

Y2 receptor deletion attenuates the type 2 diabetic syndrome of ob/ob mice

Amanda Sainsbury, Christoph Schwarzer, Michelle Couzens and Herbert Herzog

Amanda Sainsbury, Neurobiology Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia; Christoph Schwarzer, Neurobiology Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia; Michelle Couzens, Neurobiology Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia and Herbert Herzog, Neurobiology Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia

Hypothalamic neuropeptide Y (NPY) is implicated in the regulation of a variety of physiological functions, notably energy homeostasis and reproduction. Chronically elevated NPY levels in the hypothalamus, as in genetically obese ob/ob mice, are associated with obesity, a syndrome of type 2 diabetes, and infertility. However, it is not known which of the five cloned Y receptors mediate these effects. Here, we show that crossing the Y2 receptor knockout mouse (Y2^{-/-}) onto the ob/ob background attenuates the increased adiposity, hyperinsulinemia, hyperglycemia, and increased hypothalamo-pituitary-adrenal (HPA) axis activity of ob/ob mice. Compared with lean controls, ob/ob mice had elevated expression of NPY and agouti-related protein (AgRP) mRNA in the arcuate nucleus and decreased expression of proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) mRNA. Y2 deletion in ob/ob mice significantly increased the hypothalamic POMC mRNA expression, with no effect on NPY, AgRP, or CART expression. [Y2^{-/-}/ob/ob] mice were no different from ob/ob littermates with respect to food intake and body weight, and Y2 receptor deficiency had no beneficial effect on the infertility or the reduced hypothalamo-pituitary-gonadotropic function of ob/ob mice. These data demonstrate that Y2 receptors mediate the obese type 2 diabetes phenotype of ob/ob mice, possibly via alterations in melanocortin tonus in the arcuate nucleus and/or effects on the HPA axis.

Abbreviations: AgRP, agouti-related protein; BAT, brown adipose tissue; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; DEXA, dual-energy X-ray absorptiometry; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPA, hypothalamo-pituitary-adrenal; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; TRH, thyrotropin-releasing hormone; UCP-1, uncoupling protein-1; WAT, white adipose tissue.

Neuropeptide Y (NPY) in the hypothalamus is known to be a strong stimulus for food intake (1, 2) and induces many neuroendocrine and metabolic changes that favor energy storage. Such changes include decreased thermogenesis in brown adipose tissue (BAT) (3), hyperinsulinemia, insulin resistance in muscles, insulin hyperresponsiveness in white adipose tissue (WAT) (4), activation of the hypothalamo-pituitary-adrenal (HPA) axis (4,5), and decreased activity of the hypothalamo-pituitary-thyrotropic (6), -somatotropic, and -gonadotropic axes (7). All of these adipogenic neuroendocrine and metabolic effects of central NPY administration persist, even when NPY-induced hyperphagia is prevented by pair-feeding (3,4,6,8), demonstrating that hyperphagia is not the only mechanism by which central NPY increases adiposity. However, it is not clear which of the five cloned Y receptors (Y1, Y2, Y4, Y5, and y6) are responsible for these effects.

The Y2 receptor is expressed in the central and peripheral nervous system. High concentrations of Y2 receptors can be found on NPY-ergic neurons in the hypothalamic arcuate nucleus (9,10), where it is thought to act as an inhibitory autoreceptor that can regulate the expression and secretion of NPY and other neurotransmitters (11-13). These arcuate neurons are also known to express the leptin receptor (14) and are located in an area accessible to peripheral hormones (15), enabling modulation of hypothalamic circuits important in the maintenance of energy homeostasis.

Previously, we have shown that germline as well as conditional hypothalamus-specific Y2 receptor deletion in mice resulted in reduced body weight despite an actual increase in food intake (16). Since the NPY-ergic system regulates energy homeostasis by interaction with a variety of pathways and hormones, including glucocorticoids (17-19) and leptin (20), we hypothesized that Y2 receptor deletion may alter energy homeostasis by interfering with such interactions. To investigate this possibility and to see whether the Y2 receptor plays a role in mediating the obese type 2 diabetes phenotype that results from leptin deficiency, we studied hormonal and metabolic indices and expression patterns of important central regulators of energy homeostasis in mice deficient in Y2 receptors as well as leptin.

RESEARCH DESIGN AND METHODS

Generation of [Y2^{-/-}ob/ob] double knockout mice. Male and female heterozygous (OB/ob) mice on a mixed C57BL/6-129/SvJ background were crossed with Y2^{-/-} animals (16) that were on the same mixed background. Double heterozygous [Y2^{+/-}OB/ob] animals were crossed again to subsequently obtain all of the nine possible genotypes. The ob genotype was determined by restriction fragment-length polymorphism analysis using the enzyme DdeI on a 496-bp PCR product generated from genomic DNA isolated from these mice with the primer set A (5'-GAGTCAAGCATTGTGGAGT-3') and B (5'-CAGTCGGTATCCGCCAAG-3'), with 35 cycles of 94 Celsius for 45 s, 55 Celsius for 1 min, and 72 Celsius for 20 s (21).

