Energy Metabolic Profile of Mice after Chronic Activation of Central NPY Y1, Y2, or Y5 Receptors

Melanie Henry, Lorraine Ghibaudi, Jun Gao, and Joyce J. Hwa

Abstract

HENRY, MELANIE, LORRAINE GHIBAUDI, JUN GAO, AND JOYCE J. HWA. Energy metabolic profile of mice after chronic activation of central NPY Y1, Y2, or Y5 receptors. *Obes Res.* 2005;13:36–47.

Objective: Neuropeptide Y (NPY), a 36-amino acid peptide with orexigenic properties, is expressed abundantly in the central nervous system and binds to several NPY receptor subtypes. This study examines the roles of the NPY Y1, Y2, and Y5 receptor(s) in energy homeostasis.

Research Methods and Procedures: We administered intracerebroventricular NPY (3 μg/d) or selective peptide agonists for the Y1, Y2, and Y5 receptor subtypes to C57Bl/6 mice for 6 days by mini-osmotic pumps to assess the role of each receptor subtype in NPY-induced obesity. Energy expenditure (EE) and respiratory quotient (RQ) were studied using indirect calorimetry. Adiposity was measured by DXA scanning and fat pad dissection. Insulin sensitivity was tested by whole-blood glucose measurement after an insulin challenge.

Results: Central administration of the selective Y1 agonist, Y5 agonist, or NPY for 6 days in mice significantly increased body weight, adiposity, and RQ, with significant hyperphagia in the Y5 agonist- and NPY-treated groups but not in the Y1 agonist-treated group. The NPY, Y1, or Y5 agonist-treated mice had little change in total EE during ad libitum and pair-feeding conditions. Conversely, selective activation of the Y2 receptor reduced feeding and resulted in a significant, but transient, weight loss.

Discussion: Central activation of both Y1 and Y5 receptors increases RQ and adiposity, whereas only Y5 receptor activation reduces energy expended per energy ingested. Selective activation of Y2 autoreceptors leads to hypophagia and transient weight loss, with little effect on total EE. Our study indicates that all three NPY receptor subtypes may play a role in regulating energy homeostasis in mice.

Key words: adiposity, indirect calorimetry, insulin sensitivity, respiratory quotient, food intake

Introduction

Neuropeptide Y (NPY)[1] is a 36-amino acid peptide distributed widely throughout both the central and peripheral nervous systems (1,2) and has been implicated as a key regulator of energy balance due to its pro-obesity properties (3). For example, hypothalamic NPY mRNA levels are increased under conditions of both food restriction and intense exercise (4) and return to normal levels after refeeding (5). In addition, central NPY mRNA expression is increased in genetic models of obesity including *ob/ob* mice (6), Zucker rats (7), and streptozotocin-induced diabetic rats (8). Elevated NPY tone, resulting from endogenous expression or exogenous administration, correlates positively to energy intake and negatively to energy expenditure (EE), a combination that results in obesity (3).

NPY belongs to the pancreatic polypeptide family and exerts its effects through activation of G-protein-coupled receptors known as Y1, Y2, Y3, Y4, Y5, or Y6 receptors (9). Because NPY binds to the Y1, Y2, and Y5 receptors with high affinities (10), many studies have been devoted to the identification of NPY receptor subtype(s) responsible for the regulation of energy homeostasis. Through the use of subtype-selective agonists, it has been demonstrated that Y1

Received for review August 21, 2003.
Accepted in final form November 5, 2004.
The costs of publication of this article were defrayed, in part, by the payment of page charges. This article must, therefore, be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Schering-Plough Research Institute, Kenilworth, New Jersey.
Address correspondence to Joyce J. Hwa, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033.
E-mail: joyce.hwa@spcorp.com

[1] Nonstandard abbreviations: NPY, neuropeptide Y; EE, energy expenditure; ICV, intracerebroventricular; PF, pair-fed; RQ, respiratory quotient; BMC, bone mineral content; AUC, area under the curve; EI, energy intake; ad lib, ad libitum.

and/or Y5 receptor subtypes may be responsible for NPY-induced regulation of energy intake. For example, central administration of either a selective Y1 or Y5 receptor agonist stimulates feeding (11,12). Conversely, selective agonists of the NPY Y2 or Y4 receptor fail to induce a feeding response in satiated rodents, suggesting that these receptor subtypes do not mediate the orexigenic response elicited by NPY (13). However, recent studies indicate that the alleged inhibitory Y2 autoreceptor (14), which is expressed on NPY neurons in the arcuate nucleus, may actually have anorexic properties and serve to counterregulate the orexigenic characteristics of NPY. Peptide $YY_{(3-36)}$, a Y2 agonist, is released from the gut in response to a meal, and peripheral injection of $PYY_{(3-36)}$ results in decreased food intake (15,16). Consistent with the hypothesis that activation of the Y2 receptor promotes leanness, NPY2R-null mice are hyperphagic, less active, and have elevated body weight, body fat mass, and increased bone formation (17,18).

Data obtained from NPY and Y1 or Y5 receptor knockout animals have been surprising. First, mice deficient in NPY do not display a lean or anorectic phenotype, which challenges the assertion that the endogenous neuropeptide is essential for energy homeostasis. Although a role for the NPY Y1 and Y5 receptor subtypes in feeding behavior has been established through acute administration of selective agonists, studies of mice deficient for the NPY Y1 or Y5 receptor have been unsuccessful in producing a lean phenotype. In fact, mice lacking either the Y1 or Y5 receptors paradoxically develop mild late-onset obesity (19,20). The opposite phenotypes observed with genetic manipulation suggest that compensation for the absence of NPY or the Y1 vs. Y5 receptors may occur during development. For example, the agouti-related protein expression is increased in the hypothalamus of the NPY knockout mouse, and Y1-deficient mice display reduced locomotor activity (18). However, the inhibition of fasting-induced feeding observed in the Y1 receptor knockout mouse and the reduction of NPY-induced feeding in the Y5 receptor knockout mouse support a role for these receptors in feeding regulation (20,21). Addition of a Y1 antagonist to the Y5 knockout mice is required to completely abolish NPY-induced feeding, which implies that both receptor subtypes may be involved in NPY-mediated feeding responses.

Some selective antagonists of the Y1 or Y5 receptors have been reported to reduce both spontaneous and fasting-induced feeding (22–24). However, blockade of NPY-induced feeding requires very high doses of antagonists that approach levels associated with toxicity, thereby casting doubt that the feeding inhibition observed is specific. Data obtained from antisense oligodeoxynucleotides directed against either the Y1 or Y5 receptor have been questioned due to conflicting results and concerns about selectivity and toxicity (24).

The effects of NPY and its receptors on energy metabolism and nutrient partitioning have yet to be rigorously characterized. We have previously shown that acute central administration of NPY decreases thermogenesis in rats through activation of the Y5 receptor (13). The present data explore the energy metabolic profile of NPY using sustained activation of NPY and the Y1, Y2, and Y5 receptors in the brain by chronic infusion through a mini-osmotic pump. To clarify the role of the Y5 receptor subtype on energy balance, we chose the peptide reported by Beck-Sickinger et al. (25), [cPP]1-7,NPY19-23,Ala31,Aib32,Gln34]hPP (Y5 agonist). This peptide is 12- and 145-fold more selective than D-Trp^{34} or D-Trp^{32} NPY, respectively (10). In addition, this Y5 agonist has over 2000-fold greater affinity for Y5 over the Y1 or Y2 receptor and over 200-fold greater affinity for the Y4 receptor subtype, and is 3-fold more potent than the native ligand, NPY. The Y1-selective peptide chosen, D-Arg^{25} NPY (Y1 agonist), binds the Y1 receptor with 12-, 82-, and 48-fold higher affinity than the Y2, Y4, and Y5 receptor subtypes, respectively. For Y2 receptor activation, we used Ac-$NPY_{(24-36)}$,(Leu28,Leu30) (Y2 agonist), which is 5000-fold more selective for the Y2 receptor over either the Y1 or Y5 receptor subtypes and is reported to inhibit fasting-induced feeding after central injection (15). We compared the effects of NPY and selective Y1, Y2, or Y5 receptor agonists on energy intake, EE, adiposity, and insulin sensitivity after 6 days of central infusion by mini-osmotic pump.

