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Fasting Inhibits the Growth and Reproductive Axes via Distinct Y2 and Y4 Receptor-Mediated Pathways

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Neuropeptide Y, a neuropeptide abundantly expressed in the brain, has been implicated in the regulation of the hypothalamo-pituitary-somatotropic axis and the hypothalamo-pituitary-gonadotropic axis. Elevated hypothalamic neuropeptide Y expression, such as that occurs during fasting, is known to inhibit both of these axes. However, it is not known which Y receptor(s) mediate these effects. Here we demonstrate, using Y receptor knockout mice, that Y2 and Y4 receptors are separately involved in the regulation of these axes. Fasting-induced inhibition of hypothalamic GHRH mRNA expression and reduction of circulating IGF-I levels were observed in wild-type and $Y4^{-/-}$ mice but not $Y2^{-/-}$ or $Y2^{-/-}Y4^{-/-}$ mice. In contrast, fasting-induced reduction of GnRH expression in the medial preoptic area and testis testosterone content were abolished in the absence of Y4 receptors. Colocalization of Y2 receptors and GHRH in the arcuate nucleus (Arc) suggests that GHRH mRNA expression in this region might be directly regulated by Y2 receptors. Indeed, hypothalamic-specific deletion of Y2 receptors in conditional knockout mice prevented the fasting-induced reduction in Arc GHRH mRNA expression. On the other hand, fasting-induced decrease in GnRH mRNA expression in the medial preoptic area is more likely indirectly influenced by Y4 receptors because no Y4 receptors could be detected on GnRH neurons in this region. Together these data show that fasting inhibits the somatotropic axis via direct action on Y2 receptors in the Arc and indirectly inhibits the gonadotropic axis via Y4 receptors. (*Endocrinology* 148: 2056–2065, 2007)

CONTROL OF THE GH axis or hypothalamo-pituitary-somatotropic axis is of great clinical relevance because GH is currently used in the treatment for a wide range of clinical conditions, including dwarfism (1), GH deficiency (2), Prader-Willi syndrome (3), obesity (4), and short bowel syndrome (5). However, despite the increasing clinical use of GH, understanding of GH regulation is still incomplete. Whereas it is known that GH participates in the homeostatic control of its own secretion by a short-loop feedback regulation involving GHRH and somatostatin in the arcuate nucleus of the hypothalamus (Arc) (6), very few GHRH-containing neurons in this region express the GH receptor (7), indicating that other molecules and receptors are involved in the feedback regulation of the GH axis. A molecule that has received great interest in regulation of the GH axis is neuropeptide Y (NPY) because GH receptors are expressed on NPY neurons in the Arc (8). However, the link between NPY neurons and GHRH neurons in the Arc remains unknown.

Alterations in nutritional states, such as food deprivation, can markedly influence GHRH expression in the hypothalamus as well as serum GH and IGF-I levels (9–12). In the hypothalamus, neurons expressing GHRH are localized in the Arc and the ventromedial hypothalamic nuclei (VMH) (13) and exert a stimulatory effect on the production and release of GH from the pituitary gland. GH acts on adipocytes to promote lipolysis (14) and the liver to produce IGF-I, which mediates most of GH's action on growth (15). Fasting has been shown to decrease hypothalamic GHRH expression and reduce serum GH and IGF-I levels in some species (notably rats) (9), although a recent study suggests that the duration of fasting may have differential impact on hypothalamic GHRH expression (16). Fasting-induced reduction in hypothalamic GHRH expression has been proposed to be due to an increase in hypothalamic NPY expression in response to food deprivation (17–19). In keeping with this, intracerebroventricular administration of NPY to rats and mice has been shown to profoundly inhibit the somatotropic axis by reducing hypothalamic GHRH expression (20), reducing the pituitary content of GH (21), abolishing the normal pulsatile release of GH into the circulation (22), and consequently reducing the plasma concentrations of GH and its main effector in the periphery IGF-I (22–25). Recently the importance of NPY in mediating the fasting-induced reduction in hypothalamic GHRH mRNA expression was demonstrated in NPY knockout mice, which, unlike wild-type mice, showed no fasting-induced drop in hypothalamic GHRH expression (26). Despite the critical involvement of NPY in the regulation of GHRH expression, it is not known which Y receptor mediates this effect.

In addition to inhibition of the GH axis, adverse metabolic conditions are associated with reduced or abolished reproductive functions, secondary to reduction of hypothalamic

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Abbreviations: Arc, Arcuate nucleus of the hypothalamus; Cre, Cre-recombinase; MPA, medial preoptic area; MS, medial septal nucleus; NPY, neuropeptide Y; PVN, paraventricular nucleus; rAAV, recombinant adeno-associated viral.

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drive of gonadotropin secretion (27–29). Delayed sexual maturation and hypogonadism is observed under conditions of food restriction in intact rodents (11, 30). Fasting-induced inhibition of the reproductive axis has been hypothesized to be at least in part due to the fasting-induced increase in hypothalamic NPY expression because intracerebroventricular NPY administration recapitulates hypogonadism similar to that induced by fasting (20–22, 30–32). Seven-day administration of NPY to the lateral cerebral ventricle via osmotic minipumps significantly reduced pituitary weight, seminal vesicle and testis weights and circulating testosterone levels in intact male rats (22) and delayed sexual maturation, disrupted estrous cyclicity, and reduced the number of pituitary GnRH receptors in normal female rats (21, 30, 32). The fasting-induced suppression of LH levels is attenuated when the associated up-regulation of hypothalamic NPY expression is inhibited by intracerebroventricular administration of leptin or ciliary neurotropic factor (33). Furthermore, *ob/ob* mice, which are hypogonadal and infertile, show marked improvement in gonadotropic function and partial restoration of fertility when crossed with NPY-deficient mice (34), providing additional evidence that elevated hypothalamic NPY expression causes repression of gonadal function. These effects of NPY may be due to inhibition of the release of GnRH (35–37), a central neuropeptide expressed in the medial preoptic area (MPA) and medial septal nucleus (MS) (38–40) that regulates the synthesis and release of anterior pituitary gonadotropins LH and FSH. NPY terminals have been shown to synapse on GnRH neurons in the preoptic area (38), indicating a potential direct role of NPY in regulating GnRH expression.

