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Critical Interplay Between Neuropeptide Y and Sex Steroid Pathways in Bone and Adipose Tissue Homeostasis

Susan J Allison,[1,2] Paul A Baldock,[1,2] Ronaldo F Enriquez,[1] EnJu Lin,[3] Matthew During,[4] Edith M Gardiner,[1] John A Eisman,[1] Amanda Sainsbury,[3,5] and Herbert Herzog[3,5]

ABSTRACT: Important and novel roles for neuropeptide Y (NPY) signaling in the control of bone homeostasis have recently been identified, with deletion of either the Y1 or Y2 receptors resulting in a generalized increase in bone formation. Whereas the Y2 receptor-mediated anabolic response is mediated by a hypothalamic relay, the Y1-mediated response is likely mediated by osteoblastic Y1 receptors. The presence of Y1 receptors on osteoblasts and various other peripheral tissues suggests that, in addition to neuronal input, circulating factors may also interact with the Y1-mediated pathways. The skeletal and adipose tissue (peripheral and marrow) responses to Y1 receptor deficiency were examined after (1) leptin deficiency, (2) gonadectomy, and (3) hypothalamic NPY overexpression. Bone formation was consistently increased in intact $Y1^{-/-}$ mice. However, the hypogonadism of gonadectomy or leptin deficiency blocked this anabolism in male $Y1^{-/-}$ mice, whereas females remained unchanged. The Y1-mediated bone anabolic pathway thus seems to be dependent on the presence of intact androgen signaling. Y1 deficiency also led to increased body weight and/or adiposity in all experimental models, with the exception of male *ob/ob,* showing a general adipogenic effect of Y1 deficiency that is not dependent on androgens. Interestingly, marrow adipocytes were regulated differently than general adipose depots in these models. Taken together, this interaction represents a novel mechanism for the integration of endocrine and neural signals initiated in the hypothalamus and provides further insight into the coordination of bone and energy homeostasis.
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Key words: neuropeptide Y Y1 receptor, bone, fat, sex hormones, leptin

INTRODUCTION

The hypothalamus is a dominant modulator of endocrine functions by its actions on the pituitary. The hormonal products of this axis exert powerful regulatory influences throughout the body. More recently, the role of nonhormonal outputs from the hypothalamus has generated increasing interest in light of evidence of links from this region of the brain to peripheral tissues and the critical role direct neural signaling plays in peripheral tissue homeostasis. One such neural pathway is the neuropeptide Y (NPY) system. This system is made up of three ligands, NPY, peptide YY, and pancreatic polypeptide, and mediates its effects through five Y-receptors (Y1, Y2, Y4 Y5, and Y6). Whereas the NPY system has predominantly been recognized for its potent effects on energy homeostasis and food intake, it is now known that this system influences many physiological processes including behavior, immunity, and the cardiovascular system. The musculoskeletal effects of NPY have gained particular attention, because of the ability of Y2 receptors within the hypothalamus to strongly regulate the formation of bone, in part through alterations of sympathetic nervous output.[1] In addition to this central action of Y2 receptors, Y1 receptors expressed on the osteoblast have also been implicated in this process suggesting direct, local actions in these cells.[2] Examination of Y1-deficient mice showed greater bone mass and formation but also marked changes in adipose tissue homeostasis.[3] The Y1 receptor therefore seems to represent a critical site for integration of this centrally mediated neural pathway and its peripheral target cells.

Interestingly, the peripheral expression pattern and the action of Y1 receptor in the regulation of bone and adipose tissue suggest an increased potential for interaction of the Y1 receptor system with other peripherally acting regulatory pathways. Thus, Y1 receptor signaling may also represent a mechanism through which endocrine and neural hypothalamic circuits converge to modulate peripheral homeostatic processes. To study this possibility, the interaction

Dr Eisman is a consultant and has corporate appointments with Aventis, Eli Lilly and Company, Merck, Sharp & Dohme, Novartis, NPS Pharmaceuticals, Organon, Roche, and Servier. All other authors state that they have no conflicts of interest.

[1]Bone and Mineral Research Program, Garvan Institute of Medical Research, St Vincent's Hospital, Darlinghurst, Sydney, New South Wales, Australia; [2]These authors contributed equally to this study; [3]Neuroscience Research Program, Garvan Institute of Medical Research, St Vincent's Hospital, Darlinghurst, Sydney, New South Wales, Australia; [4]Department of Neurological Surgery, Weill Medical College of Cornell University, New York, New York, USA; [5]These authors contributed equally to this study.

of Y1 with a number of known regulators of bone and adipose tissue including leptin, sex steroids, and NPY was examined. The adipocyte hormone leptin is a powerful modulator of bone and adipose homeostasis. In addition to profound obesity, leptin-deficient mice (*ob/ob*)[4,5] have altered bone homeostasis, with decreased cortical production[6] and increased cancellous bone formation and osteoclast activity.[7] Sex steroids have a well-defined role in both bone and adipose homeostasis with reduced levels of sex hormones resulting in increased bone resorption[8,9] and fat accrual.[10,11] Moreover, in addition to its role in feeding behavior and adipose deposition, central NPY overexpression has also been shown to inhibit bone formation.[12]

The mode of any interaction of these regulators of bone and adipose with Y1 signaling is unknown; hence, potential interactions were examined in in vivo models. Leptin-mediated effects were studied in $Y1^{-/-}$ mice crossed onto the leptin-deficient background, producing $Y1^{-/-}$;*ob/ob* double knockout mice. The influence of sex hormone signaling was further examined by gonadectomy in germline $Y1^{-/-}$ mice. The effects of NPY overexpression, again a model for control of bone and adipose deposition was studied after injection of recombinant NPY-expressing adeno-associated viral vector into the hypothalamus of adult $Y1^{-/-}$ mice. Mice were assessed for changes in both skeletal and adipose homeostasis.

MATERIALS AND METHODS

Animal experiments were approved by the Garvan Institute of Medical Research Animal Research Authority and were conducted in accordance with relevant guidelines and regulations.

Generation of knockout mice models

Germline deletion of the Y1 receptor gene was achieved as previously described.[13] Briefly, $Y1^{-/-}$;*ob/ob* mice were generated by crossing male and female heterozygous (*ob/ob*) mice on a mixed C57/BL6–129/SvJ background with $Y1^{-/-}$ mice on the same mixed background. Double heterozygous $Y1^{+/-}$;*Ob/ob* mice were crossed again to obtain $Y1^{-/-}$;*ob/ob* homozygous double knockout mice. All single and double knockout mice were maintained on this mixed C57/BL6–129/SvJ background.

Surgical gonadectomy

Gonadectomy or sham operation was performed on germline $Y1^{-/-}$ male and female mice at 8 wk of age, as previously described.[14] Tissues were collected 8 wk after gonadectomy.

