Insulin sensitivity : modulation by neuropeptides and hormones

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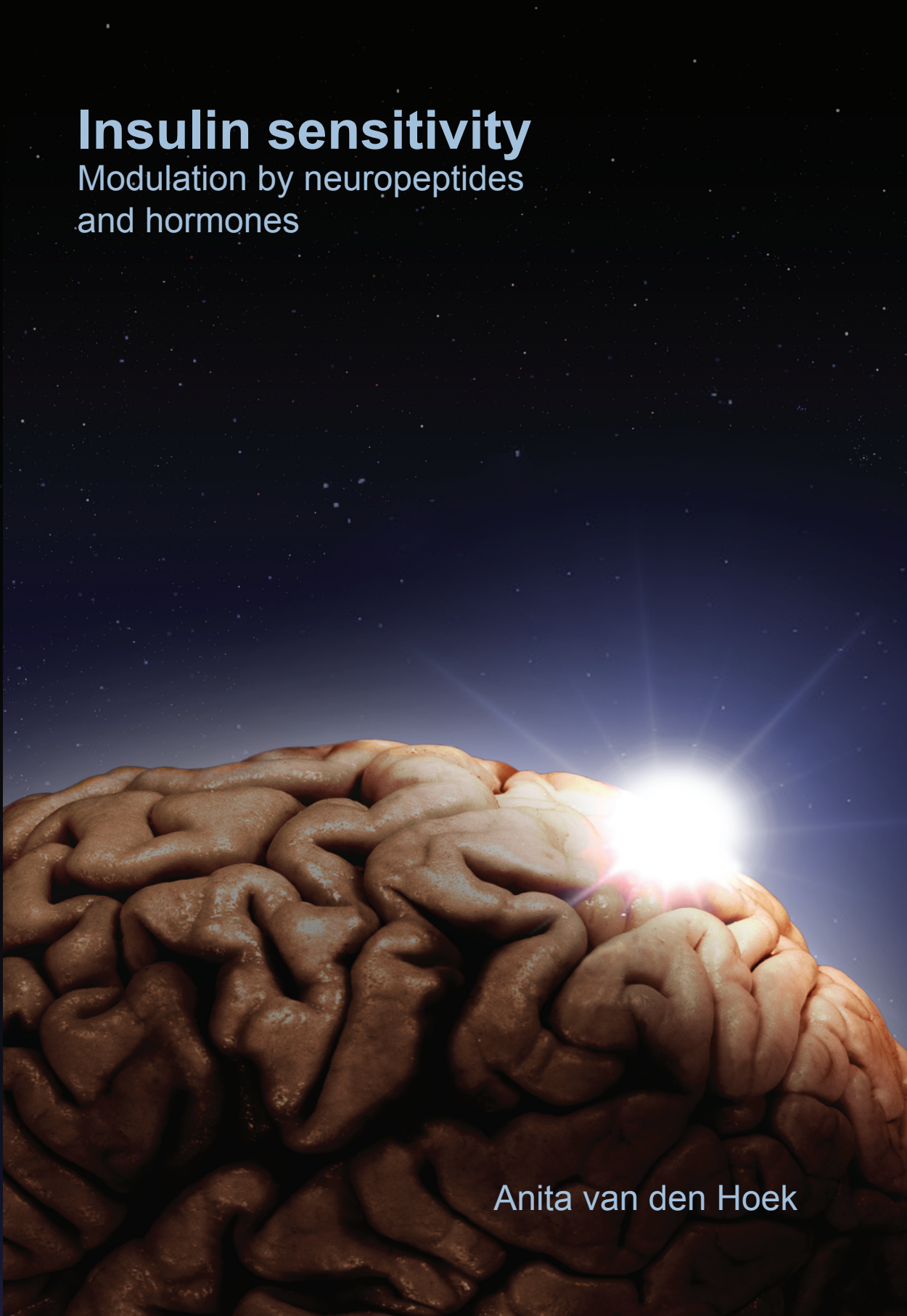

Anita van den Hoek

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The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science.
Albert Einstein

Contents

Chapter 1

General introduction

Obesity and diabetes.

Most adult animals and humans tend to keep their body weight within a relative narrow range, despite large variations in daily food intake and physical activity. This indicates that body weight is tightly regulated. However, the growing percentage of people that are overweight or obese shows that this regulatory mechanism is not flawless. There is considerable evidence that during evolution, this regulation system has evolved as a system intended for conservation of energy, seeking food in times of famine and storing energy in times of plenty. This increased the survival chance during long periods of energy deprivation. There has been little evolutionary pressure to increase energy expenditure or reduce food intake once energy stores are replete. Therefore, this regulatory system is biased strongly towards weight gain and storage of fat, with few mechanisms that encourage weight loss [1].

Nowadays, in our Western society food is in abundance and energy-rich with high levels of sugar and saturated fats. At the same time, large shifts towards less physically demanding work have been observed [2]. These environmental changes are reflected in the percentages of overweight/obese people. The prevalence of overweight and obesity is commonly assessed by using body mass index (BMI), defined as the weight in kilograms divided by the square of the height in meters (kg/m^2). A BMI over 25 kg/m^2 is defined as overweight and a BMI over 30 kg/m^2 as obese. Globally, obesity has reached epidemic proportions with more than 1 billion overweight adults, at least 300 million of them obese (World Health Organization, 2003). In The Netherlands, 47% of the adults are overweight with 11% being obese (CBS, 2004).

Overweight and obesity are caused by a disturbed balance between energy/food intake and energy expenditure. Overweight and obesity pose a major risk for chronic diseases, particularly type 2 diabetes mellitus, cardiovascular disease, hypertension, stroke and certain forms of cancer [3]. The likelihood of developing type 2 diabetes mellitus and hypertension rises steeply with increasing body fatness. Approximately 85% of patients with diabetes mellitus have type 2 and of these patients, 90% are obese or overweight (WHO, 2003). Type 2 diabetes mellitus is no immediate life threatening disease, but the increased glucose levels ultimately lead to complications, such as cardiovascular disease, retinopathy,

nephropathy and cognitive dysfunction. These complications will reduce the overall quality of life, and also form an increased risk of premature death.

Regulation of food intake.

Hypothalamic regulation of food intake.

Energy/food intake is regulated by a highly complex system, that integrates several signals concerning the metabolic status and energy expenditure, but also the availability of food, memory of food and the social situation. This regulatory mechanism involves several brain regions ranging from cortex to brainstem, but most interest has focused on the hypothalamus, which is considered as the main regulatory feeding center of the brain.

The hypothalamus consists of several nuclei, that are involved in the regulation of food intake. One of them is the arcuate nucleus, which lies around the base of the third ventricle, immediately above the median eminence. Due to this position, the neurons of the arcuate nucleus have easy access to peripheral satiety factors. First of all, peripheral signals can gain access to the arcuate nucleus from the cerebrospinal fluid (csf) in the third ventricle (either by diffusion or via receptors)[4;5]. Secondly, peripheral signals can easily reach the arcuate axon terminals, because the endothelial barrier within the median eminence lacks tight junctions [6]. Therefore, the blood-brain-barrier is not present in this region and arcuate axon terminals are in direct contact with signals from the bloodstream. The neurons of the arcuate nucleus are called first order neurons because of this direct contact with peripheral satiety factors. The arcuate nucleus contains two distinct groups of neurons with opposing effects on food intake (Fig. 1). One group consists of neurons that co-express neuropeptide Y (NPY) and agouti-related peptide (AgRP), neuropeptides, that activate appetite [7;8]. The other group consists of neurons that co-express pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), both neuropeptides that inhibit appetite [9].

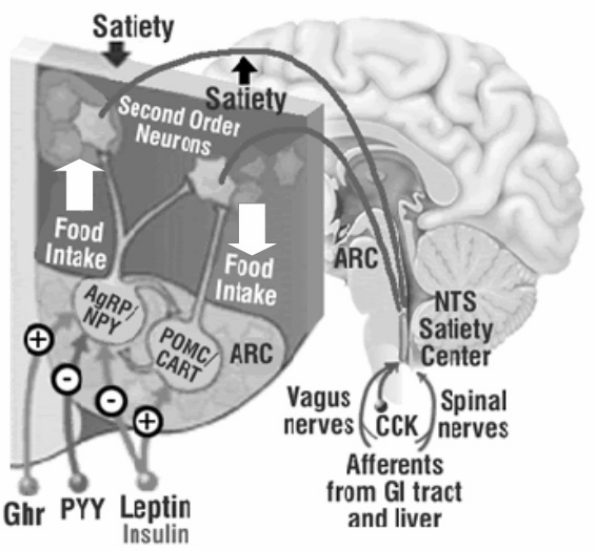

Figure 1. Central command centers. The arcuate nucleus of the brain contains two sets of neurons with opposing effects. Activation of the NPY/AgRP neurons increases appetite, whereas activation of the POMC/CART neurons has the opposite effect.
Adapted from Marx J, 2003. *Science* 299, 846-849. Republished with permission.

During fasting or a fall in the body's energy stores, the mRNA expression of the two orexigenic peptides, NPY and AgRP, is increased. NPY and AgRP will produce a shift towards a positive energy balance by increasing food intake and decreasing energy expenditure [10;11]. From the two orexigenic neuropeptides, NPY is the most potent one. Currently, six different NPY receptors have been identified, that mediate the effects of NPY [12;13]. Most of the NPY neurons (~90%) also contain AgRP [8]. AgRP acts as a high affinity antagonist of the melanocortin 3 and 4 receptors (MC3R and MC4R), 2 receptors downstream of the POMC pathway [14;15]. Furthermore, NPY/AgRP neurons can inhibit their neighbouring POMC/CART neurons by means of the neurotransmitter GABA [16].

During the fed condition or a state of positive energy balance, the mRNA expression of the two anorexigenic neuropeptides, POMC and CART is increased. These neuropeptides will produce a shift towards a negative energy balance by decreasing food intake and increasing energy expenditure [10;11]. POMC is a precursor molecule that is cleaved into several peptides that are called melanocortins (MC). Of these melanocortins, α-melanocyte-stimulating hormone (α-MSH) is considered to be the most important one for regulation of food intake. The effects of melanocortins are mediated by melanocortin receptors of which currently five are cloned. Two of them, MC3R and MC4R, are mainly expressed within the brain where they interfere with food intake. Both receptors have a high affinity for α-MSH, but also for AgRP. CART is co-localized with POMC in the arcuate nucleus. However, the mechanisms that

mediate the effects of CART are still poorly understood and until now there has not been a receptor identified.

The neurons from the arcuate nucleus project to second order neurons in the paraventricular nucleus, ventromedial nucleus, dorsomedial hypothalamic nucleus and the lateral hypothalamic area [10;11]. The second order neurons in these areas are also divided into neurons that contain orexigenic or anorexigenic neuropeptides. Second order orexigenic neuropeptides are melanin-concentrating hormone (MCH) and orexins (or hypocretins), second order anorexigenic neuropeptides are corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). The second order neurons project to different autonomic centers in the brainstem. In these areas satiety signals are processed and the hypothalamic signals are integrated with afferent information related to satiety [17].

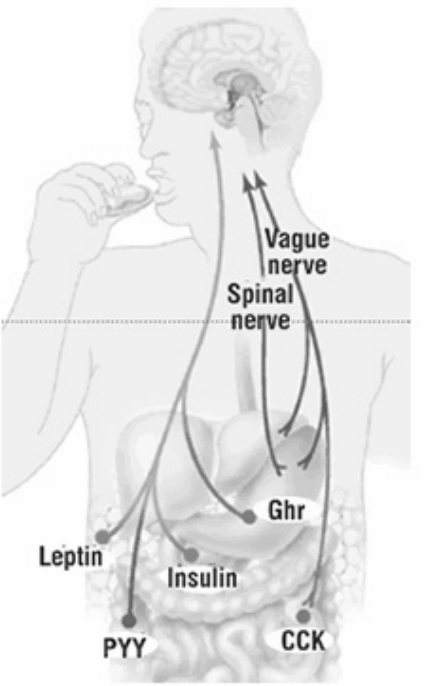

Figure 2. Appetite conrollers.
The body produces several hormones that act through the brain to regulate short- and long-term appetite.
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The hypothalamic pathways, that regulate food intake are essential for the long-term regulation of food intake and energy homeostasis. Apparently, in the obese situation these pathways are not functioning properly. Indeed, it has been shown that the balance between orexigenic and anorexigenic neuropeptides is profoundly altered in several animal models of obesity [18].

Peripheral signals that regulate food intake.

Numerous peripheral signals act on the central regulatory centers, and, thereby, contribute to the regulation of food intake and energy expenditure (Fig. 2). These peripheral signals can be divided in long-term and short-term signals [19]. Long-term signals provide information about body fat stores and the amount of energy

consumed over a more prolonged period of time. Short-term signals do not reflect body adiposity, but provide information about hunger and satiety.

Leptin and insulin are examples of long-term signals. Leptin is secreted from adipocytes in proportion to the amount of adipose tissue [20]. Although insulin is secreted from pancreatic ß–cells, the circulating concentrations of insulin are also proportional to adipose tissue [21]. However, the overall insulin concentration should be taken into account, because insulin concentration can rise rapidly in a short period of time in response to a meal, and then return to basal levels [22]. Nevertheless, insulin transport into the brain is not rapid, but occurs over a period of hours, consistent with a role for insulin as a long-term regulator of energy balance [23]. Leptin and insulin both bind to receptors located in the arcuate nucleus and thereby affect the NPY- and POMC-pathway leading to an inhibitory effect on appetite [5;24].

Ghrelin, cholecystokinin (CCK) and peptide YY (PYY) are examples of short-term signals. Ghrelin is a circulating hormone that is synthesized in the stomach and that increases food intake [25]. Ghrelin levels increase during fasting, rising sharply before and falling within one hour of a meal, suggesting that ghrelin plays a role in hunger and meal initiation [26]. CCK is a hormone that is produced in the upper part of the small intestine in response to the presence of ingested food. It is released postprandialy and inhibits food intake [27]. CCK induces satiety and decreases meal size by stimulating the vagal nerve projecting to the nucleus of the solitary tract (NTS) in the brainstem [28]. PYY is a hormone that is produces in the distal part of the gastrointestinal tract and is released into the circulation in response to a meal [29]. PYY can be cleaved into PYY_{3-36}, the isoform of PYY that inhibits food intake. PYY_{3-36} inhibits food intake by acting directly on the arcuate nucleus via the Y2R, a presynaptic inhibitory receptor on NPY neurons [30].

These long-term and short-term signals are regulated by interacting mechanisms. They cooperate, in order to integrate energy expenditure and energy intake, to ensure that energy homeostasis is maintained. In the obese situation, the mechanism fails to preserve energy homeostasis and several of these peripheral signals have been shown to be dysregulated as well in obesity [31-33].

Insulin resistance.

The metabolic syndrome comprises a cluster of anomalies that increase the risk of cardiovascular disease and type 2 diabetes mellitus: hyperglycemia, abdominal obesity, hypertriglyceridemia, hypertension and low levels of high-density lipoprotein (HDL) cholesterol [34-36]. Insulin resistance may underlie the majority of these pathologies [37] and therapies that effectively reinforce insulin action may therefore ameliorate the risk profile of metabolic syndrome patients [38;39]. Insulin resistance is defined as the requirement of an abnormally large amount of insulin (endogenous or exogenous) for a biological response [40]. Insulin resistance describes a condition that is characterized by decreased tissue sensitivity to the action of insulin and therefore affects multiple organs.

Insulin resistance in the liver leads to the failure of insulin to suppress the hepatic glucose production sufficiently. Insulin affects glucose production directly via signaling through the hepatic insulin receptor to inhibit glycogenolysis and gluconeogenesis. However, it has also been suggested that insulin suppresses glucose production indirectly through extrahepatic actions of insulin on muscle and adipose tissue to inhibit release of gluconeogenic substrates (lactate, alanine and glycerol) and gluconeogenic energy substrates (FFAs) [41-43]. In addition, insulin suppresses the hepatic production of very-low-density lipoprotein (VLDL) particles. These inhibitory effects are also directly on the liver through the effects of insulin on synthesis and secretion of VLDL [44] and indirectly because insulin affects the FFA release from adipose tissue [45].

Insulin resistance in muscle and adipose tissue leads to a diminished ability of insulin to stimulate glucose uptake in these tissues. In muscle, insulin stimulates the uptake and oxidation of glucose and the formation of glycogen. Skeletal muscle can use both glucose and FFA as energy source and the shift between these two depends primarily on the availability of FFAs and exercise level. In adipose tissue, glucose is needed for the formation of glycerol-3-phosphate, which is necessary for the formation of triglycerides (TG). Insulin stimulates the glucose uptake and therefore promotes adipocyte TG synthesis. Insulin also inhibits the rate of TG lipolysis through inhibition of the lipolytic enzyme hormone sensitive lipase.

Outline of this thesis.

The studies described in this thesis all involve the hypothesis that the hypothalamus is not only involved in the regulation of food intake, but also regulates insulin sensitivity (independent of its effects on food intake). In obesity, dysregulation of several hypothalamic neuropeptides and peripheral hormones that regulate food intake, has been observed and leads to an increased food intake. Perhaps the same dysregulation of these neuropeptides and hormones can cause insulin resistance as well. All studies described here where performed in mice.

The effects of both the NPY and POMC pathway on insulin sensitivity were studied. In **chapter 2** we describe the effects of a continuous intracerebroventricular (icv) infusion of NPY on insulin sensitivity. In **chapter 3** the effects of icv injections of MTII, an agonist of the POMC pathway, is described. In **chapter 4** the acute effects of the peripheral hormone PYY_{3-36} on insulin sensitivity are described. In **chapter 5** the long-term effects of PYY_{3-36} are investigated to examine whether PYY_{3-36} could be of use in the clinical management of obesity and insulin resistance. Finally, in **chapter 6**, the role of the peripheral hormone leptin and the role of its central signalling on insulin sensitivity is examined in *ob/ob* mice and evaluated against the contribution of the obese phenotype itself on insulin sensitivity.

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Chapter 2

Intracerebroventricular Neuropeptide Y infusion precludes inhibition of glucose and VLDL-production by insulin.

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Abstract

Recent evidence demonstrates that hypothalamic insulin signaling is required for inhibition of endogenous glucose production (EGP). The downstream mechanisms responsible for the effects of hypothalamic insulin receptor activation on hepatic fuel flux remain to be established. To establish if downregulation of Neuropeptide Y (NPY) release by insulin is mandatory for its capacity to suppress glucose production, we examined the effects of a continuous intracerebroventricular (i.c.v.) infusion of NPY (10 μg/h for 3-5 hours) on glucose flux during a hyperinsulinemic euglycemic clamp in mice. We also evaluated the effects of i.c.v. NPY administration on free fatty acid- and glycerol flux and very low-density lipoprotein (VLDL) production in this experimental context. In basal conditions, none of the metabolic parameters was affected by NPY infusion. In hyperinsulinemic conditions, peripheral glucose disposal was not different between vehicle- and NPY-infused animals. In contrast, hyperinsulinemia suppressed endogenous glucose production by approximately 8% vs. 30 % in NPY- vs. vehicle-infused mice respectively ($P<0.05$). Also, VLDL-production was significantly higher during hyperinsulinemia in NPY- compared with vehicle-infused mice (97.5 ± 18.0 vs. 54.7 ± 14.9 μmol/kg/h, $P<0.01$). These data suggest that the neurophysiological action of insulin to downregulate hypothalamic NPY release is a prerequisite for its ability to suppress hepatic fuel production, whereas it is not mandatory for its capacity to modulate glucose disposal or lipolysis.

Introduction

Insulin resistance is an important characteristic of obesity and type 2 diabetes mellitus (T2DM) [1;2]. It hampers proper suppression of endogenous glucose and very low-density lipoprotein (VLDL) production in response to food intake. Accordingly, the metabolic features of obesity and T2DM include hyperglycemia and hypertriglyceridemia.

It has recently been shown that hypothalamic insulin signaling is required for inhibition of endogenous glucose production (EGP) [3]. Indeed, intracerebroventricular (i.c.v.) infusion of insulin can suppress glucose production (by 40%) in the presence of basal circulating insulin concentrations, whereas antagonism of insulin signaling or

downregulation of insulin receptor expression in hypothalamic nuclei considerably impairs the ability of circulating insulin to inhibit EGP [3;4].