The mechanism by which NPY regulates GnRH expression remains poorly defined, and the receptors involved are controversial. Unlike wild-type mice, Y1 receptor-deficient juvenile mice submitted to food restriction proceed through puberty like normally fed animals, suggesting that the Y1 receptor is involved in the normal decrease in gonadotropic function due to decreasing energy stores (41, 42). Consistently, blockade of Y1 receptors by a Y1 antagonist accelerates the onset of puberty (43). One recent study reported a stimulatory effect of PYY (3–36), an agonist of Y2 and Y5 receptors, on gonadotropin secretion (LH, FSH) that is enhanced in fasted rats (44). On the other hand, Y4 receptor deletion in mice lead to significantly enhanced gonadotropic function and rescued fertility in the sterile *ob/ob* mice, suggesting the Y4 receptor signaling pathway may be involved in controlling the gonadotropic axis (45). Therefore, current literature on the Y receptors regulating the gonadotropic axis remains contentious, with Y1, Y2, Y4, and Y5 receptors all been implicated (44, 46, 47).

The present study set out to identify the Y receptor(s) regulating the hypothalamo-pituitary-somatotropic and gonadotropic axes using selective Y receptor knockout mice, which avoided the problem of poor specificity of Y receptor agonists and antagonists. Because mice deficient in Y2 and/or Y4 receptors ($Y2^{-/-}Y4^{-/-}$) exhibit markedly reduced adiposity and augmented bone volume (48, 49), we hypothesized that possible changes in the somatotropic and the reproductive axes of these mice may explain their phenotype. Because food deprivation reliably increases central expression of NPY and inhibits that of GHRH and GnRH, we studied wild-type, $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice in the fed and fasted state because fasting represents a good model for examining the receptor(s) involved in NPY-mediated regulation of these signaling pathways. Furthermore, we generated conditional Y2 receptor knockout mice with adult-onset hypothalamus-specific deletion of Y2 receptors to determine whether Y2 receptors regulate the somatotropic axis via actions within the hypothalamus.

Materials and Methods

Animal and tissue

Generation of the Y2 receptor conditional knockout mice and germline $Y2^{-/-}$, $Y4^{-/-}$, and $Y2^{-/-}Y4^{-/-}$ mice was described earlier (48, 49). All mice were on a mixed C57BL/6–129/SvJ background. All research and animal care procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22 C) and illumination (12-h light, 12-h dark cycle, lights on at 0700 h). Mice were fed a normal chow diet *ad libitum* (6% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal/g; Gordon's Speciality Stock Feeds, Yanderra, New South Wales, Australia). Sixteen-week-old wild-type (n = 18), $Y2^{-/-}$ (n = 12), $Y4^{-/-}$ (n = 12), and $Y2^{-/-}Y4^{-/-}$ (n = 12) male mice were used. Six mice from each group were fasted for 24 h from 0900 h, and the other six mice from each group were fed *ad libitum*. Water was available *ad libitum* for all mice. All mice were culled by cervical dislocation in the morning between 0900 and 1100 h, 2–4 h after lights on, and trunk blood was collected, allowed to clot at room temperature, centrifuged and the resultant serum collected and stored at −20 C for subsequent hormone analysis. Brains were removed and immediately frozen on dry ice. The remaining six wild-type mice were used for double labeling *in situ* hybridization to determine colocalization of Y2 receptor and GHRH mRNAs in the hypothalamus or Y4 receptor and GnRH mRNAs in the MPA and MS. Testes and pituitary glands were excised and fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) at 4 C for 16 h before embedding in paraffin.

Serum hormone levels were determined with commercial RIA kits from ICN Biomedicals (testosterone; Costa Mesa, CA), and Bioclone Australia (IGF-I; Marrickville, Australia). The lower limit of detection of these assays is 0.3 nmol/liter for testosterone and 40 ng/ml for IGF-I, and the intra- and interassay coefficient of variation was less than 10% for both assays.

Conditional Y2 receptor deletion by recombinant adeno-associated viral (rAAV) vector administration

Region-specific Y2 receptor deletion was achieved in mice containing Y2 floxed sites by Cre-recombinase (Cre) expression using a rAAV vector. $Y2^{lox/lox}$ mice were anesthetized with a single dose of ketamine/xylazine (100 and 20 mg/kg, ip) (Mavlab, Slacks Creek, Queensland, Australia; Ilium Veterinary Products, Smithfield, New South Wales, Australia) and placed on a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). One microliter of rAAV vectors containing either the Cre-recombinase gene or an empty cassette (1×10^{14} genomic copies/ml) was injected bilaterally into the hypothalamus at a rate of 0.1 μl/min using a 10-μl Hamilton syringe attached to Micro4 microsyringe pump controller (World Precision Instruments Inc., Sarasota, FL). The injection coordinates were (from bregma): anterioposterior, −2.1 mm; mediolateral, ± 0.4 mm; dorsoventral, −5.3 mm, corresponding to the Arc (50). Animals were kept on a heating pad during surgery and monitored until recovery. Three weeks after AAV injection, mice were fasted for 24 h and culled as described in previous section.

In situ hybridization

Coronal slices (20 μm) of fresh frozen brains were cut and thaw mounted on charged slides and stored at −20 C until use. For radioactive

in situ hybridization, DNA oligonucleotides complementary to mouse GHRH (5′-GCTTGTCCTCTGTCCACA TGCTGTCTTCCTGGCGGCT-GAGCCTGG-3′), GnRH (5′CAAACACACAGTCAGC AGTAGAAT-GCCGGCCATCAGTTTGAGGATC-3′), and Y2 receptor (5′-TTTGT-GCC TTCGCTGATGGTAATGGTCACTTGCAGCTCCAGGACT-3′) were labeled with [^{35}S] thio-dATP (Amersham Pharmacia, Rydalmere, New South Wales, Australia, or NEN Life Science Products, Rowville, Victoria, Australia) using terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany). The levels of GHRH, GnRH, and Y2 receptor mRNA expressions were evaluated by measuring silver grain densities over individual neurons from photoemulsion-dipped sections, as described previously (51).

