratory exchange ratio (RER) (Menéndez, *et al.* 1990). NPY does not potentiate noradrenaline-induced feeding, and the catecholamiergic system does not seem to be involved in NPY-induced feeding response unlike corticosterone (Morley, *et al.* 1987, Stanley, *et al.* 1989).

In addition, central NPY decreases overall EE in the body (Fig. 5) (Zhang, *et al.* 2010), at least partly due to its inhibitory action on BAT thermogenesis. BAT is activated by cold and sympathetic stimulus, and NPY inhibits sympathetic activation of BAT and its thermogenic capacity (Billington, *et al.* 1991, Egawa, *et al.* 1990). NPY's inhibitory role

on energy consumption in BAT is independent of its stimulatory effect on food intake (Billington, *et al.* 1991). There is no evidence that NPY would directly change the physical activity.

2.4.2 Fatty acid and cholesterol metabolism

Independent of food intake, NPY influences fatty acid and cholesterol metabolism in the body. *Central* administration of NPY increases whole body adiposity and TG levels in plasma, and activates fatty acid uptake in WAT (Fig. 5) (Stafford, *et al.* 2008, Vettor, *et al.* 1994, Zarjevski, *et al.* 1993), whereas *peripheral* NPY expands WAT mass by stimulating lipogenesis, angiogenesis (Kuo, *et al.* 2007, Xie, *et al.* 2012, Zhang, *et al.* 2014) and adipogenesis in preadipocytes (Fig. 5) (Rosmaninho-Salgado, *et al.* 2012, Yang, *et al.* 2008, Zhang, *et al.* 2015).

In the liver, NPY is involved in lipogenesis, fatty acid oxidation and VLDL-TG secretion (Stafford, *et al.* 2008, Zhang, *et al.* 2010, Zhang, *et al.* 2014). Chronic, *central* administration and stress-induced excess of NPY have been shown to stimulate lipogenesis by activating acetyl-CoA carboxylase (ACC) enzyme in the liver (Zarjevski, *et al.* 1993, Zhang, *et al.* 2014). Similarly, knockdown (KD) of Y_1-receptors has been shown to decrease ACC activity, and on the other hand to increase β-oxidation by decreasing carnitine-acetyl-transferase 1 (CPT1) levels; CPT1 is the gate-keeping enzyme in mitochondrial β-oxidation (Fig. 5) (Zhang, *et al.* 2010). An acute and prolonged, *central* NPY administration in the presence of hyperinsulinemia increases VLDL-TG levels in plasma and VLDL production in the liver, respectively (Fig. 5) (Stafford, *et al.* 2008, van den Hoek, *et al.* 2004). The mechanism of action seems to involve a loading of hepatic TGs into apoB and stabilization of apoB100, as NPY neither changes FFA levels (i.e. does not increase substrate delivery from WAT to the liver), nor (in contrast to results from Zarjevski, *et al.* (1993) and Zhang, *et al.* (2014)) stimulates hepatic lipogenesis (Stafford, *et al.* 2008). NPY-induced activation of VLDL-TG secretion is mediated via Y_1-receptors and the SNS (Bruinstroop, *et al.* 2012, Rojas, *et al.* 2015, Stafford, *et al.* 2008).

Similarly to central NPY administration, chronic, *peripheral* NPY administration increases TG levels in plasma, but also total and LDL cholesterol levels (Fig. 5) (Xie, *et al.* 2012). Peripheral Y_1- or Y_2-receptors seem to be involved in cholesterol metabolism,

as their antagonism decreases levels of TG, total and LDL cholesterol, but also HDL cholesterol levels in rats with streptozotocin (STZ)-induced diabetes (Liu, *et al.* 2013). Otherwise there is quite limited knowledge about the involvement of NPY in cholesterol metabolism.

2.4.3 Glucose metabolism

The control of NPY and insulin are interconnected, as insulin suppresses NPY secretion in the hypothalamus, whereas *central* NPY administration induces both fasting and glucose-induced hyperinsulinemia (Fig. 5) (Sainsbury, *et al.* 1997, Schwartz, *et al.* 1991, Zarjevski, *et al.* 1993). The increased NPY-induced secretion of insulin is mediated via the parasympathetic nervous system (Sainsbury, *et al.* 1997). *Centrally* administered NPY enhances glucose uptake in WAT via an insulin-dependent GLUT4 transporter, but induces IR in muscle tissues and does not exert any effect on glucose utilization in BAT (Vettor, *et al.* 1994, Zarjevski, *et al.* 1994). However, *in vitro* studies with human adipocytes (3T3-L1 cells) indicate that NPY can induce IR by attenuating GLUT4 receptor translocation into the cell membrane, which decreases glucose uptake into the cells. This effect is mediated via the Y_1-receptor (Gericke, *et al.* 2012).

In contrast to the hypothalamus-mediated actions of NPY, in the pancreas, *peripheral* NPY directly inhibits glucose-induced insulin secretion from beta cells via Y_1-receptors (Fig. 5) (Cho & Kim, 2004, Morgan, *et al.* 1988, Pettersson, *et al.* 1987). *Npy* is expressed in the pancreas, mostly in the noradrenergic nerve fibers surrounding the Islets of Langerhans (Ding, *et al.* 1991, Pettersson, *et al.* 1987), but it is also expressed in the beta cells of the islets (Jamal, *et al.* 1991). However, expression of *Npy* in beta cells seems to be limited to neonatal cells, and its expression disappears after birth (Myrsen-Axcrona, *et al.* 1997, Whim. 2011). Expressions of *Npy*- and Y_1-receptors in the pancreas are significantly decreased in DIO and in genetic (leptin deficient *ob/ob*) obesity, and it has been suggested that beta cells may compensate for obesity-associated IR by reducing the cellular NPY levels in order to increase insulin secretion (Imai, *et al*. 2007).

In the liver, *centrally* administered NPY blocks the insulin-induced inhibition of glucose production (van den Hoek *et al.* 2004), and especially NPY originating from DMH has been suggested to be responsible for the actions of NPY on hepatic glucose production (Fig. 5) (Li, *et al.* 2016). However, there is some controversy about whether SNS or vagal

efferent neurons are mediating the actions of NPY on hepatic glucose production (Li, *et al.* 2016, van den Hoek, *et al.* 2008). On the other hand, when NPY is administered directly into the portal vein, *peripheral* NPY increases hepatic glucose uptake (Fig. 5) (Nishikawa *et al.* 2008). Whole-body glucose disposal remains unaltered, an effect independent of the dosing route (Nishikawa *et al.* 2008, van den Hoek *et al.* 2004).

2.5 NPY in metabolic disorders

2.5.1 Metabolic disorders in human gain-of-function polymorphism of NPY gene (rs16139)

There are at least two human single nucleotide polymorphisms (SNPs) of the *NPY* gene (rs16139, rs17149106) known to be involved in obesity or MetS (Yeung, *et al.* 2011). Rs16139 (p.L7P) is a gain-of-function polymorphism in the signal peptide of the *NPY* gene (preproNPY), caused by a single nucleotide mutation (1128T>C), which leads to a change of amino acid 7 from leucine to proline (Karvonen, *et al.* 1998). The prevalence of rs16139 seems to be higher in northern and eastern parts of Europe, and lower in the southern and western parts. In Finland, the prevalence is 6-14% of the population (Ding. 2003, Ding, *et al.* 2005, Karvonen, *et al.* 1998), but in some parts of the world e.g. in Eastern Asia, rs16139 is absent (Makino *et al.* 2001).

Rs16139 increases NPY secretion during sympathetic activation (Kallio, *et al.* 2001, Mitchell, *et al.* 2008), but at rest, NPY and noradrenaline levels in plasma are attenuated (Jaakkola, *et al.* 2005, Kallio, *et al.* 2003). The traits of MetS are more common among carriers of rs16139; for example, the following traits are all more common in carriers of rs16139; increased body weight (van Rossum, *et al.* 2006), hypercholesterolemia (especially among obese subjects) (Karvonen, *et al.* 1998), impairment of glucose metabolism and earlier onset of T2D (Jaakkola, *et al.* 2006, Nordman, *et al.* 2005, Ukkola & Kesaniemi. 2007), as well as diabetes-induced vascular complications (Jaakkola, *et al.* 2009, Jaakkola, *et al.* 2010, Karvonen, *et al.* 2001, Masoudi-Kazemabad, *et al.* 2013, Niskanen, *et al.* 2000a, Niskanen, *et al.* 2000b) but no association to changes in food intake (Karvonen, *et al.* 2006, Yeung, *et al.* 2011).

Rs16139 has been shown to be in linkage disequilibrium with another gain-of-function SNP of the *NPY* gene, rs16147 (-399T>C), where the Pro7 allele of rs16139 always co-exists with the -399T allele of rs16147 (Jaakkola, *et al.* 2012, Zhou, *et al.* 2008). NPY levels are increased in the diplotypes with one or both of the SNPs, such that subjects with both SNPs display the highest NPY levels (Jaakkola, *et al.* 2012, Zhou, *et al.* 2008). However, the overall significance of these diplotypes is far from clear. Rs16139 is associated with metabolic disturbances, whereas rs16147 has been linked with stress and emotion (Zhou, *et al.* 2008). Overweight carriers of rs16147, but not of rs16139, carry an increased risk to develop asthma, but no similar association has been detected in the carriers of both SNPs. Instead, carriers of both SNPs have an increased risk for vascular wall thickening (Jaakkola, *et al.* 2012). Furthermore, among the subjects with asthma, NPY levels are inversely related to the risk of atherosclerosis, which has been postulated to relate to the role of NPY in suppressing Th1 type inflammatory responses (Jaakkola, *et al.* 2012).

2.5.2 OE-NPY$^{D\beta H}$ mouse model

In the transgenic OE-NPY$^{D\beta H}$ mouse model, *NPY* is overexpressed in noradrenergic neurons and in peripheral SNS under a dopamine-beta-hydroxylase (*DβH*) promoter. The OE-NPY$^{D\beta H}$ mouse model was originally created to investigate the effects of extra-hypothalamic NPY on energy metabolism, and to model rs16139 in mice and stress-induced obesity (Ruohonen, *et al.* 2008).

In heterozygous OE-NPY$^{D\beta H}$ mice, basal NPY levels are increased by 1.8-fold in adrenal glands and by 1.3-fold in the brainstem as compared to wildtype (WT) control animals, but hypothalamic and plasma NPY levels do not differ between the genotypes (Ruohonen, *et al.* 2008). After acute restraining stress, NPY levels in plasma were elevated only in female heterozygous OE-NPY$^{D\beta H}$ mice (Ruohonen, *et al.* 2009). Basal adrenaline levels are elevated in plasma, but declined in adrenal glands, indicating that increased NPY in adrenal glands enhances the secretion of catecholamines into the circulation, and thus increases sympathetic activity in heterozygous OE-NPY$^{D\beta H}$ mice (Ruohonen, *et al.* 2009). These mice display increased body adiposity starting from age of 3 months, and impaired glucose tolerance (IGT), fasting hyperinsulinemia and hepatic accumulation of TGs at 6

months of age, but body weight and food intake are similar to WT mice (Ruohonen, *et al.* 2008).

Homozygous OE-NPY$^{D\beta H}$ mice display increased protein levels of NPY in the brainstem. However, only the mRNA expression of *Npy* is increased in adrenal glands and in the hypothalamus, but not at the protein level (Vähätalo, *et al.* 2015). Transgenic expression of *NPY*, detected by staining of the reporter gene LacZ, is most intense in LC nuclei in the brainstem, but can be detected at lower levels in the hypothalamic PVN and DMH. However, the level of neuronal NPY secretion is not known, nor is there any information about how it is affected by stress or any other stimulus. Despite increased expression of *Npy* in the hypothalamus, OE-NPY$^{D\beta H}$ mice do not display hyperphagia, except after a prolonged fast (Vähätalo, *et al.* 2015). Homozygous OE-NPY$^{D\beta H}$ mice have a similar obese phenotype as heterozygous mice, but with an earlier onset and more pronounced symptoms: body weight is increased at 4 months and WAT weight at 2 months in males. They also display IGT starting from age of 3 months, and fasting hyperinsulinemia, hyperleptinemia and IR at 6 months of age (Vähätalo, *et al.* 2015). There are no changes in EE or RER, but the thermogenic activity of BAT is impaired in response to cold exposure. Nonetheless, OE-NPY$^{D\beta H}$ mice are better able than their WAT-matched mice to retain their body temperature when exposed to cold (Vähätalo, *et al.* 2015). The obese phenotype is similar in females, but less pronounced and with a later onset (Vähätalo, *et al.* 2015). Homozygous OE-NPY$^{D\beta H}$ mice display attenuated adrenaline levels in urine and decreased expression of tyrosine hydroxylase (*Th*) and *Dβh* enzymes in the brainstem (Vähätalo, *et al.* 2015). TH is the rate-limiting enzyme of catecholamine biosynthesis, catalyzing the formation of L-DOPA from L-tyrosine, and DβH catalyzes the formation of noradrenaline from dopamine. Thus, even though there seem to be similar phenotypes between heterozygous and homozygous models, homozygous OE-NPY$^{D\beta H}$ mice, in contrast to heterozygous mice, display decreased sympathetic activity (Vähätalo, *et al.* 2015). Therefore, it can be considered to follow the 'Mona Lisa hypothesis' of obesity proposed by Bray (1991).

2.5.3 The Role of NPY in other models of metabolic disorders

In addition to numerous studies where exogenously administered NPY has been shown to induce hyperphagia and obesity, there are genetic animal models, which demonstrate

that NPY plays a significant role in many metabolic disorders. Leptin or leptin receptor deficient, genetically obese mice and rats, display hyperactivity of the NPY system (Stricker-Krongrad, *et al.* 1997). Fatty Zucker rats, deficient for leptin receptor, lack a normal circadian rhythm-based fluctuation in their NPY levels and typical eating times, but instead, NPY levels remain high all the time (Stricker-Krongrad, *et al.* 1997). This finding was one of the first pieces of evidence for the involvement of NPY in eating disorders. Hyperactivity of the NPY system in baseline conditions has also been suggested to be involved in the impairment of thermogenic activity of these rats (Bing, *et al.* 1997). A deficiency of NPY has been shown to improve the metabolic status of leptin deficient *ob/ob* mice (Erickson, *et al.* 1996). Furthermore, when *ob/ob* mice were crossed with germline Y_2-receptor KO mice, the female mice had a reduced body weight, as well as decreased total and LDL cholesterol levels (Naveilhan, *et al.* 2002). Similarly, a reduced weight gain and adiposity, and improved glucose metabolism have been detected in male mice, probably due to attenuation of the hypothalamic pituitary adrenal (HPA) axis activity, which has been shown to be over-activated in *ob/ob* mice and in stress (Sainsbury, *et al.* 2002b).

In addition, NPY is involved in stress-induced obesity. Under normal conditions, NPY KO mice do not differ from WT mice in terms of body composition or glucose tolerance (Karl, *et al.* 2008), but they are resistant to stress-induced obesity (Zhang, *et al.* 2014). catNPY mice (cross of NPY KO and OE-NPY$^{D\beta H}$ mice (Zhang, *et al.* 2014)) express only transgenic *NPY* in the catecholaminergic system, but not endogenous NPY; these mice are prone to stress-induced obesity and stimulation of hepatic lipogenesis, which suggests that NPY in noradrenergic neurons and the SNS mediates the obesogenic effect of stress. Furthermore, catNPY mice are more prone to DIO when compared with WT mice (Zhang, *et al.* 2014). Another model of stress-induced obesity demonstrated that the stress-induced increase in peripheral NPY release increases WAT mass via the Y_2-receptor (Kuo, *et al.* 2007).

Y-receptor KO and conditional KD models have revealed valuable information on the effects of NPY mediated via its receptors. Although Y_1-receptors were thought to mediate the obesogenic effects on NPY in the hypothalamus, germline Y_1-receptor KO mice display late-onset obesity and basal hyperinsulinemia, but no change in basal food intake (Kushi, *et al.* 1998, Pedrazzini, *et al.* 1998). Peripheral conditional Y_1-recepor KD, instead, protects from DIO, and enhances EE and hepatic fatty acid oxidation (Zhang, *et*

al. 2010), which suggests that especially the peripheral Y_1-receptor could be a plausible target for intervention against obesity and related disorders.

Germline Y_2-receptor KO mice in a WT background had a decreased body weight and fat mass compared to WT mice and these effects were independent of the diet (Sainsbury, *et al.* 2002a, Sainsbury, *et al.* 2006). Similarly, mice with peripheral-specific Y_2-receptor KD were resistant to DIO with improved glucose metabolism (Shi, *et al.* 2011). In contrast, hypothalamus-specific Y_2-receptor KO induced hyperphagia and predisposed to obesity (Shi, *et al.* 2010), highlighting the dual role of Y_2-receptors, depending on the location (pre-or post-synaptic) and function of the receptor.

Y_4-receptor KO mouse displayed decreased body weight and fat mass, probably due to the increased PP levels (Sainsbury, *et al.* 2002c, Ueno, *et al.* 1999). As double-KO of Y_2 and Y_4-receptors led to an even leaner phenotype than a deficiency of either receptor alone, these receptors have been postulated to act synergistically and compensate for each other (Sainsbury, *et al.* 2003, Sainsbury, *et al.* 2006), similar to the situation described between Y_1- and Y_5-receptors.

Y_5-receptor KO mice, similar to Y_1-receptor KO mice, showed signs of late-onset obesity. Surprisingly, they also exhibited late-onset hyperphagia, which is suggested to be due to compensatory mechanisms mediated via the Y_1-receptor (Marsh, *et al.* 1998). Although mice with double-KO of Y_1- and Y_5-receptors displayed a reduced food intake, there are still other compensatory mechanisms, which can induce weight gain and obesity in these mice (Mashiko, *et al.* 2009, Nguyen, *et al.* 2012). Unlike KO of the Y_2-receptor, KO of Y_5-receptors did not prevent the obese phenotype of *ob/ob* mice (Marsh, *et al.* 1998).

Young y_6-receptor KO mice showed decreased weight gain and adiposity, but similar to Y_1- and Y_5-KO mice, y_6-KO mice displayed obesity when they reached a mature age (Kushi, *et al.* 1998, Marsh, *et al.* 1998, Yulyaningsih, *et al.* 2014).

2.6 Pharmacotherapy in metabolic disorders

An effective way of preventing obesity related metabolic diseases is to achieve a 5-10% weight loss by lifestyle changes (i.e. by increased exercise and healthier nutrition). However, very often pharmacological treatment is needed to treat the symptoms of the

MetS. Because of the heterogenic nature of the disease, patients are usually treated with an array of different kinds of drugs for the different symptoms, e.g. statins for hypercholesterolemia and ACE-inhibitors or AT_1 receptor blockers for hypertension. Similar to other metabolic disorders, lifestyle changes are the cornerstone for treating NAFLD or its progressive form NASH. There are no officially sanctioned drugs for NAFLD, but some of the new drugs for treating T2D have shown potential in treating NASH in patients with T2D (Sumida & Yoneda. 2018).

Hyperglycemia is a serious risk factor for vascular complications, and effective medication is essential. In addition to the glucose lowering effect, diabetes drugs are believed to reduce the risk of cardiovascular events. In the following paragraphs, the most commonly used drugs on the market for treating non-insulin demanding T2D will be introduced.

Metformin, a biguanide, is the first-line anti-hyperglycemic agent. Its main indication is to treat T2D, but it has also been shown be effective in preventing the onset of T2D (Knowler, *et al.* 2002, Li, *et al.* 1999, Ramachandran, *et al.* 2006). Metformin has been used for decades, but all of its mechanism-of-actions at the cellular level are not still fully understood, e.g. during recent years, many new sites of action have been discovered. Its main mechanism of action is the decrease in hepatic gluconeogenesis due to an inhibition of mitochondrial complex I leading to decreased levels of ATP, which produces the energy required for glucose production, and the stimulation of AMP activated protein kinase (AMPK) in hepatocytes. Attenuation of gluconeogenesis enhances insulin sensitivity and decreases blood glucose levels, whereas AMPK stimulates fatty acid oxidation (Hundal, *et al.* 2000, Madiraju, *et al.* 2014, Zhou, *et al.* 2001). Metformin, in contrast to other traditional diabetes drugs, decreases body weight, which is an advantage as these patients are usually obese. In addition, metformin enhances the secretion of GLP-1 from the gut, alters the gut microbiota (Forslund, *et al.* 2015, Mannucci, *et al.* 2004, Yasuda, *et al.* 2002), and improves lipid metabolism (Lin, *et al.* 2018). Metformin has also been suggested to possess anti-cancer properties (reviewed by (Daugan, *et al.* 2016)). Despite its multi-organ advantages in T2D patients, metformin has not been proven to cure NAFLD or NASH, if one bases the evaluation on liver histology (Li, *et al.* 2013, Sumida & Yoneda. 2018). However, metformin has been shown to attenuate the levels of liver enzymes (ALT and AST) and HOMA-IR (a calculated index for estimating IR), which are signs of improved liver function (Li, *et al.* 2013, Sumida & Yoneda. 2018).

Metformin is considered as a safe drug, with rather limited adverse effects. For example, it does not induce hypoglycemia which is a serious problem encountered with many other T2D drugs, but gastrointestinal side effects may limit its use. The main serious adverse effect is lactic acidosis. Metformin is secreted through kidneys, and severe renal failure is a contraindication for using the drug.

In recent years, many new drugs for the treatment of T2D have entered the market, and many other effective drugs are being used to supplement the effects of metformin. Sodium-glucose co-transporter 2 (SGLT2) inhibitors (gliflozines) suppress glucose reuptake in the proximal renal tubules. They are the newest group of drugs, and are believed to be beneficial not only in treating hyperglycemia but they also decrease body weight, improve the lipid profile and lower the blood pressure (Yu & Woo. 2017). SGLT2 inhibitors have shown potential in preventing hepatic TG accumulation, and even some effects to decrease inflammation and fibrosis in NASH in diabetic models in rodents, as well as reducing hepatic transaminase levels in humans (Hayashizaki Someya*, et al.* 2015, Qiang*, et al.* 2015, Sumida & Yoneda. 2018). Clinical trials with the indication of steatosis in diabetes are on-going. However, these drugs have also been shown to increase gluconeogenesis (Jia*, et al.* 2018). The most common adverse effect is an elevated risk of genital infection. Similar to metformin, SGLT2 inhibitors may cause hypoglycemia, if combined with a sulfonylurea or insulin treatment (Yu & Woo. 2017).

GLP-1 analogs mimic endogenous gut derived GLP-1. They sensitize pancreatic beta cells to glucose, enhance insulin release and decrease glucagon levels. GLP-1 analogs can be divided into short and long acting drugs in order to treat either postprandial or fasting hyperglycemia, respectively. Their adverse effects are minor, but there is still only limited knowledge about their long-term use. The need for a subcutaneus (s.c.) dosing pattern may limit their use. GLP-1 analogs are effective in supporting weight loss, and in addition to T2D, the treatment of obesity is another indication for liraglutide (Mehta*, et al.* 2017). The results of clinical studies with GLP-1 analogs have been encouraging, with evidence of improved liver metabolism in patients with T2D, but not NAFLD or NASH alone (Sumida & Yoneda. 2018).