Tissue collection and analysis. At 16-18 weeks of age, male mice were killed by cervical dislocation in the morning, 4 h after removal of food from cages, for collection of trunk blood. Mice were killed within 90 s of initial handling to avoid time-dependent increases in corticosteronemia (22). Interscapular BAT was removed and frozen until analysis for uncoupling protein-1 (UCP-1) mRNA levels, standardized with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, and expressed as a percent of control values, as previously described (23). Brain was removed and immediately frozen on dry ice, and WAT depots (right inguinal, right epididymal, right retroperitoneal, and mesenteric), pancreas, entire small intestine (flushed), liver, right kidney, and right testes were collected and weighed. The length of the small intestine was measured before flushing. Plasma insulin levels were measured by radioimmunoassay kits from Linco Research (St. Louis, MO), plasma levels of corticosterone, free T4, and testosterone were measured with radioimmunoassay kits from ICN Biomedicals (Costa Mesa, CA); plasma IGF-1 concentrations were radioimmunoassayed with a kit from Bioclone Australia (Marrickville, Australia); and glycemia, cholesterolemia, and triglyceridemia were determined with colorimetric kits (Trace Scientific, Melbourne, Australia; Roche Diagnostics, Mannheim, Germany; and Sigma Diagnostics, St. Louis, MO). A subset of mice were used for determination of lean body mass by dual-energy X-ray absorptiometry (DEXA) (pDEXA Sabre with small animal software; Norland Medical Systems, Fort Atkinson, WI).

In situ hybridization. Coronal slices (20 μ m) of frozen brain were cut and thaw-mounted on charged slides. For in situ hybridization, DNA oligonucleotides complementary to mouse NPY (5'-GAGGGTCAGTCCACACAGCCCCATTTCGCTTGTTACCTAGCAT-3'), proopiomelanocortin (POMC) (5'-TGGCTGCTCTCCAGGCACCAGCTCC ACACATCTATGGAGG-3'), cocaine- and amphetamine-regulated transcript (CART) (5'-TCCTTCTCGTGGGACGCATCATCCAC GGCAGAGTAGATGTCCAGG-3'), agouti-related protein (AgRP) (5'-AGCTTGCGGCA GTAGCAAAGGCATTGAAGAAGCGGCAGT-AGCAC-3'), corticotropin-releasing hormone (CRH) (5'-CCGATAATCTCCATCAGTTTCCTGTTGCTGTGA GCTTGCTGAGCT), and thyrotropin-releasing hormone (TRH) 5'-AACCTTACTCCT CCAGAGGTT CCCTGACCCAGG CTTCCAGTTGTG-3') mRNAs were labeled with [³⁵S] thio-dATP (Amersham, Buckinghamshire, U.K.) using terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany). Matching sections from the same portion of the hypothalamus (approximately -1.8 mm [for POMC and CART] to -1.9 mm [NPY and AgRP] from Bregma for arcuate neurons and about -0.8 mm from Bregma for the paraventricular nucleus [PVN]) of knockout and wild-type mice were assayed together, as described previously (16). Hybridization with the respective sense oligonucleotides and in the presence of an excess of unlabeled antisense oligonucleotide were included as controls.

For evaluation of in situ hybridization, digital images of the areas of interest were acquired from photoemulsion-dipped and superficially counterstained brain slices at 200x magnification

using a Zeiss Axiophot equipped with the ProgRes digital camera. Silver grain density was evaluated by an experimentally blinded observer by outlining single neurons and measuring the total neuronal area and the area covered by silver grains (black grains in brightfield image) using National Institutes of Health image software. Percent of silver grain area, compared with total area calculated for single neurons, was averaged. Data are given as percent of control silver grain density averaged from at least four sections per peptide per animal.

Radioligand-binding studies were carried out on sections consecutive to those used for in situ hybridization (approximately -2.1 from Bregma), as previously described (24), using the Y2/Y5-prefering ligand 125I-PYY(3-36). Autoradiographs were scanned, and relative optical density values were determined over the strata radiatum and oriens of hippocampal area CA3 (delineated by the lateral ventricle and fimbria [the line connecting the ends of the two blades of molecular layer], the border to the stratum lacunosum moleculare, and approximately the border to CA2) and over the arcuate nucleus (using a higher magnification) and the lateral hypothalamus (measured lateral to the axes fornix-mammillothalamic tract). Specific binding was calculated by subtracting nonspecific (obtained from sections incubated in 1,000-fold excess of cold NPY) from total binding. Nonspecific labeling was uniform and never exceeded 5% of total signal in control CA3.

Statistical analyses. Before analysis, F tests were performed on all datasets to ensure nonsignificant differences in variance among the three groups of mice, justifying the use of parametric analyses. Results were assessed by factorial ANOVA. When there was a significant overall effect of Y2 deficiency, the ob locus, or interaction effects (P values shown in RESULTS), Fisher's posthoc tests were performed to locate differences, using StatView version 4.5 (Abacus Concepts, Piscataway, NJ). For all statistical analyses, $P < 0.05$ was accepted as statistically significant.