Dual-labeling in situ hybridization

Dual-labeling *in situ* hybridization was performed by combining both radioactive and nonradioactive *in situ* hybridization protocols. For radioactive *in situ* hybridization, mouse GHRH and GnRH DNA oligonucleotides were labeled with [^{35}S] thio-dATP as described above. For nonradioactive *in situ* hybridization, DNA oligonucleotides specific for the Y2 receptor gene (5′-TTTGTGCCTTCGCTGATGGTAATGGT-CACT-TGCAGCTCCAGGAC-3′) and Y4 receptor gene (5′-GGAATC-CAATGGATTGG-TCCCATTCTTACCCTGTAGGGATCCTC-3′) were end labeled with digoxigenin (Roche Diagnostics, Mannheim, Germany) at the 3′ end using terminal transferase (Roche Diagnostics) according to the manufacturer's guide (Roche Molecular Biochemicals, Mannheim, Germany, 1995). The mixture of hybridization solution was as described above. The ^{35}S-labeled probe was diluted to 10^6 cpm/ml with hybridization solution, and digoxigenin-labeled probe was diluted to 2.5 pmol/ml. Hybridization solution (100 μl) was then applied to each section and incubated for 16 h at saline sodium citrate with 50% formamide and 100 μl 1 M dithiothreitol at 40 C and twice in 2× saline sodium citrate with 50% formamide at 40 C for 15 min each, respectively. After washes, we used nitro blue tetrazolium chloride and X-phosphate (Roche Molecular Biochemicals) to detect digoxigenin-labeled Y2 and Y4 receptor signals. Sections were air dried and dipped in 3% parlodion (Sigma, St. Louis, MO) dissolved in diethyl ether. Slides were air dried overnight, dipped in photographic emulsion (Kodak, Rochester, NY), and then stored in foil-wrapped slide boxes at 4 C for 2 wk. Slides were developed with D-19 developer (Kodak) and then immersed in distilled water and coverslipped with Aquamount (Merck KGaA, Darmstadt, Germany).

GH and testosterone staining

Fixed pituitary gland and testes (n = 5 per genotype) were embedded in paraffin and cut using a microtome into 7-μm sections. Sections were deparaffinized, rehydrated, and incubated in 1% H_20O_2 in methanol for 20 min. Sections were then rinsed in PBS and blocked with 20% normal goat serum in 1× PBS for 20 min. Rabbit antihuman GH antiserum (1:1000) (ab8490; Abcam Ltd., Cambridge, UK) or rabbit antitestosterone antibody (1:500) (ab8557; Abcam) was applied for 1 h at room temperature. Slides were rinsed in 1× PBS before incubation with a peroxidase conjugated goat antirabbit IgG (1:1000) (Zymed Laboratories, San Francisco, CA) for 30 min at room temperature. Sections were washed in 1× PBS and treated with diaminobenzidine (Dako North America Inc., Carpinteria, CA) for 5 min. Slides were rinsed in water, counterstained with hematoxylin, and dehydrated in ascending ethanol and xylene before coverslipping. Background staining was uniform across sections. Negative control sections without primary antibody application showed lack of specific immunoreactivity with minimum background staining.

Quantitation

For double labeling, cell counting was performed using a grid reticule and the ×10 objective of an Axioplan light microscope (Zeiss, Oberkochen, Germany). Double-labeled cells were counted within brain regions that were delineated through adjacent landmarks, according to the atlas of the mouse brain in stereotaxic coordinates by Franklin and Paxinos (50). Positively double-labeled cells were counted if the number of silver grains overlying identified neuron bodies (digoxigenin-labeled) were five times higher than background levels. Values represent the average of positive neurons in a given area of one hemisphere from a single section. The average cell counts per area were determined from both left and right sides of the coronal sections, and then all groups were pooled for final analyses.

For quantification of GH levels in pituitary gland, a defined area (4 × 4 grid reticule) was selected under the ×100 objective of a Zeiss Axioplan light microscope using the boundary of neurohypophysis as a landmark to maintain comparable position across sections. Cells with positive staining were counted, and the average of three sections was used as the final count for each mouse (n = 5 mice per group).

Statistics

All data are expressed as means ± SEM. Differences among groups of mice were assessed by two-way ANOVA (with genotype and fasting treatment as main effects), followed by Fisher's *post hoc* comparisons (StatView, version 4.51; Abacus Concepts, Berkeley, CA). Statistical significance was defined as $P < 0.05$.

Results

Fasting inhibited the somatotropic axis of wild-type and Y4$^{-/-}$ but not of Y2$^{-/-}$and Y2$^{-/-}$Y4$^{-/-}$ double-knockout mice

All genotypes exhibited comparable GHRH mRNA levels under basal nonfasted conditions (Table 1). Fasting had a differential effect on Arc and VMH GHRH mRNA levels in the different genotypes (two-way ANOVA, genotype/fasting interaction effect, $P < 0.05$; Table 1). In wild-type mice, GHRH mRNA level was significantly reduced by approximately 40% in the Arc and VMH after fasting (Fig. 1 and Table 1). Similarly, Y4$^{-/-}$ mice also exhibited reduced hypothalamic GHRH mRNA expression after fasting (Table 1). In contrast, fasting did not decrease GHRH mRNA expression in the Arc or VMH of Y2$^{-/-}$ or Y2$^{-/-}$Y4$^{-/-}$ mice, and GHRH mRNA expression was significantly higher than the corresponding values for fasted wild-type mice (Fig. 1 and Table 1, $P \leq 0.01$). Whereas the GHRH mRNA levels in the

TABLE 1. Expression levels of GHRH and GnRH mRNAs in the brain of Y receptor knockout mice

	Wild type		Y2$^{-/-}$		Y4$^{-/-}$		Y24$^{-/-}$	
	Nonfasted	Fasted	Nonfasted	Fasted	Nonfasted	Fasted	Nonfasted	Fasted
GHRH								
Arc	100 ± 10.0	60 ± 10.0[a]	108 ± 12.5	143 ± 22.5[b]	85 ± 12.5	63 ± 15.0	75 ± 7.5	108 ± 6.5[b]
VMH	100 ± 11.7	59 ± 11.7[a]	115 ± 14.7	129 ± 8.8[b]	91 ± 11.7	62 ± 8.8[a]	94 ± 5.9	94 ± 5.9[b]
GnRH								
MPA	100 ± 12.5	46 ± 8.3[a]	92 ± 16.7	46 ± 8.3[a]	142 ± 20.8	145 ± 29.2[b]	150 ± 25.0[c]	150 ± 12.5[b]
MS	100 ± 4.3	70 ± 8.7	122 ± 4.3	83 ± 8.7[a]	130 ± 21.7	157 ± 30.4[b]	109 ± 13.0	104 ± 13.0

Data are means ± SEM of five to six mice per group.
[a] $P \leq 0.05$ *vs.* nonfasted animals of same genotype.
[b] $P < 0.05$ *vs.* fasted wild-type mice.
[c] $P < 0.05$ *vs.* nonfasted wild-type mice.