Vector-mediated overexpression of NPY in the hypothalamus

Recombinant adeno-associated viral vector expressing NPY under the control of the neural specific enolase promoter (rAAV-NPY) was injected into the hypothalamus of 15-wk-old wildtype and $Y1^{-/-}$ mice using a stereotaxic table as previously described.[12] Brain coordinates relative to bregma were posterior 2.1 mm, lateral ± 0.4 mm, and ventral 5.3 mm. One microliter of vector (1×10^{12} genome copies/ml) was injected bilaterally over 5 min using a 26-gauge guide cannula and a 33-gauge injector (Plastics One, Roanoke, VA, USA) connected to a Hamilton Syringe and a syringe infusion pump (World Precision Instruments, Waltham, MA, USA). Control groups were injected with GFP expressing vector (AAV-GFP). The efficacy of vector injection was assessed as previously described.[12]

Tissue collection

Mice were injected with 15 mg/kg of the fluorophore calcein (Sigma Chemical Co., St Louis, MO, USA) 10 and 3 days before tissue collection to enable subsequent calculation of bone formation rate. Gonadectomized and sham-operated mice, as well as $Y1^{-/-}$;*ob/ob* mice were culled at 16 wk of age, AAV-injected mice were collected at 18 wk of age, 3 wk after viral injection. Mice were killed, and the brain was removed and immediately frozen on dry ice. White adipose tissue (WAT) depots (right inguinal, right retroperitoneal, reproductive [right ovarian or right epididymal for female and male mice, respectively], and mesenteric) were collected and weighed. Both femora were excised and fixed in 4% paraformaldehyde for 16 h.

Bone histomorphometry

The right femur was bisected transversely at the midpoint of the shaft. Distal halves were embedded, undecalcified in methyl-methacrylate (Medim-Medizinische Diagnostik, Giessen, Germany), and 5-μm sagittal sections were analyzed as previously described.[1] Sections were stained for mineralized bone, cancellous bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) were calculated. Bone formation indices (mineralizing surface [MS], mineral apposition rate [MAR], bone formation rate [BFR]), and osteoclast surface (Oc.S) were calculated as previously described,[1] using Leica QWin analysis software (Leica Microsystems, Heerberg, Switzerland). To avoid possible adverse interaction of the tetracycline-based calcein labels with AAV activity, mice receiving AAV virus did not receive calcein injections; thus, it was not possible to calculate parameters of osteoblast activity as done in the other samples. Instead, osteoid parameters were measured on Masson's trichrome-stained sections as previously described.[12] Marrow adiposity was assessed using H&E-stained sections, based on the identification of circular voids in the bone marrow made by adipocytes that had been removed during processing. These were distinguished from sinusoids and blood vessels by lack of H&E staining. Adipocyte number and area were calculated using Leica QWin analysis software and expressed as total adipocyte volume as a percentage of total marrow volume.

Statistical analysis

All data are expressed as mean ± SE. Differences between multiple groups were assessed by factorial ANOVA, followed by Fisher's posthoc comparisons, using StatView version 4.5 (Abacus Concepts, San Francisco, CA, USA).

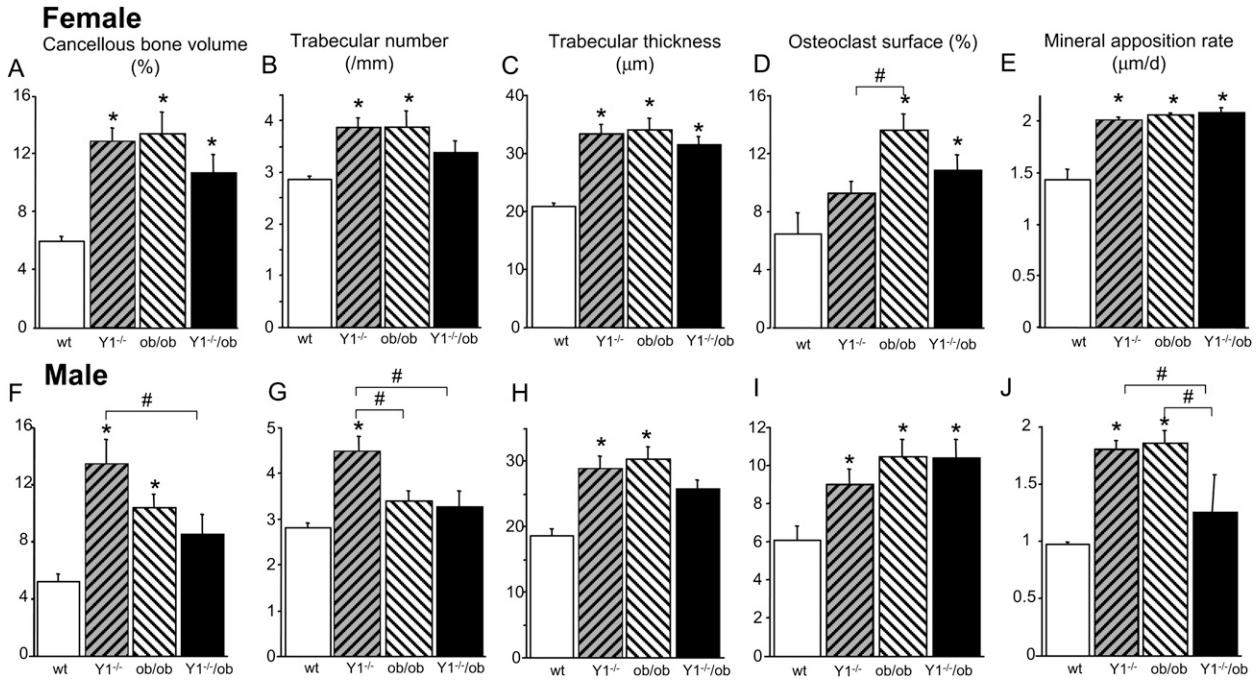

FIG. 1. Effect of Y1 receptor deletion on trabecular bone morphology and bone cell activity of *ob/ob* mice. Female (A–E), and male (F–J) mice. Cancellous bone volume (A and F), trabecular number (B and G), trabecular thickness (C and H), osteoclast surface (D and I), and mineral apposition rate (E and J). $^{*}p < 0.05$ vs. wildtype; $^{\#}p < 0.05$ as indicated. $n = 7$–10 per genotype.

For all statistical analyses, $p < 0.05$ was accepted as being statistically significant.

RESULTS

Sex-specific involvement of leptin in the anabolic effects of Y1 receptor deficiency in cancellous bone

To evaluate the potential for Y1 receptors to interact with other mediators of bone and adipose homeostasis, the effects of Y1 signaling in the leptin-deficient, hypogonadal *ob/ob* mice were examined by comparing the bone phenotypes of *Y1*$^{-/-}$ mice to those of *ob/ob* and *Y1*$^{-/-}$*;ob/ob* double mutant mice.