RESULTS

Reduced adiposity and normalization of insulinemia, glycemia, and thermogenic properties in [Y2-/-ob/ob] mice. The impact of Y2 receptor deficiency on body weight and adiposity of leptin-deficient ob/ob male mice is shown in Fig. 1. The higher body weight of ob/ob mice, as compared with wild-type mice, was unaffected by Y2 receptor deficiency (Fig. 1A). Hyperphagia of [Y2-/-ob/ob] double knockout mice at 12 weeks of age was also not different from ob/ob mice, and food intake was 6.96 ± 0.47 vs. 6.12 ± 0.12 g/day in ob/ob mice (means \pm SE of six mice per group). However, the increased combined WAT mass of ob/ob compared with wild-type mice (Fig. 1B) was significantly reduced in [Y2-/-ob/ob] double knockout mice. This effect of Y2 deletion was also observed when WAT was expressed as percent of body weight (wild-type 2.32 ± 0.13 , $n = 12$; ob/ob 8.01 ± 0.31 , $n = 15$; [Y2-/-ob/ob] 6.88 ± 0.20 , $n = 8$; overall $P < 0.0001$, posthoc $P < 0.01$ for all comparisons). It is interesting to note that these decreases in the sum of WAT mass in [Y2-/-ob/ob] mice were mostly attributable to significant decreases in epididymal and mesenteric depot weights, with no significant decrease in inguinal or retroperitoneal WAT depot weights (Fig. 1B).

Whereas ob/ob mice demonstrated significant hyperinsulinemia compared with wild-type controls, the insulinemia of [Y2-/-ob/ob] mice was 50% less than corresponding values in ob/ob mice and was not significantly different from wild-type values (Fig. 2A). Moreover, the marked hyperglycemia of ob/ob mice was significantly reduced by ablation of Y2 receptors, with [Y2-/-ob/ob] mice attaining values similar to wild-type mice (Fig. 2B).

The expression of UCP-1 mRNA in BAT, which is an index of thermogenic activity in a variety of situations (25), is substantially reduced in ob/ob mice relative to wild-type mice (Fig. 1C). Deletion of the Y2 receptor on the ob/ob background restored the UCP-1 mRNA levels to wild-type values (Fig. 2C), suggesting an increased thermogenic activity that may have contributed to the reduced fat mass in the double knockout animals.

Interestingly, the intestinal hypertrophy observed in ob/ob mice was significantly attenuated in [Y2-/-ob/ob] double knockout mice, with no significant effect on intestinal length (Table 1). Kidney size was also significantly reduced by Y2 receptor deletion in [Y2-/-ob/ob] mice (Table 1). However, cholesterolemia, triglyceridemia, and liver and pancreas weight, which are all markedly increased in ob/ob as compared with wild-type mice, were not affected by deletion of the Y2 receptor in [Y2-/-ob/ob] mice (Table 1).

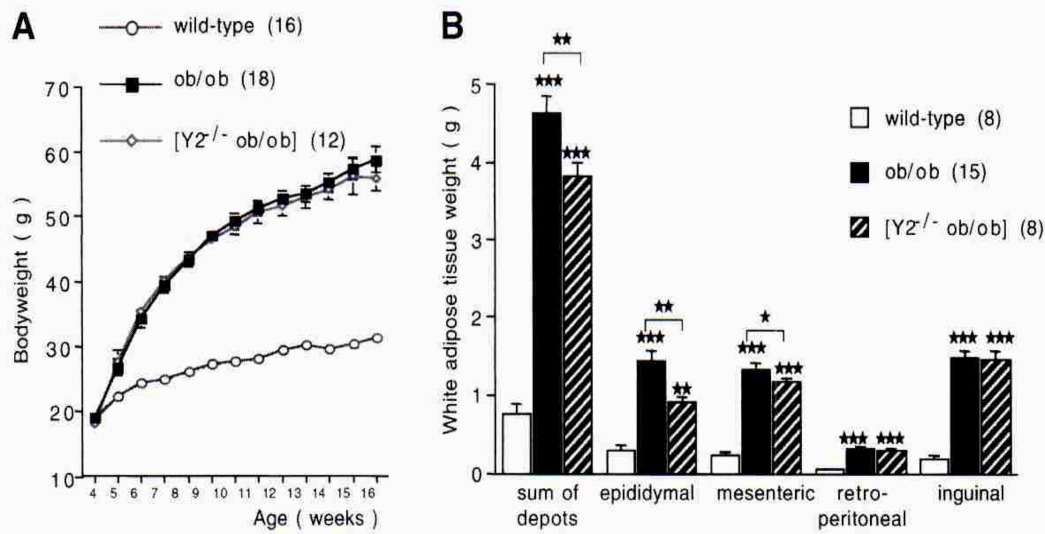


FIG. 1. Effect of germline Y2 receptor deficiency on body composition in leptin-deficient *ob/ob* mice. **A:** Body weight curve. **B:** Combined weight of right inguinal, right epididymal, right retroperitoneal, and mesenteric WAT, with the weight contribution of individual depots shown. Data are means \pm SE of the number of mice shown in parentheses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. wild-type mice (between-group differences indicated by horizontal bars above columns). Significance level of overall ANOVA analysis was $P < 0.001$ for all comparisons.

