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Y1 receptor deficiency in mice
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Y2Y4 receptor double knockout protects against obesity due to a high-fat diet or Y1 receptor deficiency in mice

Sainsbury A, Bergen HT, Boey D, Bamming D, Cooney GJ, Lin S, Couzens M, Stroth N, Lee NJ, Lindner D, Singewald N, Karl T, Duffy L, Enriquez R, Slack K, Sperk G, Herzog H

Neuropeptide Y receptors are critical regulators of energy homeostasis, but the functional interactions and relative contributions of Y receptors and the environment in this process are unknown. We measured the effects of an ad libitum diet of normal or high-fat food on energy balance in mice with single, double, or triple deficiencies of Y1, Y2, or Y4 receptors. Whereas wild-type mice developed diet-induced obesity, Y2Y4 double knockouts did not. In contrast, Y1 knockout or Y1Y2 or Y1Y4 receptor double knockout mice developed an exacerbated diet-induced obesity syndrome. Remarkably, the antiobesity effect of Y2Y4 deficiency was stronger than the obesogenic effect of Y1 deficiency, since Y1Y2Y4 triple knockouts did not develop obesity on the high-fat diet. Resistance to diet-induced obesity in Y2Y4 knockouts was associated with reduced food intake and improved glucose tolerance in the absence of changes in total physical activity. Fecal concentration of free fatty acids was significantly increased in Y2Y4 knockouts in association with a significantly reduced bile acid pool and marked alterations in intestinal morphology. In addition, hypothalamic proopiomelanocortin expression was decreased in diet-induced obesity (in both wild-type and Y1 receptor knockout mice) but not in obesity-resistant Y2Y4 receptor knockout mice fed a high-fat diet. Therefore, deletion of Y2 and Y4 receptors synergistically protects against diet-induced obesity, at least partially via changes in food intake and hypothalamic proopiomelanocortin expression.

Members of the Y receptor family, notably Y1, Y2, Y4, and Y5 receptors, are implicated in energy homeostasis and the development of obesity and insulin resistance. These receptors are activated by three endogenous ligands: neuropeptide Y, the gut-derived hormones peptide YY, and pancreatic polypeptide. Recently, there has been renewed speculation that ligands for Y receptors, such as peptide YY3-36 and pancreatic polypeptide, may be of benefit for the treatment of obesity (1).

There is considerable conflict in the literature about the role of Y receptors in the regulation of body weight. For instance, pharmacological studies suggested that Y1 receptors contribute to hyperphagia induced by increased hypothalamic neuropeptide Y secretion (2). Fasting-induced refeeding is reduced in Y1 knockout mice (3). Moreover, food intake and body weight of genetically obese ob/ob mice, in which hypothalamic neuropeptide Y-ergic activity is chronically increased, are significantly reduced by Y1 knockout (4). In contrast, Y1 knockouts develop significant increases in body weight, fat mass, and insulinemia in the absence of hyperphagia (3,5).

Similar controversies prevail regarding the role of Y2 receptors in energy homeostasis. Hypothalamus-specific or germ-line deletion of Y2 receptors resulted in significant reductions in the body weight of lean mice (6) and significant reductions in adiposity or body weight and the type 2 diabetic syndrome of ob/ob mice in the absence of reductions in food intake (7,8). In contrast, another germline Y2 receptor knockout model was shown to develop increased body weight, fat deposition, and hyperphagia (9). Furthermore, intrahypothalamic administration of the Y2 agonist neuropeptide Y[13-36] to rats significantly decreased food intake (10). Moreover, circulating peptide YY from the gut and its truncated form peptide YY3-36 (a preferential Y2 agonist) have been shown to reduce food intake, adiposity, and/or body weight after short- or long-term administration to lean or obese rodents or humans (1,11,12).

Recent work suggests that pancreatic polypeptide, a preferential Y4 receptor agonist secreted by the F cells of the islets of Langerhans, regulates energy homeostasis.

Intravenous or intraperitoneal administration of pancreatic polypeptide to mice increases metabolic rate (13) and decreases hyperglycemia, basal or glucose-induced hyperinsulinemia, insulin resistance, and hyperlipidemia in ob/ob mice, culminating in decreases in adiposity and body weight (13,14). Pancreatic polypeptide transgenic mice, which have a 20-fold increase in plasma pancreatic polypeptide levels, have reduced food intake, body weight, and fat mass as well as reduced glucose-induced insulin secretion and gastric emptying when compared with control mice (15). However, other findings do not support a role of Y4 receptors in energy homeostasis. Y4 receptor knockout had no effect on the hyperphagia, obesity, or type 2 diabetic phenotype of ob/ob mice (16).

These conflicting data make it difficult to define the true physiological and pathophysiological role of Y receptors in energy homeostasis and obesity. Moreover, it is difficult to know whether potential obesity treatments targeted to Y receptors should act as agonists or antagonists. Part of this conflict can be explained by the fact that Y receptors mediate their physiological effects via complex synergistic and redundant interactions. For instance, knockout of individual Y receptors alters ligand-binding patterns of the remaining Y receptors (17). In addition, ligands for Y receptors are renowned for their lack of specificity for individual receptor types.

To circumvent some of these complications of Y receptor biology, we made a systematic study of energy balance in mice with single, double, or triple knockout of Y1, Y2, or Y4 receptors, both under chow-fed conditions and in diet-induced obesity. In this way, we were able to clarify the interactions among these receptor types in regulating energy balance (including body weight, food intake, physical activity, thermogenesis, glucose tolerance, intestinal lipid absorption, intestinal morphology, and hypothalamic expression of neuropeptide Y and proopiomelanocortin) and to elucidate their roles in the development of diet-induced obesity. Y5 receptor knockouts were not investigated in this study because the Y5 and Y1 receptors are localized only 20 kb apart on the same chromosome, and it is therefore not plausible to obtain Y1Y5 receptor double knockout mice by cross-breeding.

