Intracerebroventricular Neuropeptide Y Infusion Precludes Inhibition of Glucose and VLDL Production by Insulin

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Recent evidence demonstrates that hypothalamic insulin signaling is required for inhibition of endogenous glucose production. The downstream mechanisms that are responsible for the effects of hypothalamic insulin receptor activation on hepatic fuel flux remain to be determined. To establish whether 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 (ICV) infusion of NPY (10 μg/h for 3–5 h) on glucose flux during a hyperinsulinemic-euglycemic clamp in mice. We also evaluated the effects of ICV NPY administration on free fatty acid and glycerol flux and 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 ~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^{-1} · h^{-1}; $P < 0.01$). 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. *Diabetes* 53:2529–2534, 2004

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CART, cocaine- and amphetamine-related transcript; EGP, endogenous glucose production; FFA, free fatty acid; ICV, intracerebroventricular; MCR3/4, melanocortin receptors 3 and 4; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; TG, triglyceride.

Insulin resistance is an important characteristic of obesity and type 2 diabetes (1,2). It hampers proper suppression of endogenous glucose and VLDL production in response to food intake. Accordingly, the metabolic features of obesity and type 2 diabetes include hyperglycemia and hypertriglyceridemia.

It was shown recently that hypothalamic insulin signaling is required for inhibition of endogenous glucose production (EGP) (3). Indeed, intracerebroventricular (ICV) 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 that are 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). ICV infusion of a melanocortin antagonist (SHU9119) does not affect the ability of hyperinsulinemia to inhibit EGP, 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 ICV infusion of NPY in Sprague-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 EGP 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 DESIGN AND METHODS

Male C57BL/6J mice were housed in a temperature-controlled room on a 12-h light-dark cycle and were fed a standard mouse diet with free access to water. All animal experiments were performed in accordance with the regulations of

TABLE 1
Plasma parameters in mice that received an ICV infusion of NPY or vehicle under basal or hyperinsulinemic conditions

	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*

Data are means ± SD for at least five mice per group. *Data based on two mice only and therefore must be considered with caution.

Dutch law on animal welfare, and the institutional ethics committee for animal procedures approved the protocol.

Surgical procedures. Mice were anesthetized 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, and 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 ICV injection of NPY (5 μg dissolved in 1 μl of sterile water; Bachem, Bubendorf, Germany).

Hyperinsulinemic-euglycemic clamp. Mice with free access to standard mouse diet 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 intracerebroventricularly 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 was determined, and in another series of experiments, free fatty acid (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), or ^{3}H-oleate (p, 2 μCi; c, 3 μCi/h; Amersham), respectively. Subsequently (after 80 min), insulin was administered in a primed (4.5 mU)-continuous (6.8 mU/h) intravenous 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 before and by the end of the clamp) to determine the plasma concentration of glucose, glycerol, FFAs, and insulin and plasma ^{14}C-glucose, ^{3}H-glycerol, and ^{3}H-oleate specific activities. At the end of the clamp, either mice were killed 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 ICV infusion of NPY (5 μg/μl) or vehicle at a rate of 2 μl/h. Mice received an intravenous injection of 500 mg/kg body wt Triton WR-1339 (Sigma, St. Louis, MO) as a 10% (wt/wt) 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 triglyceride (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 $\mu mol \cdot kg^{-1} \cdot min^{-1}$. Triton injections were given either under basal conditions (90 min after the beginning of the ICV infusion) or under hyperinsulinemic conditions (after the clamp experiment). At the end of the experiment, mice were killed, and liver samples were taken and frozen in liquid nitrogen for subsequent analysis.

