Chronic central melanocortin-4 receptor antagonism and central neuropeptide-Y infusion in rats produce increased adiposity by divergent pathways.

Baran K, Preston E, Wilks D, Cooney GJ, Kraegen EW, Sainsbury A

Katherine Baran, From the Diabetes Research Group, the Garvan Institute of Medical Research, Sydney, Australia.; Elaine Preston, From the Diabetes Research Group, the Garvan Institute of Medical Research, Sydney, Australia.; Donna Wilks, From the Diabetes Research Group, the Garvan Institute of Medical Research, Sydney, Australia.; Gregory J. Cooney, From the Diabetes Research Group, the Garvan Institute of Medical Research, Sydney, Australia.; Edward W. Kraegen, From the Diabetes Research Group, the Garvan Institute of Medical Research, Sydney, Australia. and Amanda Sainsbury, From the Diabetes Research Group, the Garvan Institute of Medical Research, Sydney, Australia.

Increased hypothalamic neuropeptide-Y (NPY) action and disruption of the melanocortin (MC)-4 receptor both result in hyperphagia and obesity. To determine whether similar hormonal and metabolic mechanisms are involved in these two obesity syndromes, we investigated the time course of effects induced by 6-day intracerebroventricular (ICV) infusion of NPY (3.5 nmol/day) or the MC4 receptor antagonist HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. The weight of white adipose tissue (WAT) deposits was increased after 6-day NPY and HS014 infusion compared with controls, and the increase was significantly greater in HS014- than in NPY-infused rats (retroperitoneal WAT: NPY 0.57 ± 0.05; HS014 0.80 ± 0.05; control 0.43 ± 0.03% body wt, n = 8 - 13, $P < 0.05$). Plasma leptin was also increased in both experimental groups (NPY 10.6 ± 1.9; HS014 4.4 ± 0.9; control 2.0 ± 0.1 ng/ml, n = 8 - 13, $P < 0.05$ for all comparisons). Basal plasma corticosterone and insulin levels were increased by ICV NPY infusion, whereas HS014-infused rats showed no significant increase in these parameters on any of 1–6 days of infusion. Both NPY and HS014 infusion potentiated intravenous glucose-induced (300 mg/kg) plasma insulin levels, and there was no difference in glycemia among groups. In NPY-infused rats, the plasma free fatty acid levels were decreased and triglyceridemia was increased compared with controls, but these parameters were unchanged in HS014-infused rats. Hepatic triglyceride content was significantly increased by HS014 but not by NPY infusion. Levels of uncoupling protein-1 mRNA in brown adipose tissue were significantly decreased after 6 days of HS014 infusion, similar to the effect of central NPY. Because ICV HS014 induced at least as great an increase in fat mass as ICV NPY and yet had divergent hormonal and metabolic effects, we conclude that MC4 receptor antagonism does not induce obesity solely by regulation of the endogenous NPY-ergic system.

Abbreviations: AgRP, agouti-related protein; BAT, brown adipose tissue; FFA, free fatty acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICV, intracerebroventricular, MC, melanocortin; NPY, neuropeptide-Y; NTS, nucleus tractus solitarius; PVN, paraventricular nucleus; TG, triglyceride; UCP-1, uncoupling protein-I; WAT, white adipose tissue.

Neuropeptide-Y (NPY) is a highly potent orexigenic peptide synthesized in the brain (1). Genetically obese ob/ob and db/db mice and fa/fa rats exhibit a lack of leptin function as their primary defect (2–5), and all exhibit increased NPY expression and secretion in the hypothalamus (6) caused by a lack of leptin's known inhibitory effect on NPY-ergic activity (7). Although other peptides are likely to be involved (1), this increase in hypothalamic NPY probably contributes to obesity in ob/ob, db/db, and fa/fa rodents because intracerebroventricular (ICV) infusions of NPY in normal rodents produce effects similar to those seen in the genetically obese rodent models. These include increased food intake, body weight, fat accumulation, plasma insulin, leptin, and corticosterone concentrations, decreased brown adipose tissue (BAT) thermogenesis, and inhibition of the somatotropic and gonadotropic axes (8–12).

Several dominant mutations at the agouti locus in the mouse also cause a syndrome of marked obesity, hyperinsulinemia, insulin resistance, and hyperglycemia (13–15). Antagonism of the hypothalamic melanocortin (MC)-4 receptor by ectopically expressed agouti is probably the cause of the agouti mouse obesity syndrome (16), because MC4 receptor knockout mice have an obesity syndrome identical to that of the agouti obese mouse (17). Agouti-related protein (AgRP), a high affinity endogenous antagonist of the MC3 and MC4 receptor (18,19), is probably involved in energy homeostasis, because hypothalamic AgRP expression is increased in fasting rats (20,21), and ICV AgRP infusions stimulate food intake in both starved and satiated rats (22,23). So far, little is known about the mechanisms and primary etiological causes of obesity in agouti mice and MC4 receptor knockout mice. Studies involving central administration of exogenous MC4 receptor antagonists have focused primarily on effects of feeding, and little is known about the effect that chronic MC4 receptor antagonism has on the peripheral hormonal and metabolic parameters known to alter energy homeostasis or the time-course of these effects. Long-term administration of the MC4 receptor antagonist HS014 has been reported to increase food intake and consequently increase body weight and adiposity (24). Additionally, chronic administration of the MC4 receptor antagonist SHU911 in rats allowed to eat ad libitum resulted in increased food intake, adiposity, plasma corticosterone, insulin, and leptin, with no changes in plasma growth hormone, insulin-like growth factor-1, leutenizing hormone, follicle-stimulating hormone, and testosterone (25). These observed effects could be a result of increased food intake, and currently no studies have observed the sequential hormonal and metabolic effects after chronic MC4 receptor antagonism in rats where increased food intake is prevented.

There is some evidence that the mechanisms contributing to obesity in genetically obese ob/ob and fa/fa rodents (which exhibit early onset obesity, early onset hyperinsulinemia, decreased linear growth, and elevated levels of plasma corticosterone) are in some ways different to the etiology of obesity in agouti and MC4 receptor knockout mice (which exhibit late-onset obesity, late-onset hyperinsulinemia, increased linear growth, and no effects on basal plasma corticosterone levels) (13,14). On the contrary, other available evidence suggests that chronic central MC4 receptor antagonism induces obesity at least partially via the NPY-ergic system (26). Agouti obese mice exhibit increased levels of NPY mRNA in the dorsomedial nucleus (27). Furthermore, the orexigenic effects of the specific MC4 receptor antagonist HS014 were partially inhibited by an NPY-Y1 receptor antagonist (28) and completely blocked by fluoxetine, which inhibits NPY release (29,30). Therefore, the available literature provides conflicting evidence as to whether MC4 receptor antagonism induces obesity and any hormonal and metabolic changes via actions on the endogenous NPY-ergic system.

