

Y4 receptor knockout rescues fertility in *ob/ob* mice

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Hypothalamic neuropeptide Y (NPY) has been implicated in the regulation of energy balance and reproduction, and chronically elevated NPY levels in the hypothalamus are associated with obesity and reduced reproductive function. However, it is not known which one of the five cloned Y receptors mediates these effects. Here we show that crossing the Y4 receptor knockout mouse (*Y4*^{-/-}) onto the *ob/ob* background restores the reduced plasma testosterone levels of *ob/ob* mice as well as the reduced testis and seminal vesicle size and morphology to control values. Fertility in the sterile *ob/ob* mice was greatly improved by Y4 receptor deletion, with 100% of male and 50% of female *Y4*^{-/-},*ob/ob* double knockout mice producing live offspring. Development of the mammary ducts and lobuloalveoli was significantly enhanced in pregnant *Y4*^{-/-} and *Y4*^{-/-},*ob/ob* females. Consistent with the improved fertility and enhanced mammary gland development, gonadotropin releasing hormone (GnRH) expression was significantly increased in *Y4*^{-/-} and *Y4*^{-/-},*ob/ob* animals. *Y4*^{-/-} mice displayed lower body weight and reduced white adipose tissue mass accompanied by increased plasma levels of pancreatic polypeptide (PP). However, Y4 deficiency had no beneficial effects to reduce body weight or excessive adiposity of *ob/ob* mice. These data suggest that central Y4 receptor signaling specifically inhibits reproductive function under conditions of elevated central NPY-ergic tonus.

[*Key Words*: Neuropeptide Y; Y4 receptor knockout; fertility; *ob/ob* mouse; gonadotropin releasing hormone; adiposity]

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Hypothalamic neuropeptide Y (NPY) is a major central regulator of sexual behavior and reproductive functions. Intracerebroventricular (ICV) administration of NPY to sex steroid-primed ovariectomized (OVX) rats increases secretion of luteinizing hormone (LH), and stimulates secretion of gonadotropin releasing hormone (GnRH) from the median eminence *in vitro*, functions that probably mediate the preovulatory surge of LH release (Sabatino et al. 1990; Urban et al. 1996; Jain et al. 1999). In contrast, when centrally administered to sex steroid-deficient OVX rats, or to intact male and female rats, NPY markedly inhibits reproductive function (Clark et al. 1985; Reznikov and McCann 1993; Xu et al. 1993; Pierroz et al. 1996). This includes decreased GnRH receptor concentration in the pituitary gland, reduced pituitary weight, and decreased plasma concentrations of prolactin, LH, follicle stimulating hormone (FSH), and testosterone. Testicular and seminal vesicle or ovarian weights are also reduced, and sexual maturation and estrous cyclicity in female rats is disrupted, leading to

drastic suppression of male and female copulatory behavior (Clark et al. 1985; Reznikov and McCann 1993; Xu et al. 1993; Pierroz et al. 1996). These inhibitory effects of NPY on reproductive function probably contribute to the decreased fertility observed under conditions of negative energy balance, such as food restriction, heavy exercise, lactation, and insulin-dependent diabetes mellitus, all of which are associated with elevated hypothalamic NPY expression (Aubert et al. 1998; Krysiak et al. 1999). In this way NPY coordinates energy availability with reproduction, inhibiting procreation during unfavorable metabolic conditions.

Interestingly, obesity is also associated with reproductive defects and reduced fertility (Caprio et al. 2001). Genetically obese *ob/ob* mice lack functional leptin, the hormone produced mainly by white adipose tissue. This mutation not only leads to hyperphagia, massive obesity, and the associated hormonometabolic defects (hypercortisolemia, hyperinsulinemia, hyperglycemia, and insulin resistance), but also leads to infertility due to insufficient hypothalamo-pituitary-gonadal drive, underdevelopment of reproductive organs, and impaired spermatogenesis (Caprio et al. 2001). In *ob/ob* mice, the lack of leptin-mediated inhibition of NPY expression and secretion in the hypothalamus (Stephens et al. 1995;

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Sainsbury et al.

Schwartz et al. 1996; Widdowson and Wilding 2000) leads to chronically elevated hypothalamic NPY-ergic activity. This secondary effect of leptin deficiency contributes to many of the associated defects. Indeed, treatment of *ob/ob* mice with leptin reduces NPY mRNA expression and peptide levels in the hypothalamus, reduces the hyperphagic, obese phenotype, and also restores fertility of male and female mice by improved function of the hypothalamo-pituitary-gonadal axis (Stephens et al. 1995; Chehab et al. 1996; Mounzih et al. 1997). Importantly, food restriction that produced a degree of weight loss similar to that produced by leptin treatment did not reduce central NPY expression and did not restore fertility in *ob/ob* mice (Mounzih et al. 1997). Further evidence that elevated central NPY-ergic tone mediates the pathology of leptin deficiency is that most of the defects of *ob/ob* mice, including infertility, are attenuated or normalized when crossed onto NPY knockout mice (Erickson et al. 1996).

The various functions of NPY are mediated by the Y receptor gene family, consisting of at least five distinct members (Y1, Y2, Y4, Y5, and y6) (Blomqvist and Herzog 1997). In addition to their involvement in reproduction and energy homeostasis, these Y-receptors when activated by their ligands, NPY, peptide YY (PYY), and pancreatic polypeptide (PP), can also modulate other important physiological functions, including circadian rhythms, gastrointestinal motility, memory, anxiety, nociception, and blood pressure (Hokfelt et al. 1998; Gehlert 1999; Kalra et al. 1999). Messenger RNAs for Y1, Y2, and Y5 are widely distributed throughout the brain (Naveilhan et al. 1998; Parker and Herzog 1999). In contrast, the Y4 receptor is predominantly expressed in the periphery including tissues such as the pancreas, intestine, colon, heart, and liver (Bard et al. 1995; Lundell et al. 1995). However, significant amounts of Y4 mRNA and specific binding sites have also been found in key areas of the hypothalamus such as the paraventricular nucleus and in certain brainstem nuclei including the area postrema and the nucleus tractus solitarius (Parker and Herzog 1999; Larsen and Kristensen 2000). Y receptors show very low primary amino acid sequence identity, yet surprisingly exhibit very similar pharmacology, with NPY and PYY being equipotent at all receptor subtypes. PP has high affinity only for the Y4 receptor, and in some species it also has moderate affinity for the Y1 and Y5 receptors.

Although NPY is known to be involved in numerous physiological and pathophysiological processes, the clarification of the functions of specific Y receptor subtypes has been severely hampered by the lack of subtype-selective agonists and antagonists. The functions of the Y4 receptor subtype and its high-affinity agonist PP are among the less well understood of the Y receptor family. However, it was recently demonstrated that ICV administration of the Y1 antagonist and Y4 agonist 1229U91 (Schober et al. 1998) to estrogen-primed OVX or intact male rats rapidly increased FSH and/or LH secretion (Jain et al. 1999; Raposo et al. 2000). These effects were attributed to Y4 activation by pharmacological analysis

of other partial Y4 agonists (Raposo et al. 2000), and also because rat PP, which is specific for the rat Y4 receptor, induced a similar profile of LH secretion in estrogen-primed OVX rats (Jain et al. 1999). Since PP is not expressed within the brain and does not cross the blood-brain barrier, it is possible that instead NPY acts as a central, albeit lower-affinity ligand for the hypothalamic Y4 receptor when expression levels are sufficiently high to agonize this receptor. We therefore hypothesized that under conditions of high hypothalamic NPY levels, as in energy deficit or leptin-deficient obesity, NPY could modulate reproductive functions through activation of Y4 receptors. To test this hypothesis we generated Y4 receptor knockout mice and crossed them with the obese *ob/ob* mutant strain and analyzed the effects on energy homeostasis and fertility.