Lack of Y1 receptors in *Y1*$^{-/-}$ mice or lack of leptin in *ob/ob* animals each resulted in marked and significant increases in cancellous bone volume and trabecular number and thickness compared with wildtype mice, with the exception of Tb.N in male *ob/ob* mice. These increases occurred despite significantly greater surface extent of osteoclasts in male *Y1*$^{-/-}$ or *ob/ob* mice and in female *ob/ob* mice. Bone formation, however, was increased in both genotypes and sexes, with significantly greater mineral apposition rate compared with wild types (Fig. 1), consistent with previous characterizations of the *Y1*$^{-/-}$ and *ob/ob* models.[3,6]

Study of *Y1*$^{-/-}$*;ob/ob* double mutant mice showed a lack of additive action of Y1 and leptin deficiency in the control of both the osteoblast and osteoclast lineages in bone (Fig. 1), as has been described previously for Y2 and leptin.[6] Moreover, in male *Y1*$^{-/-}$*;ob/ob* mice, not only were there no additive effects of Y1 and leptin deficiency on bone physiology, but the anabolic effects of Y1 or leptin deficiency were absent. This included abolition of the increases in cancellous bone volume and mineral apposition rate evident in *Y1*$^{-/-}$ and *ob/ob* mice (Fig. 1). This effect appeared cell specific, with the greater osteoclast surface of leptin deficiency still evident in male Y1$^{-/-}$;*ob/ob*. In contrast, in females, the effects of Y1 or leptin deficiency to increase trabecular bone volume and mineral apposition rate were retained in Y1$^{-/-}$;*ob/ob* mice (Fig. 1). These findings suggest that, in males, the effects of Y1 deficiency to induce anabolic effects on cancellous bone volume and mineral apposition rate require leptin or a downstream effect of leptin's actions to manifest.

Leptin-deficient obesity syndrome of ob/ob mice is modulated by Y1 signaling

In addition to its effects on bone physiology, Y1 receptor deficiency also has significant effects on energy homeostasis.[3] We therefore examined whether Y1 receptor knockout influences the obesity syndrome of genetically obese mice.

Y1 deletion alone resulted in an obesity syndrome in both sexes that is particularly apparent in female *Y1*$^{-/-}$ mice, with increased body weight and a generalized increase in white adipose tissue depots compared with wildtype (Fig. 2). Conversely, when bred onto the obese *ob/ob* background, Y1 deletion resulted in a significant decrease in body weight in *Y1*$^{-/-}$*;ob/ob* mice of both sexes (Fig. 2). In male *Y1*$^{-/-}$*;ob/ob* mice, this decrease was associated with reduced reproductive (epididymal) WAT mass, with a smaller contribution by the inguinal depot. In females, however, despite the decrease in weight in *Y1*$^{-/-}$*;*

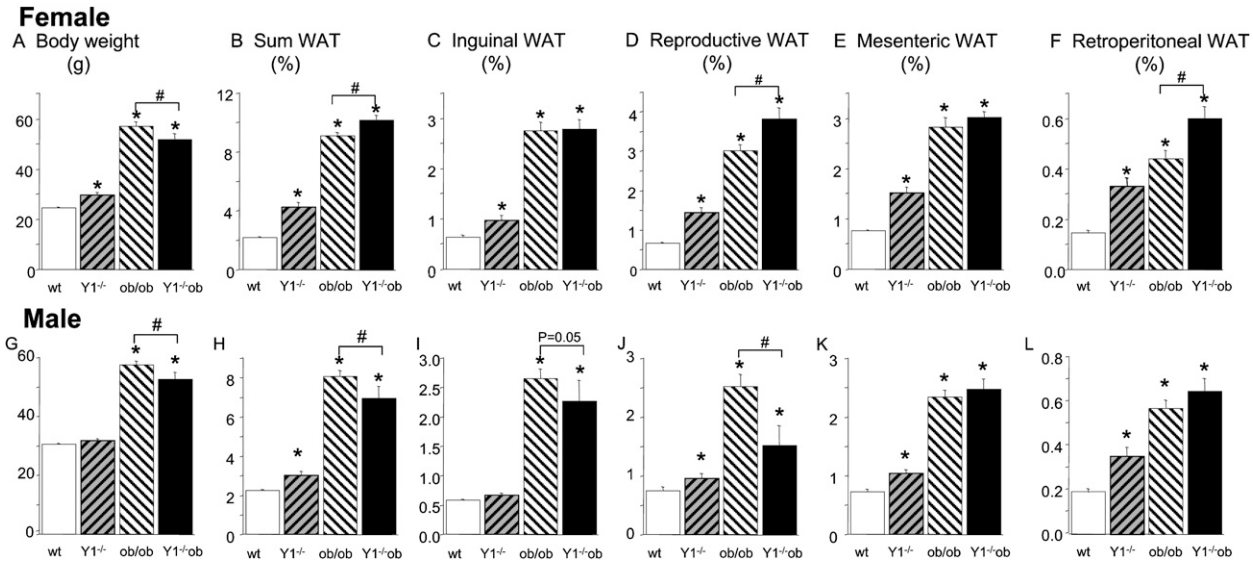

FIG. 2. Effect of Y1 receptor knockout on body weight and adiposity in lean and genetically obese (*ob/ob*) mice. Female (A–F), and male (G–L) mice. Body weight (A and G), total excised white adipose tissue (WAT) deposits (B and H), right inguinal (C and I), right reproductive (periovarian, D) or (epididymal, J) mesenteric (E and K), and right retroperitoneal (F and L) from 15- to 16-wk-old wildtype (WT), Y1 receptor knockout ($Y1^{-/-}$), *ob/ob,* or Y1 ob double mutant ($Y1^{-/-}$;*ob/ob*) mice were weighed and expressed as a percent of body weight. Data are means ± SE of seven or more female or six or more male mice per group. $^{*}p < 0.05$ vs. WT mice; $^{\#}p < 0.05$ as indicated. n = 12–16 per genotype.