Dipeptidyl peptidase 4 (DPP-4) inhibitors (gliptins) have similarities with the GLP-1 analogs, their mechanism of action is inhibition of the degradation of endogenous GLP-1. Thus, they increase insulin secretion in a glucose-dependent manner, and therefore,

they are used to combat postprandial hyperglycemia, and the risk for hypoglycemia is minimal. However, DPP-4 degrades also a variety of other peptides in addition to GLP-1 (e.g. NPY), which raises the question about the non-specificity of these inhibitors (Yu & Woo. 2017). Nonetheless, no serious adverse effects have been detected so far, and they are used as a second-line treatment (after metformin) for diabetes. There is no clear evidence that DPP-4 inhibitors would improve NAFLD or NASH in patients with T2D (Sumida, *et al.* 2017).

Other older diabetes drugs are today considered only as secondary drug treatments in cases where the first-line drugs are not suitable for the patient. Sulfonylureas and glinides increase insulin secretion from beta cells in a glucose-independent manner by blocking the cells' K^+ channels. Therefore, there is a risk of both hypoglycemia and weight gain. Glinides are used to treat postprandial hyperglycemia (Gallwitz. 2009). Glitazones act on the adipocytes as insulin sensitizers by activating peroxisome proliferator-activated receptor gamma (PPARγ), but despite their anti-hyperglycemic effect, they have not shown any ability to inhibit cardiovascular events and therefore they have only a limited use. Similar to sulfonylureas and glinides, they induce weight gain (Gallwitz. 2009). Pioglitazone has recently been recommended for the treatment of NASH in many parts of the world (Sumida & Yoneda. 2018).

3 AIMS OF THE STUDY

The aim of this thesis was to elucidate the mechanisms of increased NPY levels in the pathogenesis of the MetS, focusing on hepatic fatty acid, cholesterol and glucose metabolism. The study was conducted with a genetically obese mouse model (OE-NPY$^{D\beta H}$) overexpressing NPY in noradrenergic neurons and in the peripheral SNS. The goal of the study was to broaden the understanding of NPY-induced metabolic disturbances focusing especially on liver function. In this way, it was hoped to find novel drug targets in obesity and prediabetes, especially in case of conditions associated with excess NPY induced either by a genetic background (rs16139) or by chronic stress.

The specific aims of the study were:

1. To characterize the hepatic phenotype of obese OE-NPY$^{D\beta H}$ mice, and to study whether this phenotype predisposes mice to T2D.

2. To elucidate the mechanisms leading to hepatosteatosis, hypercholesterolemia and IR in OE-NPY$^{D\beta H}$ mice.

3. To investigate the role of hepatic glycogen on IGT in OE-NPY$^{D\beta H}$ mice, and whether treatment with the anti-hyperglycemic agent, metformin, improves glycogen metabolism and IGT.

4. To study whether peripheral NPY directly via its peripheral Y_1- or Y_2-receptors or indirectly by changing the activity of SNS in the liver is responsible for the metabolic disturbances.

5. To clarify whether obesity related metabolic disorders induced by excess peripheral NPY and / or DIO (e.g. stress combined with unhealthy diet) can be treated by pharmacological blockade of the peripheral Y_1- or Y_2-receptors.

4 MATERIALS AND METHODS

4.1 Animals

Transgenic heterozygous and/or homozygous OE-NPY$^{D\beta H}$ mice with WT control mice on a C57BL/6N genetic background were used in Study I, and homozygous OE-NPY$^{D\beta H}$ and WT mice were used in Studies II & III. Heterozygous OE-NPY$^{D\beta H}$ and WT mice were littermates and homozygous mice had originated from the same heterozygous (1st-3rd generation) breedings. The generation of the heterozygous and the homozygous transgenic OE-NPY$^{D\beta H}$ models has been described by Ruohonen, *et al.* (2008) and Vähätalo, *et al.* (2015), respectively. Mice were maintained at 21±3ºC with fixed 12-h light/darkness cycle in the Central Animal Laboratory of University of Turku. Standard rodent chow (SDS, Essex, UK) and tap water were available *ad libitum*, unless stated otherwise.

In Study I, the metabolic phenotype in obese, 4-7-month-old homozygous male or female OE-NPY$^{D\beta H}$ mice was characterized, and the mechanisms leading to these metabolic changes were studied in pre-obese, 2-month-old heterozygous and homozygous male OE-NPY$^{D\beta H}$ mice. The susceptibility of OE-NPY$^{D\beta H}$ mice to T2D was studied in 7-month-old male mice, which were consuming a high caloric diet and administered a low-dose of STZ. In drug intervention studies (II & III), 4-5-month-old homozygous male OE-NPY$^{D\beta H}$ mice were used. Mice were group housed (2-5 mice/cage), except in Study II, where the animals were housed individually to measure and guarantee the drug administration.

4.2 Ethical aspects

Animal care was in accordance with the guidelines of the International Council of Laboratory Animal Science (ICLAS), and the experimental procedures were approved by the Finnish national animal care and use committee (ELLA). The studies were planned and performed according to ethical 3R's (Reduction, Replacement, Refinement) principles. The number of animals was limited to a minimum (n = 6-12 mice / group) balancing the need to achieve both statistically significant and physiologically relevant results. The exact group sizes are reported within each study. Animals were sacrificed

while avoiding any external stress, and all relevant tissues were dissected for further analysis. Replacement of the *in vivo* studies with *in vitro* analysis would not have been possible, because the physiological and pharmacological phenomena and mechanisms in the whole body must be studied in a living organism. All the experiments were performed by avoiding excessive stress and suffering for the animal. The welfare of the animals was monitored (weight gain, food intake, visual condition) during the experiments. Any mice showing signs of illness or aggressive behavior were removed from the experiment. Pre- and post-operative analgesia (buprenorfine) and anesthesia (isoflurane) were used in the surgical procedure, where a carotid artery catheter was implanted (Study I, see chapter 4.4.3).

4.3 Drugs

4.3.1 Drugs used in drug intervention studies

STZ (Sigma-Aldrich, St. Louis, MO, USA) is a glucose analog which gains access to the pancreatic beta cells via the glucose transporter GLUT2 (Schnedl*, et al.* 1994). It was originally discovered to act as an antibiotic (Vavra*, et al.* 1959), but due to its broad range of adverse effects (e.g. genotoxicity and cytotoxic effect on pancreatic beta cells) as reviewed by (Bolzan & Bianchi. 2002), its pharmacologic indication has been limited to the induction of diabetes in rodents. STZ at high doses (50-100 mg/kg single or repeated doses) is commonly used for studying type 1 diabetes, but in order to induce T2D in Study I, a low dose of STZ (40 mg/kg repeated on 3 consecutive days, i.p.) was combined with a high caloric diet (Gilbert*, et al.* 2011).

Metformin (Enzo Life Sciences LTD, Exeter, UK) is a biguanide, which is the first line anti-hyperglycemic agent used in T2D in humans. Its basic mechanism to treat hyperglycemia is to inhibit hepatic glucose production by reducing ATP in hepatocytes, but it also possesses other beneficial mechanisms. The dose of 300 mg/kg/day (administered in the drinking water) administered in Study II was based on a previously used dose of metformin in mice (Salomäki*, et al.* 2013).

BIBO3304 trifluoroacetate (Tocris Bioscience, Bristol, UK) is a highly selective Y_1-receptor antagonist, unable to cross the BBB (Dozio*, et al.* 2007, Yuzuriha*, et al.* 2007).

The half-life of BIBO3304 is not known, but previous data on its use in a repeated, peripheral dosing pattern exists (once or twice a day), which support its relatively long half-life (Sousa, *et al.* 2012, Yuzuriha, *et al.* 2007). A dose of 1 mg/kg/day (i.p.) for BIBO3304 was selected for Study I based on Yuzuhira, *et al.* (2007) .

BIIE0246 (Tocris Bioscience) is a highly selective Y_2-receptor antagonist (Doods, *et al.* 1999), which, similar to BIBO3304, is unable to cross the BBB (Brothers, *et al.* 2010, Doods, *et al.* 1999). One limitation of BIIE0246 is its short half-life in mouse (less than 3 h) (Brothers, *et al.* 2010). However, even this is much longer than other available peripheral Y_2-receptor antagonists, and therefore BIIE0246 was considered as the most suitable antagonist for studies involving chronic administration. The dose of 2 mg/kg in acute BIIE0246 administration (Forbes, *et al.* 2012) and the same molar dose of BIBO3304 in Study I were used, when estimating an appropriate dose (1.3 mg/kg, i.p.) for BIIE0246 in repeated administration in Study III.

4.3.2 Drugs used for anesthesia and analgesia

Buprenorfine (Temgesic, Schering-Plough, Brussels, Belgium), a partial opioid agonist, was used in Study I for postsurgical analgesia (0.1 mg/kg). 4.5% inhaled isoflurane was used to anesthetize mice, and 2% isoflurane to maintain anesthesia during the surgery (Study I). Ketamine (75 mg/kg, Ketaminol, Intervet Oy, Finland) and medetomidine (1 mg/kg, Cepetor, ScanVet Oy, Finland) were used for terminal anesthesia (Studies I & III).

4.4 *In vivo* experiments

4.4.1 Body weight and composition

Body weight gain was monitored once (Studies I & II) or twice (Studies I & III) a week during the studies, and body weight was measured at sacrifice. Body composition (fat and lean mass) was analyzed *in vivo* with EchoMRI-700 (Echo Medical Systems, Houston, TX, USA) from conscious mice prior to the start and during a diet or drug intervention, and at sacrifice. Each animal was scanned twice with ±0.5 g tolerance in fat mass between

the measurements being accepted as valid. The average values for body fat and lean tissue mass, and fat and lean mass % (relative to body weight) were calculated.

4.4.2 Food intake

Food intake was monitored during drug or diet interventions once a week. In cases where the mice were group housed (Studies I & III), food intake was analyzed by calculating the total food consumption per cage and subtracting the amount of food left (on the feed hopper and in bedding) from the amount of given food, and by dividing it by the number of animals (2-5 mice/cage) in the cage; it is expressed as an average daily food intake per animal (Study I) or energy intake per cage (Study III). Food intake was measured also during experimental set-ups, i.e. during EE measurements (Study I).

4.4.3 Glucose metabolism

Tail vein blood glucose concentration was measured before sacrifice after 3 or 4 h fasting with Precision Xtra Glucose Monitoring Device (Abbott Diabetes Care, Abbott Park, CA, USA). Glucose sensitivity was analyzed *in vivo* by glucose tolerance test (GTT) (Studies I & II), where 4 h-fasted mice received intraperitoneally (i.p.) 1 g/kg body weight of glucose, and blood glucose was measured before (0 min), and 20, 40, 60 and 90 min after the administration of glucose. IR was studied by the insulin tolerance test (ITT) (Study I), where 1 h-fasted mice received (i.p.) 0.75 IU/kg of insulin (Protaphane FlexPen, Novo Nordisk, Baggvaerd, Denmark), and the blood glucose concentration was measured at 0, 20, 40 and 60 min. Hepatic glucose production (gluconeogenesis) was measured by the pyruvate tolerance test (PTT) (Study I). In PTT, 2 mg/kg of sodium pyruvate (Sigma-Aldrich) was administered to 4 h-fasted (7-month-old mice) or 6 h–fasted mice (4-month-old mice). Blood glucose concentration was measured at 0, 20, 40 and 60 min.

In order to study glucose stimulated insulinemia, serum glucose and insulin levels were measured during GTT (Study I). Three days before GTT, mice underwent surgery, where a carotid artery catheter was implanted under 2% isoflurane anesthesia. Buprenorfine (0.1 mg/kg) was administered subcutaneously (s.c.) twice a day for two days for analgesia during the recovery. In GTT, mice received glucose (1 g/kg) via a catheter after a 4 h-fast, and arterial blood samples (25 µl) were collected at 0, 5, 15, 30 and 60 min with an

automated blood sampling device (AccuSampler®μ, VeruTech, Lund, Sweden). The reason for using this technique was to avoid any possible acute stress-induced effects on blood glucose and insulin levels due to the injection. Blood glucose was measured with a glucose meter and serum insulin levels were determined as described in chapter 4.6.1.

4.4.4 Cholesterol metabolism (Study I)

Cholesterol absorption and synthesis were measured by the fecal ratio method (Wang, *et al.* 2001, Wang & Carey. 2003) from 6-month-old female OE-NPY$^{D\beta H}$ and WT mice (n = 5-10/group) (Study I). Mice received 2 μCi ^{3}H-sitostanol (American Radiolabeled Chemicals Inc, St. Louis, MO, USA) and 1 μCi ^{14}C-cholesterol (Perkin Elmer) in 150 μl of olive oil per os (p.o.), after which they were placed in individual cages with a mesh bottom for 4 days to measure food consumption and defecation. The feces were collected and dried under airflow at 60°C for 48 h. Fecal (i.e. non-absorbed) ^{14}C-cholesterol was determined in a liquid scintillation counter in order to measure cholesterol absorption. Neutral sterols and bile acids were analyzed by a gas-liquid chromatography with a 50 m capillary column from food and feces in order to measure cholesterol and bile acid synthesis (Grundy, *et al.* 1965, Miettinen. 1982). Cholesterol synthesis was calculated from these parameters using a following formula:

$$\frac{Cholesterol\ synthesis}{day} = \frac{Neutral\ sterols + bile\ acid\ in\ feces}{day} - daily\ dietary\ cholesterol$$

As the bile acid pool size is strictly constant, the daily bile acid synthesis can be directly calculated from the bile acids secreted to feces:

$$\frac{Bile\ acid\ synthesis}{day} = \frac{Bile\ acid\ in\ feces}{day}$$

4.4.5 Drug intervention studies

4.4.5.1 Type 2 diabetes model with a combination of a Western diet and streptozotocin (Study I)

In order to study susceptibility to T2D, 4-month-old OE-NPY$^{D\beta H}$ mice and WT mice (n = 7 / group) were subjected to low-doses of STZ (40 mg/kg in 7.5 mg/ml Na-Citrate solution (pH 4.5)) to interfere with the pancreatic beta cells after 3 weeks of feeding with WD (41% kcal fat, 43% kcal carbohydrate, 17% kcal protein, D12079B, Research Diets, New Brunswick, NJ, USA) to induce IR (Gilbert*, et al.* 2011). STZ was administered (i.p.) after a 4 h-fast on 3 consecutive days followed by a single dose of STZ every 4.5 weeks to maintain the hyperglycemia. Weight gain, food intake and tail vein fasting (4 h) blood glucose levels were monitored weekly, and body composition was measured prior to the start of the diet (at week -3) and at 9 weeks after the first STZ administration. Glucose metabolism was analyzed by GTT and ITT on weeks 10 and 12, respectively. Tissues were collected after 13 weeks of the first STZ administration as described in chapter 4.4.6. The study protocol is described in Fig. 6A.

4.4.5.2 Metformin (Study II)

The effects of metformin on glucose and hepatic glycogen metabolism were studied in the state preceding IGT, which was present in the 4-month-old homozygous male OE-NPY$^{D\beta H}$ mice. The aim was to elucidate the role of glycogen metabolism at the onset of prediabetes or T2D, with or without treatment with metformin. OE-NPY$^{D\beta H}$ and WT mice (n=10-11) received either metformin (300 mg/kg/day) in drinking water for 4 weeks (Met) or water without metformin (Ctr). The mice, housed individually since the age of 8 weeks, were randomly divided into treatment groups matched for their body weight, fat mass and food and water intake, which were measured one week prior to the start of the drug treatment (Week -1). The drinking bottles were changed twice a week and water intake was measured to verify the correct drug doses were being administered. Both body weight gain and food consumption were monitored once a week during the study. Body composition was analyzed at weeks 0 (prior to drug treatment) and 4 (before sacrifice). Glucose metabolism was evaluated by GTT at week 3. The study protocol is described in Fig. 6B.

4.4.5.3 Y-receptor antagonist (Studies I & III)

The effect of pharmacological blockade of peripheral Y_1- and Y_2-receptors on obesity and metabolic disorders was studied in a situation where obesity was induced by diet and / or excess NPY. Excess NPY levels such as those present in 5-month-old OE-NPY$^{D\beta H}$ mice were compared with WT controls, which were fed with regular chow diet or WD (D12079B, Research Diets). The WD was started 8 weeks before the drug administration. Mice were treated (i.p.) with the Y_1-receptor antagonist, BIBO3304 trifluoroacetate (1 mg/kg/day), the Y_2-receptor antagonist, BIIE0246 (1.3 mg/kg/day) or vehicle (DMSO, Tween® 80 (Fisher Scientific, Fair Lawn, NJ, USA) and 0.9% NaCl, 1:1:18 respectively); the injections were preceded by a 2-week habituation to the handling and injections with daily saline injections (i.p.). Mice were divided into equal treatment groups within the genotype based on their body weights at week 0. Half of the chow-fed mice (n = 7-14 / group) and all WD-fed mice (n = 7-12 / group) were treated for 2 weeks. Another half of the chow-fed mice (n = 10-13 / group) were treated for 4½ weeks.

The phenotype was observed during the study *in vivo* by measuring the body weight twice each week and average food intake per cage once a week. Body composition was measured at the initiation of the diet (week -8), prior to the habituation (week -2) and the drug treatment period (week 0), and 2- and 4½-week time points. The study protocol is described in Fig. 6C. The phenotype of chow-fed mice is mainly presented from the 4½-week cohorts because of the mild change in the phenotype during the treatments, but the mechanistic studies on mRNA level were analyzed from the 2-week cohorts to observe the primary, short-term mechanistic changes due to drug treatment.

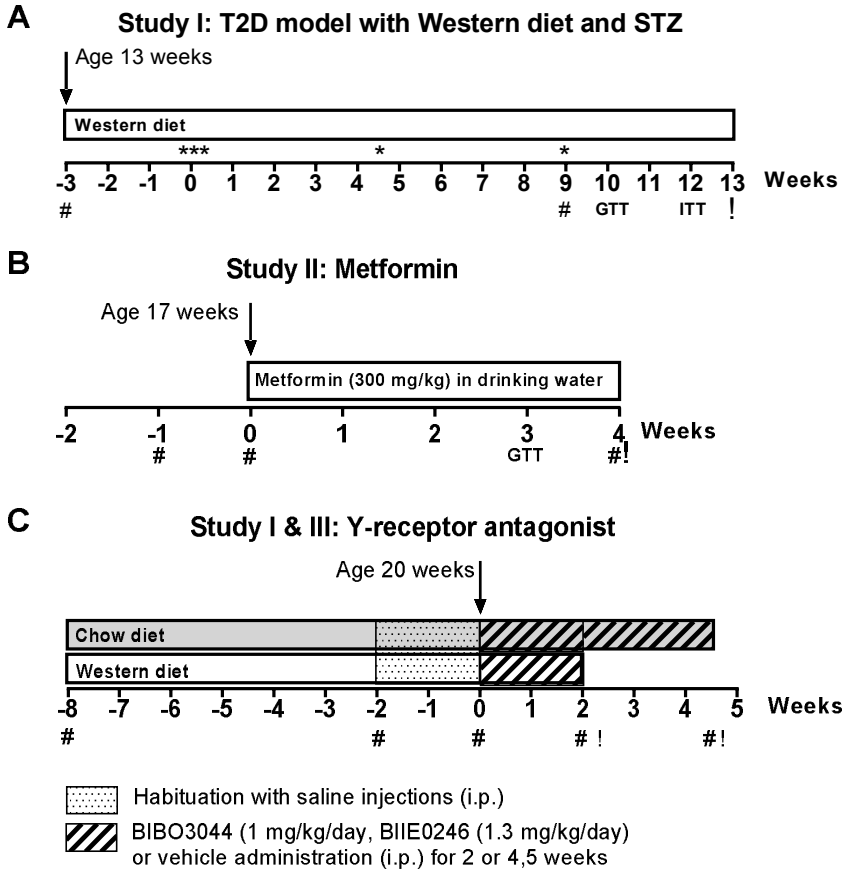

Figure 6 Experimental protocols of drug and diet intervention studies.

Homozygous OE-NPY$^{D\beta H}$ vs. wildtype mice were used in all studies. (A) Mice were subjected to a Western diet and low-dose of streptozotocin (STZ) to induce type 2 diabetes (T2D). (B) Mice on the chow diet were treated with metformin to detect its effect on the phenotype and glycogen structure. (C) Mice on the chow or Western diet were treated with Y_1- or Y_2-receptor antagonists (BIBO3044 and BIIE0246, respectively) to study the effect of drugs on their phenotype. # = Body composition measurement, * = STZ administration, ! = Sacrifice and tissue collection, GTT = Glucose tolerance test, ITT = Insulin tolerance test.

4.4.5.4 Physical activity and energy expenditure (Study I)

In order to study the effect of Y_1-receptor antagonist on energy expenditure, another set of 3-month-old WT mice (n = 4 / group) consuming a chow diet were treated with BIBO3304 or vehicle for 7 days (1 mg/kg/day, i.p.) preceded by a 7-day habituation with daily saline injections (i.p.). Total EE, usage of oxygen (VO_2), production of carbon

dioxide (VCO_2), RER and physical activity (horizontal moving and rearing) were measured with indirect calorimetry (Oxylet System, Panlab, Barcelona, Spain). After a 48 h baseline measurement, EE was monitored for 24 h after the first dose (acute administration) and for another 24 h after the last dose (chronic dosing). Body composition was measured before and after the treatments. Mice were randomly divided into the treatment groups according to their weight and fat mass, and EE, VO_2 and VCO_2 were assessed relative to lean body mass. Body weight and food intake were measured during the experiment.

4.4.6 Tissue collection

Mice were fasted for 3 (Y_1- and Y_2-receptor antagonist studies (in Studies I & III)) or 4 h (Studies I & II) before the sacrifice. In Studies I and III, mice were anesthetized with ketamine (75 mg/kg i.p.) and medetomidine (1 mg/kg i.p.) prior to obtaining terminal blood (0.5-1 ml) from vena cava or with cardiac puncture using a 25 G needle. In study II, the mice were sedated with CO_2 before blood collection. Euthanasia was performed by cervical dislocation. Blood was collected to heparin- or non-heparin-coated collection tubes to collect plasma (Study I) or serum (Studies I-III), respectively. Serum samples were incubated for 30 min at RT before centrifugation (4000 rpm for 10 min), and then stored at -70°C. The liver, WAT pads (mesenteric, epididymal, retroperitoneal and subcutaneous) and interscapular BAT were weighed. Liver sections for tissue specific analyses were dissected from the left lateral lobe. In order to isolate medial basal hypothalamus and brainstem, the brain was placed on a mouse brain block. A 3-mm section caudal to the optic nerve chiasma was cut to dissect the hypothalamus. In order to dissect the brainstem, a 3-mm section extending 2-mm caudal from the hypothalamic section was cut, and the cerebellum and cerebral cortex were excluded. Tissues for RNA isolation were placed in RNA Stabilization Reagent (RNAlater, Qiagen, Hilden, Germany) (+4ºC, o/n) (excluding WAT and BAT, which were snap-frozen in liquid nitrogen) and stored at -70ºC. Samples for histology were fixed in 10% formalin for 2-3 days, and stored in 70% (v/v) ethanol. Other samples were snap-frozen in liquid nitrogen prior to storage at -70ºC.