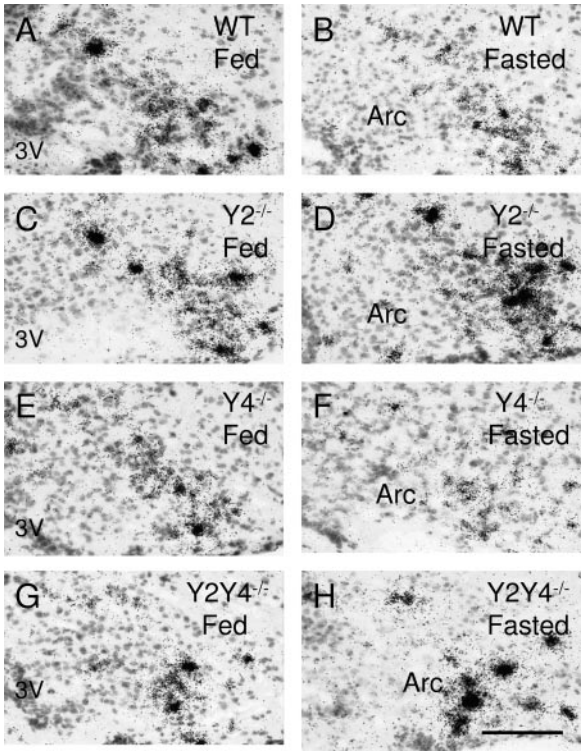

FIG. 1. GHRH mRNA expression in the Arc of fasted and nonfasted wild-type and Y receptor knockout mice. Significant reductions in Arc GHRH mRNA levels were observed in fasted wild-type (B) and $Y4^{-/-}$ mice (F), compared with nonfasted wild-type (A) and $Y4^{-/-}$ mice (E). Fasting-induced reductions in GHRH mRNA expression were not observed in fasted $Y2^{-/-}$ (D) and $Y2^{-/-}Y4^{-/-}$ mice (H), compared with nonfasted $Y2^{-/-}$ (C) and $Y2^{-/-}Y4^{-/-}$ mice (G). WT, Wild-type mice; 3V, third ventricle. *Scale bar*, 50 μm.

Arc of fasted $Y2^{-/-}$ or $Y2^{-/-}Y4^{-/-}$ mice appeared to be higher than their respective nonfasted control groups, these increases were not statistically significant ($P = 0.067$ and $P = 0.079$, respectively). Negative controls using sense probes did not result in any positive hybridization signals (data not shown).

Fasting induced significant and comparable reductions in percentage body weight in mice of all the genotypes studied (wild-type, 10.0 ± 0.8%; $Y2^{-/-}$ knockout mice, 9.9 ± 0.7%; $Y4^{-/-}$ knockout mice, 9.4 ± 1.7%; $Y2^{-/-}Y4^{-/-}$, 13.2 ± 0.6%, ANOVA, $P = 0.35$), indicating any differential effect on GHRH mRNA expression was not due to differences in body weight loss.

We observed reduced circulating IGF-I levels after fasting in wild-type and $Y4^{-/-}$ mice but not $Y2^{-/-}$ or $Y2^{-/-}Y4^{-/-}$ mice (Table 2). Basal serum IGF-I levels were comparable among genotypes.

Fasting inhibited the gonadotropic axis of wild-type and $Y2^{-/-}$ but not of $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ double-knockout mice

GnRH mRNA levels in the MPA were significantly different among genotypes in the nonfasted condition (two-way ANOVA, genotype effect, $P < 0.05$, fasting effect, $P = 0.05$) with $Y2^{-/-}Y4^{-/-}$ mice exhibiting higher levels, compared with nonfasted wild types (Table 1). A nonsignificant increase in GnRH mRNA levels in the MPA was also observed in nonfasted $Y4^{-/-}$ mice, compared with nonfasted wild-type mice ($P = 0.088$). GnRH mRNA levels in the MS were also different among genotypes (two-way ANOVA, genotype effect, $P < 0.05$), which was attributed to a higher GnRH level in the $Y4^{-/-}$ groups. After 24 h fasting, the levels of GnRH mRNA in the MPA significantly reduced by 2-fold in both wild-type and $Y2^{-/-}$ mice, compared with nonfasted animals (Fig. 2 and Table 1). A similar but less robust fasting-induced decrease in GnRH mRNA was observed in the MS of wild-type and $Y2^{-/-}$ mice (Table 1). In contrast, GnRH expression in the MPA or MS was not affected by food deprivation in $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice, compared with nonfasted animals of the same genotype (Fig. 2 and Table 1). Negative controls using sense probes did not result in any positive hybridization signals (data not shown).

Fasting resulted in nonsignificant decreases in serum testosterone levels in wild-type and $Y2^{-/-}$ mice. In contrast, fasted $Y4^{-/-}$and $Y2^{-/-}Y4^{-/-}$ mice exhibited a trend for higher serum testosterone levels, compared with nonfasted mice of the same genotype and fasted wild-type mice (Table 2).

Colocalization of Y2 receptor and GHRH mRNAs in the hypothalamus

To investigate the possibility that Y2 receptors mediate fasting-induced inhibition of the GH axis by direct actions on GHRH-expressing neurons, double-labeling experiments using ^{35}S-labeled GHRH and digoxigenin-labeled Y2 receptor probes were performed (Fig. 3, A and B). Y2 receptor mRNA was expressed in 75 ± 5.8% of Arc GHRH-expressing neurons (of which, 89 ± 1.2% were located in the lateral part of the Arc and 11 ± 1.2% located in the dorsal part of the Arc, data not shown). Very low levels of Y2 receptor and GHRH colocalization was detected in the VMH (data not show).

Y4 receptor and GnRH mRNAs were not colocalized in the MPA or MS

Similarly, to examine whether Y4 receptors mediate fasting-induced inhibition of the gonadotropic axis by direct actions on GnRH-expressing neurons in the MPA and MS,

TABLE 2. Effect of 24 h fasting on wild-type and Y knockout mice

Parameter	Wild type		$Y2^{-/-}$		$Y4^{-/-}$		$Y24^{-/-}$	
	Nonfasted	Fasted	Nonfasted	Fasted	Nonfasted	Fasted	Nonfasted	Nonfasted
Serum IGF-I (ng/ml)	238 ± 18.4	172 ± 17.0[a]	238 ± 24.1	249 ± 25.3[b]	231 ± 13.3	169 ± 14.1[a]	233 ± 17.2	233 ± 42.6
Serum testosterone (nM)	4.19 ± 1.98	3.80 ± 2.13	7.31 ± 5.06	0.94 ± 0.78	3.21 ± 1.13	9.78 ± 7.69	1.32 ± 0.46	13.6 ± 6.54[a]

Data are means ± SEM of five to 12 mice per group.
[a] $P \leq 0.05$ *vs.* nonfasted animals of same genotype.
[b] $P < 0.05$ *vs.* fasted wild-type mice.