RESEARCH DESIGN AND METHODS

Generation of the Y1, Y2, and Y4 knockout mice were previously published (6,16,18). Male double or triple knockout mice were obtained by crossing Y1, Y2, or Y4 knockout mice, respectively. All mice were on a mixed C57BL/6129/SvJ background. All research and animal care procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22°C) and illumination (12-h light cycle, lights on at 0700). Haft of the mice of each genotype were fed a normal chow diet ad libitum (6% calories from fat, 21% calories from protein, 71% calories from carbohydrate, and 2.6 kcal/g; Gordon's Speciality Stock Feeds, Yanderra, NSW, Australia). The other haft was fed ad libitum with a high-fat diet supplemented with fat and sucrose (46% calories from fat, 21% calories from protein, 33% calories from carbohydrate, and 4.72 kcal/g) from 8 weeks of age onwards. The diet was based on the composition of rodent diet catalog no. D12451 (Research Diets, New Brunswick, NJ), with the exception that safflower oil and copha were used in place of soybean oil and lard. Twenty-four-hour food intake was estimated twice a week for 4 weeks from 13 to 16 weeks of age by the average amount of food removed from the food hopper. In a subset of animals, the actual proportion of food that was consumed was calculated by subtracting the weight of the food spilt in the cage from the weight of food removed from the hopper. Body temperature was determined at 14-16 weeks of age (BAT-10 multipurpose thermometer; Physitemp Instruments, Clifton, NJ).

Determination of physical activity. Distance traveled in the open field test was determined as previously described (19). Total cage activity was recorded using a passive infrared detector (Conrad Electronics, Hanover, Germany) on top of the cage lid of single-housed animals. The signals of the passive infrared detectors were detected by an I/O interface card (PIO48 II; Conrad Electronics) and stored using custom-made software. A mean activity per day was calculated by averaging corresponding 30-min intervals of 7 consecutive days.

Glucose tolerance tests and tissue collection. At 16 weeks of age, animals were fasted overnight and then administered an intraperitoneal injection of D-glucose (60 mg/mouse).

Glucose levels were determined in tail blood samples, with glucose test strips (Accu-Check Advantage II Glucose Test Strips; Roche Diagnostics Australia, Castle Hill, NSW, Australia). At 3-8 days after the glucose tolerance test, food was removed from cage hoppers at 0900, and animals were killed 4-7 h later by cervical dislocation followed by cardiac puncture for serum collection. Brains were immediately removed and frozen on dry ice. The interscapular brown adipose tissue was removed, weighed, and stored frozen until analysis for uncoupling protein-1 mRNA levels (20). Epididymal white adipose tissue (WATe) depots were dissected and weighed. The small intestine of some mice was removed and prepared for histology as described below. In separate groups of mice, the small intestine was flushed with isotonic saline, blotted on paper towels, and weighed. In other mice, the liver, gallbladder, small intestine and contents were removed, weighed, and stored at --80°C until analysis of total bile acid pool (21).

Analysis of fecal lipids and bile acids. Fecal samples were dried to a constant weight at 90°C. Some fecal samples were used for extraction of total lipids (22). Lipids were resuspended in pure chloroform and separated by TLC (thin-layer chromatography) on Whatman Silica Gel K6 glass TLC plates (Whatman, Maidstone, England) in hexane:diethyl ether:acetic acid, 80:20:1. Standards were triolein (50 μg), dipalmitine (75 μg), and palmitine (125 μg) (Sigma, St. Louis, MO). Fractious corresponding to triglycerides, diglycerides, monoglycerides, and free fatty acids were scraped from the plate for assay of triacylglycerol (23). The free fatty acid fraction was resuspended in chloroform and assayed for free fatty acids with a colorimetric assay kit (WAKO, Saitama, Japan). Other samples of feces were used for determination of fecal bile acid concentration (21).