Results

Generation of Y4 receptor knockout mice

A targeting vector for the Y4 receptor gene was designed which allowed the production of both germ-line ($Y4^{-/-}$) and conditional ($Y4^{lox/lox}$) knockout mice (Fig. 1A). A cassette containing the neomycin resistance gene (*Neo*) flanked on either side by a 34 bp-long Cre-recombinase recognition (loxP) site oriented in the same direction was placed downstream of the Y4 receptor gene. A third loxP sequence was inserted into intron I of the Y4 receptor gene (Fig. 1A). Mouse embryonic stem (ES) cells from the strain 129/SvJ were transfected and selected under standard conditions. Positively targeted clones were identified by Southern analysis. Hybridization with the *Neo* gene was used to verify single integration (data not shown).

Two positive ES cell clones for the Y4 receptor construct were injected into blastocysts originating from C57BL/6 mice and implanted into pseudopregnant mice. Offspring with the highest percentage of agouti coat color were crossed with X chromosome-linked, oocyte-specific Cre-recombinase-expressing C57BL/6 mice (Schwenk et al. 1995) to obtain either heterozygotes carrying the floxed gene (conditional, $Y4^{+/lox}$), or heterozygotes carrying the Cre-recombinase gene and having the floxed gene already deleted (germ line $Y4^{+/-}$). DNA isolated from tail tips was used in Southern blot analysis (*EcoRI*) to confirm correct integration and/or modification of the targeted allele (Fig. 1B). Absence of the Y4 gene in $Y4^{-/-}$ mice was confirmed by Southern analysis (*EcoRV*) using a DNA fragment specific for the Y4 receptor coding sequence (Fig. 1C). All further mice generated were maintained on this 50% C57BL/6 – 50% 129/SvJ background.

Breeding of heterozygous germ-line knockout animals produced no significant deviation from the expected Mendelian ratio of genotypes (25:50:25). $Y4^{-/-}$ animals breed normally; however, the gender ratio in $Y4^{-/-}$ offspring is significantly shifted to a greater proportion of females (56%, $n = 112$, $P < 0.01$). Interestingly, male $Y4^{-/-}$ mice show very aggressive behavior with increased

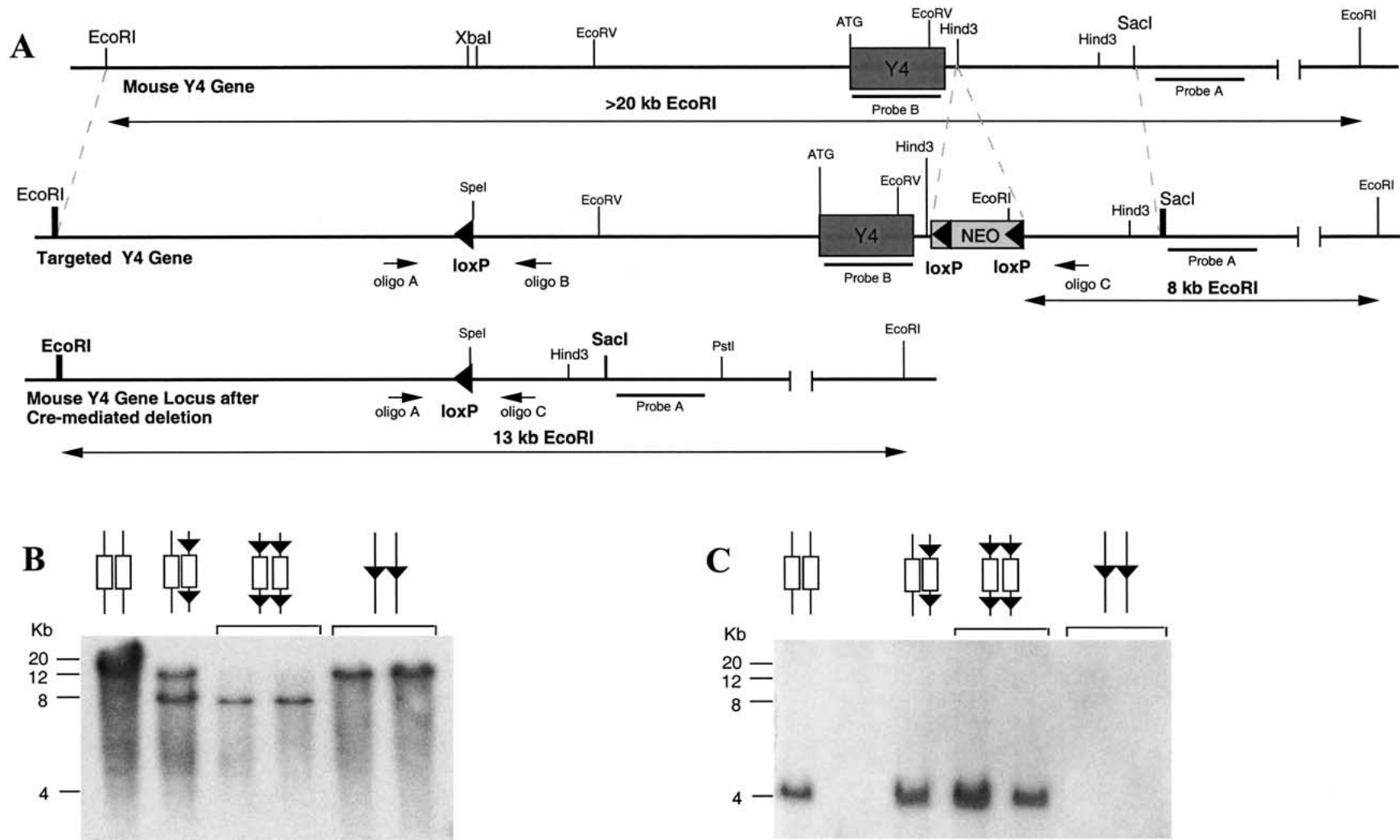


Figure 1. Generation of Y4 receptor knockout mice. (A) Targeting vector design and screening strategy. Small arrows indicate the position and orientation of oligonucleotides used in the PCR analysis, and bars indicate the position of probes used for Southern analysis of genomic DNA from targeted ES cells as well as knockout animals. (B,C) Southern analysis of genomic DNA isolated from conditional and germ line Y4 receptor knockout mice cut with *EcoRI* or *EcoRV*, using probes A and B, respectively.

Sainsbury et al.

incidences of fighting between littermates causing injuries. Female $Y4^{-/-}$ mice behave normally in early life and adulthood, but show aggressive behavior and occasionally kill littermates after 20–24 wk of age

Reduced body weight and increased plasma pancreatic polypeptide levels in $Y4^{-/-}$ mice

At 4 wk of age, the body weights of female $Y4^{-/-}$ mice were significantly less than that of $Y4^{+/+}$ controls. The body weights of male $Y4^{-/-}$ mice at that age were not significantly different from control values. Both genders of $Y4^{-/-}$ mice, particularly males, gained significantly less weight over the next 12 wk of monitoring (Fig. 2). Consistent with this is the finding that the 24-h food intake of male but not female $Y4^{-/-}$ mice was significantly less than that of wild-type controls (4.42 ± 0.10 vs. 5.64 ± 0.30 g at 8 wk, $P < 0.001$, and 4.18 ± 0.10 vs. 4.70 ± 0.28 g at 12 wk, $P < 0.05$, means \pm SEM of 9–27 mice per group).