Research Methods and Procedures

Animals

Six-week-old C57BL/6 mice (25 to 30 grams; Charles River Laboratories, Inc., Wilmington, MA) were maintained in individual cages at 22 °C on a 12-hour light/dark cycle with lights on at 7 AM. Mice had free access to food (Harlan Teklad rodent chow no. 8604; Madison, WI) and water throughout the study. All studies were conducted in a facility accredited by the American Association for Accreditation of Laboratory Animal Care following protocols approved by the Animal Care and Use Committee of the Schering-Plough Research Institute.

Surgery

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (100:20 mg/kg). A 26-gauge stainless steel obturator-filled cannula was implanted in the right lateral ventricle using Vetabond surgical glue to adhere the guide cannula and custom stainless steel adapter to the skull. Intracerebroventricular (ICV) coordinates (from bregma, 0.8 mm posterior, 1.5 mm lateral, 2.0 mm ventral) were obtained from the atlas of Franklin and Paxinos (26). After a 2-week recovery period, mice were tested for cannula placement by ICV infusion of NPY (0.1 nmol).

Satiated animals demonstrating a profound feeding effect (>0.5 grams) within 60 minutes of infusion were retained for the study.

Mini-osmotic Pump Implantation

Mini-osmotic pumps (model 2001; DURECT Corp., Palo Alto, CA), prepared under aseptic conditions contained 3 μg/d NPY, D-Arg^{25}-NPY, Ac-NPY$_{(24-36)}$, (Leu28, Leu30), [cPP 1-7, NPY 19-23, Ala 31, Aib 32, Gln 34]hPP, or vehicle (0.9% NaCl in water with 0.01% ascorbic acid filtered by a 0.5-mm membrane). All NPY-related peptides were synthesized by Anaspec Inc. (San Jose, CA). The prepared pumps were primed in saline overnight in a 37 °C water bath. Mice were anesthetized by inhalation of an isoflurane and oxygen mixture. The mini-osmotic pumps were then inserted in the subcutaneous interscapular region, and the incision was closed by suture. A line of PE60 tubing connected the minipump to the internal ICV cannula, which projected 0.3 mm from the guide cannula. The body weight and food were monitored immediately after surgery and daily for the remainder of the study.

Feeding and Body Weight Monitoring

All mice were given pre-weighed chow pellets in their home cage daily. The leftover food weight was recorded, and new pellets were placed in the bedding each day. Mice were accustomed to this routine before implant surgery to reduce the chance of variability due to novelty in procedure. Pair-fed (PF) test peptide-infused mice were monitored in the same manner as described above, with the exception that the food intake was restricted to the control mice level. Because infusion of the Y2 agonist resulted in hypophagia and weight loss, animals in the PF arm of this study were vehicle-infused, then pair fed to levels of Y2 agonist-treated mice.

Leptin and Insulin Determination

Non-fasted mice were anesthetized by inhalation of an isoflurane and oxygen mixture, and blood samples were obtained by heart puncture. Samples were centrifuged and stored as serum at −20 °C until assayed. Serum leptin and insulin levels were determined by commercially available kits: mouse leptin enzyme-linked immunosorbent assay kit (Crystal Chem Inc., Chicago, IL) and rat insulin enzyme-linked immunosorbent assay kit (Alpco Diagnostics, Windham, NH).

Intraperitoneal Insulin Tolerance Testing

Fasting glucose measurements were determined by Glucometer Elite (Bayer, Mishawaka, IN) after small blood samples were collected by the tail bleeding for time 0, after an overnight 16-hour fasting period. At time 0, animals also received a single intraperitoneal injection of human insulin (0.75U/kg$^{0.75}$), and tail blood samples were collected at 20, 40, 60, and 120 minutes post-injection.

Body Composition

After blood collection, mice were sacrificed by CO_2 gas and frozen at −20 °C until body composition was determined using DXA (pDEXA; Norland Medical Systems, Inc., White Plains, NY). Mice were thawed on the day of the scan, and fat pads were dissected out and weighed immediately after the scan procedure.

Indirect Calorimetry Method

Another group of ICV cannulated mice was used to determine the effects of EE. One week after NPY placement testing, ICV cannulated C57BL/6 mice were implanted with mini-osmotic pumps containing either vehicle or test peptide as described above. Mice were either fed ad libitum (ad lib) or pair fed for 5 days. Oxygen consumption and carbon dioxide production rates were monitored every 30 minutes (settle time, 155 seconds; measure time, 45 seconds) for 22 h/d with the use of a mouse indirect calorimeter (Oxymax; Columbus Instruments, Columbus, OH). Water and food were available ad lib in the calorimeter chamber. After mice were subjected to 16 hours of fast from the beginning of the dark cycle on Day 6, total EE and respiratory quotients (RQs) were monitored for 4 hours under fasting conditions. Measurements were taken in an airtight Oxymax chamber with an airflow rate of 0.5 L/min. RQ was calculated as the molar ratio of $V\mathrm{CO}_2$ to $V\mathrm{O}_2$. EE was calculated from heat (kilocalories) = $V\mathrm{O}_2 \times (3.815 + 1.232 \times \mathrm{RQ})$. Percentage of energy fuel derived from carbohydrate or fat oxidation was determined from $V\mathrm{O}_2$ and $V\mathrm{CO}_2$ using the methods of Elia and Livesey (27).

Statistical Analysis

Results are given as means ± SE. Statistically significant differences between vehicle and peptide-treated mice in food intake, body weight, and plasma parameters were determined by one-way ANOVA with a multiple-comparison post-test. When the group data showed unequal variances, the non-parametric Wilcoxon/Kruskal-Wallis and Dunn's multiple comparison post hoc tests were used. Correlations of body fat mass and fat pads were analyzed by linear Pearson correlation. All statistical tests were performed by GraphPad InStat version 3.01 for Windows 95 (GraphPad Software, San Diego, CA). $p < 0.05$ was considered statistically significant.

Results

Effect of NPY and the Y1 and the Y5 Agonists on Feeding and Body Weight Gain after 6 Days of Infusion

Central administration of only NPY and the Y5 agonist led to a significant increase in food intake compared with

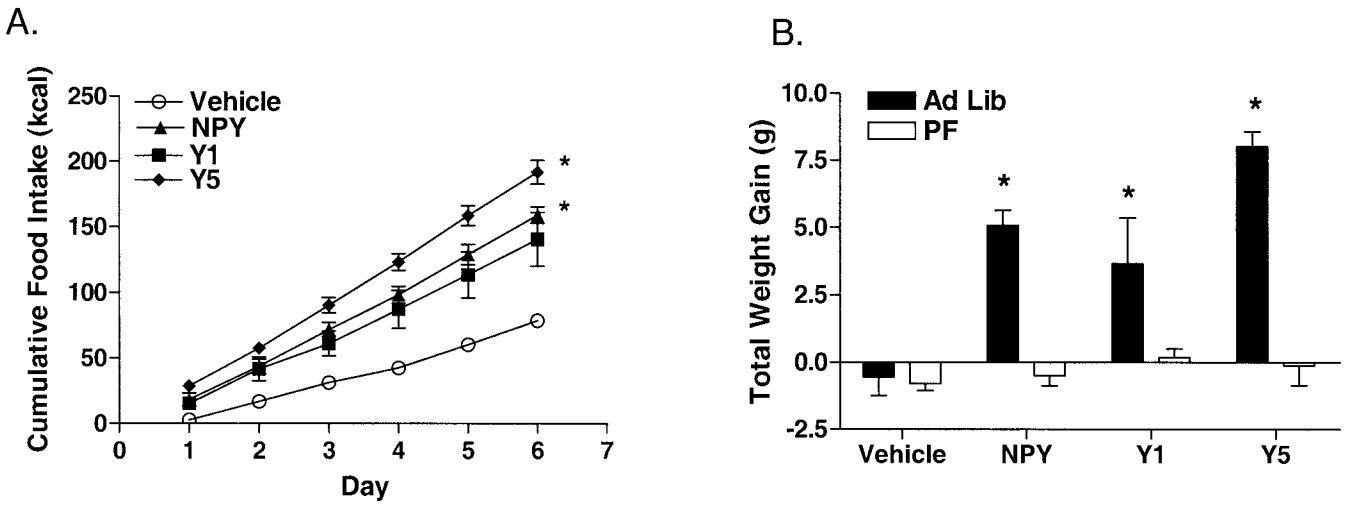

Figure 1: Effects on feeding and cumulative body weight gain after 6 days of ICV infusion of NPY or the Y1 or Y5 agonists in C57BL/6 mice. (A) Six days of infusion produced a significant increase in the cumulative EI of mice treated with NPY (▲) or the Y5 agonist (♦) compared with vehicle-treated controls (○) ($n = 5$ to 6). (*) $p < 0.05$. (B) Six days of constant ICV infusion resulted in significant body weight gain in mice treated with the NPY-, Y1-, or Y5-selective agonists compared with vehicle controls under ad lib conditions (solid bars). (*) $p < 0.01$. Mice PF to vehicle control levels had similar body weights as vehicle-treated mice at the end of the 6-day infusion (open bars). Results are means ± SE.

