The metabolic syndrome in mice overexpressing neuropeptide Y in noradrenergic neurons

Liisa Ailanen¹, Suvi T Ruohonen², Laura H Vähätalo², Katja Tuomainen², Kim Eerola², Henriikka Salomäki-Myftari¹, Matias Røyttä³, Asta Laiho⁴, Markku Ahotupa⁵, Helena Gylling⁶ and Eriika Savontaus⁷

¹Institute of Biomedicine and Turku Center for Disease Modelling; Drug Research Doctoral Program, University of Turku, Turku, Finland

²Institute of Biomedicine and Turku Center for Disease Modelling, University of Turku, Turku, Finland

³Department of Pathology, University of Turku and Turku University Hospital, Turku, Finland

⁴Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland

⁵Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland

⁶Department of Internal Medicine, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland

⁷Institute of Biomedicine and Turku Center for Disease Modelling, University of Turku; Turku University Hospital, Unit of Clinical Pharmacology, Turku, Finland

Correspondence
should be addressed
to E Savontaus
Email
eriika.savontaus@utu.fi

Abstract

A gain-of-function polymorphism in human neuropeptide Y (NPY) gene (rs16139) associates with metabolic disorders and earlier onset of type 2 diabetes (T2D). Similarly, mice overexpressing NPY in noradrenergic neurons (OE-NPY^{DBH}) display obesity and impaired glucose metabolism. In this study, the metabolic syndrome-like phenotype was characterized and mechanisms of impaired hepatic fatty acid, cholesterol and glucose metabolism in pre-obese (2-month-old) and obese (4–7-month-old) OE-NPY^{DBH} mice were elucidated. Susceptibility to T2D was assessed by subjecting mice to high caloric diet combined with low-dose streptozotocin. Contribution of hepatic Y1-receptor to the phenotype was studied using chronic treatment with an Y1-receptor antagonist, BIBO3304. Obese OE-NPY^{DBH} mice displayed hepatosteatosis and hypercholesterolemia preceded by decreased fatty acid oxidation and accelerated cholesterol synthesis. Hyperinsulinemia in early obese state inhibited pyruvate- and glucose-induced hyperglycemia, and deterioration of glucose metabolism of OE-NPY^{DBH} mice developed with aging. Furthermore, streptozotocin induced T2D only in OE-NPY^{DBH} mice. Hepatic inflammation was not morphologically visible, but upregulated hepatic anti-inflammatory pathways and increased 8-isoprostane combined with increased serum resistin and decreased interleukin 10 pointed to increased NPY-induced oxidative stress that may predispose OE-NPY^{DBH} mice to insulin resistance. Chronic treatment with BIBO3304 did not improve the metabolic status of OE-NPY^{DBH} mice. Instead, downregulation of beta-1-adrenoceptors suggests indirect actions of NPY via inhibition of sympathetic nervous system. In conclusion, changes in hepatic fatty acid, cholesterol and glucose metabolism favoring energy storage contribute to the development of NPY-induced metabolic syndrome, and the effect is likely mediated by changes in sympathetic nervous system activity.

Key Words

- ▶ neuropeptide Y
- ▶ hepatic cholesterol synthesis oxidative stress
- ▶ glucose metabolism
- ▶ type 2 diabetes NAFLD
- ▶ Y1-receptor

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Introduction

Besides its orexigenic nature in the hypothalamus (Zarjevski *et al.* 1993, Yang *et al.* 2009, Sousa-Ferreira *et al.* 2011), neuropeptide Y (NPY) induces obesity and metabolic alterations independent of hyperphagia. In humans, a gain-of-function polymorphism in NPY gene (rs16139, p.L7P), which increases NPY secretion especially upon sympathetic activation (Kallio *et al.* 2001, Mitchell *et al.* 2008), has been associated with traits of the metabolic syndrome, i.e. weight gain, hypercholesterolemia, impaired glucose tolerance (IGT), insulin resistance (IR), earlier onset of type 2 diabetes (T2D) and increased risk of diabetes-related vascular complications (Karvonen *et al.* 1998, Pihlajamaki *et al.* 2003, Nordman *et al.* 2005, van Rossum *et al.* 2006, Ukkola & Kesaniemi 2007, Jaakkola *et al.* 2009, Yeung *et al.* 2011, Masoudi-Kazemabad *et al.* 2013) without an association to increased food intake (Karvonen *et al.* 2006, Yeung *et al.* 2011).

The mechanisms whereby NPY alters energy metabolism have been elucidated in animal models. Central NPY is known to suppress the sympathetic tone, energy expenditure and brown adipose tissue (BAT) thermogenic activity, and to increase lipogenesis in white adipose tissue (WAT) (Egawa *et al.* 1991, Zarjevski *et al.* 1993, Shi *et al.* 2013). An important source of NPY in the periphery is the sympathetic nervous system (SNS), and NPY levels are increased upon stress (Ekblad *et al.* 1984). Consistently, NPY has been implicated in the stress-induced obesity, directly affecting the adipogenic and lipogenic regulation of WAT (Kuo *et al.* 2007, Zhang *et al.* 2014). In addition, chronic peripheral NPY administration induces obesity (Xie *et al.* 2012), and knockdown of peripheral NPY Y1- or Y2-receptors protects from diet-induced obesity by changing fuel oxidation (Zhang *et al.* 2010, Shi *et al.* 2011).

The mechanisms of NPY-induced hypercholesterolemia, IGT or earlier onset of T2D in humans are still unknown, but it can be hypothesized that hepatosteatosis plays a role in the NPY-induced metabolic and vascular diseases. Hepatosteatosis is suggested to be the hepatic manifestation of the metabolic syndrome and the actual cause of the related cardiovascular diseases (Lu *et al.* 2013). It predicts the incidence of T2D (Vozarova *et al.* 2002, Lee *et al.* 2003), and together with diabetes increases the risk for cardiovascular events (Targher *et al.* 2005). In various murine models, NPY has been shown to contribute to hepatic lipogenesis, VLDL-triglyceride (TG) and VLDL-cholesterol secretion, fatty

acid (FA) oxidation and increased serum cholesterol levels (Zarjevski *et al.* 1993, Stafford *et al.* 2008, Zhang *et al.* 2010, Bruinstroop *et al.* 2012, Xie *et al.* 2012, Zhang *et al.* 2014), but it is not known, whether chronic excess of NPY causes hepatosteatosis, or which hepatic mechanisms contribute to dyslipidemias and development of the metabolic syndrome.