To determine whether similar pathways are involved in the obesity syndromes induced by chronic central NPY-ergic activation and chronic MC4 receptor antagonism, we compared the hormonal and metabolic effects of 6-day ICV NPY or MC4 receptor antagonist (HS014) infusion in normal rats. All rats were pair-fed with vehicle-infused controls in order to study the effects of NPY and HS014 in the absence of their known hyperphagic effects (8,28).

RESEARCH DESIGN AND METHODS

Experimental animals. All procedures were approved by the Animal Experimentation Ethics Committee of the Garvan Institute/St Vincent's Hospital and are in keeping with the National Health and Medical Research Council of Australia's guidelines on animal experimentation. Male Wistar rats were purchased from Animal Resources Center (Perth, Australia) at weights of 250–280 g. They were housed in groups in plastic cages on pellet paper bedding and under conditions of controlled temperature (23°C) and illumination (0600–1800). They were allowed ad libitum access to standard laboratory diet (Norco Stockfeeds, South Lismore, Australia) and water, unless otherwise stated.

Placement of chronic ICV and Jugular cannulae. Animals were anesthetized with an intraperitoneal injection of ketamine and xylazine at 60 and 10 mg/kg, respectively (Mavlab, Brisbane, Australia, and Troy Laboratories, Sydney, Australia, respectively), for the placement

of a cannula in the right lateral cerebral ventricle (11), which was used for all ICV administrations. Another cannula was placed in the right jugular vein for blood sampling. Rats received an intramuscular injection of penicillin at 0.3 mg/kg (Troy Laboratories) as well as a subcutaneous analgesic, buprenorphine (Temgesic 0.025 mg/kg) (Reckitt and Coleman, Hull, U.K.), and were left to recover to presurgery weights (7–10 days) in individual cages, with daily handling to minimize stress.

Acute ICY injection of NPY and HS014. A subset of rats were used in acute (4-h) feeding studies after bolus ICV injection of porcine NPY (1.2 nmol) (Auspep, Melbourne, Australia), synthetic MC4 receptor antagonist HS014 (1.5 nmol) (Auspep), or vehicle (5 µl of 0.9% NaCl) for control rats. Injections were made over a period of 60 s, animals were then returned to their cages, and total ad libitum food intake was measured after 4 h.

Chronic ICV infusion of NPY and HS014. Rats were anesthetized with halothane (Veterinary Companies of Australia, Sydney, Australia) for implantation of subcutaneous osmotic minipumps (model 2001; Alza, Palo Alto, CA) (11), which were connected via polyethylene tubing to the ICV cannula for central infusion of porcine NPY (3.5 nmol/day), HS014 (4.8 nmol/day), or vehicle (24 µl/day of 0.9% NaCl) for control rats. Food intake of the NPY- and HS014-infused rats was restricted to that of the vehicle-infused animals (30–32 g/day) to ensure that any differences observed were not caused by increased food intake. Rats were fasted for 2–3 h (from ~0930 to 1230). Basal blood samples (0.3 ml) were collected dally from the jugular cannula between 1130 and 1230 each day using sodium citrate (6.1 mg/ml in 0.9% NaCl) as an anticoagulant. Then, 3–4 days after minipump implantation, an intravenous glucose tolerance test was performed where blood samples were taken 1 and 5 min after intravenous glucose injection (300 mg/kg). These two time points were chosen because we have previously shown that insulinemia attains a peak at 1 min after such glucose injection and returns toward baseline values within 5 min (31). Incremental areas under the resultant insulin curves were calculated Coy subtracting baseline values) between 0 and 5 min after glucose injection. All plasma samples were frozen in liquid N&sub2; and stored at -20°C until analysis. Plasma concentrations of triglyceride (TG) (Sigma Diagnostics, St. Louis, MO), nonesterified fatty acid (Wako, Osaka, Japan), and glucose (glucose oxidase method; Trace Scientific, Melbourne, Australia) were measured using commercial kits. Plasma corticosterone and leptin, basal, and glucose-stimulated insulin levels were measured by radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA, and Linco Research, St. Louis, MO, respectively).

Tissue collection from NPY- and HS014-infused rats. After 5–6 days of minipump infusion, rats were anesthetized with halothane, the interscapular BAT and liver were then removed and weighed, and portions were freeze-clamped and stored at -80°C until analysis. Epididymal and retroperitoneal fat pads were then excised and weighed.

BAT uncoupling protein-1 mRNA analysis. Total RNA was extracted from BAT samples using TRI-Reagent (Sigma) according to the manufacturer's instructions. The mouse uncoupling protein-1 (UCP-1) probe was generated by polymerase chain reaction from genomic DNA. The 182-bp long fragment corresponding to exon 3 was labeled with [α-32P]dCTP, using a megaprime DNA labeling kit (Amersham, Buckinghamshire, England), and Northern blot analysis was carried out under standard conditions. Blots were washed under stringent conditions before autoradiographic exposure to Kodak BioMax film at -80°C for 2–3 days. As a control for loading, the same blot was hybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe, and all ratios of UCP-1—to—GAPDH optical density were expressed as a percentage of control values. All films were analyzed with a computer-assisted image analysis system connected to a GS-690 Imaging Densitometer (Bio-Rad, Hercules, CA).

Liver analysis. Liver samples (50 mg) were homogenized in a glass hand-held homogenizer in a 4-ml chloroform:methanol (2:1) solution, and lipids were left to extract into the organic phase for 12–16 h at room temperature. Two milliliters of 0.6% NaCl solution was added, and then the tubes were vortexed and centrifuged for 10 rain at 4°C (825g). The lower organic phase containing lipids was transferred to glass scintillation vials and dried under nitrogen gas. The dried lipids were then resuspended in 500 µl ethanol and stored at -20°C until a TG assay was performed (Peridochrome TG kit; Roche Diagnostics). Statistics. Statistics were performed using Statview version 4.5 (Abacus Concepts). Repeated measures or factorial

analysis of variance tests were used for the statistical analysis of results, with Fisher's post hoc tests where appropriate. Data are expressed as means ± SD, and the results were considered statistically significant at $P < 0.05$.

RESULTS

Dose of NPY and HS014. To standardize the biological potency of NPY and HS014, we chose doses of these compounds that produce similar and maximal degrees of hyperphagia in ad libitum—fed rats. After acute ICV injection of a maximum dose of NPY (1.2 nmol) (32), rats consumed sevenfold as much diet as vehicle-injected rats in the ensuing 4 h (Fig. 1). A similar 4-h food intake was obtained after ICY injection of 1.5 nmol of HS014 (Fig. 1), and higher doses did not produce greater hyperphagia (data not shown). For chronic infusion studies, three times this maximally effective acute dose was infused over each 24-h period as previously described for NPY (11). Because all animals were fed the same amount as vehicle-infused controls during chronic infusions, latency to feed was used as an index of biological potency of NPY and HS014. In both NPY- and HS014-infued rats, latency to feed was <30 s on each of days 2–6 of infusion compared with >60 s in vehicle-infused controls (data not shown). If the latency to feed was >30 s, animals were excluded from the NPY- or HS014-infused groups (<10% of rats).