the vehicle control group ($n = 5$ to 6/group) after 6 days of chronic infusion (Figure 1A, Kruskal-Wallis Non-Parametric test with Dunn's multiple test, $p < 0.05$). However, NPY and the Y1 and Y5 agonists all led to significant body weight gain (Figure 1B, ANOVA with Dunnett's multiple test, $p < 0.01$). After the mini-osmotic pump implantation, a small decrease in body weight was observed in mice infused with vehicle that was likely due to surgical recuperation. Chronic ICV infusion of NPY induced a significant increase in food intake from Day 1 (Kruskal-Wallis Non-Parametric test with Dunn's multiple test, $p < 0.05$) that was sustained for the duration of the experiment. Starting from Day 3 to the end of the study, NPY and Y1 agonist-infused mice had significantly higher weight gain than the vehicle control mice. Similarly, mice infused with the Y5 agonist had a significant increase in food intake and weight gain compared with the vehicle group starting from Day 1 (for weight gain, ANOVA with Dunnett's post test, $p < 0.01$; for food intake, Kruskal-Wallis Non-Parametric test with Dunn's multiple test, $p < 0.001$), which persisted for the duration of the experiment. Unlike the rapid onset of NPY- and Y5-induced hyperphagia, the cumulative food intake of the Y1 agonist-stimulated mice was not significantly different from control mice, likely due to large variability. Mice pair fed to vehicle control levels showed no significant alterations in body weight gain after the 6-day infusion period regardless of treatment (Figure 1B).

Effect of NPY and the Y1 and Y5 Agonists on Body Composition Changes after 6 Days of Infusion

NPY and the Y5 agonist-infused mice ($n = 5$ to 6/group) had significantly elevated percentage body fat (Figure 2A, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.001$), larger fat pads in the epididymal and perirenal areas (Figure 2B, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.05$), and higher levels of leptin, a serum marker of body fat mass, compared with control mice (Figure 3, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.05$). Although DXA analysis showed that the Y1-induced increase in percentage body fat was statistically greater from that of the vehicle control group (Figure 2A, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.01$), it was still significantly lower than that of the Y5-stimulated mice (Figure 2A, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.05$). In addition, the epididymal fat pad weights and serum leptin levels of Y1-stimulated mice were significantly higher than those from the control group (Figures 2B and 3, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.05$). None of the PF groups infused with NPY agonists showed any significant differences in fat pad mass compared with controls (Figure 2C). Furthermore, treatment with NPY ($p < 0.001$) and the Y1 ($p < 0.05$) or Y5 ($p < 0.001$) agonist resulted in greater bone mass [bone mineral content (BMC)] compared with controls, and Y5-infused mice displayed greater bone mass than Y1-infused mice (Figure 4, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.05$). None of the PF groups showed any differences in bone mass compared with control.

Effects of NPY and the Y1 and Y5 Agonists on Energy Metabolism

By monitoring energy metabolism using indirect calorimetry, it was determined that NPY- and Y1 and Y5 agonist-

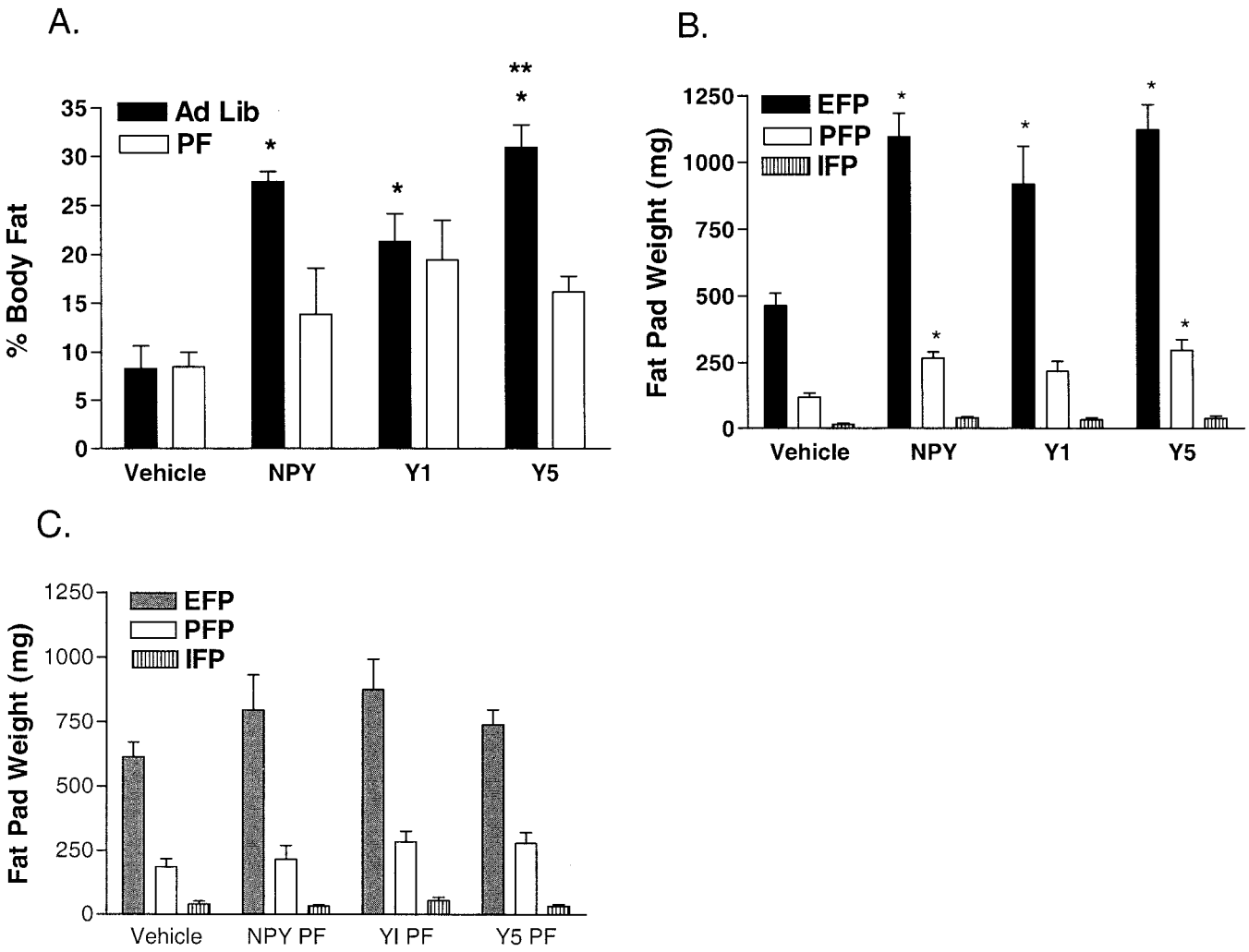

Figure 2: Chronic central activation of NPY, Y1, and Y5 receptors increases percentage fat content and fat pad weights in C57BL/6 mice. (A) Percentage of whole body fat masses determined by DXA scanning was significantly elevated in mice infused for 6 days with 3 μg/d NPY or the Y1 or Y5 agonists (solid bars, $n = 5$ to 6; $p < 0.05$ compared with vehicle controls). Mice treated with the Y5 agonist had significantly higher percentage body fat than Y1 agonist-treated mice. (**) $p < 0.05$. PF mice (open bars, $n = 5$ to 6) showed no significant differences in the percentage of body fat regardless of the peptide infused. (B) Epididymal fat pads were significantly heavier in mice infused with NPY or the selective Y1 or Y5 agonists ($n = 5$ to 6; checkered bars). (*) $p < 0.05$ compared with vehicle controls. Perirenal fat pad weights (open bars) were increased significantly in mice infused with either NPY or the Y5 agonist. No differences were observed in the weights of inguinal fat pads (striped bars) among any of the groups. (C) Regardless of the peptide infused for the 6-day period, fat pads of mice PF to treated animals were similar to those of vehicle-treated mice. Results are means ± SE. EFP, epididymal fat pad; PFP, perirenal fat pad; IFP, inguinal fat pad.