4.5 *Ex vivo* experiments: Beta oxidation assay (Study I)

In the fatty acid beta-oxidation assay, 50 mg of fresh liver tissue from homozygous 2-month-old OE-NPY$^{D\beta H}$ and WT mice (n = 6-8/group) was homogenized according to Rector, *et al.* (2008) in 200 µl of SET buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, 2 mM ATP), and the buffer volume was supplemented to yield a 20-fold (wt/vol) diluted sample. Liver tissue was homogenized on ice with a Potter S homogenizer (10 x 30 s at 1,200 rpm) in a 3-ml Potter-Elvehjem glass homogenization vessel. The assay was based on Noland, *et al.* (2007). Aliquots (40 µl) of the tissue homogenates were plated in duplicate in a 24-well cell culture plate. The reaction was started by adding 160 µl of reaction buffer (100 mM sucrose, 10 mM Tris-HCl, 10 mM KPO_4 , 100 mM KCl, 1 mM 4 $MgCl_2 \cdot 6H_2O$, 1 mM l-carnitine, 0.1 mM malate, 2 mM ATP, 0.05 mM CoA, 1 mM DTT (pH 7.4), 0.5% BSA and 200 µM [1-^{14}C]palmitate). The wells were equipped with a folded Whatman filter paper, which was injected with 200 µl of 1 N NaOH in order to collect $^{14}CO_2$. Culture plates were sealed with parafilm and allowed to incubate for 30 min at 37°C with shaking. Reactions were terminated by addition of 100 µl of 70% perchloric acid to the wells followed by 1 h-incubation at RT. The amount of oxidized $^{14}CO_2$ in the filter paper was determined by a liquid scintillation counter.

4.6 Biochemical analyses

4.6.1 Blood parameters

Insulin levels in serum were measured with an ultrasensitive mouse ELISA kit (Mercodia AB, Uppsala, Sweden), TGs with the TR0100 Serum triglyceride determination kit (Sigma-Aldrich), non-esterified fatty acids (NEFA) with the NEFA-HR(2) kit (Wako Diagnostics, Richmond, VA, USA), total cholesterol with the Cholesterol Fluorometric Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) or with Cholesterol Quantitation Kit (Biovision, Milpitas, CA, USA), and cholesterol fractions with an HDL and LDL/VLDL Cholesterol Quantification Kit (Biovision).

4.6.2 Hepatic lipids

Hepatic TGs and cholesterol were quantified with the TR0100 Serum triglyceride determination kit (Sigma-Aldrich) or with Cholesterol / CHOD-PAP Kit (Mti Diagnostics, Idstein, Germany), respectively. In order to determine TGs, the lipids in liver tissue were isolated and purified by the Folch method (Folch, *et al.* 1957) (Study I), or they were measured from the supernatant from the liver tissue (100 mg in 500 µl of PBS containing 0.1% NP-40) homogenized with Tissue Lyser (1 min 30 s; 50 Hz) and centrifuged for 2 min at full speed (Studies I & III). The hepatic cholesterol concentration was determined from this same supernatant.

4.6.3 Oxidative stress markers and cytokines (Study I)

The concentrations of serum cytokines (IL-6 and IL-10) were analyzed with the MILLIPLEX MAP Mouse Cytokine/Chemokine Multiplex Assay (MCYTOMAG-70K, Millipore Corporation, Billerica, MA, USA), resistin with the MILLIPLEX® Mouse Adipokine Multiplex Assay (MADKMAG-71K, Millipore Corporation) and adiponectin with the Mouse Adiponectin ELISA kit (EZMADP-60K, Millipore Corporation). Total peroxyl radical trapping antioxidant (TRAP) potential was measured in plasma as previously described (Ahotupa, *et al.* 1997). 8-Isoprostanes were analyzed from plasma, liver and urine with the STAT-8-Isoprostane EIA Kit (Cayman Chemical). Liver 8-isoprostane levels were normalized relative to the protein content determined by DC™ Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Urinary 8-isoprostane was normalized relative to the creatinine concentration measured by the Creatinine Colorimetric Assay Kit (Cayman Chemical). Glutathione reduced form (GSH) and glutathione oxidized form (GSSG) were measured in the liver with the Glutathione Assay Kit (Cayman Chemical), and diene conjugation as described previously (Corongiu & Milia. 1983). All of the procedures using the commercial kits were performed according to the manufacturer's instructions.

4.6.4 Histology

The samples for histology were embedded in paraffin and sectioned (5 µm) every 200 µm on microscopic slides. The morphology was characterized by hematoxylin and eosin

(H&E) staining and glycogen was visualized by periodic acid-Schiff (PAS) staining (Studies I & III). The level of hepatosteatosis in H&E stained liver samples was graded according to Cabezas, *et al.* (2012). Pancreatic areas of the Islets of Langerhans (n = 69-115/group) were measured using the Cell*A imaging software (Soft Imaging System, Münster, Germany). Lipid droplets were visualized from frozen liver samples sectioned with a cryostat (10 µm) and stained with Oil Red-O (ORO) (Sigma-Aldrich) (Study I).

4.6.5 Immunohistochemistry (Study I)

Rabbit anti-insulin (sc-9168, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) polyclonal antibody (1:500 dilution) was used for immunohistochemical staining of the beta cells. The average area of the stained islets (n = 5-15 / slide) was analyzed with ImageJ 1.48v (National Institutes of Health, USA). The number of islets in a representative slide was calculated, and the islets were grouped according to their size (ø<50µm, 50µm<ø<110µm, ø>110 µm).

4.6.6 Glycogen structure (Study II)

Glycogen structure was analyzed from primary hepatocytes isolated immediately after sacrificing the animals (n = 3-7/group) according to Kudryavtseva, *et al.* (1999). Dissected liver was perfused with 20 ml of Phosphate Buffer I (475 ml 0.066 M $Na_2HPO_4 \cdot 2H_2O$ + 25 ml 0.067 M KH_2PO_4 + 500 ml 0.15 M sucrose, pH=8.0). In this experiment, 2x2x2 mm pieces of liver were incubated in Buffer I (10 min RT) followed by another incubation (15 min) in Buffer II (400 ml 0.066 M $Na_2HPO_4 \cdot 2H_2O$ + 100 ml 0.067 M KH_2PO_4, pH=7.4) in order to isolate the hepatocytes on microscope slides.

Staining of different structures of glycogen with a fluorescent variant of the PAS reaction was based on the techniques of Bezborodkina, *et al.* (2011), Kudryavtseva, *et al.* (1999) and Kugler, *et al.* (1961). Hepatocytes were first incubated (1.5 h) in sodium periodate solution in diluted HNO_3 (200 mg of sodium periodate, 25 mL of 0.23% HNO_3), followed by staining with EtBr-SO_2 (100 mL 10^{-5} M ethidium bromide, 0.2 mL thionyl chloride) (40 min) in order to detect the labile glycogen structure (LF), and with Au-SO_2 (300 mg auramine, 100 mL water, 0.2 mL thionyl chloride) (50 min) to detect the stable glycogen structure (SF). Preparations were washed with distilled H_2O in between the stainings, and

the staining was followed by multiple washing periods with distilled H_2O (3 x 3 min), sulfurous H_2O (5 g potassium metabisulfite, 950 mL H_2O, 50 mL HCl) (3 x 3 min), and tap water (20 min), after which the samples were dehydrated with increasing concentrations of ethanol.

Samples were visualized and imaged by an Axioskop microscope (Carl Zeiss, Oberkochen, Germany), a Plan NEOFLUAR 40 × 0.75 lens, and a DFC360 FX digital black-and-white high-sensitivity CCD camera (1392 × 1400). Exciting fluorescence wavelengths for Au-SO_2 and EtBr-SO_2 were 450-490 and 546 nm and recording fluorescence wavelengths were 515-565 and 590 nm, respectively. The intensity of fluorescence from 100 cells of each sample was measured using ImageJ software. The total glycogen content (TGC) was counted as a sum of SF and LF.

In order to calculate the relative number of glucose residues in the outer tiers of the glycogen molecule, the theoretical formulation of the Whelan's model (Roach. 2002) was used by benefiting the data on the number of glucose chains. SF originates in the first eight tiers of the glycogen molecule and consists of 255 glucose chains. Therefore the ratio LF / SF ≤ 1 corresponds to the 9^{th} outer tier, the ratio LF / SF ≤ 2 corresponds to the 10^{th} outer tier, the ratio between LF / SF ≤ 4 corresponds to the 11^{th} outer tier, and the ratio between LF / SF ≤ 8 corresponds to the 12^{th} end tier of the molecule.

4.6.7 Gene expression analysis

RNA was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) (liver, WAT, BAT, intestine), RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) (liver), RNeasy Lipid tissue mini kit (Qiagen GmbH) (brain sections), or Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, CA, USA) (adrenal glands) with DNase treatment (RNase-Free DNase Set, Qiagen GmbH or Sigma DNase I, Sigma-Aldrich). The total RNA concentration and purity were ascertained spectrophotometrically (Lambda 20 UV/VIS Spectrometer, Perkin Elmer, Waltham, MS, USA) or BioSpec-nano Micro-volume UV-Vis Spectrophotometer (Shimadzu Scientific Instruments Columbia, MD, USA). mRNA was converted to cDNA with High Capacity RNA-to-cDNA Kit (Applied Biosystems) adhering to the manufacturer's instructions for conversion (37˚C 60 min, 95˚C 5 min, 4˚C ∞) on a GeneAmp PCR System 9600 (Perkin Elmer). The qPCR was performed with SYBR Green method with Kapa SYBR Fast qPCR Kit (Kapa

Biosystems, Woburn, MA, USA) with the manufacturer's instructions for qPCR program (95˚C 3 min, 40x (95˚C 3 s, 60˚C 30 s)) on 7300 Real Time PCR System (Applied Biosystems). The analysis was made using a standard curve (Study I) or $2^{-\Delta\Delta Ct}$ method (Studies I-III). mRNA expression was calculated relative to the expression of reference genes beta-actin (*Bact*) or ribosomal protein S29 (Rbs*29*). The primers used in the assay are shown in Appendix Table 1. In the microarray, mRNA samples were compared to 18918 genes on the Illumina MouseRef-8 v1.1 microchip (Study I).

4.7 Statistical analyses

The results were expressed as means ± SEM. $P<0.05$ was considered statistically significant. The results were analyzed with unpaired Student's t-test to compare the genotype differences. Two-way ANOVA test was used for comparing two genotypes and the additional challenge of drug treatment or diet intervention, and Bonferroni or Tukey's posthoc test was used to test the statistical difference between the genotypes in cases where the interaction genotype x treatment was significant or near-significant. Two-way ANOVA for repeated measures was used for analyzing parameters over time between genotypes and treatments. D'Agostino and Pearson omnibus normality test was used for analyzing the Gaussian distribution. Logarithmic transformations or non-parametric Mann-Whitney test were used, if data was not normally distributed. Correlations were analyzed with Pearson correlation coefficient. Most of the statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA) (Studies I-III). The microarray data (Study I) was quantile normalized prior to analyzing the results by *limma* of the R statistical analysis program (R language and environment for statistical computing). The filtered limit for the regulated genes was $|\log FC| = 0.5$ (FC=1.41). Pathway and cluster analysis were performed with DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp). Glycogen structure (Study II) was analyzed with SigmaPlot v 11.0 software (Systat Software Inc., San Jose, CA, USA).

5 RESULTS AND DISCUSSION

5.1 Hepatic fatty acid metabolism in OE-NPY$^{D\beta H}$ mice (Study I)

The first hypothesis of the study was that in addition to obesity and IGT (Vähätalo*, et al.* 2015), homozygous OE-NPY$^{D\beta H}$ mice would display hepatic steatosis. Liver weights, histology and adiposity were determined to characterize the hepatic phenotype of OE-NPY$^{D\beta H}$ mice. The livers of OE-NPY$^{D\beta H}$ mice were significantly enlarged compared to WT mice starting from the age of 4 months in males, and at 7 months in females (Table 3). Histological (H&E- and ORO-stainings) and biochemical analyses revealed ballooning degeneration at 4 months and hepatosteatosis at 7 months in male OE-NPY$^{D\beta H}$ mice (Fig. 7). Furthermore, microscopic infarcted areas without visible signs of inflammation or fibrosis were detected in 2 out of 4 individuals in the group of 7-month-old male OE-NPY$^{D\beta H}$ mice. Taken together, the livers of obese male OE-NPY$^{D\beta H}$ mice were graded as type 3 hepatosteatosis.

Table 3 Liver weights (g) of homozygous OE-NPY$^{D\beta H}$ in different age groups

Age	Female			Male		
	WT	OE-NPY$^{D\beta H}$	P value	WT	OE-NPY$^{D\beta H}$	P value
2 months	ND	ND	-	1.43±0.02	1.44±0.04	NS
4 months	1.19±0.06	1.26±0.05	NS	1.29±0.11	1.68±0.06	<0.05
7 months	1.05±0.02	1.14±0.07	<0.01	1.43±0.05	1.91±0.10	<0.001

Values are expressed as means ± SEM (n = 5-11/group). Statistics were analyzed with Student's t-test. ND = Not diagnosed, NS = Non-significant. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

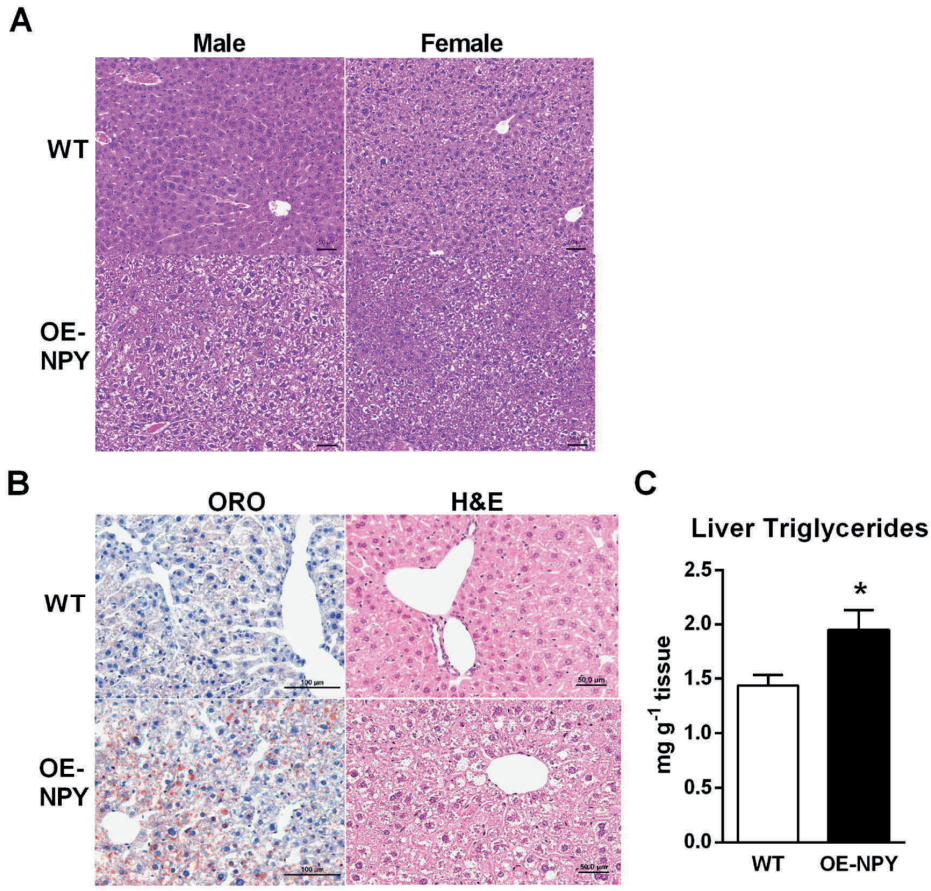

Figure 7 Hepatic histology and triglyceride accumulation of obese OE-NPY$^{D\beta H}$ mice

(A) Representative hematoxylin and eosin (H&E) stained liver specimens of homozygous 4-month-old male and female OE-NPY$^{D\beta H}$ vs. wildtype mice showing triglyceride accumulation and ballooning degeneration (scale bar 50µm). (B) Representative oil red O (ORO) (scale bar 100µm) and H&E (scale bar 50µm) stained liver specimens, and (C) quantitative triglyceride content of 7-month-old male OE-NPY$^{D\beta H}$ vs. wildtype mice (n=9-10/group) showing hepatosteatosis and ballooning degeneration. Values are expressed as means ± SEM. *P < 0.05 with Student's t-test. OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

Next, the possible mechanisms leading to hepatosteatosis were studied in pre-obese (heterozygous and homozygous) OE-NPY$^{D\beta H}$ vs. WT mice to avoid the changes in lipid metabolism induced by the obese phenotype of OE-NPY$^{D\beta H}$ mice. Expanding WAT increases hepatic fatty acid uptake, and steatosis changes both hepatic *de novo* lipogenesis and fatty acid oxidation (Postic & Girard. 2008), and may interefere with elucidating the actual mechanism of action of NPY. Male mice were used because of their more pronounced obese phenotype when they reach a mature age.

First, the hepatic gene expression profiles in the microarray analysis were compared between 2-month-old heterozygous OE-NPY$^{D\beta H}$ and WT mice. There were 27 up-regulated and 12 down-regulated genes by OE-NPY$^{D\beta H}$ genotype (Appendix Table 2). The up-regulated genes were involved in 8 biological pathways, of which the most significantly up-regulated pathways included those indicative of the recruitment of anti-oxidative and -inflammatory actions: 1) metabolism of xenobiotics by cytochrome P450, 2) drug metabolism and 3) glutathione metabolism. Down-regulated genes were involved in 5 biological pathways. The most significantly down-regulated pathways, PPAR-signaling and fatty acid metabolism pathways, indicate that hepatic fatty acid oxidation would be reduced in the livers of OE-NPY$^{D\beta H}$ mice (Table 4). In the cluster analysis, three clusters that separated the genes with similar actions from other genes were detected: 1) retinol, arachidonic acid and linoleic acid metabolism, 2) fatty acid oxidation, and 3) metabolic pathways with anti-oxidative and -inflammatory actions (discussed in chapter 5.4) (Table 5).

Table 4 Differently regulated hepatic pathways in the microarray analysis of OE-NPY$^{D\beta H}$ mice

Pathway name	Gene count	P value	FRD
Up-regulated pathways			
Metabolism of xenobiotics by cytochrome P450	6	<0.001	<0.001
Drug metabolism	6	<0.001	<0.001
Glutathione metabolism	4	<0.001	0.56
Retinol metabolism	4	0.001	1.23
Pyruvate metabolism	3	0.007	6.99
Linoleic acid metabolism	3	0.009	8.67
Down-regulated pathways			
Fatty acid metabolism	4	<0.001	0.00
PPAR signaling pathway	4	<0.001	0.00
Retinol metabolism	2	0.047	30.06

Pathway analysis was based on the microarray analysis of the mRNA in the livers of 2-month-old heterozygous OE-NPY$^{D\beta H}$ vs. wildtype mice (n=4/group). The analysis was performed with DAVID Bioinformatics Resources**.** FDR = False discovery rate (%), an adjusted P value, which estimates the probability of significant P value (< 0.05) being false. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

Table 5 Cluster analysis of hepatic up- and down-regulated genes in the microarray analysis of OE-NPY$^{D\beta H}$ mice

	Score	Gene count	P value	FDR
Cluster 1	3.97			
Retinol metabolism		6	<0.001	0.01
Arachidonic acid metabolism		5	<0.001	0.34
Linoleic acid metabolism		3	0.016	14.2
Cluster 2	3.34			
PPAR signaling pathway		6	<0.001	0.02
Fatty acid metabolism		5	<0.001	0.03
Cluster 3	2.85			
Metabolism of xenobiotics by cytochrome P450		6	<0.001	0.00
Drug metabolism		6	<0.001	0.01
Glutathione metabolism		4	0.001	1.2

Cluster analysis was based on the microarray analysis in the livers of 2-month-old heterozygous OE-NPY$^{D\beta H}$ vs. wildtype mice (n=4/group). The analysis was performed with DAVID Bioinformatics Resources**.** Biological pathways are clustered into homogenous clusters, which differ from other clusters: the higher score of the cluster, the higher homogeneity within the cluster. FDR = False discovery rate (%), an adjusted P value, which estimates the probability of significant P value (< 0.05) being false. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

Second, the most relevant genes in the microarray chip involved in fatty acid metabolism, which could explain the mechanism of hepatosteatosis of obese OE-NPY$^{D\beta H}$ mice, were selected for qPCR analysis. In addition, gate-keeping enzymes of fatty acid oxidation, (*Cpt1*) and lipogenesis (acetyl Co-A carbozylase (*Acc*)) were selected outside of the microarray results, as they have previously been shown to be targets of NPY (Zarjevski*, et al.* 1993, Zhang*, et al.* 2010). Although mechanistic changes were studied in pre-obese mice, the consequences of the obese phenotype were studied also at the mRNA level in obese homozygous OE-NPY$^{D\beta H}$ mice.

qPCR analysis showed significant decreases in the mRNA expression of the rate-limiting enzyme of peroxisomal oxidation, acyl-coenzyme A oxidase 1 (*Acox1*), and carnitine acetyltransferase (*Crat*) in pre-obese OE-NPY$^{D\beta H}$ mice, which support the finding of decreased fatty acid oxidation detected in the microarray analysis (Fig. 8A). Accordingly, an *ex vivo* beta-oxidation assay revealed decreased hepatic total palmitate oxidation to CO_2 in pre-obese homozygous OE-NPY$^{D\beta H}$ mice compared to WT mice (Fig. 8B). This confirmed the finding of decreased fatty acid oxidation preceding steatosis. The observation of decreased NPY-induced fatty acid oxidation was in line with the results of

Zhang, *et al.* (2010), which showed that KD of peripheral Y_1-receptors increased the amount of CPT1, suggesting that NPY decreases the mitochondrial fatty acid oxidation via hepatic Y_1-receptors. However, there was no significant difference in *Cpt1* expression between OE-NPY$^{D\beta H}$ and WT mice (Fig. 8A), which suggests that there are also other mechanisms involved in the way that NPY decreases fatty acid oxidation, and raises the question about the possibility of NPY-induced mitochondrial dysfunction. Furthermore, the decrease in the expression of *Acox1* suggests that the peroxisomal, i.e. very-long and branched chain fatty acid oxidation, is attenuated in OE-NPY$^{D\beta H}$ mice.