TABLE 1. EFFECT OF Y1 RECEPTOR KNOCKOUT ON CIRCULATING GLUCOSE AND HORMONE LEVELS IN LEAN AND GENETICALLY OBESE (*OB/OB*) MICE

	Female				*Male*			
	WT	*Y1*$^{-/-}$	*ob/ob*	*Y1*$^{-/-}$*ob*	*WT*	*Y1*$^{-/-}$	*ob/ob*	*Y1*$^{-/-}$*ob*
Glucose (mM)	10.1 ± 0.3	9.8 ± 0.3	16.7 ± 2.6*	10.8 ± 0.5†	9.3 ± 0.2	10.3 ± 0.3	19.5 ± 2.7*	13.4 ± 1.9†
Insulin (pM)	70 ± 10	200 ± 30	1860 ± 580*	2400 ± 1300*	130 ± 10	270 ± 80	5400 ± 1790*	2050 ± 1900
Cortico (ng/ml)	155 ± 16	121 ± 20	202 ± 37	228 ± 52	73 ± 11	79 ± 28	406 ± 109*	205 ± 67†
IGF-1 (ng/ml)	289 ± 17	343 ± 22	203 ± 44‡	304 ± 61	266 ± 26	245 ± 24	195 ± 34	190 ± 88
Intestine (g)	1.22 ± 0.07	1.21 ± 0.05	1.83 ± 0.11*	1.24 ± 0.09§	1.16 ± 0.06	1.11 ± 0.06	1.96 ± 0.07*	1.63 ± 0.25†¶
Intestine (cm)	38.6 ± 1.0	34.0 ± 0.5¶	47.0 ± 1.4*	36.8 ± 1.7§	36.9 ± 2.3	36.3 ± 0.7	46.8 ± 1.5¶	42.9 ± 2.6
Liver (g)	1.05 ± 0.03	1.25 ± 0.03	3.99 ± 0.34*	2.58 ± 0.21*§	1.34 ± 0.03	1.34 ± 0.04	4.68 ± 0.03*	4.20 ± 0.43*
Testis (g)	ND	ND	ND	ND	0.117 ± 0.003	0.106 ± 0.003‡	0.097 ± 0.007¶	0.096 ± 0.01¶
Sem V (g)	ND	ND	ND	ND	0.13 ± 0.01	0.13 ± 0.01	0.09 ± 0.01¶	0.09 ± 0.02‡
Testost (nM)	ND	ND	ND	ND	8.6 ± 2.9	10.3 ± 2.9	3.0 ± 1.1	3.2 ± 1.8

Data are means ± SE of seven or more female or six or more male mice per group.
$^{*}p < 0.001$, $^{\ddagger}p < 0.05$, and $^{\P}p < 0.01$ vs. wildtype (WT) mice of the same sex.
$^{\dagger}p < 0.05$ and $^{\S}0.001$ vs. *ob/ob* mice of the same sex.
ND, not determined.

ob/ob, adipose mass was increased, again because of a change in reproductive (ovarian) depot mass and a smaller contribution by the retroperitoneal depot.

Leptin-deficient *ob/ob* mice are characterized by hormonal and metabolic changes including elevated glucose, insulin, and corticosterone and reduced IGF-1 (Table 1). Additionally, the small intestine and liver of male and female *ob/ob* mice are markedly hypertrophied (Table 1). Crossing $Y1^{-/-}$ mice onto the *ob/ob* background rescued or partially rescued several of these features. Serum glucose and corticosterone as well as the weight and length of the small intestine were significantly reduced from *ob/ob* values and/or were not different from wildtype values in $Y1^{-/-}$;*ob/ob* mice. Interestingly, there were several sex-specific effects. Liver weight was reduced in female $Y1^{-/-}$;*ob/ob* mice only, consistent with their decrease in body weight despite greater adiposity. Whereas Y1 receptor knockout partially rescued the hyperinsulinemia of male *ob/ob* mice, female $Y1^{-/-}$;*ob/ob* mice had serum insulin levels that were at least as high as female *ob/ob* mice (Table 1).

Y1 knockout increased the mass of WAT depots in males and females (Fig. 2), but in male mice, no adipogenic effect of Y1 receptor deficiency was seen in the number, size, or total volume of adipocytes within the bone marrow (Fig. 3), consistent with differential regulation of WAT depots and marrow adipocytes. In female $Y1^{-/-}$ mice, however, there was a trend to increases in marrow adipocyte volume and number, albeit this difference

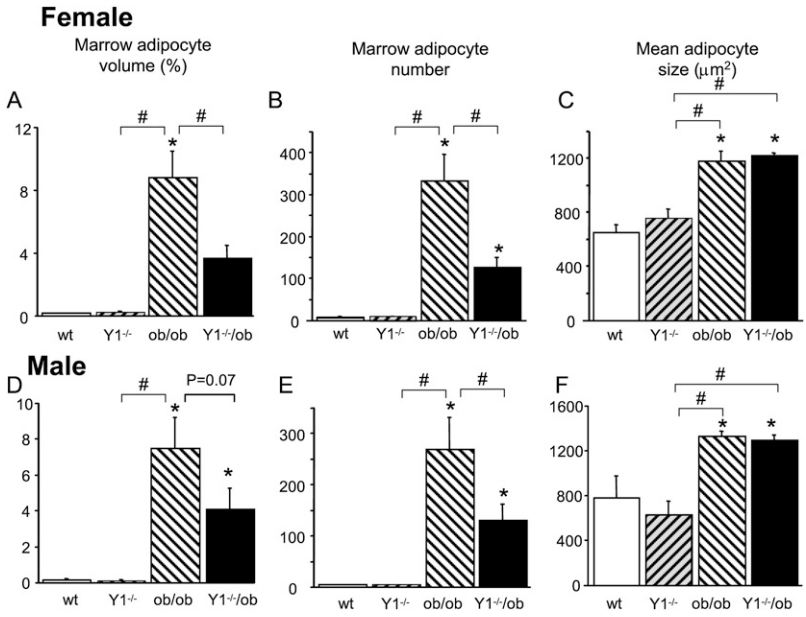

FIG. 3. Effect of Y1 receptor deletion on marrow adiposity of *ob/ob* mice. Female (A–C) and male (D–F) mice. Marrow adipocyte number (A and D), average adipocyte size (B and E), and marrow adipocyte volume (C and F). $*p < 0.05$ vs. wildtype; $^{\#}p < 0.05$ as indicated. $n = 7$–10 per genotype.

did not reach statistical significance when analyzed in conjunction with *ob/ob* mice (Figs. 3A and 3B). In contrast, *ob/ob* mice displayed markedly greater adiposity in bone marrow compared with all genotypes (Fig. 3). In *Y1*$^{-/-}$*;ob/ob* mice, marrow adipocyte number and total marrow adipocyte volume were significantly reduced compared with *ob/ob*, suggesting that the increased adiposity seen in the bone marrow microenvironment of *ob/ob* mice of both sexes is mediated at least in part by Y1 receptor signaling.

Interestingly, the infertility of *ob/ob* is not dependent on Y1 receptor signaling. None of five breeding pairs consisting of either male or female *Y1*$^{-/-}$*;ob/ob* mice mated with corresponding wildtype mice produced any offspring after a 12-wk period. Moreover, inhibition of the hypothalamopituitary gonadotropic axis seen in *ob/ob* mice was not rescued by Y1 receptor deficiency in our hands, because both *ob/ob* and *Y1*$^{-/-}$*;ob/ob* mice showed significant reductions in testis and seminal vesicle weight and marked (albeit nonsignificant) reductions in serum testosterone levels compared with wildtype values (Table 1). This contrasts with another study, in which Y1 receptor knockout normalized the low pituitary leuteinizing hormone content and seminal vesicle weights of male *ob/ob* mice.[15]

Y1 receptor knockout protects against gonadectomy-induced bone loss in female but not male mice

To examine potential interactions between circulating sex steroids and Y1 action in bone, cancellous bone was examined in gonadectomized *Y1*$^{-/-}$ and wildtype mice.