The mouse model overexpressing NPY in central and peripheral noradrenergic neurons driven by dopamine-beta-hydroxylase (*DBH*) gene promoter (OE-NPY^{DBH}) presents a model of NPY-induced metabolic syndrome-like phenotype without hyperphagia (Ruohonen *et al.* 2008, Vahatalo *et al.* 2014). Thus, it allows studying the mechanisms of NPY in SNS and brainstem in the pathogenesis of obesity and metabolic disturbances. Heterozygous OE-NPY^{DBH} mice display increased fat mass and hepatic TGs, and subsequently develop hyperinsulinemia and IGT (Ruohonen *et al.* 2008). Homozygous OE-NPY^{DBH} mice display a more severe obese phenotype, and the suggested mechanisms are direct effects of NPY on adipocytes, and decreased sympathetic activity to adipose tissues (Ruohonen *et al.* 2008, Vahatalo *et al.* 2014). In the current study, we aimed to elucidate the effects of noradrenergic NPY on hepatic FA, cholesterol and glucose metabolism, to define their contribution to the development of the hepatosteatosis, hypercholesterolemia and IGT, and finally, to characterize whether the changes are due to direct effects of NPY mediated via hepatic Y-receptors or due to changes in SNS activity.

Materials and methods

General

Generation of the transgenic heterozygous and homozygous OE-NPY^{DBH} mice has been described in detail elsewhere (Ruohonen *et al.* 2008, Vahatalo *et al.* 2014). Transgenic mice were maintained on a C57BL/6N inbred background, and either heterozygote and wild-type (WT) littermates, or 1st-3rd generation of WT and homozygous mice originating from the same heterozygous breedings were used. The mice were kept in an animal room maintained at 21 ± 3°C with fixed 12-h light:12-h darkness cycle. Standard rodent chow diet (SDS, Essex, UK) and tap water were available *ad libitum*. At termination, 4-h-fasted mice were anesthetized with ketamine (75 mg/kg i.p. Ketaminol, Intervet Oy, Finland) and medetomidine (1 mg/kg i.p. Cepetor, ScanVet Oy, Finland),

and serum was obtained by cardiac puncture. Tissues were collected and weighed, and stored in -70°C . The experimental procedures were approved by the national animal care and use committee (ELLA). Animal care was in accordance with the guidelines of the International Council of Laboratory Animal Science (ICLAS).

Study 1. Characterization of the phenotype of obese OE-NPY^{DBH} mice

Liver fat content and histology, and serum cholesterol were analyzed in 4- and 7-month-old male and female homozygous OE-NPY^{DBH} mice. These mice were previously shown to display 1.5-fold increase in WAT weight and a decrease in serum TG levels compared to WT controls. They develop IGT, IR, fasting hyperinsulinemia and hyperleptinemia, which are most pronounced in 7-month-old males (Vahatalo *et al.* 2014).

Study 2. Mechanisms leading to metabolic syndrome-like phenotype in OE-NPY^{DBH} mice

Mechanisms of hepatosteatosis, hypercholesterolemia and IGT were first elucidated by analyzing changes in hepatic mRNA expression profile preceding any metabolic disturbances (pre-obese 2-month-old heterozygous mice, $n=4-5/\text{group}$) that might suggest causal mechanisms. This was followed by *in vivo* functional assays verifying the findings in the gene expression. Homozygous male mice were used due to their more severe metabolic phenotype, except in cholesterol studies where female mice were used due to their lower level of hepatosteatosis that could induce hypercholesterolemia independent of NPY. Beta-oxidation assay (supplementary information, see section on [supplementary data](#) given at the end of this article) was carried out in 2-month-old mice ($n=6-9/\text{group}$) that do not yet show signs of hepatosteatosis. Cholesterol absorption and synthesis assay ([supplementary information](#)) was carried out in 6-month-old mice ($n=5-10/\text{group}$), and glucose-stimulated insulinemia and hepatic glucose production in early (at 4 months) and in established obese states (at 7 months) ($n=6-11/\text{group}$). Oxidative stress and inflammation as potential causes of IR were analyzed in pre-obese (at 2 months) and obese (at 5 and 7 months) states ($n=3-10/\text{group}$).

Glucose, pyruvate and insulin tolerance tests

To study insulin secretion during glucose tolerance test mice were implanted with a carotid artery catheter under

2% isoflurane anesthesia and buprenorphine analgesia (0.1 mg/kg, s.c., twice a day for 2 days, Temgesic, Schering-Plough, Brussels, Belgium). After 3-day recovery, fasted mice (4h) received glucose (1g/kg, i.p.), and arterial blood samples (25 μL) were collected using an automated blood sampling device (AccuSampler®, VeruTech, Lund, Sweden) from awake, freely moving mice. Another set of mice were challenged for glucose (1g/kg) and pyruvate (2g/kg, Sigma-Aldrich), which were injected (i.p.) after a 4 and 4 or 6h fast, respectively. Blood glucose was measured with a glucose meter (Precision Xtra Glucose Monitoring Device, Abbott Diabetes Care, Abbot Park, IL, USA) at baseline and after the injection at the indicated time points.

Study 3. Susceptibility to T2D

The susceptibility to T2D was studied by disrupting pancreatic insulin secretion with STZ in mice with IR due to high caloric diet that induces obesity in a similar manner in OE-NPY^{DBH} and WT mice, but is not sufficient to induce T2D in OE-NPY^{DBH} mice (Ruohonen *et al.* 2012). Homozygous male OE-NPY^{DBH} of 16-week-old and WT mice ($n=7/\text{group}$) subjected to western diet (41% kcal fat, 43% kcal carbohydrate, 17% kcal protein, D12079B, Research Diets, New Brunswick, NJ, USA) for 3 weeks were administered a low-dose [40 mg/kg in 7.5 mg/ml Na-Citrate solution (pH 4.5), i.p.] of STZ (Sigma-Aldrich) after a 4-h fast on 3 consecutive days (week 0), followed by a single dose every 4.5 weeks to maintain hyperglycemia. Weight gain and fasting (4h) tail vein blood glucose were monitored weekly and fat mass by quantitative NMR (EchoMRI-700, Echo Medical Systems, Houston, TX, USA) at weeks -3 and 9. Glucose (GTT) (1g/kg glucose i.p. after a 4-h fast) and insulin tolerance tests (ITT) (1 IU/kg insulin i.p. after a 1-h fast) were performed at weeks 10 and 12, respectively. Tissues were collected at week 13.