As fatty acid synthase (*Fas*) was up-regulated in the microarray analysis, and ACC has previously been shown to be activated by NPY (Zarjevski*, et al.* 1993), increased lipogenesis was considered as another possible mechanism to cause hepatosteatosis. However, there was only a tendency of increased expression of *Fas* ($P = 0.06$), and no difference in the expression of *Acc* in pre-obese mice. In obese OE-NPY$^{D\beta H}$ mice, the expression of *Fas* was increased by 2.5-fold, and there was a tendency towards increased expression of *Acc* ($P = 0.06$) (Fig. 8A) as well. However, enhancement of lipogenesis in obese OE-NPY$^{D\beta H}$ mice is more likely a consequence of hepatosteatosis and hyperinsulinemia than the actual cause, as they have previously been shown to increase *de novo* lipogenesis (Assimacopoulos-Jeannet*, et al.* 1995, Donnelly*, et al.* 2005, Matsuzawa-Nagata*, et al.* 2008).

Other mechanisms (increased uptake of FFA and secretion of VLDL-TG into other tissues) were not assessed or considered as a probable mechanism for hepatosteatosis, because other previous findings in OE-NPY$^{D\beta H}$ mice did not support these possibilities. Simultaneous accumulation of TGs into the liver and WAT, and suppressed lipolysis in WAT decrease the supply of FFAs (Vähätalo*, et al.* 2015), and thus it seems unlikely that there would have been any increased flux of lipids from WAT to the liver.

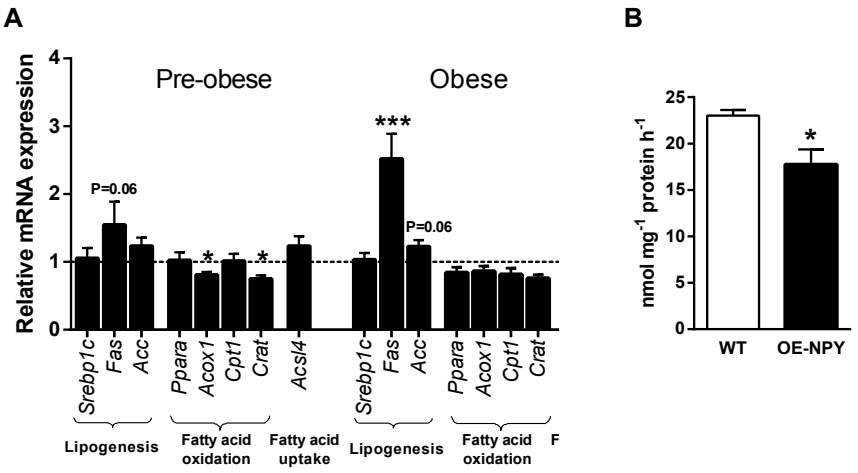

Figure 8 Hepatic fatty acid metabolism in OE-NPY$^{D\beta H}$ mice

(A) mRNA expression of selected genes involved in fatty acid metabolism in 2-month-old heterozygous (n=5/group) and 7-month-old homozygous (n=10/group) OE-NPY$^{D\beta H}$ mice (pre-obese and obese, respectively). Values are analyzed with a standard curve method, normalized to β-actin expression and expressed as a fold change relative to wildtype mice. (B) Total palmitate oxidation of 2-month-old homozygous OE-NPY$^{D\beta H}$ vs. wildtype mice (n=6-9/group). Values are expressed as means ± SEM. *P < 0.05 and ***P < 0.001 with Student's t-test. *Srebp1c* = Sterol regulatory element binding transcription factor1, *Fas* = Fatty acid synthase, *Acc* = Acetyl-coenzyme A carboxylase, *Ppara* = Peroxisome proliferator-activated receptor alpha, *Acox1* = Acyl-coenzyme A oxidase 1, *Cpt1* = Carnitine palmitoyltransferase1, *Crat* = Carnitine acetyltransferase, *Acsl4* = Acyl-coenzyme A synthetase long-chain family member 4, OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

5.2 Cholesterol metabolism in OE-NPY$^{D\beta H}$ mice (Study I)

As peripheral NPY administration has previously been shown to cause obesity accompanied with increased serum cholesterol levels in rats (Xie*, et al.* 2012), and furthermore, hypercholesterolemia and atherosclerosis have been shown to associate to *NPY* variant rs16139 (Karvonen*, et al.* 1998, Karvonen*, et al.* 2001), it was hypothesized that obese OE-NPY$^{D\beta H}$ mice would also display hypercholesterolemia as a symptom of the MetS. As expected, male OE-NPY$^{D\beta H}$ mice displayed significantly increased total cholesterol levels at 7 months with a tendency towards increased cholesterol at 4 months (P = 0.08). In females, hypercholesterolemia was less pronounced and with a later onset (P = 0.07 at 7 months) (Table 6). HDL, which is the main component of circulating cholesterol in mice, was significantly increased already at 4 months in both sexes. Cholesterol metabolism between humans and mice is somewhat different, and in mice, elevated total cholesterol levels in serum together with elevated HDL cholesterol values

associate with T2D and atherosclerosis (Kozarsky, *et al.* 2000, Nishina, *et al.* 1994), which suggests that the increased HDL concentration in OE-NPY$^{D\beta H}$ mice would not be beneficial. The VLDL/LDL fraction, which is normally considered as "bad cholesterol" in humans, was not changed in OE-NPY$^{D\beta H}$ mice. However, as TG is the main component of VLDL, and TG levels are decreased in serum of OE-NPY$^{D\beta H}$ mice (Vähätalo, *et al.* 2015) this may compensate for the level of VLDL/LDL to the level of control mice.

Table 6 Cholesterol levels in serum of obese mice (Original publication I)

	4 months			7 months		
Female	**WT**	**OE-NPY$^{D\beta H}$**	**P value**	**WT**	**OE-NPY$^{D\beta H}$**	**P value**
Total Cholesterol (µg/µl)	1.65±0.15	1.90±0.14	0.12	0.42±0.08	0.59±0.08	0.07
HDL Cholesterol (µg/µl)	1.17±0.08	1.40±0.06	<0.05	0.34±0.02	0.40±0.02	<0.05
LDL/VLDL Cholesterol (µg/µl)	ND	ND	-	0.17±0.01	0.13±0.03	0.27

	4 months			7 months		
Male	**WT**	**OE-NPY$^{D\beta H}$**	**P value**	**WT**	**OE-NPY$^{D\beta H}$**	**P value**
Total Cholesterol (µg/µl)	1.64±0.12	2.08±0.20	0.08	1.44±0.12	2.03±0.12	<0.01
HDL Cholesterol (µg/µl)	1.46±0.05	1.68±0.06	<0.05	0.73±0.03	0.83±0.07	<0.05
LDL/VLDL Cholesterol (µg/µl)	0.37±0.06	0.25±0.02	0.08	0.11±0.02	0.11±0.02	0.98

The data is represented from 4- and 7-month-old homozygous OE-NPY$^{D\beta H}$ vs. wildtype (WT) mice (n=7-11/group). Values are expressed as means ± SEM. Statistics were analyzed with Student's t-test. HDL = High-density lipoprotein, LDL = Low-density lipoprotein, VLDL = Very low-density lipoprotein, ND = Not diagnosed. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

To elucidate the mechanism of NPY to cause hypercholesterolemia, the whole body cholesterol metabolism (i.e. intestinal cholesterol absorption, and hepatic cholesterol and bile acid synthesis) was studied *in vivo* by the fecal ratio method. Obese female OE-NPY$^{D\beta H}$ mice were used in the study, as they display hypercholesterolemia but the obese phenotype is otherwise less pronounced. Hepatic cholesterol synthesis has been previously reported to be activated by hepatosteatosis and the MetS (Gylling, *et al.* 2007, Simonen, *et al.* 2011), and the aim of using the female mice in this study, was to avoid the changes in cholesterol metabolism induced by the obese phenotype.

OE-NPY$^{D\beta H}$ mice displayed accelerated cholesterol and bile acid synthesis (P = 0.06) compared to WT mice *in vivo* (Fig. 9A, B), and the enhancement of cholesterol synthesis was confirmed by increased expression of cholesterol synthetizing enzymes, 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), farnesyl-diphosphate farnesyl transferase 1

(*Fdft1*) and 7-dehydrocholesterol reductase (*Dhcr7*), both in pre-obese and obese states (Fig. 9C). Furthermore, the tendency towards decreased (P = 0.08) expression of HDL-receptor (*Srb1*) in obese male OE-NPY$^{D\beta H}$ mice may explain increased HDL levels in serum. The expression of LDL-receptor (*Ldlr*) was not different between the genotypes (Fig. 9C). Cholesterol absorption, measured *in vivo* by the fecal ratio method, was not changed, and accordingly, the expression of cholesterol influx or efflux proteins in the intestine was similar between the genotypes (Original publication I).

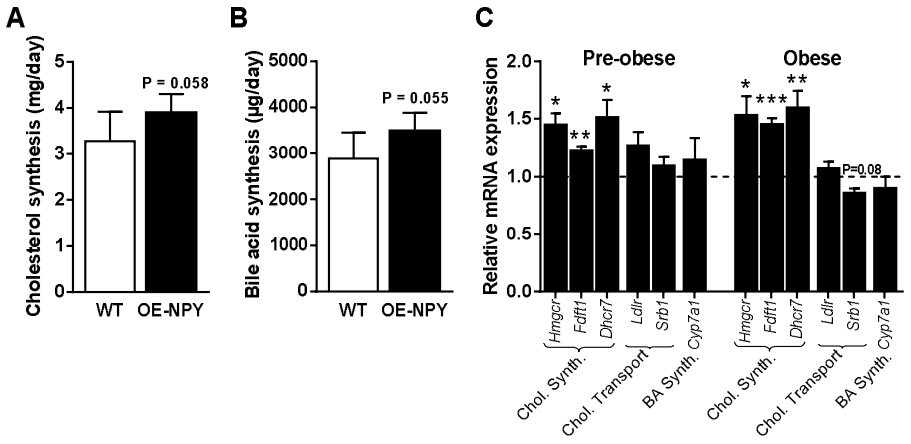

Figure 9 Cholesterol metabolism in OE-NPY$^{D\beta H}$ mice

(A) Cholesterol and (B) bile acid synthesis in homozygous female OE-NPY$^{D\beta H}$ vs. wildtype mice at 6 months measured by fecal ratio method (n = 5-10/group). (C) mRNA expression of cholesterol (Chol.) and bile acid (BA) synthesis enzymes in the livers of 2-month-old heterozygous (n=5/group) and 7-month-old homozygous (n=10/group) OE-NPY$^{D\beta H}$ mice (pre-obese and obese, respectively). Values are expressed as means ± SEM. mRNA expression values were analyzed with standard curve method, normalized to β-actin expression and expressed as a fold change relative to wildtype mice. *P < 0.05, **P < 0.01 and ***P < 0.001 with Student's t-test. *Hmgcr* = 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *Fdft1* = Farnesyl diphosphate farnesyl transferase 1, *Dhcr7* = 7-dehydrocholesterol reductase, *Srb1* = Scavenger receptor, class B, type 1, *ldlr* = LDL-receptor, *Cyp7a1* = Cytochrome P450 7a1, OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice.

5.3 Glucose metabolism in OE-NPY$^{D\beta H}$ mice (Study I)

5.3.1 Glucose metabolism in early and established impaired glucose tolerance

Male OE-NPY$^{D\beta H}$ mice have been previously shown to display IGT starting from the age of 4 months, and IR and fasting hyperinsulinemia at 7 months (Vähatalo*, et al.* 2015). In order to examine whether this resulted from an inhibition of insulin release induced by increased peripheral NPY (Cho & Kim 2004), or whole body IR, insulin levels in serum were measured during GTT from 4-month-old OE-NPY$^{D\beta H}$ and WT mice (n = 7/group). Glucose levels were similar between the genotypes during GTT, but OE-NPY$^{D\beta H}$ mice displayed higher insulin levels (Fig. 10A-B), which indicates IR already at 4 months. Furthermore, the Islets of Langerhans in the pancreas were enlarged in 7-month-old OE-NPY$^{D\beta H}$ mice (WT 0.013±0.001 mm^2; OE-NPY$^{D\beta H}$ 0.020±0.002 mm^2; P<0.001; n=69-115 islets/group), which is in line with the previously detected fasting hyperinsulinemia detected when the mice were at a mature age (Vähätalo*, et al.* 2015).

In order to characterize the hepatic IR, a situation in which there is known to be accelerated hepatic gluconeogenesis, OE-NPY$^{D\beta H}$ and WT mice were challenged with pyruvate, which activates gluconeogenesis in the liver, and glucose. Four-month-old OE-NPY$^{D\beta H}$ mice displayed normal glucose tolerance and decreased gluconeogenic rate, whereas their 7-month-old counterparts showed clear evidence of impaired glucose clearance but a similar gluconeogenic rate as compared to WT mice (Fig. 10C-D). These results do not suggest that OE-NPY$^{D\beta H}$ mice have hepatic IR. Instead, all the results together indicate that the age of 4 months in OE-NPY$^{D\beta H}$ mice is a threshold age, when the first signs of IGT appear and IR is evident. However, elevated blood glucose and IR are still effectively compensated by increased insulin secretion and by storing excess energy (e.g. pyruvate) into the liver (and WAT). As the energy accumulates with aging (at 7 months), IGT with enlarged pancreatic islets and fasting hyperinsulinemia become established.

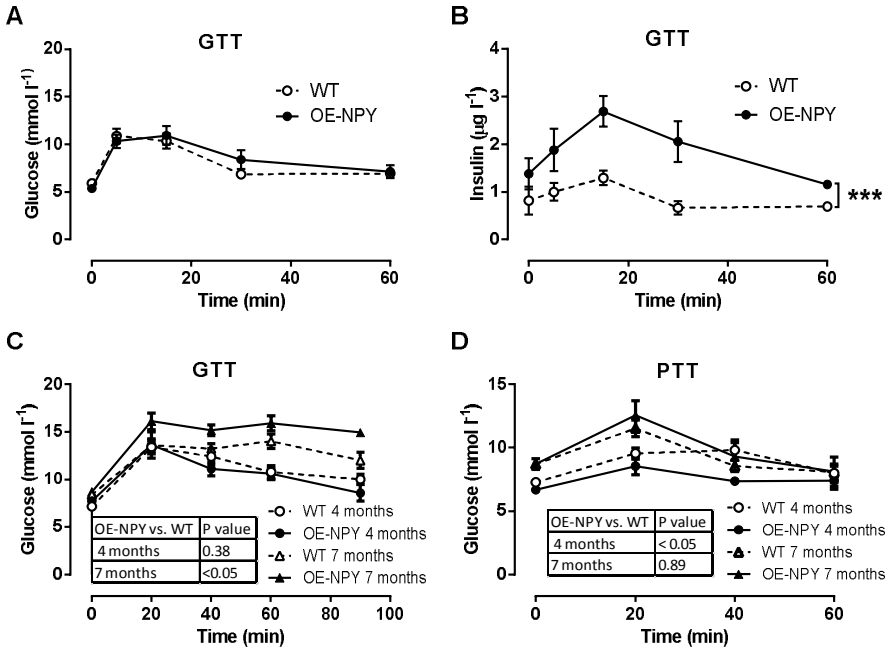

Figure 10 Glucose metabolism in OE-NPY$^{D\beta H}$ mice

(A) Glucose tolerance test (GTT) and (B) glucose-induced insulinemia in 4-month-old homozygous OE-NPY$^{D\beta H}$ vs. wildtype mice (n = 7/group). (C) GTT and (D) pyruvate tolerance tests (PTT) of 4- and 7-month-old homozygous OE-NPY$^{D\beta H}$ vs. wildtype mice (n = 6-11/group). Values are expressed as means ± SEM. ***P<0.001 with repeated measures ANOVA. OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice.

5.3.2 Susceptibility to type 2 diabetes (Study I)

Hepatosteatosis, IGT and IR increase the risk of the onset of T2D. As the incidence of T2D is known to be more common among the carriers of *NPY* variant (rs16139) as compared to non-carriers (Jaakkola, *et al.* 2006, Nordman, *et al.* 2005, Ukkola & Kesaniemi. 2007), it was hypothesized that OE-NPY$^{D\beta H}$ mice would also be more prone to T2D than their WT counterparts. In order to study the susceptibility of OE-NPY$^{D\beta H}$ mice to T2D, mice were exposed to WD combined with low doses of STZ. Feeding with WD induces similar obesity and IR in both genotypes, but on its own it is not sufficient to induce T2D in OE-NPY$^{D\beta H}$ mice (Ruohonen, *et al.* 2012). This may be due to their ability to effectively compensate for the increased glucose levels by enhancing insulin secretion, as discussed above. This discrepancy was overcome with low-dose of STZ,

which disturbs insulin secretion without leading to complete deficiency of insulin (Gilbert, *et al.* 2011).

The mice consuming the WD gained weight and fat mass similarly during the study, but food intake was increased in OE-NPY$^{D\beta H}$ mice throughout the study (Fig. 11). The difference in food intake between the genotypes was especially apparent after each STZ administration, as the reduction in food intake was lower in OE-NPY$^{D\beta H}$ mice. There are several options for explaining this phenomenon: despite their similar baseline food intake, stress may trigger hyperphagia in OE-NPY$^{D\beta H}$ mice (Vähätalo, *et al.* 2015). It is also possible that STZ-induced acute hypoglycemia was stronger in OE-NPY$^{D\beta H}$ mice, which could explain the difference in food intake particularly after the injections. However, as glucose levels were not measured after the administration of STZ, and the hypoglycemia is only based on the observations of the mouse behavior, this possible explanation remains only as a speculation.

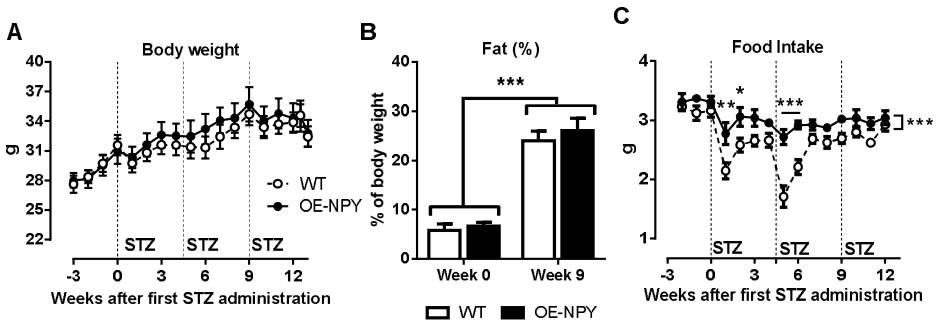

Figure 11 Body composition and food intake in OE-NPY$^{D\beta H}$ mice subjected to Western diet and low-dose streptozotocin

(A) Body weight gain, (B) adiposity and (C) food intake in homozygous OE-NPY$^{D\beta H}$ and wildtype mice subjected to Western diet at age of 13 weeks and 3 weeks later to low-dose of streptozotocin (STZ) (40 mg/kg, i.p.) (n = 7/group). Values are expressed as means ± SEM. *P<0.05 and ***P<0.001 with two-way ANOVA (B) or with repeated measures ANOVA (C). OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice.

OE-NPY$^{D\beta H}$ mice responded to the destruction of beta cells more strongly by displaying fasting (4 h) hyperglycemia with diabetic levels (over 13.8 mmol/l in mice) after 4 weeks of the first STZ administration (Fig. 12A). Furthermore, they displayed an augmented response in GTT compared to WT mice (Fig. 12B), despite one OE-NPY$^{D\beta H}$ mouse (out of 8) having to be removed from the test because of its extremely high fasting blood glucose level (> 28 mmol/l), and two mice (out of the rest 7 mice) reaching blood glucose

values over the detection level (28 mmol/l) at the 20 min time point. In ITT, both genotypes displayed equal diet-induced IR (Fig. 12C). However, it is possible that the dose of insulin (0.75 IU/kg) was not adequate for WD-fed mice, and it is possible that there would have been a difference between the genotypes, if a higher dose had been used. Fasting insulin levels in serum were similarly low in both genotypes (Fig. 12D), as were the amount, size and intensity of insulin antibody stained areas in the pancreas (Original publication I), suggesting that STZ-induced beta cell loss had been equivalent in both genotypes. There was a tendency that the livers of OE-NPY$^{D\beta H}$ mice were enlarged (WT 1.59±0.08 g; OE-NPY$^{D\beta H}$ 1.90±0.14 g; P = 0.08), and the relative liver weights correlated positively with mean fasting blood glucose values (P < 0.001, r^2 = 0.62). Thus, OE-NPY$^{D\beta H}$ mice displayed increased susceptibility to T2D, when beta cell function was disturbed. Furthermore, it seems that liver function is involved in the disease, as the liver weight associates with the accumulation of energy (in the form of TGs and glycogen) into the liver.

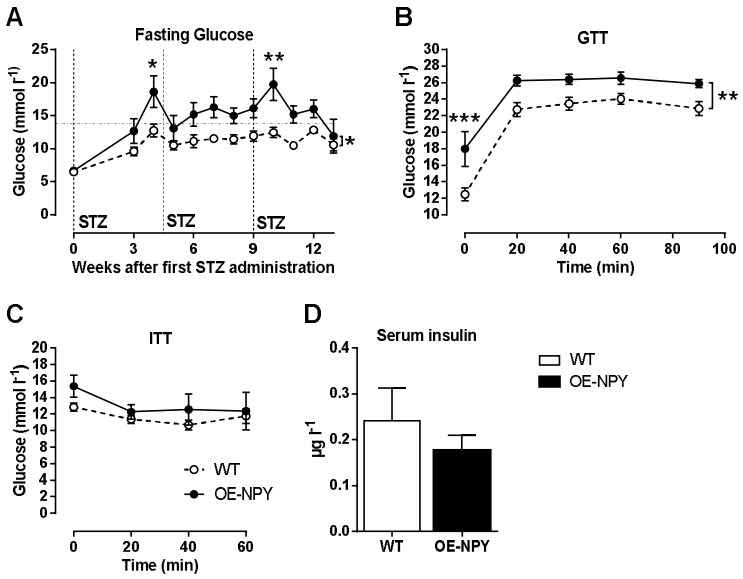

Figure 12 Glucose metabolism in OE-NPY$^{D\beta H}$ mice consuming a Western diet and administered low-dose streptozotocin

(A) Fasting blood glucose, (B) glucose (GTT) and (C) insulin tolerance test (ITT), and (D) serum insulin levels in homozygous OE-NPY$^{D\beta H}$ and wildtype mice (n = 7/group) consuming a Western diet at age of 13 weeks and 3 weeks later administered a low-dose of streptozotocin (STZ) (40 mg/kg, i.p.) (n = 7/group). Values are expressed as means ± SEM. *P<0.05, **P<0.01 and ***P<0.001 with repeated measures ANOVA. Serum insulin is analyzed with Student's t-test. OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice.