Plasma levels of PP (the endogenous high-affinity ligand for Y4 receptors) were increased by two- to threefold in both male and female $Y4^{-/-}$ mice compared to wild-type controls (Fig. 3A), with male mice having significantly greater basal plasma PP concentrations than

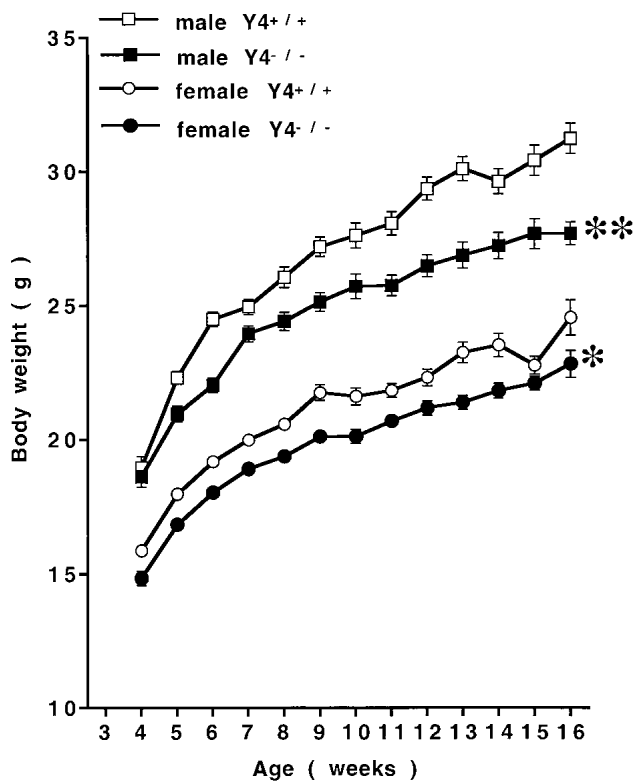


Figure 2. Body weight of Y4 receptor knockout mice. Body weight of male $Y4^{-/-}$ (■) and female $Y4^{-/-}$ (●) mice compared with combined wild-type ($Y4^{+/+}$) and heterozygous ($Y4^{+/-}$) controls (males □, females ○). Values are means \pm SEM of 20–32 mice per group. * $P < 0.05$ and ** $P < 0.01$ versus curve for same-sex controls.

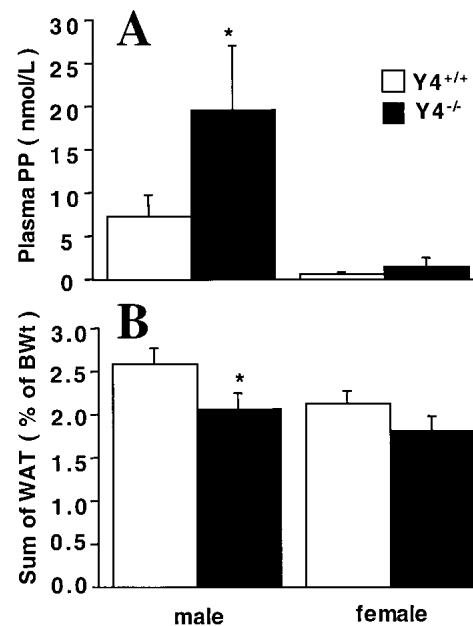


Figure 3. Plasma pancreatic polypeptide concentrations and adiposity of Y4 receptor knockout mice. (A) Plasma levels of pancreatic polypeptide, and (B) weight (as percent of body weight) of combined right inguinal, right epididymal or periovarian, right retroperitoneal, and mesenteric white adipose tissue (WAT) of germ line $Y4^{-/-}$ mice (filled bars) versus $Y4^{+/+}$ control mice (open bars). Values are means \pm SEM of 15–31 mice per group. (*) $P < 0.05$ versus same-sex $Y4^{+/+}$ controls.

females (Fig. 3A, $P < 0.0001$). The combined mass of white adipose tissue (WAT) deposits was significantly lower in $Y4^{-/-}$ mice than in wild types, significantly so in male mice (Fig. 3B). Interestingly, when individual WAT deposits were investigated, mesenteric WAT was the major contributor to decreased WAT weight in male and female $Y4^{-/-}$ mice (0.65 ± 0.06 vs. 0.84 ± 0.05 % of body weight in male mice, $P < 0.01$, and 0.53 ± 0.06 vs. 0.76 ± 0.05 % of body weight in female mice, $P < 0.05$, means \pm SEM of 28–37 mice per group), with no significant effect of Y4 deletion on the weight of epididymal or periovarian WAT, nor on inguinal or retroperitoneal WAT (data not shown). $Y4^{-/-}$ mice showed no significant difference from wild types with respect to plasma concentrations of leptin, insulin, glucose, or testosterone but had a tendency to reduced levels of corticosterone ($P = 0.055$; Table 1). The weights (as a percentage of body weight) of the pancreas, stomach, small intestine, liver, kidney, and testis or ovary of $Y4^{-/-}$ mice were not significantly different from those of wild-type controls (Table 1).

Decreased corticosteronemia in $Y4^{-/-}$,*ob/ob* double knockout mice

Male and female heterozygous (*OB/ob*) mice on a C57BL/6 background were crossed with $Y4^{-/-}$ animals. Double heterozygous ($Y4^{+/-}$,*OB/ob*) animals were

Table 1. Plasma hormone and metabolite concentrations, and organ weights in $Y4^{-/-}$ mice

		$Y4^{+/+}$	$Y4^{-/-}$
Leptin (ng/mL)	male	5.3 ± 0.8	4.5 ± 0.8
	female	5.7 ± 0.6	5.1 ± 0.7
Insulin (pM)	male	110 ± 14	89 ± 10
	female	58 ± 7	50 ± 5
Glucose (mM)	male	9.9 ± 0.3	10.5 ± 0.5
	female	10.0 ± 0.3	10.7 ± 0.3
Corticosterone (ng/mL)	male	76 ± 16	57 ± 16
	female	165 ± 25	111 ± 15
Testosterone (nM)	male	10.4 ± 3.3	15.9 ± 4.3
	female	ND	ND
Gonad weight (% BWt)	male	0.75 ± 0.03	0.80 ± 0.03
	female	0.08 ± 0.01	0.07 ± 0.01

Data are means ± SEM of 7–21 mice per group. ND, Not determined.

crossed again to subsequently obtain all of the nine possible genotypes. The food intake of male and female $Y4^{-/-},ob/ob$ double knockout mice was not significantly different from that of the *ob/ob* mice (data not shown), suggesting that Y4 receptors are not directly involved in the regulation of feeding behavior in these animals. At 4 wk of age, male and female $Y4^{-/-},ob/ob$ double knockout mice had body weights similar to those of the *ob/ob* mice, but male double knockouts gained significantly more weight in the following 12 wk of monitoring compared to *ob/ob* controls (Fig. 4). A tendency for increased body weight was also apparent in $Y4^{-/-},OB/ob$ males compared to *OB/ob* or wild-type controls (Fig. 4). Female $Y4^{-/-},ob/ob$ double knockout mice showed no significant difference from *ob/ob* mice with respect to body weight over the 12 wk of monitoring (data not shown).

There was a significant interaction effect between Y4 and leptin deficiency on WAT mass ($P < 0.01$). Whereas Y4 deficiency significantly increased the WAT mass of lean *OB/ob* mice, the increased WAT mass of obese *ob/ob* mice was not further increased in $Y4^{-/-},ob/ob$ double knockout mice (Table 2). Insulinemia and glycemia of *ob/ob* mice were significantly elevated over wild-type values, and the absence of Y4 receptors in $Y4^{-/-},ob/ob$ double knockout mice had no significant effect on these parameters (Table 2). Plasma levels of PP in *ob/ob* mice were strongly reduced compared to wild-type mice (Fig. 3A). Y4 deletion restored plasma PP levels in $Y4^{-/-},ob/ob$ mice to values not significantly different from those of control mice (Fig. 3A; Table 2). Furthermore, the hypercorticosteronemia of *ob/ob* mice was significantly attenuated in $Y4^{-/-},ob/ob$ double knockout mice (Fig. 5A).