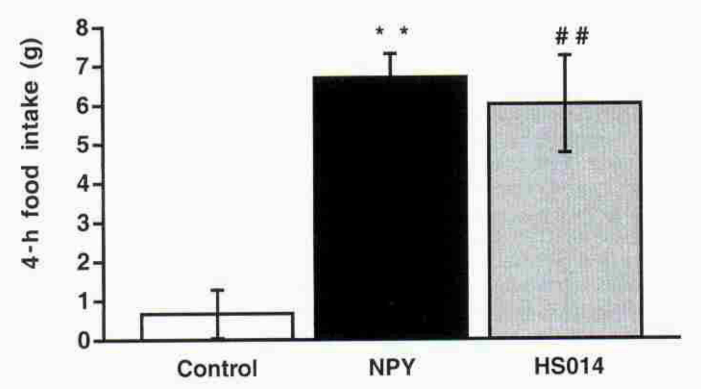

FIG. 1. Total ad libitum food intake in the 4 h after acute ICV injection of NPY (1.2 nmol) or HS014 (1.5 nmol) compared with ICV vehicle-injected control rats (5.0 μl of 0.9% NaCl). Plotted values are means ± SD of 4–7 rats per group. $^{**}P < 0.01$ NPY vs. control; $\#\#P < 0.01$ HS014 vs. control.

Body weight and adiposity of NPY- and HS014- infused rats. There was no significant difference among NPY-, HS014-, and saline-infused rats with respect to body weight before infusion (NPY 354 ± 8, HS014 372 ± 7, and control 367 ± 6 g, n = 9 - 16, NS) or with respect to body weight gain during the 6 days of infusion (Fig. 2A). This is consistent with data showing that whereas chronic ICV NPY or MC4 receptor antagonist infusion significantly increases body weight in ad libitum—fed rats (8,24,25,33), no such increase is observed when hyperphagia is prevented by pair-feeding with vehicle-infused controls (11,33,34). However, 6 days of central infusion of NPY or HS014 with pair-feeding to control rats significantly increased fat accumulation in the epididymal and retroperitoneal white adipose tissue (WAT) depots compared with saline-infused controls (Fig. 2B). Furthermore, the retroperitoneal fat depot weight was significantly greater in HS014-infused than in NPY-infused rats (Fig. 2B). The 6 days of ICV infusion of both NPY and HS014 resulted in significant increases in plasma leptin levels compared with controls, but the effect was significantly greater in NPY-infused rats than in rats infused with HS014 (Fig. 2C).

Plasma corticosterone, insulin, and glucose levels during chronic ICV NPY or HS014 infusions. The plasma concentrations of corticosterone during the 6 days of ICV infusion are shown in Fig. 3A. Compared with the vehicle-infused control rats, pair-fed NPY-infused rats had an increase in plasma corticosterone levels during the first 2 days of infusion. In contrast, long-term ICV infusion of the MC4 receptor antagonist HS014 with pair-feeding to control rats had no significant effect on corticosteronemia at any time point. NPY-infused rats exhibited

significantly greater plasma insulin levels compared with control and HS014-infused rats over the 6 days of central infusion (Fig. 3B). Rats chronically ICV-infused with HS014 exhibited no significant difference from control values in plasma insulin levels over the 6 days of infusion (Fig. 3B). Levels of glycemia were not significantly changed from control values in ICV NPY- or HS014-infused rats (Fig. 3C).

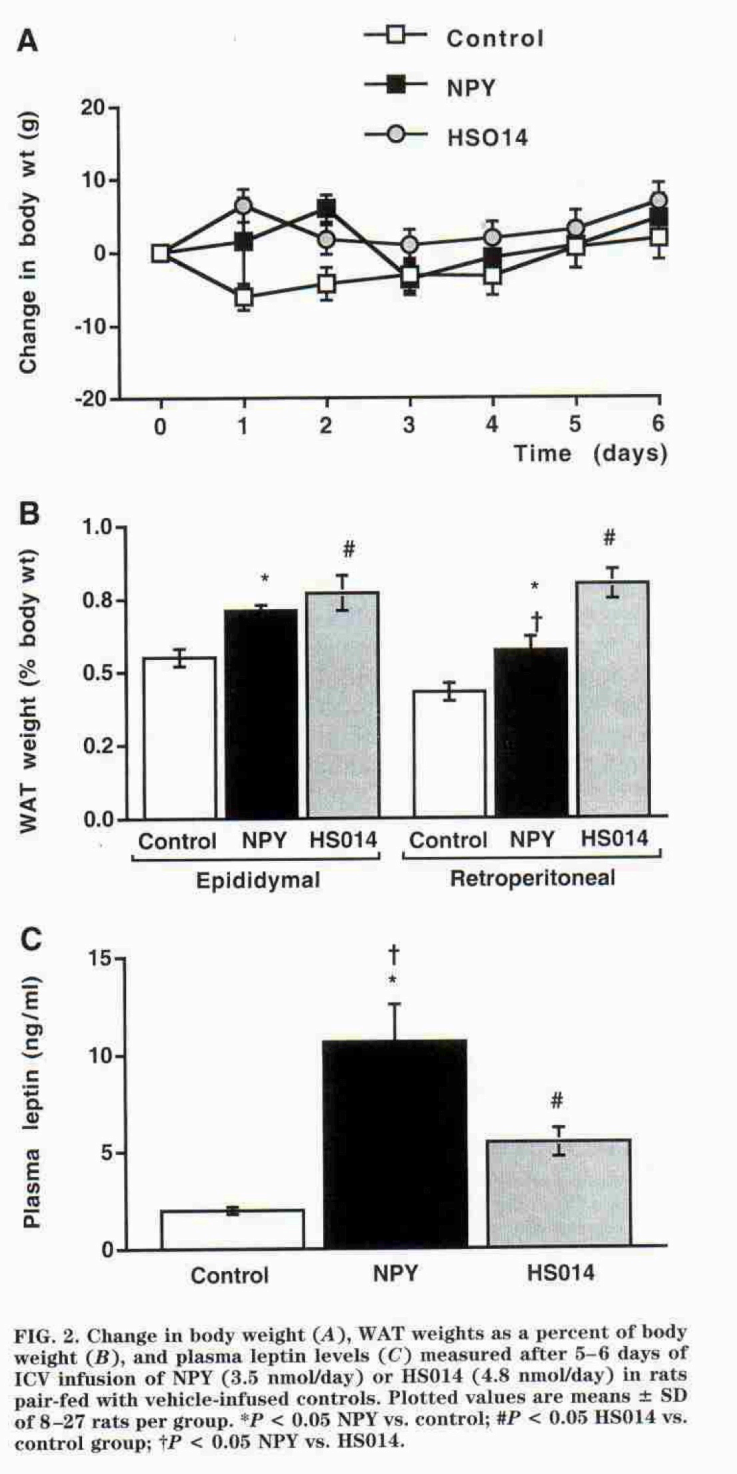

FIG. 2. Change in body weight (*A*), WAT weights as a percent of body weight (*B*), and plasma leptin levels (*C*) measured after 5–6 days of ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. Plotted values are means ± SD of 8–27 rats per group. **P* < 0.05 NPY vs. control; #*P* < 0.05 HS014 vs. control group; †*P* < 0.05 NPY vs. HS014.

