Y2 and Y4 Receptor Signalling Attenuates the Skeletal Response of Central NPY

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Abstract
Both the neuropeptide Y (NPY) and the leptin systems have been shown to be important central mediators of bone metabolism. However, the interaction between these two systems is complex and not fully understood. Here, we show that a unique interaction exists between Y2 and Y4 receptors in the regulation of bone homeostasis that is not evident when combined with lack of Y1 signalling. Despite the hypoleptinaemia shown in male Y2/Y4 double knockout (Y2−/−Y4−/−) mice, when on the leptin-deficient *ob/ob* background, these mice display reduced cancellous bone mass. However, combined Y2/Y4 deletion enhances the effect of leptin deficiency on the cortical bone compartment. By replicating the enhanced central NPY expression evident in *ob/ob* mice using virally mediated overexpression of NPY in the hypothalamus of Y receptor knockout mice, we demonstrate that Y2−/−Y4−/− mice have an exaggerated response to the anti-osteogenic effects of elevated hypothalamic NPY in both cancellous and cortical bone and that this effect appears to be dependent on Y1 receptor signalling. This study highlights the complex interaction between Y receptors in the control of bone mass. Moreover, it suggests that the reduction in cortical bone observed in the absence of leptin is due to the anti-osteogenic effect of elevated hypothalamic NPY levels.

Introduction
The understanding of the important role that neuronal signals play in the regulation of skeletal tissue has increased markedly in recent years following the identification of several powerful, centrally mediated pathways linking the brain to the control of bone mass, through the modulation of osteoblast activity. One such example involves neuropeptide Y (NPY)-mediated signalling through Y2 receptors expressed in neurons of the hypothalamus (Baldock et al. 2002). A second pathway involves leptin signalling via its hypothalamic receptor (Ducy et al. 2000) to β2 adrenergic receptors in the osteoblast (Takeda et al. 2002). Y2 and leptin receptors are co-expressed on NPY neurons in the arcuate nucleus and have been shown to interact in mediating leptin's effects on energy homeostasis (Baskin et al. 1999); however, their relationship in the control of bone mass is uncertain. Studies in the leptin-deficient state have returned conflicting results. Y2 receptor and leptin-deficient double mutant mice (Y2−/−;*ob/ob*) displayed no additive effect on cancellous bone volume or osteoblast activity, suggesting the two pathways may share certain common elements in the control of bone homeostasis (Baldock et al. 2005). However, a subsequent study provided clear evidence that independent actions also exist, as greater cortical bone formation was observed in Y2−/−;*ob/ob* compared to *ob/ob* mice, while bone resorption was elevated in the leptin-deficient models but was unchanged in Y2−/− mice (Baldock et al. 2006). Adding to this complexity, as circulating leptin levels increase, the independence of the two pathways becomes more apparent. Particularly at normal or elevated leptin levels, the lack of Y2 receptor signalling induces a clear increase in bone formation compared to Y2 intact controls (Baldock et al. 2005).

The source of this varied interaction between the NPY and leptin pathways may relate, in part, to the complexity of the NPY system. Superficially, NPY's effect on bone reveals a relatively simple inverse relationship. Mice deficient in NPY display enhanced bone mass in both the cancellous and cortical compartments due to increased osteoblast activity whilst viral-mediated overexpression of NPY specifically in the hypothalamus of mice leads to decreased bone mass and osteoblast activity, despite markedly increased body weight and adiposity (Baldock et al. 2009). However, NPY is known to act through at least five different Y receptors, which have varied and widespread expression. Studies using germline knockout mice have revealed a definitive role for both Y1 and Y2 receptors in the regulation of bone mass. Whilst Y2 receptors act in the hypothalamus to inhibit osteoblast activity (Baldock et al. 2002), Y1 receptors inhibit bone metabolism via direct actions on bone cells (Baldock et al. 2007; Lee et al. 2010). Furthermore, no additive effect on bone mass was observed with deletion of both Y1 and Y2 receptors, suggesting that they may act at different points along the same pathway with respect to the control of bone formation (Baldock et al. 2007).

However, the role of Y receptors in the regulation of bone mass is more complex. Y2 receptors have been shown to be inhibitory autoreceptors (Zhang et al. 1997). Thus, the loss of Y2 receptors increases NPY production in the hypothalamus, a change even more apparent in double Y2 and Y4 knockout (Y2−/−Y4−/−) mice (Sainsbury et al. 2003). This increased NPY expression in the arcuate nucleus of the hypothalamus would be expected to decrease bone formation, but bone formation is in fact increased in Y2−/− mice and even more so in Y2−/−Y4−/− mice (Sainsbury et al. 2003). The mechanism behind this seeming contradiction is unclear. Whilst the deletion of Y4 receptors had no obvious effect on bone homeostasis in mice (Sainsbury et al. 2003), the combined deletion of both Y2 and Y4 receptors produced a further increase in bone compared to Y2−/− mice (Sainsbury et al. 2003). Interestingly, this synergism was evident only in male Y2−/−Y4−/− mice and was coincident with a gender-specific lean phenotype and a threefold decrease in serum leptin (Sainsbury et al. 2003). Leptin deficiency is associated with increased cancellous bone volume (Ducy et al. 2000) but reduced cortical bone mass (Baldock et al. 2006). Similarly, the Y2−/−Y4−/−-mediated reduction in circulating leptin levels was coincident with an increase in cancellous bone volume and a decrease in cortical bone mass compared to normoleptinaemic Y2−/− mice (Sainsbury et al. 2003). Moreover, an effect of combined Y2 and Y4 receptor deficiency on femoral bone mineral density and bone mineral content was also evident in both male and female *ob/ob* mice (Lee et al. 2008a). Thus, the Y2−/−Y4−/− model as well as its gender-specific effect on leptin production presents an opportunity to dissect in more detail the relationship between Y receptor and leptin signalling to bone and presents a model of an additive interaction between the Y receptor and leptin pathways that was not evident in the Y2−/−;*ob/ob* model. It also presents an opportunity to examine the conflicting relationship between elevated NPY levels and Y receptor deletion in the hypothalamus.