The effects of Y1 receptor deficiency to increase cancellous bone volume, trabecular number and thickness, and mineral apposition rate observed in intact animals (Fig. 1) were also observed in sham-operated *Y1*$^{-/-}$ mice (Fig. 4). There was no change in mineralizing surface with Y1 receptor knockout. However, the resultant bone formation rate was significantly greater in female but not male sham-operated *Y1*$^{-/-}$ mice (Fig. 4).

In wildtype mice, gonadectomy halved cancellous bone volume in association with the loss of trabecular number and increase in osteoclast surface (Fig. 4). Despite the increase in bone resorption, no change in mineralizing surface, mineral apposition rate, or bone formation rate was evident postgonadectomy. In *Y1*$^{-/-}$ mice, gonadectomy-induced bone loss was evident in both sexes, with loss of both cancellous bone volume and trabecular number, but the extent of bone loss varied between males and females. In female mice, cancellous bone volume was reduced by a similar proportion in both genotypes (wildtype, 48%; *Y1*$^{-/-}$, 41%), and cancellous bone volume and trabecular number and thickness remained significantly greater in ovariectomized *Y1*$^{-/-}$ mice compared with ovariectomized wildtype mice (Fig. 4), similar to previously described effects of ovariectomy in *Y2*$^{-/-}$ mice.[14] This maintenance of greater cancellous bone volume in ovariectomized *Y1*$^{-/-}$ mice was associated with a nonsignificant increase in osteoclast surface and a consistently greater mineral apposition rate and bone formation rate (Fig. 4). These data indicate that loss of Y1 receptor signaling partially protects against ovariectomy-induced osteopenia in female mice.

In male mice, however, Y1 deletion did not protect against orchidectomy-induced osteopenia. The significantly greater cancellous bone volume, trabecular number, and mineral apposition rate of sham-operated *Y1*$^{-/-}$ mice were abolished by orchidectomy (Fig. 4). Loss of cancellous bone volume was more extensive in *Y1*$^{-/-}$ compared with wildtype (wildtype, 50%; *Y1*$^{-/-}$, 69%). The elevation in osteoclast surface was no different between *Y1*$^{-/-}$ and wildtype mice after orchidectomy (Fig. 4). In the absence of differing resorption, the decrease in mineral apposition

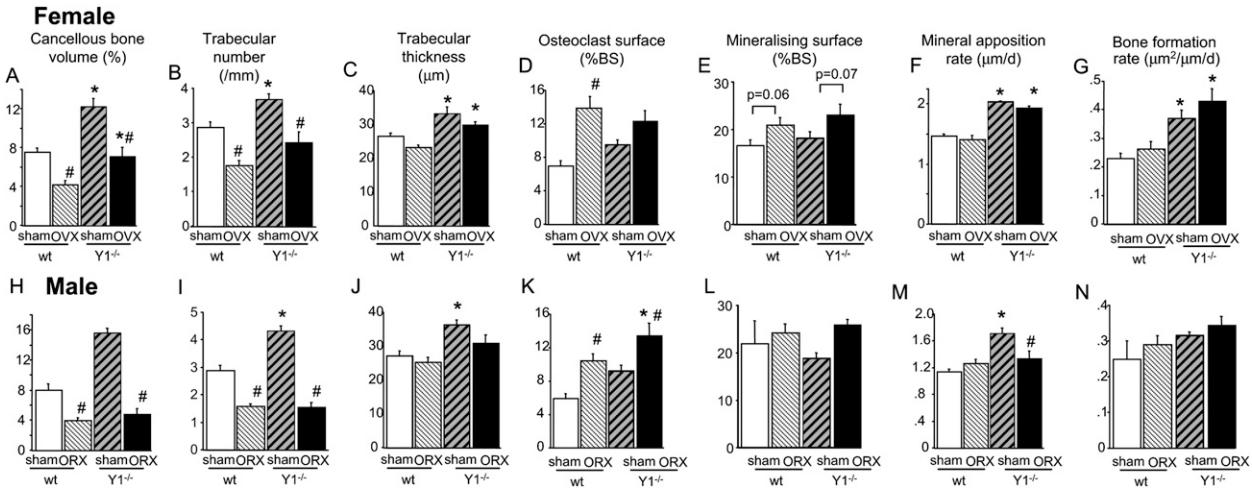

FIG. 4. Effect of gonadectomy (ovariectomy [OVX] or orchidectomy [ORX]) and germline Y1 receptor deletion on cancellous bone of female (A–G) and male (H–N) mice. Cancellous bone volume (A and H), trabecular number (B and I), trabecular thickness (C and J), osteoclast surface (D and K), mineralizing surface (E and L), mineral apposition rate (F and M), and bone formation rate (G and N). $^{*}p < 0.05$ vs. wildtype within equivalent operation; $^{\#}p < 0.05$ vs. sham within the same genotype. $n = 8$–13 per operation/genotype.