5.3.3 *Glycogen metabolism (Studies I & II)*

In order to study the role of glycogen on IGT of OE-NPY$^{D\beta H}$ mice, liver samples of obese (7-month-old) male OE-NPY$^{D\beta H}$ mice were first stained with PAS, which revealed glycogen accumulation (Fig. 13A). This, together with the accumulation of TGs, may explain the enlargement of the livers of OE-NPY$^{D\beta H}$ mice. Glycogen metabolism is regulated by phosphorylation of the glycogen forming enzyme, glycogen synthase, and glycogen degrading enzyme, glycogen phosphorylase. Physiologically, glycogen accumulates during the postprandial state, and it is degraded to glucose during fasting. In case of OE-NPY$^{D\beta H}$ mice, glycogen accumulation seems to be a result of the overall acceleration of glycogen cycling in the pre-obese state, as mRNA expression of hepatic glycogen synthase (*Gys2*) and phosphorylase (*Pygl*) enzymes were both increased in pre-obese (2-month-old heterozygous) OE-NPY$^{D\beta H}$ mice (Fig. 13B). Instead in obese state (at 7 months), when accumulation of glycogen was already present, there was no significant change in the expression of these enzymes. This data is in line with the phenotype of OE-NPY$^{D\beta H}$ mice, as both accumulation of glycogen and increased glycogen metabolism are associated with diabetes (Brereton, *et al.* 2016, Hundal, *et al.* 2010).

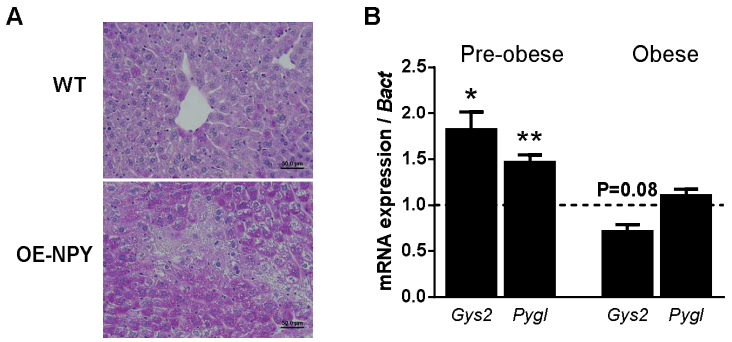

Figure 13 Glycogen accumulation and metabolism in OE-NPY$^{D\beta H}$ mice

(A) Representative periodic acid-Schiff (PAS) stained liver specimens showing glycogen accumulation into the hepatocytes of 7-month-old homozygous OE-NPY$^{D\beta H}$ vs. wildtype mice (scale bar 50µm). (B) mRNA expression of glycogen synthase and phosphorylase enzymes in 2-month-old heterozygous (n = 5/group) and 7-month-old homozygous (n = 10/group) OE-NPY$^{D\beta H}$ mice (pre-obese and obese, respectively). mRNA expression values were analyzed with $2^{-\Delta\Delta Ct}$ method, normalized to β-actin expressions and expressed as a fold change relative to wildtype mice. Values are expressed as means ± SEM. *P<0.05 and **P<0.01 compared to WT mice with Student's t-test. *Gys2* = Glycogen synthase 2, *Pygl* = Liver glycogen phosphorylase, OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice.

Glycogen metabolism and its possible role on the glucose metabolism of OE-NPY$^{D\beta H}$ mice were further elucidated by treating mice with a well-known anti-hyperglycemic agent, metformin. First, the effect of metformin was studied *in vivo* to characterize, whether it could improve their phenotype. Then, the hepatocytes were isolated and the different glycogen structures were analyzed *ex vivo* from naïve and metformin treated mice. The age of the mice in this study was 4 months, the previously shown borderline age for the first symptoms of IGT. OE-NPY$^{D\beta H}$ and WT mice fed on chow diet were treated with metformin for 4 weeks and compared to mice without drug treatment.

Due to single housing, which induced mild obesity also in WT mice, OE-NPY$^{D\beta H}$ mice were not different from WT mice with respect to body weight or composition prior to metformin treatment (Original publication II). Over the treatment period, metformin significantly decreased body weight and fat mass gain in both genotypes. It seems that the metformin-induced decline in fat gain was mainly due to visceral fat and more pronounced in WT mice, as the mesenteric WAT depot was significantly decreased in WT mice, and there was a tendency towards a decreased retroperitoneal WAT depot ($P = 0.075$) in both genotypes in the groups treated with metformin. Food intake was similar in all groups over the treatment period (Original publication II).

Blood glucose in fasting (4 h) and during GTT was similar between the treatment groups and genotypes, showing IGT in all groups (Fig. 14A-B), which is in line with the similar elevated body weights and adiposity between the genotypes. However, metformin inhibited glucose increase during the first 20 min period (Area under curve (AUC) 0-20 min) as compared to control treated mice with no effect on the clearance rate of glucose (at 20-90 min) (Fig. 14C-D). WT mice displayed a better improvement in glucose tolerance, probably due to their larger decrease in visceral fat. Metformin did not change the expressions of its known gluconeogenic targets (Petersen, *et al.* 2017), *Pck1*, *G6pc* or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1a*) (Original publication II), probably due to lack of IR or T2D in these mice.

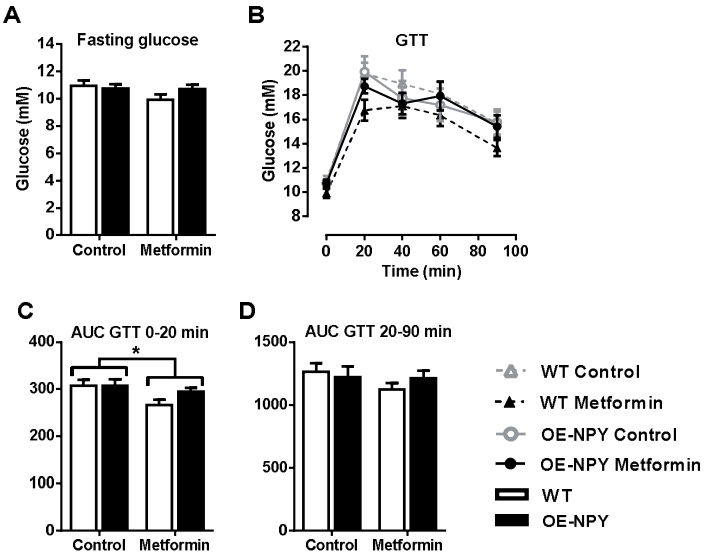

Figure 14 Glucose metabolism in OE-NPY$^{D\beta H}$ mice treated with metformin

(A) Fasting (4h) blood glucose, (B) glucose tolerance test (GTT), and (C) area under curve (AUC) of GTT at 0-20 min and (D) 20-90 min periods in 5-month-old homozygous OE-NPY$^{D\beta H}$ and wildtype mice (n = 9-11/group) treated with metformin for 3 weeks vs. control treatment. Values are expressed as means ± SEM. Values are expressed as means ± SEM. * $P<0.05$ between treatment groups with two-way ANOVA. GTT was analyzed with repeated measures ANOVA. OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice.

Glycogen metabolism, the total amount of glycogen (TGC) and its different structures (LF and SF) were analyzed by a cytofluorometric technique from the hepatocytes of OE-NPY$^{D\beta H}$ and WT mice. The hepatocytes analyzed from the naïve mice (i.e. without metformin treatment) differed with respect to the structure of glycogen between the genotypes, but the TGC was not changed. The changes in the structure, i.e. decreased content of the mature form of glycogen, LF, and increased content of the intermediate form of glycogen, SF, resulted in a reduced LF/SF ratio (Table 7). This indicates that there was an enhancement of glycogen synthesis in OE-NPY$^{D\beta H}$ mice as compared with WT mice. Similar pathogenic structural changes leading to a more stable glycogen accumulation have previously been detected in liver cirrhosis (Bezborodkina, *et al*. 2011, Kudryavtseva, *et al*. 1998). In contrast, with respect to the physiological type of glycogen synthesis (i.e. when glucose is administered after a prolonged fast), at first the content of SF increases quickly, and the LF increases more slowly and is responsible for the actual physiological accumulation of glycogen (Kudryavtseva, *et al.* 2003, Kudryavtseva, *et al.* 2001, Kudryavtseva, *et al.* 1996, Kudryavtseva, *et al.* 1998).

The filling of glucose molecules on the outer tiers of the glycogen molecule was analyzed by Whelan's model (Roach. 2002). The filling of the 10th outer tier by 81.7% in WT mice was interpreted as a constant regulation of blood glucose, but the significantly decreased filling (58.4%) of the 10th tier in OE-NPY$^{D\beta H}$ mice suggested enhanced activity of glycogen phosphorylase (Table 8), i.e. increased degradation of glycogen to glucose, which may induce IGT with aging. Together with the decreased LF/SF ratio and decreased filling of the 10th outer tier, it is postulated that there is an overall increase of glycogen metabolism, which is in agreement with the results detected at the mRNA level in pre-obese OE-NPY$^{D\beta H}$ mice.

Table 7 The contents of glycogen structures in the hepatocytes of OE-NPY$^{D\beta H}$ mice treated with metformin

Genotype	Treatment	SF	LF	TGC	LF / SF
WT	Ctr	10.6±0.4	17.6±0.6	28.5±1.0	1.6±0.0
	Met	9.3±0.4	15.4±0.6	25.4±1.4	1.5±0.0
OE-NPY	Ctr	14.3±0.5**	12.9±0.4**	30.9±1.0	0.6±0.0***
	Met	5.5±0.2$^{***\#\#\#}$	10.7±0.4$^{***\#}$	16.5±0.7$^{***\#\#\#}$	1.7±0.0$^{\#\#\#}$

The data is represented as mean ± SEM (n = 3 mice / group). **P < 0.01 and ***P < 0.001 compared to WT-Ctr mice, and $^{\#}$ P < 0.05, $^{\#\#\#}$ P < 0.001 compared to OE-NPY$^{D\beta H}$–Ctr mice with Student's t-test. LF = labile structure of glycogen, SF = stable structure of glycogen, TGC = total glycogen content, Ctr = Control group, receiving water without metformin, Met = Metformin treated group, OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice. Reprinted with permission from Ailanen *et al.* Pharmacol Res Perspect. 2018, 6(2):e00389.

Table 8 The filling of outer tiers of glycogen molecules with respect to the content of glucose residues (%) in the hepatocytes of OE-NPY$^{D\beta H}$ mice treated with metformin

Genotype	Treatment	Outer tier		
		9th	10th	11th
WT	Ctr	100	81.7	-
	Met	100	83.7	-
OE- NPY	Ctr	100	58.4	-
	Met	100	100	52.5

The calculations were performed from 100 hepatocytes with Whelan's model. Ctr = control group, receiving water without metformin, Met = metformin treated group, OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice. Reprinted with permission from Ailanen *et al.* Pharmacol Res Perspect. 2018, 6(2):e00389.

Treatment with metformin decreased the TGC in OE-NPY$^{D\beta H}$ mice, as well as both glycogen structures so that the LF/SF ratio was normalized to the level of the WT mice (Table 7). A similar treatment effect was not detected in WT mice, probably because the glycogen content was normal. Furthermore in the hepatocytes of metformin treated OE-NPY$^{D\beta H}$ mice, there was an increase in the glycogen residues in the 10th outer tier and a composition also on the 11th outer tier of the glycogen molecule, which suggested that there was a reduction in the activity of glycogen phosphorylase (Table 8). This was supported by the decreased expression of *Pygl* in metformin treated OE-NPY$^{D\beta H}$ mice (Fig. 15). This points to metformin's ability to inhibit glucose production from glycogen. Similar findings have been previously observed in T2D (Chu, et al. 2000, Wang, et al. 2016, Xu, et al. 2015).

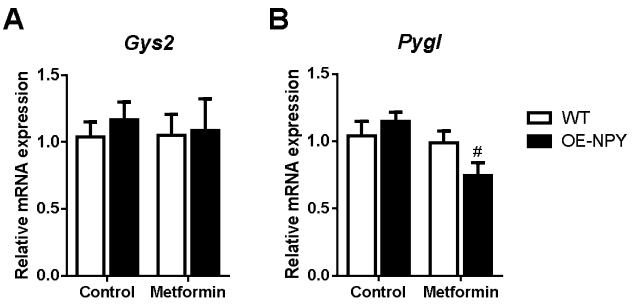

Figure 15 mRNA expression of enzymes involved in glycogen metabolism in OE-NPY$^{D\beta H}$ mice treated with metformin

(A) mRNA expression of glycogen synthase and (B) phosphorylase enzymes in the livers of 5-month-old homozygous OE-NPY$^{D\beta H}$ and wildtype mice after 4 weeks of receiving water with or without metformin (300mg/kg/day) (n = 9-11 / group). mRNA expression values were analyzed with $2^{-\Delta\Delta Ct}$ method, normalized to geometrical mean of expressions of β-actin and S29, and expressed as a fold change relative to control treated wildtype mice. Values are expressed as means ± SEM. $^{\#}$ P<0.05 between treatment groups in OE-NPY$^{D\beta H}$ mice with Bonferroni posthoc test, after detecting a significant genotype x treatment interaction in two-way ANOVA . *Gys2* = Glycogen synthase 2, *Pygl* = Liver glycogen phosphorylase, OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice. Reprinted with permission from Ailanen *et al.* Pharmacol Res Perspect. 2018, 6(2):e00389.

These results suggest that overactivity of glycogen metabolism and changes in its structure confirm the ability of NPY to store energy, similarly to the situation detected for PTT and fatty acid metabolism. In established obesity, also changes in glycogen metabolism have been associated with the IGT of OE-NPY$^{D\beta H}$ mice, and may also be associated with their increased susceptibility to T2D. However, metformin seems to be a plausible treat-

ment also when glycogen is considered as an important component of the disease. Furthermore, metformin's ability to delay or prevent the onset of T2D may be attributable to its actions on glycogen metabolism.

5.4 Inflammation and oxidative stress in OE-NPY$^{D\beta H}$ mice (Study I)

Inflammation and oxidative stress are associated with obesity and they may enhance the development of IR. The microarray analysis suggested that anti-inflammatory and anti-oxidative actions had been recruited in the livers of pre-obese OE-NPY$^{D\beta H}$ mice (chapter 5.1. Tables 4 and 5). The recruitment of anti-inflammatory and anti-oxidative mechanisms are usually a sign of activation of defense pathways against an abnormal condition in the body. However, as this was detected in pre-obese OE-NPY$^{D\beta H}$ mice, which were still healthy, the inflammatory and oxidative responses together with the increased adiposity could reinforce the onset of IR in these mice. Therefore, cytokine levels and oxidative stress markers were assayed in blood, liver and urine. In pre-obese OE-NPY$^{D\beta H}$ mice, the levels of the anti-inflammatory cytokine IL10, which has been shown to protect from hepatosteatosis and IR (Dagdeviren, *et al.* 2016, Paredes-Turrubiarte, *et al.* 2016), were decreased in serum ($P = 0.06$) and this decline may enhance the inflammatory response (Table 9).

In obese male OE-NPY$^{D\beta H}$ mice, there were no differences in the levels of IL6, IL10, adiponectin or markers of oxidative stress (8-isoprostane and TRAP) in blood, but resistin levels were increased. As stated before, resistin is known to induce IGT and IR (Rajala, *et al.* 2003, Steppan, *et al.* 2001) and to associate with obesity, inflammation and T2D (Fu, *et al.* 2006, McTernan, *et al.* 2003, Shetty, *et al.* 2004). Although an expanded adipose tissue may increase resistin levels, NPY also independently stimulates its production (Kuo, *et al.* 2007, Yuzuriha, *et al.* 2003). Thus, also high resistin levels seem to enhance the development of IR in OE-NPY$^{D\beta H}$ mice. In the livers of obese OE-NPY$^{D\beta H}$ mice, 8-isoprostane levels were increased, which indicates that there was lipid peroxidation and this may impair liver function. However, the concentrations of other markers of oxidative stress (i.e. diene conjugates, GSH or GSSG levels, or GSH/GSSG ratio) in the liver, serum or urine were not changed. Serum resistin and adiponectin levels were analyzed also in female OE-NPY$^{D\beta H}$ mice, which displayed no difference in resistin

levels, but higher adiponectin levels; this may explain their less prominent metabolic disturbances (Table 9).

Table 9 Cytokines and oxidative stress markers in OE-NPY$^{D\beta H}$ mice

	Pre-obese			Obese		
Plasma	**WT**	**OE-NPY**	**P value**	**WT**	**OE-NPY**	**P value**
IL6 (pg/ml)	10.8±3.7	10.6±7.4	0.87	3.3±0.0	8.7±3.2	0.78
IL10 (pg/ml)	17.8±5.1	8.8±3.4	0.06	20.6±9.3	23.8±6.1	0.58
TRAP	ND	ND	-	216.4±14.3	231.3±8.0	0.39
8-Isoprostane (pg/ml)	ND	ND	-	326.3±16.6	347.3±13.8	0.35
Urine						
8-Isoprostane / Creatinine	ND	ND	-	0.05±0.00	0.04±0.01	0.33
Liver						
Diene conjugation (pmol/ml)	ND	ND	-	651.7±16.2	672.1±7.9	0.51
8-Isoprostane (pg/mg)	ND	ND	-	70.4±3.1	83.4±4.5	<0.05
GSH (pg/mg)	ND	ND	-	30.3±1.6	31.7±0.7	0.45
GSSG (pg/mg)	ND	ND	-	7.9±0.5	8.0±0.4	0.87
GSH / GSSG (pg/mg)	ND	ND	-	0.26±0.01	0.26±0.01	0.55
mRNA expression						
Il1b	0.92±0.33	0.70±0.11	0.28	0.97±0.18	0.43±0.05	<0.01
Tnfa	1.24±0.40	0.55±0.12	0.06	1.14±0.19	0.48±0.13	<0.01
Tgfb1	1.01±0.07	0.77±0.10	<0.05	1.04±0.09	0.56±0.07	<0.001
	Female			**Male**		
Serum	**WT**	**OE-NPY**	**P value**	**WT**	**OE-NPY**	**P value**
Resistin (ng/ml)	3.4±0.4	3.4±0.3	0.99	2.0±0.2	3.0±0.4	<0.05
Adiponectin (pg/ml)	14.7±0.9	18.5±0.8	<0.01	9.7±0.6	10.5±0.6	0.40

Cytokine levels in serum were analyzed from homozygous male OE-NPY$^{D\beta H}$ vs. wildtype mice at 2 (pre-obese) (n = 3-9/group) and 7 months (obese) (n = 4-8/group), and oxidative stress markers in plasma, urine and liver were measured at 5 months (n = 8-9/group). Hepatic mRNA expression was analyzed from 2-month-old heterozygous (pre-obese) (n = 5/group) and 7-month-old homozygous (obese) (n = 10/group) male OE-NPY$^{D\beta H}$ vs. wildtype mice with $2^{-\Delta\Delta Ct}$ method, normalized to β-actin expression and expressed as a fold change relative to wildtype mice. Serum resistin and adiponectin were measured from homozygous female and male OE-NPY$^{D\beta H}$ vs. wildtype mice at 7 months (n = 8-10/group). Values are expressed as means ± SEM. Statistics were analyzed with Student's t-test. ND = Not diagnosed, IL6 = Interleukin 6, IL10 = Interleukin 10, TRAP = Total peroxyl radical trapping antioxidant potential, GSH = Glutathione, GSSG = Glutathione disulfide, *Il1b* = Interleukin 1 beta, *Tnfa* = Tumor necrosis factor alpha, *Tgfb1*= Transforming growth factor beta 1, OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

Interestingly, the expressions of hepatic pro-inflammatory cytokines, transforming growth factor beta1 (*Tgfb1*), tumor necrosis factor alpha (*Tnfa*) and interleukin 1beta (*Il1b*), were decreased both in pre-obese and obese states (Table 9), which is opposite to the other signs of inflammation. Therefore, these results, together with previous findings (Ferreira, *et al.* 2010, Sigala, *et al.* 2013, Wheway, *et al.* 2005, Zhu, *et al.* 2015), suggest that NPY has a dual role in inflammation, being simultaneously both inflammatory and anti-inflammatory. Thus, the decreased IL10 and increased resistin levels in serum together with elevated 8-isoprostane levels in the liver may enhance the development of IR in OE-NPY$^{D\beta H}$ mice. Instead, the decreased cytokine expression is not in line with other findings. One explanation may be that decreased expression of cytokines in the liver does not correlate with the secretion of cytokines into the circulation, or alternatively oxidative stress in OE-NPY$^{D\beta H}$ mice may be cytokine independent.

5.5 Contribution of peripheral NPY via Y_1- and Y_2-receptors to the obese phenotype of OE-NPY$^{D\beta H}$ mice (Studies I & III)

Last, the mechanism of action of NPY to induce the metabolic disturbances in OE-NPY$^{D\beta H}$ mice was studied. The first hypothesis was that they are caused by increased NPY mediating directly via peripheral Y_1- and/or Y_2-receptors, as their contribution to metabolic disturbances has previously been shown (Kuo, *et al.* 2007, Liu, *et al.* 2013, Shi, *et al.* 2011, Zhang, *et al.* 2010). In order to study their role specifically in the liver, mRNA expressions of *Y1r* and *Y2r* were first studied in pre-obese (2-month-old) heterozygous and obese (7-month-old) homozygous OE-NPY$^{D\beta H}$ mice as well as being compared with WT mice. Hepatic *Y1r* expression was detected, but it was similar in the genotypes in both cohorts (pre-obese OE-NPY$^{D\beta H}$ 1.3±0.4 vs. WT 1.5±0.6, P = 0.83; obese OE-NPY$^{D\beta H}$ 1.3±0.4 vs. WT 1.4±0.5, P = 0.96). This suggests that NPY may change the hepatic metabolism of fatty acids, glucose and cholesterol by activating Y_1-receptors, although no change in the expression level of the receptor was detected. We did not detect any expression of *Y2r* in the liver, suggesting that NPY is unlikely to exert any direct effects on the liver via the Y_2-receptor, although we cannot exclude the possibility that Y_2-receptor still could indirectly mediate the actions of NPY also in the liver.

The role of peripheral Y_1- and Y_2-receptors on the phenotype of OE-NPY$^{D\beta H}$ mice was studied in more detail by treating OE-NPY$^{D\beta H}$ and WT mice with specific peripheral Y_1-

and Y_2-receptor antagonists (BIBO3304 and BIIE0246, respectively). In addition, one aim of these studies was to elucidate the pharmacologic potential of the antagonists for treating obesity due to genetic *Npy* overexpression and/or DIO and the related disorders. Therefore, the mice were studied both while consuming chow and a WD. Prior to the treatments, OE-NPY$^{D\beta H}$ mice on chow diet displayed increased body weight and fat mass and decreased lean mass as compared with WT mice (Appendix Fig. 1A, C, E). When on the WD, body weight and composition were similar between the genotypes (Appendix Fig. 1B, D, F). The phenotype was in line with the previous observations in these mice (Ruohonen, *et al.* 2012, Vähätalo, *et al.* 2015), and the body compositions were similar between the treatment groups within the same genotype (Appendix Fig. 1). In contrast to previous results, which have detected no difference in food intake between the genotypes (Vähätalo, *et al.* 2015), baseline energy intake was slightly increased in chow-fed OE-NPY$^{D\beta H}$ mice as compared to their WT counterparts (Appendix Fig. 1G). This is probably due to the mild stress during habituation caused by the saline injections, as OE-NPY$^{D\beta H}$ mice seem to be more stress-sensitive (unpublished observations). In the following sections, the effects of Y_1- and Y_2-receptor antagonists on each genotype and diet will be described.