Study 4. Peripheral Y1R-antagonist treatment

To study whether hepatic Y-receptors, or modulation of SNS activity, mediate the effects of noradrenergic NPY, expressions of hepatic Y- and adrenergic receptors were analyzed. The contribution of Y1-receptors was studied using a peripherally acting Y1R-antagonist, BIBO3304 trifluoroacetate (Tocris Bioscience, Bristol, UK) (1 mg/kg/day, i.p.) (Dozio *et al.* 2007, Yuzuriha *et al.* 2007) or vehicle (DMSO, Tween® 80 (Fisher Scientific, Fair Lawn, NJ, USA) and 0.9% NaCl, 1:1:18), which were administered to 20-week-old homozygous male OE-NPY^{DBH} and WT

mice ($n=10\text{--}13/\text{group}$) over 30 days preceded by a 2-week habituation with daily saline injections. Food intake was monitored weekly and weight gain twice a week. Body composition was measured on days -14, 0 and 30 by quantitative NMR followed by tissue collection (after 3-h fast). As peripheral Y1-knockdown was shown to reduce respiratory exchange ratio (RER) without significantly affecting adiposity (Zhang *et al.* 2010), the effects of BIBO3304 vs vehicle (for 7 days preceded by 7 days habituation) were elucidated on 24-h energy expenditure (EE), RER and activity in 3-month-old WT mice ($n = 4/\text{group}$) with an indirect calorimetry (Oxylet System, Panlab, Barcelona, Spain).

Biochemical assays

Serum insulin levels were determined with Ultrasensitive Mouse Insulin ELISA Kit (Mercodia AB, Uppsala, Sweden), non-esterified fatty acids (NEFA) with the NEFA-HR(2) kit (Wako Diagnostics, Richmond, VA, USA), and total cholesterol and cholesterol fractions with a Cholesterol Quantitation Kit and with a HDL and LDL/VLDL Cholesterol Quantification Kit (Biovision) (Study 1) or Cholesterol Fluorometric Assay kit (Cayman Chemical) (Study 4). Serum and liver TGs were measured with TR0100 Serum triglyceride determination kit (Sigma-Aldrich). The hepatic TGs were analyzed from lipids purified with Folch method (Folch *et al.* 1957) (Study 1), or directly from the liver tissue homogenized in PBS containing 0.1% NP-40 (Study 4).

Oxidative stress markers were analyzed in plasma, liver and urine. Total peroxyl radical trapping antioxidant potential in plasma was measured as previously described (Ahotupa *et al.* 1997). Hepatic diene conjugation was measured according to a previously published method (Corongiu & Milia 1983). Glutathione reduced form (GSH) and glutathione oxidized form (GSSG) were measured by Glutathione Assay Kit (Cayman Chemical). 8-Isoprostanes were determined by STAT-8-Isoprostane EIA Kit (Cayman Chemical) and urinary creatinine by Creatinine Colorimetric Assay Kit (Cayman Chemical). Urinary 8-isoprostane was normalized relative to creatinine concentration. Serum cytokines were analyzed with MILLIPLEX MAP Mouse Cytokine/Chemokine Multiplex Assay (MCYTOMAG-70K, Millipore Corporation, Billerica, MA, USA), resistin with MILLIPLEX® Mouse Adipokine Multiplex Assay (MADKMAG-71K) and adiponectin with Mouse Adiponectin ELISA kit (EZMADP-60K, Millipore Corporation).

Gene expression analyses

RNA was extracted with Trizol Reagent (Invitrogen) or RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) with DNase treatment (RNase-Free DNase Set, Qiagen GmbH). Total RNA concentration and purity were ascertained spectrophotometrically (Lambda 20 UV/VIS Spectrometer, Perkin Elmer, Waltham, MS, USA). Microarray was performed using the Illumina MouseRef-8 v1.1 microchip, where RNA samples were compared to 18918 genes on the chip. For the quantitative real-time PCR analysis (qPCR), RNA was converted to cDNA with High Capacity RNA-to-cDNA Kit (Applied Biosystems) on a GeneAmp PCR System 9600 (Perkin Elmer). The qPCR was performed with SYBR Green method using Kapa Sybr Fast qPCR Kit (Kapa Biosystems, Woburn, MA, USA) and 7300 Real-Time PCR System (Applied Biosystems) or CFX96 Real-time PCR Detection System (Bio-Rad). The primers used in the assay are shown in [Supplementary Table 1](#). Beta actin (*Bact*) was used as an endogenous control.

Histology

Liver histology was analyzed from formalin-fixed samples embedded in paraffin, sectioned (5 μm) every 200 μm on microscopic slides, stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS), and analyzed under a standard light microscope. Hepatosteatosis was graded according to (Cabezas *et al.* 2012). Liver cryosections (10 μm) were stained with Oil Red O (ORO) to visualize the lipid droplets. Pancreatic sections were stained with H&E. The areas of the islets of Langerhans ($n=69\text{--}115/\text{group}$) were measured using the Cell*A imaging software (Soft Imaging System, Münster, Germany). Rabbit anti-insulin (sc-9168, Santa Cruz Biotechnology, Inc.) polyclonal antibody (1:500 dilution) was used for immunohistochemical staining of the beta cells. The average area of the stained islets ($n=5\text{--}15/\text{slide}$) was analyzed with ImageJ 1.48v (National Institute of Health, USA). The number of islets in a representative slide was calculated, and the islets were grouped according to their size.

Statistical analyses

The results were analyzed with unpaired Student's *t*-test to compare the genotype differences or two-way ANOVA and Bonferroni *post hoc* test to compare two genotypes and an additional challenge. Two-way ANOVA for repeated measures was used to analyze parameters over

Table 1 Liver weights (g) of OE-NPY^{DBH} mice in different age groups.

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

Liver weights were measured from 2- ($n = 6-9/\text{group}$), 4- ($n = 5-6/\text{group}$) and 7-month-old ($n = 9-11/\text{group}$) homozygous female and male OE-NPY^{DBH} vs wild-type mice. Values are expressed as means ± S.E.M. Statistics were analyzed with Student's *t*-test. ND=Not diagnosed, NS=non-significant.

time between genotypes. Logarithmic transformations or non-parametric Mann-Whitney test were used if data was not normally distributed (D'Agostino and Pearson omnibus normality test). Correlations were analyzed with Pearson correlation. These analyses were performed with GraphPad Prism 6.0 software (GraphPad Software, Inc.). The microarray results were analyzed by *limma* of the R statistical analysis program (R language and environment for statistical computing). Pathway and cluster analysis were performed with David Bioinformatics Database (<http://david.abcc.ncifcrf.gov/home.jsp>). The results are expressed as means ± S.E.M. $P < 0.05$ was considered statistically significant.

Results

Study 1. OE-NPY^{DBH} induces hepatosteatosis and hypercholesterolemia

Hepatosteatosis in OE-NPY^{DBH} mice develops with age. The livers of OE-NPY^{DBH} male mice were heavier than those of WT controls at 4 and 7 months, but only at 7 months in females (Table 1). Hepatic lipid accumulation

was detected in both sexes, and ballooning degeneration in 4- and 7-month-old male OE-NPY^{DBH} mice (Fig. 1A and B). Hepatosteatosis was confirmed at 7 months by ORO staining and biochemical TG analysis (Fig. 1B and C). Infarcted areas (microscopic) were detected in two of four OE-NPY^{DBH} mice, but no visible inflammation or fibrosis. Liver of obese male OE-NPY^{DBH} mice was graded as type 3 hepatosteatosis.