Plasma insulin and glucose levels after intravenous glucose injection. Immediately before glucose injection, basal insulinemia was greater than control values in NPY- but not HS014-infused rats (Fig. 4A). Intravenous glucose-induced plasma insulin levels were more elevated than control values for both NPY- and HS014-infused rats at 1 and 5 rain

postglucose injection (Fig. 4A), and the difference was significant at 5 min. The incremental areas under the insulin curves shown in Fig. 4 are NPY 3,960 ± 440, P = 0.08 vs. control; HS014 5,130 ± 330, P < 0.001 vs. control; and control 2,750 ± 470 pmol/1 × 5 min, with no significant difference between NPY- and HS014-infused rats. Plasma glucose levels of NPY- and HS014-infused rats were no different from control values either before or at any time after glucose injection (Fig. 4B).

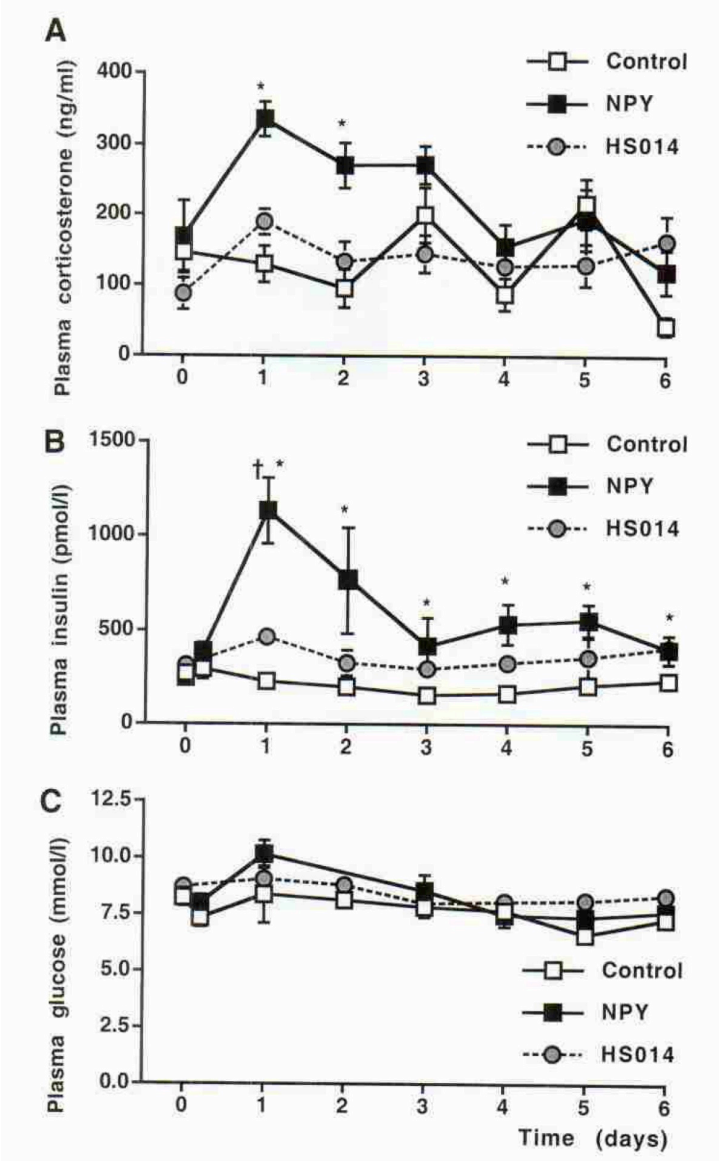

FIG. 3. Plasma levels of corticosterone (*A*), insulin (*B*), and glucose (*C*) measured during 6-day ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. Plotted values are means ± SD of 5–15 rats per group. **P* < 0.05 NPY vs. corresponding control; †*P* < 0.05 NPY versus corresponding HS014.

Plasma lipid concentrations during chronic ICV NPY or HS014 infusion. The plasma concentrations of free fatty acids (FFAs) and TGs are shown in Fig. 5. Plasma FFA levels in NPY-infused rats were significantly reduced compared with saline and HS014-infused rats for the first 3 days of ICV infusion (Fig. 5A). Plasma FFA levels in HS014-infused rats were not significantly different from control levels for the entire 6 days of infusion (Fig. 5A). Plasma TG levels of NPY-infused rats were significantly elevated compared with control and HS014-infused rats on days 1–5 of central infusion (Fig. 5B). Plasma TG levels of HS014-infused rats were not significantly different relative to controls during the infusion period (Fig. 5B).

Liver TG content after chronic ICV NPY or HS014 infusion. Hepatic TG content was significantly increased over control values in HS014- but not NPY-infused rats (Fig. 5C). There was no significant difference among the three groups with respect to liver weight after

5–6 days of infusion (NPY 13.2 ± 0.3, HS014 12.6 ± 0.5, and control 12.0 ± 0.3 g, n = 4–8, NS).