The downstream mechanisms responsible for the apparent impact of hypothalamic insulin receptor activation on hepatic fuel flux remain to be established. The arcuate nucleus of the hypothalamus is a major target of insulin in the brain. This nucleus contains two insulin sensitive populations of neurons that exert powerful, opposing effects on fuel flux: pro-opiomelanocortin (POMC) neurons (stimulated by insulin), guiding a catabolic adaptive response to environmental cues, and NPY neurons (inhibited by insulin), that primarily promote anabolic adaptations [5]. I.c.v. infusion of a melanocortin antagonist (SHU9119) does not affect the ability of hyperinsulinemia to inhibit endogenous glucose production, which suggests that the POMC pathway is not involved in the acute effects of insulin on hepatic fuel flux [3]. In regard to the other major insulin sensitive neural route, it was reported that subchronic i.c.v. infusion of NPY in Spraque-Dawley rats and mice induces hyperinsulinemia, hyperglycemia and dyslipidemia [6;7]. These findings led us to hypothesize that downregulation of central (hypothalamic) NPY neuronal activities by insulin is critical for its ability to control endogenous glucose and lipid production. To test this hypothesis, we examined whether infusion of NPY into the lateral cerebral ventricle precludes proper inhibition of endogenous fuel production during a hyperinsulinemic euglycemic clamp in mice.

Research designs and methods

Animals. Male C57BL/6J mice were housed in a temperature-controlled room on a 12-hour light-dark cycle and were fed a standard mouse chow diet with free access to water. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

Surgical procedures. Mice were anaesthetized with 0.5 ml/kg Hypnorm (Janssen pharmaceutica, Beerse, Belgium) and 12.5 mg/kg midazolam (Genthon, Nijmegen, the Netherlands). A 25-gauge guide cannula was stereotaxically implanted into the left lateral ventricle using the following coordinates from Bregma: 0.46 mm posterior, 1.0 mm lateral end 2.2 mm ventral [8]. The guide cannula was secured with two screws and dental cement (AgnTho's, Lidingö, Sweden) to the skull surface. After a recovery

period of 1 week, adequate placement of the cannulae was tested with the feeding response to an i.c.v. injection of NPY (5 μg dissolved in 1 μl sterile water) (Bachem, Bubendorf, Germany).

Hyperinsulinemic euglycemic clamp. Mice with free access to standard mouse chow and water until the beginning of the clamp experiment were used. Hyperinsulinemic clamps were performed under Hypnorm/Midazolam anesthesia as described earlier [9-13]. During the entire experiment (basal and hyperinsulinemic period) NPY (5 μg/μl) or vehicle was administered i.c.v. at a rate of 2 μl/h (via an injection cannula) using an infusion pump and a 10 μl Hamilton syringe. In one series of experiments glucose and glycerol turnover were determined and in another series of experiments FFA turnover was determined. First, basal rates of glucose, glycerol or FFA turnover were determined by giving a primed (p) continuous (c) infusion of ^{14}C-glucose (p: 0.2 μCi, c: 0.3 μCi/h) (Amersham, Little Chalfont, U.K.), ^{3}H glycerol (p: 0.6 μCi, c: 0.9 μCi/h) (Amersham, Little Chalfont, U.K) or ^{3}H-oleate (p: 2 μCi, c: 3 μCi/h) (Amersham, Little Chalfont, U.K) respectively. Subsequently, (after 80 min) insulin was administered in a primed (4.5 mU) continuous (6.8 mU/h) i.v. infusion for ~1.5 h to attain steady state circulating insulin levels of ~4 ng/ml. A variable infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10 min intervals via tail bleeding (< 3 μl)(Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). Blood samples (60 μl) were taken during the basal period (after 60 and 80 min) and during the clamp period (20 min prior to- and by the end of the clamp) to determine the plasma concentration of glucose, glycerol, FFA and insulin and plasma ^{14}C-glucose, ^{3}H-glycerol and ^{3}H-oleate specific activities. At the end of the clamp, mice were either sacrificed and their livers isolated and frozen in liquid nitrogen for subsequent analysis, or mice were used to determine VLDL-production.

VLDL-production. Mice were given a continuous i.c.v. infusion of NPY (5 μg/μl) or vehicle at a rate of 2 μl/h. Mice were intravenously injected with 500 mg of Triton WR-1339 (Sigma, St. Louis, MO, USA) per kg body weight as a 10% (w/w) solution in sterile saline. Serum VLDL clearance is virtually completely inhibited under these circumstances [14]. Blood samples (20 μl) were taken on t=0, 30, 60 and 90 min after Triton injection and used for determination of plasma triglycerides (TG) concentration. Plasma TG concentrations were related to body weight and hepatic VLDL-TG production was calculated from the slope of the curve and expressed as

μmol/kg/min. Triton injections were given either under basal conditions (90 min after the beginning of the i.c.v. infusion) or under hyperinsulinemic conditions (after the clamp experiment). At the end of the experiment, mice were sacrificed and liver samples were taken and frozen in liquid nitrogen for subsequent analysis.

Analytical procedures. Plasma levels of glucose, glycerol, FFA, TG and corticosterone were determined using commercially available kits (Sigma, St. Louis, MO, USA; Boehringer Mannheim, Mannheim, Germany and Wako, Neuss, Germany; Alpco, Windham, NH, USA). Plasma insulin, glucagon and NPY concentration were measured by radioimmunoassay (Linco Research Inc., St. Charles, MO, USA; Alpco, Windham, NH, USA; Peninsula Laboratories, San Carlos, CA, USA). Total plasma ^{14}C-glucose and ^{3}H-glycerol was determined in 10 μl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water. Total plasma ^{3}H-oleate was determined in 7.5 μl plasma after extraction of lipids by a modification of Bligh and Dyer's method [15]. In short, 7.5 μl plasma was dried and resolved in 100 μl water. Then 1.1 ml demi-water and 4.5 ml methanol:chloroform (2:1) was added and mixed thoroughly, after which 1.5 ml chloroform was added and mixed and finally, 1.5 ml demi-water was added and mixed. After centrifugation, the chloroform layer was collected and FFA fraction was separated from the other lipid components by thin-layer chromatography (TLC) on silica gel plates. Content of TG in liver was determined as described before [16]. Briefly, 10-20 μg of tissue was homogenized in phosphate-buffered saline (PBS) and samples were taken for measurement of protein content [17]. Lipids were extracted and TG fraction was separated from the other lipid components by high performance thin-layer chromatography (HPTLC) on silica gel plates.

Calculations. Turnover rates of glucose, FFA and glycerol (μmol/min/kg) were calculated during the basal period and during the steady-state portion of the clamp as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{14}C-glucose, ^{3}H-oleate or ^{3}H-glycerol (dpm/μmol). The ratio was corrected for body weight. EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the infusion rate of glucose.

Statistical analysis. Differences between groups were determined by Mann-Whitney non-parametric test for 2 independent samples. A P-value < 0.05 was considered statistically significant. All values shown represent mean ± SD.

Results

Plasma parameters. Body weight, plasma glucose, FFA, glycerol, insulin, glucagon and corticosterone in basal and hyperinsulinemic conditions are shown in table 1. In basal conditions, no differences in plasma parameters were detected between vehicle- and NPY-infused animals. In steady-state clamp conditions, insulin, glucagon and corticosterone levels and plasma glucose concentrations were similar in both groups. Hyperinsulinemia suppressed both FFA and glycerol levels to a similar extent in vehicle- and NPY-infused mice. Plasma NPY levels at the end of the clamp period were similar in both groups (4.0 ± 2.0 ng/ml in vehicle-infused mice *vs.* 5.1 ± 2.4 ng/ml in NPY-infused animals).

Table 1. Plasma parameters in mice that received an i.c.v.-infusion of NPY or vehicle under basal or hyperinsulinemic conditions. Values represent mean ± SD for at least 5 mice per group. [1] These data are based on 2 mice only and therefore have to be considered with caution.

	Basal		Hyperinsulinemic	
	Vehicle	NPY	Vehicle	NPY
Body weight (g)	23.3 ± 1.2	22.2 ± 1.2	23.3 ± 1.2	22.3 ± 1.4
Glucose (mmol/l)	7.0 ± 1.4	6.8 ± 1.8	7.5 ± 1.2	7.7 ± 1.2
FFA (mmol/l)	0.7 ± 0.2	0.7 ± 0.3	0.2 ± 0.1	0.3 ± 0.1
Glycerol (mmol/l)	0.7 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Insulin (ng/ml)	0.8 ± 0.3	1.0 ± 0.6	3.5 ± 0.9	3.9 ± 1.1
Glucagon (pmol/l)	100.0 ± 12.4	99.1 ± 22.0	71.3 ± 14.5	66.8 ± 22.2
Corticosterone (ng/ml)	29.6 ± 5.4	21.0 ± 9.9	26.2 ± 7.2	25.6 ± 17.6[1]

Glucose turnover. The rate of glucose infusion necessary to maintain euglycemia during insulin infusion was significantly lower in NPY-infused mice than in vehicle-infused animals (28.6 ± 8.6 *vs.* 59.8 ± 12.8 μmol/min/kg, $P<0.01$; Figure 1), indicating that i.c.v. NPY administration acutely induces insulin resistance. In basal conditions, glucose disposal was similar in NPY- and vehicle-infused mice (146.2 ± 40.9 *vs.* 138.1 ± 30.0 μmol/min/kg, respectively; Figure 2).

Hyperinsulinemia barely increased glucose disposal and the subtle increase it brought about was of similar magnitude in NPY- and vehicle-infused animals (163.2 ± 22.8 *vs.* 151.1 ± 24.8 *μ*mol/min/kg, respectively). In contrast, endogenous glucose production (EGP), which was similar in basal conditions, was adequately suppressed by insulin in vehicle-infused animals (by ~30%, P<0.01), whereas it was much less affected in NPY-infused mice (~8%, P=NS; P<0.05 for difference between NPY- and vehicle-infused animals; Figure 2).

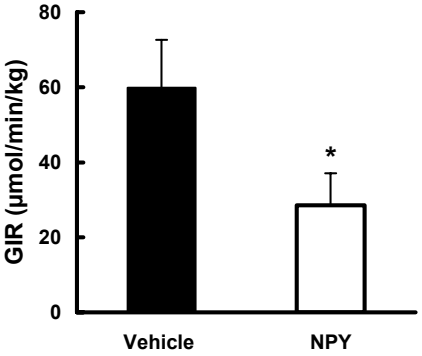

Figure 1. Glucose infusion rate (GIR) in mice that received an i.c.v.-infusion of NPY or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 5 mice per group. *P<0.01 *vs.* vehicle.

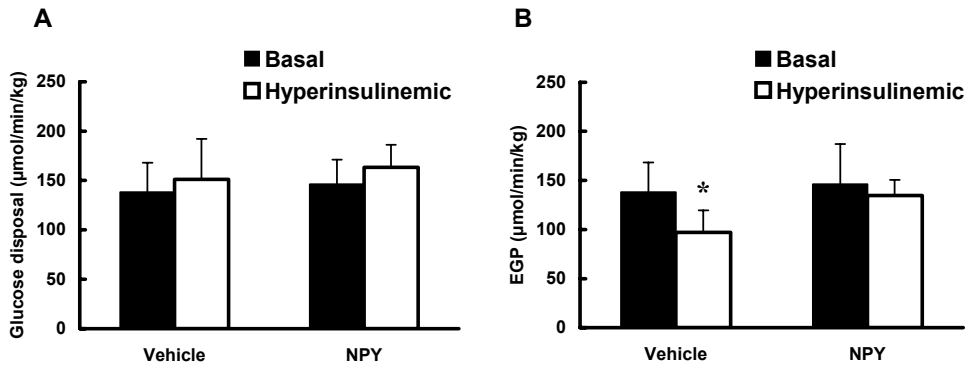

Figure 2. Glucose disposal (a) and endogenous glucose production (b) in mice that received an i.c.v.-infusion of NPY or vehicle before (basal) and after (hyperinsulinemic) the initiation of a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 5 mice per group. *P<0.05 *vs.* basal.

FFA and glycerol turnover. Basal rates of FFA (16.6 ± 6.5 *vs.* 18.1 ± 7.8 *μ*mol/min/kg) and glycerol turnover (7.3 ± 3.5 *vs.* 6.8 ± 1.4 *μ*mol/min/kg) were not different between vehicle and NPY infused animals (Figure 3). Hyperinsulinemia suppressed both FFA and glycerol turnover to a similar extent in both groups (6.6 ± 2.3 *vs.* 9.0 ± 4.8 *μ*mol/min/kg and 4.6 ± 1.6 *vs.* 4.3 ± 1.0 *μ*mol/min/kg in vehicle and NPY-infused animals for FFA and glycerol turnover, respectively).

VLDL-production. VLDL-production was similar in both groups in basal conditions (82.5 ± 20.4 (vehicle) *vs.* 68.8 ± 34.9 (NPY) *μ*mol/kg/h; Figure 4), whereas it remained significantly higher in hyperinsulinemic conditions during NPY infusion (97.5 ± 18.0 *vs.* 54.7 ± 14.9 *μ*mol/kg/h, P<0.01; Figure 4).

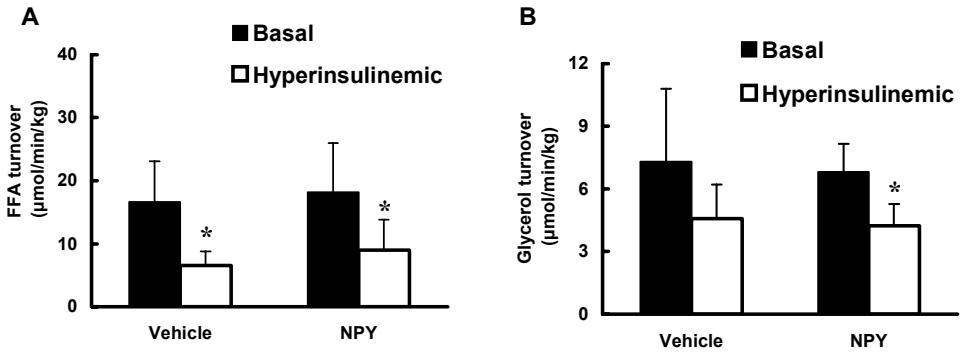

Figure 3. Free fatty acids (FFA) turnover (a) and glycerol turnover (b) in mice that received an i.c.v.-infusion of NPY or vehicle before (basal) and after (hyperinsulinemic) the initiation of a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 5 mice per group. *P<0.05 *vs.* basal.

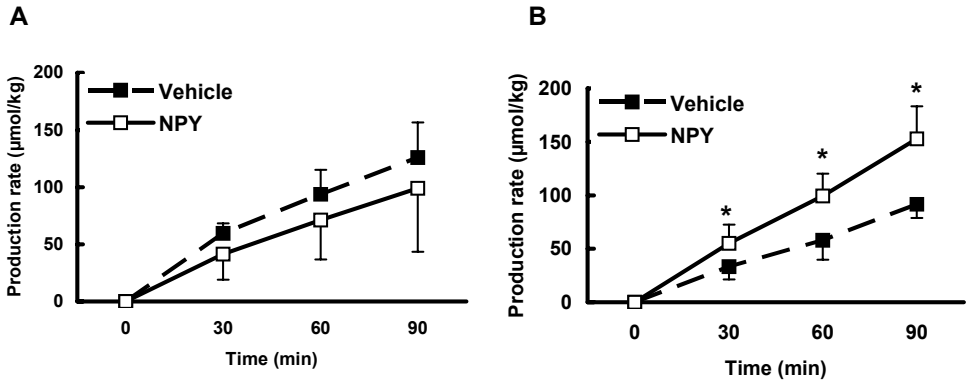

Figure 4. VLDL production rate in mice that received an i.c.v.-infusion of NPY or vehicle under basal (a) or hyperinsulinemic (b) conditions. Values represent mean ± SD for at least 5 mice per group. *P<0.01 *vs.* vehicle.

Discussion

This study demonstrates that i.c.v. infusion of NPY acutely impairs the ability of insulin to inhibit glucose and VLDL production. In contrast, NPY administration did not affect insulin's stimulatory action on glucose disposal and inhibitory effect on lipolysis. We infer that suppression of central NPY neuronal activities by insulin may be pivotal for its ability to suppress endogenous glucose and VLDL production.

One of the major targets of insulin in the brain is an intricate neuronal circuit in the arcuate nucleus that plays a critical role in the regulation of energy balance and fuel flux. This circuit comprises a catabolic regulatory pathway, primarily consisting of neurons co-expressing POMC and Cocaine and Amphetamine Related Transcript (CART). These POMC/CART neurons effectively counterbalance the actions of an anabolic pathway, comprising NPY/Agouti related protein (AgRP) neurons [5]. Insulin has reciprocal regulatory effects on these neurons: it stimulates the activity of POMC neurons, while it inhibits neuronal NPY release. POMC conveys its catabolic message via α-melanocyte stimulating hormone (α-MSH), a derivative peptide, which activates melanocortin 3 and 4 receptors (MCR3/4). Acute i.c.v. infusion of a potent MCR3/4 antagonist did not affect the ability of circulating insulin to inhibit glucose production [3], which indicates that the inhibitory action of insulin on glucose production does not require the stimulatory impact of hypothalamic insulin receptors on melanocortin neurons (although subchronic administration of a MCR3/4 antagonist

does impair insulin action in rats, probably via effects on food intake and body fat content [18]). To explain the acute effects of hypothalamic insulin signaling on endogenous glucose production [3], we explored the impact of i.c.v. NPY infusion on the metabolic effects of hyperinsulinemia during a euglycemic clamp. Our data clearly show that NPY impairs the ability of hyperinsulinemia to suppress endogenous (primarily hepatic) glucose production in this experimental context. Furthermore, insulin does not only suppress hepatic glucose production, but also inhibits VLDL production [19;20] and our results indicate, that i.c.v. NPY administration hampers this metabolic action of insulin as well. We infer that the primary neurophysiological effect of insulin to inhibit neuronal NPY release may be critical for its capacity to inhibit (hepatic) glucose and VLDL production.

In contrast to its apparent impact on glucose and VLDL production, NPY administration did not alter the effects of hyperinsulinemia on glucose disposal or lipolysis. The latter observation supports the notion that the effect of NPY on the ability of insulin to modulate VLDL metabolism was a direct hepatic effect and not mediated via enhanced flux of free fatty acids to the liver, brought about by any potential impact of NPY on lipolysis. The former finding agrees with data reported by Obici [3], which indicate that hypothalamic insulin signaling does not (acutely) affect insulin mediated glucose disposal (despite its clear inhibitory effect on hepatic insulin action). Collectively, the current knowledge suggests that downregulation of hypothalamic NPY by insulin may be a prerequisite for its acute inhibitory impact on endogenous glucose and VLDL production, whereas it does not directly affect fuel flux in other peripheral tissues.

NPY receptors are not only present in the brain, but in many peripheral tissues as well [21-23]. To dismiss the possibility that i.c.v. NPY infusion modulated insulin sensitivity via activation of peripheral receptors (after leakage through the blood brain barrier into the circulation), we measured plasma NPY levels at the end of the i.c.v.-infusion period. NPY concentrations were similar in vehicle and NPY-infused animals, demonstrating that the effects of NPY on glucose and VLDL-production that we observed were not due to activation of peripheral NPY receptors.

It is apt to consider that anesthesia may impact on the neuromodulatory effects of peptides. However, our findings agree with and corroborate a similar study performed in conscious unrestrained rats without access to food [24]. The similarity of

the results of either study supports the position that anesthesia did not affect our data to a major extent and adds further credibility to our main message.

It is important to recognize, that we probably infused a pharmacological dose of NPY, which precludes a definite inference as to whether NPY is a second messenger downstream the brain insulin receptor involved in the physiological control of fuel metabolism. Also, the present study does not rule out the possibility that i.c.v. NPY administration hampers the capacity of insulin to suppress glucose and VLDL production via other mechanistic routes than those downstream its arcuate receptor. Indeed, NPY has a variety of neuroendocrine effects that may also be involved. For example, it stimulates the activity of the pituitary adrenal ensemble [25] and adrenalectomy was shown to prevent or reduce some metabolic effects of subchronic i.c.v. NPY administration, like hyperphagia, weight gain and hyperinsulinemia [25-28]. Corticosteroids enhance endogenous glucose production primarily via stimulation of gluconeogenesis without affecting glycogenolysis [29]. However, circulating levels of corticosterone were not affected by NPY administration in the present study, which obviously argues against the position that the pituitary adrenal ensemble is involved in the acute effects of NPY on hepatic insulin sensitivity. We also checked if NPY enhances plasma glucagon concentrations to stimulate EGP, but glucagon levels did not differ between NPY and vehicle treated animals. Thus, it remains a challenge to unveil the messengers that relay NPY signals from the brain to the liver to control glucose and VLDL production.