In order to further investigate possible interactions between the different Y receptor-mediated bone anabolic pathways, we initially compared Y2−/−Y4−/− mice with Y1−/−Y2−/−, Y1−/−Y4−/− and Y1−/−Y2−/−Y4−/− mice to examine the possibility of a unique response in the Y2−/−Y4−/− model. Subsequently, the interaction with leptin signalling was investigated by the deletion of various Y receptor combinations in the absence of leptin using *ob/ob* mice and in the presence of excess leptin by employing viral-mediated overexpression of NPY in the hypothalamus.

Materials and Methods

Generation of Germline Y Receptor Knockout Mice

Germline deletion of Y1, Y2 and Y4 receptor genes was achieved as previously described (Sainsbury et al. 2002a, b; Howell et al. 2003). Double or triple knockout mice were obtained by crossing Y1, Y2 or Y4 knockout mice, respectively. Different Y receptor knockout mice were then crossed with heterozygous *Ob/ob* mice to obtain Y2−/−;*ob/ob*, Y4−/−;*ob/ob* and Y2−/−Y4−/−;*ob/ob* mice. All mice generated were maintained on a mixed C57/BL6-129/SvJ background. Mice were group-housed under conditions of controlled temperature (22°C) and illumination (12-h light cycle, lights on at 07:00 h) and were maintained on standard rodent chow (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal/g; Gordon's Speciality Stock Feeds, Yanderra, NSW, Australia) ad libitum. 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.

Virus Injections

Adult mice were anaesthetised with a single dose of ketamine/xylazine (100 mg/kg and 20 mg/kg; ip) and injected with adenoassociated virus (AAV) expressing either NPY or an empty vector control as previously described (Lin et al. 2006).

Tissue Collection and Analysis

Mice were injected with calcein (20 mg/kg) 10 and 3 days prior to tissue collection and killed by cervical dislocation at 16 weeks of age. Femurs and tibias were excised, fixed overnight in

4% paraformaldehyde in phosphate-buffered saline at 4°C and then stored in 70% ethanol at 4°C before undergoing processing.

Bone Densitometry Analysis

Whole body bone mineral density and bone mineral content were measured in anaesthetised mice using a dedicated mouse dual X-ray absorbitometry (DXA) (Lunar Piximus II, GE Medical Systems, Madison, WI, USA) 3 days prior to tissue collection. In addition, excised individual femora and tibiae were scanned following collection, fixation and removal of soft tissue.

Histomorphometry

Following fixation, the right femora were bisected transversely at the midpoint of the shaft, and the distal halves embedded and undecalcified in methyl-methacrylate (APS Chemicals, Sydney, Australia). Five-micrometre sagittal sections were analysed as previously described (Baldock et al. 2002). Briefly, cancellous bone volume (BV/TV, percent), trabecular number (number/millimetre) and trabecular thickness (micrometre) were calculated from von Kossa-stained sections whilst osteoclast surface (percent) was used to estimate bone resorption from sections stained for tartrate-resistant acid phosphatase activity. Mineral apposition rate (MAR, micrometre/day) was used to estimate bone formation from unstained sections. All analysis was carried out using Leica QWin analysis software (Leica Microsystems Ltd., Heerberg, Switzerland).

Statistical Analyses

All data are expressed as means±SEM. Differences between groups were assessed by ANOVA, followed by Tukey's post hoc tests if an overall significance was detected by ANOVA. Statistical analyses were performed with SPSS for Mac OS X, version 17.0 (SPSS Inc., Chicago, IL, USA). For all statistical analyses, $p < 0.05$ was accepted as being statistically significant.

Results

Greater Elevation of Cancellous Bone Volume in Male But not Female Y2−/−Y4−/− Mice

In order to delineate the interactions between various Y receptors on the control of bone mass, cancellous bone of the distal femoral metaphyses was examined in wild-type, double Y1−/−Y2−/−, Y1−/−Y4−/−, Y2−/−Y4−/− and triple Y1−/−Y2−/−Y4−/− knockout mice. Firstly, it is important to note that previous studies investigating the effects of multiple Y receptor deficiencies on energy homeostasis have shown that body weight under chow-fed conditions is similar in all groups of mice investigated here, except for Y1−/−Y4−/− mice which showed significant increases in body weight compared to wild-type (Sainsbury et al. 2006). In addition, both Y2−/−Y4−/− and Y1−/−Y2−/−Y4−/− mice showed decreased adiposity (Sainsbury et al. 2003, 2006), although this was more pronounced in the Y2−/−Y4−/− mice which also showed decreased serum leptin levels. Importantly, however, serum levels of corticosterone, free T4, glucose, testosterone and insulin-like growth factor-1 were all similar between groups and were not different from wild-type levels (Sainsbury et al. 2006).