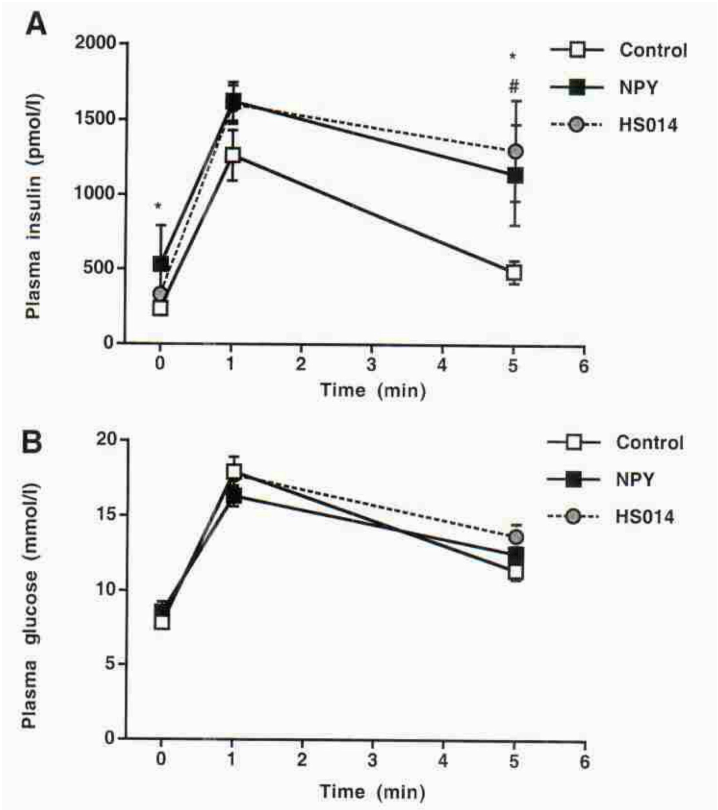

FIG. 4. Plasma levels of insulin (*A*) and glucose (*B*) measured at 1 and 5 min after an intravenous glucose injection (300 mg/kg) in rats ICV-infused for 3–4 days with NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) and pair-fed with vehicle-infused controls. Plotted values are means ± SD of 4–14 rats per group. **P* < 0.05 NPY vs. corresponding control; #*P* < 0.05 HS014 vs. corresponding control.

BAT weight and UCP-1 mRNA levels after chronic ICV NPY or HS014 infusion. BAT weight was significantly increased over control values after 5–6 days of ICV NPY or HS014 infusion (NPY 0.36 ± 0.05, HS014 0.33 ± 0.02, and control 0.16 ± 0.02 g, n = 5–6, both P < 0.01 vs. control). Both NPY- and HS014-infused rats exhibited a significantly lower level of UCP-1 mRNA in the BAT compared with that of saline-infused controls, with no significant difference between the NPY- and HS014-infused groups (Fig. 6).

DISCUSSION

This work investigated whether aspects of the obesity syndromes induced by chronically increased NPY-ergic activity or central MC4 receptor antagonism are mediated by independent or common pathways. To this end, we compared the time course of hormono-metabolic consequences of chronic ICV infusion of NPY and HS014 (an analogue of the endogenous MC3/MC4 receptor antagonist AgRP) in rats, where hyperphagia had been prevented by pair-feeding.

It was demonstrated that 6 days of central infusion of either NPY or HS014 in rats resulted in significant increases in WAT mass, even though NPY- and HS014-infused rats were prevented from overeating by pair-feeding with vehicle-infused control rats. As with another very recent study using 7-day ICV AgRP injections (33), we demonstrated that MC4 receptor antagonist-infused rats show an increase in WAT mass in the absence of increased food

intake. Our study also extends this observation by making a direct comparison of the effects of central HS014 infusion with those of NPY and by time course comparison of endocrine and metabolic effects. Because ICV HS014 induced at least as great an increase in fat mass as ICV NPY and yet had divergent hormonal and metabolic effects, we conclude that MC4 receptor antagonism does not induce obesity solely by regulation of the endogenous NPY-ergic system.

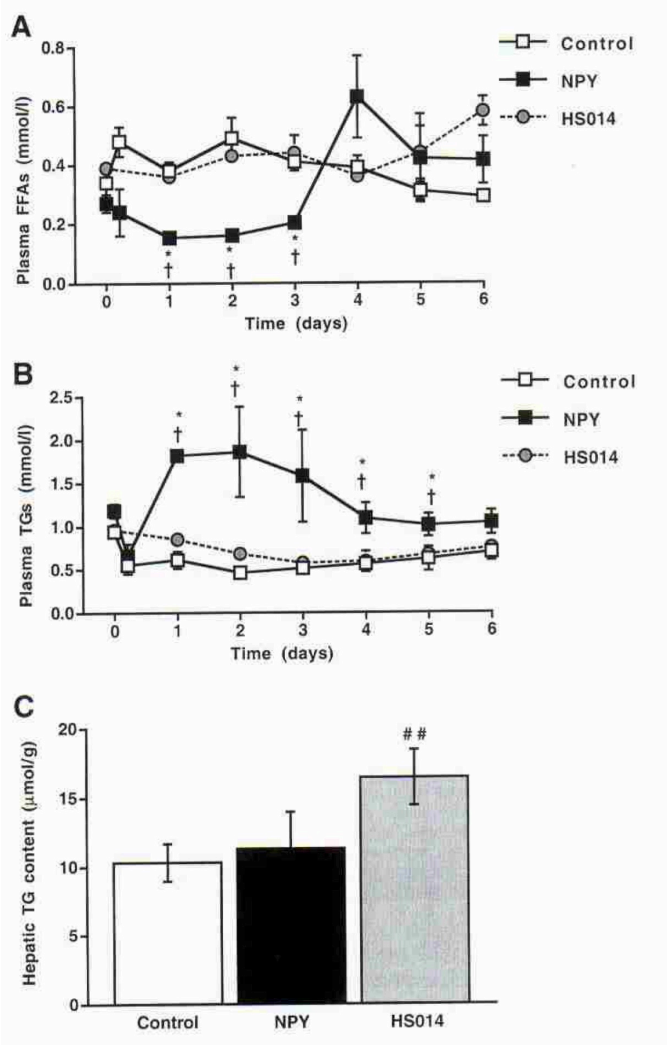

FIG. 5. Plasma levels of FFAs (*A*), TGs (*B*), and hepatic TG content (*C*) measured after 6 days of ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. Plotted values are means ± SD of 4–15 rats per group. **P* < 0.05 NPY vs. corresponding control; ##*P* < 0.01 HS014 vs. corresponding control; †*P* < 0.05 NPY vs. corresponding HS014.

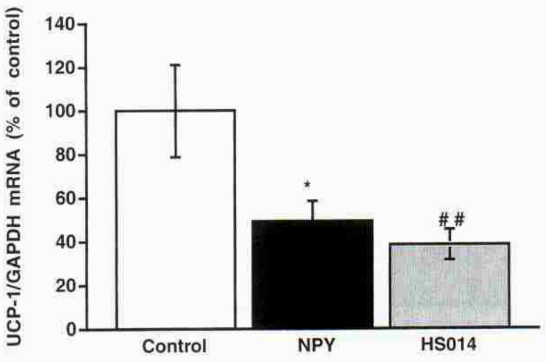

FIG. 6. Levels of mRNA for UCP-1 in BAT after 6 days of ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. UCP-1 mRNA levels were standardized against those of GAPDH, and expressed as a percentage of controls. Plotted values are means ± SD of 5–7 rats per group. *$P < 0.05$ NPY versus corresponding control; ##$P < 0.01$ HS014 vs. corresponding control.