Serum total cholesterol of 7-month-old male OE-NPY^{DBH} mice was significantly increased and there was a similar trend already at 4 months in males and at 7 months in females (Table 2). LDL cholesterol was not changed, but HDL cholesterol was increased (Table 2).

Study 2. Mechanisms leading to metabolic syndrome-like phenotype in OE-NPY^{DBH} mice

OE-NPY^{DBH} attenuates hepatic FA oxidation To study the possible mechanisms leading to hepatosteatosis, hepatic gene expression profiles were compared between pre-obese OE-NPY^{DBH} and WT mice by microarray analysis, which showed 27 upregulated and 12 downregulated genes in OE-NPY^{DBH} livers (Table 3)

Figure 1

Liver histology and triglyceride accumulation of obese OE-NPY^{DBH} mice. (A) Representative hematoxylin and eosin stained liver sections from 4-month-old homozygous female and male OE-NPY^{DBH} vs wild-type mice showing ballooning degeneration and triglyceride accumulation in males (scale bar 50µm). (B) Representative Oil red O (scale bar 100µm) and hematoxylin and eosin (scale bar 50µm) stained liver sections, and (C) liver triglyceride content of 7-month-old homozygous male OE-NPY^{DBH} vs wild-type mice ($n = 9-10/\text{group}$) showing hepatosteatosis. Values are expressed as means ± S.E.M. * $P < 0.05$ with Student's *t*-test. H&E = hematoxylin and eosin staining, ORO = Oil red O staining, WT = wild-type mice, OE-NPY = OE-NPY^{DBH} mice.

Table 2 Serum cholesterol levels of obese OE-NPY^{DBH} mice.

	4 months			7 months		
	WT	OE-NPY ^{DBH}	P value	WT	OE-NPY ^{DBH}	P value
Female						
Total Cholesterol ($\mu\text{g } \mu\text{L}^{-1}$)	1.65 $\pm$ 0.15	1.90 $\pm$ 0.14	0.12	0.42 $\pm$ 0.08	0.59 $\pm$ 0.08	0.07
HDL Cholesterol ($\mu\text{g } \mu\text{L}^{-1}$)	1.17 $\pm$ 0.08	1.40 $\pm$ 0.06	<0.05	0.34 $\pm$ 0.02	0.40 $\pm$ 0.02	<0.05
LDL/VLDL Cholesterol ($\mu\text{g } \mu\text{L}^{-1}$)	ND	ND	-	0.17 $\pm$ 0.01	0.13 $\pm$ 0.03	0.27
Male						
Total Cholesterol ($\mu\text{g } \mu\text{L}^{-1}$)	1.64 $\pm$ 0.12	2.08 $\pm$ 0.20	0.08	1.44 $\pm$ 0.12	2.03 $\pm$ 0.12	<0.01
HDL Cholesterol ($\mu\text{g } \mu\text{L}^{-1}$)	1.46 $\pm$ 0.05	1.68 $\pm$ 0.06	<0.05	0.73 $\pm$ 0.03	0.83 $\pm$ 0.07	<0.05
LDL/VLDL Cholesterol ($\mu\text{g } \mu\text{L}^{-1}$)	0.37 $\pm$ 0.06	0.25 $\pm$ 0.02	0.08	0.11 $\pm$ 0.02	0.11 $\pm$ 0.02	0.98

Serum cholesterol levels were measured from 4- ($n = 7-11/\text{group}$) and 7-month-old ($n = 7-10/\text{group}$) homozygous female and male OE-NPY^{DBH} vs wild-type mice. Values are expressed as means $\pm$ S.E.M.. Statistics were analyzed with Student's *t*-test. ND=Not diagnosed.

contributing in eight upregulated and five downregulated biological pathways, respectively (Table 4). Downregulated PPAR signaling and FA metabolism pathways pointed to decreased hepatic FA oxidation in OE-NPY^{DBH} mice. In the cluster analysis, three clusters that separated the genes with similar actions were detected: retinol, arachidonic acid and linoleic acid metabolism; FA oxidation; metabolic pathways with anti-inflammatory actions (Table 5).

The most relevant genes involved in FA metabolism were selected for qPCR analysis to validate attenuated FA oxidation in OE-NPY^{DBH} mice. Oxidative enzymes acyl-Coenzyme A oxidase 1 (*Acox1*) and carnitine acetyltransferase (*Crat*) were significantly decreased in pre-obese OE-NPY^{DBH} mice (Fig. 2A). Decreased FA oxidation was confirmed by beta-oxidation assay (Fig. 2B). In contrast, in obese hepatosteatotic OE-NPY^{DBH} mice an increase in *Fas* and a tendency in acetyl Co-A carboxylase (*Acc*) expression suggested increased lipogenesis, but enzymes of beta-oxidation were not significantly different (Fig. 2A).

Oxidative stress precedes hepatosteatosis in OE-NPY^{DBH} mice In the hepatic microarray analysis, pathways involved in metabolism of xenobiotics by cytochrome P450, drug metabolism and glutathione metabolism were upregulated (Table 4), indicating that anti-inflammatory actions were recruited in the pre-obese liver. Accordingly, mRNA expression of proinflammatory cytokines, transforming growth factor beta 1 (*Tgfb1*), tumor necrosis factor alpha (*Tnfa*) and interleukin 1 beta (*Il1b*), were decreased. In the steatotic livers, proinflammatory cytokine expression was still downregulated fitting with the absence of inflammatory cells and fibrosis despite of steatosis. In contrast, 8-isoprostane, a marker of lipid peroxidation, was increased. Other markers of oxidative stress, diene conjugates, GSH or GSSG levels or GSH/GSSG

ratio were not changed. Anti-inflammatory cytokine IL10 tended to be decreased ($P = 0.06$) in serum from pre-obese male OE-NPY^{DBH} mice. In obese male mice resistin levels were increased, but IL6, IL10, adiponectin or markers of oxidative stress were not changed. Interestingly, adiponectin was increased in female OE-NPY^{DBH} mice, which may explain their less prominent metabolic disturbances. Urinary oxidative stress markers were unchanged (Table 6).

Enhanced cholesterol synthesis precedes hypercholesterolemia in OE-NPY^{DBH} mice Obese female OE-NPY^{DBH} mice, representing a phenotype with hypercholesterolemia but less pronounced hepatosteatosis than the males, showed a tendency to accelerated cholesterol and bile acid synthesis in fecal analysis (Fig. 3A and B), which directly measures the whole body cholesterol metabolism. Enhanced cholesterol synthesis was confirmed by increased mRNA expression of cholesterol synthesis enzymes 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), farnesyl diphosphate farnesyl transferase 1 (*Fdft1*) and 7-dehydrocholesterol reductase (*Dhcr7*) in both pre-obese and obese male OE-NPY^{DBH} mice. mRNA expression of LDL-receptor (*Ldlr*) was not changed, but HDL transporter, scavenger receptor B1 (*Srb1*), was slightly decreased ($P = 0.08$) in obese mice (Fig. 3C). Cholesterol absorption was not changed (Fig. 3D and E).