In male mice, all Y receptor models investigated displayed elevated cancellous bone volume, although it failed to reach statistical significance in the Y1−/−Y2−/−Y4−/− line (Fig. 1a). This increase in bone volume was associated with significantly increased trabecular thickness (Fig. 1c) and mineral apposition rate (Fig. 1d) compared to wild-type mice. In addition, the greatest elevation in cancellous bone volume was evident in male Y2−/−Y4−/− double knockout mice (Fig. 1a). This marked increase in cancellous bone volume in male Y2−/−Y4−/− mice was associated with greater trabecular number (Fig. 1b) and mineral apposition rate (Fig. 1d) compared with wild-type and other double and triple knockout mice investigated. However, no interaction was evident in any of the other models investigated where cancellous bone volume and mineral apposition rate were increased only to levels similar to that previously shown in single Y1−/− and Y2−/− mice (Baldock et al. 2002, 2007). Interestingly, the marked effects of double Y2 and Y4 receptor deletion on cancellous bone

volume (Fig. 1a), trabecular number (Fig. 1b) and osteoblast activity (Fig. 1d) were no longer evident when combined with Y1 receptor deletion.

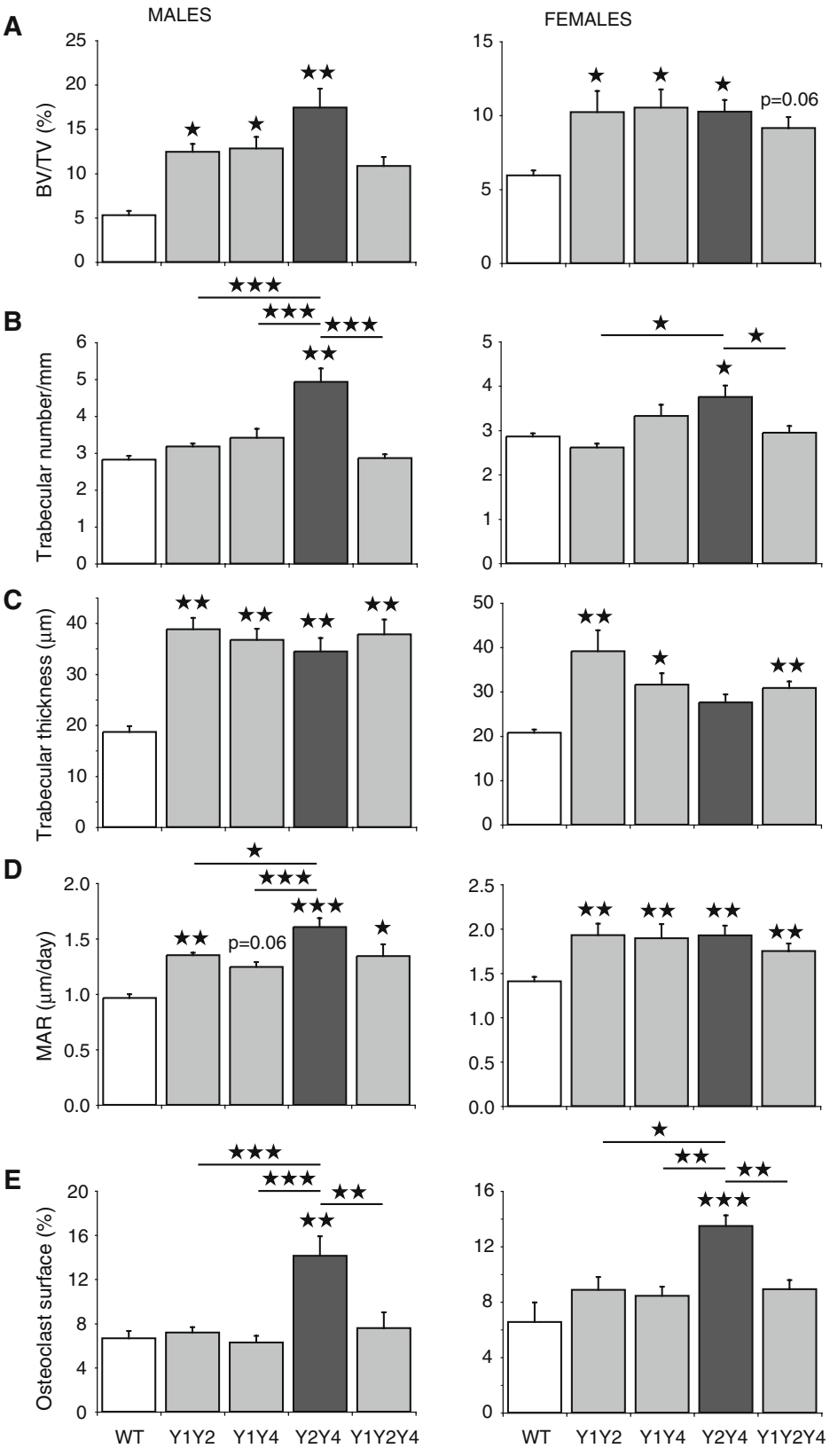

Figure 1 Effect of Y receptor deficiencies on cancellous bone volume, osteoblast activity and osteoclast activity. **a** Cancellous bone volume (BV/TV), **b** trabecular number, **c** trabecular thickness, **d** mineral apposition rate (*MAR*) and **e** osteoclast surface in male and female mice at 16 weeks of age. Mean±SEM of 3–16 mice per group are shown. ⋆$p<0.05$, ⋆⋆$p<0.01$, ⋆⋆⋆$p<0.001$ compared to wild-type or as indicated. *WT*, wild-type; *Y1Y2*, $Y1^{-/-}Y2^{-/-}$; *Y1Y4*, $Y1^{-/-}Y4^{-/-}$; *Y2Y4*, $Y2^{-/-}Y4^{-/-}$; *Y1Y2Y4*, $Y1^{-/-}Y2^{-/-}Y4^{-/-}$