Analytical procedures. Plasma levels of glucose, glycerol, FFAs, TGs, and corticosterone were determined using commercially available kits (Sigma; Boehringer Mannheim, Mannheim, Germany; Wako, Neuss, Germany; and Alpco, Windham, NH). Plasma insulin, glucagon, and NPY concentrations were measured by radioimmunoassay (Linco Research, St. Charles, MO; Alpco; and Peninsula Laboratories, San Carlos, CA). Total plasma ^{14}C-glucose and ^{3}H-glycerol was determined in 10 μl of 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 of plasma after extraction of lipids by a modification of Bligh and Dyer's method (15). In short, 7.5 μl of plasma was dried and resolved in 100 μl of water. Then 1.1 ml of demiwater and 4.5 ml of methanol:chloroform (2:1) was added and mixed thoroughly, after which 1.5 ml of chloroform was added and mixed, and finally 1.5 ml of demiwater was added and mixed. After centrifugation, the chloroform layer was collected and FFA fraction separated from the other lipid components by thin-layer chromatography on silica gel plates. Content of TGs in liver was determined as described before (16). Briefly, 10–20 μg of tissue was homogenized in 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 on silica gel plates.

Calculations. Turnover rates of glucose, FFAs, and glycerol ($\mu mol \cdot min^{-1} \cdot kg^{-1}$) 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 nonparametric test for two independent samples. $P < 0.05$ was considered statistically significant. All values shown represent mean ± SD.

RESULTS

Plasma parameters. Body weight, plasma glucose, FFAs, 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).

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 $\mu mol \cdot min^{-1} \cdot kg^{-1}$, respectively; $P < 0.01$; Fig. 1), indicating that ICV 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 $\mu mol \cdot min^{-1} \cdot kg^{-1}$, respectively; Fig. 2). Hyperinsulinemia barely increased glucose disposal, and the subtle increase that it brought about was of similar magnitude in NPY- and vehicle-infused animals (163.2 ± 22.8 vs. 151.1 ± 24.8 $\mu mol \cdot min^{-1} \cdot kg^{-1}$, respectively). In contrast, 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-

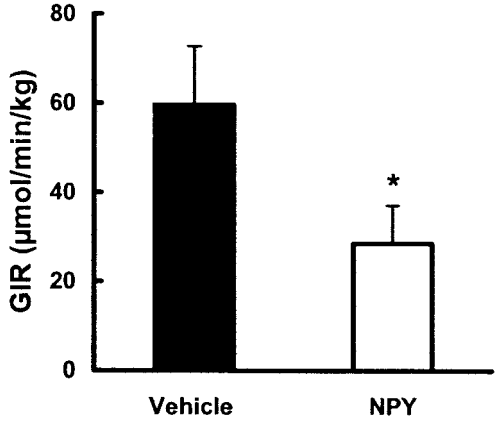

FIG. 1. Glucose infusion rate (GIR) in mice that received an ICV infusion of NPY or vehicle during a hyperinsulinemic-euglycemic clamp. Values represent mean ± SD for at least five mice per group. *$P < 0.01$ vs. vehicle.

infused mice (~8%, NS; $P < 0.05$ for difference between NPY- and vehicle-infused animals; Fig. 2).

FFA and glycerol turnover. Basal rates of FFA (16.6 ± 6.5 vs. 18.1 ± 7.8 μmol · min^{-1} · kg^{-1}) and glycerol turnover (7.3 ± 3.5 vs. 6.8 ± 1.4 μmol · min^{-1} · kg^{-1}) were not different between vehicle- and NPY-infused animals (Fig. 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^{-1} · kg^{-1} and 4.6 ± 1.6 vs. 4.3 ± 1.0 μmol · min^{-1} · kg^{-1} in vehicle- and NPY-infused animals for FFA and glycerol turnover, respectively).