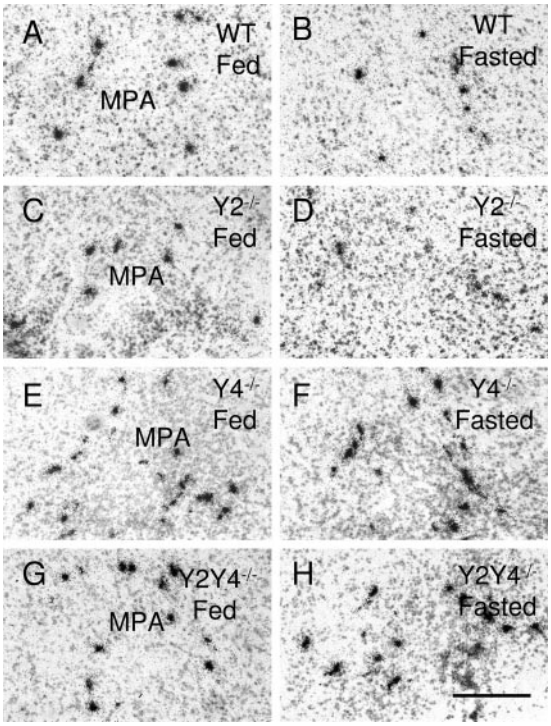

FIG. 2. GnRH mRNA expression in the MPA of fasted and nonfasted wild-type and Y receptor knockout mice. Significant reductions in MPA GnRH mRNA levels were observed in fasted wild-type (B) and $Y2^{-/-}$ mice (D), compared with nonfasted wild-type (A) and $Y2^{-/-}$ mice (C). Fasting-induced reductions in GHRH mRNA expression were not observed in fasted $Y4^{-/-}$ (F) and $Y2^{-/-}Y4^{-/-}$ mice (H), compared with nonfasted $Y4^{-/-}$ (E) and $Y2^{-/-}Y4^{-/-}$ mice (G). WT, Wild-type mice. *Scale bar*, 50 μm.

double-labeling experiments using ^{35}S-labeled GnRH and digoxigenin-labeled Y4 receptor probes were performed. Our results revealed a lack of cells labeled with the Y4 receptors probes in the MPA or MS, and thus, we find no evidence of GnRH and Y4 receptor mRNAs colocalization in the MPA (Fig. 3, C and D) or MS (data not shown).

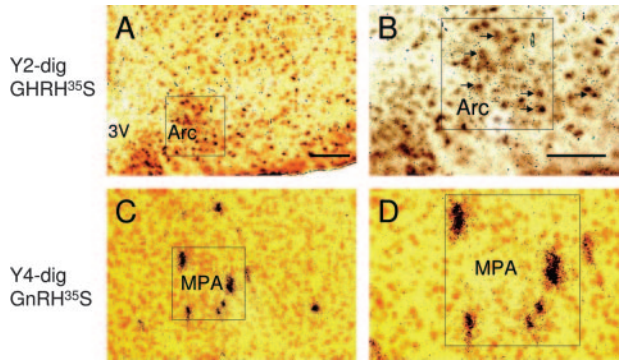

FIG. 3. Representative sections showing double labeling of Y2 receptor and GHRH in the Arc and lack of Y4 receptor and GnRH double labeling in the MPA. Colocalization of Y2 receptors and GHRH mRNAs in the Arc (A and B) or Y4 receptors and GnRH mRNAs in the MPA (C and D) was determined by double labeling using digoxigenin-labeled Y2 or Y4 receptor oligoprobes and ^{35}S-labeled GHRH or GnRH oligoprobes. B and D are higher magnifications of A and C, respectively. *Scale bars*, 40 μm (A and C) and 25 μm (B and D).

Enhanced immunoreactivity of GH in the pituitary gland of fasted $Y2^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ receptor double-knockout mice

To determine whether effects of Y2 receptor knockout on GHRH mRNA expression in the Arc and VMH has a functional impact on GH downstream of the hypothalamo-pituitary-somatotropic axis, we examined GH immunoreactivity in the pituitary gland of fasted animals. Fasting induced a differential change in pituitary gland GH immunoreactivity between genotypes (two-way ANOVA, genotype/fasting interaction effect, $P < 0.0001$). Fasting induced a significant reduction in the GH immunopositive cells in the pituitary glands of wild-type and $Y4^{-/-}$ mice ($P < 0.001$ *vs.* respective fed controls). In contrast, there was a significantly greater number of GH immunopositive cells in the pituitary glands of fasted $Y2^{-/-}$ and $Y2^{-/-}Y4^{-/-}$, compared with their respective nonfasted control groups ($P < 0.001$ for both comparisons, Figs. 4 and 5). Interestingly, $Y2^{-/-}Y4^{-/-}$ mice exhibited significantly less GH-positive cells than wild-type mice under nonfasted conditions ($P < 0.01$).