In female mice, cancellous bone volume was similarly elevated in all Y receptor-deficient models compared to control, with no differences between models (Fig. 1a). This increase was associated with enhanced bone formation as demonstrated by significantly increased mineral apposition rate in all models compared to wild-type (Fig. 1d). Despite the lack of a greater effect in female Y2−/−Y4−/− mice compared to other knockout models studied, the elevated cancellous bone volume in female Y2−/−Y4−/− mice was associated with increased trabecular number (Fig. 1b) whereas in the other Y receptor-deficient models it was associated with increased trabecular thickness (Fig. 1c).

Interestingly, bone resorption was also elevated in both male and female double Y2−/−Y4−/− knockout mice as demonstrated by significantly increased osteoclast surface compared to wild-type and other Y receptor-deficient models investigated (Fig. 1e). Again, this effect was no longer evident when combined with Y1 receptor deletion. Thus, these data reveal a unique interaction between Y1, Y2 and Y4 receptor signalling in both the osteoblastic and osteoclastic regulation of cancellous bone mass, albeit one that is more pronounced in male animals.

Combined Lack of Y2, Y4 and Leptin Signalling Does not Increase Cancellous Bone Mass

The data presented above suggest a unique skeletal response to combined Y2 and Y4 receptor deletion (in the absence of Y1 deficiency) resulting in enhanced cancellous bone mass. Previous studies also identified a reduction in cortical bone mass in these mice (Sainsbury et al. 2003). This opposing response between the two bone envelopes is consistent with the skeletal effects of hypoleptinaemia known to be present in male Y2−/−Y4−/− mice (Sainsbury et al. 2003). To examine the dependence of the Y2−/−Y4−/− skeletal phenotype upon coincident leptin deficiency, Y2−/−, Y4−/− and Y2−/−Y4−/− mice were crossed onto the leptin-deficient, *ob/ob* background. In contrast to findings in leptin-intact models above (Fig. 1), in the complete absence of leptin, cancellous bone volume was not greater in Y2−/−Y4−/− mice than in other models (Fig. 2a), nor was trabecular thickness or trabecular number (data not shown). Moreover, the levels of cancellous bone volume in leptin-deficient models was not greater than that shown in leptin-intact models in Fig. 1a.

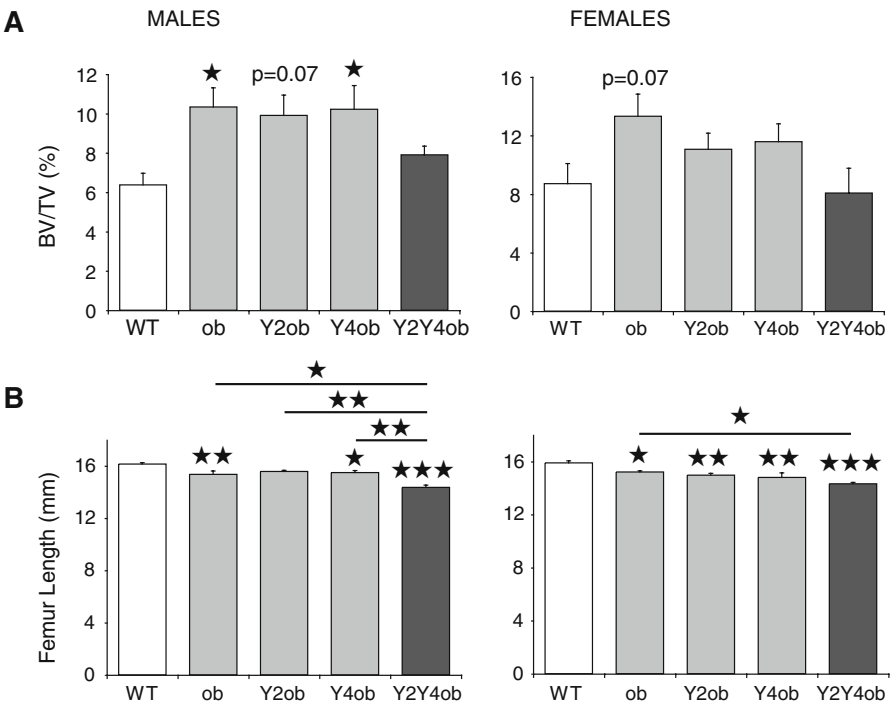

Figure 2 Effect of Y2, Y4 and double Y2, Y4 receptor deficiency on cancellous bone volume and femur length. **a** Cancellous bone volume (BV/TV), **b** femur length in male and female mice at 16 weeks of age. Mean±SEM of 4–10 mice per group are shown. ⋆$p<0.05$, ⋆⋆$p<0.01$, ⋆⋆⋆$p<0.001$ compared to wild-type or as indicated. *WT*, wild-type; *ob*, *ob/ob*; *Y2ob*, $Y2^{-/-}$;*ob/ob*; *Y4ob*, $Y4^{-/-}$;*ob/ob*; *Y2Y4ob*, $Y2^{-/-}Y4^{-/-}$;*ob/ob*

Interestingly, the cancellous bone volume of Y2−/−Y4−/−;*ob/ob* mice was not significantly different to wild-type levels (Fig. 2a), suggesting that the unique increase in cancellous bone volume and osteoblast activity in male Y2−/−Y4−/− mice (Fig. 1) may not involve a direct response to hypoleptinaemia. However, it does still indicate the potential for unique signalling to bone in the absence of both Y2 and Y4 receptors.