Our data imply that insulin resistant neural circuits and related NPY neuronal activities may be involved in the pathogenesis of some of the features of the metabolic syndrome. High fat diet-induced obesity syndromes in rodents (and many genetically engineered obesity models as well) are marked by hyperglycemia and hypertriglyceridemia. Human obesity is also frequently complicated by these adverse metabolic sequelae, which are partly brought about by impaired ability of insulin to suppress endogenous glucose and VLDL production. High fat feeding was shown to induce both insulin resistance and (as a corollary) high NPY expression levels in the arcuate nucleus of the rodent brain [30;31]. Other obese animal models are also characterized by high NPY neuronal activity [32-35]. Given the effects of hypothalamic insulin on hepatic fuel flux, it is conceivable that brain insulin resistance and unleashed NPY neuronal activity are involved in the pathogenesis of hyperglycemia and hypertriglyceridemia as sequelae of high fat feeding and obesity. In this scenario,

NPY receptor antagonistic drugs may be appropriate tools to treat these metabolic anomalies, which predispose to type 2 diabetes mellitus and cardiovascular disease.

In summary, we here provide evidence that i.c.v. NPY administration precludes the inhibition of hepatic glucose and VLDL production by circulating insulin. This finding may imply that the increased hypothalamic NPY levels that are typically observed in various obese animal models underlie hepatic insulin resistance and associated metabolic anomalies in these models. NPY receptor antagonists may therefore be useful therapeutical tools in the clinical management of insulin resistance and type 2 diabetes.

Acknowledgements

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Chapter 3

Intracerebroventricular administration of melanotan II increases insulin sensitivity of glucose disposal in mice.

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Abstract

Aims/hypothesis. The present study was conducted to evaluate the effects of central administration of melanotan II (MTII), a MC3/4 receptor agonist, on hepatic and whole body insulin sensitivity, independent of food intake and body weight.

Methods. 225 ng of MTII was injected in 3 aliquots over 24 hours into the left lateral ventricle in male C57Bl/6 mice without access to food. The control group received 3 distilled water injections. Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic-euglycaemic clamp in combination with ^{3}H-glucose infusion. GLUT-4 mRNA expression was measured in skeletal muscle.

Results. Plasma glucose and insulin concentrations during basal and hyperinsulinaemic conditions were similar in MTII- and placebo-treated mice. Endogenous glucose production (EGP) and glucose disposal in the basal state were significantly higher in MTII-treated mice compared to the control group (71±22 *vs.* 43±12 μmol/min/kg, $p<0.01$). During hyperinsulinaemia, glucose disposal was significantly higher in MTII-treated mice (151±20 *vs*. 108±20 μmol/min/kg, $p<0.01$). In contrast, the inhibitory effect of insulin on EGP was not affected by MTII (relative decrease of EGP: 45±27 *vs.* 50±20%). GLUT-4 mRNA expression in skeletal muscle was significantly increased in MTII-treated mice (307±94 *vs.* 100±56%, $p<0.01$).

Conclusions/interpretation. Intracerebroventricular administration of MTII acutely increases insulin-mediated glucose disposal, whereas it does not affect insulin's capacity to suppress EGP in C57Bl/6 mice. These data indicate that central stimulation of MC3/4 receptors modulates insulin sensitivity in a tissue specific manner, independent of its well-known impact on feeding and body weight.

Introduction

The hypothalamus integrates a multitude of behavioural and metabolic adaptations to food intake and starvation, necessary to maintain fuel homeostasis despite profound environmental variations in nutrient availability [1]. Two types of neurons in the arcuate nucleus of the hypothalamus are of major importance for the control of these processes: neurons co-expressing Agouti related protein (AgRP) and neuropeptide Y (NPY), and neurons expressing pro-opiomelanocortin (POMC), the molecular precursor of alpha-melanocyte stimulating hormone (α-MSH) [2]. α-MSH binds to and stimulates melanocortin (MC) receptors. AgRP can bind to MC receptors as well, and

thereby inhibit the POMC pathway [3]. NPY and POMC neuropeptides exert opposing effects on food intake and fuel homeostasis. NPY acts to promote anabolic pathways, whereas α-MSH counteracts the effects of NPY [4-6]. For instance, during food deprivation NPY/AgRP neuronal activity is high, whereas POMC/α-MSH expression levels are low [5], and this setting of the arcuate neuronal circuitry strongly stimulates food intake and reduces energy expenditure [7].

Apart from its impact on food intake, intracerebroventricular (icv) administration of NPY acutely hampers insulin's capacity to inhibit hepatic glucose and VLDL production in C57Bl/6 mice, whereas insulin sensitivity of muscle and adipose tissue remains unaffected [8]. Conversely, chronic (7 days) icv infusion of α-MSH enhances peripheral and hepatic insulin sensitivity in rats through stimulation of the MC3/4 receptor [9] and POMC gene overexpression ameliorates insulin resistance in leptin-deficient mice via a mechanism that is independent of its effects on food intake and body weight [10]. In the latter studies, the effects on insulin sensitivity occur in the presence of a concomitant reduction in food intake and fat mass, which precludes distinction of putative *direct* effects of α-MSH or MC4 receptor on insulin sensitivity from *indirect* effects via feeding and body composition.

In addition to the effect of MC4 receptor activation on insulin sensitivity, Fan et al documented decreased insulin concentration after central activation of the melanocortin neuronal circuitry and increased levels of insulin in MC4 receptor knockout mice, even before the onset of detectable hyperphagia or obesity [11]. In humans, MC4 receptor mutations are associated with obesity [12;13].

The aim of the present study was to document the *direct* effects of activation of MC3/4 receptors on insulin sensitivity (i.e. *via* other mechanistic routes than feeding and fat mass). Therefore, we injected melanotan II (MTII) [14], an agonist of the MC3/4 receptor [15] icv, and quantified hepatic and peripheral insulin sensitivity of glucose metabolism during a hyperinsulinaemic euglycaemic clamp in mice without access to food.

Research designs and methods

Animals. Male, 3 months old C57Bl/6 mice (originated from the Jackson Laboratories and bred in our own animal facility) were housed in a temperature and humidity controlled room on a 12-hour-light-dark cycle with free access to standard

mouse chow and water. All animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

Surgical procedures. Mice were anaesthetised with 0.5 mg/kg Medetomidine (Pfizer, Capelle a/d IJssel, the Netherlands), 5 mg/kg Midazolam (Roche, Mijdrecht, the Netherlands), and 0.05 mg/kg Fentanyl (Janssen-Cilag, Tilburg, the Netherlands). A 25-gauge guide cannula was stereotactically implanted into the left lateral ventricle using the following coordinates from Bregma: 0.46 mm posterior, 1.0 mm lateral end 2.2 mm ventral [16;16]. The guide cannula was secured with two screws and dental cement (AgnTho's, Lidingö, Sweden) to the skull surface. After a recovery period of 1 week, adequate placement of the cannulae was tested by measuring the feeding response to an acute icv injection of NPY (5 μg dissolved in 1 μl sterile water) (Bachem, Bubendorf, Germany).

Hyperinsulinaemic euglycaemic clamp. Mice fasted for 24 hours (with food withdrawn at 09.00 am the day before the experiment) were used. At 9.00 hours and 17.00 hours the day before the experiment and at 8.45 hours on the day of the experiment mice were given 75 ng (in 1.5 μl distilled water) MTII (PhoenixEurope GmbH, Karlsruhe, Germany) or 1.5 μl distilled water (control group) icv. This dose of MTII was based on data from Murphy *et al* [17]), who showed inhibition of food intake using this dose. During icv injections, mice were lightly anaesthetised with isoflurane. All experiments were performed at 09.00 hours. Hyperinsulinaemic euglycaemic clamps were performed as described earlier [18;19]. During the experiments, mice were sedated with 6.25 mg/kg Acepromazine (Sanofi sante animale, Libourne Cedex, France) 6.25 mg/kg Midazolam (Roche, Mijdrecht, the Netherlands), and 0.3125 mg/kg Fentanly (Janssen-Cilag, Tilburg, the Netherlands).

Basal rates of glucose turnover were measured by giving a primed (0.7 μCi) continuous (1.2 μCi/h) infusion of ^{3}H-glucose (Amersham, Little Chalfont, UK) for 80 min. Subsequently, insulin was administered in a primed (4.1 mU) continuous (6.8 mU/h) i.v. infusion for 2 to 3 hours to attain steady state circulating insulin levels of about 4 ng/ml. The ^{3}H-glucose infusion (1.2μCi/h) was continued. A variable infusion of 12.5% D-glucose (in PBS) solution was also started and adjusted to maintain blood euglycaemia (measured at 10 minute intervals via tail bleeding, Freestyle,

TheraSense, Disetronic Medical Systems BV, Vianen, the Netherlands). Bloodsamples (60 μl) were taken during the basal period (after 60 and 80 minutes) and during the clamp period (when glucose levels in the blood were stable and 20 and 40 minutes later) for determination of plasma glucose, NEFA, insulin and ^{3}H-glucose specific activity.

mRNA expression of GLUT-4. A real time polymerase chain reaction (RT-PCR) was used to measure mRNA expression levels of GLUT-4 in skeletal muscle. Skeletal muscle was taken out in additional groups of mice directly at 10.30 hours. after a 24h fast and 3 icv injections with either MTII or vehicle (injections at the same time-points as in the hyperinsulinaemic euglycaemic clamp experiment). Muscle was homogenised in 1.2 ml RNA-Bee (Tel-Test, Inc, Texas, US) and total RNA was extracted according to Chomzcinsky and Sacchi [20]. The amount of RNA was determined by spectrophotometry (ND-1000 spectrophotometer, Nanodrop®) at a wavelength of 260 nm. The quality was checked by the ratio of absorption at 260 nm and absorption at 280 nm. Complementary DNA (cDNA) was obtained of total RNA. For RT-PCR, forward and reverse primers and TaqMan probe were designed from mice specific sequence data (*Entrez*, National Institutes of Health; and *Ensembl*, Sanger Institute) using computer software (Primer Express, Applied Biosystems). For each of the genes a Blast Search was done to reveal that sequence homology was obtained only for the target gene. Forward, reverse primers and TaqMan probe (5' CCATGAGATCTGAGGCCACA 3'; 5' GTATTTGCCGAAGTTGTAGCCG 3'; 5' CAAGGGCAAGATCATCATGCACGACC 3') of GLUT-4 were used. The TaqMan probe was 5'-6-carboxyfluorescein (FAM) and 3'- (BHQ1) labelled. GAPDH (5' VIC and 3' BHQ1, Applied Biosystems) and Cyclophilin (5' TET and 3' BHQ1, 5' CAAATGCTGGACCAAACACAA 3'; 5' GCCATCCAGCCATTCAGTCT 3'; 5' CCGGTTCCCAGTTTTTTATCTGCACTGCC 3') were used as housekeeping genes. PCR amplification was performed in a total reaction volume of 12.5 µl. The reaction mixture consisted of qPCR™ MasterMix (Eurogentec, Belgium), the optimal primer and probe concentrations of GLUT-4 and the endogenous control, nuclease free water and cDNA. An identical cycle profile was used for all genes: 50°C for 2 min + 95°C for 10 min + [95°C for 15 sec + 60°C for 1 min] * 40 cycles. Data were analysed using a comparative critical threshold (Ct) method where the amount of target normalised to the amount of endogenous control (GAPDH/cyclophilin) and relative to

the control sample is given by $2^{-\Delta\Delta Ct}$ (Applied Biosystems). All samples were run together allowing relative comparisons of the samples.

Analytical procedures. Plasma glucose and NEFA levels were determined using a commercially available kit (Instruchemie, Delfzijl, The Netherlands; Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin and corticosterone concentrations were measured by Elisa (both ALPCO Diagnostics, Windham, NH, USA). For the determination of plasma ^{3}H glucose, plasma was deproteinised with 20% trichloroacetic acid, dried to remove water, resuspended in distilled water and counted with scintillation fluid (Ultima Gold, Packard, Meridien, Connecticut, USA).

Calculations. Turnover rate of glucose (μmol/min/kg) was calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{3}H-glucose (dpm/*μ*mol). The ratio was corrected for body weight. EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Statistical analysis. Data are presented as mean ± standard deviation. Differences between groups were determined by Mann-Whitney *U* test for 2 independent samples. A P-value <0.05 was considered statistically significant.

Results

Plasma parameters. Body weight, plasma corticosterone, glucose, NEFA and insulin concentrations in basal and hyperinsulinaemic conditions are shown in Table 1. In the basal state, these parameters did not differ between MTII- and vehicle treated animals. In steady state hyperinsulinaemic conditions, plasma NEFA levels decreased ~2-fold while insulin concentrations increased ~10 fold as expected. No differences were observed in plasma glucose, insulin and NEFA levels between MTII- and vehicle-treated mice during hyperinsulinaemia.

Table 1. Body weight, plasma corticosterone, NEFA, glucose and insulin concentration in vehicle (n=10) and MTII (n=8) mice. Values are expressed as means ± SD. n.d. is not determined.

	Basal		Hyperinsulinemic	
	Vehicle	MTII	Vehicle	MTII
Body weight (g)	17.9 ± 1.7	18.9 ± 1.6	-	-
Corticosterone (mmol/l)	33.3 ± 17.5	37.8 ± 12.4	n.d.	n.d.
Glucose (mmol/l)	5.8 ± 1.0	6.7 ± 1.2	8.4 ± 1.0	8.2 ± 2.4
NEFA (mmol/l)	0.55 ± 0.17	0.62 ± 0.17	0.26 ± 0.14	0.24 ± 0.08
Insulin (ng/ml)	0.31 ± 0.11	0.31 ± 0.07	3.8 ± 2.9	3.7 ± 2.7

Glucose turnover. In basal conditions, EGP (and thereby glucose disposal) was significantly higher in MTII treated animals compared to vehicle treated mice (71 ± 22 *vs.* 43 ± 10 *μ*mol/min/kg, respectively, $p<0.01$). During the hyperinsulinaemic period, the rate of glucose infusion necessary to maintain euglycaemia was significantly higher in MTII- than in vehicle-treated animals (114 ± 23 *vs.* 85 ± 20 μmol/min/kg, $p<0.05$). Accordingly, the glucose disposal rate was significantly higher in MTII treated animals (151 ± 20 *vs.* 108 ± 20 *μ*mol/min/kg, resp., $p<0.01$, Figure 1a). In contrast, hyperinsulinaemia suppressed EGP to a similar extent in MTII- *vs.* vehicle-treated animals (45 ± 27% *vs.* 50 ± 20%, ns, Figure 1b).

mRNA expression of GLUT-4. GLUT-4 mRNA expression in skeletal muscle was higher in the MTII treated group compared to vehicle-treated mice (307 ± 94 *vs.* 100 ± 56 %, p<0.01, Figure 2).

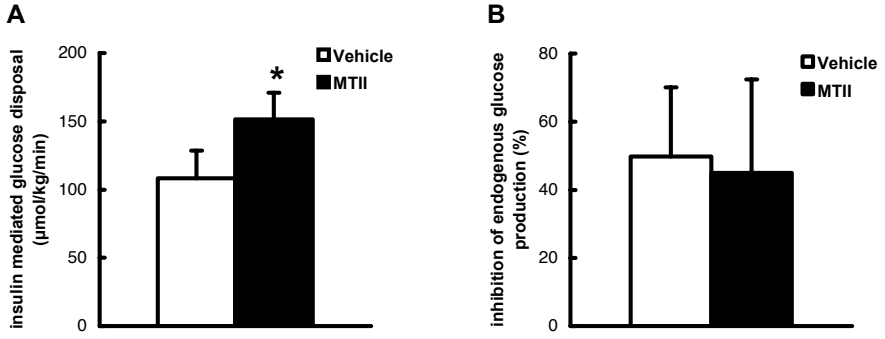

Figure 1. Insulin mediated glucose disposal (a) and inhibition of endogenous glucose production (b) by insulin in 24 hours fasted mice that received icv injections of MTII (n=10) or vehicle (n=8). Values represent mean ± SD. *P<0.01 *vs.* vehicle.

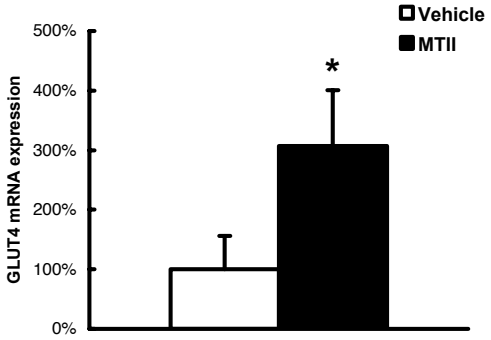

Figure 2. GLUT4 mRNA expression levels in 24 hours fasted mice that received injections of MTII (n=6) or vehicle (n=7) in basal conditions. *P<0.01 *vs.* vehicle.

Discussion

This study shows, that activation of MC3/4 receptors enhances whole body sensitivity of glucose metabolism for insulin action in mice via other mechanistic routes than feeding and fat mass. In particular, MTII promotes insulin mediated glucose disposal, whereas it leaves the capacity of insulin to suppress EGP unaffected. These observations are in line with the emerging notion, that neural circuits control insulin action in peripheral tissues.

Interestingly, GLUT-4 mRNA was increased in muscle of MTII treated animals, which suggests, that activation of MC3/4 receptors enhances GLUT-4 gene-expression to promote glucose uptake. The downstream mechanisms that actuate the effects of hypothalamic neuronal circuits on muscle GLUT-4 mRNA expression remain to be fully elucidated. It cannot be ruled out that MTII increased locomotor activity and subsequently GLUT-4 mRNA expression in muscle. However, this seems unlikely since other studies did not observe any increase in locomotor activity after central administration of MTII [14;17]. Additional studies are required to elucidate the mechanisms involved in the modulation of insulin sensitivity by central administration of MTII.

Glucose production in the basal state was higher in mice treated with MTII, whereas MTII did not affect the capacity of insulin to suppress EGP. Thus, central melanocortin pathways appear to directly impact endogenous glucose output. Although Fan *et al*. [11] documented decreased plasma insulin concentrations after icv administration of MTII, we did not find significant changes in basal plasma insulin concentrations as a potential explanation for the observed increase in basal glucose production. As Fan *et al* injected more than ten times the amount we injected, this discrepancy may be explained by the difference in doses used. Since MTII has been shown to activate the sympathetic nervous system [21;22] and sympathetic outflow can promote glucose production [23], it is conceivable that the autonomic nervous system relays signals from the hypothalamus to peripheral tissues to modulate glucose metabolism. Unfortunately, our study does not provide data to evaluate this possibility. Alternatively, the brain interacts with the liver *via* the hypothalamo-pituitary-adrenal axis (HPA). Corticosteroids stimulate glucose production [24] and HPA activity is under strict control of the hypothalamus. In fact, corticotrophin releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus express MC4 receptors and central MTII injection acutely enhances CRH and corticosterone release in rats [25]. However, MTII did not modify circulating corticosterone concentrations in our experimental setting, which refutes the thesis that central melanocortin pathways modulated the HPA axis to enhance glucose output in this study. Interestingly, short-term fasting is accompanied by a decrease in EGP [26;27], and POMC/α-MSH mRNA expressions decrease concomitantly [5]. It is therefore tempting to speculate, that blunted melanocortin signalling is involved in the

decrease in EGP during fasting. In this scenario, administration of MTII may have prevented the normal decline in EGP associated with fasting in the present study.

Although MTII increased basal EGP, it did not appear to affect insulin's capacity to suppress it. A previous paper [9] reports that chronic (7 days) icv infusion of α-MSH reinforces insulin action on glucose production (as well as on glucose disposal) in rats. However, this effect occurred in the presence of concomitant diminutions of food intake and body adiposity and both of these long-term sequelae of MTII administration can impact insulin sensitivity. Our data indicate that activation of melanocortin circuits, through a mechanism that is independent of food intake and body weight, enhances insulin sensitivity and that insulin action on glucose disposal is more sensitive to manipulation of MC3/4 receptors than its capacity to suppress EGP.

We recently showed that icv infusion of NPY in C57Bl/6 mice acutely hampers insulin's inhibitory effect on EGP, whereas it does not appear to affect insulin mediated glucose disposal [8]. We now show, that activation of melanocortin circuits reinforces insulin action on glucose disposal, while suppression of glucose production remains unaffected. NPY and melanocortin circuits in the arcuate nucleus play critical roles in the control of fuel homeostasis in the face of fluctuations in nutrient availability. NPY neurons, active during fasting, stimulate feeding and inhibit energy expenditure, whereas melanocortin circuits, suppressed in fasting conditions, counteract NPY to exert opposing effects on energy balance. These behavioural and metabolic actions serve to protect the body against the perils of famine. Our data suggest that the brain also modulates glucose metabolism to further reinforce the line of defence: enhanced activity of NPY neurons promotes glucose production, whereas reduced melanocortin activity hampers glucose disposal in fasting conditions, keeping glucose available as pivotal fuel for the brain. Conversely, diminished NPY'ergic and increased melanocortin signalling allow insulin to appropriately suppress glucose output and promote glucose disposal in response to food intake. The current findings may imply that MC-3/4 receptor agonists can serve as "insulin sensitisers" in the treatment of the metabolic syndrome and type 2 diabetes mellitus. However, tachyphylaxis to chronic MTII administration has been observed in mice and rats [28;29]. In addition, the present study shows that MTII increases basal EGP. Thus, the impact of chronic MTII administration on glucose metabolism in (insulin resistant) animal models and humans remains to be established.