Increased testosterone levels in the Leydig cells of testes of fasting $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ receptor double-knockout mice

To determine whether the effects of Y4 receptor deficiency on GnRH mRNA expression in the MPA and MS has a

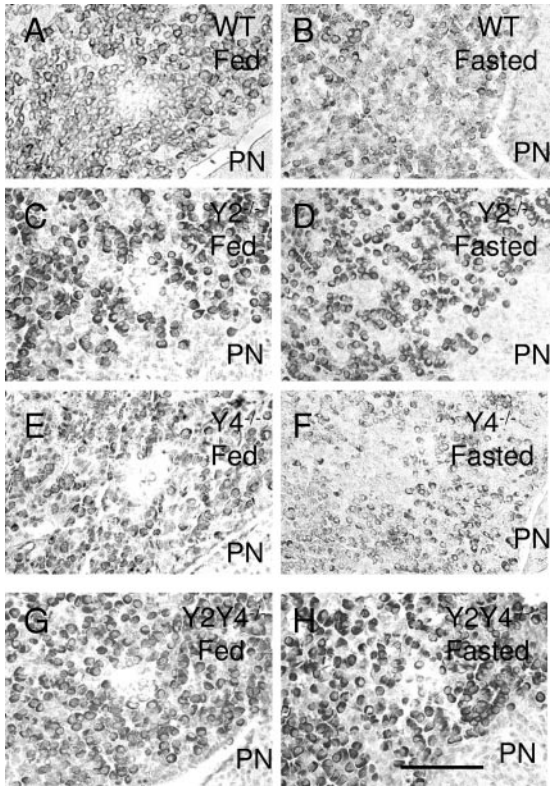

FIG. 4. GH immunostaining in the pituitary gland of fasted wild-type and Y receptor knockout mice. Significant reductions in pituitary GH immunoreactivity were observed in fasted wild-type (B) and $Y4^{-/-}$ mice (F), compared with nonfasted wild-type (A) and $Y4^{-/-}$ mice (E). Fasting-induced reductions in pituitary GH immunoreactivity were not observed in fasted $Y2^{-/-}$ (D) and $Y2^{-/-}Y4^{-/-}$ mice (H), compared with nonfasted $Y2^{-/-}$ (C) and $Y2^{-/-}Y4^{-/-}$ mice (G). WT, Wild-type mice; PN, pars nervosa. *Scale bar*, 25 μm.

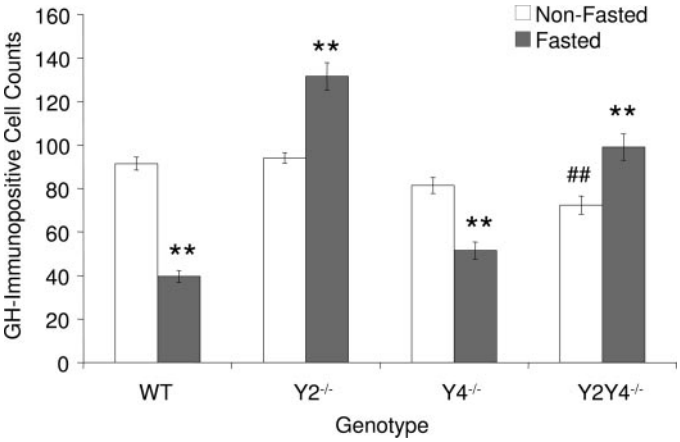

FIG. 5. Comparison of fasting-induced effect on pituitary GH immunoreactivity in wild-type (WT) and Y receptor knockout mice. The number of GH-immunopositive cells in a defined area in the anterior pituitary gland was significantly and differentially altered by fasting in the wild-type and Y receptor knockout mice. **, $P < 0.01$ *vs.* nonfasted mice of the same genotype; ##, $P < 0.01$ *vs.* nonfasted wild-type mice.

functional effect on peripheral testosterone levels, we examined testosterone immunostaining in the Leydig cells of the testes of these mice. Fasting induced similar reductions in testosterone staining in wild-type and $Y2^{-/-}$ mice but not $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice (Fig. 6).

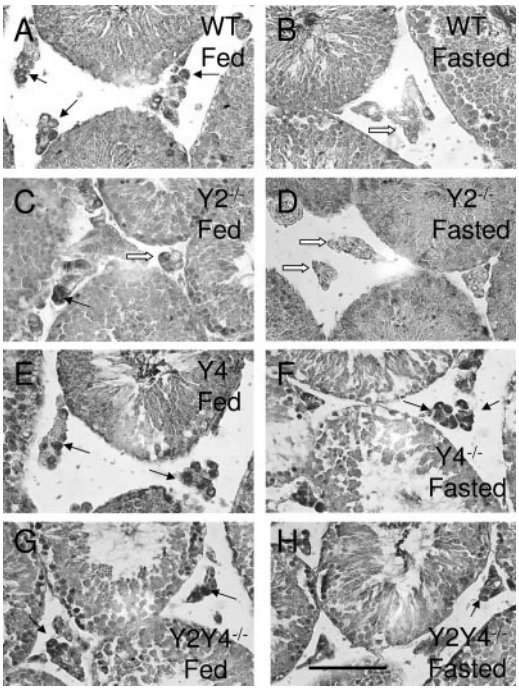

FIG. 6. Testosterone immunostaining in Leydig cells of testes of fasted and nonfasted wild-type (WT) and Y receptor knockout mice. Representative testes sections showing testosterone immunostaining in nonfasted wild-type (A), $Y2^{-/-}$ (C), $Y4^{-/-}$ (E), and $Y2^{-/-}Y4^{-/-}$ (G) mice and fasted wild-type (B), $Y2^{-/-}$ (D), $Y4^{-/-}$ (F), and $Y2^{-/-}Y4^{-/-}$ (H) mice. *Black* and *white arrows* indicate strongly or weakly stained Leydig cells, respectively. Note the preponderance of strongly stained Leydig cells in fasted $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice, compared with other fasted groups. *Scale bar*, 25 μm.

Altered expression levels of GHRH mRNA in the Arc of fasting hypothalamic-specific Y2 receptor conditional knockout mice

To more specifically analyze the role of hypothalamic Y2 receptors in regulating GHRH signaling in the Arc, we studied expression of GHRH mRNA in conditional knockout mice with adult-onset selective focal deletion of Y2 receptor in the hypothalamus. This conditional knockout strategy circumvents any effects due to global Y2 receptor deletion and avoids any potential complication during development. Conditional Y2 receptor knockout mice received rAAV-Cre vector into the Arc via stereotaxic injection. Recombinant AAV vectors have been shown to transduce neuronal cells with high efficiency and stability and showed no toxicity (52). *In situ* hybridization showed lower Y2 receptor mRNA expression in the Arc of Y2 receptor conditional knockout mice, compared with rAAV-empty injected controls (Fig. 7), indicating successful rAAV-Cre-induced knockdown via action of Cre-recombinase. Specificity of Y2 receptor probes has been confirmed in Y2 receptor germline knockout mice (data not shown).