5.5.1 Pharmacological blockade of Y_1-receptors (Study I and unpublished observations)

Germline Y_1-receptor KO mice have been shown to be prone to late-onset obesity (Kushi, *et al.* 1998), but the peripheral adult-onset KD of Y_1-receptors has been suggested to inhibit DIO and improve fatty acid metabolism (Zhang, *et al.* 2010). Therefore, the contribution of peripheral Y_1-receptors to the obese phenotype of OE-NPY$^{D\beta H}$ mice was studied in animals consuming either chow or WD; it was hypothesized that NPY's ability to activate Y_1-receptors would be one potential mechanism inducing the hepatic disturbances. OE-NPY$^{D\beta H}$ mice had an increased body weight and fat mass gain on both diets, but pharmacological blockade of Y_1-receptors with BIBO3304 did not change body weight gain or composition, or energy intake in either genotype or diet (Fig. 16).

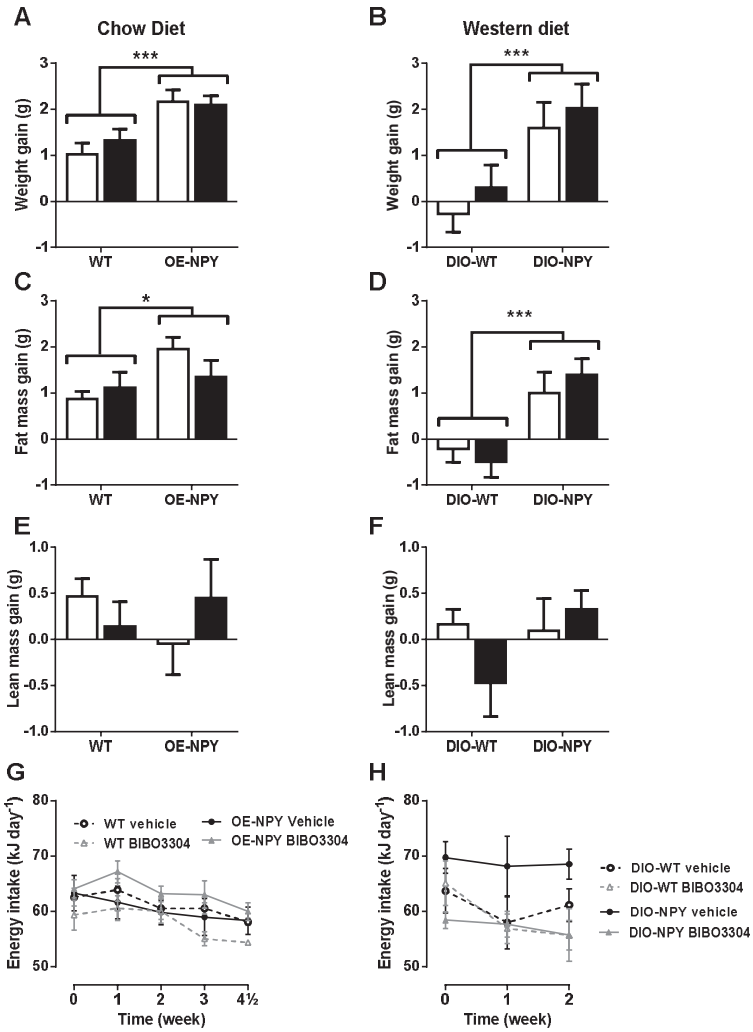

Figure 16 Effects of treatment with a peripheral Y_1-receptor antagonist on body composition

A, B) Body weight, C, D) fat mass and E, F) lean mass gain, and G, H) caloric intake in chow (A, C, E, G) or Western diet (B, D, F, H) fed homozygous OE-NPY$^{D\beta H}$ and wildtype mice treated with Y_1-receptor antagonist (BIBO3304) for 4½ or 2 weeks, respectively (n = 7-14 mice / group (A, C, E), n = 7-11 mice / group (B, D, F) and n = 3-6 cages / group (G, H)). Values are expressed as means ± SEM. * $P < 0.05$ and *** $P < 0.001$ comparing the different genotypes with two-way ANOVA. Energy intake is analyzed with repeated measures ANOVA. WT = Wildtype mice on chow diet, NPY = OE-NPY$^{D\beta H}$ mice on chow diet, DIO-WT = Wildtype mice on Western diet, DIO-NPY= OE-NPY$^{D\beta H}$ mice on Western diet.

OE-NPY$^{D\beta H}$ mice on both diets displayed hyperinsulinemia and an increased HOMA-IR index indicative of IR when compared with the WT mice. In addition, chow-fed OE-NPY$^{D\beta H}$ mice displayed hypercholesterolemia, and hepatic accumulation of TGs and

cholesterol, and BIBO3304 did not prevent these changes (Table 10). Surprisingly, BIBO3304 increased cholesterol in the liver and decreased TG levels in serum of chow-fed WT mice, and increased cholesterol levels in serum in both genotypes in DIO (Table 10). Unlike in humans, decreased TG levels in serum do not seem to be beneficial, as decreased TG levels have previously been detected in high fat diet-fed mice, as well as in obese OE-NPY$^{D\beta H}$ mice (Gao, *et al.* 2010, Vähätalo, *et al.* 2015). These results thus suggest that BIBO3304 exerts no effect on body composition, but may have undesirable effects on fatty acid and cholesterol metabolism, especially in mice with normal NPY levels.

Table 10 Biochemical parametres in serum and the liver in Y_1-receptor antagonist treated mice on chow and Western diet

	Chow diet			
	WT (n = 10-13)		OE-NPY (n = 11-13)	
	Vehicle	BIBO3304	Vehicle	BIBO3304
Serum				
Glucose (mM)	7.8±0.4	7.5±0.5	8.3±0.5	8.5±0.4
Insulin (µg/l)	0.28 ± 0.03	0.30 ± 0.05	0.47 ± 0.04***	0.51 ± 0.07***
HOMA-IR	2.5±0.4	3.0±0.6	4.3±0.4**	5.7±1.0**
Cholesterol (mM)	2.3±0.1	2.3±0.1	2.6±0.1***	2.7±0.1***
Triglycerides (mg/ml)	0.33±0.03	0.22±0.02#	0.35±0.02	0.34±0.03
Liver				
Triglycerides (mg/g tissue)	12.8±0.8	18.0±3.1	23±1.8**	21.8±2.8**
Cholesterol (mg/g tissue)	3.0±0.1	3.6±0.1#	3.5±0.2P=0.07	3.6±0.1 P=0.07
8-isoprostane (mg/g tissue)	45.1±1.4	51.6±4.3	51.1±2.0	48.1±3.4

	Western diet			
	DIO-WT (n =7-11)		DIO-NPY (n =7-8)	
	Vehicle	BIBO3304	Vehicle	BIBO3304
Serum				
Glucose (mM)	9.3±0.4	9.6±1.0	9.5±0.6	9.7±0.4
Insulin (µg/l)	0.8±0.2	0.6±0.1	1.0±0.3*	1.2±0.2*
HOMA-IR	7.6±1.4	7.0±1.9	9.8±1.8**	12.7±1.6
Cholesterol (mM)	6.1±0.4	7.2±0.4#	6.5±0.7	7.4±0.5#

The data is presented from chow and Western diet-fed cohorts of homozygous OE-NPY$^{D\beta H}$ and wildtype mice after treatment with a Y_1-receptor antagonist (BIBO3304) or vehicle for 4½ and 2 weeks, respectively. Values are expressed as means ± SEM. *P<0.05, **P<0.01 and ***P<0.001 comparing the genotypes with two-way ANOVA. #P<0.05 comparing BIBO3304 and vehicle treatment within the same genotype with Bonferroni post-hoc test following a near-statistically significant interaction between treatment and genotype in two-way ANOVA. OE-NPY = OE-NPY$^{D\beta H}$ mice, WT = Wildtype mice.

Despite the almost undetectable effects of BIBO3304 on the phenotype, its effects on physical activity and EE in WT mice were studied, as NPY has been suggested to inhibit EE via peripheral Y_1-receptors (Zhang, *et al.* 2010). However, neither acute (after first dose) (unpublished observations) nor repeated dosing (daily injections for 7 days) of BIBO3304 had any effect on EE (relative to lean mass), RER or total activity (Original Publication I).

Taken together, it seems highly unlikely that the metabolic disorders detected in OE-NPY$^{D\beta H}$ mice are mediated via peripheral Y_1-receptors. Furthermore, at odds with the working hypothesis, peripheral Y_1-receptor antagonism did not prevent the obesity or hepatic disorders induced by excess NPY and/or DIO. In fact, it seems that BIBO3304 may even have undesirable effects on TG and cholesterol metabolism, when NPY levels are normal.

5.5.2 Pharmacological blockade of Y_2-receptors (Study III)

As previous studies have shown that peripheral Y_2-receptors mediate the obesogenic effects of NPY (Kuo, *et al.* 2007, Shi, *et al.* 2011), a peripheral Y_2-receptor antagonist was assessed as another potential drug candidate for treating obesity and related disorders. The effect of Y_2-receptor antagonist BIIE0246 on body composition was studied in mice consuming a chow diet or against DIO for 4½ or 2 weeks, respectively. In animals fed the chow diet, BIIE0246 increased the weight gain in both genotypes without causing any change in fat mass gain. However, the differential effect of the Y_2-receptor antagonist on the lean mass gain between the genotypes (treatment x genotype interaction $P = 0.05$) indicated that the increased lean mass gain explains the body weight gain in OE-NPY$^{D\beta H}$ mice, but not in WT mice (Fig. 17A-C). In DIO, BIIE0246 had opposite effects on the phenotypes (treatment x genotype interaction in body weight $P < 0.05$, in fat mass $P < 0.001$ and in lean mass $P < 0.05$). In OE-NPY$^{D\beta H}$ mice, it inhibited fat mass gain without causing any change in body weight or lean mass gain. In contrast, in WT mice, BIIE0246 increased weight and fat mass gain, and lean mass gain tended to decline (Fig. 17D-F).

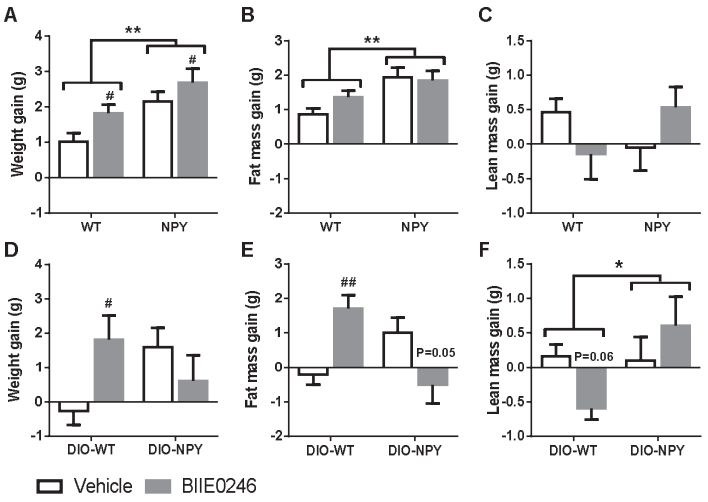

Figure 17 Effects of peripheral Y_2-receptor antagonist on body composition

(A, D) Body weight, (B, E) fat mass and (C, F) lean mass gain in homozygous OE-NPY$^{D\beta H}$ and wildtype mice on chow (n=11-12/group) (A-C) or Western diet (n=7-11/group) (D-F) treated with the Y_2-receptor antagonist (BIIE0246) or vehicle for 4½ or 2 weeks, respectively. Values are expressed as means ± SEM. *P<0.05 and **P<0.01 comparing the different genotypes with two-way ANOVA, and $^{\#}$P<0.05, $^{\#\#}$P<0.01, P=0.05 and P=0.06 comparing BIIE0246 treatment and vehicle treatment with two-way ANOVA (A), or with Bonferroni post-hoc test following a significant interaction between treatment and genotype observed in the two-way ANOVA (D, E, F). WT = Wildtype mice on chow diet, NPY = OE-NPY$^{D\beta H}$ mice on chow diet, DIO-WT = Wildtype mice on Western diet, DIO-NPY= OE-NPY$^{D\beta H}$ mice on Western diet. Reprinted with permission from Ailanen *et al.* Front Pharmacol. 2018, 9:319.

OE-NPY$^{D\beta H}$ mice displayed the basic obese phenotype of IR and hypercholesterolemia when compared to WT mice. In both genotypes of mice fed a chow diet, BIIE0246 increased serum insulin levels and the HOMA-IR index (Table 11). Furthermore, a 2-week treatment with BIIE0246 induced hypercholesterolemia in WT mice (Original publication III). In DIO, BIIE0246 did not change the glucose parameters in either genotype (Table 11). However, in OE-NPY$^{D\beta H}$ mice (but not in WT mice) in both treatment groups, serum cholesterol levels correlated positively with body fat mass (DIO-NPY vehicle $R^2 = 0.90$, $P < 0.01$; DIO-NPY BIIE0246 $R^2 = 0.96$, $P < 0.001$), and the slope of the regression curve of cholesterol and fat mass was significantly lower in the group treated with BIIE0246 as compared with the vehicle-treated group (Fig. 18). Thus, the serum cholesterol level in OE-NPY$^{D\beta H}$ mice seems to be highly dependent on body fat mass unlike the situation in WT mice. Furthermore, serum cholesterol levels seem to be lower relative to body adiposity after treatment with BIIE0246. As Y_2-receptor antagonism has previously been shown to decrease serum cholesterol levels (Liu, *et al.*

2013), it can be speculated that there might have been an improvement also in absolute cholesterol levels, if the treatment had continued for a longer period of time.

Table 11 Glucose and lipids in serum of Y_2-receptor antagonist treated mice

	Chow diet			
	WT (n = 11-13)		NPY (n = 11-12)	
	Vehicle	BIIE0246	Vehicle	BIIE0246
Glucose (mM)	8.4±0.6	8.0±0.5	8.6±0.4$^{P=0.05}$	8.9±0.3$^{P=0.05}$
Insulin (µg l^{-1})	0.28±0.03	0.44±0.04$^{\#}$	0.47±0.04***	0.51±0.06$^{***\#}$
HOMA-IR	2.5±0.3	3.6±0.3$^{\#}$	4.3±0.4***	5.1±0.5$^{***\#}$
Cholesterol (mM)	2.3±0.1	2.5±0.1	2.6±0.1**	2.7±0.1**

	Western diet			
	DIO-WT (n = 7-12)		DIO-NPY (n = 6-7)	
	Vehicle	BIIE0246	Vehicle	BIIE0246
Glucose (mM)	9.3±0.4	9.3±0.6	9.4±0.6	9.5±0.6
Insulin (µg l^{-1})	0.75±0.14	0.76±0.10	1.03±0.25$^{P=0.06}$	1.51±0.55$^{P=0.06}$
HOMA-IR	7.6±1.4	6.7±0.9	9.8±1.8*	14.8±4.8*
Cholesterol (mM)	6.1±0.4	6.4±0.4	6.5±0.5	5.8±0.8

The data is presented from chow and Western diet-fed cohorts of homozygous OE-NPY$^{D\beta H}$ and wildtype mice after treatment with the Y_2-receptor antagonist (BIIE0246) or vehicle for 4½ and 2 weeks, respectively. Values are expressed as means ± SEM. *P<0.05, **P<0.01, ***P<0.001, P=0.05 and P=0.06 comparing the genotypes, and $^{\#}$P<0.05 comparing BIIE0246 and vehicle treatment within the same genotype with two-way ANOVA. WT = Wildtype mice on chow diet, NPY = OE-NPY$^{D\beta H}$ mice on chow diet, DIO-WT= Wildtype mice on Western diet, DIO-NPY= OE-NPY$^{D\beta H}$ mice on Western diet. Reprinted with permission from Ailanen *et al.* Front Pharmacol. 2018, 9:319.

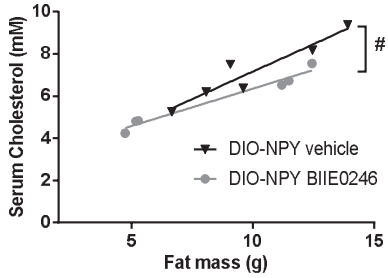

Figure 18 Positive correlation between body fat mass and cholesterol levels in serum of OE-NPY$^{D\beta H}$ mice on Western diet

Western diet-fed homozygous OE-NPY$^{D\beta H}$ mice were treated with the Y_2-receptor antagonist (BIIE0246) or vehicle for 2 weeks (n=6-7/group). Values are expressed as means ± SEM. $^{\#}$P<0.05 comparing the slopes of correlation curves between BIIE0246 and vehicle treatment with linear regression analysis. DIO-NPY= OE-NPY$^{D\beta H}$ mice on Western diet. Reprinted with permission from Ailanen *et al.* Front Pharmacol. 2018, 9:319.

In the liver, the effect of BIIE0246 was different between the genotypes. When fed the chow diet, OE-NPY$^{D\beta H}$ mice had increased contents of TGs and cholesterol, and similarly treatment with BIIE0246 tended to increase the contents of lipids in WT mice, but not in OE-NPY$^{D\beta H}$ mice (interaction $P < 0.05$ and $P = 0.05$, respectively) (Fig. 19A, B). In DIO, the contents of TGs and cholesterol were not changed, but BIIE0246 induced ballooning degeneration in the hepatocytes of WT mice (Fig. 19C, E), similarly as previously detected in the naïve OE-NPY$^{D\beta H}$ mice (Fig. 7). The ballooning degeneration was confirmed as glycogen accumulation with PAS staining (Fig. 19E). On the contrary, ballooning degeneration and glycogen content were less pronounced in OE-NPY$^{D\beta H}$ mice treated with BIIE0246 compared to vehicle-treated mice (Fig. 19E). mRNA expressions of glycogen metabolism related genes were not changed, and BIIE0246 did not have any influence on the weights of the liver (unpublished observations).

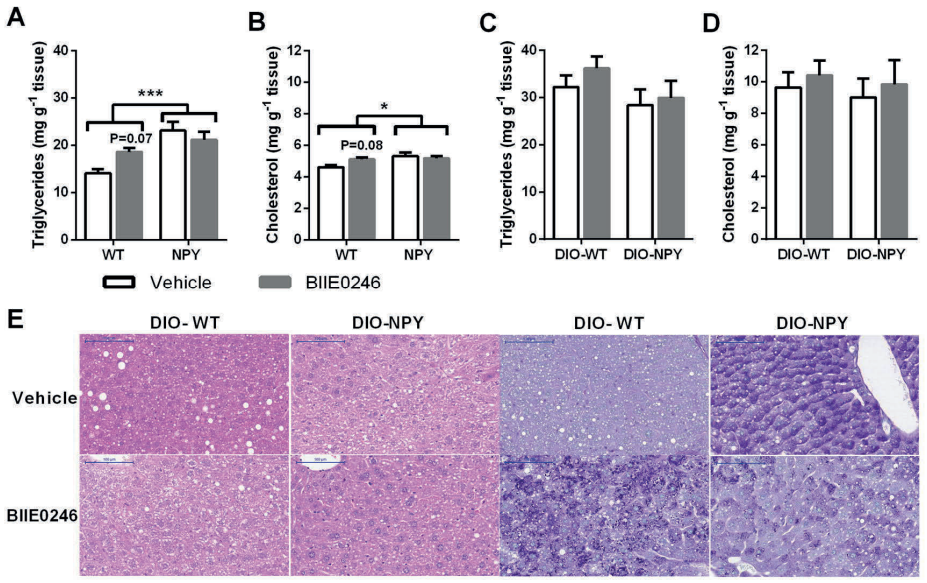

Figure 19 Hepatic phenotype of mice treated with a Y_2-receptor antagonist

(A) Contents of triglycerides and (B) cholesterol in the livers of mice on chow diet treated with Y_2-receptor antagonist (BIIE0246) or vehicle for 4½ weeks (n=8-12/group). (C) Triglycerides and (D) cholesterol in the livers (n=7-11/group), and (E) representative hematoxylin and eosin (right panel) and Periodic-acid Schiff stainings (left panel) of liver slides with x20 magnification (scale bar 100 µm) in mice consuming a Western diet treated with BIIE0246 or vehicle for 2 weeks. Values are expressed as means ± SEM. *P<0.05 and ***P<0.001 comparing the different genotypes with two-way ANOVA. P-values represent a comparison of BIIE0246 with vehicle treatment within WT mice with the Bonferroni post-hoc test following a significant or near-significant interaction between treatment and genotype in two-way ANOVA. WT = Wildtype mice consuming a chow diet, NPY = OE-NPY$^{D\beta H}$ mice consuming a chow diet, DIO-WT= Wildtype mice consuming a Western diet, DIO-NPY= OE-NPY$^{D\beta H}$ mice consuming a Western diet.

Thus, the results of Y_2-receptor antagonist study suggest that blockade of Y_2-receptors is not on its own, able to prevent the obese phenotype of OE-NPY$^{D\beta H}$ mice, and Y_2-receptors do not mediate the metabolic changes induced by *Npy* overexpression in noradrenergic neurons. However, when DIO is combined with excess NPY, Y_2-receptor antagonism prevents obesity, decreases hepatic glycogen accumulation and relative serum cholesterol levels to body adiposity, and thus, pharmacological blockade of peripheral Y_2-receptors in this situation could show some potential in treating obesity. On the contrary, when NPY levels are normal, Y_2-receptor antagonism, similar to Y_1-receptor antagonism, even seems to exert undesirable effects on the metabolic status, especially when the subject is consuming a healthy diet.

In order to explain why the treatment with BIIE0246 leads to the discrepant findings between the genotypes in DIO and induces an OE-NPY$^{D\beta H}$ –like phenotype in chow-fed WT mice, the mRNA expressions of relevant genes were first studied in the appropriate peripheral tissues, i.e. in retroperitoneal WAT and in adrenal glands at the 2-week time point. The goal was to identify the primary effects of the drug on mRNA expression, prior to the effect on the phenotype. The changes in the expressions of adipogenic and angiogenic enzymes in WAT could not explain the opposite phenotypes between the genotypes in DIO (Original publication III)). Furthermore, changes in expressions of *Npy* and *Y2r* in adrenal glands were not detected (unpublished observations). Therefore, the central effects of BIIE0246 in the hypothalamus and in the brainstem, the parts of the brain which have an ineffective BBB, were considered as other possible targets. The results show (Fig. 20) that in mice with DIO, peripherally administered BIIE0246 slightly, but significantly increased the expression of *Npy* in the hypothalamus, and tended to decrease the expression of *Pomc*. This effect was especially pronounced in WT mice, and may account for their obesity. Instead, when animals were consuming chow, treatment with BIIE0246 decreased the expression of *Th*, the rate-limiting enzyme in the synthesis of noradrenaline, which suggests that there is a decreased sympathetic tone, which may cause obesity, similarly to the situation detected in naïve OE-NPY$^{D\beta H}$ mice (Vähätalo*, et al.* 2015). Finally, the effect of BIIE0246 on the expression of hypothalamic auto-inhibitory *Y2r* was studied; BIIE0246 significantly increased the expression of *Y2r* in OE-NPY$^{D\beta H}$ mice on both diets, whereas no change was detected in WT mice (treatment x genotype interaction $P < 0.05$ (chow) and $P < 0.01$ (DIO)). This may explain the milder adverse metabolic effects in chow-fed OE-NPY$^{D\beta H}$ mice and the beneficial metabolic effects OE-NPY$^{D\beta H}$ mice against DIO as compared to WT mice.