In conclusion, the present study shows that activation of central melanocortin-3/4 receptors by melanotan II enhances insulin sensitivity of whole body glucose disposal, independent of food intake and fat mass, whereas it does not affect insulin's ability to suppress EGP. These observations are in line with the emerging notion, that neural circuits, apart from their effects on feeding, modulate insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability.

Acknowledgements

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Chapter 4

PYY_{3-36} reinforces insulin action on glucose disposal in mice fed a high fat diet.

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Abstract

PYY_{3-36} is released by the gut in response to nutrient ingestion. It modulates the activities of orexigenic neuropeptide Y (NPY) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons in the hypothalamus to inhibit food intake. As both NPY and POMC have been shown to also impact insulin action, we wondered whether PYY_{3-36} could improve insulin sensitivity. To address this question, we examined the acute effect of intravenous PYY_{3-36} on glucose and free fatty acid (FFA) flux during a hyperinsulinemic euglycemic clamp in mice maintained on a high fat diet for 2 weeks prior to the experiment. We also evaluated the effects of PYY_{3-36} infusion on glucose uptake in muscle and adipose tissue in this experimental context. In basal conditions, none of the metabolic parameters was affected by PYY_{3-36}. In hyperinsulinemic conditions, glucose disposal was significantly increased in PYY_{3-36}-infused- compared with vehicle-infused mice (103.8 ± 10.9 *vs.* 76.1 ± 11.4 μmol/min/kg, respectively, P=0.001). Accordingly, glucose uptake in muscle and adipose tissue was greater in PYY_{3-36}-treated animals, although the difference with controls did not reach statistical significance in adipose tissue (muscle: 2.1 ± 0.5 *vs.* 1.5 ± 0.5 μmol/ g tissue, P=0.049; adipose tissue: 0.8 ± 0.4 *vs.* 0.4 ± 0.3 μmol/ g tissue; P=0.08). In contrast, PYY_{3-36} did not impact insulin action on endogenous glucose production or FFA metabolism. These data indicate that PYY_{3-36} reinforces insulin action on glucose disposal in mice fed a high fat diet, through a mechanism that is independent of food intake and body weight. In contrast, it leaves glucose production and lipid flux largely unaffected in this experimental context.

Introduction

Peptide YY (PYY) belongs to a family of peptides that is critically involved in the regulation of appetite. It is synthesized in specialized cells (L-cells) that are found primarily in the distal gastro-intestinal tract. Circulating PYY levels rise within 15 minutes after a meal in proportion to the amount of calories ingested and remain elevated for about 6 hours [1]. The two natural forms of this peptide, PYY_{1-36} and PYY_{3-36}, have opposing effects on food intake [2]: PYY_{1-36} stimulates appetite whereas PYY_{3-36} (the major circulating form) inhibits feeding [3-5].

PYY_{3-36} reduces food intake by acting on appetite regulation centers in the hypothalamus [3;6]. Specifically, PYY_{3-36} is an agonist of the presynaptic NPY Y2

receptor (Y2R), which inhibits NPY neuronal activity in the arcuate nucleus and thereby activates adjacent pro-opiomelanocortin (POMC) neurons [3]. In addition to their critical role in the control of feeding, both NPY and POMC affect insulin action. Intracerebroventricular infusion of NPY induces hyperinsulinemia and insulin resistance in rats [7;8]. In contrast, central administration of α-melanocyte stimulating hormone (α-MSH), a splice-product of the POMC peptide, enhances insulin sensitivity [9]. In view of the fact that PYY_{3-36} inhibits NPY- and activates POMC neuronal activity, we wondered whether it could improve insulin sensitivity "directly" (i.e. through a mechanism independent of food intake and body weight). To address this question, we infused PYY_{3-36} intravenously and quantified its acute effects on glucose and fatty acid flux during a hyperinsulinemic euglycemic clamp in mice without access to food during the experiment.

Methods

Animals. Male C57BL/6J mice were housed in a temperature-controlled room on a 12-hour light-dark cycle and were fed a high fat diet (43 energy% fat derived from bovine lard) with free access to water for 2 weeks to induce insulin resistance. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

Hyperinsulinemic euglycemic clamp. Thirty-six mice were fasted overnight with food withdrawn at 05.00 pm the day prior to the study. The next day, hyperinsulinemic euglycemic clamps were performed as described earlier [10]. PYY or vehicle was administered as a primed (0.15 μg) continuous (0.25 μg/h) intravenous (i.v.) infusion during the whole experiment (basal and hyperinsulinemic period). In one series of experiments glucose turnover was quantified and in another series FFA turnover was determined. First, basal rates of glucose or FFA turnover were measured by giving a primed (p) continuous (c) infusion of ^{3}H-glucose (p: 0.7 μCi, c: 1.2 μCi/h) (Amersham, Little Chalfont, U.K.) or ^{14}C-palmitate (p: 1.8 μCi, c: 3 μCi/h) (Amersham, Little Chalfont, U.K) respectively for 80 min. Subsequently, insulin was administered in a primed (4.1 mU) continuous (6.8 mU/h) i.v. infusion for 2 to 3 hours to attain steady state circulating insulin levels of ~6 ng/ml. A variable infusion of 12.5% D-glucose was used to maintain euglycemia (measured at 10 min intervals via

tail bleeding, Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). Blood samples (75 μl) were taken during the basal period (after 60 and 80 minutes) and during the clamp period (when glucose levels were stable and 20 and 40 minutes later) for determination of plasma glucose, FFA and insulin concentrations and ^{3}H-glucose and ^{14}C-palmitate specific activities.

To assess insulin-mediated glucose uptake in individual tissues, 2-deoxy-D-[^{3}H] glucose (2-[^{3}H]DG; Amersham, Little Chalfont, UK) was administered as a bolus (1μCi), 40 minutes before the end of the clamp experiments in which FFA turnover was measured. At the end of the clamp, mice were sacrificed and muscle and adipose tissue were isolated and frozen in liquid nitrogen for subsequent analysis.

Analytical procedures. Plasma levels of glucose and FFA were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands and Wako, Neuss, Germany). Plasma insulin and PYY_{3-36} concentrations were measured by a mouse insulin ELISA and PYY_{3-36} RIA (Alpco, Windham, NH, USA; Phoenix pharmaceuticals, Belmont, CA, USA). Total plasma ^{3}H-glucose was determined in 7.5 μl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water. Total plasma ^{14}C-palmitate was determined in 7.5 μl plasma after extraction of lipids by a modification of Bligh and Dyer's method [11]. Briefly, 7.5 μl plasma was dried and resolved in 100 μl water. Then 1.1 ml demi-water and 4.5 ml methanol:chloroform (2:1) was added and mixed thoroughly, after which 1.5 ml chloroform was added and mixed and finally, 1.5 ml demi-water was added and mixed. After centrifugation, the chloroform layer was collected and the FFA fraction was separated from other lipid components by thin-layer chromatography (TLC) on silica gel plates.

Tissue analysis. For determination of tissue 2-DG uptake, the homogenate of muscle and adipose tissue was boiled and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG as described previously [10;12;13].

Calculations. Turnover rates of glucose and FFA (μmol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{3}H-glucose or ^{14}C-palmitate (dpm/μmol). The ratio was corrected for body weight. EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Tissue-specific glucose uptake in muscle and adipose tissue was calculated from tissue 2-DG content, corrected for plasma specific activity and expressed as μmol per gram of tissue.

Statistical analysis. Differences between groups were determined by Mann-Whitney non-parametric test for 2 independent samples. A P-value < 0.05 was considered statistically significant. All values shown represent mean ± SD.

Results

Animals. Mice were 16-18 weeks old during the experiments. Body weight was 23.3 ± 1.2 gram in the control group and 22.8 ± 1.4 gram in the PYY group.

Plasma parameters. Plasma glucose, FFA, insulin and PYY_{3-36} concentrations in basal and hyperinsulinemic conditions are shown in table 1. In the basal state, plasma parameters did not differ between PYY- and vehicle-infused animals, except for the plasma PYY_{3-36} concentration. In steady state clamp conditions, plasma insulin and glucose concentrations were similar in both groups. Hyperinsulinemia suppressed FFA levels to a similar extent in PYY- and vehicle-infused mice.

Table 1. Plasma parameters in overnight fasted mice that received an i.v.-infusion of PYY or vehicle under basal or hyperinsulinemic conditions. Values represent mean ± SD for at least 7 mice per group.

	Basal		Hyperinsulinemic	
	Vehicle	PYY	Vehicle	PYY
Glucose (mmol/l)	4.9 ± 0.6	5.0 ± 0.6	9.0 ± 0.9	9.2 ± 2.0
FFA (mmol/l)	0.9 ± 0.2	1.0 ± 0.2	0.4 ± 0.1	0.5 ± 0.1
Insulin (ng/ml)	0.3 ± 0.1	0.3 ± 0.1	5.2 ± 2.9	6.7 ± 2.7
PYY_{3-36} (pg/μl)	< 1	3.8 ± 0.7	< 1	3.2 ± 0.7

Glucose turnover. In the basal condition, glucose disposal was similar in PYY- and vehicle-infused mice (39,5 ± 10.9 *vs.* 45.9 ± 12.6 μmol/min/kg, respectively; P=0.19, Figure 1a). The rate of glucose infusion necessary to maintain euglycemia during insulin infusion was significantly higher in PYY-infused mice than in vehicle-infused animals (89.1 ± 7.1 *vs.* 50.9 ± 13.6 μmol/min/kg, P=0.001), indicating that i.v. PYY_{3-36}

administration acutely enhances insulin sensitivity. Hyperinsulinemia increased glucose disposal in both groups. However, the disposal rate was significantly higher in PYY-infused animals compared with vehicle-infused controls (103.8 ± 10.9 *vs.* 76.1 ± 11.4 *μ*mol/min/kg, respectively, *P*=0.001, Figure 1a). In contrast, endogenous glucose production (EGP), which was similar in PYY and vehicle treated mice in basal conditions, was suppressed by insulin to the same extent in both groups. (by 62 ± 29 *vs.* 42 ± 18% from basal respectively; *P*=0.30, Figure 1b).

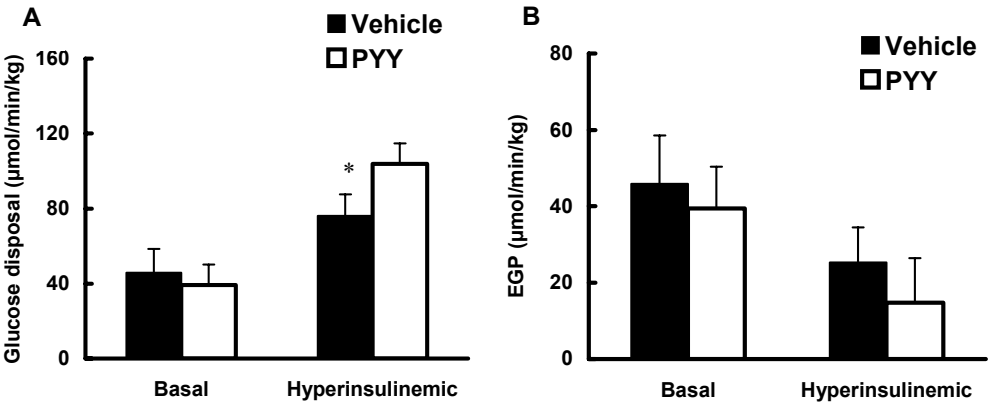

Figure 1. Glucose disposal (a) and endogenous glucose production (b) in overnight fasted mice before (basal) and during (hyperinsulinemic) a hyperinsulinemic euglycemic clamp. Prior to and during insulin infusion the animals received an i.v.-infusion of PYY or vehicle. Values represent mean ± SD for at least 7 mice per group. *P<0.01 *vs.* vehicle.

Tissue-specific glucose uptake. Insulin-mediated 2-deoxy-glucose uptake in muscle and adipose tissue was greater in PYY-treated animals, but the difference with controls did not reach statistical significance in adipose tissue (muscle: 2.1 ± 0.5 *vs.* 1.5 ± 0.5 *μ*mol/ g tissue, *P*=0.049; adipose tissue: 0.8 ± 0.4 *vs.* 0.4 ± 0.3 *μ*mol/ g tissue; *P*=0.08, Figure 2).

FFA turnover. Basal free fatty acid turnover (38.0 ± 14.2 *vs.* 42.3 ± 9.9 *μ*mol/min/kg) was not different between PYY- and vehicle-infused animals and was suppressed to a similar extent by insulin in both groups (22.4 ± 12.3 *vs.* 21.3 ± 10.9 *μ*mol/min/kg in PYY- and vehicle-infused animals, respectively; Figure 3).

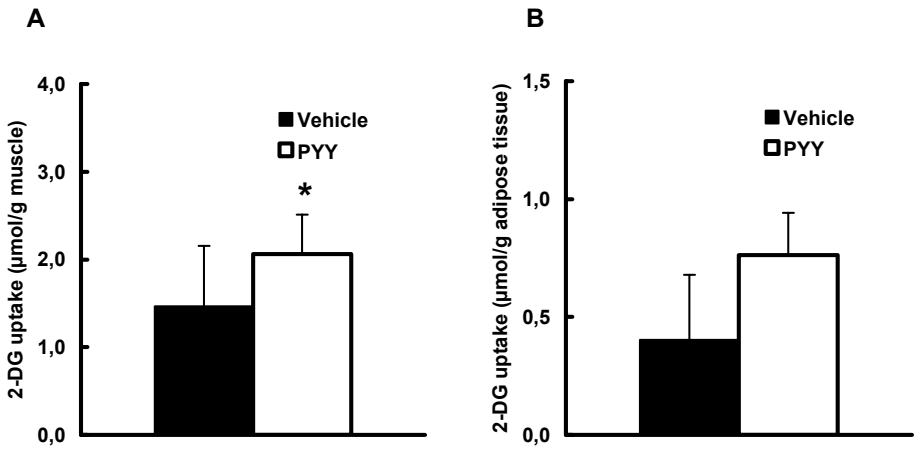

Figure 2. Muscle-specific (a) and adipose tissue-specific (b) glucose uptake under hyperinsulinemic conditions in overnight fasted mice that received an i.v.-infusion of PYY or vehicle. Values represent mean ± SD for at least 7 mice per group. *P<0.05 *vs.* vehicle.

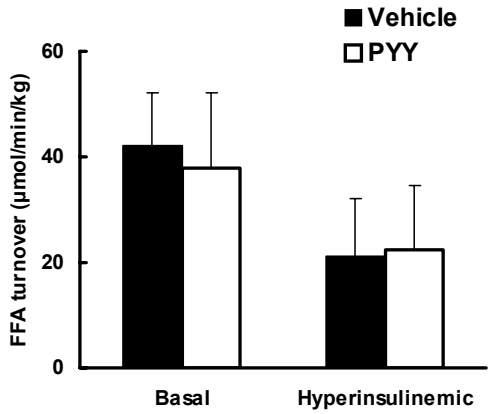

Figure 3. Free fatty acids (FFA) turnover in overnight fasted mice before (basal) and during (hyperinsulinemic) a hyperinsulinemic euglycemic clamp. Prior to and during insulin infusion the animals received an i.v.-infusion of PYY or vehicle. Values represent mean ± SD for at least 9 mice per group.

Discussion

Here we show that intravenous PYY_{3-36} administration acutely reinforces insulin action on glucose disposal in overnight fasted mice maintained on a high fat diet. In contrast, PYY_{3-36} does not appear to impact the effect of insulin on endogenous glucose production or FFA metabolism in this experimental context.

PYY_{3-36} clearly enhanced insulin-induced glucose disposal as determined by tracer dilution methodology. Accordingly, 2-DG uptake in muscle and adipose tissue

in hyperinsulinemic conditions was higher during PYY_{3-36} infusion, albeit the difference with control attained statistical significance only for muscle. In contrast, PYY_{3-36} did not significantly impact the capacity of insulin to inhibit endogenous glucose production. Insulin action on FFA metabolism was not affected by PYY_{3-36} either, as indicated by similar fatty acid turnover rates during hyperinsulinemia in PYY_{3-36} and saline infused animals. In light of the current experimental context, these data suggest that PYY_{3-36} reinforces the impact of insulin on glucose disposal through a mechanism that is independent of food intake and body weight. In contrast, it appears to leave insulin action on glucose production and FFA turnover largely unaffected.

The plasma PYY_{3-36} concentration rose to 3.2 ± 0.7 pg/μl in response to PYY infusion. Relatively few studies have looked at the physiology of circulating PYY_{3-36} in rodents. According to a recent paper by Batterham et al.[3], postprandial PYY_{3-36} concentrations amount to 112 pmol/l (~0.4 pg/μl) in freely feeding rats. In a study by Lee et al [14] plasma PYY levels in fasting mice were 0.18 pg/µl, which accords with our data (table 1). We are not aware of any study measuring postprandial PYY_{3-36} concentrations in mice. Thus, the dose of PYY_{3-36} we administered may have induced supraphysiological PYY_{3-36} levels. Therefore, our data do not allow a definitive inference as to whether the postprandial rise of circulating PYY_{3-36} can reinforce insulin action.

Postprandial PYY_{3-36} release is decreased in obese compared with lean subjects and circulating levels correlate negatively with body mass index. Conversely, intravenous PYY_{3-36} infusion significantly reduces food intake in humans [15] and repeated administration of PYY_{3-36} attenuates weight gain in rodents [3]. These findings suggest that diminished PYY_{3-36} release may contribute to the pathogenesis of obesity in animals and man. The observations presented here allow us to hypothesize that low circulating PYY_{3-36} levels may also compromise insulin action in obese subjects. Moreover, perhaps even more important, the data suggest that PYY_{3-36} or synthetic analogues of this peptide may be useful tools in the clinical management of obesity and insulin resistance.

It remains to be established whether PYY_{3-36} acts through hypothalamic neural circuits (in analogy of the mechanism guiding its effects on appetite) to enhance insulin-mediated glucose disposal. As PYY_{3-36} is a high affinity agonist to the Y2R [2] and the distribution of this receptor subtype is largely confined to the central nervous

system (particularly the arcuate nucleus of the hypothalamus) [16], it is most likely that PYY_{3-36} modulates insulin action by activation of Y2R in the brain. As alluded to earlier, Y2R signaling inhibits NPY neuronal activity in the arcuate nucleus of the hypothalamus and thereby activates adjacent POMC neurons [3]. Hypothalamic overexpression of NPY induces obesity and insulin resistance in mice [7;8], whereas activation of melanocortin receptor subtypes 3 and 4 in the brain enhances insulin sensitivity [9]. Thus, the present data are in keeping with the contention that PYY_{3-36} modulates insulin action via hypothalamic Y2R. The downstream mechanisms that actuate the effects of hypothalamic neuronal circuits on muscle and adipose tissue remain to be fully elucidated. At this point, vagotomy was shown to prevent the hyperinsulinemic effects of NPY, which suggests that the autonomic nervous system may be involved [17]. Also, adrenalectomy prevents the obesity syndrome produced by chronic central NPY infusion and reverses the obese phenotype in leptin-deficient ob/ob mice [18;19], indicating that the pituitary-adrenal ensemble may also serve as a second messenger. Thus, neural and/or endocrine mechanistic routes may convey signals from hypothalamic nuclei to peripheral tissues to orchestrate energy balance and fuel flux. It is conceivable that similar routes partake in the control of insulin action by PYY_{3-36}.

In conclusion, PYY_{3-36} reinforces insulin action in mice maintained on a high fat diet through a mechanism that is independent of food intake and body weight. In this context, PYY_{3-36} appears to predominantly impact insulin-mediated glucose disposal, whereas it leaves insulin action on glucose production largely unaffected. These novel data suggest that PYY_{3-36} or synthetic analogues of this peptide may be valuable assets to our armamentarium of drugs designed to battle insulin resistance and type 2 diabetes mellitus.

Acknowledgements

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Chapter 5

Chronic PYY_{3-36} treatment ameliorates insulin resistance in C57BL6-mice on a high fat diet.