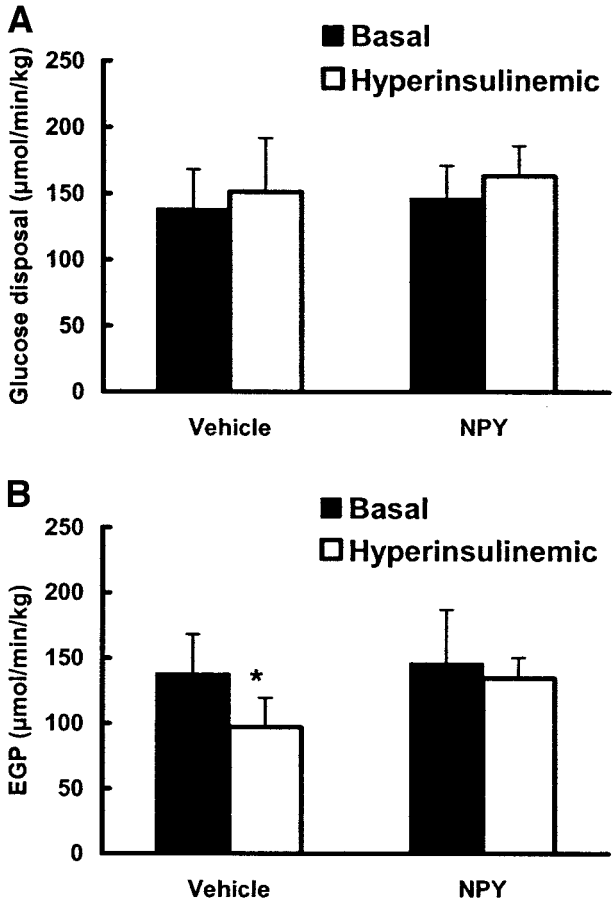

FIG. 2. Glucose disposal (*A*) and EGP (*B*) in mice that received an ICV infusion of NPY or vehicle before (basal) and after (hyperinsulinemic) the initiation of a hyperinsulinemic-euglycemic clamp. Values represent mean ± SD for at least five mice per group. *$P < 0.05$ vs. basal.

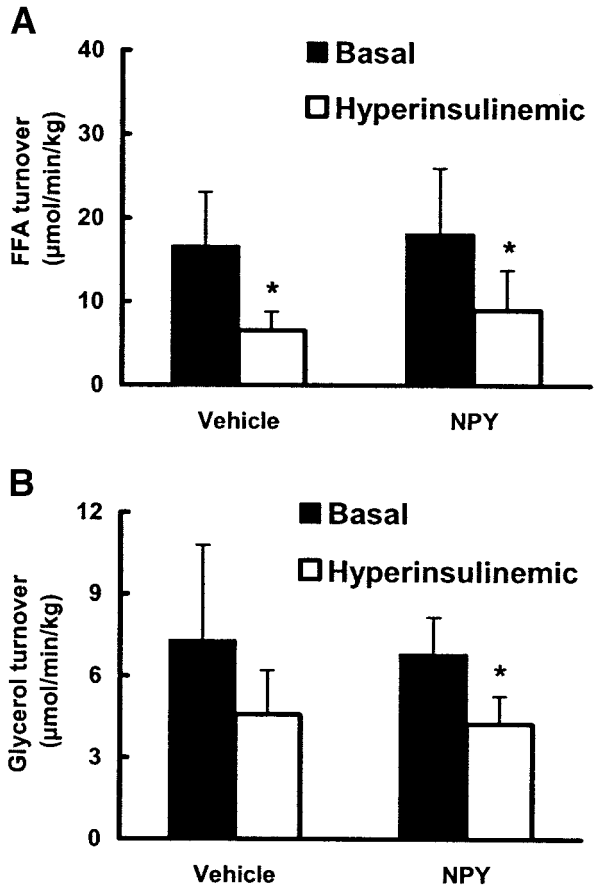

FIG. 3. FFA turnover (*A*) and glycerol turnover (*B*) in mice that received an ICV infusion of NPY or vehicle before (basal) and after (hyperinsulinemic) the initiation of a hyperinsulinemic-euglycemic clamp. Values represent mean ± SD for at least five mice per group. *$P < 0.05$ vs. basal.