OE-NPY^{DBH} mice show glucose-induced hyperinsulinemia and hepatic glycogen accumulation Glucose-induced insulinemia was analyzed *in vivo* to study whether the previously observed IGT resulted from NPY-induced inhibition of insulin release or whole body IR. Insulin levels were markedly higher in OE-NPY^{DBH} mice when glucose levels were still comparable to WT supporting IR (Fig. 4A and B). In pyruvate challenge, OE-NPY^{DBH} mice showed

Table 3 Hepatic up- and downregulated genes in pre-obese OE-NPY^{DBH} mice.

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

Up- and downregulated genes were measured in the livers of 2-month-old heterozygous OE-NPY^{DBH} vs wild-type mice ($n = 4/\text{group}$) by *limma* of the R statistical analysis program. Genes were considered significantly up- or downregulated when $P < 0.05$. FC = fold change.

decreased gluconeogenesis when statistically significant difference in GTT was not yet revealed, but when IGT was established, there was no difference in PTT or expression of gluconeogenic enzymes (Fig. 4C and E). The average size of islets of Langerhans in obese OE-NPY^{DBH} mice was enlarged (WT $0.013 \pm 0.001 \text{ mm}^2$; OE-NPY^{DBH} $0.020 \pm 0.002 \text{ mm}^2$; $P < 0.001$) supporting previously detected hyperinsulinemia. Furthermore, obese OE-NPY^{DBH} mice displayed hepatic glycogen accumulation (Fig. 4F) preceded by increased expression of both glycogen synthase (*Gys2*) and phosphorylase

(*Pygl*) (Fig. 4E), which suggest acceleration of glycogen cycling.

Study 3. OE-NPY^{DBH} increases susceptibility to STZ-induced diabetes

Susceptibility to T2D was studied by subjecting 16-week-old OE-NPY^{DBH} and WT mice to high caloric diet and low doses of STZ. Both genotypes gained weight and fat mass similarly (Fig. 5A and B). Fasting (4h) glucose was significantly increased in OE-NPY^{DBH} mice and showed

Table 4 Pathway analysis of hepatic up- and downregulated genes in pre-obese OE-NPY^{DBH} mice.

Pathway name	Number of genes	P value	FRD
Upregulated 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
Arachidonic acid metabolism	3	0.030	24.42
Citrate cycle (TCA cycle)	2	0.098	61.06
Downregulated pathways			
Fatty acid metabolism	4	<0.001	0.00
PPAR signaling pathway	4	<0.001	0.00
Retinol metabolism	2	0.047	30.06
Arachidonic acid metabolism	2	0.057	35.40
Vascular smooth muscle contraction	2	0.081	46.94

Pathway analysis was based on the microarray analysis in the livers of 2-month-old heterozygous OE-NPY^{DBH} vs wild-type mice ($n = 4/\text{group}$). 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.

diabetic levels (over 13.8 mmol L⁻¹ in mice) after 4 weeks of the first STZ administration (Fig. 5C). Glucose clearance in GTT was decreased in OE-NPY^{DBH} when compared to

Table 5 Cluster analysis of hepatic up- and downregulated genes in pre-obese OE-NPY^{DBH} mice.

	Score	Number of genes	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^{DBH} vs wild-type mice ($n = 4/\text{group}$). 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 (%), adjusted P value, which estimates the probability of significant P value (<0.05) being false.

WT mice (Fig. 5D) but diet-induced IR was evident in both genotypes (Fig. 5E). Fasting serum insulin levels were equally low (Fig. 5F), and there was no difference between the genotypes in the amount, size or intensity of insulin antibody stained areas in pancreas (Fig. 5G and H). Livers of OE-NPY^{DBH} mice were non-significantly enlarged (WT 1.59 ± 0.08 g; OE-NPY^{DBH} 1.90 ± 0.14 g; $P = 0.08$) and relative liver weights correlated positively with mean fasting blood glucose values ($P < 0.001$, $r^2 = 0.62$). Both genotypes showed severe hepatosteatosis, but not steatohepatitis (data not shown).

Study 4. Hepatic beta1-adrenoceptors but not Y-receptors contribute to the phenotype of OE-NPY^{DBH} mice

Contribution of increased sympathetic NPY and modulated catecholamine release on the phenotype of OE-NPY^{DBH} mice was first evaluated by analyzing hepatic mRNA expression of Y1 (*Y1r*), Y2 (*Y2r*), beta1- (*Adrb1*) and beta2-adrenergic (*Adrb2*) receptors. *Y1r* and *Adrb2* expressions were similar between genotypes, but *Adrb1* expression was decreased in OE-NPY^{DBH} mice (Fig. 6A). *Y2r* was undetected.

Y1-receptor mediated actions of NPY were further elucidated with chronic peripheral Y1R-antagonist treatment. Y1R-antagonist did not prevent the phenotype, i.e. obesity, hepatosteatosis, IR, hyperinsulinemia, hypercholesterolemia or oxidative stress, in OE-NPY^{DBH} mice, nor had an effect on food intake (Fig. 6B–I and Table 7). However, in WT mice it decreased serum TGs and increased liver cholesterol (Fig. 6E and H). EE (relative to lean mass), RER and total activity were similar in both treatment groups after 1- (data not shown) and 7-day-treatment (Fig. 6J, K and L).

Discussion

We have previously shown that homozygous OE-NPY^{DBH} mice develop obesity, IGT and IR (Vahatalo *et al.* 2014). In this study, we show that they also develop hepatosteatosis and hypercholesterolemia, and that this phenotype renders them susceptible to T2D. We previously published that the mechanism of obesity in OE-NPY^{DBH} mice lies mostly in decreased sympathetic signaling in adipose tissues (Vahatalo *et al.* 2014). The current work shows that changes in hepatic lipid, glucose and cholesterol metabolism, and oxidative stress precede obesity, and suggests that decreased sympathetic signaling also in the

Figure 2

Hepatic fatty acid metabolism in OE-NPY^{DBH} mice. (A) mRNA expression of selected genes involved in fatty acid metabolism in 2-month-old heterozygous (pre-obese) ($n = 5/\text{group}$) and 7-month-old homozygous (obese) ($n = 10/\text{group}$) OE-NPY^{DBH} mice relative to wild-type mice. Beta actin was used as endogenous control. (B) Total palmitate oxidation in the livers of 2-month-old homozygous OE-NPY^{DBH} vs wild-type mice ($n = 6-9/\text{group}$). Values are expressed as means $\pm$ S.E.M. * $P < 0.05$ and *** $P < 0.001$ with Student's t -test. *Srebp1c*=Sterol regulatory element binding transcription factor 1, *Fas*=fatty acid synthase, *Acc*=acetyl-CoA carboxylase, *Ppara*=peroxisome proliferator-activated receptor alpha, *Acox1*=acyl-Coenzyme A oxidase 1, *Cpt1*=carnitine palmitoyltransferase 1, *Crat*=carnitine acetyltransferase, *Acs14*=acyl-CoA synthetase long-chain family member 4, WT=wild-type mice, OE-NPY=OE-NPY^{DBH} mice.

liver importantly contributes to the development of the metabolic disturbances in OE-NPY^{DBH} mice.