infused mice all had significantly elevated RQ under ad lib conditions compared with control mice. Mice infused with the Y5 agonist also displayed elevated RQ under PF conditions (Table 1, Kruskal-Wallis Non-Parametric test with Dunn's multiple test, $p < 0.05$). However, all groups of mice had similar RQs under fasting conditions (Table 1). Total EE of mice infused with NPY or the Y1 or the Y5 agonists was similar to that of the vehicle control group under ad lib, PF, or fasting conditions (Table 1). Compared with the control group, only the Y5 agonist-infused mice had a significantly lower ratio of EE to energy intake (EI), indicating a reduction of calories metabolized compared with calories consumed (Table 1, Kruskal-Wallis Non-Parametric test with Dunn's multiple test, $p < 0.05$).

Effect of NPY and the Y1 and Y5 Agonists on Insulin Sensitivity

In a separate set of mice ($n = 11$/group), sensitivity to insulin was assessed after an overnight fast on Day 6 of ICV infusion. Insulin sensitivity of NPY- or Y1 agonist-treated mice was similar to that of vehicle control mice (Figure 5A). However, Y5 agonist-stimulated mice were significantly

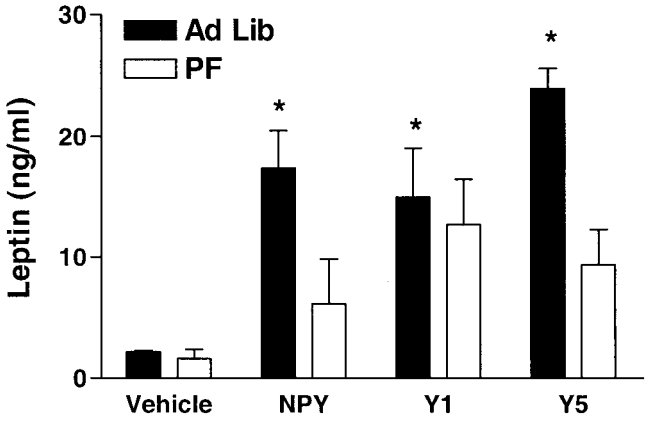

Figure 3: Elevated serum leptin after 6 days of central NPY, Y1, or Y5 activation. Leptin was significantly increased on Day 7 in satiated C57BL/6 mice treated with NPY or the Y1 or Y5 agonists (n = 5 to 6; solid bars). (*) $p < 0.05$ compared with vehicle controls. No statistically significant differences were observed among PF groups (n = 5 to 6, open bars). Results are means ± SE.

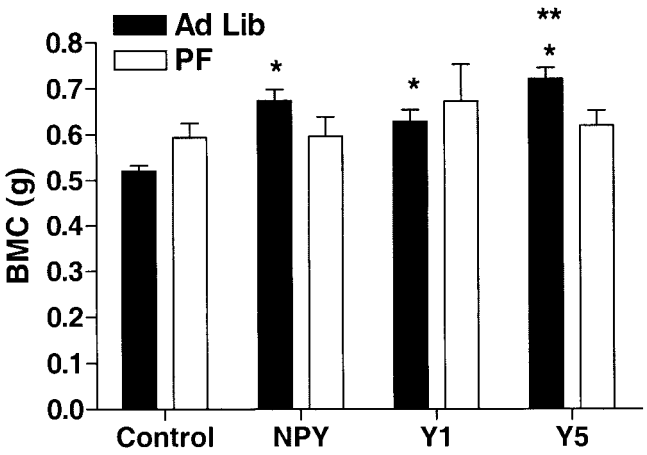

Figure 4: Elevated bone mass after 6 days of ICV infusion with 3 μg/d NPY, the Y1 or Y5 agonists, or vehicle. BMC determined by DXA scanning was significantly increased in mice infused for 6 days with 3 μg/d NPY or the Y1 or Y5 agonists under ad lib conditions (solid bars, n = 5 to 6). (*) $p < 0.05$ compared with vehicle controls. Mice treated with the Y5 agonist had significantly greater BMC than that of Y1-treated animals. (**) $p < 0.05$. However, BMC measures of PF mice (open bars, n = 5 to 6) were not affected by the peptide infusion. Results are means ± SE.

less sensitive to the insulin challenge, with elevated area under the curve (AUC) compared with vehicle control mice (Figure 5B, ANOVA with Dunnett's post test, $p < 0.01$).

Effect of the Y2 Agonist on Feeding and Body Weight Gain after 6 Days of Infusion

In another set of mice, a significant decrease in daily food intake was observed in mice (n = 8/group) infused with the Y2 agonist starting from Day 1, which gradually returned to similar levels of vehicle control mice. By Day 6, cumulative food intake remained significantly lower in Y2-infused mice than in control mice (Figure 6A, Student's t test, $p < 0.05$). Treatment with the Y2 agonist also caused a marked decrease in body weight for the first three postoperative days before returning to within control levels (Figure 6B, Student's t test, $p < 0.05$). In a separate set of mice (n = 6/group) that included a PF group, Y2-infused mice exhibited a similar pattern of weight loss to that observed in the first study. Vehicle-infused mice pair fed to levels of Y2-infused mice showed minimal weight loss, similar to that of vehicle-infused control mice (Figure 6C).

Effect of the Y2 Agonist on Body Composition Changes after 6 Days of Infusion

DXA analysis indicated that Y2-infused mice (n = 8/group) had reduced percentage body fat (Figure 7A, Student's t test, $p < 0.05$) and significantly smaller fat pads in the inguinal, epididymal, and perirenal areas (Figure 7B, Student's t test, $p < 0.05$). However, there were no significant differences in leptin levels or bone mass concentration between Y2-infused mice and control mice (data not shown). We observed an overall higher level of body fat in this set of mice compared with the mice used in the other NPY peptide studies. We repeated the Y2 experiments in a leaner, more comparable set and included a PF arm. In this set of mice (n = 5 to 6/group), Y2-infused mice had significantly reduced percentage body fat compared with vehicle-infused PF mice (Figure 7C, ANOVA with Tukey-Kramer multiple comparison test, $p < 0.05$). This reduction in body fat was consistent with the previous study but was not statistically significant compared with vehicle-infused mice due to smaller sample size and multiple group comparison. However, fat pads from all groups were not significantly affected in this study (Figure 7D), nor were serum leptin levels or bone mass concentration (data not shown).

Effect of the Y2 Agonist on Energy Metabolism

Using the indirect calorimetry method to assess energy metabolism, total EI and total EE were similar among the vehicle, Y2 agonist, and PF groups (n = 5 to 6/group) under both ad lib and fasting conditions. Furthermore, the average RQ of Y2 agonist-infused mice was equivalent to that of the control mice at all time-points. However, the RQ of mice pair fed to the Y2 agonist group was significantly greater than that of vehicle or Y2-infused mice (Table 2, Kruskal-Wallis Non-Parametric test with Dunn's multiple test, $p < 0.05$).