Analysis of intestinal morphology. One-centimeter pieces of small intestine were taken from the following three locations: duodenum, 3 cm distal from the stomach-duodenal junction; jejunum, at the midpoint of the small intestine; and ileum, 3 cm proximal from the ileocaecal junction. Intestinal pieces were fixed for 16-18 h in Bouin's solution (Sigma), embedded in paraffin, sectioned at 7 μm, mounted on charged slides (SuperFrost Plus; MenzelGlaser, Braunschweig, Germany), and stained with periodic acid Schiff. Villus length (from base of the crypt to tip of the villus) was measured in at least 15 villi from three mice in each group, using a light microscope (Axiophot; Zeiss, Oberkochen, Germany) connected to a Leica camera and Leica Image Manager software (Leica, Heerbrugg, Switzerland). The number of goblet cells per villus (excluding crypts) were counted in at least 15 villi from five mice in each group. All analyses were done by investigators unaware of genotype.
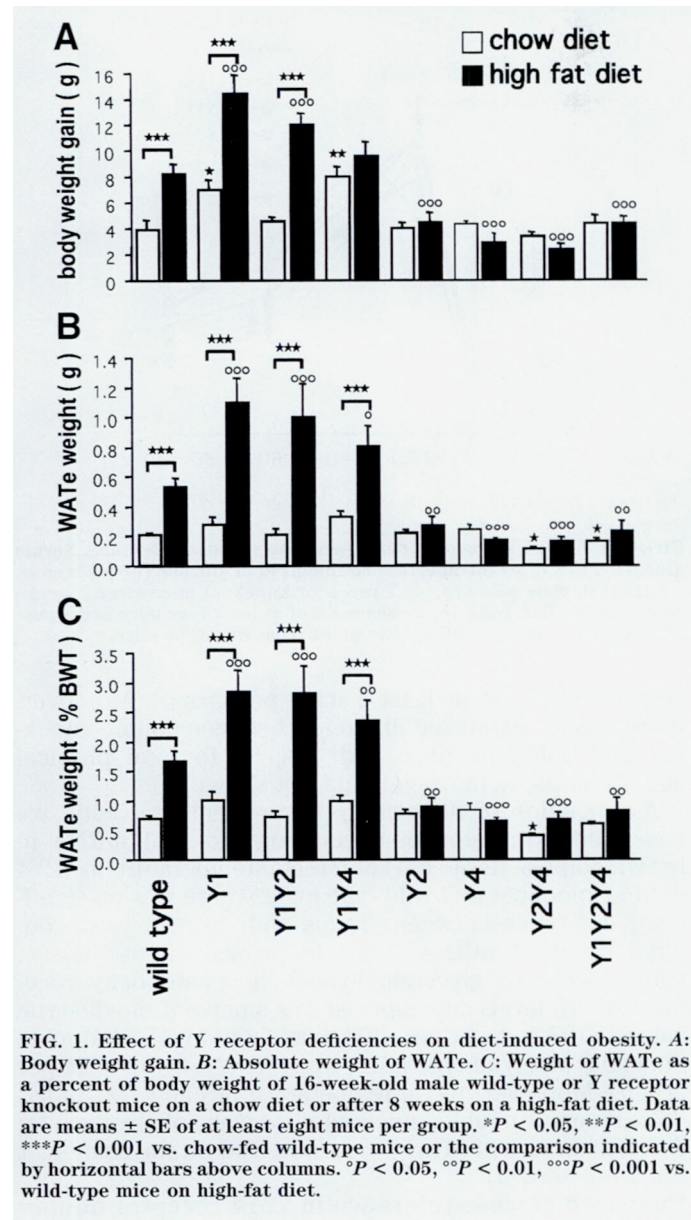
Neurochemical analyses. Coronal brain slices (20 μm) at the level of the hypothalamic arcuate nucleus were hybridized using DNA oligonucleotides complementary to mouse neuropeptide Y and proopiomelanocortin (POMC) (7).

Serum analyses. Serum hormone levels were determined with commercial radioimmunoassay kits from Linco Research (St. Louis, MO) (leptin and insulin), ICN Biomedicals (Costa Mesa, CA) (corticosterone, free T4, and testosterone), and Bioclone Australia (Marrickville, Australia) (IGF-1). Basal serum glucose levels were determined with a glucose oxidase kit (Trace Scientific, Melbourne, Australia).

Statistical analysis. All data are expressed as means ± SE. Differences among groups of mice were assessed by ANOVA or repeated-measures ANOVA, followed by Fisher's post hoc comparisons, if appropriate (StatView version 4.51; Abacus Concepts, Berkeley, CA). Statistical significance was defined as $P < 0.05$.
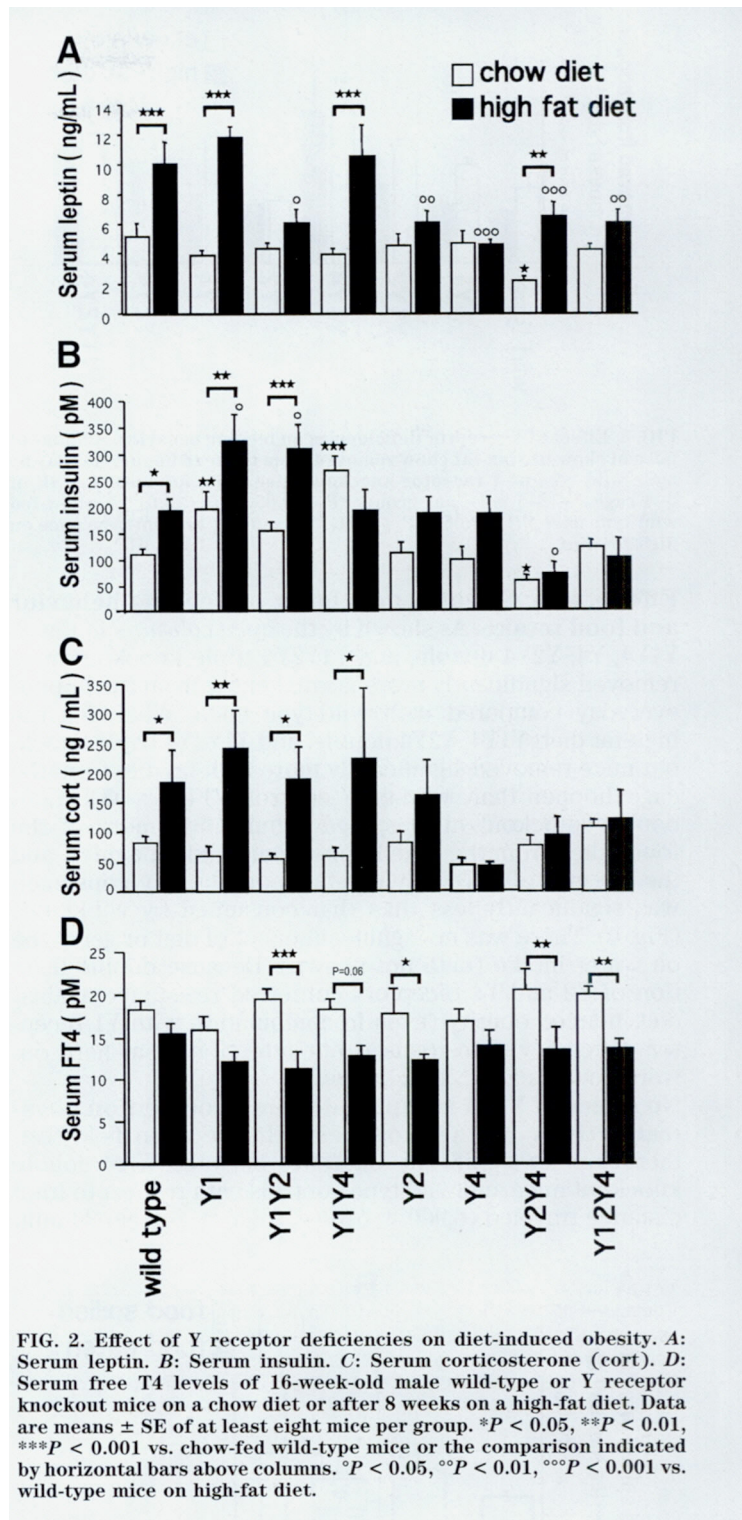