The mechanisms of hepatosteatosis in OE-NPY^{DBH} mice were studied in pre-obese state in order to elucidate the causal factors and to avoid the TG accumulation-induced disturbances on FA metabolism, (i.e. increased lipogenesis and FA oxidation). The results show that decreased FA

oxidation precedes hepatosteatosis OE-NPY^{DBH} mice. Instead, lipogenesis is significantly increased in steatotic livers, and is likely a consequence of hepatosteatosis and hyperinsulinemia that are known to activate lipogenic enzymes and lipogenesis (Assimakopoulos-Jeannet *et al.* 1995, Donnelly *et al.* 2005, Matsuzawa-Nagata *et al.* 2008). Hepatic FA uptake or VLDL secretion were not measured,

Table 6 Oxidative stress markers and cytokines in OE-NPY^{DBH} mice.

	Pre-obese			Obese		
	WT	OE-NPY ^{DBH}	P value	WT	OE-NPY ^{DBH}	P value
Blood						
IL6 (pg mL ⁻¹)	10.83 $\pm$ 3.74	10.60 $\pm$ 7.38	0.87	3.25 $\pm$ 0.01	8.67 $\pm$ 3.19	0.78
IL10 (pg mL ⁻¹)	17.75 $\pm$ 5.14	8.80 $\pm$ 3.35	0.06	20.63 $\pm$ 9.29	23.80 $\pm$ 6.13	0.58
TRAP	ND	ND	-	216.40 $\pm$ 14.32	231.30 $\pm$ 7.97	0.39
8-Isoprostane (pg mL ⁻¹)	ND	ND	-	326.3 $\pm$ 16.60	347.3 $\pm$ 13.80	0.35
Urine						
8-Isoprostane/Creatinine	ND	ND	-	0.047 $\pm$ 0.00	0.042 $\pm$ 0.01	0.33
Liver						
Dienconjugation (pmol mg ⁻¹)	ND	ND	-	651.70 $\pm$ 16.20	672.10 $\pm$ 7.90	0.51
8-Isoprostane (pg mg ⁻¹)	ND	ND	-	70.40 $\pm$ 3.10	83.40 $\pm$ 4.50	<0.05
GSH (pg mg ⁻¹)	ND	ND	-	30.30 $\pm$ 1.60	31.70 $\pm$ 0.70	0.45
GSSG (pg mg ⁻¹)	ND	ND	-	7.9 $\pm$ 0.5	8.00 $\pm$ 0.40	0.87
GSH/GSSG (pg mg ⁻¹)	ND	ND	-	0.26 $\pm$ 0.00	0.26 $\pm$ 0.01	0.55
mRNA expression						
<i>Il1b</i>	0.92 $\pm$ 0.33	0.70 $\pm$ 0.11	0.28	0.97 $\pm$ 0.18	0.43 $\pm$ 0.05	<0.01
<i>Tnfa</i>	1.24 $\pm$ 0.40	0.55 $\pm$ 0.12	0.06	1.14 $\pm$ 0.19	0.48 $\pm$ 0.13	<0.01
<i>Tgfb1</i>	1.01 $\pm$ 0.07	0.77 $\pm$ 0.10	<0.05	1.04 $\pm$ 0.09	0.56 $\pm$ 0.07	<0.001
	Female			Male		
Blood	WT	OE-NPY^{DBH}	P value	WT	OE-NPY^{DBH}	P value
Resistin (ng mL ⁻¹)	3.35 $\pm$ 0.37	3.35 $\pm$ 0.30	0.99	1.98 $\pm$ 0.20	3.04 $\pm$ 0.39	<0.05
Adiponectin (pg mL ⁻¹)	14.70 $\pm$ 0.85	18.50 $\pm$ 0.82	<0.01	9.70 $\pm$ 0.58	10.47 $\pm$ 0.61	0.40

Serum cytokine levels and hepatic mRNA expression were analyzed from 2- (pre-obese) ($n = 3-9/\text{group}$) and 7-month-old (obese) ($n = 4-10/\text{group}$) male OE-NPY^{DBH} vs wild-type mice. Oxidative stress markers in plasma, urine and liver were measured from 5-month-old male OE-NPY^{DBH} vs wild-type mice ($n = 8-9/\text{group}$). Serum resistin and adiponectin were measured from 7-month-old female and male OE-NPY^{DBH} vs wild-type mice ($n = 8-10/\text{group}$). Values are expressed as means $\pm$ S.E.M. 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.

Figure 3

Cholesterol metabolism in OE-NPY^{DBH} mice. (A) Cholesterol and (B) bile acid synthesis in 6-month-old homozygous female OE-NPY^{DBH} vs wild-type mice measured by fecal ratio method ($n = 5-10$ /group). (C) mRNA expression of cholesterol and bile acid synthesis enzymes in 2-month-old heterozygous (pre-obese) ($n = 5$ /group) and 7-month-old homozygous (obese) ($n = 10$ /group) OE-NPY^{DBH} mice relative to wild-type mice. Beta actin was used as endogenous control. (D) Cholesterol absorption measured by fecal ratio method and (E) mRNA expression of jejunal cholesterol influx and efflux proteins in 6-month-old homozygous female OE-NPY^{DBH} mice relative to wild-type mice ($n = 7-9$ /group). Beta actin was used as endogenous control. Values are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ with Student's t -test. *Hmgcr* = 3-hydroxy-3-methylglutaryl-CoA reductase, *Fdft1* = farnesyl diphosphate farnesyl transferase 1, *Dhcr7* = 7-dehydrocholesterol reductase, *Ldlr* = LDL-receptor, *Srb1* = Scavenger receptor, class B, type 1, *Cyp7a1* = cytochrome P450 7a1, *Npc1l1* = Niemann-Pick C1-Like 1, *Acat2* = acetyl-Coenzyme A acetyltransferase 2, *Abca1* = ATP-binding cassette A1, *Abcg5* = ATP-binding cassette G5, *Abcg8* = ATP-binding cassette G8, WT = wild-type mice, OE-NPY = OE-NPY^{DBH} mice.

but suppressed lipolysis, decreased serum TG and non-esterified FA levels and simultaneous accumulation of TGs into the liver and WAT argue against increased flux of lipids from WAT to the liver (Vahatalo *et al.* 2014).