Conversely, cortical bone mass of Y2−/−Y4−/−;*ob/ob* mice was decreased, as indicated by long bone length, known to be reduced in *ob/ob* mice (Hamrick et al. 2004), which was further reduced following the additional loss of both Y2 and Y4 receptor signalling in both male and female mice (Fig. 2b). This is in addition to previously described significant reductions in femoral bone mineral density and bone mineral content in both sexes of Y2−/−Y4−/−;*ob/ob* mice compared to wild-type, even in the absence of correction for body weight (Lee et al. 2008a). Critically, this reduction was also evident compared to *ob/ob* mice and was not apparent in either Y2−/−;*ob/ob* or Y4−/−;*ob/ob* mice (Fig. 2b), suggesting that even when leptin levels were constant Y2−/−Y4−/− mice responded more strongly than other genotypes.

Y2−/−Y4−/− Mice Exhibit Enhanced Responses to Hypothalamic NPY

The cortical effects of leptin deficiency seen in *ob/ob* mice, which act in opposition to cancellous changes, have been postulated to result from the elevated hypothalamic NPY levels resulting from loss of leptin signalling (Lee et al. 2008b). Thus, the greater cortical changes evident in Y2−/−Y4−/−;*ob/ob* mice may represent an enhanced response to hypothalamic NPY signalling rather than a generalised effect of leptin deficiency. Moreover, the reduced cancellous bone seen in Y2−/−Y4−/−;*ob/ob* mice further indicates that loss of Y2 and Y4 receptor signalling induces elevated responses to hypothalamic NPY. This hypothesis was investigated by specifically overexpressing NPY in the hypothalamus of male wild-type, Y2−/−, Y2−/−Y4−/− and Y1−/−Y2−/−Y4−/− knockout mice using a virally mediated technique. Increased central NPY expression reduced tibial bone mass (as determined by DXA on isolated bones) in wild-type and Y2−/− mice as previously reported (Fig. 3 and Baldock et al. 2005). However, tibial bone mineral density (Fig. 3a) and bone mineral content (Fig. 3b) were reduced to significantly lower levels in Y2−/−Y4−/− mice compared to the other genotypes investigated, despite equivalent NPY expression (data not shown). Indeed, the percentage change in both tibial bone mineral density and tibial bone mineral content in NPY-treated mice compared to empty control was two- to threefold greater in Y2−/−Y4−/− mice than other genotypes (Fig. 3c), indicating the existence of a unique response pattern in these mice which is no longer apparent when combined with Y1 receptor deletion.

It is interesting to note that these decreases in bone mass following NPY overproduction occurred despite marked (50–70%) increases in body weight (Lin et al. 2006). That bone mass increases consistently with body weight is one of the most fundamental relationships in skeletal biology (Reid 2008). Thus, the loss of bone following NPY overproduction is evidence of the power of the anti-osteogenic pathway induced by central NPY signalling. As with skeletal changes, body weight changes were attenuated between Y2−/−Y4−/− and Y1−/−Y2−/−Y4−/− mice, further indicating the importance of Y1 receptor signalling in the absence of Y2 and Y4 receptors (Lin et al. 2006).

We next investigated whether the unique skeletal effect of elevated hypothalamic NPY in Y2−/−Y4−/− mice is also evident in cancellous bone. As shown in Fig. 4, increased central NPY expression did not alter cancellous bone volume, trabecular thickness or trabecular number in wild-type mice. However, it did abolish the elevated cancellous bone volume (Fig. 4a) and trabecular thickness (Fig. 4b) evident in all Y receptor knockout models investigated. Trabecular number, only elevated in the Y2−/−Y4−/− model, was also significantly reduced in the NPY overproducing Y2−/−Y4−/− group (Fig. 4c). Interestingly, the magnitude of change in cancellous bone volume caused by elevated NPY levels in the Y2−/−Y4−/− double knockout mice (−82%) was double that exhibited by Y2−/− and Y1−/−Y2−/−Y4−/− mice (−44% and −45%, respectively).

Together, these data show that combined Y2 and Y4 receptor deletion in mice leads to an enhanced response to central NPY levels in both cortical and cancellous bone. Interestingly, the additional deletion of the Y1 receptor eliminates this unique response.