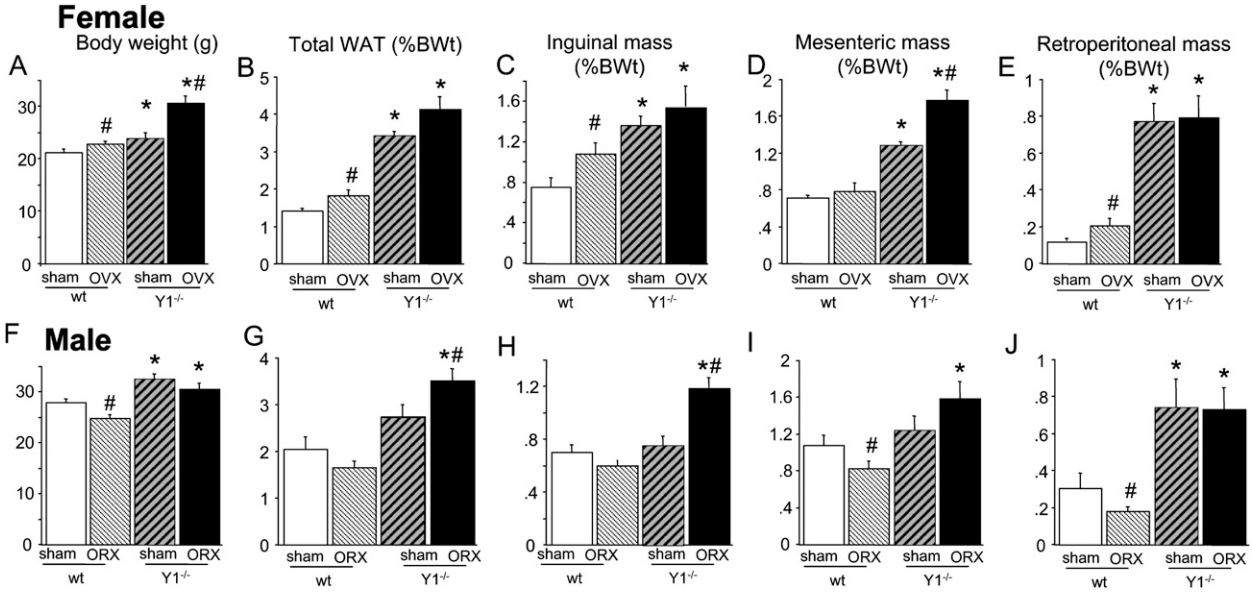

FIG. 5. Effect of gonadectomy and germline Y1 receptor deletion on body composition of female (A–E) and male (F–J) mice. Body weight (A and F), total WAT (B and G) inguinal (C and H), mesenteric (D and I), and retroperitoneal (E and J) mass. $^{*}p < 0.05$ vs. wildtype within equivalent operation; $^{\#}p < 0.05$ vs. sham within the same genotype. $n = 8$–13 per operation/genotype.

rate in hypogonadal male $Y1^{-/-}$ seems critical to the exaggerated bone loss after orchidectomy.

Y1 receptor deletion increases weight gain and fat accumulation after gonadectomy

Sham-operated $Y1^{-/-}$ mice exhibited increases in body weight and WAT mass compared with sham-operated wildtype mice, and this effect was most evident in females (Fig. 5). In wildtype mice, gonadectomy led to sex-specific effects on body weight and WAT mass. Whereas female mice showed ovariectomy-induced increases in body weight and fat mass, male mice showed decreases in body weight and adiposity after orchidectomy (Fig. 5).

Y1 receptor deficiency, however, led to significant gonadectomy-induced increases in body weight and/or WAT mass over and above values seen in gonadectomized wildtype or intact Y1 knockout animals in specific sites, namely the inguinal and mesenteric WAT depots (Fig. 5). This was the same in both male and female mice. Interestingly, unlike female $Y1^{-/-}$ mice in which ovariectomy significantly increased body weight, no such increase in body weight was seen in male $Y1^{-/-}$ orchidectomized mice.

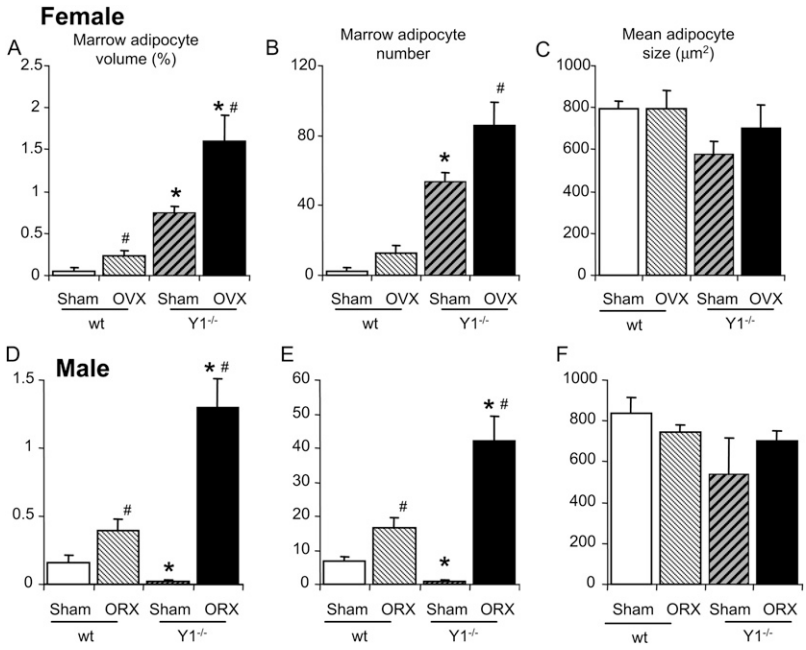

FIG. 6. Effect of hypothalamic NPY overexpression and germline Y1 receptor deletion on body composition, cancellous bone, and marrow adiposity of female mice. Female (A–C), and male (D–F) mice. Marrow adipocyte volume (A and D), marrow adipocyte number (B and E), and mean adipocyte size (C and F). $^{*}p < 0.05$ vs. Sham within genotype; $^{\#}p < 0.05$ vs. WT within operation. n = 8–13 per operation/genotype.

In the marrow, intact $Y1^{-/-}$ mice showed a sex-specific difference in adiposity, with significantly greater volume and number of marrow adipocytes in females, and significant reductions in marrow adipocyte volume and number in males (Fig. 6). Consistent with effects in peripheral fat stores, marrow adipocyte volume was increased by gonadectomy in $Y1^{-/-}$ mice, with marked increases in adipocyte number (Fig. 6). This increase was most evident in males, despite a significantly lower adipocyte volume and number in sham-operated $Y1^{-/-}$ compared with sham-operated wildtype mice. In wildtype mice, significant, albeit smaller, gonadectomy-induced increases in adipocyte volume and number were evident. Adipocyte size was not altered in any group.

Lack of effect of Y1 receptor deletion on reduced bone formation induced by hypothalamic NPY overexpression

A critical factor to the changes evident in leptin-deficient mice is the increased expression of NPY in the hypothalamus, with many of the physiological effects in *ob/ob* mice attenuated in the absence of NPY.[16] To study the influence of elevated hypothalamic NPY in the absence of Y1 receptor signaling, an adeno-associated viral vector expressing NPY was injected into the hypothalamus of wildtype and $Y1^{-/-}$ mice.

AAV-NPY–induced significant increases in body weight and adiposity in wildtype mice (Fig. 7). $Y1^{-/-}$ mice treated with AAV-NPY for 3 wk showed marked increases in body weight and adiposity over GFP vector-injected $Y1^{-/-}$ control mice, and these values were significantly greater than those seen in wildtype AAV-NPY–treated animals (Fig. 7).

Comparing wildtype and $Y1^{-/-}$ mice with hypothalamic injection, cancellous bone volume was greater in AAV-GFP $Y1^{-/-}$ mice compared with AAV-GFP wildtype (Fig. 7). This difference was, however, abolished after hypothalamic injection of AAV-NPY, with a reduction in cancellous bone volume in $Y1^{-/-}$ mice. Cancellous bone volume of wildtype mice was not reduced by AAV-NPY injection. However, osteoblast activity, estimated using osteoid width, was markedly reduced in both genotypes after AAV-NPY injection. These data show that, despite a reduction in cancellous bone volume, AAV-NPY–treated $Y1^{-/-}$ mice maintained their elevated rate of osteoblast activity relative to wildtype levels.