Hepatic TG accumulation in obese OE-NPY^{DBH} mice develops into type 3 hepatosteatosis characterized by lipid droplets and ballooning degeneration without inflammation and fibrosis. However, markers of inflammation were detected both in pre-obese and obese states, which together with hepatosteatosis may contribute to the development of IR and T2D in OE-NPY^{DBH} mice. The upregulated anti-inflammatory pathways in pre-obese livers, usually considered a secondary response to oxidative stress, may be an early sign of disruptions in hepatic FA metabolism. 8-Isoprostane, a marker for oxidative stress (reviewed by (Roberts & Morrow 2000)), was elevated in steatotic livers. Decreased levels of anti-inflammatory cytokine IL10 in pre-obese state may also promote metabolic disturbances, as IL10 has been shown to protect from hepatosteatosis and IR (Dagdeviren *et al.* 2016, Paredes-Turrubiarte *et al.* 2016). Furthermore, although inflammatory cells were not present in expanding WAT (Vahatalo *et al.* 2014), we detected elevated levels of resistin, whose production has previously been shown to be stimulated by NPY in

WAT (Yuzuriha *et al.* 2003, Kuo *et al.* 2007). Resistin can independently induce IGT and IR (Steppan *et al.* 2001, Rajala *et al.* 2003), and it associates with T2D, obesity and inflammation (McTernan *et al.* 2003, Shetty *et al.* 2004, Fu *et al.* 2006). Interestingly, we also detected decreased expression of cytokines (*Il1b*, *Tnfa* and *Tgfb1*), usually associated with the development of steatohepatitis. Decreased FA oxidation, (i.e. decreased production of ROS) or cytokine-independent oxidative stress may explain this discrepancy to our other results. Presumably NPY has anti-inflammatory effects (Wheway *et al.* 2005, Ferreira *et al.* 2010), but it has also been shown to be upregulated in human hepatosteatosis and to be involved in the development of fibrosis (Sigala *et al.* 2013, Zhu *et al.* 2015). Together with our results they suggest that NPY has a dual role in inflammation.

NPY variant (rs16139) is associated with hypercholesterolemia and atherosclerosis (Karvonen *et al.* 1998, 2001), and in consistence, obese OE-NPY^{DBH} mice showed elevated serum total and HDL cholesterol together with a tendency of decreased HDL-receptor, *Srb1*, expression, which all associate with T2D or atherosclerosis in mice (Nishina *et al.* 1994, Kozarsky *et al.* 2000). VLDL/LDL fraction was not changed, which may be influenced by decreased serum TGs, the main component of VLDL,

Figure 4

Glucose metabolism in OE-NPY^{DBH} mice. (A) Glucose tolerance test and (B) glucose-induced hyperinsulinemia in 4-month-old homozygous OE-NPY^{DBH} vs wild-type mice ($n = 7/\text{group}$). (C) Glucose (D) and pyruvate tolerance tests of 4-month-old and 7-month-old homozygous OE-NPY^{DBH} vs wild-type mice ($n = 6\text{--}11/\text{group}$). (E) mRNA expression of selected genes involved in glucose metabolism in 2-month-old heterozygous (pre-obese) ($n = 5/\text{group}$) and 7-month-old homozygous (obese) ($n = 10/\text{group}$) OE-NPY^{DBH} mice relative to wild-type mice. Beta actin was used as endogenous control. (F) Representative periodic acid-Schiff stained liver slides showing glycogen accumulation into the hepatocytes in 7-month-old OE-NPY^{DBH} vs wild-type mice (scale bar 50 μm). Values are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ with two-way ANOVA of repeated measures (A–D) or Student's t -test (E). GTT = glucose tolerance test, PTT = pyruvate tolerance test, Pck1 = phosphoenolpyruvate carboxykinase 1, Pgc1a = peroxisome proliferative activated receptor, gamma, coactivator 1 alpha, Gys2 = Glycogen synthase 2, Pygl = liver glycogen phosphorylase, WT = wild-type mice, OE-NPY = OE-NPY^{DBH} mice.

previously observed in these mice (Vahatalo *et al.* 2014). The mechanism of hypercholesterolemia in OE-NPY^{DBH} mice is elevated cholesterol synthesis without a change in cholesterol absorption. As this is detected already in pre-obese state, thus without agitation of hepatosteatosis or the metabolic syndrome (Gylling *et al.* 2007, Simonen *et al.* 2011), we suggest that the hepatic cholesterol synthesis is elevated by noradrenergic NPY and explains the hypercholesterolemia of obese OE-NPY^{DBH} mice.

The mechanism of IGT, previously detected in OE-NPY^{DBH} mice (Vahatalo *et al.* 2014), was scrutinized in states of early and in established obesity. In the early stage, OE-NPY^{DBH} mice seem to compensate elevated blood glucose levels effectively by increasing insulinemia and by restoring excess energy to glycogen and TGs by the liver. This was demonstrated by almost absent pyruvate-induced increase in glucose levels, and by increased glycogen metabolism known to associate with T2D (Hundal *et al.* 2000). Based on previous findings (Cho & Kim 2004, Machida *et al.* 2014), NPY unlikely increases pancreatic insulin secretion directly, and glucose-induced hyperinsulinemia in OE-NPY^{DBH} mice is rather secondary to systemic IR. With aging, IGT, IR and fasting hyperinsulinemia (Vahatalo *et al.* 2014) with enlarged pancreatic islets, and hepatic

glycogen accumulation, as shown here, become evident. To test whether this phenotype similar to carriers of rs16139 (Ukkola & Kesaniemi 2007, Jaakkola *et al.* 2009, Nordman *et al.* 2005) renders OE-NPY^{DBH} mice susceptible to T2D, the mice were exposed to low-dose STZ to interfere with pancreatic beta cells less than in the traditional T1D STZ-model, and high caloric diet to induce IR (Gilbert *et al.* 2011). Only OE-NPY^{DBH} mice reached diabetic fasting blood glucose levels, thus showing higher susceptibility to T2D. They also showed augmented response in GTT, despite comparably low fasting insulin levels and severe IR as WT mice. However, it is presumable that glucose-stimulated insulin release was impaired in OE-NPY^{DBH} mice, and likely with a higher insulin dose or a more sensitive method to assess insulin sensitivity, more severe IR would have been detected in OE-NPY^{DBH} mice.