Increased fertility in $Y4^{-/-},ob/ob$ double knockout mice

The most profound effects of Y4 deletion on *ob/ob* mice were observed upon reproductive functions. Figure 5B–D shows plasma testosterone levels, and the absolute weight of testis and seminal vesicle of heterozygous *OB/ob*

ob and homozygous *ob/ob* mice with or without Y4 receptor deletion. *OB/ob* mice that were $Y4^{+/+}$ showed no significant difference from wild-type mice with respect to any of the parameters shown in Figure 5, nor in any other of the parameters measured (see Materials and Methods), with the exception of a significant increase in relative and absolute hepatic weight. In *ob/ob* mice, plasma testosterone concentrations were markedly depressed compared to wild-type or heterozygous *OB/ob* animals, in keeping with the significantly reduced weight of testis and seminal vesicle in these animals (Fig. 5B–D). Notably, crossing the Y4 receptor knockout onto *ob/ob* mice significantly increased the plasma testosterone levels of males compared to *ob/ob* animals, and significantly increased absolute testis and seminal vesicle weights to values not significantly different from those of *OB/ob* or wild-type control mice (Fig. 5B–D). The observed increase in body weight of male $Y4^{-/-},ob/ob$ compared to *ob/ob* mice (Fig. 4) is probably in part due to this increase in plasma testosterone levels. Figure 6 shows the improvement in testis and seminal vesicle size in *ob/ob* mice with Y4 receptor deficiency, since the organs of $Y4^{-/-},ob/ob$ double knockout mice resembled those of wild types. Under light microscopy, the morphology of testis and seminal vesicle from $Y4^{-/-},ob/ob$ double knockout mice was similar to that of wild-type

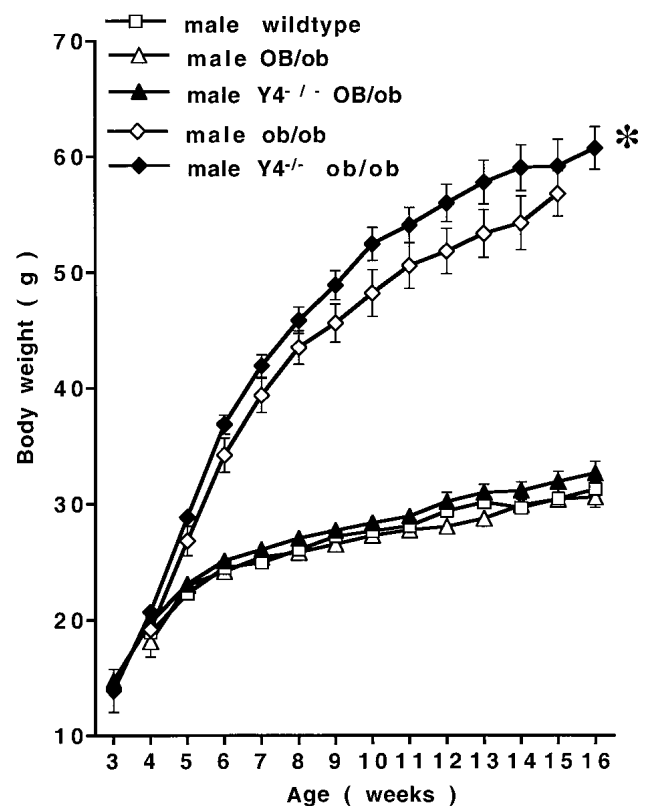


Figure 4. Body weight of $Y4^{-/-},ob/ob$ double knockout mice. Body weight of male $Y4^{-/-},ob/ob$ (◆) compared with *ob/ob* (◇), $Y4^{-/-},ob/ob$ (▲), *OB/ob* (△) and wild-type (□) mice. Values are means ± SEM of 20–32 mice per group. * $P < 0.05$ versus curve for *ob/ob* mice.

Table 2. Weight of white adipose tissue deposits, and plasma hormone and metabolite concentrations in male $Y4^{-/-}, ob/ob$ double knockout mice and controls

	<i>OB/ob</i>	$Y4^{-/-}OB/ob$	<i>ob/ob</i>	$Y4^{-/-}ob/ob$
Sum of WAT (%)	2.48 ± 0.22	3.51 ± 0.33 ^a	8.19 ± 0.29 ^c	7.51 ± 0.30 ^c
Insulin (pmol/L)	89 ± 16	201 ± 41	7420 ± 2230 ^c	9800 ± 2300 ^b
Glucose (mmol/L)	10.5 ± 0.5	12.8 ± 0.7	18.3 ± 4.0 ^b	13.7 ± 2.0
PP (nmol/L)	ND	ND	1.18 ± 1.04	6.45 ± 1.70 ^d

Data are means ± SEM of 6–18 mice per group. Sum of WAT, combined weight of right inguinal, right epididymal or periovarian, right retroperitoneal, and mesenteric white adipose tissue as a percent of body weight; PP, pancreatic polypeptide; ND, not determined.

^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ versus *OB/ob* control mice. ^d $P < 0.05$ versus *ob/ob* mice.

organs. Leydig cell and mature sperm densities were increased over *ob/ob* levels (Fig. 6).

These changes were also reflected in fertility. To test the fertility of male $Y4^{-/-}, ob/ob$ double knockouts, they were paired with female $Y4^{-/-}, OB/ob$ mice which were previously proven to be fertile by their ability to produce live offspring with male wild-type mice. The same strategy was applied to test fertility in female $Y4^{-/-}, ob/ob$ mice. For comparison, male or female *ob/ob* mice were paired with fertile *OB/ob* animals of the opposite sex. All eight breeding pairs consisting of $Y4^{-/-}, ob/ob$ males and $Y4^{-/-}, OB/ob$ females produced live offspring. In contrast, only one of the eight *ob/ob* males was able to produce offspring. Interestingly, four of the eight $Y4^{-/-}, ob/ob$ females were able to establish pregnancies and deliver live pups, two of them twice, whereas none of the eight *ob/ob* females tested were capable of doing so.

To identify possible abnormalities in estrous cycles, estrous cyclicity was assessed by daily vaginal smear cy-

tology in $Y4^{-/-}, ob/ob$, and $Y4^{-/-}, ob/ob$ mice. $Y4^{-/-}$ animals exhibited normal vaginal opening and onset of normal estrous cyclicity, with proestrous, estrous, metestrous, and diestrous stages all present, and a cycle length of 4–5 d. In *ob/ob* animals, the vaginal opening was very small and a diestrous-like swab was observed until 9 wk of age, when cycles lasting from 11 to 15 d commenced. An estrous-like cytology lasted 6–8 d and was characterized by a small number of poorly clumped superficial epithelial cells, large quantities of cellular debris, and increased mucous. In fertile $Y4^{-/-}, ob/ob$ animals, the vaginal opening was complete by 9 wk of age but the total cycle length remained extended, from 11 to 16 d with an estrous lasting up to 7 d. This extended estrous showed plentiful clumped superficial epithelial cells, and the female was receptive in this state and mating could produce a pregnancy. Infertile $Y4^{-/-}, ob/ob$ animals showed a persistent small vaginal orifice and vaginal cytology similar to *ob/ob* animals, characterized by an ab-