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Abstract

PYY_{3-36} is a gut-derived hormone, that acts on hypothalamic nuclei to modulate energy metabolism. We recently showed, that PYY_{3-36} acutely reinforces insulin action on glucose disposal in insulin resistant mice. Because the long-term effects of PYY_{3-36} on insulin sensitivity are still unknown, we examined the effects of chronic PYY_{3-36} administration (2.5 μg/day s.c. for 7 days) on glucose turnover during a hyperinsulinemic-euglycemic clamp in C57BL6 mice maintained on a high fat diet for 16 weeks before the experiment. In addition, metabolic efficacy of continuous *vs.* intermittent administration of PYY_{3-36} was evaluated. Under hyperinsulinemic conditions, glucose disposal was significantly increased in PYY_{3-36} treated mice *vs.* vehicle-treated mice (78.8 ± 13.3 *vs.* 63.4 ± 15.5 μmol/min/kg, respectively, P=0.012). Tissue specific glucose uptake was significantly increased in adipose tissue (0.5 ± 0.2 *vs.* 0.2 ± 0.1 μmol/ g tissue; P=0.006), but not in muscle (2.2 ± 1.4 *vs.* 1.6 ± 0.8 μmol/ g tissue for PYY_{3-36} and vehicle-treated animals, respectively, P=0.38) of PYY_{3-36} treated animals. In contrast, insulin action on endogenous glucose production was not significantly affected. Furthermore, none of these metabolic parameters were affected by the mode of PYY_{3-36} administration (continuous or intermittent).

In conclusion, chronic PYY_{3-36} administration enhances the ability of insulin to promote glucose disposal, whereas it does not significantly affect endogenous glucose production in C57BL6 mice maintained on a high fat diet for 16 weeks. In addition, this study shows that continuous and intermittent administration are equally effective in this respect.

Introduction

The metabolic syndrome comprises a cluster of anomalies that increase the risk of cardiovascular disease and type 2 diabetes mellitus: hyperglycemia, abdominal obesity, hypertriglyceridemia, hypertension and low levels of high-density lipoprotein (HDL) cholesterol [1-3]. Insulin resistance may underlie the majority of these pathologies [4] and therapies that effectively reinforce insulin action may therefore ameliorate the risk profile of metabolic syndrome patients [5;6].

Diet-induced obese, insulin resistant C57BL6-mice have increased levels of neuropeptide Y (NPY) and decreased levels of pro-opiomelanocortin (POMC) in

hypothalamic nuclei [7-9]. These features of hypothalamic neural circuits may be involved in the pathogenesis of the metabolic syndrome, as intracerebroventricular (icv) administration of NPY or antagonists of POMC induce insulin resistance [10-13]. Therefore, antagonists of NPY and/or agonists of POMC signalling may be useful tools in the clinical management of this syndrome. Peptide YY_{3-36} (PYY_{3-36}) is released in response to food intake by L-cells in the distal gastrointestinal tract. It acts via Y2 receptors on NPY neurons in the arcuate nucleus to inhibit NPY neuronal activity and thereby activates adjacent POMC neurons [14;15]. We recently found that PYY_{3-36} administration acutely reinforces insulin action on glucose disposal through a mechanism that is independent of food intake and body weight [16]. This finding suggests that PYY_{3-36} may be used as a therapeutic tool in the clinical management of insulin resistance and the metabolic syndrome. However, the metabolic effects of long-term PYY_{3-36} administration are currently unknown, and waning of early impact may occur during chronic treatment through down regulation of receptor expression or function [17;18]. Therefore, the aim of this study was to investigate the long-term effects of PYY_{3-36} on insulin action by administering PYY_{3-36} subcutaneously for 7 days in mice fed a high-fat diet, and quantifying the effects on glucose production and disposal during a hyperinsulinemic euglycemic clamp study. As the physiology of PYY_{3-36} entails intermittent release in response to food intake, we also examined whether continuous and intermittent administration of PYY_{3-36} impact glucose metabolism differentially in this experimental context.

Research designs and methods

Animals. Male C57BL6 mice were housed in a temperature-controlled room on a 12-hour light-dark cycle and were fed a high fat diet (43 energy% fat derived from bovine lard) with free access to water for 16 weeks to induce insulin resistance. After 15 weeks of high fat diet, osmotic minipumps (Alzet minipump, model 2001, Charles River, Maastricht, The Netherlands) were placed subcutaneously in the back region under light isoflurane anesthesia. All mice received a saline (n = 15) or PYY_{3-36} (2.5 μg/day, n = 5) infusion via the osmotic minipump at a rate of 0.5 μl/h for 7 days. In addition, daily subcutaneous injections (50 μl at 09.00 am) of saline or PYY_{3-36} (2.5 μg) were given, where mice receiving continuous PYY_{3-36} treatment were additionally injected with saline, and mice receiving saline by minipump were assigned to receive

either saline (n = 8) or PYY_{3-36} (n = 7) by injection. Thus, glucose kinetics were determined in 2 experimental groups: 1) receiving saline and 2) receiving PYY_{3-36}, where PYY_{3-36} was administered continuously by minipump or intermittently by daily subcutaneous injection. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

Hyperinsulinemic euglycemic clamp. Mice were fasted overnight with food withdrawn at 05.00 pm the day prior to the study. The next day, hyperinsulinemic euglycemic clamps were performed as described earlier [19]. First, basal rates of glucose turnover were measured by giving a primed (0.7 μCi) continuous (1.2 μCi/h) infusion of ^{14}C-glucose (Amersham, Little Chalfont, U.K.) for 80 min. Subsequently, insulin was administered in a primed (4.1 mU) continuous (6.8 mU/h) i.v. infusion for 2 to 3 hours to attain steady state circulating insulin levels of ~3.5 ng/ml. A variable infusion of 12.5% D-glucose was used to maintain euglycemia (measured at 10 min intervals via tail bleeding, Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). Blood samples (75 μl) were taken during the basal period (after 60 and 80 minutes) and during the clamp period (when glucose levels were stable and 20 and 40 minutes later) for determination of plasma glucose, FFA, insulin and PYY_{3-36} concentrations and ^{14}C-glucose specific activities.

To assess insulin-mediated glucose uptake in individual tissues, 2-deoxy-D-[^{3}H] glucose (2-[^{3}H]DG; Amersham, Little Chalfont, UK) was administered as a bolus (1μCi), 40 minutes before the end of the clamp experiments. At the end of the clamp, mice were sacrificed and muscle and adipose tissue were isolated and frozen in liquid nitrogen for subsequent analysis.

Analytical procedures. Plasma levels of glucose and FFA were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands and Wako, Neuss, Germany). Plasma insulin and PYY_{3-36} concentrations were measured by a mouse insulin ELISA and PYY_{3-36} RIA (Mercodia, Uppsala, Sweden; Phoenix pharmaceuticals, Belmont, CA, USA; sensitivity of 1 pg/μl for the PYY_{3-36} RIA). Total plasma ^{14}C-glucose was determined in 7.5 μl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water.

Tissue analysis. For determination of tissue 2-DG uptake, the homogenate of muscle and adipose tissue was boiled and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG as described previously [19-21].

Calculations. Turnover rates of glucose (*μ*mol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{14}C-glucose (dpm/*μ*mol). The ratio was corrected for body weight. EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Tissue-specific glucose uptake in muscle and adipose tissue was calculated from tissue 2-DG content, corrected for plasma specific activity and expressed as *μ*mol per gram of tissue.

Statistical analysis. Differences between groups were determined by Mann-Whitney non-parametric test for 2 independent samples. A P-value < 0.05 was considered statistically significant. All values shown represent means ± SD.

Results

Animals. Body weight did not differ between PYY_{3-36} and vehicle-infused animals (after 7 days of PYY_{3-36}/saline administration: 28.0 ± 3.7 gram in the PYY_{3-36} group and 28.3 ± 1.5 gram in the control group, P=0.68). Overnight food intake was measured at day 2 and day 5 of PYY_{3-36}/saline administration and was similar in both groups (day 2: 2.37 ± 0.68 *vs.* 2.32 ± 0.33 gram, P=0.96; day 5: 2.76 ± 0.54 *vs.* 2.75 ± 0.43 gram, P=0.97 in PYY_{3-36} and vehicle-treated animals, respectively). Furthermore, body weight and overnight food intake was not different in groups receiving continuous or intermittent PYY_{3-36} treatment (body weight: 29.5 ± 3.9 *vs.* 26.9 ± 3.4 gram, P=0.20; food intake day 2: 2.14 ± 0.98 *vs.* 2.48 ± 0.56 gram, P=0.38; day 5: 2.53 ± 0.69 *vs.* 2.92 ± 0.39 gram, P=0.27 for continuous and intermittent administration, respectively).

Plasma parameters. Plasma glucose, FFA, and insulin concentrations in basal and hyperinsulinemic conditions are shown in table 1. Plasma glucose and insulin concentrations did not differ between vehicle and PYY_{3-36} treated animals under basal and steady state clamp conditions. Furthermore, continuous and intermittent PYY_{3-36} administration had similar impact on these parameters, except for the plasma glucose levels under basal conditions, which were slightly but significantly higher in

the group that received continuous PYY_{3-36} administration (basal glucose: 9.3 ± 0.9 *vs.* 7.7 ± 1.5 mmol/l, P=0.048; hyperinsulinemic glucose: 9.9 ± 0.8 *vs.* 9.1 ± 0.6 mmol/l, P=0.073; basal insulin: 0.9 ± 0.4 *vs.* 0.5 ± 0.3 ng/ml, P=0.073; hyperinsulinemic insulin: 3.9 ± 1.0 *vs.* 3.4 ± 0.6 ng/ml, P=0.43). Plasma FFA concentrations were slightly, but significantly, lower in PYY_{3-36} treated mice in basal (P=0.025) and steady state clamp (P=0.031) conditions, where continuous and intermittent PYY_{3-36} administration did not have differential effects (basal FFA: 0.9 ± 0.3 *vs.* 0.9 ± 0.1 mmol/l, respectively, P=0.76; hyperinsulinemic FFA: 0.5 ± 0.1 *vs.* 0.4 ± 0.1 mmol/l, respectively, P=0.073). Plasma PYY_{3-36} concentrations in basal and hyperinsulinemic conditions were below the detection level in all groups (<1 pg/μl), except for the basal condition of the mice that received intermittent PYY_{3-36} administration (3.7 ± 0.8 pg/μl).

Table 1. Plasma parameters under basal or hyperinsulinemic conditions in overnight fasted mice that received PYY_{3-36} (n=12) or vehicle (n=8) for 7 days. Data are the means ± SD. * <0.05 *vs.* vehicle.

	Basal		Hyperinsulinemic	
	Vehicle	PYY_{3-36}	Vehicle	PYY_{3-36}
Glucose (mmol/l)	7.7 ± 1.3	8.4 ± 1.5	8.4 ± 1.2	9.4 ± 0.8
FFA (mmol/l)	1.1 ± 0.2	0.9 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
Insulin (ng/ml)	0.7 ± 0.3	0.7 ± 0.4	3.2 ± 0.9	3.6 ± 0.8

Glucose turnover. In basal conditions, glucose disposal was similar in PYY_{3-36} and vehicle-treated mice (52.0 ± 10.5 *vs.* 50.4 ± 10.4 μmol/min/kg, respectively, P=0.68). The rate of glucose infusion necessary to maintain euglycemia during insulin infusion was significantly higher in PYY_{3-36} treated mice than in vehicle-treated animals (54.0 ± 11.4 *vs.* 33.4 ± 11.6 μmol/min/kg, *P*=0.000), indicating that chronic PYY_{3-36} administration enhances whole body insulin sensitivity. Continuous and intermittent administration of PYY_{3-36} had similar effects on the glucose infusion rate (54.7 ± 9.2 *vs.* 53.6 ± 10.2 μmol/min/kg, respectively, P=0.27). Hyperinsulinemia increased glucose disposal in both groups. However, the disposal rate was significantly higher

in PYY_{3-36} treated animals compared with vehicle-treated controls (78.8 ± 13.3 *vs.* 63.4 ± 15.5 *μ*mol/min/kg, respectively, *P*=0.012, Figure 1a) and was similar in animals treated by continuous and intermittent administration (81.2 ± 13.8 *vs.* 77.1 ± 13.7 *μ*mol/min/kg, respectively, P=0.64).

Endogenous glucose production was similar in PYY_{3-36} and vehicle-treated mice in basal conditions and was suppressed by insulin to the same extent in both groups (by 54 ± 18 *vs.* 40 ± 26% from basal in PYY_{3-36} *vs.* vehicle treated groups, respectively; *P*=0.27, Figure 1b), where percent inhibition did not differ between animals receiving continuous or intermittent PYY_{3-36} treatment. (52 ± 25 *vs.* 55 ± 12% from basal, respectively, P=0.53).

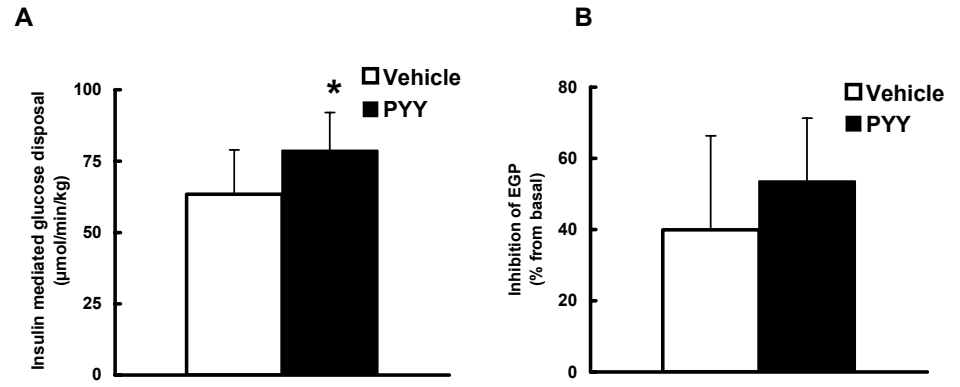

Figure 1. Insulin mediated glucose disposal (a) and inhibition of endogenous glucose production (EGP) by insulin (b) in overnight fasted mice before (basal) and during (hyperinsulinemic) a hyperinsulinemic euglycemic clamp study. Prior to the clamp experiment the animals received PYY_{3-36} (n=12) or vehicle (n=8) for 7 days. Values represent the means ± SD. *P<0.05 *vs.* vehicle.

Tissue-specific glucose uptake. Insulin-mediated 2-deoxy-glucose uptake was measured in muscle and adipose tissue. In muscle, 2-deoxy-glucose was similar in both groups (2.2 ± 1.4 *vs.* 1.6 ± 0.8 *μ*mol/ g tissue for PYY_{3-36} and vehicle-treated animals, respectively, *P*=0.38). In adipose tissue 2-deoxy-glucose uptake was significantly increased in PYY_{3-36} treated animals compared with vehicle treated mice (0.5 ± 0.2 *vs.* 0.2 ± 0.1 *μ*mol/ g tissue; *P*=0.006, Figure 2).

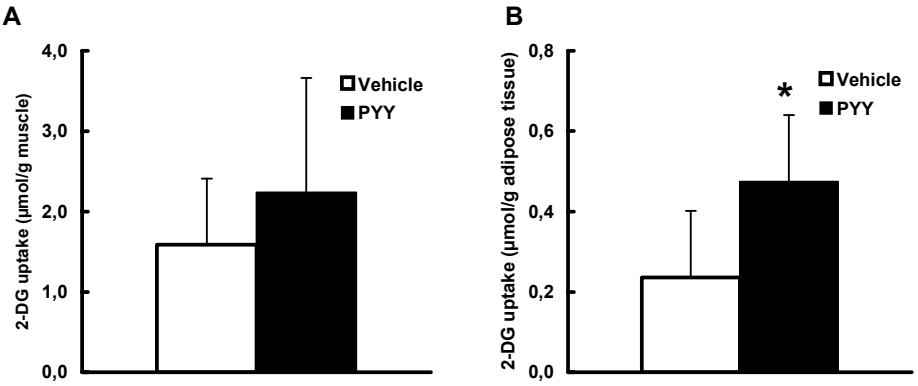

Figure 2. Muscle-specific (a) and adipose tissue-specific (b) glucose uptake under hyperinsulinemic conditions in overnight fasted mice that received PYY_{3-36} (n=11) or vehicle (n=7) for 7 days. Values represent the means ± SD. *P<0.05 *vs.* vehicle.

Discussion

Here we show that chronic PYY_{3-36} administration improves whole body insulin sensitivity of glucose metabolism in C57BL6 mice maintained on a high fat diet for 16 weeks. In particular, PYY_{3-36} treatment enhances the ability of insulin to promote glucose disposal via mechanistic routes that are independent of food intake or body weight. In addition, this study documents that continuous and intermittent administration of PYY_{3-36} reinforce insulin action to a similar extent.

These data corroborate our previous findings, which unveil similar acute effects of PYY_{3-36} administration on insulin action [16], and support the emerging concept of neural circuits controlling fuel flux, independent of their impact on food intake and body weight. In addition, our data indicate that the effects of PYY_{3-36} on glucose metabolism do not wane during chronic treatment, which suggests that this peptide may be a novel asset in the battle against insulin resistance and the metabolic syndrome.

Although PYY_{3-36} enhanced insulin-induced glucose disposal, it did not significantly affect the ability of insulin to inhibit endogenous glucose production. Nonetheless, we can not exclude the possibility that the experimental group size may have limited the statistical power necessary to detect a subtle influence of PYY_{3-36} on hepatic glucose metabolism. Alternatively, PYY_{3-36} exerts differential, tissue specific, effects on insulin action.

The mechanism by which PYY_{3-36} affects insulin-mediated glucose metabolism remains to be elucidated. Perhaps, PYY_{3-36} modulates insulin action via the hypothalamic Y2 receptor, in analogy with the mechanism guiding its effects on appetite. Y2-receptor mediated inhibition of NPY and stimulation of POMC neuronal activity by PYY_{3-36} potentially reinforces insulin action on glucose metabolism indeed [10;11;13].

Circulating PYY_{3-36} levels in fasting conditions remained below the level of detection (< 1 pg/μl) during continuous treatment, and rose to 3.7 ± 0.8 pg/μl approximately one hour after i.p injection. During hyperinsulinemia (3-3.5 hours after injection), PYY_{3-36} levels were undetectable by our assay in all animals. Thus, in spite of the fact that continuous PYY_{3-36} treatment did not produce measurable plasma concentrations and intermittent administration induced a merely transitory increase of circulating PYY_{3-36}, both treatments significantly facilitated insulin mediated glucose disposal in high fat fed animals. Relatively few papers report plasma PYY_{3-36} concentrations in rodents. Postprandial levels may be in the range of 112 pmol/L (~ 0.4 pg/μl) and 0.18 pg/μl in freely feeding normal weight rats and mice respectively [14;22], whereas fasting levels are considerably lower, as PYY_{3-36} is primarily released in response to food intake [14;23]. Plasma PYY_{3-36} concentrations in high fat fed mice are unknown, but may be significantly reduced, as obese humans have clearly diminished circulating PYY_{3-36} levels [24]. Taken together, our data suggest, that even a relatively low dose of PYY_{3-36} (in view of the low circulating PYY_{3-36} levels during treatment) can reinforce insulin action. Further dose-response experiments are warranted to evaluate the potential efficacy of PYY_{3-36} in the treatment of the metabolic syndrome.

Food intake and body weight were not affected by PYY_{3-36} administration in our study. These findings agree with data from Challis *et al.*, indicating that 7 days of PYY_{3-36} administration did not affect food intake and body weight in $POMC^{-/-}$ and wild type mice [25]. In contrast, Batterham *et al.* reported that PYY_{3-36} acutely inhibits food intake [14], an observation that could not be reproduced by Tschöp and coworkers [26;27]. To take this issue further, we compared the acute effects of a single intraperitoneal (2.5 μg) injection of PYY_{3-36} (n = 8) or vehicle (n = 8) at 09.00 am on food intake in our animals, and found that cumulative food intake in 4 hours after injection was significantly inhibited by 21% in overnight fasted mice (P=0.028), whereas

subsequent feeding over 24 hours was not affected by PYY_{3-36}. These data suggest that this dose of PYY_{3-36} has a short-term inhibitory impact on food intake in overnight fasted C57BL6 mice, whereas consumption over 24 hours is not affected, probably as a result of a rebound compensatory increase of appetite [14;15].

In conclusion, the present study shows that chronic PYY_{3-36} administration reinforces insulin action on glucose disposal in mice maintained on a high fat diet, whereas it also tends to enhance the ability of insulin to suppress endogenous glucose production. These observations suggest that PYY_{3-36} or potential analogues may be a useful treatment for insulin resistance and the metabolic syndrome.

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Chapter 6

Leptin deficiency *per se* dictates body composition, insulin action and insulin clearance in ob/ob mice.