Effect of the Y2 Agonist on Insulin Sensitivity

In a separate set of mice (n = 6/group), sensitivity to insulin was assessed on Day 6 of the ICV infusion after an

Table 1. Effects of NPY, the Y1 agonist, and the Y5 agonist on energy metabolism

Treatment	Vehicle	NPY	Y1	Y5
Ad lib RQ	0.884 ± 0.006	1.021 ± 0.010*	0.965 ± 0.028*	1.049 ± 0.013*
Ad lib EE	60.3 ± 1.6	64.2 ± 1.2	66.2 ± 3.3	66.9 ± 1.3
Ad lib EI	78.7 ± 3.6	158.9 ± 6.4*	140.5 ± 20.4	191.9 ± 9.2*
Ad lib EE/EI	0.77 ± 0.04	0.41 ± 0.01	0.57 ± 0.07	0.35 ± 0.01*
PF RQ	0.898 ± 0.007	0.911 ± 0.010	0.931 ± 0.009	0.936 ± 0.014*
PF EE	64.9 ± 2.0	59.7 ± 3.7	51.9 ± 3.7	53.8 ± 2.6
PF EI	78.4 ± 2.0	80.2 ± 3.1	85.1 ± 0.6	82.2 ± 4.1
PF EE/EI	0.83 ± 0.03	0.74 ± 0.05	0.61 ± 0.05	0.67 ± 0.06
Fasting RQ	0.753 ± 0.011	0.767 ± 0.013	0.755 ± 0.007	0.784 ± 0.016
Fasting EE/h	0.344 ± 0.012	0.370 ± 0.020	0.336 ± 0.010	0.343 ± 0.017

Six days of infusion with NPY, the Y1, and the Y5 agonist significantly elevated RQ, a relative measure of carbohydrate vs. lipid oxidation, under ad lib conditions only. However, only mice treated with the Y5 agonist had elevated RQ levels under PF conditions ($n = 5$ to 6). No differences were observed for any groups under fasting conditions.
* $p < 0.05$.

overnight fast. Insulin sensitivity of Y2 agonist-treated mice was similar to that of vehicle control mice at all time-points; therefore, the AUC of Y2-infused mice was similar to that of vehicle control mice (data not shown).

Discussion

Obesity derives from a chronic imbalance between EI and EE. NPY is one of the key neuropeptides synthesized in the hypothalamus that can integrate metabolic, neuronal, and hormonal signals to regulate energy intake and expenditure to attain energy homeostasis. It has been reported that chronic central infusion of NPY into the brain ventricles of rodents induces hyperphagia, hyperleptinemia, and insulin resistance, a profile that resembles the obesity syndrome of genetically and diet-induced obese animals (28–30). Because NPY binds to the Y1, Y2, and Y5 receptor subtypes

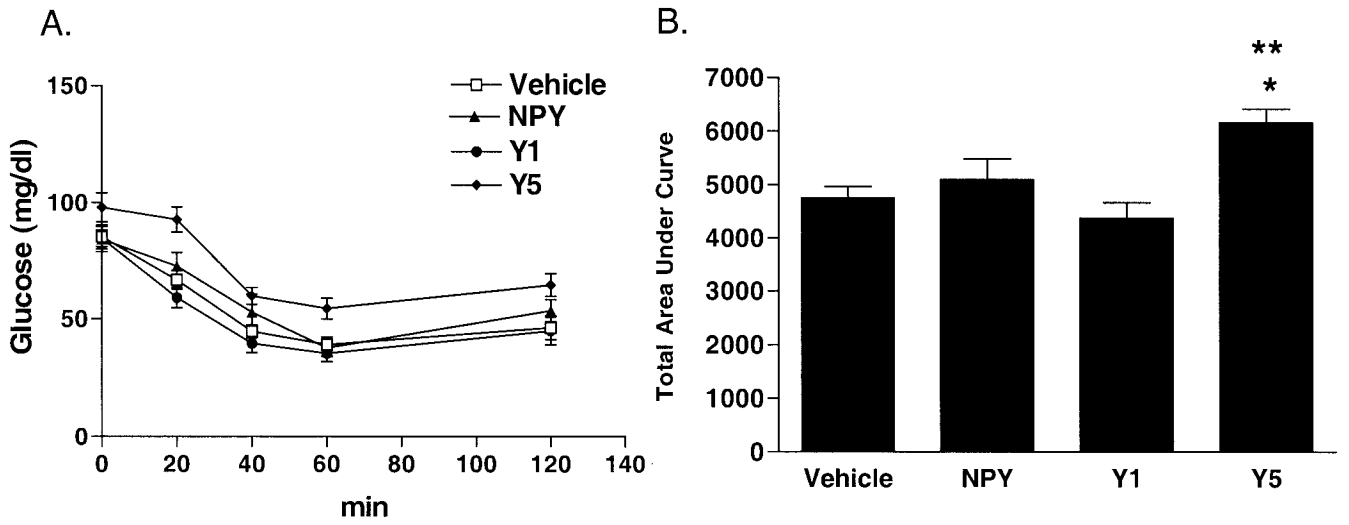

Figure 5: (A) Intraperitoneal insulin tolerance test in C57BL/6 mice after 6 days of ICV infusion with 3 μg/d NPY, the Y1 or Y5 agonists, or vehicle. Insulin sensitivity was assessed after an overnight fast in mice infused with NPY (▲), the Y1 agonist (●), the Y5 agonist (♦), or vehicle (□) for 6 days ($n = 11$/group). At time 0, fasting glucose was similar for all groups. (B) Total AUC was significantly greater in mice treated with the Y5 agonist compared with vehicle-infused controls. (**) $p < 0.01$. (*) Mice infused with the Y1 agonist, $p < 0.001$. Results are means ± SE.