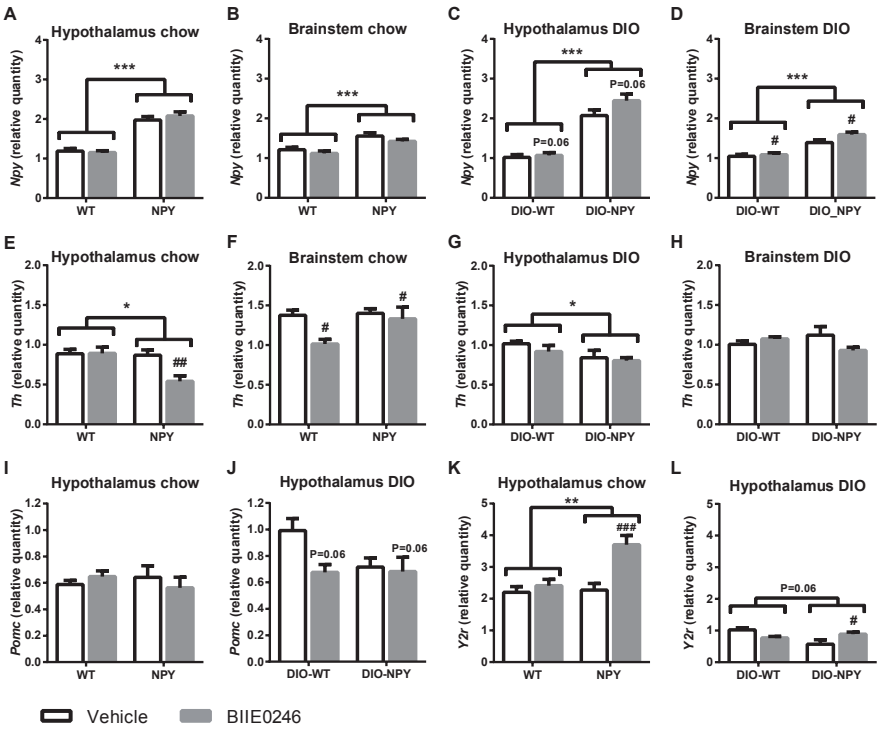

Figure 20 Central sympathetic and noradrenergic systems after treatment with Y_2-receptor antagonist

(A-D) mRNA expression of *Npy*, (E-H) *Th* (I-J) *Pomc* and (K-L) *Y2r* in the hypothalamus and the brainstem of chow- (A, B, E, F, I and K) or Western-diet-fed (C, D, G, H, J and L) homozygous OE-NPY$^{D\beta H}$ and wildtype mice (n=6-12/group) after 2-week Y_2-receptor antagonist (BIIE0246) or vehicle treatment. mRNA expression was analyzed with $2^{-\Delta\Delta Ct}$ method, normalized to β-actin expression and expressed as a fold change relative to wildtype mice. Values are expressed as means ± SEM. *P<0.05, **P<0.01, ***P<0.001 and P=0.06 comparing the different genotypes with two-way ANOVA, and $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and P=0.06 comparing BIIE0246 treatment and vehicle treatment with two-way ANOVA (C, D, F J), or with Bonferroni post-hoc test following a significant interaction between treatment and genotype in two-way ANOVA (E, K, L). *Npy* = Neuropeptide Y, *Pomc* = pro-opiomelanocortin, *Th* = Tyrosine hydroxylase, *Y2r* = Y_2-receptor, WT = Wildtype mice on chow diet, NPY = OE-NPY$^{D\beta H}$ mice on chow diet, DIO-WT= Wildtype mice on Western diet, DIO-NPY= OE-NPY$^{D\beta H}$ mice on Western diet. Reprinted with permission from Ailanen *et al.* Front Pharmacol. 2018, 9:319.

5.6 Contribution of sympathetic nervous system to the obese phenotype of OE-NPY$^{D\beta H}$ mice (Study I)

As the studies with Y_1- and Y_2-receptor antagonists suggested that the liver related metabolic disorders in OE-NPY$^{D\beta H}$ mice are not mediated via hepatic Y_1- and Y_2-receptors, changes in the levels of sympathetic activity in the liver were considered as

another option. This hypothesis was supported by previous findings of changes in sympathetic activity in OE-NPY$^{D\beta H}$ mice (Ruohonen, *et al.* 2009, Vähätalo, *et al.* 2015). Furthermore, the liver is surrounded by NPY-containing nerve fibers (Ding, *et al.* 1991), but there is no evidence about the functions of Y-receptors in the hepatocytes. Therefore SNS seems as a potential mediator of NPY-induced changes on liver metabolism.

As discussed earlier, in heterozygous mice, catecholamine levels are increased, but in contrast, elevated NPY levels in homozygous animals tend to reduce the catecholamine levels. Nonetheless, both models display obesity and metabolic disturbances, although these are more pronounced in the homozygous model (Ruohonen, *et al.* 2008, Vähätalo, *et al.* 2015). In addition to the central decrease of sympathetic tone, sympathetic activity is attenuated at the tissue level in WAT and BAT, which by inhibiting lipolysis and activating lipogenesis expands the adipose tissue (Vähätalo, *et al.* 2015). This led to a hypothesis that a change in SNS activity could also explain the hepatic disturbances. In the liver, mRNA expression of adrenoceptor beta 1 (*Adrb1*) was decreased both in pre-obese, heterozygous and obese, homozygous OE-NPY$^{D\beta H}$ mice, but the expression of *Adrb2* was not changed (Fig. 21). The decrease in the expression of *Adrb1* suggests that independent of overall sympathetic activity (i.e. increased activity in heterozygous and decreased activity in homozygous OE-NPY$^{D\beta H}$ mice (Ruohonen, *et al.* 2009, Vähätalo, *et al.* 2015), *Npy* overexpression in noradrenergic neurons attenuates hepatic sympathetic activity, and this leads to similar degrees of hepatic accumulation of TGs with aging in both *Npy* overexpression models (Ruohonen, *et al.* 2008). The contribution of Adrb1-receptors to the metabolic disturbances had been previously studied in the Adrb1 KO model and in the adipose tissue-specific overexpression model of *Adrb1*. Similar to OE-NPY$^{D\beta H}$ mice, *Adrb1*-deficiency induced IGT and IR, and predisposed to DIO and hepatosteatosis (Ueta, *et al.* 2012), whereas adipose tissue-specific overexpression of *Adrb1* protected from DIO (Soloveva, *et al.* 1997). These studies support our final proposal of an important role of Adrb1-receptors and SNS activity on the metabolic outcome observed in OE-NPY$^{D\beta H}$ mice.

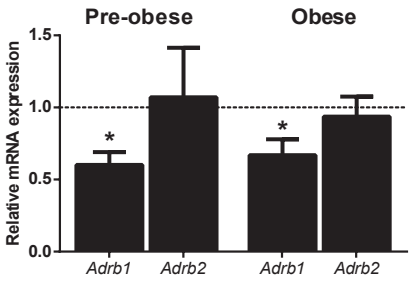

Figure 21 Sympathetic tone in the liver of OE-NPY$^{D\beta H}$ mice.

(A) mRNA expression of adrenoceptors beta 1 (*Adrb1*) and 2 (*Adrb2*) in 2-month-old heterozygous (pre-obese) (n = 4-5/group) and 7-month-old homozygous (obese) (n = 6-7/group) male OE-NPY$^{D\beta H}$ mice. mRNA expression values were analyzed with $2^{-\Delta\Delta Ct}$ method, normalized to the expression of β-actin and expressed as a fold change relative to wildtype mice. Values are presented as mean ± SEM. *P<0.05 difference between genotypes with Student's t-test.

5.7 The role of NPY expressed within noradrenergic neurons inducing the metabolic syndrome

5.7.1 Summary

According to the observed results with OE-NPY$^{D\beta H}$ mice, it can be concluded that increased noradrenergic NPY predisposes to the MetS-like phenotype, characterized by obesity and IGT with IR and hyperinsulinemia (Vähätalo *et al.* 2015), hypercholesterolemia, hepatosteatosis and hepatic glycogen accumulation. Furthermore, this phenotype renders the mice susceptible to T2D, when glucose metabolism is disturbed by consumption of a high-caloric diet and by destruction of pancreatic beta cells. The outcome in the animals is similar to that observed in the human gain-of-function polymorphism in the *NPY* gene (rs16139) (Jaakkola, *et al.* 2006, Karvonen, *et al.* 1998, Nordman, *et al.* 2005, Ukkola & Kesaniemi. 2007, van Rossum, *et al.* 2006).

Fig. 22 shows the postulated liver-related actions of NPY to induce the MetS-like phenotype in OE-NPY$^{D\beta H}$ mice. Simultaneously, as TGs accumulate into WAT (Vähätalo *et al.* 2015), NPY induces the storage of TGs and glycogen in the liver. NPY thus seems to stimulate the storage of all of the available energy substrates. In a pre-obese state, hepatic fatty acid oxidation is decreased, leading to the accumulation of TGs. The

mechanism of action of *NPY* overexpression in noradrenergic neurons seems to be decreased SNS activity in the liver due to decreased expression of *Adrb1*. However, although in this study, Y_1-receptor antagonism was not able to prevent the metabolic disturbances present in OE-NPY$^{D\beta H}$ mice, it cannot be ruled out that NPY acting directly on hepatic Y_1-receptors would also decrease fatty acid oxidation. Peripheral KD of Y_1-receptors has been shown to increase the amount of CPT1, the gate-keeping enzyme of fatty acid oxidation, which was interpreted to mean that NPY can exert direct actions on the liver (Zhang *et al.* 2010). Therefore, future studies could include examining whether the NPY-induced decrease in fatty acid oxidation is due to mitochondrial dysfunction, a condition known to be present in established NAFLD.

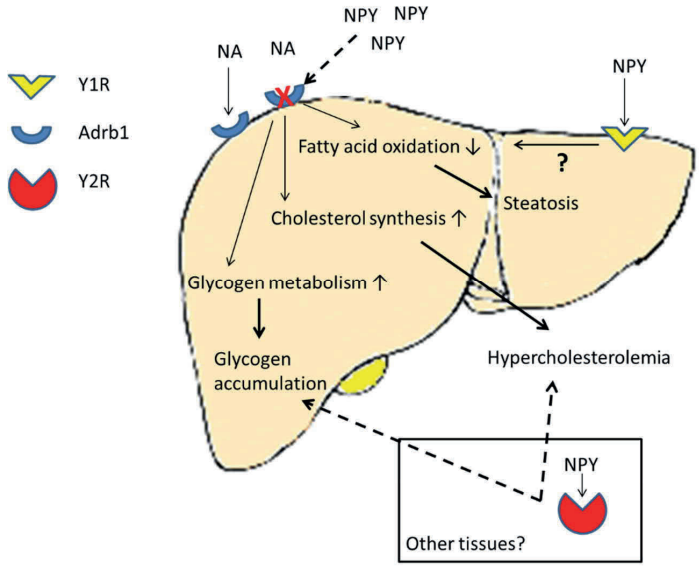

Figure 22 The role NPY co-expressed with catecholamines in the sympathetic nervous system.

Excess neuropeptide Y (NPY) decreases the expression of beta1-adrenoceptors (*Adrb1*) leading to an attenuation of sympathetic tone in the liver. This results in decreased fatty acid oxidation, and increased glycogen metabolism and cholesterol synthesis evoking triglyceride and glycogen accumulation and hypercholesterolemia. Furthermore, peripheral NPY acting via Y_1-receptors may cause an inhibition of fatty acid oxidation. Y_2-receptors are not present in the liver, but they seem to be indirectly (probably via adipose tissue) involved with glycogen and cholesterol metabolism. Y1R = Y_1-receptor, Y2R = Y_2-receptor, NA = Noradrenaline.

Furthermore, NPY decreases gluconeogenesis and increases glycogen metabolism, leading eventually to hepatic glycogen accumulation, which together with the

accumulation of TGs, supports the idea of an NPY-induced increase in energy storage. As this condition is combined with effective insulin secretion from the pancreas, the fasting blood glucose levels remains normal for a prolonged period. However, ultimately IGT and IR become established with aging and obesity. Treatment with the widely used anti-hyperglycemic agent, metformin, seems to slow down the development of established IGT by normalizing the glycogen structure and by attenuating glycogenolysis.

Decreased hepatic SNS activity may also contribute to the hypercholesterolemia, induced by accelerated cholesterol synthesis in the liver, but without any change in intestinal cholesterol absorption. Since especially the HDL component of the total cholesterol was increased and there was also a tendency towards decreased expression of hepatic HDL-receptor, *Srb1* may be involved. Liu, *et al.* (2013) have previously shown that antagonism of both peripheral Y_1- or Y_2-receptors decreases total, LDL and HDL cholesterol levels in rats with STZ-induced diabetes. In contrast, our results suggest that treatment with a Y_1-antagonist in DIO actually augments the hypercholesterolemia, an effect independent of NPY levels, which suggests that Y_1-receptors are not involved in mediating the NPY-induced hypercholesterolemia. However, cholesterol levels in serum were decreased relative to the body adiposity after treatment with a Y_2-receptor antagonist, which suggests that the actions of NPY on hepatic cholesterol metabolism are mediated via Y_2-receptors. However, as Y_2-receptors were not detected in the liver, cholesterol metabolism is unlikely to be directly affected by NPY via Y_2-receptors in that tissue, but by other mechanisms. Similarly, we suggest that Y_2-receptors indirectly influence glycogen metabolism, as treatment with a Y_2-receptor antagonist decreased glycogen accumulation and reduced the amount of ballooning degeneration in DIO.

5.7.2 Comparison between the OE-NPY$^{D\beta H}$ mouse model and human NPY polymorphism rs16139

As stated previously, the carriers of the human gain-of-function polymorphism in the *NPY* gene (rs16139) display increased body weight in populations with normal BMI, and carry an increased risk for metabolic disorders, e.g. hypercholesterolemia, susceptibility to T2D and cardiovascular diseases in an obese population (Jaakkola, *et al.* 2009, Karvonen, *et al.* 1998, Masoudi-Kazemabad, *et al.* 2013, Nordman, *et al.* 2005, Pihlajamaki, *et al.* 2003, Ukkola & Kesaniemi. 2007, van Rossum, *et al.* 2006, Yeung, *et al.* 2011). The

presence of hepatic steatosis in carriers of rs16139 has not been studied, but as steatosis is known to be closely related to these other metabolic disorders, it is likely that the risk for steatosis could be also increased. The obese phenotype of the OE-NPY$^{D\beta H}$ mice includes IGT and IR (Vähätalo, *et al.* 2015), and according to the present study, also hypercholesterolemia, hepatic steatosis and increased susceptibility to T2D, which is similar to the phenotype of carriers of rs16139. This makes the OE-NPY$^{D\beta H}$ model a valuable tool for elucidating the mechanisms of NPY-induced metabolic disturbances among rs16139, and may give valuable information about how the disorders could be targeted in this complicated set of symptoms.

Obviously, the genetic background of the mouse model is not the same as in rs16139, as overexpression of *NPY* is induced in a different manner. This may raise the question of whether the mechanism of action of NPY is the same in both scenarios. In the case of the human polymorphism, increased NPY levels have been detected during sympathetic activity (Kallio*, et al.* 2001, Mitchell*, et al.* 2008). On the other hand, at rest both noradrenaline and NPY levels are lower in carriers of the polymorphism (Jaakkola, *et al.* 2005). In the OE-NPY$^{D\beta H}$ model, transgenic overexpression of *NPY* induces induction (heterozygous mice) or suppression of sympathetic tone (homozygous mice) (Ruohonen*, et al.* 2009, Vähätalo*, et al.* 2015), and therefore any comparison of these two models is fraught with difficulties. Despite the different effects of *Npy* overexpression on sympathetic activity, the phenotype of the heterozygous and homozygous mice is similar. However, homozygous OE-NPY$^{D\beta H}$ mice are better in modelling rs16139, as they both display decreased sympathetic tone at baseline, and their metabolic phenotype is similar to the human carriers of the polymorphism , i.e. IGT, IR, increased susceptibility to T2D, hypercholesterolemia, inhibition of lipolysis (Jaakkola, *et al.* 2005, Jaakkola*, et al.* 2009, Karvonen*, et al.* 1998, Masoudi-Kazemabad*, et al.* 2013, Nordman*, et al.* 2005, Pihlajamaki*, et al.* 2003, Ukkola & Kesaniemi. 2007, van Rossum*, et al.* 2006, Vähätalo, *et al.* 2015, Yeung*, et al.* 2011).

Indeed, when observing the liver, it is interesting that the hepatic phenotype and the mechanism seem to be the same in both models: the mice in both models display hepatic TG accumulation, with an earlier onset in the homozygous mice. Despite the opposite effects on catecholamine levels in these models, mice in both models display decreased expression of *Adrb1* in the liver, indicative of reduced hepatic sympathetic tone. Furthermore, studies with both models suggest that TGs accumulate into hepatocytes due

to decreased fatty acid oxidation. Therefore, with respect to their impact on liver metabolism, both models of OE-NPY$^{D\beta H}$ could be used for modelling the hepatic metabolism in rs16139 carriers.

5.8 Methodological aspects

5.8.1 Relevance of OE-NPY$^{D\beta H}$ mice as a disease model

As stated above, the phenotype of OE-NPY$^{D\beta H}$ mice is rather similar to the human gain-of-function polymorphism of the *NPY* gene (rs16139), which makes the OE-NPY$^{D\beta H}$ model a valuable tool for elucidating the properties of NPY inducing the metabolic disturbances observed in carriers of the polymorphism. In addition, a pharmacological study with Y_2-receptor antagonist BIIE0246 indicated that the OE-NPY$^{D\beta H}$ mice are a good prediabetic model for examining the actions of novel drug candidates, especially in scenarios when obesity is combined with high NPY levels. Although there is no clear evidence that stress directly induces hepatosteatosis, overexpression of *NPY* within noradrenergic neurons has been shown to play a significant role in stress-induced changes on hepatic fatty acid metabolism (Zhang, *et al.* 2014). In addition, chronic stress has been shown to impair liver function by causing inflammation and subjecting individuals to serious liver diseases (i.e. hepatitis, cirrhosis and hepatocarcinoma) (Chida, *et al.* 2006, Vere, *et al.* 2009). Therefore OE-NPY$^{D\beta H}$ mice could have potential as a disease model of the impaired liver function induced by stress. Furthermore, when mice are forced to consume a high caloric diet supplemented by administration of a low-dose of STZ, OE-NPY$^{D\beta H}$ mice seem to have potential to be used as a model for T2D in studying anti-hyperglycemic drug candidates.

However, there are limitations to the use of OE-NPY$^{D\beta H}$ mice as a disease model. The failure to observe any improvement in IGT after treatment with metformin may question its usefulness as a model for drug development, at least in cases when high NPY levels or changes in the activity of the SNS are not relevant with respect to the induction of disease. Furthermore, species differences have to be taken into account, e.g. cholesterol metabolism is substantially different in mice and humans. Another limitation relates to our unpublished observations which show that OE-NPY$^{D\beta H}$ mice tend to be quite stress sensitive, and this needs to be taken into account when planning the study protocols. They

may lose their obese phenotype, if they are handled too intensively or subjected to stress-inducing techniques. Males also may show aggressive behavior against other male mice, which may limit their use with the available methods.

5.8.2 Study methods

The methods used in this study have been previously published and widely used, which makes the observed results reliable and convincing. However, the methods and study conditions are not fully standardized, which may lead to variations in the results and complicate comparison between different studies. In addition, mimicking of some standard clinical studies of glucose metabolism (e.g. hyperglycemic clamp test) would demand special (surgical) operations in mice, which limits their use. Furthermore, there has been some debate about the methodology of GTT in rodents, e.g. whether the proper glucose dose should be based on the whole body weight or lean mass, whether the optimal dosing route should be i.p. or p.o., and what is the optimal duration for fasting prior to glucose dosing. Instead, in ITT, it is sometimes difficult to estimate the appropriate insulin dose. For example, the administered dose of 0.75 IU/kg of insulin was possibly too low dose for mice with diet-induced IR (Study I).

In study II, mice received the metformin in their drinking water, which is a non-invasive and unstressful way of dosing. However, in order to confirm the adequacy of drug dosing, mice were separated into individual cages, which does not promote their wellbeing and may have evoked some stress. As a result, all mice, including WT mice, were obese, and there was no difference in body composition between the genotypes, unlike the normal situation present in 4-month-old mice. However, it is interesting that despite the similar obese phenotype between the genotypes, a difference in the structure of liver glycogen was still observable. This confirms our other findings that NPY has direct effects on the liver metabolism independent of its effects on WAT.

When studying the effects of Y_1- and Y_2-receptor antagonist, mice were first habituated for injections for 2 weeks, and then continued with drug treatments for 2 or 4½ weeks. The dosing method-induced stress is definitely a limitation in these studies, but by providing time for habituation, the stress-induced changes in the phenotype were minimized. The other limitation of the study is the short half-life of BIIE0246 (< 3 h) and the unknown half-life of BIBO3044, which may have caused fluctuations of the drug

concentration over time and incomplete blockade of the receptors at some times. As the dosing was performed in the morning (9:00-11:00 a.m.), the inadequate concentrations of the drug may have taken place at night, during the active and feeding hours of the mice. However, as the pharmacokinetic profiles of the drugs are unknown, one can only speculate on the accumulation of the drugs into the tissues. Compared to other available Y_2-antagonists, BIIE0246 has the longest half-life (Brothers, *et al.* 2010), which made it the most suitable drug for the current Y_2-receptor antagonist study. Furthermore, BIBO3044 has previously been used in repeated dosing (Sousa, *et al.* 2012, Yuzuriha, *et al.* 2007), which supported its use in the current Y_1-receptor antagonist study. The estimation of the proper dose was also demanding, because there were no or very few studies with chronic, peripheral dosing with these drugs, and no studies investigating energy metabolism. If the dose was too low, no drug response would have been detected, whereas too high a dose might have led to toxicity or non-specific effects of the drug. However, the estimation of the doses (based on (Forbes, *et al.* 2012)) seems to have been successful, as significantly different effects were detected between the phenotypes of the mice from different treatment groups.

The short half-life of the drug(s) and stress caused by daily injections could have been overcome by subcutaneously implanted osmotic minipumps, which constantly release the drug throughout the day. However, implantation would have demanded surgery under anesthesia, which in turn causes stress, and leads to enormous weight loss during the recovery period, especially in DIO (unpublished observations). Therefore, the challenges of the dosing method would not have been completely overcome by adopting an osmotic minipump based administration technique.