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Abstract

Many obese humans appear to be both insulin- and leptin resistant. Since leptin may impact glucose metabolism directly, it is conceivable that lack of leptin signal transduction critically contributes to insulin resistance in these individuals. Furthermore, at this time, it remains unclear if leptin affects glucose metabolism via peripheral and/or central mechanistic routes. To explore the contribution of *cerebral* leptin deficiency to insulin resistance in leptin deficient *ob/ob* mice, we infused leptin i.c.v. in *ob/ob* mice. To also evaluate the impact of adiposity on insulin action in these animals, another group of young *ob/ob* animals were subjected to severe calorie restriction, so that their body weight became similar to that of wild type mice. Hyperinsulinemic euglycemic clamps and isotope dilution techniques were used to determine insulin sensitivity of hepatic glucose production (HGP) and disposal (GD). Leptin infusion (i.c.v., 2.5 μg/h for 3 hours) acutely increased both the hepatic insulin sensitivity index and glucose disposal index (9.1±2.4. *vs.* 5.0±2.7 %·nmol^{-1} and 25.6±5.6. *vs.* 13.6±4.8 μmol·min^{-1}·kg^{-1}·nmol^{-1}, respectively; $P<0.05$ for both comparisons) in *ob/ob* mice. Furthermore, i.c.v. leptin administration acutely reduced circulating insulin levels during continuous insulin infusion.

Food restriction barely affected body composition although it profoundly curtailed body weight. Insulin suppressed HGP clearly more in the lean *ob/ob* mice than in their obese counterparts, but its impact remained less than in wild-type mice (% suppression: 11.8±8.9 *vs.* 1.3±1.1 *vs.* 56.6±13.0 %·nmol^{-1}, respectively; $P<0.05$). The glucose disposal index of lean *ob/ob* mice was also in between that of obese *ob/ob* and lean wild-types (37.5±21.4 *vs.* 25.1±14.6 *vs.* 59.6±17.3 μmol·min^{-1}·kg^{-1}·nmol^{-1}, respectively; $P<0.05$ wild-type mice *vs. ob/ob* mice). In conclusion, leptin deficiency *per se* is not just responsible for hyperphagia and obesity in *ob/ob* mice, but critically determines body composition, insulin action and insulin clearance from the circulation in these animals. The data suggest that leptin resistance in obese humans may contribute to insulin resistance and hyperinsulinemia in these individuals.

Introduction

Leptin conveys signals pertaining to the size of bodily energy stores to the brain, where it orchestrates behavioral and metabolic adaptations meant to maintain energy

balance in the face of environmental fluctuations in nutrient availability [1-3]. Leptin may also affect body composition, as suggested by the fact that it specifically reduces fat mass (leaving lean body mass untouched) in rodents [4].

Leptin deficiency produces severe obesity, insulin resistance and impaired glucose tolerance in *ob/ob* mice [5;6]. The ratio of adipose over lean tissue is significantly elevated in these animals [7]. Since *in vivo* measures of insulin sensitivity correlate strongly with total and regional fat mass in animals and humans [8;9], it is tempting to attribute at least part of the metabolic anomalies of *ob/ob* mice to their altered body composition. However, there is also evidence to suggest that leptin impacts glucose metabolism independently of its effects on food intake and body weight. Accordingly, intraperitoneal administration of leptin acutely reduces glycemia and insulinemia and restores glucose tolerance without affecting body weight in *ob/ob* mice. Injection of a low dose of leptin into the ventromedial hypothalamus of lean rats promotes basal (insulin independent) glucose uptake in various tissues, suggesting that the central nervous system is a critical target of leptin in the control of glucose metabolism [10;11]. Leptin deficient *ob/ob* mice are an ideal model to study the (metabolic) impact of leptin, as exogenously administered leptin inevitably competes with endogenous peptide in wild-type animals. We therefore used *ob/ob* mice to evaluate the effects of i.c.v. leptin infusion on insulin sensitivity. To determine the simultaneous impact of the obese phenotype of these animals on insulin action, we also subjected young *ob/ob* animals to severe calorie restriction, so that their body weight became similar to that of wild type mice. In both experimental settings, hyperinsulinemic euglycemic clamps and isotope dilution techniques were used as measures of in vivo insulin action on glucose metabolism.

Research designs and methods

Animals. Wild-type and *ob/ob* mice were obtained from our breeding colony of C57BL6 ob+/- mice. Mice were individually housed in a temperature-controlled room on a 12:12h light-dark cycle and were fed a standard mouse chow diet with free access to water. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

I.c.v. leptin experiment. Male *ob/ob* mice were anesthetized with 0.5 mg/kg Medetomidine (Pfizer, Capelle a/d IJssel, The Netherlands), 5 mg/kg Midazolam (Roche, Mijdrecht, The Netherlands) and 0.05 mg/kg Fentanyl (Janssen-Cilag, Tilburg, The Netherlands). A 25-gauge guide cannula was stereotaxically implanted into the left lateral ventricle using the following coordinates from Bregma: 0.46 mm posterior, 1.0 mm lateral and 2.2 mm ventral [12]. The guide cannula was secured with dental cement (Oral Hygiene Center BV, Amersfoort, The Netherlands) to the skull surface. After a recovery period of 1 week, adequate placement of the cannulae was tested with the feeding response to an i.c.v. injection of NPY (5 μg dissolved in 1 μl of sterile water; Bachem, Bubendorf, Germany). After overnight fasting, a hyperinsulinemic euglycemic clamp experiment was performed under 6.25 mg/kg Acepromazine (Sanofi sante animale, Libourne Cedex, France), 6.25 mg/kg Midazolam (Roche, Mijdrecht, The Netherlands) and 312.5 μg/kg Fentanyl (Janssen-Cilag, Tilburg, The Netherlands) anesthesia. During the entire experiment (basal and hyperinsulinemic period) leptin (1 μg/μl; Bachem, Bubendorf, Germany) or vehicle was infused into the left lateral ventricle at a rate of 2.5 μl/h.

Food restriction experiment. Male and female mice were divided into the following experimental groups: 1) ad libitum-fed *ob/ob* mice, 2) *ob/ob* mice food restricted to 55% of the ad libitum wild-type food intake, 3) ad libitum-fed wild-type mice. Three nights preceding the experiments, all groups were food restricted for two nights to 55 % of the wild-type food intake (this was done to avoid potential bias of the results attributable to differences in daily caloric intake) followed by overnight fast. The next morning, a hyperinsulinemic euglycemic clamp was performed under 0.5 ml/kg Hypnorm (Janssen pharmaceutica, Beerse, Belgium) and 12.5 mg/kg Midazolam (Roche, Mijdrecht, The Netherlands) anesthesia.

Hyperinsulinemic euglycemic clamp. Hyperinsulinemic clamps were performed at 9.00 a.m. as described earlier [13;14]. Basal rates of glucose turnover were determined by administering a primed (P) continuous (C) infusion (P: 0.8 μCi, C: 0.02 μCi/min) of 3-^{3}H-glucose (Amersham, Little Chalfont, U.K.). After 60 min, insulin was given as a prime (10 mU) followed by continuous infusion of 0.25 mU/min. A variable infusion of 12.5% D-glucose was used to maintain euglycemia (measured at 10 min intervals via tail bleeding, Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). Blood samples (60 μl) were taken at the end of the basal period and during the clamp (40 and 20 min before and by the end of the clamp) for

determination of 3-^{3}H-glucose specific activity and plasma insulin, glucose and free fatty acid (FFA) concentrations. At the end of the clamp, mice were sacrificed and tissue samples were taken within 5 min, frozen immediately in liquid nitrogen and stored at –20°C for subsequent analysis. Carcasses were stored at –20 °C for body composition analysis.

Analytical procedures. Plasma levels of glucose and free fatty acids (FFAs) were determined using commercially available enzymatic kits (Sigma, St. Louis, MO;Sigma, St. Louis, MO and Wako Chemicals, Neuss, Germany, respectively). Plasma insulin concentration was measured by radioimmunoassay (Linco Research Inc., St. Charles, MO). Total plasma 3-^{3}H-glucose was determined in 7.5 μl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water. Content of triglycerides (TG) in liver and muscle tissue was determined as described before [15]. Briefly, 10-20 μg of tissue was homogenized in phosphate buffered saline (PBS) and samples were taken for measurement of protein content [16]. Lipids were extracted and TG fraction was separated from the other lipid components by high performance thin layer chromatography (HPTLC) on silica gel plates.

Body composition analysis. Carcasses were dehydrated at 65 °C until constant mass was achieved and hydrolyzed in 100 ml ethanolic potassium hydroxide (3M in 65% ethanol). Aliquots were taken, diluted with PBS and used for determination of body triglyceride content (as detailed in [17;18] by enzymatic measurement of glycerol (Sigma, St. Louis, MO).

Calculations. Turnover rates of glucose (μmol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of 3-^{3}H-glucose (dpm/μmol). The ratio was corrected for lean body mass. Hepatic glucose production (HGP) was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

The hepatic insulin sensitivity index was calculated as the ratio of the relative suppression of HGP during the hyperinsulinemic condition to plasma insulin levels during hyperinsulinemic conditions. The glucose disposal index was calculated as the ratio of glucose disposal to plasma insulin levels during hyperinsulinemic conditions.

Statistical analysis. Differences between groups were determined by Kruskall-Wallis and Mann-Whitney nonparametric tests for independent samples. A P-value < 0.05 was considered statistically significant. All values shown represent means ± SD.

Results

I.c.v. leptin experiment.

Animals. *Ob/ob* mice were 4 months old at the time of the experiments. Body weight was 44.6 ± 6.7 gram in the control group and 44.7 ± 2.2 gram in the leptin group.

Plasma parameters. Plasma glucose, FFA and insulin concentrations in basal and hyperinsulinemic conditions are shown in Table 1. In the basal state, plasma parameters did not differ between leptin- and vehicle-infused animals. In steady state clamp conditions, plasma glucose and FFA concentrations were similar in both groups. However, circulating insulin levels were significantly lower in leptin-infused compared to vehicle-infused animals, despite equal insulin infusion rates.

Glucose turnover. Hyperinsulinemia suppressed the hepatic glucose production to a different extent in both groups. To correct for disparate insulin levels during the clamp, we calculated the hepatic insulin sensitivity index. This index was significantly higher in leptin-infused animals compared to vehicle-infused animals (9.1 ± 2.4. *vs.* 5.0 ± 2.7 %·nmol^{-1}, respectively; P<0.05, Fig. 1).

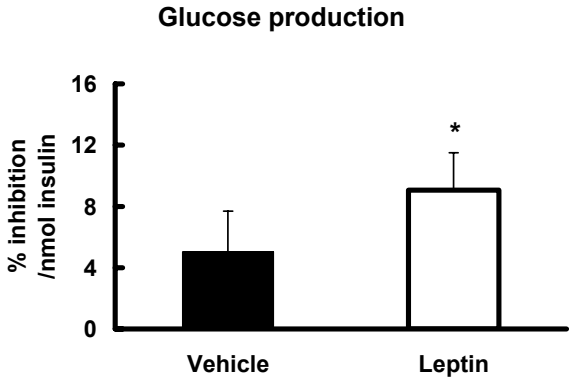

Figure 1. Percentual inhibition of the endogenous glucose production (EGP) per nmol insulin as determined by hyperinsulinemic clamp in *ob/ob* mice that received an icv infusion of vehicle or leptin. Values represent the mean ± SD of at least 5 mice per group. * $P < 0.05$ *vs.* vehicle.

Hyperinsulinemia increased glucose disposal in both groups. However, the glucose disposal index was significantly higher in leptin-infused- compared to vehicle-infused animals (25.6 ± 5.6. *vs.* 13.6 ± 4.8 $\mu mol \cdot min^{-1} \cdot kg^{-1} \cdot nmol^{-1}$, respectively; $P<0.05$, Fig. 2).

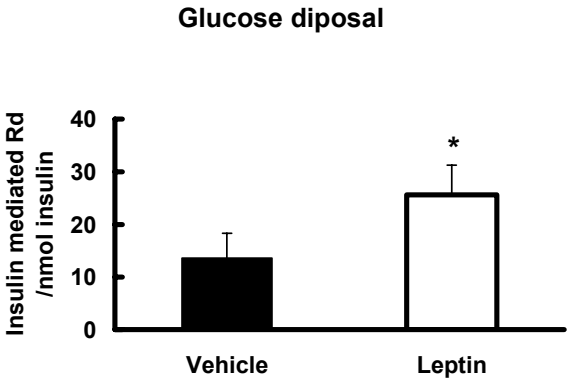

Figure 2. Insulin mediated glucose disposal (Rd) per nmol insulin as determined by hyperinsulinemic euglycemic clamp in obese *ob/ob* mice that received an icv infusion of vehicle or leptin. Values represent the mean ± SD of at least 5 mice per group. * $P < 0.05$ *vs.* vehicle.

Food restriction experiment.

Body weight. Food restriction of *ob/ob* mice was initiated at the age of 2 months to keep them lean. However, even at 2 months, *ob/ob* mice were heavier than wild-type mice (Fig. 3). After 4 months of severe food restriction (55% of wild-type intake), body weight of *ob/ob* mice was similar to that of wild-type mice, while body weight of ad libitum-fed *ob/ob* mice was clearly higher.

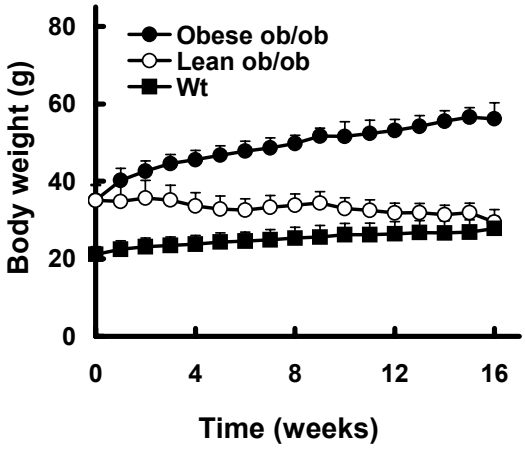

Figure 3. Growth curves of ad libitum-fed *ob/ob* mice (obese *ob/ob*), food restricted *ob/ob* mice (lean *ob/ob*) and ad libitum-fed wild-type mice (wt). At 2 months of age *ob/ob* mice were food restricted to 55% of ad libitum wild-type food intake to keep them lean. Values represent the mean ± SD of at least 5 mice per group.

Body composition. Body weight, lean body mass and the amount of water and lipid of lean *ob/ob*, obese *ob/ob* and wild-type mice are shown in Table 2. Although body weight of lean *ob/ob* mice was similar to that of wild-type animals, their body composition (lipid and lean mass expressed as percentage of body weight) was more like that of ad libitum fed *ob/ob* counterparts. Lean body mass of lean *ob/ob* mice was significantly lower than that of obese *ob/ob* and wild-type animals. Absolute amounts of TG in lean *ob/ob* mice were significantly higher than in wild-type -, but significantly lower than in obese *ob/ob* mice.

Tissue TG. Hepatic TG content of lean *ob/ob* mice was very low as compared to obese *ob/ob* mice and similar to wild-type mice (35.3 ± 16.0, 141.7 ± 51.4, 47.1 ± 35.3 μmol/g protein, respectively; $P<0.05$ lean *ob/ob vs.* obese *ob/ob*, Fig. 4A). TG content of muscle tended to be lower in lean *ob/ob* mice compared to obese *ob/ob* mice, but was significantly higher ($P<0.05$) compared to wild-type mice (173.2 ± 90.2, 257.7 ± 86.7 and 59.0 ± 37.0 μmol/g protein for lean *ob/ob*, obese *ob/ob* and wild-type mice respectively, Fig. 4B).

Plasma parameters. Plasma glucose, FFA and insulin concentrations under basal and hyperinsulinemic euglycemic clamp conditions are shown in Table 3. In the basal state plasma parameters of lean *ob/ob* mice were not different from obese *ob/ob* mice. Both groups of *ob/ob* mice were hyperinsulinemic as compared to wild-type animals. Obese *ob/ob* mice had significantly higher plasma glucose concentrations as compared to wild-type mice, while circulating glucose levels in lean *ob/ob* mice were in between those in obese *ob/ob* and wild types.

In steady state clamp conditions, circulating insulin levels were significantly higher in *ob/ob* mice than in wild-type mice, despite equal insulin infusion rates. Glucose levels in steady state were not significantly different between lean and obese *ob/ob* mice, but glucose levels in wild-type mice were significantly lower than glucose levels in both groups of *ob/ob* mice. FFA concentrations were diminished during hyperinsulinemia (as compared to baseline) by ~25% in obese *ob/ob*, ~50% in lean *ob/ob* and ~70% in wild-type mice.

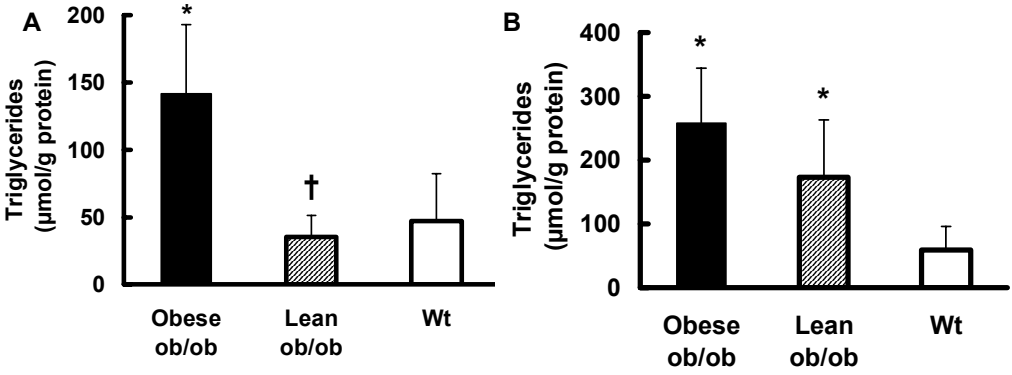

Figure 4. Triglyceride levels in liver (A) and muscle (B) determined in obese *ob/ob*, lean *ob/ob* and wild-type (wt) mice. Values represent the mean ± SD of at least 5 mice per group. * $P < 0.05$ *vs.* wt; † $P < 0.05$ *vs.* obese *ob/ob*.

Glucose turnover. Hyperinsulinemia suppressed hepatic glucose production to a different extent in the three groups. The hepatic insulin sensitivity index was significantly higher in lean *ob/ob* mice compared to obese *ob/ob* mice, but significantly lower compared to wild-type mice (11.8 ± 8.9, 1.3 ± 1.1 and 56.6 ± 13.0 %·nmol^{-1} for lean *ob/ob*, obese *ob/ob* and wild-type mice respectively; P<0.05, Fig. 5).

Insulin infusion enhanced glucose disposal in all groups. The glucose disposal index was significantly reduced in obese *ob/ob* mice as compared to wild-type, while the index of lean *ob/ob* mice was in between those of obese *ob/ob* and lean wild-types (37.5 ± 21.4, 25.1 ± 14.6 and 59.6 ± 17.3 µmol·min^{-1}·kg^{-1}·nmol^{-1} for the lean *ob/ob*, obese *ob/ob* and wild-type mice, respectively; P<0.05 wild-type mice *vs. ob/ob* mice, Fig. 6).

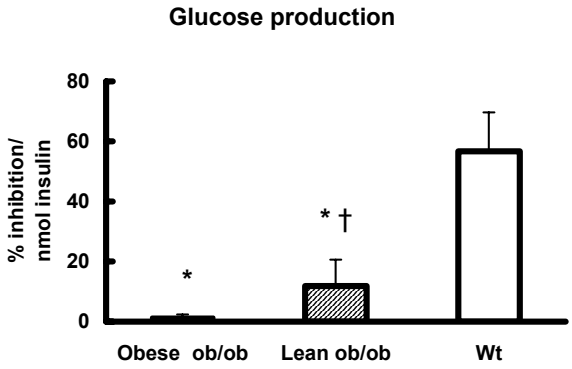

Figure 5. Percentual inhibition of the endogenous glucose production (EGP) per nmol insulin as determined by hyperinsulinemic clamp in obese *ob/ob*, lean *ob/ob* and wild-type mice. Values represent the mean ± SD of at least 5 mice per group. * $P < 0.05$ *vs.* wt.; † $P < 0.05$ *vs.* obese *ob/ob*.

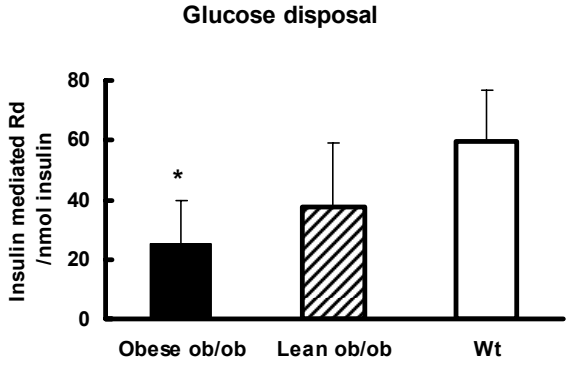

Figure 6. Insulin mediated glucose disposal (Rd) per nmol insulin as determined by hyperinsulinemic euglycemic clamp in obese *ob/ob*, lean *ob/ob* and wild-type mice. Values represent the mean ± SD of at least 5 mice per group. * $P < 0.05$ *vs.* wt.