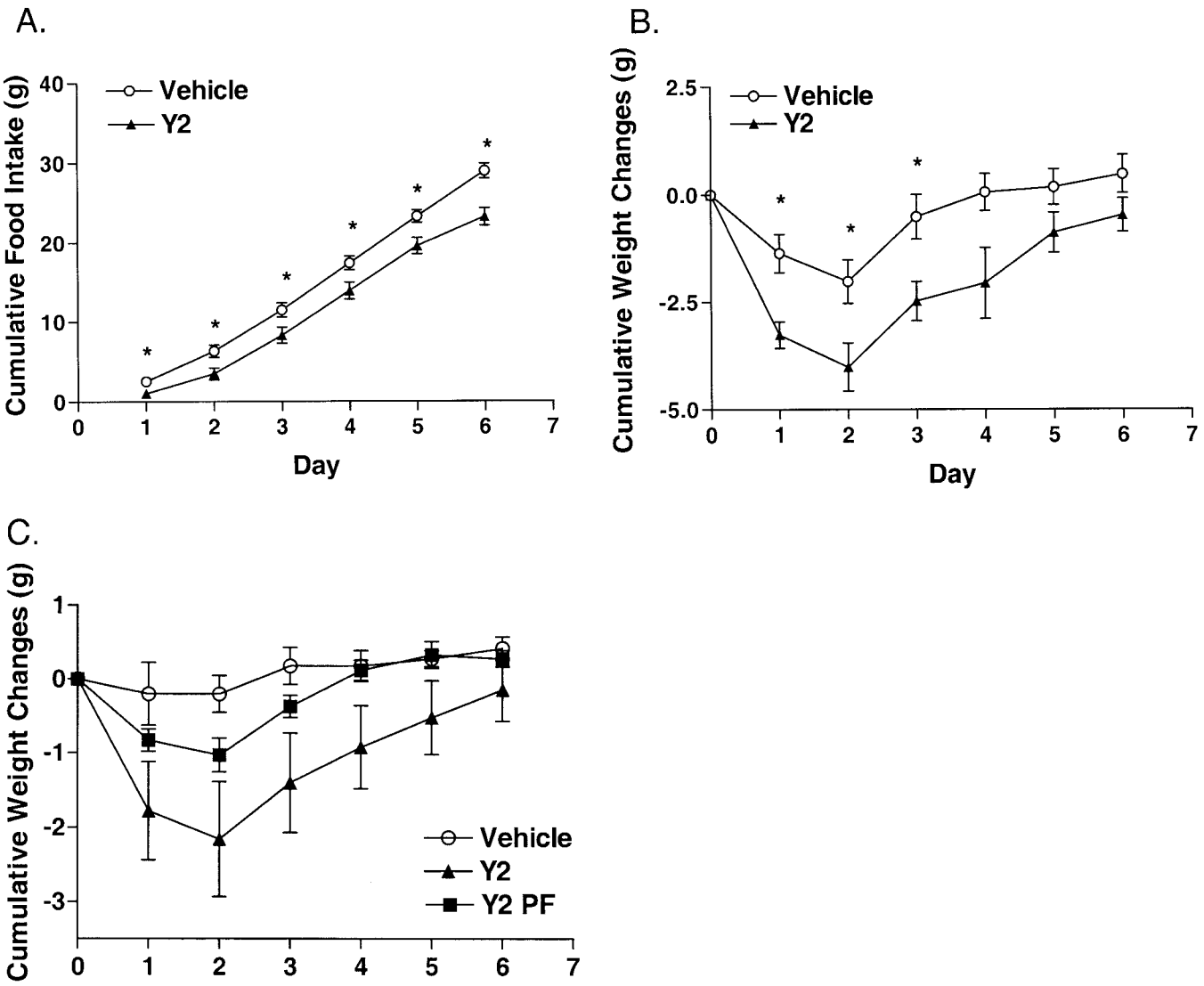

Figure 6: Effects on feeding and body weight after 6 days of ICV infusion of the Y2 agonist in C57BL/6 mice. (A) Mice treated with the Y2 agonist (▲) consumed less than controls throughout the 6-day period ($n = 5$ to 6). (*) $p < 0.05$ compared with vehicle controls (○). (B) Infusion of the Y2-selective agonist (▲) resulted in significant body weight loss during the first 3 days of infusion, but by Day 4, and continuing until Day 6, body weight gain was similar to that of vehicle control mice (○) ($n = 5$ to 6). (*) $p < 0.05$. (C) In a separate set of mice that included a PF group (■), the weight loss pattern of Y2-infused mice (▲) mimicked that of the previous experiment; however, the PF group showed comparable weight loss with that of controls (○) ($n = 5$ to 6/group). Results are means ± SE.

with high affinities, and all subtypes of the NPY receptor are localized in hypothalamic nuclei regulating energy homeostasis (31), it is important to determine the roles of each NPY receptor subtype in the regulation of EI vs. EE and nutrient partitioning. Our data indicate that central Y1, Y2, and Y5 receptors differentially affect energy homeostasis through various mechanisms. For example, chronic activation of central Y5 receptors can lead to weight gain through a combination of hyperphagic and nutrient partitioning effects, whereas chronic activation of central Y1 receptors causes weight gain by altering nutrient partitioning alone. In contrast, chronic activation of central Y2 receptors causes transient weight loss mainly through hypophagia.

Using the indirect calorimetric method, we found that activation of central Y5 receptors with NPY or selective Y5 agonists significantly increased RQ in mice under ad lib and PF conditions. However, central infusion of NPY or the Y1 or Y5 receptor agonists did not significantly change total EE. It has been determined that elevated 24-hour RQ, the ratio of carbon dioxide production and oxygen consumption, is one of the risk factors for weight gain, and it correlates positively with energy balance and inversely with

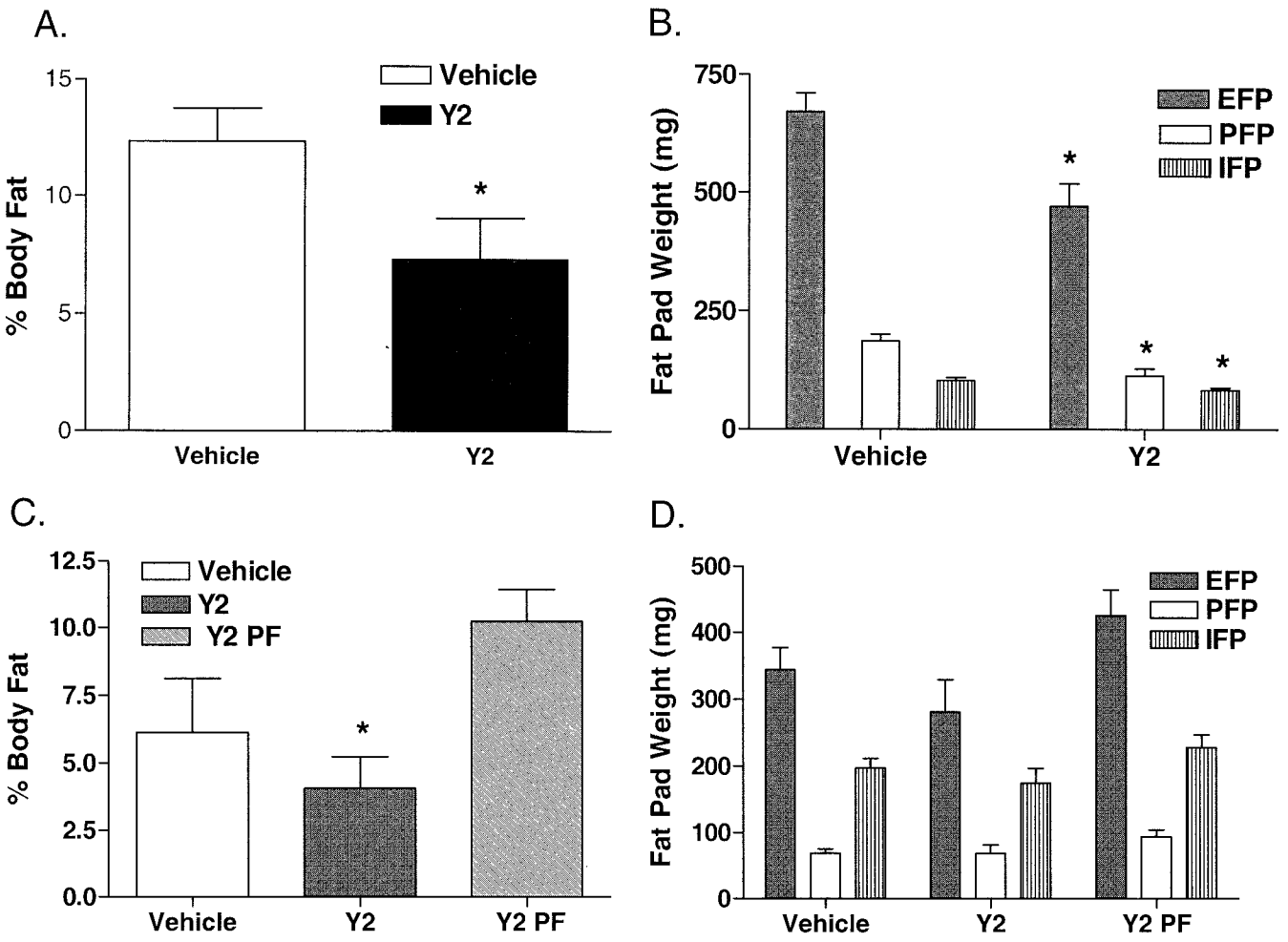

Figure 7: Chronic central activation of NPY Y2 receptors reduces percentage fat content and fat pad weights in C57BL/6 mice. (A) Mice treated with the Y2 agonist (hatched bars, $n = 8$; *, $p < 0.05$) had a significantly lower percentage of body fat mass compared with controls (open bars; $n = 8$) after 6 days of infusion. (B) The weights of all three fat pads were reduced significantly in mice treated with the Y2 agonist compared with vehicle-treated controls ($n = 8$). (*) $p < 0.05$. (C) In a separate set of mice that included a PF arm, Y2 agonist-infused mice (checked bars) had significantly reduced percentage body fat than that of vehicle-infused mice PF to Y2 levels (hatched bars, $n = 5$ to 6). (*) $p < 0.05$. (D) No fat pad differences were observed among the vehicle, Y2 agonist infused, and vehicle infused in this study. Results are means ± SE. EFP, epididymal fat pad; PFP, perirenal fat pad; IFP, inguinal fat pad.

muscle sympathetic nerve activity (32,33). Our data revealed that chronic infusion of NPY-, Y1-, or Y5-selective agonists in the brain under ad lib conditions significantly increased mean daily RQ. The increase in RQ observed in mice infused with selective Y1 or Y5 agonists suggests that central activation of either Y1 or Y5 receptors can modify nutrient partitioning by reducing energy substrates derived from lipid oxidation and/or increasing lipogenesis. When mice infused with NPY, Y1, or Y5 agonists were pair fed, the differences in RQ among the treated groups vs. the vehicle group were significantly reduced compared with ad lib condition. Only the Y5 agonist-infused mice showed a significant elevation of mean RQ under PF conditions, suggesting that central Y1, but not Y5, receptor subtypes modulate nutrient partitioning mainly by regulating EI.