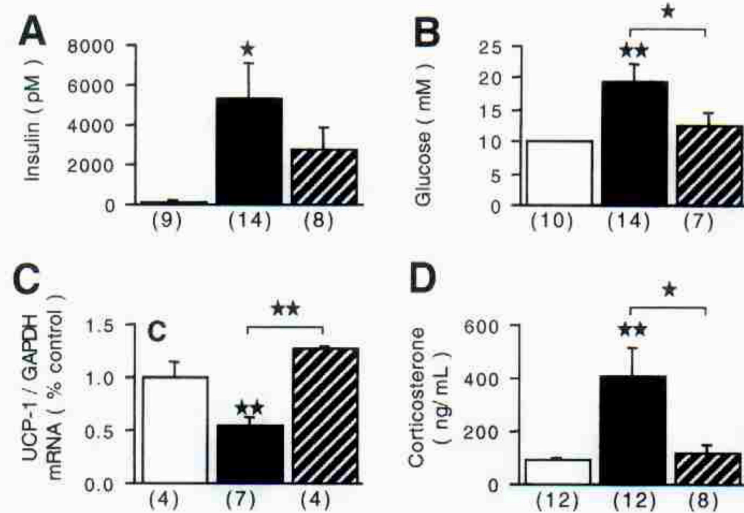


FIG. 2. Effect of germline Y2 receptor deficiency on hormonal and thermoregulatory parameters in leptin-deficient *ob/ob* mice. Insulinemia (**A**), glycemia (**B**), and UCP-1 mRNA levels (**C**) in BAT, standardized to GAPDH mRNA levels and expressed as a percent of wild-type values. **D:** Corticosteronemia. Data are means \pm SE of the number of mice shown in parentheses below each column. \square , wild-type mice; \blacksquare , *ob/ob* mice; \square with diagonal lines, [Y2^{-/-} *ob/ob*] mice. * $P < 0.05$ and ** $P < 0.01$ vs. wild-type mice (between-group differences indicated by horizontal bars above columns). Significance level of overall ANOVA analysis was $P < 0.05$ (**A** and **B**) and $P < 0.01$ (**C** and **D**).

Inhibitory effect of germline Y2 receptor knockout on the HPA axis of ob/ob mice. The effect of leptin and/or Y2 receptor deficiency on the HPA axis is depicted in Fig. 2D, Fig. 3, and Table 2. Y2 knockout per se significantly reduced the expression of CRH mRNA in the PVN compared with wild-type mice (Fig. 3 and Table 2), accompanied by a tendency to decrease in corticosteronemia (64 ± 17 ng/ml in Y2^{-/-} on the lean background versus 102 ± 32 ng/ml in wild-type mice, $n = 10$ -18 male mice per group). Compared with wild-type, *ob/ob* mice had significant increases in both CRH mRNA expression levels in the PVN (Fig. 3 and

TABLE 1

Comparison of plasma hormone and metabolite concentrations and organ weights in [Y2^{-/-}ob/ob] and control mice

	Wild type	ob/ob	[Y2 ^{-/-} ob/ob]	Overall P
Intestine (g)	1.03 ± 0.05 (13)	1.89 ± 0.10 (11)*	1.61 ± 0.05 (7)*†	<0.0001
Intestine (cm)	32.7 ± 4.8 (10)	45.8 ± 1.7 (10)‡	43.0 ± 1.3 (7)§	<0.01
Kidney (g)	0.41 ± 0.02 (13)	0.54 ± 0.03 (14)*	0.46 ± 0.02 (8)	<0.01
Cholesterol (mmol/l)	3.54 ± 0.59 (13)	5.74 ± 0.41 (15)‡	6.56 ± 0.60 (6)‡	<0.01
Triglyceride (mmol/l)	1.62 ± 0.21 (10)	2.48 ± 0.19 (15)‡	2.86 ± 0.24 (6)‡	<0.01
Liver (g)	1.44 ± 0.04 (13)	4.56 ± 0.31 (15)*	4.45 ± 0.28 (7)*	<0.0001
Pancreas (g)	0.29 ± 0.01 (16)	0.41 ± 0.02 (16)*	0.37 ± 0.01 (7)§	<0.001
IGF-1 (mg/dl)	322 ± 22 (24)	218 ± 40 (12)§	290 ± 40 (9)	<0.05
Free T4 (pmol/l)	20.6 ± 2.3 (9)	19.7 ± 2.6 (6)	18.9 ± 3.0 (6)	NS
Testosterone (nmol/l)	14.2 ± 3.9 (16)	4.2 ± 2.0 (13)§	5.2 ± 1.4 (7)§	<0.05
Testes (g)	0.33 ± 0.02 (13)	0.19 ± 0.01 (14)*	0.20 ± 0.02 (8)*	<0.05

Means ± SE of the number of mice shown in parentheses. *P < 0.05 vs. wild-type mice; †P < 0.01 vs. ob/ob mice; ‡P < 0.01, §P < 0.05 vs. wild-type mice; ||P < 0.05 vs. ob/ob mice. Overall P: the level of significance of ANOVA analysis. NS, not significant.

