Y4 receptor knockout rescues fertility in ob/ob mice

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Abstract
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.

Keywords
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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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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 hypothalamic-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 tonus 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 O VX or intact male rats rapidly increased FSH and/or LH secretion [Jain et al. 1999; Raposinho et al. 2000]. These effects were attributed to Y4 activation by pharmacological analysis of other partial Y4 agonists (Raposinho 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 O VX 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 low-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 (Y4lox/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 bred 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.
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 ± 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 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 ± SEM of 28–37 mice per group), with no significant effect of Y4 deletion on the weight of 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 ± SEM of 15–31 mice per group. [*] P < 0.05 versus same-sex Y4−/+ controls.

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
Y4 receptor KO rescues fertility in ob/ob mice

Table 1. Plasma hormone and metabolite concentrations, and organ weights in Y4−/− mice

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

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 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 of the 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.
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 cytology 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-

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

<table>
<thead>
<tr>
<th></th>
<th>OB/ob</th>
<th>Y4−/−OB/ob</th>
<th>ob/ob</th>
<th>Y4−/−ob/ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of WAT [%]</td>
<td>2.48 ± 0.22</td>
<td>3.51 ± 0.33*</td>
<td>8.19 ± 0.29*</td>
<td>7.51 ± 0.30*</td>
</tr>
<tr>
<td>Insulin [pmol/L]</td>
<td>89 ± 16</td>
<td>201 ± 41</td>
<td>7420 ± 2300*</td>
<td>9800 ± 2300b</td>
</tr>
<tr>
<td>Glucose [mmol/L]</td>
<td>10.5 ± 0.5</td>
<td>12.8 ± 0.7</td>
<td>18.3 ± 4.0b</td>
<td>13.7 ± 2.0</td>
</tr>
<tr>
<td>PP [nmol/L]</td>
<td>ND</td>
<td>ND</td>
<td>1.18 ± 1.04</td>
<td>6.45 ± 1.70d</td>
</tr>
</tbody>
</table>

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. *P < 0.05, **P < 0.01, ***P < 0.001 versus OB/ob control mice. #P < 0.05 versus ob/ob mice.

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.
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 $ob/ob$ 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 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×.

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×.

Figure 8. Effect of $Y4$ receptor deletion on mammary gland development in $Y4^{-/-}$ and $Y4^{-/-}, ob/ob$ double knockout mice. Whole mounts (5× original magnification) and corresponding hematoxylin/eosin-stained histology (insets, 100× 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).
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 ± 0.66% area of 4th gland in Y4−/− animals compared to 15.79 ± 0.86% in wild-type animals, \( P < 0.05 \), means ± 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.

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

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Neurons in the arcuate nucleus</th>
<th>Y4−/−</th>
<th>ob/ob</th>
<th>Y4−/−, ob/ob</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild type</td>
<td>100 ± 5.48 (5)</td>
<td>98 ± 3.22 (5)</td>
<td>178 ± 7.44 (5)c</td>
</tr>
<tr>
<td>NPY</td>
<td></td>
<td>100 ± 4.70 (4)</td>
<td>95 ± 3.65 (5)</td>
<td>167 ± 7.28 (5)c</td>
</tr>
<tr>
<td>AgRP</td>
<td></td>
<td>100 ± 3.91 (5)</td>
<td>99 ± 4.84 (4)</td>
<td>33 ± 3.00 (5)c</td>
</tr>
<tr>
<td>POMC</td>
<td></td>
<td>100 ± 2.21 (5)</td>
<td>103 ± 2.67 (5)</td>
<td>23 ± 1.26 (5)c</td>
</tr>
<tr>
<td>CART</td>
<td></td>
<td>100 ± 6.63 (5)</td>
<td>123 ± 2.10 (5)c</td>
<td>104 ± 4.33 (5)</td>
</tr>
<tr>
<td>Med. Sept. n.</td>
<td></td>
<td>100 ± 6.42 (5)</td>
<td>120 ± 4.33 (5)c</td>
<td>103 ± 4.62 (5)</td>
</tr>
<tr>
<td>Preopt. a.</td>
<td></td>
<td>100 ± 4.20 (5)</td>
<td>98 ± 5.29 (5)</td>
<td>98 ± 6.04 (5)</td>
</tr>
<tr>
<td>Neurons in the cortex</td>
<td>NPY</td>
<td>100 ± 3.36 (4)</td>
<td>102 ± 4.24 (5)</td>
<td>96 ± 2.38 (5)</td>
</tr>
<tr>
<td>CART</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent mean labeling intensity of neurons given as percentage of wild type ± SEM [number of animals]; \( *P < 0.05 \), \( **P < 0.01 \) and \( ***P < 0.001 \) versus wild-type; \( \#P < 0.05 \) and \( \#P < 0.01 \) versus ob/ob. Med. Sept. n. = medial septal nucleus; Preopt. a. = preoptic area.

**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-
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 µm.

Genetic analysis of our male and female Y4−/− mice also showed a severe-fald 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 anorexigenic 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
Materials and methods

Targeting vector construction and gene disruption

A 129/SvJ mouse genomic BAC library (GenomSys) 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 pBlueScript 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 Xbal 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 cells. 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 (Y4lox/+) 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 (Y4lox/lox). Breeding the respective heterozygous mice generated all five possible genotypes (Y4lox/lox, Y4lox/+, 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’-ATCCTTCCCTGGCTCTATG-3’), oligonucleotide B (5’-GGA TAATACC AGCATGGC-3’), and oligonucleotide C (5’-GCA TCTGGTACTGAGTGGC-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 perivisceral, 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 [125I]PP (Auspep). Plasma leptin and insulin levels were measured by radioimmunoassay kits from Linco Research, corticosterone 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 superfast 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 [1998] with slight variations [Tsushima et al. 1997]. In short, DNA oligonucleotides complementary to mouse NPY (5’-GAGGTCAGTC CACACAGCCCCATGCGCTTGTTACCTAGCAT-3’), mouse POMC (5’-TGCTGTCCTCAGGACCCACGACGCTCCACACT CATTGAGC-3’), mouse GnRH (5’-AACACACAGTCAG CAGTAGATGCCGCCTACATTTAGAGAT-3’), mouse CART (5’-TCTCTTCGTGGACCCATCATCAGCCCGCA GATGATGTCAGG-3’), and mouse AGRP (5’-AGCTGGC GCAGTACGAAAAACCTTGGTTAAGGCCAGTACGCAC 3’) mRNAs were labeled with [35S]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 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 fmol (0.6 × 106 cpm) of labeled oligonucleotide probe in 50 µL hybridization buffer. The hybridization buffer consisted of 50% formamide, 5× SSC (1× SSC is 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 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 fmol (0.6 × 106 cpm) of labeled oligonucleotide probe in 50 µL hybridization buffer. The hybridization buffer consisted of 50% formamide, 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate at pH 7.2), 500 µg/mL salmon sperm DNA, 250 µg/mL yeast tRNA, 1× 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× SSC (42°C, 15 min), cooled to room temperature for 30 min, rinsed in 1× 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.
unpublished as further controls in some experiments.

Autoradiographs were scanned, and relative optical densities 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 Axiopt 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-µm 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-µm 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 µm, 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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References