Interestingly, marrow adipocyte volume did not respond to central NPY administration, with the greater levels in $Y1^{-/-}$ compared with wildtype mice unchanged by AAV-NPY injection, despite a decrease in adipocyte size in $Y1^{-/-}$ AAV-NPY–injected mice.

DISCUSSION

These studies showed a key role for Y1 receptor signaling in the regulation of bone and adipose homeostasis and show critical interactions between this pathway and sex steroids in the regulation of both tissues. In bone, the presence of androgens was necessary for the anabolic activity of the Y1-deficient pathway. In adipose tissue, lack of Y1 signaling produced sex-specific changes in adiposity in genetically obese *ob/ob* mice, and Y1 receptor deficiency increased fat mass in both male and female gonadectomized mice. Taken together, these data showed that Y1 receptors represent a novel site of interaction between classic endocrine and neural pathways in the regulation of both bone and adipose tissues.

The initial findings linking Y receptor signaling and bone homeostasis were made in Y2 receptor-deficient models and showed that hypothalamic Y2 receptors were responsible for tonic inhibition of osteoblast activity.[1] Importantly, the recent identification of Y1 receptors on osteoblastic cells may explain the generalized bone anabolic

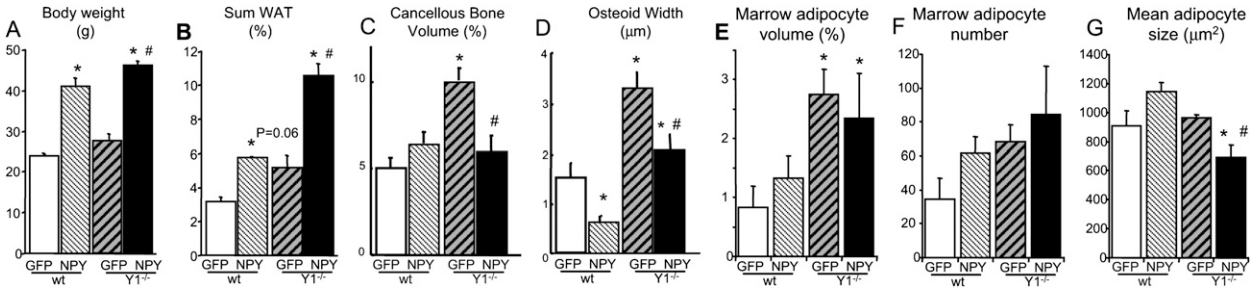

FIG. 7. Effect of hypothalamic NPY overexpression and germline Y1 receptor deletion on body composition, cancellous bone, and marrow adiposity of female mice. Final body weight (A), white adipose tissue (B), cancellous bone volume (C), osteoid width (D), marrow adipocyte volume (E), marrow adipocyte number (F), and mean adipocyte size (G). $^{*}p < 0.05$, vs. GFP within genotype; $^{\#}p < 0.05$ within treatment. $n = 5–7$ per injection/genotype.

response in germline but not hypothalamus-specific $Y1^{-/-}$ mice.[3] In addition, the effects of NPY on osteoblast-like cultures were absent in $Y1^{-/-}$ cells, indicating a role for direct control of anabolism by Y1 signaling. The NPY pathway is known to interact with a number of humeral factors such as leptin,[17] glucocorticoids,[18] and insulin.[19] In such a manner, osteoblastic Y1 receptors may represent a point of integration between neural and humeral signals.

Leptin deficiency is associated with numerous endocrine changes including hypogonadism[4,5] and marked changes in the regulation of both bone and adipose tissue.[5,7,12] Interestingly, cancellous bone volume in $Y1^{-/-}$*;ob/ob* mice showed a sex-specific response compared with $Y1^{-/-}$ and *ob/ob* mice. Whereas the elevated cancellous bone volume seen in the single mutants was still evident in female $Y1^{-/-}$*; ob/ob* mice, it was absent in males. This was consistent with an attenuation of the anabolism of $Y1^{-/-}$ and *ob/ob* models rather than reductions in bone absorption. In contrast, the anabolic response in female $Y1^{-/-}$*;ob/ob* mice remained largely intact. Interestingly, Y1 deletion was unable to rescue the hypogonadism of *ob/ob* mice, with both male and female *ob/ob* and $Y1^{-/-}$*;ob/ob* mice remaining infertile and male mice remaining hypogonadal, in contrast to another report.[15] These findings implicate reduced testosterone levels in the inhibition of bone formation in this model and therefore a potential requirement for androgenic steroids in Y1 signaling in bone.

Isolation of the hypogonadism of the *ob/ob* phenotype using gonadectomy in otherwise intact $Y1^{-/-}$ mice considerably simplified the endocrine aspects of the model and showed a sex-specific response to the absence of gonadal steroids in $Y1^{-/-}$ mice. Gonadectomy produced marked bone loss in all groups, consistent with the known effects of sex hormone deficiency in these models.[14] Importantly, however, a marked reduction in osteoblast activity was evident after the loss of gonadal steroids in male $Y1^{-/-}$ but not females. This decrease was not reflected in mineralizing surface, consistent with normal osteoblastogenesis, but rather, a decrease in mineral apposition rate, suggesting a reduction in osteoblast activity. Indeed, mineral apposition rate of orchidectomized $Y1^{-/-}$ mice was not different to wildtype and significantly reduced from intact $Y1^{-/-}$ mice. In contrast to the response in males, loss of Y1 receptor signaling in female mice provided some protection against gonadectomy-induced bone loss in the distal femur, as previously shown in both sexes of $Y2^{-/-}$ mice.[14]

It has been shown that an increase in hypothalamic NPY levels, as in leptin-deficient mice or in mice with hypothalamic administration of NPY, leads to decreased bone mass.[6,12,20] As male Y1 receptor knockout mice have a significant reduction in NPY mRNA expression in the arcuate nucleus of the hypothalamus,[21] it is possible that a reduction in central NPY-ergic signaling could contribute to their anabolic bone phenotype. To test this possibility, we used viral vector-mediated overexpression of NPY in $Y1^{-/-}$ mice. Increased hypothalamic NPY, while capable of reducing osteoblast activity in both wildtype and Y1 receptor knockout mice, did not abolish the greater osteoblast activity of the $Y1^{-/-}$ model. Thus, it seems that other signals—possibly through androgens—and not solely changes in central NPY-ergic activity are critical to the anabolic activity associated with $Y1^{-/-}$ mice.