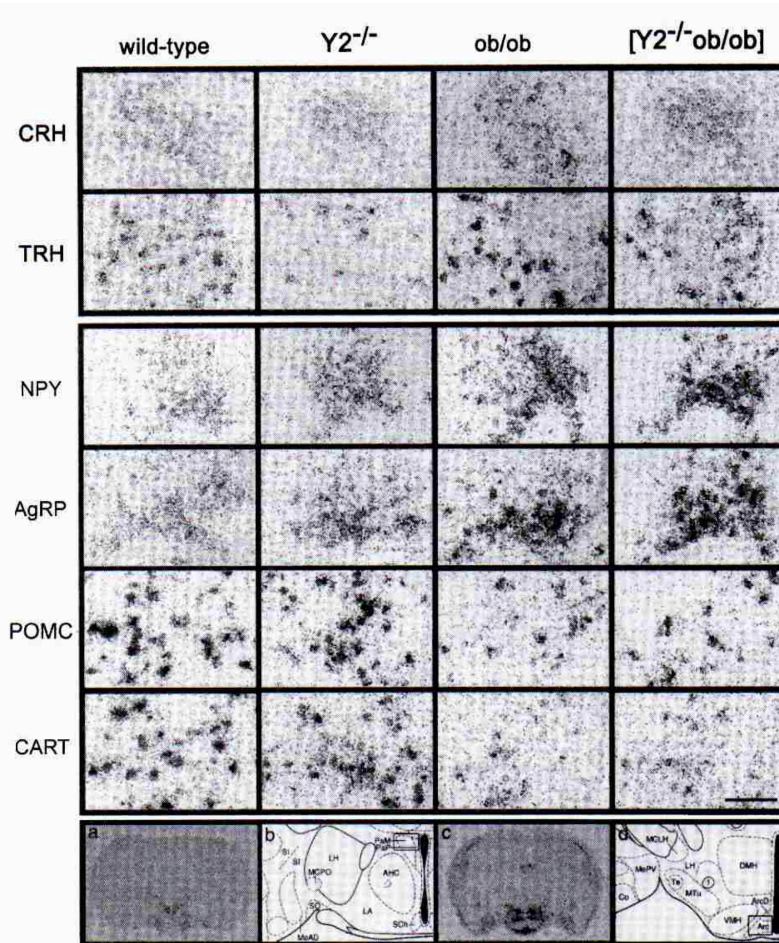


FIG. 3. Effect of germline Y2 receptor deletion on CRH and TRH mRNA levels in neurons of PVN (upper panel) and on NPY, AgRP, POMC, and CART mRNA levels in the arcuate nucleus (Arc; middle panel). High-power bright-field photomicrographs of dipped sections obtained from wild-type, Y2^{-/-}, ob/ob, and [Y2^{-/-}ob/ob] double knockout mice after in situ hybridization for the respective mRNA. Note the difference in POMC mRNA levels in [Y2^{-/-}ob/ob] double knockout compared with ob/ob mice. Scale bar = 100 μm. The PVN was depicted at about -0.8 mm from Bregma and the Arc at about -1.8 to -1.9 mm from Bregma, depicted as low magnification film autoradiograph for TRH in a and CART in c. Maps are modified from Franklin and Paxinos, schematically depicting the hypothalamus at the level of the PVN (b) and Arc (d). The areas represented by the high magnification photomicrographs are outlined as black rectangles. ArcD, arcuate nucleus, dorsal; AHC, anterior hypothalamic area central; Co, cortical amygdaloid nucleus; DMH, dorsomedial hypothalamic nucleus; f, fornix; LA, lateral hypothalamic area; MCLH, magnocellular nucleus lateral hypothalamus; MCPO, magnocellular preoptic nucleus; MeAD, medial amygdaloid nucleus, anterodorsal; MePV, medial amygdaloid nucleus, posterodorsal; Mtu, medial tubular nucleus; PaM, PVN magnocellular part; PaP, PVN parvocellular part; Sch, supra-chiasmatic nucleus; SI, substantia innominata; SO, supraoptic nucleus; Te, terete hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; 3, third ventricle.

Table 2) and marked hypercorticism (Fig. 2D). Y2 receptor knockout in [Y2^{-/-}ob/ob] mice not only reduced CRH mRNA levels in the PVN to values significantly less than wild-type controls (Fig. 3 and Table 2) but also normalized plasma concentrations of corticosterone compared with wild-type values (Fig. 2D).

Effect of germline Y2 receptor knockout on the hypothalamo-pituitary-somatotropic axis of ob/ob mice. Since germline Y2 receptor deficiency significantly reduced fat mass in [Y2^{-/-}ob/ob] mice compared with ob/ob controls in the absence of any reduction in body weight, we investigated whether changes in activity of the hypothalamo-pituitary-somatotropic axis may have occurred. Plasma levels of IGF-1, the main growth effector of the somatotrophic axis, were significantly reduced in ob/ob mice compared with wild-type mice (Table 1), which