In the nonfasted state, there was no significant difference between hypothalamic Y2 receptor knockout mice and control mice (Fig. 8 and Table 3). After fasting, a differential effect on GHRH mRNA expression was observed between hypothalamic Y2 receptor knockout mice and the rAAV-empty vector injected controls (two-way ANOVA, genotype/fasting interaction effect, $P < 0.001$). Whereas fasted rAAV-empty injected control mice had significantly reduced GHRH mRNA levels, compared with nonfasted mice ($P < 0.01$, Fig. 8 and Table 3), fasting induced a significant increase in GHRH expression in the hypothalamic Y2 receptor knockout mice, compared with their nonfasted counterparts (Fig. 8 and Table 3). Thus, there is a significant difference between hypothalamic Y2 receptor knockout mice and control mice in the fasted state ($P < 0.0001$).

Discussion

This study shows that the fasting-induced inhibition of GHRH expression in the Arc and VMH and reduction in serum IGF-I levels in mice are likely mediated by Y2 receptors because these effects were not evident in mice with Y2 receptor ablation. Substantial colocalization of Y2 receptors and GHRH in the Arc suggests that Y2 receptors may exert a direct regulatory action on GHRH expression in these neurons. Moreover, hypothalamus-specific deletion of Y2 receptors in adult mice also prevented the fasting-induced reduc-

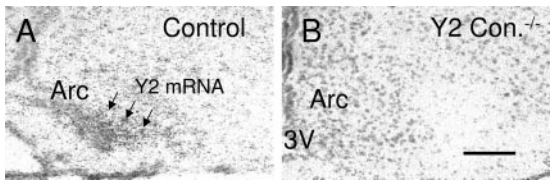

FIG. 7. Y2 receptor mRNA expressions in the Arc of adult-onset hypothalamus-specific Y2 receptor conditional knockout mice. Reduced Y2 receptor mRNA was observed in the Arc of Y2 receptor conditional (Con) knockout mice that received rAAV-Cre (B), compared with rAAV-empty injected control mice (A). 3V, Third ventricle. *Scale bar*, 40 μm.

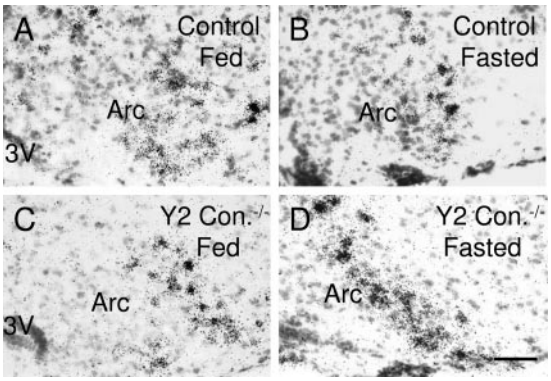

FIG. 8. GHRH mRNA expression in the Arc of fasted and nonfasted adult-onset hypothalamus-specific Y2 receptor conditional (Con) knockout mice. Fasting induced a significant reduction in Arc GHRH mRNA expression in control mice that received rAAV-empty injection (A *vs.* B). In contrast, GHRH mRNA expression was higher in the Arc of fasted Y2 receptor conditional knockout mice (rAAV-Cre injected) (D), compared with nonfasted Y2 receptor conditional knockout mice (C). 3V, Third ventricle. *Scale bar*, 40 μm.

tion in Arc GHRH expression, in keeping with a direct hypothalamic effect of Y2 receptors in regulation of GHRH expression and activity of the hypothalamo-pituitary-somatotropic axis under fasting condition. Consistent with GHRH expression levels, pituitary GH level was higher in fasted $Y2^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice than fasted wild-type and $Y4^{-/-}$ mice. In contrast, the receptor responsible for the fasting-induced reduction in GnRH mRNA expression in the MPA and to a lesser extent MS is most likely to be Y4 and not Y2. Reduction of GnRH mRNA levels in the MPA was observed in fasting wild-type and $Y2^{-/-}$ mice but not $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice. Lack of Y4 receptor expression and lack of colocalization of Y4 receptors with GnRH in the MPA rules out a direct effect of Y4 receptors on these neurons. In correlation with the levels of GnRH, serum and testicular testosterone levels was higher in fasted $Y4^{-/-}$ and $Y2^{-/-}$ $Y4^{-/-}$ mice, compared with wild-type and $Y2^{-/-}$ mice.

Previously, chronic infusion of a Y2 receptor agonist NPY (13–36) into the lateral ventricle of fed adult male rats was shown not to have any effect on the GH axis (22). However, the current study, by using fasting as a model of elevated hypothalamic NPY expression, revealed a definitive role of Y2 receptors in the regulation of this axis under conditions of elevated hypothalamic NPYergic expression and secretion. This is consistent with our observations that Y2 receptor deletion in *ob/ob* mice (in which hypothalamic NPY expression is chronically elevated) increased the low IGF-I levels of *ob/ob* mice and increased lean mass (51). In addition, both germline and hypothalamus-specific deletion of Y2 receptors lead to markedly increased bone volume (53), in keeping with Y2-mediated regulation on the somatotropic axis.

TABLE 3. Expression levels of GHRH mRNA in the Arc of fasting Y2 receptor conditional knockout mice

mRNA region	Control (rAAV-empty)		Y2 receptor conditional knockout (rAAV-Cre)	
	Nonfasted	Fasted	Nonfasted	Fasted
GHRH Arc	100.0 ± 2.0	84.7 ± 3.1[a]	96.9 ± 1.0	110.2 ± 2.0[a,b]

Data are means ± SEM of three to eight mice per group.
[a] $P \leq 0.01$ *vs.* nonfasted animals of same genotype.
[b] $P < 0.001$ *vs.* fasted wild-type mice.

GH is known to exert feedback on hypothalamic somatostatin and GHRH neurons. GH stimulates the synthesis and secretion of somatostatin from neurons in the periventricular and paraventricular nucleus (PVN) and inhibits the synthesis and release of GHRH from Arc neurons (6, 54). Whereas GH receptors are expressed in the majority of somatostatin neurons, there are few GHRH neurons in the Arc that coexpress GH receptors (7). The finding that most of the Arc NPY-expressing neurons coexpress GH receptors indicates that these neurons are a target for the direct action of GH (8). The fact that hypophysectomy significantly decreased hypothalamic NPY mRNA levels and that GH replacement restored the levels to those of intact animals demonstrates that GH increases NPY levels (8). Because these NPY neurons reside in close proximity to GHRH-expressing neurons in the Arc, they have been proposed to be the link between GH and GHRH-expressing neurons (8). Our novel demonstration of marked colocalization of Y2 receptors and GHRH in the Arc demonstrate for the first time how these neurons may be functionally linked and suggest that Y2 receptors may be involved in the negative feedback to GH secretion under conditions of excess NPY and/or GH. Thus, GH acts on the GH receptors present on Arc NPY neurons to stimulate NPY release. NPY may then act on Y2 receptors expressed on GHRH-containing neurons in an inhibitory fashion to reduce GHRH release. The observation that GHRH mRNA expression was not significantly altered by Y2 receptor deletion under nonfasted condition indicates that other Y receptors or regulatory mechanisms are involved in basal homeostatic control of GH axis. The observation that some Arc GHRH neurons did not express Y2 receptors supports this proposition. In addition, a previous report that fasting-induced rise in NPY mRNA level was not accompanied by a fall in GHRH mRNA in the GH-deficient spontaneous dwarf rats (10) suggests regulation of GHRH mRNA expression is more complex than a simple negative association between NPY and GHRH expression.