RESULTS

Effects of Y receptor deficiency on diet-induced obesity. The body weights of mice at 8 weeks of age (before the introduction of the high-fat diet) were not significantly different from each other, except for Y1Y2Y4 triple knockout mice, which were lighter (wild-type, 23.7 ± 0.3 g; Y1, 22.8 ± 0.6 g; Y1Y2, 24.0 ± 0.4 g; Y1Y4, 24.2 ± 0.4 g; Y2, 24.5 ± 0.6 g; Y4, 24.4 ± 0.3 g; Y2Y4, 24.8 ± 0.4 g; and Y1Y2Y4, 22.1 ± 0.5 g, $P < 0.05$ vs. wild-type, n = at least 11 mice per genotype). Figure 1A, ↔ shows that under chow-fed conditions, body weight gain from 8 weeks until 16 weeks of age was similar in all groups of mice, except for Y1 knockout and Y1Y4 double knockout mice. The absolute and relative weights of the WATe depot and serum

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FIG. 1. Effect of Y receptor deficiencies on diet-induced obesity. *A:*
Body weight gain. *B:* Absolute weight of WATe. *C:* Weight of WATe as
a percent of body weight of 16-week-old male wild-type or Y receptor
knockout mice on a chow diet or after 8 weeks on a high-fat diet. Data
are means ± SE of at least eight mice per group. *P < 0.05, **P < 0.01,
***P < 0.001 vs. chow-fed wild-type mice or the comparison indicated
by horizontal bars above columns. °P < 0.05, °°P < 0.01, °°°P < 0.001 vs.
wild-type mice on high-fat diet.

leptin levels were similar in all groups of mice under chow-fed conditions, except for Y2Y4
double and Y1Y2Y4 triple knockout mice, which showed significant decreases (Fig. 1B and C,
Fig. 2A, ⇔). Under chow-fed conditions, serum insulin levels were significantly increased over
wild-type values in Y1 and Y1Y4 double knockout mice, and significantly decreased relative to
wild-type in Y2Y4 double knockouts (Fig. 2B, ⇔). In chow-fed animals, there were no
significant differences among groups with respect to serum levels of corticosterone or free T4
(Fig. 1C and D), glucose, testosterone, or IGF-1 (data not shown). When fed ad libitum for 8
weeks on the high-fat diet, wild-type mice developed a distinct obesity syndrome
characterized by increased body weight gain (Fig. 1A), increased WATe weight (Fig. 1B and
C), and increases in serum leptin, insulin, and corticosterone levels (Fig. 2A-C), with a
tendency to decreased free T4 levels (Fig. 2D). Y1 receptor deficiency, either alone or in
combination with Y2 or Y4 receptor deficiency (i.e., Y1, Y1Y2, and Y1Y4 receptor knockout
mice), exacerbated this diet-induced obesity syndrome (Figs. 1A-C and 2B). Y1, Y1Y2, and
Y1Y4 receptor knockout mice also developed hyperleptinemia, hypercorticosteronemia, and a
tendency to decreased free T4 levels after high-fat feeding, but these responses were not
significantly different from wild-type animals (Fig. 2A,C,D). In contrast to the exacerbating
effect of Y1 deficiency on diet-induced obesity, Y2 or Y4 deletion (in Y2, Y4, Y2Y4 double,

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and Y1Y2Y4 triple knockout mice) conferred protection against diet-induced obesity (Figs. 1 and 2). However, similar to wild-type mice, Y2, Y2Y4 double, and Y1Y2Y4 triple knockout mice showed marked decreases in serum free T4 levels in response to high-fat feeding (Fig. 2D).

FIG. 2. Effect of Y receptor deficiencies on diet-induced obesity. *A:* Serum leptin. *B:* Serum insulin. *C:* Serum corticosterone (cort). *D:* Serum free T4 levels of 16-week-old male wild-type or Y receptor knockout mice on a chow diet or after 8 weeks on a high-fat diet. Data are means ± SE of at least eight mice per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. chow-fed wild-type mice or the comparison indicated by horizontal bars above columns. °$P < 0.05$, °°$P < 0.01$, °°°$P < 0.001$ vs. wild-type mice on high-fat diet.