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^{-1} · h^{-1}; Fig. 4), whereas it remained significantly higher in hyperinsulinemic conditions during NPY infusion (97.5 ± 18.0 vs. 54.7 ± 14.9 μmol · kg^{-1} · h^{-1}; $P < 0.01$; Fig. 4).

DISCUSSION

This study demonstrates that ICV 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 EGP 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 coexpressing POMC and cocaine- and amphetamine-related transcript (CART). These POMC/CART neurons effectively counterbalance

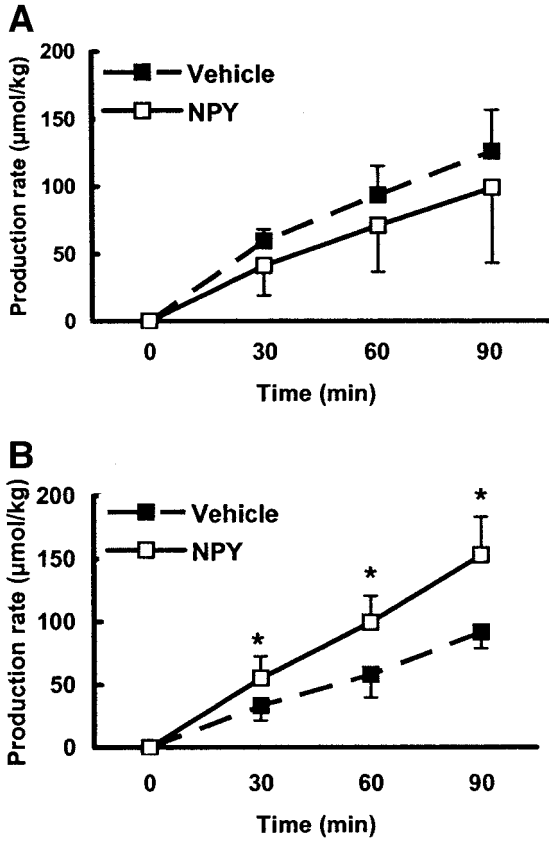

FIG. 4. VLDL production rate in mice that received an ICV infusion of NPY or vehicle under basal (*A*) or hyperinsulinemic (*B*) conditions. Values represent mean ± SD for at least five mice per group. **P* < 0.01 vs. vehicle.

the actions of an anabolic pathway, comprising NPY/agouti-related protein 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, a derivative peptide that activates melanocortin 3 and 4 receptors (MCR3/4). Acute ICV 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 an 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 EGP (3), we explored the impact of ICV 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 EGP (primarily hepatic) in this experimental context. Furthermore, insulin not only suppresses hepatic glucose production but also inhibits VLDL production (19,20), and our results indicate that ICV 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 FFAs to the liver, brought about by any potential impact of NPY on lipolysis. The former finding agrees with data reported by Obici et al. (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 EGP and VLDL production, whereas it does not directly affect fuel flux in other peripheral tissues.

NPY receptors are present not only in the brain but also in many peripheral tissues (21–23). To dismiss the possibility that ICV 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 ICV 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 appropriate to consider that anesthesia may have an 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 primary point.

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 of the brain insulin receptor involved in the physiological control of fuel metabolism. Also, the present study does not rule out the possibility that ICV NPY administration hampers the capacity of insulin to suppress glucose and VLDL production via other mechanistic routes than those downstream of 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 ICV NPY administration, such as hyperphagia, weight gain, and hyperinsulinemia (25–28). Corticosteroids enhance EGP 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 whether 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 suggest 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 EGP 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 and cardiovascular disease.

In summary, we provide evidence that ICV NPY administration precludes the inhibition of hepatic glucose and VLDL production by circulating insulin. This finding may suggest 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 therefore may be useful therapeutic tools in the clinical management of insulin resistance and type 2 diabetes.

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