TABLE 2
 Expression levels of neuropeptide mRNAs in hypothalamic nuclei

mRNA	Wild type	Y2 ^{-/-}	ob/ob	[Y2 ^{-/-} ob/ob]
Paraventricular nucleus				
CRH	100 ± 3.5 (5)	71 ± 2.0 (5)*	113 ± 2.3 (4)†	72 ± 4.9 (4)‡§
TRH	100 ± 2.6 (6)	60 ± 4.5 (5)‡	117 ± 5.9 (5)†	68 ± 7.7 (5)‡§
Lateral hypothalamic area				
TRH	100 ± 6.2 (5)	103 ± 3.6 (5)	115 ± 2.9 (5)†	120 ± 2.2 (5)*
Arcuate nucleus				
NPY	100 ± 4.2 (10)	110 ± 10.7 (5)	180 ± 7.5 (5)‡	184 ± 22.6 (5)‡
AgRP	100 ± 3.3 (9)	112 ± 1.8 (5)	167 ± 7.3 (5)‡	178 ± 11.6 (3)‡
POMC	100 ± 2.7 (10)	93 ± 2.6 (5)	33 ± 3.0 (5)‡	57 ± 4.6 (5)‡
CART	100 ± 2.7 (10)	84 ± 4.2 (5)*	23 ± 1.3 (5)‡	24 ± 2.9 (5)‡

Data represent mean labeling intensity of neurons, given as percent of wild-type ±SE of the number of mice shown in parentheses. **P* < 0.01, †*P* < 0.05, ‡*P* < 0.001 vs. wild-type mice; §*P* < 0.001, ||*P* < 0.01 vs. ob/ob mice. Significance level of overall ANOVA analysis was *P* < 0.001 for all comparisons.

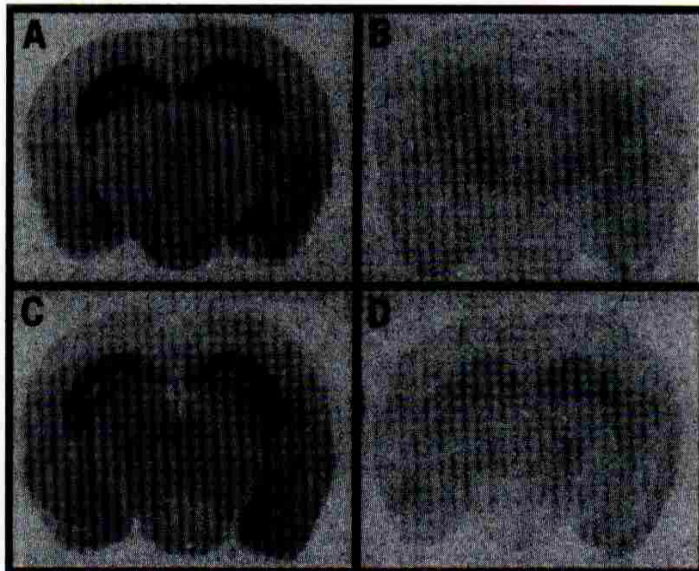


FIG. 4. Film autoradiographs of ¹²⁵I-PYY(3-36) binding on brain sections of control (A), Y2^{-/-} (B), ob/ob (C), and [Y2^{-/-}ob/ob] double knockout mice (D). Sections (20 μm) taken at ~2.1 mm posterior to bregma are depicted. Note the almost entire loss of ¹²⁵I-PYY(3-36) binding sites in both groups of Y2-deficient mice.

is in agreement with the known reduction in lean body mass in these mice (26). By contrast, in [Y2^{-/-}ob/ob] mice, the concentrations of IGF-1 were not significantly different from values of wild-type mice (Table 1). The whole-body lean mass (determined by DEXA) of [Y2^{-/-}ob/ob] mice was greater than that of ob/ob controls, at 19.8 g (42.4% of body wt) vs. 16.0 g (32.7% of body wt), albeit lean body mass was less than that of wild-type mice, 25.2 g (87.3% of body wt), in both groups of obese animals.

Effect of germline Y2 receptor knockout on the hypothalamo-pituitary-thyrotropic and -gonadotropic axes of ob/ob mice. We also investigated the effect of Y2 receptor knockout on other hypothalamo-pituitary axes that are known to be dysregulated by hypothalamic NPY administration or leptin deficiency. Figure 3 and Table 2 show that the expression of TRH mRNA in the PVN was significantly increased in ob/ob mice versus wild-type controls and that these values were significantly reduced in both Y2^{-/-} and [Y2^{-/-}ob/ob] mice. Elevated TRH mRNA expression was also observed in ob/ob mice in the lateral hypothalamus, but in contrast to the PVN, no influence of Y2 deficiency was evident in the lateral hypothalamus (Table 2). These changes in central TRH expression were not reflected by changes in plasma concentrations of free T4 (Table 1), which showed no significant difference among the groups of mice investigated. Leptin-deficient ob/ob mice exhibit profound inhibition of the hypothalamo-pituitary-gonadotropic axis, exemplified in this study by the significant reductions in plasma testosterone levels, testicular weight (Table 1), and sterility (only one of eight male

ob/ob mice paired with fertile OB/ob females were able to produce live offspring). Y2 receptor knockout had no impact on these parameters of reproductive function in [Y2^{-/-}/ob/ob] mice (Table 1), and male [Y2^{-/-}/ob/ob] animals paired with fertile [Y2^{-/-}/OB/ob] females were unable to produce offspring (n = 5 breeding pairs).