Effects of Y receptor deficiency on feeding behavior and food intake. As shown by the open columns in Fig. 3, Y1Y4, Y4, Y2Y4 double, and Y1Y2Y4 triple knockout mice removed significantly more normal chow from the hopper everyday compared with wild-type mice. When fed the high-fat diet, Y1Y4, Y2Y4 double, and Y1Y2Y4 triple knockout mice removed

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significantly more high-fat diet from the cage hopper than wild-type controls (Fig. 3, ■). Y2Y4 double knockout mice spilled significantly more of the food taken from the cage hopper than wild-type mice, and the amount of chow or high-fat food actually consumed was significantly less than that consumed by wild-types (Fig. 4). There was no significant effect of diet or genotype on water intake (data not shown). Because double deletion of Y2 and Y4 receptors conferred resistance against diet-induced obesity (even in combination with Y1 receptor deficiency), we focused our attention from here onwards on the Y2Y4 knockouts.
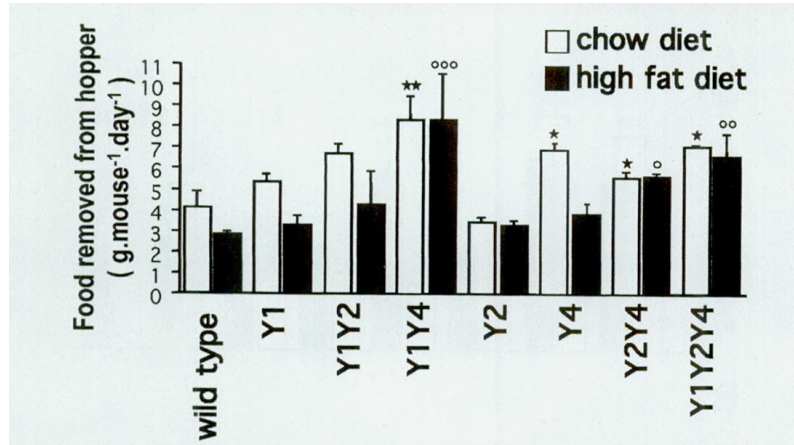
FIG. 3. Effect of Y receptor deficiencies on feeding behavior. Amount of normal chow or high-fat chow removed from the feed hopper per day by male wild-type or Y receptor knockout mice. Data are means ± SE of 2–4 cages of 2–5 mice per group. *$P < 0.05$, **$P < 0.01$ vs. chow-fed wild-type mice. °$P < 0.05$, °°$P < 0.01$, °°°$P < 0.001$ vs. wild-type mice on high-fat diet.
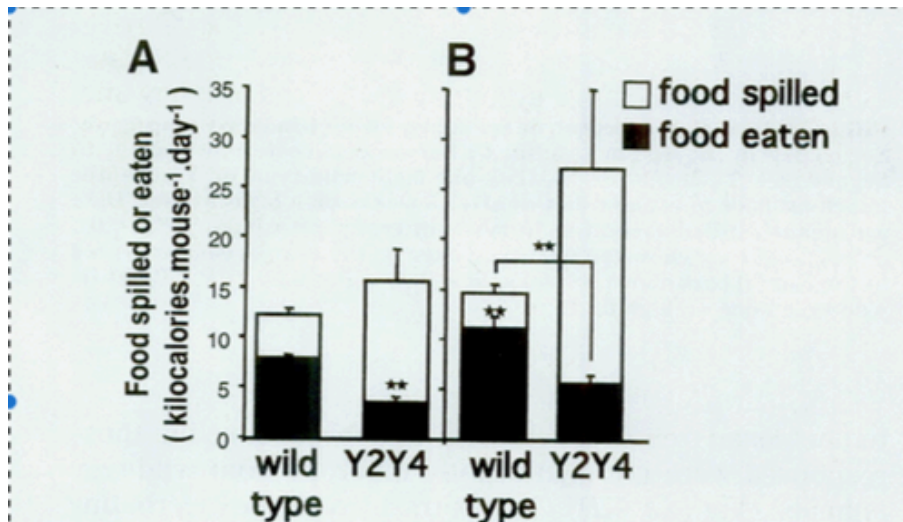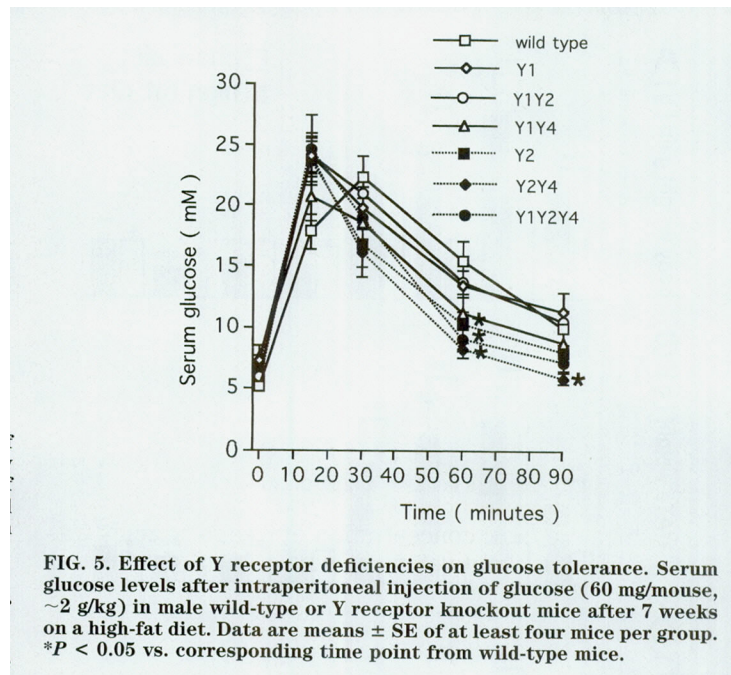

FIG. 4. Effect of Y receptor deficiencies on the amount of food eaten or spilled. The proportion of total food removed from the feed hopper (total column height) that was spilled in the cage bedding (upper, open columns) versus the calculated amount eaten (■) in 16-week-old male wild-type or Y2Y4 receptor knockout mice on a chow diet (*A*) or after 8 weeks on a high-fat diet (*B*). Data are means ± SE of at least four mice per group. *$P < 0.05$ or ***$P < 0.001$ vs. the amount of food spilled or eaten by wild-type mice on the chow diet.