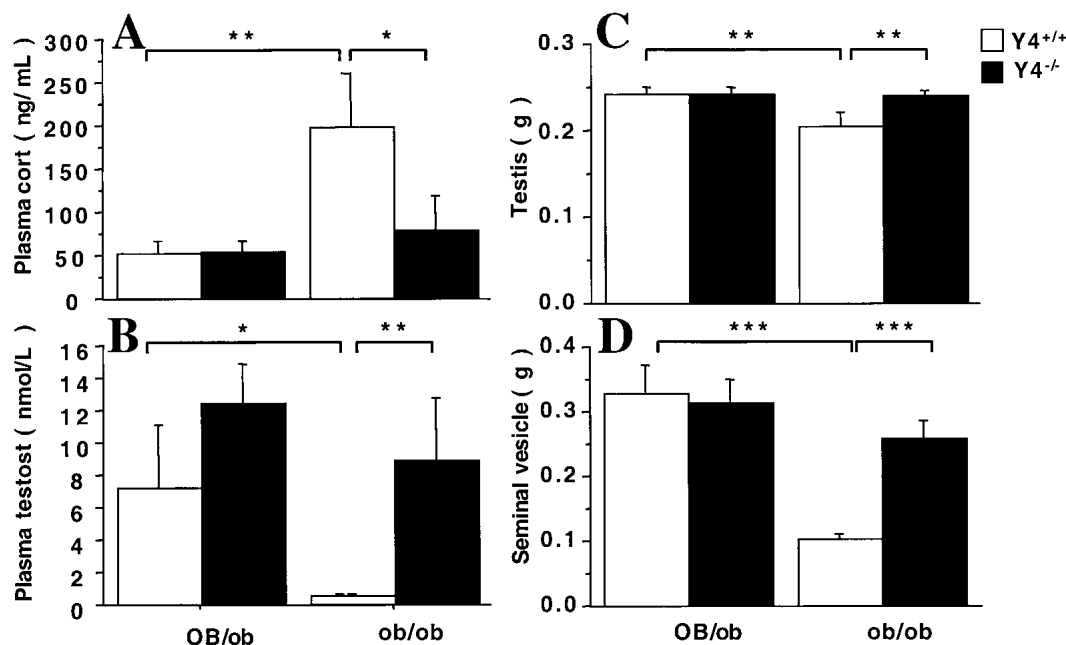


Figure 5. Effect of *Y4* deletion on plasma corticosterone and testosterone levels, and testis and seminal vesicle weight in mice with an *ob/ob* background. Plasma concentrations of (A) corticosterone and (B) testosterone (C) testis weight and (D) seminal vesicle weight of $Y4^{+/+}$ mice (open bars) and $Y4^{-/-}$ mice (filled bars) on an *OB/ob* or *ob/ob* background. Values are means ± SEM of 6–17 mice per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for the comparison indicated by horizontal bars.

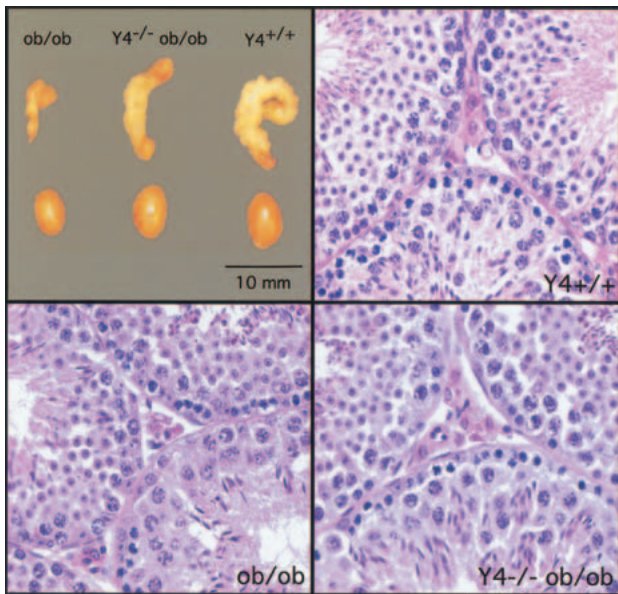


Figure 6. Effects of Y4 receptor deletion on testis and seminal vesicle morphology in $Y4^{-/-}$ and $Y4^{-/-}, ob/ob$ double knockout mice. Lower panels show hematoxylin/eosin-stained sections from testis of the indicated genotypes. Three seminiferous tubules were chosen that displayed different stages of sperm production. Leydig cells are seen at the center of the panels. Original magnification, 200 \times .

sence of large numbers of clumped superficial epithelial cells during an extended estrous. Ovarian morphology of the four different genotypes was also assessed (Fig. 7). Hematoxylin/eosin-stained sections show normal histology in $Y4^{-/-}$ and wild-type (WT) mice, characterized by the presence of multiple primary and secondary follicles and well developed corpora lutea. Ovaries from

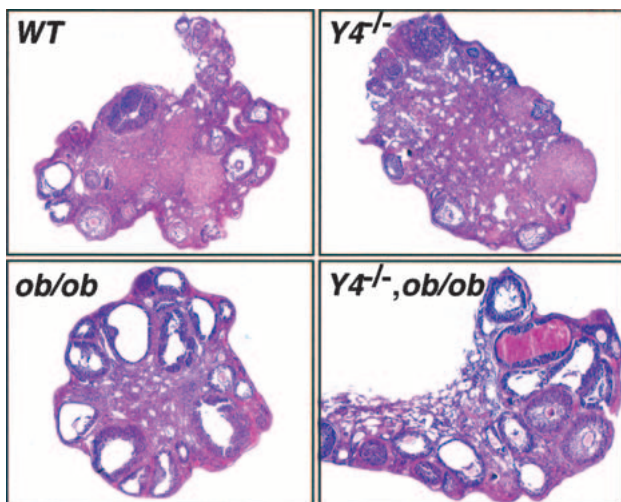


Figure 7. Effects of Y4 receptor deletion on ovary morphology. Hematoxylin/eosin-stained sections of ovaries at estrous from wild-type (WT) and $Y4^{-/-}$ animals, or extended estrous in *ob/ob* and $Y4^{-/-}, ob/ob$ animals. Original magnification, 100 \times .

ob/ob mice, taken during their extended estrous, showed very few primary or secondary follicles. Many large empty follicles were seen and no proper developed corpora lutea were present. Loss of Y4 on the *ob/ob* background resulted in an increase in the number of corpora lutea and primary and secondary follicles, the extent of which was variable among individuals, consistent with the partial rescue of fertility in this genotype. Large empty follicles, characteristic of the *ob/ob* genotype, continued to be present in all animals.

Mammary gland development examined in $Y4^{+/+}$, $Y4^{-/-}$, *ob/ob*, and $Y4^{-/-}, ob/ob$ double knockout females showed significant changes (Fig. 8). In $Y4^{-/-}$ females, ductal development proceeded normally at puberty to

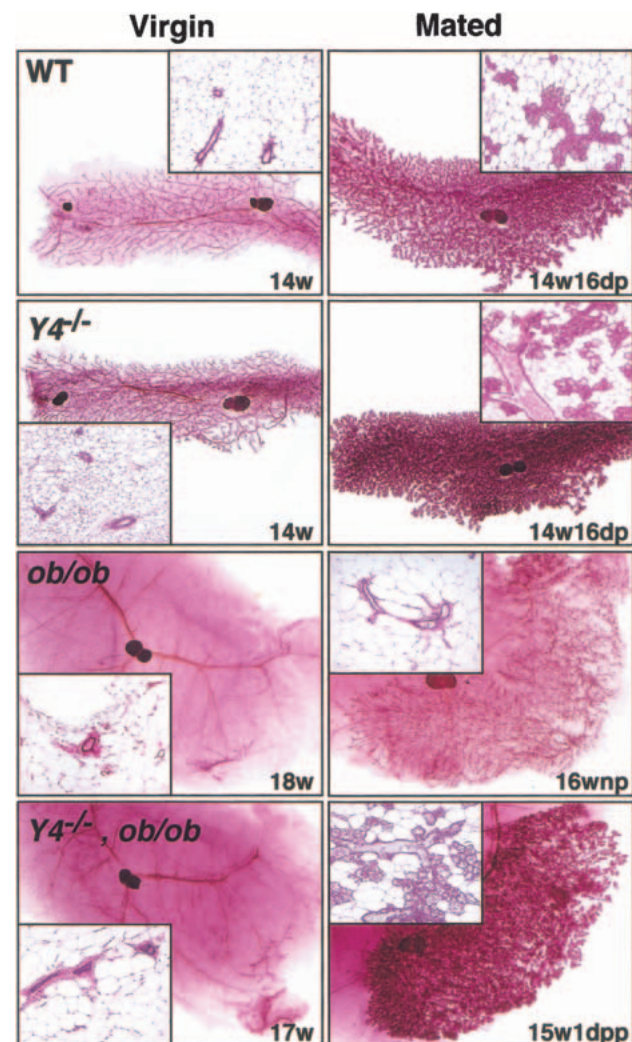


Figure 8. Effect of Y4 receptor deletion on mammary gland development in $Y4^{-/-}$ and $Y4^{-/-}, ob/ob$ double knockout mice. Whole mounts (5 \times original magnification) and corresponding hematoxylin/eosin-stained histology (*insets*, 100 \times original magnification) of the 4th inguinal mammary gland from females of the indicated genotypes, either virgins or after pairing with a fertile male (mated). Animal age is indicated in weeks (w) and reproductive state for the mated group as days of pregnancy (dp), days postpartum (pp), or nulliparous (np).