Because plasma concentrations of leptin are positively correlated with percent body fat (35), increased plasma leptin levels in NPY- and HS014-infused rats probably reflected the increased adiposity. However, plasma leptin levels of rats infused with the MC4 receptor antagonist HS014 were significantly lower than those of NPY-infused rats, even though adiposity in the HS014 group was at least as great. A similar outcome was recently reported in comparison of effects of ICV SHU9119 and NPY in ad libitum—fed rats (25). This highlights the fact that leptinemia is regulated by many factors besides fat mass (35). Corticosterone and insulin, plasma levels of which were shown to be increased in NPY- but not HS014-infused rats, are both known to positively regulate plasma leptin levels (35). Additionally, other researchers have shown that plasma testosterone levels are significantly reduced by NPY but not by SHU9119 infusion (12,25), and because testosterone is an inhibitor of leptin secretion (35), this difference may also contribute to the lower plasma leptin levels of the HS014 group.

It has recently been reported that chronic central infusion of the MC3/MC4 receptor antagonist SHU9119 increased plasma corticosterone levels in ad libitum—fed rats (25). This effect was probably mediated by SHU9119-induced hyperphagia because feeding is known to increase corticosteronemia (36), and because in the present study MC4 receptor antagonist infusion with concomitant pair-feeding caused no such hypercorticosteronemia. Our finding is in keeping with reports that animal models with chronic MC4 receptor antagonism, such as MC4 receptor knockout mice and agouti obese yellow mice, do not have elevated basal plasma corticosterone levels (17).

High plasma levels of insulin are known to contribute to obesity because attenuation of the hyperinsulinemia of genetically obese rodents (37,38) reduced the obese phenotype in the absence of significant effects on feeding. Furthermore, administration of exogenous insulin to animals and humans results in increased fat accumulation and other metabolic defects characteristic of obesity (39–41). NPY-infused rats exhibited two- to threefold increases over controls in basal plasma insulin levels over the infusion period. This hyperinsulinemia, in addition to reduced thermogenesis (9,42–45), is the likely primary mechanism by which NPY-infused rats become obese in the absence of hyperphagia. In contrast to the NPY-infused group, rats that received HS014 infusions did not exhibit any significant change in insulinemia relative to control rats, indicating that hyperinsulinemia may not be the only contributor to the observed increase in adiposity. Glucose-stimulated plasma insulin concentrations were elevated in both NPY- and HS014-infused rats in the absence of any difference from controls in glycemia, an indication of insulin resistance. This increased insulin response to glucose in both obesity models may be mediated by insulin resistance, which is commonly observed in situations of increased fat mass, as in ICV NPY-infused rats (11). Therefore, the late-onset hyperinsulinemia observed in MC4 receptor knockout and agouti obese yellow mice is unlikely to result from a primary defect in insulin secretion, and hyperinsulinemia probably

develops secondary to insulin resistance.

In addition to the hormonal differences discussed above, differences in metabolic profiles were also observed between ICV NPY- and MC4 receptor antagonist-induced increases in adiposity, suggesting independent etiologies. Whereas central NPY infusion significantly reduced plasma FFA and increased plasma TG levels (possibly mediated by hyperinsulinemia and/or reduced sympathetic nervous activity), the plasma concentrations of FFAs and TGs in HS014-infused rats were not significantly different from controls, reflective of the unchanged plasma insulin profile. Unlike NPY-infused rats, the total liver TG content observed in rats infused with HS014 was increased relative to controls, despite no change in tissue weight.

It is well established that central NPY administration decreases sympathetically mediated thermogenic activity in BAT (9,42–45), most likely by decreasing the expression of UCP-1 (9). This effect is likely to contribute to NPY-induced obesity because BAT ablation per se also results in an obesity syndrome (46). This study has shown that UCP-1 mRNA levels are also decreased in BAT after 6 days of central MC4 receptor antagonism with HS014, extending a recent report that 7-day ICV AgRP injection with pair-feeding decreased the levels of UCP-1 protein in BAT (33). Furthermore, the extent of BAT UCP-1 mRNA downregulation in HS014-infused rats was at least as great as that induced by 6-day ICV NPY infusion. Recently, it was reported that ICV NPY infusion or antagonism of central MC3 and MC4 receptors with AgRP could reduce the plasma levels of thyroid-stimulating hormone and T4 (33,47,48). Reduced thyroid function could reduce wholebody energy expenditure and contribute to increased energy retention as fat.

The heterogeneity of hormonal and metabolic consequences of central NPY infusion or MC4 receptor antagonism shown in this study are supported by a recent comparison of brain sites of c-Fos activation in response to NPY or the MC3/MC4 receptor antagonist AgRP-(83–132) (49). c-Fos immunoreactivity was significantly increased over control values in the hypothalamic PVN of both NPY- and AgRP-injected rats, measured at 2 h after ICV injection. In contrast, only NPY significantly increased c-Fos activation in the NTS at this time point, although AgRP did produce a lesser degree of c-Fos activation in this site at 24 h after injection. Only AgRP resulted in significantly increased c-Fos immunoreactivity in the accumbens shell and the lateral septum (49). The PVN is implicated as a site regulating BAT thermogenesis (9) and activity of the thyrotropic axis (48), whereas the NTS is implicated in mediation of NPY-induced insulin secretion (50). Collectively, these data fit the hypothesis that both NPY and MC receptor antagonism reduce thyroid function (47,48) and BAT UCP-1 expression by effects on the PVN, yet only NPY has significant effects on basal hyperinsulinemia, mediated by neuronal activation in the NTS.

In conclusion, the differences in hormonal and metabolic perturbations that develop during the early stages of ICV NPY and HS014 infusions, using doses that produce similar increases in adiposity, suggest that NPY and MC4 receptor antagonism induce increases in fat mass via at least partially divergent pathways. In the NPY obesity model, hypercorticosteronemia and hyperinsulinemia are early onset factors. In contrast, the increase in adiposity induced by chronic central MC4 receptor antagonism does not appear to involve hypercorticosteronemia or basal hyperinsulinemia. However, it is likely that the etiology of both obesity models involves increased glucose-induced insulin secretion, possibly secondary to increased fat mass and insulin resistance, and downregulation of BAT UCP-1 mRNA expression, which may be mediated by changes in thyroid function as recently reported (33,47,48).

ACKNOWLEDGMENTS

The National Health and Medical Research Council of Australia has supported this work via a Center Grant to the Garvan Institute and a postdoctoral fellowship awarded to A. Sainsbury (Peter Doherty Fellowship 987122).

We thank Dr. Herbert Herzog, Marjorie Liu, Michelle Couzens, and Elizabeth Smith (Garvan Institute, Sydney, Australia) for provision of the UCP-1 and GAPDH probes and help with Northern analysis. We thank Dr. Julie Ferguson and Dr. Jenny Kingham for their invaluable veterinary advice and the staff of the Garvan Institute Biological Testing Facility.