Altered expression levels of neuropeptides in the arcuate nucleus of [Y2^{-/-}/ob/ob] double knockout mice. Because Y2 receptors are known to be expressed on NPY neurons in the arcuate nucleus, where they can modulate the expression and release of several important neurotransmitters, we determined the expression level of several hypothalamic peptides in the different models. All genetic modifications caused alterations in mRNA expression of the neuropeptides investigated in the arcuate nucleus (Fig. 3 and Table 2). Y2 deficiency increases NPY and AgRP mRNA levels, while POMC and CART mRNA levels are downregulated in the arcuate nucleus in Y2^{-/-} compared with wild-type mice (Fig. 3 and Table 2). As expected, leptin-deficient ob/ob mice exhibited marked increases in NPY and AgRP mRNA levels, accompanied by a strong suppression of POMC and CART mRNA expression (Fig. 3 and Table 2). [Y2^{-/-}/ob/ob] animals displayed mRNA levels for NPY, AgRP, and CART in the arcuate nucleus that were similar to those of ob/ob mice; however, POMC mRNA levels were doubled compared with ob/ob mice, suggesting a direct or indirect regulation of expression through Y2 receptors (Fig. 3 and Table 2).

Altered binding levels for 125I-PYY(3-36) in [Y2^{-/-}/ob/ob] double knockout mice. To investigate whether Y2 receptor deletion mediates the observed alterations on hypothalamic functions via alteration in the expression of other Y receptors, we performed radioligand-binding assays on brain slices. In wild-type and ob/ob mice, the binding of 125I-PYY(3-36), a Y2/Y5-preferring ligand, was highest in the strata radiatum and oriens of CA3 region in the hippocampus (Fig. 4 and Table 3). The binding levels in the hypothalamus of these groups were markedly lower than in the CA3 but were still well above the nonspecific binding, in line with previous studies (27). Binding levels were somewhat lower in the lateral hypothalamus of ob/ob than wild-type mice (Table 3). In contrast, both Y2-deficient mice groups displayed a pronounced drop in 125I-PYY(3-36) binding, rarely exceeding levels of nonspecific binding, confirming successful Y2 receptor deletion (Fig. 4 and Table 3). Interestingly, this finding also demonstrates a very low level of Y5 receptor expression in Y2^{-/-} and [Y2^{-/-}/ob/ob] mice.

TABLE 3
¹²⁵I-PYY(3-36) binding in hippocampal area CA3 and hypothalamic nuclei

Area	Wild type	Y2 ^{-/-}	ob/ob	[Y2 ^{-/-} /ob/ob]
CA3	0.233 ± 0.0155	0.016 ± 0.0063*	0.212 ± 0.0318	0.029 ± 0.0092*
Lateral hypothalamus	0.079 ± 0.0070	0.004 ± 0.0032*	0.067 ± 0.0041†	0.008 ± 0.0045*
Arcuate nucleus	0.050 ± 0.0042	0.004 ± 0.0050*	0.055 ± 0.0035	0.002 ± 0.0012*

Data represent mean labeling intensity as relative optical densities ±SE of five mice per group. **P* < 0.001, †*P* < 0.05 vs. wild-type mice. Significance level of overall ANOVA analysis was *P* < 0.001 for all comparisons.

DISCUSSION

Here, we demonstrate that the obese type 2 diabetes phenotype of leptin-deficient ob/ob mice is partially mediated by signaling through Y2 receptors, since crossing our Y2 receptor knockout onto the ob/ob strain attenuated the increased adiposity, hyperinsulinemia, hyperglycemia, and increased HPA axis activity of these mice.

This study and our previous work have revealed an important regulation of the HPA axis by Y2 receptors. On a lean background as well as on the ob/ob background, Y2 receptor deficiency reduced CRH mRNA expression in the hypothalamic PVN and reduced plasma corticosterone concentrations. In addition to modulating output of the HPA axis, Y2 receptors also mediate responses to glucocorticoids. Whereas in wild-type mice corticosterone administration caused an obesity syndrome, Y2 knockout mice were not affected by this treatment, suggesting that excess glucocorticoids mediate their effects on energy balance, at least in part, through the Y2 receptor (16).

The improvement in hormonal and metabolic parameters in [Y2^{-/-}/ob/ob] versus ob/ob mice may be due to these reductions in HPA axis activity and responsiveness to endogenous glucocorticoids. Adrenalectomy is known to attenuate aspects of the obese type 2 diabetes

syndrome of leptin-deficient mice and rats, and the syndrome can be restored by specific administration of exogenous glucocorticoids (28-30), demonstrating a causal role of excess glucocorticoids in this syndrome. It is thought that adrenalectomy mediates improvements in hormonal and metabolic indices in obese rodents by normalizing activity of hypothalamic peptide systems that regulate hormonal-metabolic status and energy balance (31,32), notably by increased melanocortin tonus (33). Indeed, adrenalectomy normalized the elevated AgRP and reduced POMC mRNA levels in the hypothalamus of ob/ob mice to wild-type levels, with no significant effect on the increased NPY mRNA expression of these mice (33). Similarly, in [Y2^{-/-}/ob/ob] mice, the normalization of corticosteronemia may have contributed to the increased hypothalamic POMC expression compared with ob/ob animals, which may have improved hormonal and metabolic parameters by increasing melanocortin agonism by a melanocyte-stimulating hormone.