Previously, the effects of NPY on hepatic metabolism have mostly been attributed to central, hypothalamic effects (Zarjevski *et al.* 1993, Stafford *et al.* 2008), but also peripheral effects are possible (Zhang *et al.* 2010, Xie *et al.* 2012). In order to elucidate whether the observed changes in OE-NPY^{DBH} mice are due to NPY's direct effect in the liver, or altered SNS activity, hepatic NPY and adrenergic receptor expressions were analyzed. As presence of hepatic

Figure 5

Type 2 diabetes model in OE-NPY^{DBH} mice. (A) Weight gain, (B) whole body fat mass %, (C) fasting blood glucose, (D) glucose and (E) insulin tolerance tests, (F) fasting serum insulin levels, (G) quantitated pancreatic insulin intensity, and (H) the amount and size of beta cells in homozygous OE-NPY^{DBH} vs wild-type mice ($n = 7/\text{group}$) subjected to high caloric diet from age of 13 weeks and low-dose streptozotocin from age of 16 weeks (Week 0) onwards. Values are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ with two-way ANOVA and Bonferroni *post hoc* test (B) or two-way ANOVA of repeated measures (C–D). Gray dashed line = glucose 13.8 mmol L⁻¹, the limit for diabetes in mice, STZ = streptozotocin, GTT = glucose tolerance test, ITT = Insulin tolerance test, WT = wild-type mice, OE-NPY = OE-NPY^{DBH} mice.

Y1-receptors (but not Y2-receptors) was detected, and peripheral Y1-receptor knockdown has previously shown beneficial effects on hepatic FA metabolism (Zhang *et al.* 2010), their contribution was studied by chronic treatment with a peripheral Y1R-antagonist, BIBO3044. Opposite to our hypothesis, Y1R-antagonist was unable to prevent the metabolic phenotype of OE-NPY^{DBH} mice. Thus, in the model of excess noradrenergic NPY, peripheral Y1-receptors seem to play a minor role in the phenotype. Furthermore, in WT mice, peripheral Y1-receptor antagonism did not produce similar beneficial effects, e.g. increased FA oxidation, as Y1-receptor knockdown. In contrast, reduced serum TGs and increased hepatic cholesterol point to metabolic disturbances similar to

what is seen in OE-NPY^{DBH} mice and high fat diet-fed mice (Gao *et al.* 2010).

Attenuated SNS activity in adipose tissue was previously shown to contribute to obesity in OE-NPY^{DBH} mice, and therefore similar actions in the liver were considered as another probable mechanism. A significant decrease in *Adrb1* expression both in heterozygous and homozygous OE-NPY^{DBH} mice was detected, although they display opposite effects of NPY overexpression on catecholamine levels with heterozygous showing increased and homozygous mice decreased sympathetic tone (Ruohonen *et al.* 2008, Vahatalo *et al.* 2014). Despite this discrepancy, the metabolic phenotype is similar, albeit more pronounced in the homozygous model.

Figure 6

Roles of NPY and adrenergic receptors in OE-NPY^{DBH} mice. (A) mRNA expression of liver NPY- and adrenergic receptors in 2-month-old heterozygous (pre-obese) ($n=4-5$ /group) and 7-month-old homozygous (obese) ($n=6-7$ /group) OE-NPY^{DBH} mice relative to wild-type mice. Beta actin was used as endogenous control. (B) Weight gain, (C) fat mass gain, (D) cumulative food intake, (E-F) serum lipids and (G-I) liver 8-isoprostane in OE-NPY^{DBH} vs wild-type mice ($n=10-13$ /group) treated with Y1R-antagonist BIBO3044 or vehicle for 4 weeks. (J) Energy expenditure, (K) respiratory exchange ratio and (L) physical activity of wild-type mice ($n=4$ /group) treated with Y1R-antagonist or vehicle for 1 week. Values are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ difference between genotypes with Student's *t*-test (A, H and I) or with two-way ANOVA and Bonferroni *post hoc* test (B-D and F-G). # $P < 0.05$ and ## $P < 0.01$ difference between treatments within a same genotype with two-way ANOVA and Bonferroni *post hoc* test (E and H), or with two-way ANOVA of repeated measures (L). *Adrb1*=adrenergic-beta1-receptor, *Adrb2*=adrenergic-beta2-receptor, *Y1r*=Y1-receptor, EE=energy expenditure, RER=respiratory exchange ratio, WT=wild-type mice, OE-NPY=OE-NPY^{DBH} mice.

Table 7 Glucose metabolism in Y1R-antagonist treated OE-NPY^{DBH} mice.

	WT		OE-NPY ^{DBH}	
	Vehicle	Y1R	Vehicle	Y1R
Glucose (mmol L ⁻¹)	7.76 $\pm$ 0.38	7.45 $\pm$ 0.46	8.29 $\pm$ 0.46	8.51 $\pm$ 0.39
Insulin (μ g L ⁻¹)	0.28 $\pm$ 0.03	0.30 $\pm$ 0.05	0.47 $\pm$ 0.04**	0.51 $\pm$ 0.07
HOMA-IR	2.54 $\pm$ 0.34	3.04 $\pm$ 0.60	4.30 $\pm$ 0.44**	5.72 $\pm$ 1.04
HOMA-B (%)	37.96 $\pm$ 7.84	38.49 $\pm$ 8.10	42.03 $\pm$ 4.68	39.56 $\pm$ 5.17
QUICKY	0.33 $\pm$ 0.01	0.34 $\pm$ 0.01	0.31 $\pm$ 0.01*	0.31 $\pm$ 0.01

Glucose metabolism parameters were measured from 6-month-old homozygous male OE-NPY^{DBH} vs wild-type mice ($n=10-13$ /group) treated with Y1R-antagonist (BIBO3044) or vehicle for 4 weeks. Values are expressed as means $\pm$ S.E.M. * $P < 0.05$ and ** $P < 0.01$ between genotypes in vehicle treated groups with Student's *t*-test.

However, decreased *Adrb1* expression points to suppressed sympathetic action in the liver, which may explain the locally induced metabolic changes. Low catecholamines have been reported to increase lipogenesis in WAT, and *Adrb1* deficiency to induce IGT and IR, and to increase susceptibility to obesity and hepatosteatosis on a high fat diet (Ueta *et al.* 2012) providing additional support for our theory.

In conclusion, OE-NPY^{DBH} mice develop obesity, IGT, IR, hepatosteatosis and hypercholesterolemia, and when pancreatic insulin production is disrupted, also T2D. The model reveals that NPY expressed in the noradrenergic neurons has potent metabolic effects in the WAT and the liver, which support energy conservation and storage. Our results suggest that the effects on WAT and liver are mediated mostly via inhibition of SNS in OE-NPY^{DBH} mice, and that peripheral Y1-receptor mediated actions are less important, at least in the liver. The findings from OE-NPY^{DBH} mice may be relevant to the mechanisms of stress-induced obesity and the metabolic disorders associated with the gain-of-function *NPY* polymorphisms.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0223>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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