GRAPH: FIG. 1. Total ad libitum food intake in the 4 h after acute ICV injection of NPY (1.2 nmol) or HS014 (1.5 nmol) compared with ICV vehicle-injected control rats (5.0 µl of 0.9%

NaCl). Plotted values are means ± SD of 4-7 rats per group. **P < 0.01 NPY vs. control; ##P < 0.01 HS014 vs. control.

GRAPH: FIG. 2. Change in body weight (A), WAT weights as a percent of body weight (B), and plasma leptin levels (C) measured after 5–6 days of ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. Plotted values are means ± SD of 8–27 rats per group. *P < 0.05 NPY vs. control; #P < 0.05 HS014 vs. control group; †P < 0.05 NPY vs. HS014.

GRAPH: FIG. 3. Plasma levels of corticosterone (A), insulin (B), and glucose (C) measured during 6-day ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. Plotted values are means ± SD of 5–15 rats per group. *P < 0.05 NPY vs. corresponding control; †P < 0.05 NPY versus corresponding HS014.

GRAPH: FIG. 4. Plasma levels of insulin (A) and glucose (B) measured at 1 and 5 min after an intravenous glucose injection (300 mg/kg) in rats ICV-infused for 3–4 days with NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) and pair-fed with vehicle-infused controls. Plotted values are means ± SD of 4–14 rats per group. *P < 0.05 NPY vs. corresponding control; #P < 0.05 HS014 vs. corresponding control.

GRAPH: FIG. 5. Plasma levels of FFAs (A), TGs (B), and hepatic TG content (C) measured after 6 days of ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. Plotted values are means ± SD of 4–15 rats per group. *P < 0.05 NPY vs. corresponding control; ##P < 0.01 HS014 vs. corresponding control; †P < 0.05 NPY vs. corresponding HS014.

GRAPH: FIG. 6. Levels of mRNA for UCP-1 in BAT after 6 days of ICV infusion of NPY (3.5 nmol/day) or HS014 (4.8 nmol/day) in rats pair-fed with vehicle-infused controls. UCP-1 mRNA levels were standardized against those of GAPDH, and expressed as a percentage of controls. Plotted values are means ± SD of 5–7 rats per group. *P < 0.05 NPY versus corresponding control; ##P < 0.01 HS014 vs. corresponding control.

REFERENCES

1. Kalra SP, Dube MG, Pu SY, Xu B, Horvath TL, Kaita PS: Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocrine Rev 20:68–100, 1999
2. Coleman DL: Diabetes-obesity syndromes in mice. Diabetes 31 (Suppl. 1):1–6, 1982
3. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432, 1994
4. Chen H, Charlat O, Tartaglia LA, Wooff EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP: Evidence that the diabetes gene encodes the leptin receptor, identification of a mutation in the leptin receptor gene in db/db mice. Cell 84:491–495, 1996
5. Lee GH, Proenca R, Montes JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM: Abnormal splicing of the leptin receptor in diabetic mice. Nature 379:632–635, 1996
6. Dryden S, Frankish H, Wang Q, Williams G: Neuropeptide Y and energy balance. One way ahead for the treatment of obesity?. Eur J Clin Invest 24:293–308, 1994
7. Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, MacKellar W, Rosteck PR, Schoner B, Smith D, Tinsley FC, Zhang XY, Heiman M: The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature 377:530–532, 1995
8. Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF: Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. Peptides 7:1189–1192, 1986
9. Kotz CM, Briggs JE, Grace MK, Levine AS, Billington CJ: Divergence of the feeding and thermogenic pathways influenced by NPY in the hypothalamic PVN of the Rat. Am J Physiol 44:R471–R477, 1998
10. Zarjevski N, Cusin I, Vettor R, Rohner-Jeanrenaud F, Jeanrenaud B: Intracerebroventricular administration of neuropeptide Y to normal rats has divergent effects on glucose utilization by adipose tissue and skeletal muscle. Diabetes 43:764–769, 1994

11. Sainsbury A, Rohner-Jeanrenaud F, Cusin I, Zakrzewska KE, Halban PA, Gaillard RC, Jeanrenaud B: Chronic central neuropeptide Y infusion in normal rats: status of the hypothalamo-pituitary-adrenal axis, and vagal mediation of hyperinsulinaemia. Diabetologia 40:1269–1277, 1997
12. Pierroz DD, Catzeflis C, Aebi AC, Rivier JE, Aubert ML: Chronic administration of neuropeptide Y into the lateral ventricle inhibits both the pituitary-testicular axis and growth hormone and insulin-like growth factor I secretion in intact adult male rats. Endocrinology 137:3–12, 1996
13. Yen TT, Gill AM, Frigeri LG, Barsh GS, Wolff GL: Obesity, diabetes, and neoplasia in yellow A(vy)/— mice: ectopic expression of the agouti gene. Faseb J 8:479–488, 1994
14. Boston BA, Blaydon KM, Varnerin J, Cone RD: Independent and additive effects of central POMC and leptin pathways on murine obesity. Science 278:1641–1644, 1997
15. Graham M, Shutter JR, Sarmiento U, Sarosi I, Stark KL: Overexpression of AGRT leads to obesity in transgenic mice. Nat Genet 17:273–274, 1997
16. Harrold JA, Widdowson PS, Williams G: Altered energy balance causes selective changes in melanocortin-4 (MC4-R), but not melanocortin-3 (MC3-R), receptors in specific hypothalamic regions: further evidence that activation of MC4-R is a physiological inhibitor of feeding. Diabetes 48:267–271, 1999
17. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson PA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F: Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88:131–141, 1997
18. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS: Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein [published erratum appears in Science. 281:1615, 1998]. Science 278:135–138, 1997
19. Fong TM, Mao C, MacNeil T, Kalyani R, Smith T, Weinberg D, Tota MR, Van der Ploeg LH: ART (protein product of agouti-related transcript) as an antagonist of MC-3 and MC-4 receptors. Biochem Biophys Res Commun 237:629–631, 1997
20. Mizuno TM, Mobbs CV: Hypothalamic agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. Endocrinol 140:814–817, 1999
21. Korner J, Wardlaw SL, Liu SM, Conwell IM, Leibel RL, Chua SC Jr: Effects of leptin receptor mutation on Agrp gene expression in fed and fasted lean and obese (LA/N-faf) rats. Endocrinology 141:2465–2471, 2000
22. Hagan MM, Rushing PA, Pritchard LM, Schwartz MW, Strack AM, Van Der Ploeg LH, Woods SC, Seeley RJ: Long-term orexigenic effects of AgRP-(83-132) involve mechanisms other than melanocortin receptor blockade. Am J Physiol 279:R47–R52, 2000
23. Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DGA, Small CJ, Edwards CMB, Heath MM, Stanley SA, Seal LJ, Bhatti JR, Smith DM, Ghatei MA, Bloom SR: Hypothalamic localization of the feeding effect of agouti-related peptide and α-melanocyte-stimulating hormone. Diabetes 49:177–182, 2000
24. Kask A, Pahkla R, Irs A, Rago L, Wikberg JE, Schioth HB: Long-term administration of MC4 receptor antagonist HS014 causes hyperphagia and obesity in rats. Neuroreport 10:707–711, 1999
25. Raposinho PD, Castillo E, d'Alleves V, Broqua P, Pralong FP, Aubert ML: Chronic blockade of the melanocortin 4 receptor subtype leads to obesity independently of neuropeptide Y action, with no adverse effects on the gonadotropic and somatotropic axes. Endocrinology 141:4419–4427, 2000
26. Marsh DJ, Miura GI, Yagaloff KA, Schwartz MW, Barsh GS, Palmiter RD: Effects of neuropeptide Y deficiency on hypothalamic agouti-related protein expression and responsiveness to melanocortin analogues. Brain Res 848:66–77, 1999
27. Kesterson RA, Huszar D, Lynch CA, Simerly RB, Cone RD: Induction of neuropeptide Y gene expression in the dorsal medial hypothalamic nucleus in two models of the agouti obesity syndrome. Mol Endocrinol 11:630–637, 1997
28. Kask A, Schioth HB, Harro J, Wikberg JE, Ragu L: Orexigenic effect of the