Many studies have attempted to identify the NPY receptor subtypes involved in the regulation of EI (for review, see 24). It has been clearly demonstrated that acute or chronic administrations of NPY analogs that activate the Y5 receptor elicit robust feeding behavior. However, data obtained from selective small molecule Y5 receptor antagonists have been equivocal. For example, CGP71683A, a selective Y5 receptor antagonist with high affinity for the muscarinic receptor and serotonin uptake sites, was reported to diminish spontaneous feeding and fasting-induced feeding (34,35). However, several other selective Y5 antagonists did not have any effect on EI in natural or fasting conditions, which is consistent with the phenotype observed in mice deficient for the Y5 receptor (20,36). Using a Y5-selective agonist, we demonstrated that central activation of

Table 2. Effects of the Y2 agonist on energy metabolism

Treatment	Vehicle	Y2	PF
RQ	0.918 ± 0.005	0.914 ± 0.015	0.959 ± 0.003*
EE	58.3 ± 2.2	51.3 ± 3.4	52.1 ± 2.7
EI	90.1 ± 5.7	80.9 ± 6.5	80.8 ± 0.5
EE/EI	0.66 ± 0.05	0.64 ± 0.03	0.64 ± 0.03
Fasting RQ	0.831 ± 0.010	0.833 ± 0.015	NA
Fasting EE/h	0.306 ± 0.010	0.288 ± 0.015	NA

Six days of infusion with the Y2 agonist produced comparable measures of EE and RQ compared with vehicle-infused mice under both ad lib and fasting conditions. However, only mice treated with vehicle and PF to the levels of Y2 agonist-infused mice had elevated RQ levels under ad lib conditions (n = 5 to 6/group). No differences in energy metabolism were observed for any groups under fasting conditions. * *$p < 0.05$.

the Y5 receptor can increase feeding significantly without changing total EE, resulting in a significant reduction of calories metabolized per calories ingested. In contrast, a number of selective Y1 antagonists have been shown to decrease EI in genetically obese rodents (37,38) but not in Y1 knockout mice. Evidence supports a role for the Y1 receptor in modulating feeding behavior when NPY tone is elevated after fasting or in genetically obese rodents (21). Nevertheless, Y1 agonist-treated mice did not significantly increase feeding due to large variability in our study. Our data confirm that chronic infusion of NPY or selective Y5 receptor agonists can lead to hyperphagia, whereas infusion of the selective Y2 agonist causes transient hypophagia. The activation of Y2 autoreceptors acts mainly to inhibit endogenous NPY release from hypothalamic neurons, which can explain why we did not observe the Y2-induced inhibitory phenotype during chronic infusion of exogenous NPY. Although NPY can activate Y1, Y2, and Y5 receptors, the hyperphagic effect induced by chronic infusion of NPY was similar to that of Y1- or Y5-selective agonists at the same dose. The possibility remains that there may be interplay among NPY Y1, Y2, and Y5 receptor signaling in mediating the effects of NPY on EI.

Body adiposity is regulated by integrating circulating long-term signals such as leptin and insulin to communicate the size and the distribution of body fat and short-term satiety factors from the gastrointestinal tract that signal the brain to adjust food intake and EE to maintain a constant adipose depot. It is known that hypothalamic NPY/agouti-related protein and proopiomelanocortin/cocaine- and amphetamine-regulated transcript neurons are sensitive to alterations of nutrient status and energy balance and, thus, play a key role in the central nervous system regulating system. Our data suggest that chronic activation of central Y5 receptors by NPY or a selective Y5 agonist increases adiposity, especially in the visceral fat pads, but not in the subcutaneous fat pad, whereas chronic activation of central Y1 receptors shows only a trend toward increased adiposity. In contrast, chronic activation of Y2 autoreceptors transiently reduced body weight, and Y2 agonist-treated mice showed a modest trend to have lower body weight compared with the control mice. These data are consistent with a recent publication that reported transient decreases in body weight and food intake in C57BL/6 mice after 7 days of peripheral administration of the Y2 agonist $PYY_{(3-36)}$ by mini-osmotic pumps (39). However, fat mass of the Y2 agonist-infused mice, determined by both DXA and fat pad dissection, were significantly decreased compared with the control mice.

In addition to the well-known role of NPY and its receptor(s) in obesity, further studies using knockout mice have suggested that this peptide is a modulator of bone development (40). Mice deficient in the Y2 receptor have increased rates of bone mineralization and formation, and this effect is exacerbated in the absence of the Y4 receptor (41). Despite significant decreases in adiposity observed after central Y2 agonist infusion, no changes were observed in the BMC of these mice, possibly due to the limited duration of the study or, perhaps, due to the animals' growth maturity at the time of treatment. Conversely, mice infused centrally with NPY or the Y1 or Y5 agonists showed significant elevations of BMC in a condensed time frame, most likely as a necessary physical support for the increased body fat mass consequent to treatment.

Elevated NPY tone in the brain has been shown to be associated with increased basal insulinemia, marked muscular insulin resistance, and hypersensitivity to insulin in white adipose tissue (29). In that study, muscle insulin sensitivity was not related to GLUT4 expression but was inversely correlated with an increase in myocellular triglyceride content (29). In the current study, only mice chronically infused with the selective Y5 agonist were significantly more resistant to insulin than control mice, even though mice infused with NPY or the Y1 or Y5 agonists all

significantly increased weight gain and body adiposity. Recently, Mashiko et al. (42) showed that chronic infusion of the selective Y5 agonist, D-Trp^{34} NPY, significantly raised myocellular triglyceride levels and reduced liver glycogen content in mice, suggesting that Y5 agonist treatment can alter nutrient partitioning by preferentially using dietary carbohydrates for lipid synthesis, rather than glycogen synthesis. Our RQ data also agree with their observation that activation of central Y5 receptors can change nutrient partitioning under both ad lib and PF conditions. These data suggest that activating the central Y5 receptor subtype may reduce insulin sensitivity by shifting nutrient partitioning through preferential use of carbohydrate for fat storage.

To differentiate the primary effects of activation of Y1 or Y5 receptors with the secondary effects induced by elevated EI, groups of mice chronically infused with NPY or Y1- or Y5-selective agonists were pair fed with the average daily food consumed by the vehicle group. Comparing the PF groups with ad lib groups of mice infused with NPY-, Y1-, or Y5-selective agonists, we found that the weight gain induced by chronic activation of central Y1 or Y5 receptors was directly related to hyperphagia. In our study, however, Y5 agonist-infused PF mice continued to demonstrate significantly increased RQ, suggesting that Y5 receptor-induced change in nutrient partitioning was not totally dependent on hyperphagia. The observation that Y5-induced weight gain in mice can be abolished by pair feeding is consistent with other studies in mice chronically infused with D-Trp^{34}NPY (42). Although D-Trp^{34} NPY-infused PF mice are reported to have significantly more epididymal and retroperitoneal fat pads than control mice, our study showed only a trend toward increased adiposity and percentage fat mass in mice chronically infused with NPY or the Y1 or Y5 agonists under pair-feeding conditions.

In conclusion, NPY is an anabolic neuropeptide that can induce positive energy balance through the activation of multiple NPY receptor subtypes in the hypothalamus. Stimulation of central Y1 receptors using a selective Y1 agonist increases weight gain and adiposity by changing nutrient partitioning but has little effect on EE. In contrast, activation of central Y5 receptors by a selective Y5 agonist not only increases EI and adiposity but also alters nutrient partitioning by promoting fat accumulation at the expense of metabolizing carbohydrate and decreases energy metabolized per energy ingested. In addition, selective excitation of Y2 autoreceptors leads to hypophagia and transient weight loss, while having little effect on EE. Our studies indicate that all three NPY receptor subtypes may play a role in regulating energy homeostasis in mice through activation of multiple receptor subtypes.

Acknowledgments

There was no outside support/funding for this study.