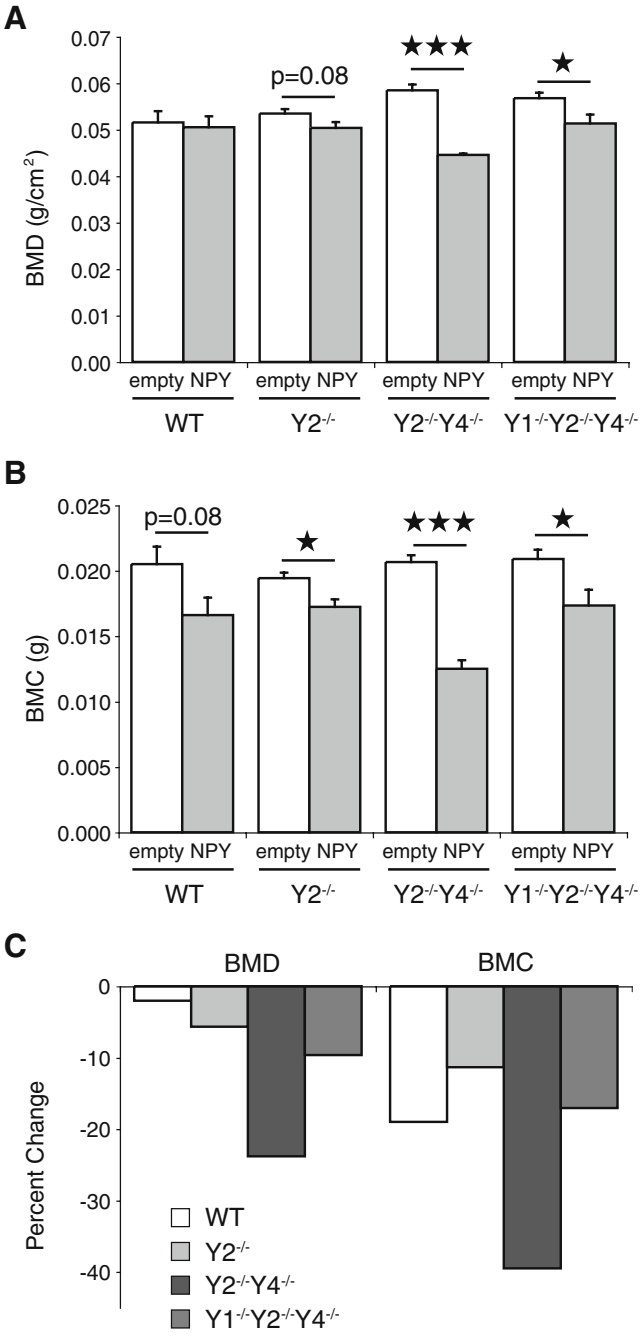

Figure 3 Effect of Y receptor deficiencies on the cortical bone response to elevated hypothalamic NPY. **a** Tibial bone mineral density (*BMD*) and **b** tibial bone mineral content (*BMC*) in male mice 3 weeks after AAV vector injection. **c** Percent change in tibial BMD and BMC in NPY overexpressing mice compared to empty controls. Mean±SEM of 3–12 mice per group are shown. ⋆$p<0.05$, ⋆⋆⋆$p<0.001$ as indicated. *WT*, wild-type; *Y2*, $Y2^{-/-}$; *Y2Y4*, $Y2^{-/-}Y4^{-/-}$; *Y1Y2Y4*, $Y1^{-/-}Y2^{-/-}Y4^{-/-}$