melanocortin MC4 receptor antagonist HS014 is inhibited only partially by neuropeptide Y Y1 receptor selective antagonists. Can J Physiol Pharmacol 78:143–149, 2000
29. Kask A, Rago L, Korrovits P, Wikberg JE, Schioth HB: Evidence that orexigenic effects of melanocortin 4 receptor antagonist HS014 are mediated by neuropeptide Y. Biochem Biophys Res Commun 248:245–249, 1998
30. Dryden S, Frankish HM, Wang Q, Pickavance L, Williams G: The serotonergic agent fluoxetine reduces neuropeptide Y levels and neuropeptide Y secretion in the hypothalamus of lean and obese rats. Neuroscience 72:557–566, 1996
31. Sainsbury A, Wilks D, Cooney GJ: Interaction between adrenal glucocorticoids and parasympathetic activation in mediating hyperinsulinemia during long-term central neuropeptide Y infusion in rats. Diabetologia 43:859–865, 2000
32. Clark JT, Kalra PS, Kalra SP: Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. Endocrinology 117:2435–2442, 1985
33. Small CJ, Kim MS, Stanley SA, Mitchell JRD, Murphy K, Morgan DGA, Ghatei MA, Bloom SR: Effects of chronic central nervous system administration of agouti-related protein in pair-fed animals. Diabetes 50:248–254, 2001
34. Zarjevski N, Cusin I, Vettor R, Rohner-Jeanrenaud F, Jeanrenaud B: Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity. Endocrinology 133:1753–1758, 1993
35. Dagogo-Jack S: Regulation and possible significance of leptin in humans: leptin in health and disease. Diabetes Rev 7:23–38, 1999
36. Dallman MF, Strack AM, Akana SF, Bradbury MJ, Hanson ES, Scribner KA, Smith M: Feast and famine: critical role of glucocorticoids with insulin in dally energy iow. Front Neuroendocrinol 14:303–347, 1993
37. Assimacopoulos-Jeannet F, Jeanrenaud B: The hormonal and metabolic basis of experimental obesity. Clin Endocrinol Metab 5:337–365, 1976
38. Standridge M, Alemzadeh R, Zemel M, Koontz J, Moustaid-Moussa N: Diazoxide down-regulates leptin and lipid metabolizing enzymes in adipose tissue of Zucker rats. FASEB J 14:455–460, 2000
39. Cusin I, Terrettaz J, Rohner-Jeanrenaud F, Zarjevski N, Assimacopoulos-Jeannet F, Jeanrenaud B: Hyperinsulinemia increases the amount of GLUT4 mRNA in white adipose tissue and decreases that of muscles: a clue for increased fat depot and insulin resistance. Endocrinology 127: 3246–3248, 1990
40. Marangou AG, Weber KM, Boston RC, Aitken PM, Heggie JCP, Hirsner RLG, Best JD, Alford FP: Metabolic consequences of prolonged hyperinsulinemia in humans: evidence for induction of insulin insensitivity. Diabetes 35:1383–1389, 1986
41. Pontiroli AE, Alberetto M, Pozza G: Patients with insulinoma show insulin resistance in the absence of arterial hypertension. Diabetologia 35: 294–295, 1992
42. Hwa JJ, Witten MB, Williams P, Ghibaudi L, Gao J, Salisbury BG, Mullins D, Hamud F, Strader CD, Parker EM: Activation of the NPYY5 receptor regulates both feeding and energy expenditure. Am J Physiol 277: R1428–R1434, 1999
43. Currie PJ, Coscina DV: Dissociated feeding and hypothermic effects of neuropeptide Y in the paraventricular and perifornical hypothalamus. Peptides 16:599–604, 1995
44. Szreder Z, Hori T, Kaizuka Y: Thermoregulatory effect of intacerebral injections of neuropeptide Y in rats at different environmental temperatures. Gen Pharmacol 25:85–91, 1994
45. Egawa M, Yoshimatsu H, Bray GA: Neuropeptide Y suppresses sympathetic activity to interscapular brown adipose tissue in rats. Am J Physiol 260:R328–R334, 1991
46. Tritos NA, Elmquist JK, Mastaitis JW, Flier JS, Maratos-Flier E: Characterization of expression of hypothalamic appetite-regulating peptides in obese hyperleptinemic brown adipose tissue-deficient (uncoupling protein-promoter-driven diphtheria toxin A) mice. Endocrinology 139: 4634–4641, 1998
47. Kim MS, Small CJ, Stanley SA, Morgan DG, Seal LJ, Kong WM, Edwards CM, Abusnana S, Sunter D, Ghatei MA, Bloom SR: The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. J Clin Invest 105:1005–1011, 2000
48. Fekete C, Kelly J, Mihaly E, Sarkar S, Rand WM, Legradi G, Emerson CH, Lechan

RM: Neuropeptide Y has a central inhibitory action on the hypothalamic- pituitary-thyroid axis. Endocrinology 142:2606–2613, 2001
49. Hagan MM, Benoit SC, Rushing PA, Pritchard LM, Woods SC, Seeley RJ: Immediate and prolonged patterns of agouti-related peptide-(83–132)-induced c-Fos activation in hypothalmic and extrahypothalamic sites. Endocrinology 142:1050–1056, 2001
50. Dunbar JC, Ergene E, Barraco RA: Neuropeptide-Y stimulation of insulin secretion is mediated via the nucleus tractus solitarius. Horm Metab Res 24:103–105, 1992