Appreciation of the skeletal effects of leptin has increased the need for greater understanding of the regulation of adipose tissue and its relationship to bone homeostasis. Y1 signaling, in addition to its effects in bone, also regulates adipose tissue, with increased fat mass in both sexes of $Y1^{-/-}$ mice.[22] As in bone, this activity was not induced by deletion of hypothalamic Y1 receptors, suggesting local regulation.[3] The findings of reduced body weight in $Y1^{-/-}$*;ob/ob* mice relative to *ob/ob* mice are in accordance with previous reports for Y1[15] and other Y receptors.[17,23] However, there were sex-specific differences in the influence of Y1 signaling on fat, suggesting modulation of Y1 actions by endocrine factors. In females, deletion of Y1 receptors increased WAT mass in normal mice, as well as accentuated fat accrual in the hypogonadal and leptin-deficient *ob/ob* models. Of note, the significantly greater fat mass but reduced body weight of female $Y1^{-/-}$*;ob/ob* compared with *ob/ob* mice showed that the weight reduction to be through loss of lean mass. This is most likely because of change in organ weight, such as liver and intestine rather than muscle mass, because IGF-1 levels in female $Y1^{-/-}$*;ob/ob* are not reduced relative to wildtype values as are female *ob/ob* levels.

In contrast to females, Y1 receptor deficiency in male *ob/ob* mice resulted in significant reductions in body weight and adiposity. It is noteworthy that the significant hyperinsulinemia of *ob/ob* mice was attenuated in male but not female $Y1^{-/-}$;*ob/ob* mice. Because insulin is lipogenic and promotes

partitioning of fuels toward WAT and away from muscle,[24,25] the reduction in serum insulin levels seen in male *Y1*$^{-/-}$;*ob/ob* mice may have contributed to their associated reduction in adiposity. The net effect of germline Y1 receptor knockout on serum insulin levels is likely a balance between effects of Y1 deficiency in the hypothalamus versus Y1 deficiency on the pancreas. In the hypothalamus, NPY stimulates insulin secretion, possibly through actions on Y1 and Y5 receptors in the ventromedial hypothalamus.[26] In the pancreas in contrast, NPY inhibits insulin secretion,[27] possibly through direct action on Y1 receptors expressed on β cells.[28] Therefore, germline lack of Y1 receptors may be expected to promote low serum insulin levels (as seen in male Y1$^{-/-}$;*ob/ob* mice) through actions in the brain but to promote high serum insulin levels (as seen in male and female Y1$^{-/-}$ and female Y1$^{-/-}$;*ob/ob* mice) through actions in pancreatic tissue. It is not clear why central effects of Y1 receptor deficiency seem to predominate over peripheral (pancreatic) effects of Y1 receptor deficiency on serum insulin in male *ob/ob* mice, but this difference may be related to differential effects of male and female gonadal steroids on the hypothalamus.[29,30]

With the exception of male *ob/ob* mice in which fat mass was reduced by Y1 deletion as discussed above, Y1 receptor deletion significantly increased body weight and/or adiposity in every other experimental model under study (male and female Y1$^{-/-}$ mice, male and female gonadectomized mice, female *ob/ob* mice, and female mice with hypothalamic overexpression of NPY). Interestingly, although androgens seem to be permissive for the effects of Y1 deficiency to promote bone anabolic effects, there is no such permissive role of androgens for the Y1-mediated regulation of fat mass, because orchidectomized male mice also exhibit increased adiposity. The adipogenic effects of Y1 deficiency are likely related to increased circulating insulin levels seen in Y1 receptor knockout models from this and other laboratories.[21,31–33]

Interestingly, the adipogenic effects of Y1 receptor deficiency were strong enough to overcome the weight loss and fat loss induced by orchidectomy in male mice. Estrogen deficiency increases fat mass and is evident after menopause or after surgical ovariectomy.[34,35] Central NPY levels have been implicated in both of these processes,[36–38] with hypothalamic NPY expression being elevated after ovariectomy[29,30] but is reduced by estradiol administration.[39] In contrast, central NPY-ergic expression is increased by testosterone and reduced by orchidectomy.[40] Consistent with this relationship, fat mass was increased by ovariectomy and reduced by orchidectomy in wildtype mice. However, this effect of orchidectomy to reduce body weight and fat mass was abolished in Y1$^{-/-}$ mice, orchidectomized Y1 deficient mice being significantly fatter than sham-operated knockouts.

The understanding of the influence of adipocytes on bone mass has recently been expanded, with identification of marrow adiposity as a risk factor for fracture.[41] Leptin[20,42] and sex steroids[43] are known regulators of marrow adiposity, through control of apoptosis. Consistently, in this study, leptin-deficient and gonadectomized mice displayed increased marrow adiposity in both male and female mice, through marked increases in marrow adipocyte number. Moreover, Y1 deficiency altered these process, reducing in marrow adiposity in male and female *Y1*$^{-/-}$*;ob/ob* mice but increasing marrow adiposity in gonadectomized *Y1*$^{-/-}$ mice relative to wildtype mice. Whereas this presents a relatively simple picture, the regulation of marrow adipose is complex. For example, leptin deficiency increases long bone adiposity but does not alter axial sites.[20] The increase in marrow adiposity in *ob/ob* mice can be reversed with peripheral[44] or central[42,45] leptin treatment, indicating multiple regulatory sites. Similarly, sex hormone deficiency, increased long bone adiposity in the metaphyseal but not epiphyseal region, through varied responses to β3 adrenergic signaling.[46] Interestingly, hypothalamic NPY overexpression did not alter marrow adiposity, despite marked increases in peripheral fat accrual,[12] indicating specific regulatory axes to marrow versus other fat stores. The regulation of marrow adiposity therefore seems to be different, at least in part, from that of other adipose stores. Given the presence of a number of adipokine receptors on bone cells, these differences in marrow adipocyte regulation and therefore local signaling in the bone microenvironment may have important consequences for bone mass.

The genetic, surgical, and pharmacological techniques used in this study have enabled a more defined view of the key role for Y1 receptors in the control of both bone and adipose homeostasis. The studies described here indicate a requirement for androgens in the activation of the bone anabolic response to lack of Y1 signaling. This finding is of particular importance, given that an age-related decline of androgen levels is associated with a gradual decline in bone mass in males.[47] These studies also support an important role for Y1 receptors in the control of adipose deposition in a variety of circumstances (leptin deficiency, gonadal steroid deficiency, and hypothalamic overexpression of NPY). Moreover, these studies have shown for the first time the ability of Y1 receptor deletion to modify adipose production within the bone microenvironment. This study thereby showed the diverse modes of action of Y1 receptor signaling in the control of bone and adipose homeostasis opening up potential new ways to control these processes.

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Address reprint requests to:
P Baldock, PhD
Bone and Mineral Research Program
Garvan Institute of Medical Research
St Vincent's Hospital
384 Victoria Street
Darlinghurst, Sydney, NSW 2010, Australia
E-mail: p.baldock@garvan.org.au

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