Distinct to the inhibitory action of Y2 receptors on the response of the hypothalamo-pituitary-somatotropic axis to fasting, our data show that Y4 receptor is an important mediator of the effects of fasting on the hypothalamo-pituitary-testicular axis. In contrast to the reduction of GnRH in the MPA and MS seen in fasting wild-type and $Y2^{-/-}$ mice, $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice showed no reduction in GnRH levels. In fact, these mice even have higher levels of basal GnRH, suggesting Y4 receptor may play an important role regulating the secretion of GnRH under nonfasted conditions, in agreement with a previous study hypothesizing Y4 receptors mediate the basal release of LH (47). Consistent with the changes in GnRH expression level in the MPA and MS, testosterone production in the testicular Leydig cells was greater in $Y4^{-/-}$ and $Y2^{-/-}Y4^{-/-}$ mice, compared with wild-type and $Y2^{-/-}$ mice. The pattern of variation in serum testosterone levels was in agreement with involvement of Y4 receptors in the regulation of this axis, although due to insensitivity of the test kit and sample size, the differences observed in circulating testosterone levels failed to reach

statistical significance. Difficulty in assessing significance of any variation in serum testosterone due to the highly pulsatile release pattern has also been commented by other research groups (22). Previously, we have shown that the reproductive deficits in *ob/ob* mice are mediated at least in part by Y4 receptors (45). The present results show that fasting-induced inhibition of the GnRH axis is also mediated by Y4 receptors.

The lack of Y4 receptor expression in the MPA or colocalization of Y4 receptors with GnRH in this area suggests that the effect of fasting to reduce GnRH expression is an indirect effect of Y4 receptor signaling. The identification of intermediate targets linking Y4 receptors and GnRH neurons in the MPA is an area of further study. Approximately half of the NPY-expressing afferents to GnRH neurons in the MPA originate from the Arc, whereas another substantial proportion originates from the noradrenergic/adrenergic cell populations of the brain stem (55). There are also additional NPYergic fibers projecting to MPA GnRH neurons with as-yet-unidentified origins (55). Previous binding studies using the Y4 receptor selective ligand [^{125}I]PP revealed conflicting results with two studies showing Y4 receptor binding sites in the MPA, PVN, Arc, olfactory tubercle, interpeduncular nucleus, and the solitary tract nucleus (56, 57) with another study demonstrating binding restricted to the parvocellular PVN in the rat hypothalamus (58). The latter data were supported by the observation that hypothalamic Y4 receptor mRNA expression was found only in the parvocellular PVN (58, 59). This is consistent with our current demonstration that Y4 receptors are not expressed in MPA neurons. However, it is possible that Y4 receptors (and hence detectable by binding studies) may be expressed in terminals originating from regions such as the Arc or the brain stem that synapse onto MPA GnRH neurons and thus mediate indirect regulation.

Previously we have shown that $Y2^{-/-}Y4^{-/-}$ mice exhibit a markedly lean phenotype with reductions in body weight and white adipose tissue mass and increased bone volume that are more robust than effects of either Y2 or Y4 receptor deletion alone (48, 60). Our current data suggest that Y2 receptor knockout mice maintain reduced body weight and adiposity via effects on the GH axis, whereas the lean phenotype of Y4 receptor knockout mice may be due to an active GnRH axis. The present study shows that, particularly in the fasted state, $Y2^{-/-}Y4^{-/-}$ receptor double-knockout mice have relative activation of both of these axes, compared with wild-type mice. Because rodents go through a period of semifasting during the light phase and expression of NPY in the hypothalamus shows a daily rhythmic pattern (61) and because GH, IGF-I, and sex hormones promote lean mass at the expense of fat mass (15, 62, 63), the combined action of the increased levels of GH, IGF-I, and testosterone observed particularly in fasted $Y2^{-/-}Y4^{-/-}$ receptor double-knockout mice may contribute to the extremely reduced adiposity and enhanced bone volume observed in these animals.

In summary, this is the first study to show localization of Y2 receptors on GHRH-expressing neurons in the Arc and provides clear evidence for a direct link between Arc NPY and GHRH neurons via Y2 receptors. We propose that GH acts on GH receptors known to be expressed on Arc NPY neurons to stimulate the release of NPY, which in turn activates Y2 receptors expressed on GHRH-containing neurons to reduce GHRH release, thereby inhibiting GH and IGF-I secretion as demonstrated here under fasting conditions. This study also provides clear evidence that fasting regulates the somatotropic and gonadotropic axes via distinct Y2 and Y4 receptor pathways, respectively. However, it cannot be completely excluded that other Y receptors may also play a role in these processes. The current findings may have implications in the treatment of human obesity because inhibition of Y2 and Y4 signaling may circumvent the suppression of IGF-I and sex hormone levels axes that have been observed in overweight people during weight loss interventions (64, 65). In addition, abdominal obesity is associated with markedly blunted GH secretion (66–68) and can be treated with GH replacement, which reduce abdominal visceral fat and increase lean mass (4). By enhancing the somatotropic and gonadotropic axes, combination treatment with Y2/Y4 antagonists may represent a novel treatment strategy for obesity.

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Erratum

In the article "Chronic Intermittent Psychosocial Stress (Social Defeat/Overcrowding) in Mice Increases the Severity of an Acute DSS-Induced Colitis and Impairs Regeneration" by S. O. Reber, F. Obermeier, H. R. Straub, W. Falk, and I. D. Neumann (*Endocrinology* **147:**4968-4976), the third author's name was incorrectly set in the printed paper. The correct spelling should be R. H. Straub. *The printer regrets the error.*