Besides an indirect effect via reduced HPA axis output, the increased hypothalamic POMC expression of [Y2^{-/-}/ob/ob] mice may be mediated by release of inhibitory effects of Y2 receptors on POMC neurons within the hypothalamus. NPY-expressing neurons, which are known to coexpress the Y2 receptor (9) and contain GABA, make inhibitory synaptic contact with POMC neurons in the arcuate nucleus (34). It is possible that Y2 activation induces effects by inhibiting GABA-ergic transmission from NPY/GABA neurons, thereby releasing the inhibition of POMC neurons by GABA (34).

Y2 receptor deletion had no beneficial effect on the hyperphagia of ob/ob mice. Although increased hypothalamic NPY-ergic transmission mediates at least part of the hyperphagia of ob/ob mice (20), this effect is mediated via other receptors besides Y2, probably the Y1 receptor. Hyperphagia was significantly reduced in ob/ob mice that had a null mutation in the Y1 receptor gene (35) but not in Y5 receptor-deficient ob/ob mice (36). Further evidence that Y1 receptors mediate hyperphagia in situations of elevated endogenous NPY-ergic tonus are the observations that fasting-induced refeeding is attenuated in Y1^{-/-} (37) but not in Y5^{-/-} (36) mice. In contrast, Y2 agonism actually decreases food intake. Acute or chronic administration of the Y2-preferring agonist NPY (13-36) to normal rats decreased overnight feeding (38) and daily food intake (7). More importantly, it has been shown that the gut-derived peptide PYY (3-36), released postprandially, reduces food intake in humans and rodents via activation of Y2 receptors (39). This hypophagic effect of Y2 receptor agonism may be mediated by decreased hypothalamic expression and secretion of NPY and AgRP (12,16). NPY release from hypothalamic slices is specifically inhibited by Y2 agonism (12). Furthermore, Y2 deletion increases NPY and AgRP mRNA levels in the arcuate nucleus (16). The lack of effect of Y2 receptor knockout on hypothalamic NPY or AgRP expression, or on food intake in ob/ob mice, is likely due to already maximal levels of these parameters.

Although food intake was unaffected by Y2 receptor deficiency in [Y2^{-/-}/ob/ob] mice, it is likely that efficiency of nutrient absorption from the gut was reduced in [Y2^{-/-}/ob/ob] versus ob/ob mice, which probably contributed to the reduced fat mass of the double knockout mice. Evidence for this is our observation of reduced intestinal mass in [Y2^{-/-}/ob/ob] compared with ob/ob mice and the previous finding that increases in intestinal mass in ob/ob mice correlate with increased absorption of all nutrient groups (40).

Despite the significant reductions in fat mass, Y2 receptor deletion did not reduce the increased body weight of ob/ob mice. Changes in adipose tissue depot weights are not always associated with commensurate changes in body weight in experimental animals (23, 37). This may be because changes in fat mass are often associated with opposite changes in lean mass. For instance, leptin-deficient and NPY-infused rodents have increased fat mass but decreased lean mass or naso-anal length and/or downregulation of the hypothalamo-pituitary somatotrophic axis (7,26,41). The reduced fat mass of [Y2^{-/-}/ob/ob] versus ob/ob mice was associated with increased plasma concentrations of IGF-1, suggesting improved function of the somatotrophic axis, which may have contributed to an increase in lean mass in the former group. It is interesting to note that Y2 receptor deficiency on the lean background markedly increased bone density (42), in keeping with a role of Y2 receptors in the regulation of lean body mass.

This study shows that Y2 receptors are involved in a direct or indirect regulation of hypothalamic TRH expression, since TRH mRNA levels in the PVN were downregulated in Y2^{-/-} and [Y2^{-/-}/ob/ob] mice compared with wild-type and ob/ob controls. This was not

associated with any effect on plasma concentrations of free T4. It is not clear how this change in central TRH expression affected functional activity of the hypothalamo-pituitary-thyroid axis, or whether any changes in thyrotropic function contributed to any of the observed metabolic changes in [Y2-/-ob/ob] mice, such as the increased expression of BAT UCP-1, which is regulated by thyroid hormones (43).

It is noteworthy that Y2 receptor deficiency attenuates aspects of the leptin-deficient phenotype that pertain to the metabolic syndrome, with no effect on the infertility and defective hypothalamo-pituitary-gonadotropic function of ob/ob mice. In contrast, Y4 receptor knockout specifically rescued the infertility of ob/ob mice, with no effect on fat mass, insulinemia, or glycemia. Null mutation of Y1 receptors in ob/ob mice attenuated not only the hyperphagia and increased body weight, but also the reduced gonadotropic function of these mice (35). These data clearly show that although increased hypothalamic NPY signaling contributes to the hyperphagia, obesity, hypercorticosteronemia, decreased somatotrophic axis activity, and infertility of ob/ob mice (20), these different effects of leptin deficiency are mediated by distinct Y receptors.

In summary, Y2 receptor deletion attenuates the obese type 2 diabetes phenotype of ob/ob mice, without any effects on fertility. This may be mediated by increased central melanocortin agonism, indicated by increased POMC mRNA expression in the arcuate nucleus, which may be a direct effect of Y2 deletion, or secondary to reduced HPA axis activity in [Y2-/-ob/ob] mice. Y2-specific therapeutics may therefore be beneficial in the clinical management of obesity and type 2 diabetes, as well as in the treatment of osteoporosis, without side effects on reproductive function.

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