Sainsbury et al.

produce a branched ductal tree of a complexity seen in mixed 129/SvJ-C57BL/6 animals. During pregnancy, lobuloalveolar development was accelerated as demonstrated by the significantly increased lobuloalveolar content of the glands at day 16 of pregnancy ($18.74 \pm 0.66\%$ area of 4th gland in $Y4^{-/-}$ animals compared to $15.79 \pm 0.86\%$ in wild-type animals, $P < 0.05$, means \pm SEM of 5 mice per group) (Fig. 8). In virgin *ob/ob* animals, no ductal development occurred, with the exception of one animal from the mated group which showed increased ductal development. However, this development was abnormal, as the gland consisted of only finely branched ducts without the extensive underlying network of major ducts that were seen in wild-type animals (Fig. 8), consistent with development during a prior pseudo- or failed-pregnancy. In nulliparous $Y4^{-/-}, ob/ob$ females, the loss of Y4 failed to rescue the development of the mammary ductal tree and the gland remained in the prepubescent state, consisting of a few short ducts emanating from the sinus beneath the nipple (Fig. 8). In $Y4^{-/-}, ob/ob$ females that experienced a full-term pregnancy, the degree of mammary development varied. Some females showed minimal ductal elongation and produced a few sparse lobules, while others produced large areas of lobules that showed the presence of milk and oil droplets (Fig. 8). However, irrespective of the histological appearance of these glands, the mothers were incapable of ensuring survival of the pups.

Altered neuropeptide expression in $Y4^{-/-}, ob/ob$ double knockout mice

To assess possible central mechanisms for changes in energy balance and reproductive function in $Y4^{-/-}$ and $Y4^{-/-}, ob/ob$ mice, we measured central expression of peptides or their precursors known to regulate energy balance [NPY, agouti-related peptide (AgRP), proopiomelanocortin (POMC), and cocaine and amphetamine-regulated transcript (CART)] (Kalra et al. 1999) as well as reproduction (NPY and GnRH) (Pierroz et al. 1996; Jain

et al. 1999). In situ hybridization was performed on coronal brain sections obtained from male mice, using specific radiolabeled antisense DNA oligonucleotides for these mRNAs. Background labeling was uniform and never exceeded 5% of specific signal.

$Y4^{-/-}$ mice did not display altered NPY, AgRP, POMC, or CART mRNA levels in any area investigated (Table 3). Typically, increased NPY and AgRP mRNA expression accompanied by decreased POMC and CART mRNA levels were observed in the arcuate nucleus of *ob/ob* mice. Notably, $Y4^{-/-}, ob/ob$ double knockout mice displayed significantly lower NPY and AgRP levels than *ob/ob* animals, although the levels were still markedly augmented compared to wild-type animals (Table 3). Most importantly however, $Y4^{-/-}$ mice as well as $Y4^{-/-}, ob/ob$ double knockouts showed significantly increased GnRH mRNA levels in forebrain neurons compared to wild-type values (Table 3; Fig. 9). These data are consistent with the enhanced reproductive function observed in $Y4^{-/-}, ob/ob$ mice, and suggest that the increase in GnRH expression caused by removal of Y4 receptor signaling is a major contributor to the establishment of fertility in male *ob/ob* mice. This hypothesis is further supported by results obtained from another Y receptor knockout model generated by us (Baldock et al. 2002). In $Y2^{-/-}$ mice, the lack of Y2 receptors did not alter the expression levels of GnRH mRNA in forebrain neurons compared to wild-type values ($104 \pm 2.11\%$, $n = 5$, $P < 0.002$). More importantly, $Y2^{-/-}, ob/ob$ double knockout animals were completely infertile, as none of the six male or four female mice tested produced any offspring.

Discussion

This study shows that the infertility of leptin-deficient *ob/ob* mice involves signaling through Y4 receptors, since crossing our Y4 receptor knockout onto the *ob/ob* strain conferred the ability to produce live births in breeding pairs consisting of male or female $Y4^{-/-}, ob/ob$ double knockout mice. This was accompanied by signifi-

Table 3. Expression levels NPY, AgRP, POMC, and CART mRNAs

mRNA	wild type	$Y4^{-/-}$	<i>ob/ob</i>	$Y4^{-/-}, ob/ob$
Neurons in the arcuate nucleus				
NPY	100 \pm 5.48 (5)	98 \pm 3.22 (5)	178 \pm 7.44 (5) ^c	155 \pm 0.57 (3) ^{c,d}
AgRP	100 \pm 4.70 (4)	95 \pm 3.65 (5)	167 \pm 7.28 (5) ^c	136 \pm 8.38 (3) ^{b,c}
POMC	100 \pm 3.91 (5)	99 \pm 4.84 (4)	33 \pm 3.00 (5) ^c	39 \pm 0.45 (3) ^c
CART	100 \pm 2.21 (5)	103 \pm 2.67 (5)	23 \pm 1.26 (5) ^c	26 \pm 3.27 (4) ^c
GnRH neurons in the forebrain				
Med. Sept. n.	100 \pm 6.63 (5)	123 \pm 2.10 (5) ^a	104 \pm 4.33 (5)	122 \pm 8.81 (4) ^{a,d}
Preopt. a.	100 \pm 6.42 (5)	120 \pm 4.33 (5) ^a	103 \pm 4.62 (5)	120 \pm 3.85 (4) ^{a,d}
Neurons in the cortex				
NPY	100 \pm 4.20 (5)	98 \pm 5.29 (5)	98 \pm 6.04 (5)	97 \pm 7.72 (4)
Neurons in the paraventricular nucleus				
CART	100 \pm 3.36 (4)	102 \pm 4.24 (5)	96 \pm 2.38 (5)	101 \pm 3.10 (3)

Data represent mean labeling intensity of neurons given as percentage of wild type \pm SEM (number of animals); ^a $P < 0.05$, ^b $P < 0.01$ and ^c $P < 0.001$ versus wild-type; ^d $P < 0.05$ and ^e $P < 0.01$ versus *ob/ob*. Med. Sept. n. = medial septal nucleus; Preopt. a. = preoptic area.