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FIG. 5. Effect of Y receptor deficiencies on glucose tolerance. Serum glucose levels after intraperitoneal injection of glucose (60 mg/mouse, ~2 g/kg) in male wild-type or Y receptor knockout mice after 7 weeks on a high-fat diet. Data are means ± SE of at least four mice per group. *$P < 0.05$ vs. corresponding time point from wild-type mice.

No effect of Y2Y4 receptor double knockout on physical activity or thermogenesis. In the open field test, there was no significant difference between Y2Y4 double knockout mice and wild-type controls with respect to total distance traveled (6,960 ± 510 vs. 5,830 ± 240 cm/30 min, respectively; n = at least 4 mice per group). Moreover, there was no significant difference between double knockout and wild-type mice with respect to total physical activity in the home cage (data not shown).

As an index of nonactivity thermogenic capacity, we measured expression of uncoupling protein-1 mRNA in brown adipose tissue (24) and rectal temperature in Y2Y4 double knockout and wild-type mice. There was no significant difference between groups with respect to uncoupling protein-1 mRNA levels in brown adipose tissue, standardized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and expressed as a percent of wild-type values (107.3 ± 6.7 vs. 100.0 ± 7.4% in Y2Y4 double knockout and wild-type mice, respectively; n = 5 mice per group). Moreover, rectal temperature of chow- or fat-fed Y2Y4 receptor double knockout mice was not significantly different from that of wild-type mice fed on the same diet (data not shown).

Enhanced glucose tolerance in Y2Y4 receptor double knockout mice. There was no significant difference among groups with respect to peak glycemia following intraperitoneal glucose injection. However, in Y2, Y2Y4 double, and Y1Y2Y4 triple knockout mice, there was a significant reduction in serum glucose levels at 60 and 90 min postinjection compared with wild-type mice (Fig. 5). All other groups of mice (Y1, Y1Y2 double, Y1Y4 double, and Y4 knockout mice) showed glucose clearance curves indistinguishable from wild-type mice (Fig. 5).

Decreased intestinal lipid absorption in Y2Y4 receptor double knockout mice. Fig. 6 shows the concentration of triglycerides, diglycerides, and free fatty acids in feces from wild-type and Y2Y4 receptor double knockout mice, either under chow-fed (Fig. 6A) or high-fat-fed (Fig. 6B) conditions. In both groups of mice, free fatty acids were the most abundant fecal lipid species, and undigested or partially digested lipids (triglycerides and diglycerides) were 6- to 30-fold less abundant. Monoglycerides were not detectable in the fecal samples investigated. These findings indicate that triglyceride digestion to free fatty acids proceeds efficiently in both wild-type and Y2Y4 receptor double knockout mice. However, absorption of digested lipids may be impaired in Y2Y4 double knockout mice, since the fecal concentration of free fatty acids was increased in knockout versus wild-type mice, significantly so during high-fat feeding (Fig. 6B). Dally fecal output (dried weight) from Y2Y4 double knockout mice

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on a chow diet was significantly less than that of wild-type controls (0.64 ± 0.12 vs. 1.23 ± 0.04 g · mouse-1 · day-1, n = 3-4 cages of 2-4 mice per group). However, there was no significant difference in dally fecal output between knockout and wild-type mice on the high-fat diet (0.22 ± 0.02 vs. 0.25 ± 0.04 g · mouse-1 · day-1 in wild-types, n 3-4 cages of 2-4 mice per group).
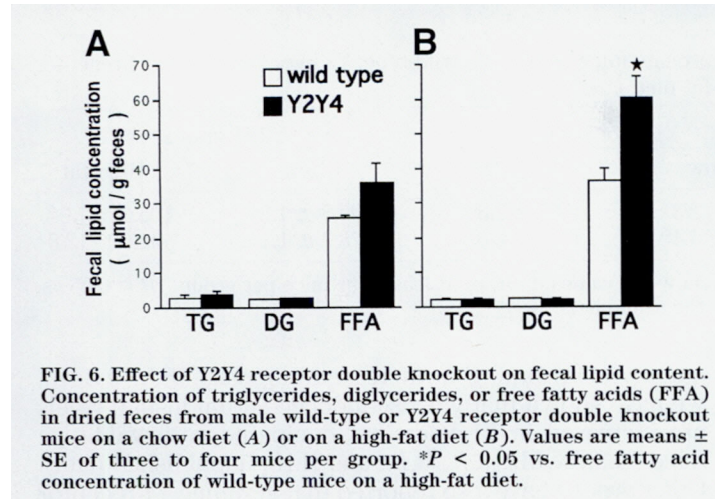
FIG. 6. Effect of Y2Y4 receptor double knockout on fecal lipid content. Concentration of triglycerides, diglycerides, or free fatty acids (FFA) in dried feces from male wild-type or Y2Y4 receptor double knockout mice on a chow diet (*A*) or on a high-fat diet (*B*). Values are means ± SE of three to four mice per group. *$P < 0.05$ vs. free fatty acid concentration of wild-type mice on a high-fat diet.

Reduced bile pool and differences in intestinal morphology in Y2Y4 receptor double knockout mice. We determined the total bile pool because intestinal lipid absorption is dependent on bile production. The total bile pool of Y2Y4 knockouts was significantly reduced compared with wild-type mice (20.0 ± 3.6 vs. 41.0 ± 3.0 μmol total bile, respectively; and 88 ± 17 vs. 142 ± 10 *μ*mol/100 g body wt, respectively; means ± SE of 12-13 mice per group, $P < 0.01$). Fecal total bile acid concentration was similar in Y2Y4 knockout and wild-type mice (33 ± 9 vs. 34 ± 2 *μ*mol/g dried feces, respectively, means ± SE of at least three mice per group). Since dally fecal output was reduced by 50% in Y2Y4 versus wild-type mice, total bile acid output of Y2Y4 knockouts was therefore half that of wild-type counterparts, in keeping with the smaller total bile acid pool size.