Table 1. Plasma parameters in *ob/ob* mice that received an i.c.v. infusion of vehicle or leptin under basal and hyperinsulinemic conditions. Values represent the mean ± SD of at least 5 mice per group. * $P < 0.05$ *vs.* vehicle.

	Glucose (mmol/l)		FFA (mmol/l)		Insulin (nmol/l)	
	Basal	Hyperinsulinemic	Basal	Hyperinsulinemic	Basal	Hyperinsulinemic
Vehicle	11.5 ± 4.5	10.1 ± 2.9	1.2 ± 0.4	0.8 ± 0.4	1.0 ± 0.3	8.3 ± 2.3
Leptin	11.2 ± 3.9	9.3 ± 2.2	1.2 ± 0.3	0.7 ± 0.2	0.8 ± 0.2	5.1 ± 1.1*

Table 2. Body composition of obese *ob/ob*, lean *ob/ob* and wild-type (wt) mice. Values represent the mean ± SD of 9 obese *ob/ob* mice, 12 lean *ob/ob* mice and 12 wild-type mice. * $P < 0.05$ *vs.* wt; † $P < 0.05$ *vs.* obese *ob/ob*.

	BW	Lean bm	Water		Lipid	
	(g)	(g)	(g)	(% of BW)	(g)	(% of BW)
Obese *ob/ob*	50.3 ± 2.8*	23.8 ± 2.4	14.7 ± 1.6*	29.3 ± 3.1*	26.5 ± 3.8*	52.5 ± 5.6*
Lean *ob/ob*	29.7 ± 2.5	16.5 ± 2.9*†	10.6 ± 1.4*†	35.9 ± 5.6*†	13.2 ± 2.5*†	44.6 ± 8.0*†
Wt	26.3 ± 4.6	24.4 ± 3.5	17.7 ± 2.8	67.5 ± 3.6	1.9 ± 1.5	6.7 ± 4.4

Table 3. Plasma parameters in obese *ob/ob*, lean *ob/ob* and wild-type (wt) mice under basal and hyperinsulinemic conditions. Values represent the mean ± SD of at least 5 mice per group. * $P < 0.05$ *vs.* wt.

	Glucose (mmol/l)		FFA (mmol/l)		Insulin (nmol/l)	
	Basal	Hyperinsulinemic	Basal	Hyperinsulinemic	Basal	Hyperinsulinemic
Obese *ob/ob*	6.6 ± 1.2*	7.2 ± 1.4*	0.8 ± 0.2	0.6 ± 0.2*	1.8 ± 0.2*	7.0 ± 3.9*
Lean *ob/ob*	5.5 ± 1.8	8.8 ± 3.6*	0.8 ± 0.1	0.4 ± 0.2*	1.4 ± 0.9*	4.6 ± 2.1*
Wt	4.5 ± 0.8	5.3 ± 0.9	0.7 ± 0.3	0.2 ± 0.1	0.1 ± 0.02	1.4 ± 0.2

Discussion

Here we show that intracerebroventricular administration of minute amounts of leptin acutely ameliorates insulin resistance in *ob/ob* mice. Chronic calorie restriction, despite curtailing body weight to that of wild type controls, barely affects body composition of these animals. However, it does considerably blunt lipid accumulation in muscle and liver and also clearly reinforces insulin action on glucose production and disposal. As the mice were pair-fed for 3 days prior to the experimental procedures, we believe that the metabolic benefits we report here are primarily due to the constraint of adipose mass and tissue lipid storage, and not to calorie restriction *per se*. We infer that both leptin deficiency and the obese phenotype contribute to insulin resistance in *ob/ob* mice. Finally, circulating insulin levels during insulin infusion were consistently higher in *ob/ob* animals than in normal weight controls and i.c.v. leptin administration acutely reduced these levels, which suggests that leptin promotes (hepatic) insulin clearance via activation of cerebral leptin receptors.

The current literature pertaining to the acute effects of leptin on glucose metabolism is rather confusing. Nevertheless, our finding that i.c.v. leptin administration acutely facilitates insulin action in *ob/ob* mice corroborates and complements various previous reports. Kamohara and coworkers were the first to show that both intravenous (i.v.) and i.c.v. leptin acutely enhance glucose turnover in wild-type C57/BL6 mice [10]. Subsequently, Sivitz et al demonstrated, that i.v. leptin administration for 48 hours enhances the glucose infusion rate necessary to maintain euglycemia during insulin in normal weight Sprague Dawley rats [19]. Rossetti et al reported that i.v. leptin for 6 hours enhances insulin's ability to inhibit endogenous glucose production, whereas it does not affect peripheral insulin action in lean Sprague-Dawley rats [20]. They went on to show that i.c.v infusion of leptin redirects intrahepatic glucose flux, whereas it does not significantly impact on insulin's capacity to suppress HGP in these animals [21]. In apparent contrast, Cusin et al. showed that subchronic (4 days) i.c.v. leptin treatment reinforces insulin action on glucose disposal, but not production, in lean heterozygous Zucker *FA/fa* rats [22]. However, leptin treated animals had lost weight at the time hyperinsulinemic clamps were performed in their experiment, which probably explains the metabolic impact of the intervention as pair-fed animals, which were not treated with leptin, exhibited similar

metabolic features. We now demonstrate that i.c.v. leptin infusion acutely reinforces insulin action on glucose production and disposal in leptin deficient *ob/ob* mice. We believe that this model is ideally suitable for the study of acute metabolic effects of leptin, as exogenous leptin inevitably competes with endogenous peptide in other models. The mechanism that is responsible for the acute metabolic impact of i.c.v. leptin remains to be established. However, a single i.c.v. injection of leptin acutely activates AMPK in skeletal muscle of FVB mice [23] and downstream biochemical corollaries can stimulate glucose uptake [24].

As far as we are aware, the impact of curtailing body weight by calorie restriction on insulin sensitivity has never been studied before in *ob/ob* mice. However, a wealth of data supports the position that calorie restriction prevents the onset of insulin resistance in various animal species [25], and weight loss promotes insulin action in humans ([26] and references herein). The size of adipose depots, in particular the visceral fat mass, has an inverse correlation with insulin sensitivity, and the magnitude of beneficial change of insulin action in response to weight loss correlates with the percent decrease of visceral fat in humans [26]. Moreover, (visceral) obesity is often accompanied by accumulation of lipids in tissues that are not designed to store triglycerides (i.e. muscle and liver) and this may impair insulin action in these tissues [27]. Calorie restriction precluded lipid storage in liver and muscle to a large extent in our study. Thus, calorie restriction facilitates insulin action on glucose production and disposal in *ob/ob* mice, probably because it constrains the growth of adipose depots and largely prevents lipid storage in muscle and liver. Notably, all mice were pair-fed for 3 days prior to the experiments. Therefore, we believe that the metabolic benefits we report here are not due to calorie restriction *per se*.

Our data showing that prolonged calorie restriction does not alter body composition (despite a reduction of body weight) corroborate previous reports [28-30]. They strongly support the notion that leptin not just controls energy balance, but also directs fuel flux away from adipose tissue. In keeping with this, subchronic leptin administration specifically reduces (visceral) fat content in rats [4].

Insulin clearance from the circulation may be controlled by leptin through activation of cerebral receptors. *Ob/ob* animals had consistently higher circulating insulin concentrations than wild-type controls during insulin infusion at an equal rate. This observation corroborates earlier papers, reporting that insulin clearance is reduced in

obese individuals [31]. Insulin, when released by beta cells into the portal vein, is primarily cleared by the liver. The kidney is largely responsible for clearance of (exogenous) insulin from the systemic circulation [32]. If insulin is not cleared by liver or kidney, basically all insulin sensitive tissues can internalize and degrade insulin, where muscle has a primary role [32]. Elevated lipids and free fatty acids hamper insulin processing at the cellular and whole body level [33-35], perhaps through inhibition of insulin degrading enzyme [36]. Thus, storage of excess TG in insulin degrading tissues (e.g. liver and kidney) may diminish insulin clearance in obesity [36]. We were surprised to find that i.c.v. administration of leptin significantly reduced circulating insulin concentrations during continuous insulin infusion. This finding strongly suggests that leptin controls insulin clearance through activation of its receptors in the brain.

What are the potential (patho)physiological implications of our findings? Growing evidence implies that obesity is a state of cerebral leptin resistance in many individuals [37]. Disrupted leptin signal transduction in the brain may 1) produce obesity and favor accumulation of adipose tissue rather than lean body mass; 2) hamper insulin action to inhibit glucose production and stimulate glucose disposal; and 3) restrain insulin clearance. If so, drugs designed to impact signals downstream the leptin receptor conveying its metabolic messages (i.e. neuropeptide Y, pro-opiomelanocortin [38]) may be useful tools in the battle against obesity and type 2 diabetes.

In summary, we here show that leptin deficiency *per se* is deeply involved in the genesis of the metabolic phenotype of *ob/ob* mice. It is not just responsible for hyperphagia and obesity, but critically determines body composition, insulin action and insulin clearance from the circulation in these animals. The data suggest that putative cerebral leptin resistance in obese humans may underlie various features of the metabolic syndrome and therefore molecular messengers downstream the leptin receptor may be useful targets for the treatment of obesity and type 2 diabetes.

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Chapter 7

General discussion

Obesity has now reached epidemic proportions globally and has become a worldwide public health problem. It can lead to several chronic diseases, including cardiovascular disease and type 2 diabetes mellitus. The problem of obesity arises when food intake exceeds energy expenditure. A complex system has evolved to regulate food intake and energy homeostasis, but this is biased towards weight gain. Several peripheral signals act within the central nervous system to give information about short-term and long-term energy stores. The integration of this multitude of signals occurs in the hypothalamus, which contains a large number of neuropeptides that influence food intake.

The general hypothesis that is used throughout this thesis is, that the neuropeptides of this hypothalamic regulatory site of food intake and energy homeostasis are not only involved in regulation of food intake, but are also regulating insulin sensitivity, independently of the effects on food intake and body weight. Therefore, the aim of the studies described in this thesis was to investigate the effects of some of these neuropeptides and of some of the peripheral signals, which affect these neuropeptides, on insulin action.

All experiments described in this thesis were performed in mice. Usually wild-type (C57BL\6) mice were used, except for chapter 6, in which ob/ob mice were used as well. Sometimes a high fat diet was used to induce insulin resistance. All experiments had a similar set-up consisting of administration of a certain hypothalamic neuropeptide or compound/hormone which can affect the hypothalamic regulatory center of food intake. Subsequently, insulin sensitivity was measured by a hyperinsulinemic euglycemic clamp technique. Mice were clamped in the fasted or fed state, depending on the (an)orexigenic nature of the administered neuropeptide/hormone. To ensure low physiological levels of the used neuropeptide/hormone, mice were clamped in the fed state for orexigenic agents and the fasted state for the anorexigenic agents. This way we could artificially raise the neuropeptide/hormone of our interest in the experimental group and evaluate the results against the low levels in the control group.

Using this method, the effects of an icv infusion of the neuropeptide NPY on insulin sensitivity were studied in **chapter 2**. The results of this chapter show, that NPY can cause insulin resistance, specifically in the liver. Insulin-mediated glucose disposal was not affected by NPY, implying that organs like adipose tissue and muscle, were not affected.

The effect of the POMC pathway (the counter-regulating pathway of NPY) was studied in **chapter 3** with the administration of MTII, an agonist of the POMC pathway. The results of that chapter show, that the POMC pathway can improve insulin-mediated glucose disposal and does not affect hepatic insulin sensitivity. Therefore, both pathways are not completely opposing each other's effects, but seem to have a different tissue-specific effect.

In **chapters 4 and 5** the results on insulin sensitivity are shown for the acute (chapter 4) or chronic (chapter 5) administration of the gut hormone PYY. Both chapters show, that PYY improves insulin sensitivity with respect to insulin mediated glucose disposal and there seems to be a tendency towards an improved hepatic insulin sensitivity as well.

Finally, in **chapter 6,** the role of central leptin signalling on insulin sensitivity is examined in ob/ob mice and evaluated against the contribution of the obese phenotype itself on insulin sensitivity. The results show that both the obese phenotype and the lack of leptin signals in the brain, *per se*, contribute to the insulin resistance of ob/ob mice.

As mentioned before, the general hypothesis, that is used as the basis for the experiments described in this thesis, is that hypothalamic neuropeptides regulate insulin sensitivity, in addition to and independently of their role in regulating food intake. However, it is important to remember that other brain regions also play an important role in the regulation of food intake. Especially the brainstem, with the nucleus of the solitary tract in particular, but also parts of the limbic system, the amygdala and the cerebral cortex play a role [1]. The results described in this thesis are in keeping with the hypothalamic model that we used, but it is important to keep in mind that they don't prove the involvement of the hypothalamus. NPY, by example, is one of the most abundant neurotransmitters of the brain and its receptors are widely distributed throughout the brain [2]. MTII, which is used in chapter 3, is an agonist of the MC 3 and 4 receptors. Although the MC3 receptor is mainly expressed in the hypothalamus, the MC4 receptor is expressed in virtually all major brain regions, including the brainstem [3]. Similarly, the leptin receptor is also expressed in the brainstem [4]. The icv infusion/injection that we used in our experiments could therefore affect parts of the brainstem as well, since the brainstem also has easy access to the csf via the fourth ventricle. Therefore, to evaluate the role of the

hypothalamus, additional experiments should be done with intra-hypothalamic injections.

The downstream mechanism via which the neuropeptides/hormones described in this thesis affect (insulin mediated) glucose turnover remains to be elucidated. First of all, the effects could be mediated via an endocrine mechanistic pathway. There are several hormones, like glucagon, growth hormone, corticosterone and epinephrine, which can affect glucose turnover [5-8]. NPY and MTII, by example, have been shown to stimulate adrenocortical secretion via increased release of corticotrophin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) [9-11]. NPY is also able to increase glucagon concentration [12]. Although, we did not detect a modification of these hormones in our experimental settings (corticosterone and glucagon were measured in chapter 2 and corticosterone in chapter 3), the involvement of some of these hormones in the studies described in this thesis cannot be ruled out and could be a possible mechanism. Secondly, the effects could be mediated via a neural mechanistic pathway. Direct multisynaptic connections have been shown between the hypothalamus and peripheral organs that take up glucose, like liver, adipose tissue and muscle [13-17]. Many studies show that parasympathetic input to these peripheral tissues is important for glucose uptake [15;17-20]. Sympathetic neural activity in general stimulates hepatic glucose production [21-23]. However, in addition to the sympathetic stimulation of hepatic glucose production, vagal input to the liver also modulates hepatic glucose production [24]. Therefore, the effects on glucose turnover described in this thesis can also be mediated via a neural mechanistic pathway or perhaps a combination of neural and endocrine mechanistic pathways control glucose turnover.

When we compare the effects of the different neuropeptides/hormones described in this thesis, we observe that the NPY pathway causes hepatic insulin resistance, whereas the POMC pathway (MTII) improves peripheral insulin sensitivity. Furthermore, MTII was shown to stimulate basal glucose production. PYY_{3-36} and leptin are both known to inhibit the NPY- and stimulate the POMC pathway and would therefore be expected to improve insulin sensitivity. Indeed, PYY_{3-36} improved peripheral insulin sensitivity and had a small tendency to improve hepatic insulin sensitivity as well and leptin improved both hepatic and peripheral insulin sensitivity. However, MTII also affected basal glucose production, an effect that was not seen with PYY_{3-36} or leptin. This discrepancy can have several reasons.

First of all, we cannot rule out that some of the effects described in this thesis are not mediated via the hypothalamus but perhaps involve different brain regions, like the brain stem. The icv injections of MTII possibly affect the MC receptors in a more wide-spread brain area than the iv infusion of PYY_{3-36} does. Secondly, it could be a simple dose effect. In the studies described in this thesis a single dose was used (based on available literature that showed effects on food intake) and we therefore cannot rule out the possibility that we might see additional effects (including an effect on basal glucose production) if a higher dose was used. Finally, other neuropeptides, like AgRP and CART, could be involved as well. The effects of these neuropeptides on insulin sensitivity are barely investigated and they might be differentially affected by PYY_{3-36} and leptin. PYY_{3-36} and leptin might have an additional effect on these neuropeptides as well, as they are coexpressed with NPY and POMC neurons respectively. Currently, it is unknown how these neuropeptides affect insulin sensitivity. Therefore, the possibility exists that PYY_{3-36} and leptin additionally affect these neuropeptides, which can subsequently counteract the increase in basal glucose production that was caused by MTII.

The experiments described in this thesis show the dual involvement of neuropeptides/hormones in the regulation of both food intake and insulin sensitivity. The physiological significance of this dual regulation could consist of an extra supplemental mechanism in the body's attempt to maintain glucose homeostasis and a fine-tuning of this mechanism through the possibility of regulating insulin sensitivity in a tissue specific way. Hypothalamic NPY levels, by example, will be increased during fasting, which will lead to hepatic insulin resistance. This could be an additional mechanism to the low insulin levels that are present during the fasted state, ensuring that the hepatic glucose production will be maintained at a high level. The opposite pathway, the POMC pathway, will be increased in the fed state and will lead to an increased insulin sensitivity of peripheral tissues like muscle and adipose tissue. This will facilitate the glucose uptake by these tissues leading to an effective decrease in blood glucose levels. Leptin and PYY are both hormones that can influence both pathways. Leptin reflects the size of fat depots and therefore acts as a long-term regulator, while PYY is increased immediately after a meal and therefore acts as a short-term regulator that will decrease food intake and simultaneously increase insulin sensitivity to decrease blood glucose levels acutely.

The central regulatory center for food intake and energy metabolism is extremely important in maintaining energy supply during times of plenty, but especially during times of famine. The experiments described in this thesis show that the neuropeptides and hormones that are involved in this regulation system, are not only involved in the regulation of food intake, but also in the regulation of insulin sensitivity. One could therefore hypothesize that disturbances in the regulation system itself, will lead to disturbances in both food intake and insulin sensitivity. Disturbances like leptin resistance and/or decreased PYY levels that were seen in obese subjects can therefore lead to a disturbed balance between the NPY and POMC pathway and can contribute to the increased food intake and insulin resistance. However, at present the role of these neuropeptides/hormones in the pathogeneses of obesity and type 2 diabetes in humans is still unknown.

Implications for human pathophysiology

Leptin was long thought of as the new therapeutic cure for obesity after successful experiments in several obese rodent models. However except for a few rare cases, obese humans turned out to be leptin resistant instead of leptin deficient. However, leptin resistance can eventually lead to the same metabolic consequences as leptin deficiency. We show in chapter 6 that leptin deficiency in *ob/ob* mice, particularly leptin deficiency in the brain, can lead to insulin resistance. For obese humans these findings imply that leptin resistance, particularly leptin resistance of the brain, can ultimately contribute to insulin resistance as well.

For PYY_{3-36}, human studies have shown that obese subject respond to PYY_{3-36} by reduced food intake and are therefore not PYY_{3-36} resistant [25]. Furthermore, it was shown that obese subjects have decreased PYY_{3-36} levels compared to lean subjects. For NPY and the POMC pathway, there are only a few studies that measured NPY or α-MSH levels in plasma or csf in obese and lean subjects.

For NPY, some studies find higher NPY levels in obese subjects [26;27] and some studies find no differences [28;29]. There is one study in which the distinction between obese non-diabetic and obese diabetic subjects was made and plasma NPY levels were found to be significantly higher in the diabetic subjects [30].

For α-MSH, there are a few studies that find higher plasma levels in obese subjects and a negative correlation with insulin resistance [31;32]. However, very little is known regarding the function of circulating α-MSH, as currently a great deal of

attention has focused on the central role of α-MSH and its antagonism at the MC4 receptor by AgRP. There is one study that measured α-MSH in csf in obese and lean subjects, but no difference was found [28]. However, the csf-concentrations do not reflect hypothalamic concentrations, as NPY and α-MSH are not only confined to the hypothalamic region. There is one study that examined the hypothalamic NPY protein in normal and obese subjects and they did not find any differences [33]. However that study was based on four obese subjects only and did not make any distinction between insulin sensitive or insulin resistant subjects. Therefore, the possibility exists that obese and insulin resistant subjects, like mice susceptible to diet induced obesity, have increased hypothalamic NPY levels and decreased POMC levels, which could be of consequence in the pathogenesis of obesity and type 2 diabetes mellitus.