Another potential drawback of the peripheral administration of Y-receptor antagonists were the potential central effects of the drugs. Despite the fact that BIBO3044 and BIIE0246 are both very specific for their receptors and unable to cross the BBB (Brothers, *et al.* 2010, Doods, *et al.* 1999, Dozio, *et al.* 2007), hypothalamus and brainstem are some of the few brain regions that lack an effective BBB (Rodriguez, *et al.* 2010, Wang, *et al.* 2008). Therefore, the central effects of the drugs had to be taken into account, and the original goal of blocking only peripheral Y_1- and Y_2-receptors was not completely achieved.

5.9 **Significance**

In conclusion, overexpression of *NPY* in noradrenergic neurons decreases systemic and tissue specific sympathetic activity, which predisposes to obesity and metabolic disorders. The NPY-induced changes in liver metabolism seem to be crucial for the progression of hepatosteatosis, IGT and hypercholesterolemia, and as hepatosteatosis has recently been shown to co-exist with the MetS and to be a relevant risk factor for cardiovascular diseases, this could also predispose to cardiovascular events in subjects with excess NPY levels. As the phenotype is rather similar between OE-NPY$^{D\beta H}$ mice and human carriers of the polymorphism of the *NPY* gene (rs16139), it can be suspected that the mechanism is also the same (discussed in more detail in chapter 5.7.2). Therefore, this study may reveal novel drug targets in the liver to combat certain metabolic disorders. Furthermore, as changes in hepatic glycogen metabolism play a major role in IGT, and these changes can be normalized with metformin treatment, especially the carriers of the polymorphism could benefit from metformin treatment to prevent the development of T2D. Instead, targeting peripheral Y_1- or Y_2-receptors by their specific antagonists seems to be more complicated and less likely to achieve the desired therapeutic goals.

6 CONCLUSIONS

This study with the OE-NPY$^{D\beta H}$ mouse model shows that in addition to the previous findings of obesity and IGT, *NPY* overexpression in catecholaminergic system predisposes to hepatosteatosis, hypercholesterolemia and T2D. The following conclusions can be made according to the studies included in this thesis based on the aims:

1. Obese OE-NPY$^{D\beta H}$ mice displayed hepatosteatosis with ballooning degeneration, but without fibrosis or inflammation (type 3 steatosis). The mice also displayed IGT, IR and hypercholesterolemia, which together with steatosis, predisposed them to T2D, when beta cell function was disrupted.

2. Decreased fatty acid oxidation and increased cholesterol synthesis preceded hepatosteatosis and hypercholesterolemia, respectively. Recruitment of inflammatory actions may have enhanced the development of IR in the OE-NPY$^{D\beta H}$ mice.

3. Obese OE-NPY$^{D\beta H}$ mice displayed hepatic glycogen accumulation preceded by increased glycogen metabolism, which suggests that this effect is involved in the IGT encountered in OE-NPY$^{D\beta H}$ mice. Treatment with metformin inhibited glycogenolysis in OE-NPY$^{D\beta H}$ mice, but did not significantly improve IGT.

4. The studies with Y_1- and Y_2-receptor antagonists suggested that the direct actions of NPY in the liver mediated by Y_1- and Y_2-receptors are not responsible for the metabolic disturbances in OE-NPY$^{D\beta H}$ mice. Instead, the decreased expression of *Adrb1* suggested that there is an attenuation of sympathetic tone in the liver which could lie behind the changes observed in the OE-NPY$^{D\beta H}$ mice.

5. Pharmacological blockade of peripheral Y_1- receptors did not improve the metabolic status of OE-NPY$^{D\beta H}$ mice, but may have even worsened it in the WT mice. Antagonism of Y_2-receptors was beneficial, when DIO was combined with excess NPY, whereas in situations when the levels of NPY were normal, similarly to Y_1-receptor antagonism, the drug treatment impaired the metabolic status independent of the diet. Therefore, Y_2-receptor antagonism seems to be a plausíble treatment for obesity, but only in situations when excess NPY (e.g. due to stress) is combined with an unhealthy diet.

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APPENDICES

Table 1 Primer sequences used in qPCR studies

Gene name	Primer sequence	Tissue	Ref. gene	Study
Acetyl-Coenzyme A acetyltransferase 2 *(Acat2*)	Forw: 5'-ATTCCAGCCATAAAGCAAGC-3'	Intestine	*Actb*	I
	Rev: 5'-TTTAGCTATTGCCGCAGACA-3'			
*A*cetyl-CoA carboxylase 1 *(Acc1)*	Forw: 5'-GCGTCGGGTAGATCCAGTT-3'	Liver	*Actb*	I
	Rev: 5'-CTCAGTGGGGCTTAGCTCTG-3'			
Acyl-Coenzyme A oxidase 1, palmitoyl, (*Acox1*)	Forw: 5'-GCCCAACTGTGACTTCCATC-3'	Liver	*Actb*	I & III
	Rev: 5'-GCCAGGACTATCGCATGATT-3'			
Acyl-CoA synthetase long-chain family member 4 *(Acsl4)*	Forw: 5'-GAAATTCACAGCATGCAATCAG-3'	Liver	*Actb*	I
	Rev: 5'-TCTACTTGGAGGAACGCTCAA-3'			
Adrenergic receptor, beta 1 *(Adrb1)*	Forw: 5'-GTGGGTAACGTGCTGGTGAT -3'	Liver	*Actb*	I & III
	Rev: 5'-GAAGTCCAGAGCTCGCAGAA-3'			
Adrenergic receptor, beta 2 *(Adrb2)*	Forw: 5'-TGGTTGGGCTACGTCAACTC-3'	Liver	*Actb*	I & III
	Rev: 5'-TCCGTTCTGCCGTTGCTATT-3'			
ATP-binding cassette transporter A1 *(Abca1*)	Forw: 5'-GCAGATCAAGCATCCCAACT-3'	Intestine	*Actb*	I
	Rev: 5'-CCAGAGAATGTTTCATTGTCCA-3'			
ATP-binding cassette transporter G5 *(Abcg5)*	Forw: 5'-TCCTGCATGTGTCCTACAGC-3'	Intestine	*Actb*	I
	Rev: 5'-ATTTGCCTGTCCCACTTCTG-3'			
ATP-binding cassette transporter G8 *(Abcg8*)	Forw: 5'-AACCCTGCGGACTTCTACG-3'	Intestine	*Actb*	I
	Rev: 5'-CTGCAAGAGACTGTGCCTTCT-3'			
β-actin (*Actb*)	Forw: 5'-TCCATCATGAAGTGTGACGT-3'		-	I & III
	Rev: 5'- GAGCAATGATCTTGATCTTCA-3'			
Carboxylesterase 3 (*Ces3*)	Forw: 5'-TGGTATTTGGTGTCCCATCA-3'	WAT/r	*Rps29*	III
	Rev: 5'-GCTTGGGCGATACTCAAACT-3'			
Carnitine acetyltransferase (*Crat*)	Forw: 5'-GCCATTGCTATGCACTTCAAC-3'	Liver	*Actb*	I & III
	Rev: 5'-GGTCCGAAGAACATGACACA-3'			
Carnitine palmitoyltransferase 1a (*Cpt1*)	Forw: 5'-GCTGTCAAAGATACCGTGAGC-3'	Liver	*Actb*	I & III
	Rev: 5'-TCTCCCTCCTTCATCAGTGG-3'			
Cytochrome P450 7a1 *(Cyp7a1)*	Forw: 5'-GATCCTCTGGGCATCTCAAG-3'	Liver	*Actb*	I
	Rev: 5'-AGAGGCTGCTTTCATTGCTT-3'			
Farnesyl diphosphate farnesyl transferase 1 *(Fdft1)*	Forw: 5'-CCAAACAGGACTGGGACAAG-3'	Liver	*Actb*	I
	Rev: 5'-GACGAGAAAGGCCAATTCC-3'			
Fatty acid binding protein 4 (*Fabp4*)	Forw: 5'-GGATGGAAAGTCGACCACAA-3'	WAT/r	*Rps29*	III
	Rev: 5'-TGGAAGTCACGCCTTTCATA-3'			
Fatty acid synthase (*Fas*)	Forw: 5'-GCTGCTGTTGGAAGTCAGC-3'	Liver	*Actb*	I & III
	Rev: 5'-AGTGTTCGTTCCTCGGAGTG-3'			
Glucose-6-phosphatase *(G6pc)*	Forw: 5'-CGACTCGCTATCTCCAAGTGA-3'	Liver	*Rps29*	II
	Rev: 5'-GGGCGTTGTCCAAACAGAAT-3'			
Glycogen synthase 2 *(Gys2)*	Forw: 5'-CGATGCTGTCAGAAAAGCTG-3'	Liver	*Actb, Rps29*	I, II & III
	Rev: 5'-AGCCATCTTCCAAAATGCAC-3'			
Glycogen phosphorylase, liver *(Pygl)*	Forw: 5'-CCAGAGTGCTCTACCCCAAT-3'	Liver	*Actb, Rps29*	I, II & III
	Rev: 5'-CCACCACAAAGTACTCCTGTTTC-3'			

Hormone sensitive lipase (*Lipe*)	Forw: 5'-GCGCTGGAGGAGTGTTTTT-3'	WAT/r	*Rps29*	III
	Rev: 5'-CGCTCTCCAGTTGAACCAAG-3'			
Interleukin 1 beta *(Il1b)*	Forw: 5'-TGTAATGAAAGACGGCACACC-3'	Liver	*Actb*	I
	Rev: 5'-TCTTCTTTGGGTATTGCTTGG-3'			
Lipoprotein lipase (*Lpl*)	Forw: 5'-CTCGCTCTCAGATGCCCTAC-3'	WAT/r	*Rps29*	III
	Rev: 5'-AGGCCTGGTTGTGTTGCTT-3'			
LDL receptor *(Ldlr)*	Forw: 5'-CAAGAGGCAGGGTCCAGA-3'	Liver	*Actb*	I
	Rev: 5'-CCAATCTGTCCAGTACATGAAGC-3'			
Matrix metallopeptidase (*Mmp3*)	Forw: 5'-TTGTTCTTTGATGCAGTCAGC-3'	WAT/r	*Rps29*	III
	Rev: 5'-GATTTGCGCCAAAAGTGC-3'			
Neuropeptide Y (*Npy*)	Forw: 5'-CCGCTCTGCGACACTACAT-3'	Brain	*Actb*	III
	Rev: 5'-TGTCTCAGGGCTGGATCTCT-3'			
Neuropeptide Y (*Npy*)	Forw: 5'-CTCCGCTCTGCGACACTAC-3'	Adr. gland	*Actb*	III
	Rev: 5'-GGAAGGGTCTTCAAGCCTTGT-3'			
Niemann pick c1 like 1 *(Npc1l1)*	Forw: 5'-TACACGGCCTGGTCTTCCT-3'	Intestine	*Actb*	I
	Rev: 5'-CAGTACCAGAGCTTGGTTAACATC-3'			
Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha *(Ppargc1a)*	Forw: 5'-TATGGAGTGACATAGAGTGTGCT-3'	Liver	*Actb, Rps29*	I & II
	Rev: 5'-CCACTTCAATCCACCCAGAAAG-3'			
Peroxisome proliferator activated receptor alpha *(Ppara)*	Forw: 5'-CACGCATGTGAAGGCTGTAA-3'	Liver	*Actb*	I
	Rev: 5'-GCTCCGATCACACTTGTCG-3'			
Phosphoenolpyruvate carboxykinase 1, cytosolic (*Pck1)*	Forw: 5'-ATGTGTGGGCGATGACATT-3'	Liver	*Actb, Rps29*	I & II
	Rev: 5'-AACCCGTTTTCTGGGTTGAT-3'			
Pro-opiomelanocortin (*Pomc*)	Forw: 5'-CAAGCCGGTGGGCAACAAACG-3'	Hypothalamus	*Actb*	III
	Rev: 5'-CTAATGGCCGCTCGCCTTCAAG-3'			
Ribosomal protein S29 (*Rps29*)	Forw: 5'-ATGGGTCACCAGCAGCTCTA-3'	WAT/r	-	II & III
	Rev: 5'-AGCCTATGTCCTTCGCGTACT-3'			
Sterol regulatory element binding transcription factor 1 *(Srebp1c)*	Forw: 5'-GATGTGCGAACTGGACACAG -3'	Liver	*Actb*	I
	Rev: 5'-CATAGGGGGCGTCAAACAG -3'			
Scavenger receptor class B type I *(Srb1)*	Forw: 5'-GCCCATCATCTGCCAACT-3'	Intestine	*Actb*	I
	Rev: 5'-TCCTGGGAGCCCTTTTTACT-3'			
Transforming growth factor beta 1 *(Tgfb1)*	Forw: 5'-TGGAGCAACATGTGGAACTC-3'	Liver	*Actb*	I
	Rev: 5'-CAGCAGCCGGTTACCAAG-3'			
Tumor necrosis factor alfa *(Tnfa)*	Forw: 5'-CTGAACTTCGGGGTGATCGG-3'	Liver	*Actb*	I
	Rev: 5'-GGCTTGTCACTCGAATTTTGAGA-3'			
Tyrosine hydroxylase (*Th*)	Forw: 5'-CCCAAGGGCTTCAGAAGAG-3'	Brain	*Actb*	III
	Rev: 5'-GGGCATCCTCGATGAGACT-3'			
Uncoupling protein 1 (*Ucp1*)	Forw: 5'-ACTGCCACACCTCCAGTCATT-3'	BAT	*Actb*	III
	Rev: 5'-CTTTGCCTCACTCAGGATTGG-3'			
Y1 receptor *(Yr1)*	Forw: 5'-TCACAGGCTGTCTTACACGACT-3'	Liver, Brain, Adr. Gland	*Actb*	I & III
	Rev: 5'-TTTCTCCTTTTCAAGCGAATG-3'			
Y2receptor *(Y2r)*	Forw: 5'-AACGCGCAAGAGTCAATACA-3'	Liver, Brain	*Actb*	I & III
	Rev: 5'-CCATAGGGCTCCACTTTCACT-3'			
3-hydroxy-3-methylglutaryl-Coenzyme A reductase *(Hmgcr)*	Forw: 5'-TGATTGGAGTTGGCACCAT-3'	Liver	*Actb*	I
	Rev: 5'-TGGCCAACACTGACATGC-3'			
7-dehydrocholesterol reductase *(Dhcr7)*	Forw: 5'-GAGGCGTCCAAGAAGGTG-3'	Liver	*Actb*	I
	Rev: 5'-GCAGCCCATTCACCTCATAC-3'			

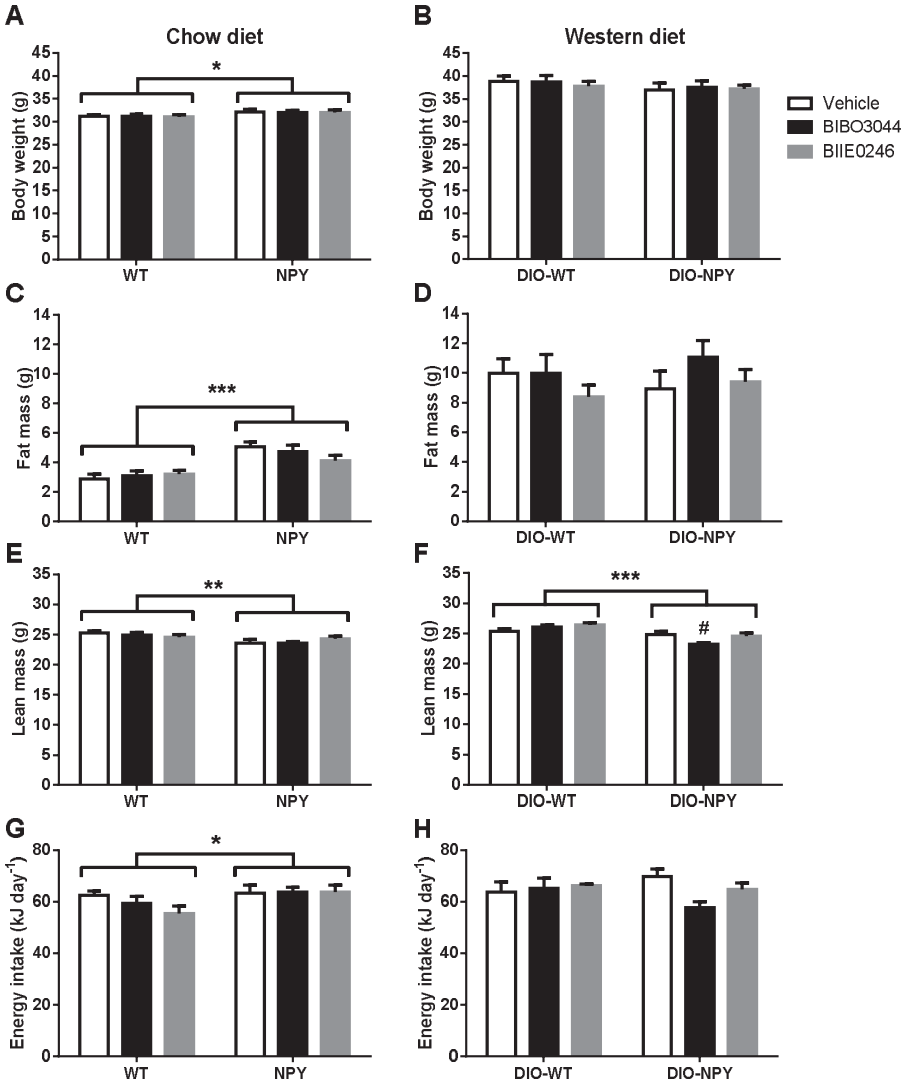
BAT = Brown adipose tissue, WAT/r = Retroperitoneal white adipose tissue, Brain = Brainstem and hypothalamus

Table 2 Up- and down-regulated genes in the hepatic microarray of pre-obese OE-NPY$^{D\beta H}$ mice

ENTREZ ID		FC
Up-regulated genes		
11677	aldo-keto reductase family 1, member B3 (aldose reductase) *(Akr1b3)*	2.45
14864	glutathione S-transferase, mu 3 *(Gstm3)*	1.47
67106	zinc finger and BTB domain containing 8 opposite strand *(Zbtb8os)*	1.44
232174	cytochrome P450, family 26, subfamily b, polypeptide 1 *(Cyp26b1)*	2.01
13095	cytochrome P450, family 2, subfamily c, polypeptide 29 *(Cyp2c29)*	1.65
17448	malate dehydrogenase 2, NAD (mitochondrial) *(Mdh2)*	1.64
14629	glutamate-cysteine ligase, catalytic subunit *(Gclc)*	1.45
18534	phosphoenolpyruvate carboxykinase 1, cytosolic *(Pck1)*	1.63
20893	basic helix-loop-helix domain containing, class B2 *(Bhlhb2)*	1.72
105387	aldo-keto reductase family 1, member C14 *(Akr1c14)*	1.53
67758	arylacetamide deacetylase (esterase) *(Aadac)*	1.70
68041	Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish)) *(Mid1ip1)*	1.48
66270	RIKEN cDNA 1810015C04 gene (*1810015C04Rik)*	1.50
107141	cytochrome P450, family 2, subfamily c, polypeptide 50 *(Cyp2c50)*	1.44
50790	acyl-CoA synthetase long-chain family member 4 *(Acsl4)*	1.49
14104	fatty acid synthase *(Fasn)*	1.41
12452	cyclin G2 *(Ccng2)*	1.52
14857	glutathione S-transferase, alpha 1 (Ya) *(Gsta1)*	1.68
14858	glutathione S-transferase, alpha 2 (Yc2) *(Gsta2)*	1.48
14960	histocompatibility 2, class II antigen A, alpha *(H2-Aa)*	1.63
66046	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5 *(Ndufb5)*	1.42
72082	cytochrome P450, family 2, subfamily c, polypeptide 55 *(Cyp2c55)*	1.46
27400	hydroxysteroid (17-beta) dehydrogenase 6 *(Hsd17b6)*	1.74
16648	karyopherin (importin) alpha 3 *(Kpna3)*	1.42
67603	dual specificity phosphatase 6 *(Dusp6)*	1.62
12116	betaine-homocysteine methyltransferase *(Bhmt)*	1.47
14828	heat shock protein 5 *(Hspa5)*	1.50
Down-regulated genes		
11430	acyl-Coenzyme A oxidase 1, palmitoyl *(Acox1)*	-1.49
12908	carnitine acetyltransferase *(Crat)*	-1.43
13119	cytochrome P450, family 4, subfamily a, polypeptide 14 *(Cyp4a14)*	-1.59
74147	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase *(Ehhadh)*	-1.48
11520	adipose differentiation related protein *(Adfp)*	-1.42
17068	lymphocyte antigen 6 complex, locus D (*Ly6d)*	-1.45
13117	cytochrome P450, family 4, subfamily a, polypeptide 10 *(Cyp4a10)*	-1.43
16427	inter alpha-trypsin inhibitor, heavy chain 4 *(Itih4)*	-1.11
16803	lipopolysaccharide binding protein *(Lbp)*	-1.62
66058	transmembrane protein 176A *(Tmem176a)*	-1.44
16819	lipocalin 2 *(Lcn2)*	-4.29
15439	haptoglobin *(Hp)*	-1.47

Microarray was performed in the livers of 2-month-old heterozygous OE-NPY$^{D\beta H}$ vs. WT mice (n=4/group) by *limma* of the R statistical analysis program**.** Genes were considered significantly up- or downregulated when $P < 0.05$. FC=fold change. Reprinted with permission from Ailanen *et al.* J Endocrinol 2017, 234(1):57-72.

Figure 1 Baseline phenotype of chow or Western diet-fed OE-NPY$^{D\beta H}$ and wildtype mice prior to treatment with Y_1- or Y_2-receptor antagonists



(A-B) Baseline body weight, (C-D) fat mass and (E-F) lean mass of 16-week-old homozygous OE-NPY$^{D\beta H}$ and wildtype mice on a chow (n=19-25 mice/group) and Western diet (n=7-11 mice/group). (G-H) Baseline daily average caloric intake in the chow (n =7-10 cages/group) and Western diet-fed (n=3-4 cages/group) OE-NPY$^{D\beta H}$ and wildtype mice. The phenotype was analyzed after a 2-week habituation with daily saline injections (i.p.), prior to dosing of Y_1- or Y_2-receptor antagonists (BIBO3044 and BIIE0246, respectively), or vehicle. Values are expressed as means ± SEM. *P<0.05, **P<0.01 and ***P<0.001 comparing the different genotypes with two-way ANOVA. # P<0.05 comparing BIBO3044 group with vehicle group within OE-NPY$^{D\beta H}$ mice with Tukey's post-hoc test following a significant interaction between treatment and genotype in two-way ANOVA. WT = Wildtype mice on chow diet, NPY = OE-NPY$^{D\beta H}$ mice on chow diet, DIO-WT= Wildtype mice on Western diet, DIO-NPY= OE-NPY$^{D\beta H}$ mice on Western diet.

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