Although there was no significant difference between wild-type and knockout mice with regards to either weight (1.1 ± 0.05 vs. 1.1 ± 0.05 g, respectively; means ± SE of 22-24 mice per group) or length (39.4 ± 0.8 vs. 37.6 ± 0.6 cm, respectively; means ± SE of 25-27 mice per group) of the small intestine, the average length of small intestinal villi was significantly decreased in the duodenum of Y2Y4 double knockout versus wild-type mice (431 ± 2 vs. 525 ± 6 *μ*m, respectively, n = 3 mice per genotype, $P < 0.05$) (Fig. 7A and B). This difference in villus length was not observed in the jejunum (295 ± 16 vs. 287 ± 6 *μ*m; Y2Y4 and wild-type mice, respectively, n = 3 mice per genotype) or ileum (204 ± 24 vs. 184 ± 15 *μ*m in Y2Y4 and wild-type mice, respectively, n = 3 mice per genotype). Y2Y4 receptor double knockout mice had an increase over wild-type mice in the number of goblet cells in the duodenum (23.7 ± 1.7 vs. 20.6 ± 1.0 goblet cells per villus in wild-type mice, n = 5 mice per group, NS), the jejunum (21.6 ± 1.1 vs. 18.0 ± 0.6 goblet cells per villus in wild-type mice, n = 5 mice per group, $P < 0.05$), and the ileum (23.1 ± 1.4 vs. 18.2 ± 0.6 goblet cells per villus in wild-type mice, n = 5 mice per group, $P < 0.05$). Representative villi from the ileum of wild-type and Y2Y4 receptor knockout mice are shown in Fig. 7C and D.
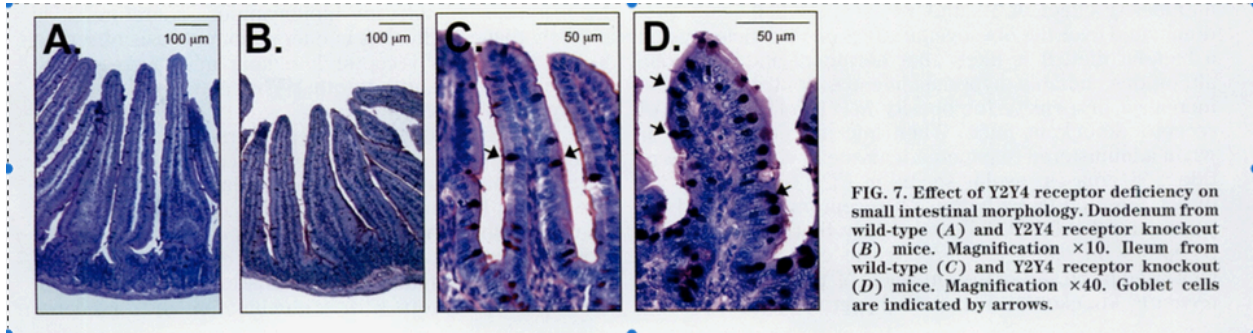
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FIG. 7. Effect of Y2Y4 receptor deficiency on small intestinal morphology. Duodenum from wild-type (*A*) and Y2Y4 receptor knockout (*B*) mice. Magnification ×10. Ileum from wild-type (*C*) and Y2Y4 receptor knockout (*D*) mice. Magnification ×40. Goblet cells are indicated by arrows.

Altered hypothalamic expression of neuropeptide Y and POMC in Y receptor knockout mice. In wild-type mice, the high-fat diet resulted in significant decreases in neuropeptide Y and POMC mRNA levels in the arcuate nucleus (Table 1). Increased weight gain in both chow-fed and high-fat--fed Y1 receptor knockout mice, compared with wild-types, was associated with decreased neuropeptide Y and POMC mRNA levels compared with wild-type values (Table 1). In addition, in Y1 receptor knockout mice, diet-induced obesity was associated with decreased neuropeptide Y and POMC mRNA levels compared with chow-fed mice (Table 1). In contrast, in Y2Y4 receptor knockout mice, neuropeptide Y and POMC mRNA levels were not significantly decreased following ingestion of a high-fat diet compared with chow-fed double knockouts. Rather, neuropeptide Y and POMC mRNA levels were increased in Y2Y4 receptor knockout mice compared with wild-type mice fed the same diet (Table 1).

TABLE 1
Expression levels of neuropeptide Y mRNA or POMC mRNA in the arcuate nucleus of male wild-type, Y1 knockout, or Y2Y4 receptor double knockout mice on a chow diet or after 8 weeks on a high-fat diet

	Wild-type		Y1 knockout		Y2Y4 knockout	
	Chow	High fat	Chow	High fat	Chow	High fat
Neuropeptide Y	100.0 ± 1.8	82.4 ± 5.3*	69.3 ± 2.3†	62.5 ± 2.3*†	109.3 ± 1.8†	105.3 ± 7.0†
POMC	100.0 ± 7.4	74.5 ± 4.3*	75.7 ± 5.4†	64.9 ± 3.6*†	78.7 ± 1.1†	92.6 ± 12.8†

Data are labeling intensity of neurons given as a percent of wild-type chow-fed values $\pm$ SE of at least four mice per group. *$P < 0.05$ vs. chow-fed mice of the same genotype. †$P < 0.05$ vs. wild-type mice on the same diet.

DISCUSSION

These data demonstrate that ablation of Y2 and Y4 receptors protects against diet-induced obesity, probably via reduced food intake. In contrast, deletion of Y1 receptors significantly exacerbates diet-induced obesity. Moreover, the antiobesity effect of double Y2 and Y4 receptor deficiency overrides the obesogenic effect of Y1 receptor deficiency and a high-fat diet (as seen in Y1Y2Y4 triple knockout mice). These observations provide evidence that the molecular system that regulates energy homeostasis, including the Y receptor system, has less redundancy and capacity for compensation to favor positive over negative energy balance than previously thought.