Additional studies should be done that unravel the mechanism(s) by which the brain is capable of regulating insulin sensitivity. Both the endocrine or neural (sympathetic and parasympathetic) mechanistic pathways should be investigated. In addition, there are other neuropeptides and hormones that will play a role in the regulation of food intake and insulin sensitivity and these should be explored as well. It should be investigated in more detail whether disturbances in the balance of the NPY and POMC pathway or disturbances in other neuropeptides/hormones of this regulation system play a role in the pathogenesis of obesity or insulin resistance in humans. Furthermore, the possibility of (ant)agonists of these neuropeptides/hormones as a tool in the battle against obesity, type 2 diabetes and the metabolic syndrome should be investigated. There is still a lot of research that has to be done and several questions that still arise. The research described in this thesis is therefore a starting-point showing that neuropeptides/hormones that are involved in the regulation of food intake also, and independently of their effect on food intake, affect insulin sensitivity.

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Summary / Samenvatting

Summary

Nowadays, in our Western society food is in abundance and energy-rich with high levels of sugar and saturated fats. At the same time, large shifts towards less physically demanding work have been observed. These environmental changes are reflected in the large number of overweight/obese people. Obesity has now reached epidemic proportions globally and has become a worldwide public health problem. It can lead to several chronic diseases, including cardiovascular disease and insulin resistance/type 2 diabetes mellitus. The problem of obesity arises when food intake exceeds energy expenditure.

Food intake or energy intake is regulated by a highly complex system, that integrates several signals concerning the metabolic status and energy expenditure. This regulation mechanism consists of several central regulation centers, which are situated in several different brain regions, particularly in the hypothalamus, which is considered as the main feeding center of the brain. In the hypothalamus there are two opposing pathways that regulate food intake: one pathway stimulates food intake and consists of neurons, that produce the neuropeptides NPY (neuropeptide Y) and/or AgRP (agouti-related peptide), the other pathway inhibits food intake and consists of neurons that produce the neuropeptides POMC (pro-opiomelanocortin) and/or CART (cocaine- and amphetamine-regulated transcript).

In addition to these central regulation centers of food intake, there are numerous peripheral signals, which are also involved in the regulation of food intake. These factors are derived from different organs like stomach, gut, pancreas and adipose tissue, by example. These hormones act on the central regulation centers, and, thereby, contribute to the regulation of food intake by providing the brain information about hunger, satiety and body fat stores.

The hypothesis, that is used throughout this thesis, is that the neuropeptides/hormones that are involved in the regulation of food intake, also regulate insulin sensitivity (besides and independent of their role in regulating food intake). In obesity, dysregulation of several hypothalamic neuropeptides and peripheral hormones that regulate food intake, has been observed and will lead to an increased food intake. Perhaps the same dysregulation of these neuropeptides and hormones can cause insulin resistance as well.

In **chapter 2** the effects of NPY, the most important neuropeptide of the pathway that stimulates food intake, on insulin sensitivity were studied, independent of the effects on food intake or body weight. NPY was continuously infused in the lateral ventricle of the brain (icv) of mice and insulin sensitivity was simultaneously determined by means of a hyperinsulinemic euglycemic clamp technique. The results of this chapter show increased hepatic glucose and VLDL production under hyperinsulinemic conditions, and thereby provide evidence, that icv NPY administration precludes the inhibition of hepatic glucose and VLDL production by circulating insulin. This finding may imply, that the increased hypothalamic NPY levels, that are typically observed in various obese animal models may underlie hepatic insulin resistance and associated metabolic anomalies in these models.

The aim of **chapter 3** was to evaluate the effects of central administration of melanotan II (MTII), on hepatic and whole-body insulin sensitivity, independent of food intake and body weight. MTII is an agonist of the melanocortin receptors 3 and 4, which are both receptors of the POMC pathway, the pathway that inhibits food intake. The results of this chapter show an increase in the basal glucose production and uptake after central administration of MTII. Furthermore, hyperinsulinemic glucose uptake was increased in the mice that received MTII, whereas the inhibitory effect of insulin on hepatic glucose production was not affected by MTII. We, therefore, conclude, that icv administration of MTII acutely increases insulin-mediated glucose disposal, but does not affect insulins capacity to suppress the hepatic glucose production in mice. These data indicate, that central stimulation of melanocortin-3/4 receptors modulates insulin sensitivity in a tissue-specific manner, independent of its well-known impact on feeding and body weight.

In **chapter 4**, the acute effects of PYY_{3-36} on insulin sensitivity were investigated. PYY_{3-36} is a hormone, that is released by the gut in response to nutrient ingestion. It modulates the activities of orexigenic NPY neurons and anorexigenic POMC neurons in the hypothalamus to inhibit food intake. As both NPY and POMC have been shown to also impact insulin action, we wondered whether PYY_{3-36} could improve insulin sensitivity. PYY_{3-36} was therefore intravenously infused in mice during a hyperinsulinemic euglycemic clamp. The results of this chapter show, that in hyperinsulinemic conditions, glucose disposal was significantly increased in PYY_{3-36}-infused- compared with vehicle-infused mice. We therefore conclude, that PYY_{3-36}

improves insulin sensitivity, in particular the ability of insulin to enhance glucose disposal.

Because the long-term effects of PYY_{3-36} on insulin sensitivity are still unknown, the effects of chronic PYY_{3-36} administration on insulin sensitivity were examined in **chapter 5**. In addition, metabolic efficacy of continuous *vs.* intermittent administration of PYY_{3-36} was evaluated. Mice therefore received PYY_{3-36} subcutaneously for 7 days by means of an osmotic minipump or daily subcutaneous injections, after which insulin sensitivity was determined with the hyperinsulinemic euglycemic clamp technique. In hyperinsulinemic conditions, glucose disposal was significantly increased in PYY_{3-36} treated mice *vs.* vehicle-treated mice. In contrast, insulin action on hepatic glucose production was not significantly affected, although there was a tendency towards enhanced ability of insulin to suppress hepatic glucose production. Furthermore, none of these metabolic parameters was affected by the mode of PYY_{3-36} administration (continuous or intermittent). These observations suggest, that PYY_{3-36} or potential analogues may be a useful treatment for insulin resistance.

Finally, in **chapter 6**, the role of the adipose tissue-derived hormone leptin and the role of its central signalling on insulin sensitivity is examined in *ob/ob* mice and evaluated against the contribution of the obese phenotype itself on insulin sensitivity. To explore the contribution of *cerebral* leptin deficiency to insulin resistance in leptin deficient *ob/ob* mice, we infused leptin icv in *ob/ob* mice. To also evaluate the impact of adiposity on insulin action in these animals, another group of young *ob/ob* animals were subjected to severe calorie restriction, so that their body weight became similar to that of wild type mice. The hyperinsulinemic euglycemic clamp technique was subsequently used to determine insulin sensitivity. Icv leptin infusion acutely increased both the hepatic insulin sensitivity and insulin-mediated glucose disposal in *ob/ob* mice. Furthermore, icv leptin administration acutely reduced circulating insulin levels during continuous insulin infusion (hyperinsulinemic period). Food restriction barely affected body composition although it profoundly curtailed body weight. Insulin suppressed the hepatic glucose production clearly more in the lean *ob/ob* mice than in their obese counterparts, but its impact remained less than in wild-type mice. Insulin-mediated glucose disposal of lean *ob/ob* mice was also in between that of obese *ob/ob* and lean wild-types. In conclusion, leptin deficiency *per se* is not just responsible for hyperphagia and obesity in *ob/ob* mice, but critically determines body

composition, insulin action and insulin clearance from the circulation in these animals. The data suggest that leptin resistance in obese humans may contribute to insulin resistance and hyperinsulinemia in these individuals.

In conclusion, the studies presented in this thesis show the effects of several neuropeptides/hormones on insulin sensitivity. As there is still a lot of research that has to be done, the research described in this thesis can be considered as a starting-point showing that neuropeptides/hormones that are involved in the regulation of food intake also, and independently of their effect on food intake, affect insulin sensitivity.

Samenvatting

Tegenwoordig is in onze Westerse samenleving voedsel in overvloed aanwezig en bovendien energierijk, met hoge suikergehaltes en veel verzadigde vetten. Tegelijkertijd worden er enorme verschuivingen waargenomen naar fysiek minder veeleisend werk. Deze veranderingen in onze omgeving worden gereflecteerd in het percentage mensen dat kampt met overgewicht/obesitas. Obesitas heeft inmiddels globaal gezien epidemische proporties aangenomen en is een wereldwijd gezondheidsprobleem geworden. Het kan tot verschillende chronische ziekten leiden, waaronder cardiovasculaire aandoeningen en insuline resistentie/type 2 diabetes mellitus. Het probleem van obesitas ontstaat op het moment dat de voedselinname het energieverbruik overschrijdt.

Voedselinname of energie-inname wordt gereguleerd door een complex systeem dat de verschillende signalen omtrent de metabole status en het energieverbruik integreert. Dit regulatiesysteem bestaat uit verschillende centrale regulatiecentra die gesitueerd zijn in verschillende hersengebieden, met name in de hypothalamus, het gebied dat wordt beschouwd als het belangrijkste voedingscentrum van de hersenen. In de hypothalamus zijn er twee tegenovergestelde paden die voedselinname reguleren: één pad stimuleert voedselinname en bestaat uit neuronen die de neuropeptiden NPY (neuropeptide Y) en/of AgRP (agouti-gerelateerd peptide) produceren, het andere pad inhibeert voedselinname en bestaat uit neuronen die de neuropeptiden POMC (pro-opiomelanocortine) en/of CART (cocaïne- en amfetamine gereguleerd transcript).

Naast deze centrale regulatiecentra van voedselinname, zijn er ook veel perifere signalen die ook betrokken zijn bij de regulatie van voedselinname. Deze factoren komen vanuit verschillende organen zoals maag, darm, pancreas en vetweefsel, bijvoorbeeld. Deze hormonen werken op de centrale regulatiecentra en dragen daarmee bij aan de regulatie van voedselinname door de hersenen te voorzien van informatie betreffende honger, verzadiging en vet-voorraden.

De hypothese, waarop de studies uit dit proefschrift gebaseerd zijn, is dat de neuropeptiden/hormonen die betrokken zijn bij de regulatie van voedselinname, ook de insulinegevoeligheid reguleren (naast en onafhankelijk van hun rol in de regulatie van voedselinname). Bij obesitas is er een disregulatie waargenomen van verschillende hypothalame neuropeptiden en perifere hormonen die voedselinname

reguleren. Wellicht kan dezelfde disregulatie van deze neuropeptiden en hormonen ook insulineresistentie veroorzaken.

In **hoofdstuk 2** werden de effecten van NPY, het belangrijkste neuropeptide van het pad dat voedselinname stimuleert, op insulinegevoeligheid onderzocht, onafhankelijk van de effecten op voedselinname of lichaamsgewicht. NPY werd continu geïnfuseerd in het laterale ventrikel van de hersenen (icv) van muizen en de insulinegevoeligheid werd tegelijkertijd bepaald met behulp van de hyperinsulinemische euglycemische clamp techniek. De resultaten van dat hoofdstuk laten onder hyperinsulinemische condities een verhoogde hepatische glucose- en VLDL-produktie zien en tonen daarmee aan, dat icv NPY toediening de inhibitie van de hepatische glucose- en VLDL-produktie door insuline kan voorkomen. Deze bevinding impliceert, dat de toegenomen hypothalame NPY levels, die worden waargenomen in verschillende obese diermodellen, wellicht ten grondslag liggen aan de hepatische insulineresistentie en de daarmee geassocieerde metabole afwijkingen van deze modellen.

Het doel van **hoofdstuk 3** was om de effecten van centrale toediening van melanotan II (MTII), op hepatische en totale insulinegevoeligheid te onderzoeken, onafhankelijk van voedselinname en lichaamsgewicht. MTII is een agonist van de melanocortine receptoren 3 en 4, dat beiden receptoren zijn van de POMC route, welke voedselinname inhibeert. De resultaten van dat hoofdstuk laten een toename zien in de basale glucose produktie en opname na centrale toediening van MTII. Bovendien was de hyperinsulinemische glucose opname toegenomen in de muizen die MTII hadden ontvangen, terwijl MTII geen effect had op het inhiberende effect van insuline op de hepatische glucose produktie. We concluderen hieruit, dat icv toediening van MTII acuut de insuline-gemedieerde glucose opname verhoogt, maar geen effect heeft op het vermogen van insuline om de hepatische glucose produktie te onderdrukken. Deze data geven aan, dat centrale stimulatie van de melanocortine 3/4 receptoren de insulinegevoeligheid moduleert op een weefselspecifieke manier en onafhankelijk van het al bekende effect op voedselinname en lichaamsgewicht.

In **hoofdstuk 4** werden de acute effecten van PYY_{3-36} op insulinegevoeligheid onderzocht. PYY_{3-36} is een hormoon, dat wordt afgescheiden door de darm in respons op een maaltijd. Het moduleert de activiteiten van de orexigene NPY neuronen en de anorexigene POMC neuronen in de hypothalamus om zo voedselinname te inhiberen. Aangezien zowel NPY als POMC een effect heeft op de

insulinewerking, vroegen wij ons af of PYY_{3-36} de insulinegevoeligheid zou kunnen verbeteren. PYY_{3-36} werd daarom intraveneus geïnfuseerd bij muizen tijdens een hyperinsulinemische euglycemische clamp. De resultaten van dat hoofdstuk laten zien dat onder hyperinsulinemische condities, de glucose opname significant is toegenomen in PYY-geïnfuseerde dieren in vergelijking met vehicle-geïnfuseerde muizen. We concluderen hieruit, dat PYY_{3-36} de insulinegevoeligheid verbetert, met name het vermogen van insuline om de glucose opname te stimuleren.

Aangezien de lange-termijn effecten van PYY_{3-36} op insulinegevoeligheid nog onbekend zijn, werden de effecten van chronische PYY_{3-36} toediening op insulinegevoeligheid onderzocht in **hoofdstuk 5**. Daarnaast werd ook de metabole efficiëntie van continue *vs.* onderbroken toediening van PYY_{3-36} onderzocht. Muizen kregen daarvoor 7 dagen lang subcutaan PYY_{3-36} toegediend dmv een osmotische minipomp die onderhuids werd geplaatst of dmv dagelijkse subcutane injecties, waarna vervolgens de insulinegevoeligheid werd gemeten met de hyperinsulinemische euglycemische clamp techniek. Onder hyperinsulinemische condities was de glucose opname significant toegenomen in de PYY-behandelde dieren *vs.* vehicle-behandelde dieren. De insulinewerking op de hepatische glucose produktie was daarentegen niet significant veranderd, alhoewel er een tendens was tot een toegenomen vermogen van insuline om de hepatische glucose produktie te onderdrukken. Bovendien werd geen van deze metabole parameters beïnvloed door de wijze van PYY_{3-36} toediening (continu of onderbroken). Deze observaties suggereren dat PYY_{3-36} of potentiele analogen een nuttige behandeling kunnen vormen tegen insulineresistentie.

Ten slotte werd in **hoofdstuk 6** de rol van het door vetweefsel geproduceerde hormoon leptine en de rol van de centrale signalling hiervan op insulinegevoeligheid onderzocht in *ob/ob* (leptine deficiente) muizen en afgezet tegen de bijdrage van het obese fenotype zelf op insulinegevoeligheid. Om de bijdrage van cerebrale leptine deficientie op insulineresistentie te onderzoeken, werd leptine icv geïnfuseerd in *ob/ob* muizen. Om ook de impact van de hoeveelheid vet op de insuline werking te onderzoeken, werd een andere groep jonge *ob/ob* muizen op strenge calorie restrictie gezet, zodat hun lichaamsgewicht gelijk werd aan dat van de wild-type muizen. De hyperinsulinemische euglycemische clamp techniek werd vervolgens gebruikt om de insulinegevoeligheid te bepalen. Icv leptine infusie verhoogde acuut zowel de hepatische insulinegevoeligheid als de insuline gemedieerde glucose

opname. Bovendien reduceerde icv leptine acuut de circulerende insuline concentraties gedurende continue insuline infusie (hyperinsulinemische periode). Voedselrestrictie had nauwelijks invloed op de lichaamssamenstelling ondanks de aanzienlijke vermindering van lichaamsgewicht. Insuline onderdrukte de hepatische glucose produktie duidelijk meer in de dunne *ob/ob* muizen in vergelijking met de dikke *ob/ob* muizen, maar dit effect bleef verminderd in vergelijking met de wild-type muizen. Insuline-gemedieerde glucose opname van de dunne ob/ob muizen lag ook in tussen die van de obese *ob/ob* en de dunne wild-type muizen. We concluderen hieruit dat leptine deficientie *per se* niet slechts verantwoordelijk is voor de hyperfagie en obesitas in *ob/ob* muizen, maar bepalend is voor lichaamssamenstelling, insulinewerking en insulineklaring vanuit de circulatie in deze dieren. De data suggereert dat leptineresistentie in obese patienten mogelijk bij kan dragen aan de insulineresistentie en hyperinsulinemie van deze individuen.

Concluderend, de studies in dit proefschrift laten de effecten zien van verschillende neuropeptiden/hormonen op insulinegevoeligheid. Aangezien er nog veel onderzoek gedaan moet worden hieraan, kan het hier beschreven onderzoek beschouwd worden als een beginpunt dat laat zien, dat neuropeptiden/hormonen die betrokken zijn bij de regulatie van voedselinname ook, en onafhankelijk van hun effect op voedselinname, insulinegevoeligheid beïnvloeden.

Curriculum vitae

Curriculum vitae

Anita Mariska van den Hoek is geboren op 7 oktober 1976 in Bennekom. In 1993 behaalde zij haar HAVO diploma en in 1995 haar VWO diploma aan het Johannes Fontanus College in Barneveld. In datzelfde jaar begon zij aan de studie Biologie aan de Universtiteit Utrecht. Hiervoor werd in 1996 de propedeuse behaald. Zij vervolgde met het doctoraal examen van de studie Fundamentele Biomedische Wetenschappen, waarvoor in 2000 het doctoraal examen werd behaald. In het kader van het doctoraalexamen heeft zij stage gelopen bij de vakgroep Vergelijkende Fysiologie, project-groep Neuro-Ethologie aan de Universiteit Utrecht onder begeleiding van dr. M.J. Lankheet en Prof. dr. W.A. van de Grind. Vervolgens werd een 2^{e} stage gelopen bij het Nederlands Instituut voor Hersenonderzoek in Amsterdam onder begeleiding van dr. D.F. Fischer.

In december 2000 werd zij voor 4 jaar aangesteld als promovendus bij de afdeling Endocrinologie van het Leids Universitair Medisch Centrum waarbij zij gedetacheerd was bij de afdeling Biomedisch Onderzoek van TNO-Kwaliteit van Leven in Leiden. Gedurende deze periode werd onder begeleiding van dr. H. Pijl, Prof. dr. J.A. Romijn en Prof. dr. L.M. Havekes het in dit proefschrift beschreven onderzoek verricht.

Vanaf december 2004 werd zij vervolgens voor 1 jaar aangesteld bij de afdeling Endocrinologie van het Leids Universitair Medisch Centrum waarbij een vervolgonderzoek werd uitgevoerd van het hier beschreven onderzoek.

Vanaf januari 2006 werkt zij als onderzoeker Diabetes bij de afdeling Biomedisch Onderzoek van TNO-Kwaliteit van Leven in Leiden.

List of publications

List of publications

Van den Hoek, A.M., van Heijningen, C., Havekes, L.M., Romijn, J.A., Kalsbeek, A and Pijl, H.. Intracerebroventricular administration of neuropeptide Y hampers insulin action to suppress endogenous glucose production via the sympathetic nervous system. *In preparation.*

Van den Hoek, A.M., Heijboer, A.C., Voshol, P.J., Havekes, L.M., Romijn, J.A., Corssmit, E.P., and Pijl, H.. Chronic PYY_{3-36} treatment promotes fat oxidation and ameliorates insulin resistance in C57BL6-mice. *In preparation.*

Heijboer A.C., Pijl, H, van den Hoek A.M., Havekes L..M., Romijn J.A., Corssmit E.P. Review: Gut-brain axis: regulation of glucose metabolism. *Submitted.*

Van den Hoek, A.M., Teusink, B, Voshol, P.J., Havekes, L.M., Romijn, J.A., and Pijl, H. Leptin deficiency *per se* dictates body composition, insulin action and plasma insulin kinetics in ob/ob mice. *Submitted.*

Heijboer A.C., van den Hoek A.M., Parlevliet E.T., Havekes L.M., Romijn J.A., Pijl H, Corssmit E.P. Intravenous administration of ghrelin differentially affects hepatic and muscle insulin sensitivity. *Diabetologia, in press*.

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