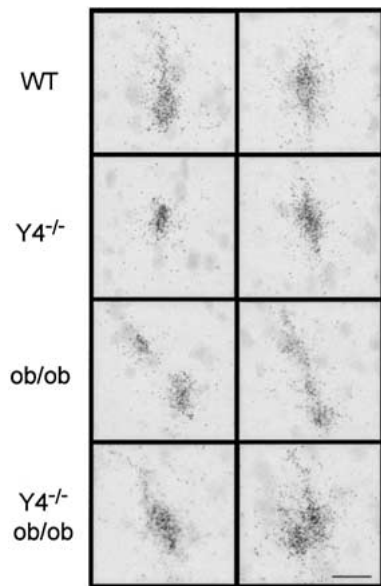


Figure 9. Effect of Y4 receptor deletion on GnRH mRNA levels in neurons of the medial septal nucleus. High-power bright-field photomicrographs of dipped sections obtained from wild-type, $Y4^{-/-}$, ob/ob and $Y4^{-/-}, ob/ob$ double knockout mice after *in situ* hybridization for GnRH. Bar, 10 μ m.

cant improvements in gonadotropic function in male mice, partial restoration of estrous cycling in females, and increased GnRH expression in the forebrain, in keeping with mediation by the brain centers that regulate reproduction. Since PP, the only known endogenous high-affinity Y4 receptor agonist, does not cross the blood-brain barrier and is not found in the brain, we propose that the high hypothalamic NPY levels present in *ob/ob* mice are sufficient to inhibit reproductive functions by agonism of central Y4 receptors. In support of this hypothesis, crossing the *ob/ob* strain onto NPY-deficient mice has also been shown to rescue fertility in male and female $NPY^{-/-}, ob/ob$ double knockout mice (Erickson et al. 1996). Furthermore, fasting of *ob/ob* animals, which reduces their body weight without affecting the high NPY levels, does not restore fertility (Mounzih et al. 1997).

Although Y4 receptor deletion in *ob/ob* mice can restore fertility and lead to improved mammary gland development, pups born of $Y4^{-/-}, ob/ob$ females die from dehydration and/or starvation within 1–2 d after birth. The primary cause appears to be failed lactogenesis, but could also be due to the defective maternal behavior of these females as seen by the scattering of pups in the cage rather than collection within the nest. A similar situation is found in leptin-treated female *ob/ob* mice, which can conceive and deliver live pups, but also fail to lactate (Chehab et al. 1996). Furthermore, mice with knockout of genes involved in prolactin release or the prolactin signaling pathway show a similar lactational and behavioral defect (Liu et al. 1997; Ormandy et al. 1997; Wynick et al. 1998). This suggests that although Y4 receptor deletion can partially restore gonadotropic

function in *ob/ob* females, pituitary prolactin release may only be restored to a level sufficient to maintain the corpus luteum but insufficient to allow lactogenesis and correct maternal behavior. Two $Y4^{-/-}, ob/ob$ mothers had second litters and both again failed to lactate. This raises the possibility that in addition to leptin's hypothalamic action to reduce NPY signaling, binding to its receptor in the mammary gland may also be required for full development and normal onset of lactogenesis.

In addition to lack of leptin, the genetic background of C57BL/6 *ob/ob* mice has also been considered to contribute to infertility. Male animals deficient in the leptin gene on a mixed C57BL/6J-BALB/cJ genetic background showed improved fertility, with 41% being able to produce offspring, although none of the *ob/ob* females on this genetic background were able to do so (Ewart-Toland et al. 1999). It has been suggested that inheritance of so-called modifier genes is responsible for the improvement in male fertility in these animals. Interestingly, one of the loci found to be associated with high testosterone levels in the present study (D14MIT113) is very close to the location of the Y4 receptor gene (D14MIT14), making the Y4 receptor a strong candidate for such a modifier gene. In our studies, the improved fertility of $Y4^{-/-}, ob/ob$ mice was not due to breeding onto the mixed C57BL/6-129/SvJ background, because our *ob/ob* control mice on the same genetic background were effectively sterile by comparison.

Analysis of our male and female $Y4^{-/-}$ mice also showed a several-fold increase in plasma concentrations of PP compared to wild-type values. PP is released from pancreatic F cells in response to stimuli such as food intake and hypoglycemia, mainly by vagal muscarinic activation (Schwartz 1983; Havel et al. 1993). It is thought to act on Y4 receptors in the brain stem accessible to plasma-borne factors to enhance digestive events such as gastric secretion, motility, and emptying by subsequent activation of vagal cholinergic pathways (McTigue et al. 1997). It is therefore possible that the loss of Y4 receptor function contributed to the reduced body weight gain and reduced adiposity in $Y4^{-/-}$ mice by an inefficiency of nutrient uptake during digestion. It is also possible that the reduced body weight of $Y4^{-/-}$ mice was mediated by the high plasma PP concentrations acting on other peripheral Y receptors besides Y4. In support of this, PP transgenic mice, which have a 20-fold increase in plasma PP levels, have a metabolic phenotype similar to that of our $Y4^{-/-}$ mice. This includes reduced food intake, body weight, and fat mass, as well as reduced glucose-induced insulin secretion and gastric emptying, with effects more evident in male compared to female transgenic mice (Ueno et al. 1999). It appears unlikely that changes in hypothalamic neuropeptide expression are involved in the manifestation of the reduced body weight in $Y4^{-/-}$ mice, because the expression levels of the orexigenic and anorexic peptides (NPY, AgRP, POMC, and CART) were no different in $Y4^{-/-}$ mice compared to controls. In *ob/ob* mice, Y4 deficiency increased plasma PP levels from the low levels characteristic of congenitally obese rodents (Jia and Taylor 1984) to levels

Sainsbury et al.

not significantly different from those of wild-type mice. However, this increase was probably of insufficient magnitude to reduce the body weight and adiposity of $Y4^{-/-}, ob/ob$ mice relative to ob/ob mice.

In conclusion, Y4 receptors are specifically involved in the down-regulation of reproductive function and impaired fertility observed in leptin-deficient ob/ob mice. This is likely to occur by NPY-mediated activation of Y4 receptors in the brain regions that control the gonadotropic axis. In contrast, other aspects of obesity in ob/ob mice, such as defects in lactation, as well as hyperphagia, hyperinsulinemia, hyperglycemia, and increased fat mass, are most likely not mediated by Y4 receptor agonism. In normal physiology, the Y4 receptor may contribute to reducing reproductive capacity when hypothalamic NPY expression is increased, such as in food restriction, heavy exercise, lactation, and type 1 diabetes mellitus, representing a possible new target for the treatment of infertility.

Materials and methods

Targeting vector construction and gene disruption

A 129/SvJ mouse genomic BAC library (GenomSystems) was screened under low stringency for the Y4 receptor gene using a human Y4 cDNA probe (Lundell et al. 1995). Positively hybridizing clones were isolated and mapped. A 12 kb *HindIII* fragment and a 4.5 kb *SacI* fragment were subcloned into pBlue-script and used to generate the targeting construct. A loxP-flanked Neo cassette was inserted into the *HindIII* site downstream of the Y4 gene and a third loxP sequence was introduced by cloning two complementary 46 mer oligonucleotides into an *XbaI* site 5 kb upstream of the Y4 receptor gene initiation codon. A 1.2 kb *HindIII/EcoRI* fragment 5' to the Y4 targeting construct was used to screen for positively targeted ES cell clones. Two of these clones were injected into C57BL/6 blastocysts. Chimeric mice were bred either with C57BL/6 mice to generate heterozygous conditional knockout mice ($Y4^{lox/+}$) or with C57BL/6 transgenic animals containing the Cre-recombinase gene under an oocyte-specific promoter to obtain heterozygous germ line Y4 receptor knockout mice ($Y4^{-/-}$). Breeding the respective heterozygous mice generated all five possible genotypes ($Y4^{lox/lox}$; $Y4^{lox/+}$; $Y4^{+/+}$; $Y4^{-/-}$; $Y4^{-/+}$). The genotype of mice was determined by Southern blot analysis as described in the Results section, and by PCR using oligonucleotide A (5'-ATCCTTCTGCTCTATG-3'), oligonucleotide B (5'-GGA TAATACC AGCATGGC-3'), and oligonucleotide C (5'-GCA TCTGTACTGAGTGGC-3'), with 35 cycles of 94°C for 45 sec, 60°C for 1 min, and 72°C for 20 sec.