We recently showed that Y2 and Y4 receptor knockout synergistically increases bone volume in conjunction with a significant increase in bone formation rate (25). It is therefore conceivable that in addition to decreased food intake, this threefold increase in bone volume could contribute to the lean phenotype observed in Y2Y4 receptor double knockout mice.

The current work suggests that Y2Y4 receptor double deficiency decreases absorption of fat from the digestive tract. This change was not associated with any decrease in efficiency of triglyceride digestion to free fatty acids. The reduced intestinal fat absorption in Y2Y4 receptor knockout mice is likely to be mediated, at least in part, by the reduced total bile pool observed in these animals. Indeed, fat absorption and energy extraction from food is positively correlated with the concentration of functional bile acids (26,27). It is unlikely however that decreased intestinal lipid absorption contributes to the lean phenotype of Y2Y4 receptor knockout mice, since the calorie content of the extra free fatty acids excreted in the

feces of these mice represents <1% their total dally calorie intake. Previous work has shown that both peptide YY and pancreatic polypeptide inhibit gallbladder emptying (28) or gallbladder bile output in pigs and/or humans (28, 29), presumably via interactions with Y receptors in the dorsovagal complex of the brain stem and subsequent vagal-dependent pathways (28,30). The current work extends these data to show the unambiguous involvement of Y2 and Y4 receptors in the regulation of total bile pool.

Reduced intestinal absorption of lipids in Y2Y4 receptor double knockout mice may be influenced by the observed decrease in villus length of the duodenum. It is also possible that the increased number of goblet cells observed in the small intestine of Y2Y4 receptor double knockout mice may have led to increased production of intestinal mucus, which could accelerate intestinal transit and impair absorption of nutrients (31).

In contrast to the protective effects of Y2 and Y4 receptor deficiency against diet-induced obesity, animals deficient in Y1 receptors had a tendency for increased adiposity under chow-fed conditions and had an exacerbated obesity syndrome in response to the high-fat diet. This was observed in single Y1 receptor knockout animals as well as in Y1Y2 and Y1Y4 double knockout animals. In Y1Y2Y4 receptor triple knockout animals however, the antiobesity effect of Y2 and Y4 receptor deficiency predominated over the obesogenic effect of Y1 deficiency and a high-fat diet. It is likely that hormonal and metabolic alterations, notably hyperinsulinemia, contribute to the increased propensity for obesity in Y1, Y1Y2, and Y1Y4 receptor knockout mice. When injected peripherally or when administered to pancreatic tissue in vitro, neuropeptide Y decreases insulin secretion (32,33), probably via direct interaction with the Y1 receptors expressed on pancreatic β-cells (34,35). It is likely that the lack of Y1 receptors on pancreatic islet tissue contributed to the increased basal serum insulin levels of Y1, Y1Y2, and Y1Y4 receptor knockout mice, with hyperinsulinemia subsequently contributing to increased adiposity (36). Indeed, we have shown that glucose-induced serum insulin levels are increased in Y1 knockout compared with wild-type mice (A.S., N.J.L., K.S., R.E., and H.H., unpublished data). Other groups have also reported that germline Y1 receptor knockout induces obesity and basal hyperinsulinemia (3, 5, 37) and that hyperinsulinemia is likely to be a key etiological feature in the obese phenotype of these mice (37).

High-fat feeding resulted in decreased expression of neuropeptide Y in the arcuate nucleus of wild-type mice and Y1 receptor knockout mice, possibly as an adaptive response to inhibit further weight gain. This is most likely mediated via increased circulating concentrations of leptin and the gut peptide peptide YY3-36, release of which is strongly increased by dietary fat (38, 39), subsequently acting on leptin or Y2 receptors in the arcuate nucleus to reduce neuropeptide Y expression levels and thereby reducing feeding (1,40,41). Expression of POMC in the arcuate nucleus of wild-type and Y1 receptor knockout mice fed a high-fat diet was also reduced, a change that may contribute to the diet-induced obesity observed in these mice by decreasing secretion of α-melanocyte stimulating hormone. Consistent with this hypothesis, rodent obesity models of genetic (42,43) or dietary (44) origin exhibit decreased expression of POMC in the arcuate nucleus. It was of interest that the decreases in hypothalamic expression of neuropeptide Y and POMC induced by the high-fat diet were absent in obesity-resistant Y2Y4 receptor knockout mice and were exacerbated in obesityprone Y1 receptor knockout mice. These findings suggest a possible causative role of changes in POMC signaling in the development or resistance to diet-induced obesity in wild-type, Y1 knockout, and Y2Y4 receptor double knockout mice.

We recently demonstrated that single, double, or triple deletion of Y1, Y2, or Y4 receptors alters the expression or binding patterns of the remaining Y receptors in the brain (17). However, the major changes observed are located in areas not likely to participate in the regulation of energy homeostasis, notably the hippocampus. Therefore, it is unlikely that the changes in energy homeostasis observed in our panel of Y receptor knockout mice were due to compensatory changes in other Y receptors remaining in the hypothalamus.

In conclusion, these data demonstrate that Y2 and Y4 receptors are necessary for diet-induced obesity, at least in part via effects on food intake and hypothalamic POMC expression. In contrast, Y1 receptors inhibit diet-induced obesity, probably via direct inhibition of insulin secretion from pancreatic β-cells. These data clearly indicate the possibility that simultaneously inhibiting and/or activating different Y receptors with a combination of Y receptor antagonists and agonists could provide new treatments to improve the efficiency of

lifestyle interventions for obesity.

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