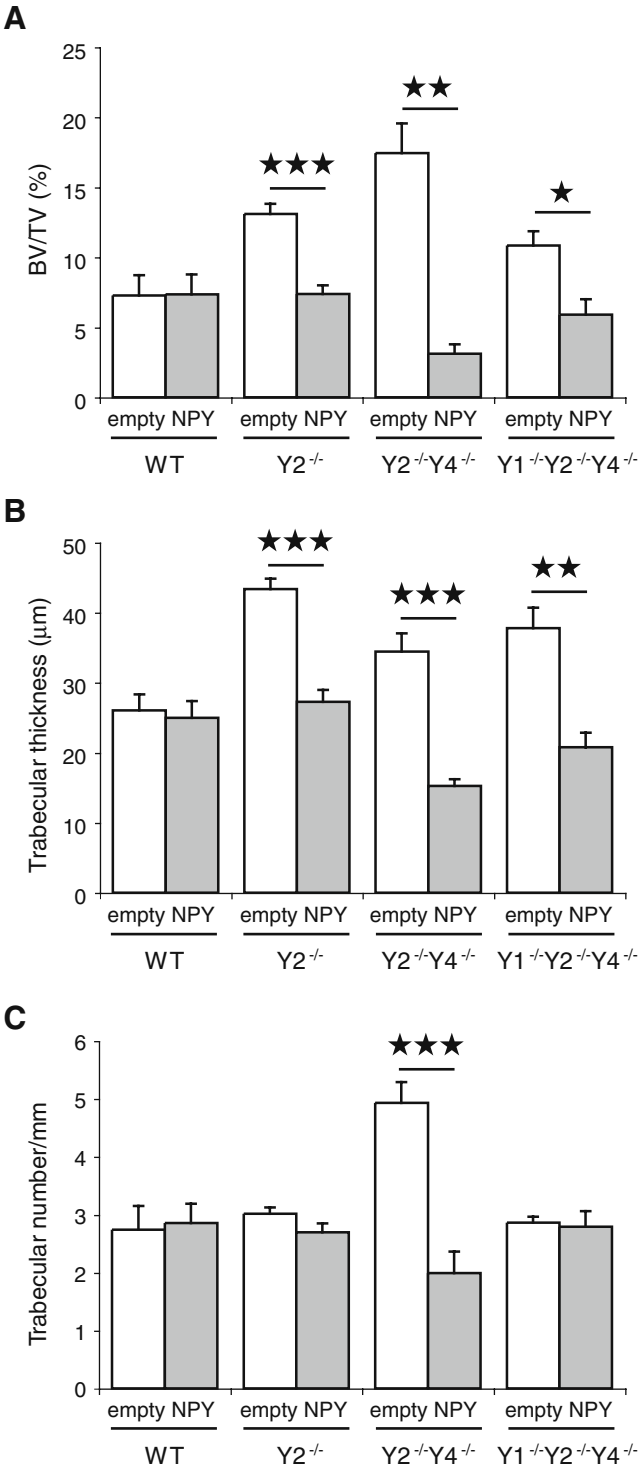

Figure 4 Effect of Y receptor deficiencies on the cancellous bone response to elevated hypothalamic NPY. **a** Cancellous bone volume (BV/TV), **b** trabecular thickness and **c** trabecular number in male mice 3 weeks after AAV vector injection. Mean±SEM of 3–13 mice per group are shown. ⋆$p<0.05$, ⋆⋆$p<0.01$, ⋆⋆⋆$p<0.001$ as indicated. *WT*, wild-type; *Y2*, $Y2^{-/-}$; *Y2Y4*, $Y2^{-/-}Y4^{-/-}$; *Y1Y2Y4*, $Y1^{-/-}Y2^{-/-}Y4^{-/-}$

Discussion

Results from this study help to delineate the complex interaction between the NPY and the leptin systems on bone homeostasis. In particular, we show that the unique interaction between Y2 and Y4 receptors is not seen with other Y receptor combinations such as Y1/Y2 double knockout as well as Y1/Y4 double knockout. Importantly, these data also demonstrate that the major effects of Y2 and Y4 deletion on bone are lost when combined with Y1 receptor deletion. Furthermore, the combined lack of Y2 and Y4 receptor signalling reduces the potent effect of leptin deficiency to promote cancellous bone mass whilst exacerbating the effect of leptin deficiency to decrease cortical bone. This phenotype is consistent with an exaggerated response to the anti-osteogenic actions of increased hypothalamic NPY that is secondary to the loss of leptin as demonstrated by the extreme loss of both cancellous and cortical bone shown in Y2−/−Y4−/− mice with viral overexpression of hypothalamic NPY.

The NPY-mediated regulation of bone mass has been shown to involve signalling via central Y2 receptors (Baldock et al. 2002) and osteoblastic Y1 receptors (Lee et al. 2010). In contrast, the Y4 receptor and its primary ligand pancreatic polypeptide (PP) do not appear to play a role in bone (Sainsbury et al. 2003; Wortley et al. 2007). However, the unique skeletal changes in Y2−/−Y4−/− mice present an opportunity to examine the system more closely: in particular, the interaction between NPY and leptin-deficient regulation of bone mass. Hypoleptinaemic male Y2−/−Y4−/− mice display a unique increase in cancellous bone volume coincident with reduced cortical bone mass (Sainsbury et al. 2003). This phenotype was not present with any other combination of Y receptor deletion investigated and suggests an interaction between the NPY-deficient (Baldock et al. 2009) and β2 adrenergic-deficient (Takeda et al. 2002) regulation of osteoblast activity, as evident in NPY knockout and leptin-deficient *ob/ob* mice, respectively.

Previous studies examining crosses of either Y1 or Y2 receptor null mice with leptin-deficient models have failed to identify additive effects in cancellous bone, whilst activities were opposing in cortical bone (Baldock et al. 2005, 2006; Allison et al. 2009). In contrast to these previously published models, the Y2−/−Y4−/−;*ob/ob* mice showed a generalised reduction of bone mass in both cortical and cancellous bone. Such a phenotype is consistent with an exaggerated response to the anti-osteogenic actions of increased hypothalamic NPY that is secondary to the loss of leptin (Wilding et al. 1993). Indeed, in addition to the exaggerated loss of cortical bone (Lee et al. 2008a), the reduction in cancellous bone volume shown here in Y2−/−Y4−/−;*ob/ob* mice indicates that the elevation of central NPY is capable of overcoming the pro-osteogenic actions of the adrenergic pathway. Interestingly, the enhanced reduction in cortical bone mass in these mice suggests that the loss of cortical bone in *ob/ob* mice is the result of central NPY signalling.

Y2−/−Y4−/− mice, whether leptin intact or leptin deficient, display a complex skeletal phenotype suggesting unique responses in these mice. Importantly, however, endocrine changes do not explain the unique responses in Y2−/−Y4−/− mice (Sainsbury et al. 2003). However, previous studies have identified marked changes in Y receptor binding in the hypothalamus in response to the loss of individual Y receptors (Lin et al. 2005), suggesting that neural processes may be altered in these mice. Moreover, the Y2−/−Y4−/− phenotype was absent in Y1−/−Y2−/−Y4−/− mice, reinforcing the likelihood of a neural mechanism. In order to examine the possible changes in signalling present in the hypothalamus of Y2−/−Y4−/− mice, a locus-specific elevation in NPY expression was induced in mature mice, using a virally mediated approach. This method has the advantage of minimising developmental changes associated with leptin deficiency. Increased NPY expression in the arcuate nucleus has been shown to induce marked anti-osteogenic activities in bone (Baldock et al. 2005) and results were consistent with previous studies. However, Y2−/−Y4−/− mice again displayed a unique response, with markedly greater reductions in both cancellous and cortical bone mass, despite equivalent increases in NPY. In this manner, the mechanism responsible for the unique skeletal response can be isolated to the hypothalamus and further to signalling involving arcuate NPY. Importantly, the exaggerated response to arcuate NPY was again normalised in Y1−/−Y2−/−Y4−/− mice. This result indicates a critical role of central Y1 receptors in this context, likely through modulation of Y1 expression in the paraventricular nucleus, the downstream region for signalling of NPY produced in the arcuate (Jhanwar-Uniyal et al. 1993).

