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.

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. 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. 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. 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. 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. 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. 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. 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. Briefly, 10-20 µg of tissue was homogenized in phosphate-buffered saline (PBS) and samples were taken for measurement of protein content. 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.
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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. ' These data are based on 2 mice only and therefore have to be considered with caution.

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<tr>
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<th>Basal</th>
<th>Hyperinsulinemic</th>
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<tr>
<td></td>
<td>Vehicle</td>
<td>NPY</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>23.3 ± 1.2</td>
<td>22.2 ± 1.2</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>7.0 ± 1.4</td>
<td>6.8 ± 1.8</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Glycerol (mmol/l)</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.8 ± 0.3</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>Glucagon (pmol/l)</td>
<td>100.0 ± 12.4</td>
<td>99.1 ± 22.0</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>29.6 ± 5.4</td>
<td>21.0 ± 9.9</td>
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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 µmol/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 µmol/min/kg) and glycerol turnover (7.3 ± 3.5 vs. 6.8 ± 1.4 µmol/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 µmol/min/kg and 4.6 ± 1.6 vs. 4.3 ± 1.0 µmol/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) µmol/kg/h; Figure 4), whereas it remained significantly higher in hyperinsulinemic conditions during NPY infusion (97.5 ± 18.0 vs. 54.7 ± 14.9 µmol/kg/h, P<0.01; Figure 4).

![Figure 3](image-url)

**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.
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. 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, 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...
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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 and adrenalectomy was shown to prevent or reduce some metabolic effects of subchronic i.c.v. NPY administration, like hyperphagia, weight gain and hyperinsulinemia. Corticosteroids enhance endogenous glucose production primarily via stimulation of gluconeogenesis without affecting glycogenolysis. 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. Other obese animal models are also characterized by high NPY neuronal activity. 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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