References

1. **Tatemoto K, Carlquist M, Mutt V.** Neuropeptide Y: a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature*. 1982;296:659–60.
2. **Lundberg JM, Terenius L, Hokfelt T, et al.** Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. *Acta Physiol Scand*. 1982;116:477–80.
3. **Billington CJ, Briggs JE, Harker S, et al.** Neuropeptide Y in hypothalamic paraventricular nucleus: a center coordinating energy metabolism. *Am J Physiol*. 1994;266:R1765–70.
4. **Lewis DE, Shellard L, Koeslag DG, et al.** Intense exercise and food restriction cause similar hypothalamic neuropeptide Y increases in rats. *Am J Physiol*. 1993;264:E279–84.
5. **Beck B, Jhanwar-Uniyal M, Burlet A, et al.** Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status. *Brain Res*. 1990;528:245–9.
6. **Wilding JP, Gilbey SG, Bailey CJ, et al.** Increased neuropeptide-Y messenger ribonucleic acid (mRNA) and decreased neurotensin mRNA in the hypothalamus of the obese (ob/ob) mouse. *Endocrinology*. 1993;132:1939–44.
7. **Sanacora G, Kershaw M, Finkelstein JA, et al.** Increased hypothalamic content of preproneuropeptide Y messenger ribonucleic acid in genetically obese Zucker rats and its regulation by food deprivation. *Endocrinology*. 1990;127:730–7.
8. **Williams G, Gill JS, Lee YC, et al.** Increased neuropeptide Y concentrations in specific hypothalamic regions of streptozocin-induced diabetic rats. *Diabetes*. 1989;38:321–7.
9. **Blomqvist AG, Herzog H.** Y-receptor subtypes: how many more? *Trends Neurosci*. 1997;20:294–8.
10. **Parker EM, Balasubramaniam A, Guzzi M, et al.** [D-Trp(34)] neuropeptide Y is a potent and selective neuropeptide Y Y(5) receptor agonist with dramatic effects on food intake. *Peptides*. 2000;21:393–9.
11. **Gerald C, Walker MW, Criscione L, et al.** A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature*. 1996;382:168–71.
12. **Mullins D, Kirby D, Hwa J, et al.** Identification of potent and selective neuropeptide Y Y(1) receptor agonists with orexigenic activity in vivo. *Mol Pharmacol*. 2001;60:534–40.
13. **Hwa JJ, Witten MB, Williams P, et al.** Activation of the NPY Y5 receptor regulates both feeding and energy expenditure. *Am J Physiol*. 1999;277:R1428–34.
14. **King PJ, Widdowson PS, Doods HN, et al.** Regulation of neuropeptide Y release by neuropeptide Y receptor ligands and calcium channel antagonists in hypothalamic slices. *J Neurochem*. 1999;73:641–6.
15. **Batterham RL, Cowley MA, Small CJ, et al.** Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature*. 2002; 418:650–4.
16. **Batterham RL, Cohen MA, Ellis SM, et al.** Inhibition of food intake in obese subjects by peptide YY3-36. *N Engl J Med*. 2003;349:941–8.
17. **Naveilhan P, Hassani H, Canals JM, et al.** Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. *Nat Med*. 1999;5:1188–93.
18. **Baldock PA, Sainsbury A, Couzens M, et al.** Hypothalamic Y2 receptors regulate bone formation. *J Clin Invest*. 2002;109: 915–21.

19. **Kushi A, Sasai H, Koizumi H, et al.** Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice. *Proc Natl Acad Sci USA*. 1998;95:15659–64.
20. **Marsh DJ, Hollopeter G, Kafer KE, et al.** Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nat Med*. 1998;4:718–21.
21. **Pedrazzini T, Seydoux J, Kunstner P, et al.** Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. *Nat Med*. 1998;4:722–6.
22. **Kanatani A, Ishihara A, Asahi S, et al.** Potent neuropeptide Y Y1 receptor antagonist, 1229U91: blockade of neuropeptide Y-induced and physiological food intake. *Endocrinology*. 1996;137:3177–82.
23. **Schaffhauser AO, Stricker-Krongrad A, Brunner L, et al.** Inhibition of food intake by neuropeptide Y Y5 receptor antisense oligodeoxynucleotides. *Diabetes*. 1997;46:1792–8.
24. **Chamorro S, Della-Zuana O, Fauchere JL, et al.** Appetite suppression based on selective inhibition of NPY receptors. *Int J Obes Relat Metab Disord*. 2002;26:281–98.
25. **Cabrele C, Langer M, Bader R, et al.** The first selective agonist for the neuropeptide YY5 receptor increases food intake in rats. *J Biol Chem*. 2000;275:36043–8.
26. **Messier C, Emond S, Ethier K.** New techniques in stereotaxic surgery and anesthesia in the mouse. *Pharmacol Biochem Behav*. 1999;63:313–8.
27. **Elia M, Livesey G.** Energy expenditure and fuel selection in biological systems: the theory and practice of calculations based on indirect calorimetry and tracer methods. *World Rev Nutr Diet*. 1992;70:68–131.
28. **Raposinho PD, Pierroz DD, Broqua P, et al.** Chronic administration of neuropeptide Y into the lateral ventricle of C57BL/6J male mice produces an obesity syndrome including hyperphagia, hyperleptinemia, insulin resistance, and hypogonadism. *Mol Cell Endocrinol*. 2001;185:195–204.
29. **Zarjevski N, Cusin I, Vettor R, et al.** Intracerebroventricular administration of neuropeptide Y to normal rats has divergent effects on glucose utilization by adipose tissue and skeletal muscle. *Diabetes*. 1994;43:764–69.
30. **Beck B, Stricker-Krongrad A, Nicolas JP, et al.** Chronic and continuous intracerebroventricular infusion of neuropeptide Y in Long-Evans rats mimics the feeding behaviour of obese Zucker rats. *Int J Obes Relat Metab Disord*. 1992;16:295–302.
31. **Parker RM, Herzog H.** Regional distribution of Y-receptor subtype mRNAs in rat brain. *Eur J Neurosci*. 1999;11:1431–48.
32. **Snitker S, Tataranni PA, Ravussin E.** Respiratory quotient is inversely associated with muscle sympathetic nerve activity. *J Clin Endocrinol Metab*. 1998;83:3977–9.
33. **Zurlo F, Lillioja S, Esposito-Del Puente A, et al.** Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ. *Am J Physiol*. 1990;259:E650–7.
34. **Criscione L, Rigollier P, Batzl-Hartmann C, et al.** Food intake in free-feeding and energy-deprived lean rats is mediated by the neuropeptide Y5 receptor. *J Clin Invest*. 1998;102:2136–45.
35. **Della Zuana O, Sadlo M, Germain M, et al.** Reduced food intake in response to CGP 71683A may be due to mechanisms other than NPY Y5 receptor blockade. *Int J Obes Relat Metab Disord*. 2001;25:84–94.
36. **Kanatani A, Ishihara A, Iwaasa H, et al.** L-152,804: orally active and selective neuropeptide Y Y5 receptor antagonist. *Biochem Biophys Res Commun*. 2000;272:169–73.
37. **Kanatani A, Kanno T, Ishihara A, et al.** The novel neuropeptide Y Y(1) receptor antagonist J-104870: a potent feeding suppressant with oral bioavailability. *Biochem Biophys Res Commun*. 1999;266:88–91.
38. **Kanatani A, Hata M, Mashiko S, et al.** A typical Y1 receptor regulates feeding behaviors: effects of a potent and selective Y1 antagonist, J-115814. *Mol Pharmacol*. 2001;59:501–5.
39. **Tschop M, Casteneda TR, Joost HG, et al.** Does gut hormone PYY_{3-36} decrease food intake in rodents? *Nature*. 2004;8;430:1–4.
40. **Baldock PA, Sainsbury A, Couzens M, et al.** Hypothalamic Y2 receptors regulate bone formation. *J Clin Invest*. 2002;109:915–21.
41. **Sainsbury A, Baldock PA, Schwarzer C, et al.** Synergistic effects of Y2 and Y4 receptors on adiposity and bone mass revealed in double knockout mice. *Mol Cell Biol*. 2003;23:5225–33.
42. **Mashiko S, Ishihara A, Iwaasa H, et al.** Characterization of neuropeptide Y (NPY) Y5 receptor-mediated obesity in mice: chronic intracerebroventricular infusion of D-Trp(34)NPY. *Endocrinology*. 2003;144:1793–801.