Measurement of food intake and body weight

Groups of 16–21 animals per genotype originating from 3–4 different breeding pairs were group-housed and fed standard chow. Body weight was monitored at the same time each week from 4 wk of age onwards. Food intake was measured over 7 d in individually-housed mice at 8 and 12 wk of age.

Tissue collection and analysis

At 16–18 wk of age, $Y4^{+/+}$ and $Y4^{-/-}$ mice derived from crosses of $Y4^{-/+}$ parents, as well as six of the nine possible genotypes obtained from crosses of double heterozygous animals ($Y4^{+/+}, OB/$

OB ; $Y4^{-/-}, OB/OB$; $Y4^{+/+}, OB/ob$; $Y4^{-/-}, OB/ob$; $Y4^{+/+}, ob/ob$; and $Y4^{-/-}, ob/ob$) were killed by cervical dislocation between 10.00–15.00 h for collection of trunk blood, and brains and plasma were immediately frozen and stored at -80°C . Food was removed from cages 1–3 h before death, which occurred within 90 sec of initial handling and removal from cages to avoid time-related increases in stress hormones (corticosterone) (Sakakura et al. 1976). White adipose tissue depots (right inguinal, right epididymal or periovarian, right retroperitoneal, and mesenteric), pancreas, stomach, small intestine, liver, kidney, heart, ovary, testis, and seminal vesicle were removed and weighed. For the morphology investigation, the third and fourth inguinal mammary glands were dissected out, whole-mounted on glass slides, and fixed in Bouin's solution. Plasma PP was radioimmunoassayed using guinea pig anti-rat PP serum and second Antibody Precipitating System from Linco Research and ^{125}I -PP (Auspep). Plasma leptin and insulin levels were measured by radioimmunoassay kits from Linco Research, corticosteronemia and plasma testosterone concentrations were measured with kits from ICN Biomedicals, and glycemia was determined with a glucose oxidase assay kit (Trace Scientific, Melbourne, Australia).

In situ hybridization

Coronal brain sections (20 μm) were cut on a cryostat and thaw-mounted on superfrost slides. The sections were kept desiccated at -80°C until their use in the respective experiments. Matching sections from the same coronal brain level of knockout and control mice (4–5 male mice per group) were assayed together, generally following the method of Young (1989) with slight variations (Tsunashima et al. 1997). In short, DNA oligonucleotides complementary to mouse NPY (5'-GAGGGTCAGTC CACACAGCCCCATTCGCTTGTACCTAGCAT-3'); mouse POMC (5'-TGGCTGCTCTCCAGGCACCAGCTCCACACAT CTATGGAGG-3'); mouse GnRH (5'-CAAACACACAGTCAG CAGTAGAATGCCGGCCATCAGTTTGGAGGATC-3'); mouse CART (5'-TCCTTCTCGTGGGACGCATCATCCACGGCAG AGTAGATGTCCAGG-3'); and mouse AGRP (5'-AGCTTGGCG CAGATAGCAAAAAGGCATTGAAGAAGCGGCAGTACCAC-3') mRNAs were labeled with [^{35}S]thio-dATP (1300 Ci/mmol, Amersham) by reaction with terminal deoxynucleotidyltransferase (Roche) and precipitated with ethanol/sodium chloride. Frozen sections were rapidly immersed in 2% paraformaldehyde in 150 mM NaCl and 10 mM phosphate buffer (pH 7.2; phosphate buffered saline, PBS) for 10 min in an ice bath, rinsed in PBS, immersed in 0.25% acetic anhydride in 0.1 M triethylamine hydrochloride (pH 8.2 in saline) for 10 min, dehydrated by ethanol series, and delipidated with chloroform. Air-dried sections were hybridized at 42°C for 18 h with 30 fmole (0.6×10^6 cpm) of labeled oligonucleotide probe in 50 μL hybridization buffer. The hybridization buffer consisted of 50% formamide, 5 \times SSC (1 \times SSC is 150 mM NaCl, 15 mM sodium citrate at pH 7.2), 500 $\mu\text{g}/\text{mL}$ salmon sperm DNA, 250 $\mu\text{g}/\text{mL}$ yeast tRNA, 1 \times Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 10% dextran sulfate, and 20 mM dithiothreitol. Slides were washed four times in 50% formamide in 2 \times SSC (42°C, 15 min), cooled to room temperature for 30 min, rinsed in 1 \times SSC, and dipped briefly in water. Sections were then dipped in 70% ethanol, dried, and exposed to Kodak Biomax MR films for 1–5 d. Subsequently the slides were dipped in Kodak NTB-2 photosensitive emulsion (diluted 1:1 with distilled water), air dried, and exposed for 6 to 14 d. Films and dipped slides were developed with Kodak D19 developer. Sections were counterstained superficially with hematoxylin, dehydrated, and coverslipped with Aquamount

(BDH). The corresponding radiolabeled sense DNAs were used to exclude nonspecific hybridization of the probe. Sections hybridized with a 50 times excess of unlabeled probe were included as further controls in some experiments.

Autoradiographs were scanned, and relative optical densities (ROD) were measured over the arcuate nucleus, the medial septal nucleus, or the paraventricular nucleus. Background measured over white matter was deducted. For evaluation of mRNA levels in scattered neurons, images from dipped sections were digitized using a ProgRes 3008 camera (Zeiss) mounted on a Zeiss Axiophot microscope. Silver grain density over single neurons was evaluated using NIH-Image 1.61 software (written by Wayne Rasband and available from anonymous FTP at zippy.nimh.nih.gov).

Histology of mammary gland, vaginal smear, ovary, testis, and seminal vesicle

Mammary development was investigated using carmine-stained whole mounts followed by paraffin embedding and staining of 5- μ m sections using hematoxylin/eosin. Lobuloalveoli in hematoxylin/eosin-stained sections were quantified using digital photography and automated area measurement by a macro running the NIH Image analysis software (<http://rsb.info.nih.gov/nih-image/>). The success of lactation and suckling was assessed by examination of the stomach contents of pups; milk where present was visible through the skin. Estrous cyclicity was studied using DiffQuick staining of vaginal swabs spread on glass slides. Ovaries were fixed in 4% paraformaldehyde and embedded in paraffin before examination of 5- μ m hematoxylin/eosin-stained sections. For examination of morphology, seminal vesicle was separated from the coagulating gland and cut from the urethra at their junction. The epididymis/vas deferens was removed from the testis. Testes were fixed in Bouin's fixative, transferred to 10% glucose solution, paraffin embedded, sectioned at 5 μ m, and stained with hematoxylin-eosin.

Statistical analyses

Results for body weight were compared among groups by repeated measures ANOVA followed by Fisher's post-hoc tests. Differences in food intake were assessed by Student's *t*-tests. Differences between *Y4*^{-/-} and *Y4*^{+/+} mice in tissue and organ weights and plasma hormone and metabolite concentrations were assessed by 2-way ANOVA (effect of *Y4* deletion and gender, with subsequent Fisher's post-hoc tests). Comparison of these parameters among male *Y4*^{+/+},*OB/OB*; *Y4*^{-/-},*OB/OB*; *Y4*^{+/+},*OB/ob*; *Y4*^{-/-},*OB/ob*; *Y4*^{+/+},*ob/ob*; and *Y4*^{-/-},*ob/ob* mice was made by 2-way ANOVA (effect of *Y4* deletion and *ob* mutation). When the *Y4* or *Y4***ob* interaction effect was significant, Fisher's post-hoc tests were used to locate significant differences between groups. Alterations in neuronal neuropeptide mRNA expression were assessed by ANOVA with Fisher's post-hoc tests. StatView version 4.5 (Abacus Concepts) was used for all statistical analyses, and *P* < 0.05 was accepted as being statistically significant.

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Y4 receptor knockout rescues fertility in *ob/ob* mice

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