The interaction between Y receptors in the hypothalamus is clearly complex and appears context dependent. In particular, arcuate-specific overexpression of exogenous NPY using a virally mediated process highlights the unique skeletal response of Y2−/−Y4−/− mice

and the role of Y1 expression in this model. Y2−/−Y4−/− mice showed markedly increased anti-osteogenic responses to NPY injection that were corrected by loss of Y1. These interrelationships between the receptors of the NPY system and their effects upon peripheral tissue homeostasis represent an important consideration in the development of potential therapeutic agents. In addition to consideration of the receptor specificity of any such agent, the compensatory response by those remaining receptors must also be evaluated.

References

Allison SJ, Baldock PA, Enriquez RF et al (2009) Critical interplay between neuropeptide Y and sex steroid pathways in bone and adipose tissue homeostasis. J Bone Miner Res 24:294–304

Baldock PA, Sainsbury A, Couzens M et al (2002) Hypothalamic Y2 receptors regulate bone formation. J Clin Invest 109:915–921

Baldock PA, Sainsbury A, Allison S et al (2005) Hypothalamic control of bone formation: distinct actions of leptin and Y2 receptor pathways. J Bone Miner Res 20:1851–1857

Baldock PA, Allison S, McDonald MM et al (2006) Hypothalamic regulation of cortical bone mass: opposing activity of Y2 receptor and leptin pathways. J Bone Miner Res 21:1600–1607

Baldock PA, Allison SJ, Lundberg P et al (2007) Novel role of Y1 receptors in the coordinated regulation of bone and energy homeostasis. J Biol Chem 282:19092–19102

Baldock PA, Lee NJ, Driessler F et al (2009) Neuropeptide Y knockout mice reveal a central role of NPY in the coordination of bone mass to body weight. PLoS One 4:e8415

Baskin DG, Breininger JF, Schwartz MW (1999) Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes 48:828–833

Ducy P, Amling M, Takeda S et al (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 100:197–207

Hamrick MW, Pennington C, Newton D, Xie D, Isales C (2004) Leptin deficiency produces contrasting phenotypes in bones of the limb and spine. Bone 34:376–383

Howell OW, Scharfman HE, Herzog H, Sundstrom LE, Beck-Sickinger A, Gray WP (2003) Neuropeptide Y is neuroproliferative for post- natal hippocampal precursor cells. J Neurochem 86:646–659

Jhanwar-Uniyal M, Beck B, Jhanwar YS, Burlet C, Leibowitz SF (1993) Neuropeptide Y projection from arcuate nucleus to parvocellular division of paraventricular nucleus: specific relation to the ingestion of carbohydrate. Brain Res 631:97–106

Lee NJ, Enriquez RF, Boey D et al (2008a) Synergistic attenuation of obesity by Y2- and Y4-receptor double knockout in ob/ob mice. Nutrition 24:892–899

Lee NJ, Wong IP, Baldock PA, Herzog H (2008b) Leptin as an endocrine signal in bone. Curr Osteoporos Rep 6:62–66

Lee NJ, Doyle KL, Sainsbury A et al (2010) Critical role for Y1 receptors in mesenchymal progenitor cell differentiation and osteoblast activity. J Bone Miner Res (in press)

Lin S, Boey D, Couzens M, Lee N, Sainsbury A, Herzog H (2005) Compensatory changes in [125I]-PYY binding in Y receptor knockout mice suggest the potential existence of further Y receptor(s). Neuropeptides 39:21–28

Lin EJ, Sainsbury A, Lee NJ et al (2006) Combined deletion of Y1, Y2, and Y4 receptors prevents hypothalamic neuropeptide Y overexpression-induced hyperinsulinemia despite persistence of hyperphagia and obesity. Endocrinology 147:5094–5101

Reid IR (2008) Relationships between fat and bone. Osteoporos Int 19:595–606

Sainsbury A, Schwarzer C, Couzens M et al (2002a) Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. Proc Natl Acad Sci USA 99:8938–8943

Sainsbury A, Schwarzer C, Couzens M et al (2002b) Y4 receptor knockout rescues fertility in ob/ob mice. Genes Dev 16:1077–1088

Sainsbury A, Baldock PA, Schwarzer C et al (2003) Synergistic effects of Y2 and Y4 receptors on adiposity and bone mass revealed in double knockout mice. Mol Cell Biol 23:5225–5233

Sainsbury A, Bergen HT, Boey D et al (2006) Y2Y4 receptor double knockout protects against obesity due to a high-fat diet or Y1 receptor deficiency in mice. Diabetes 55:19–26

Takeda S, Elefteriou F, Levasseur R et al (2002) Leptin regulates bone formation via the sympathetic nervous system. Cell 111:305–317

Wilding JP, Gilbey SG, Bailey CJ et al (1993) Increased neuropeptide- Y messenger ribonucleic acid (mRNA) and decreased neuro- tensin mRNA in the hypothalamus of the obese (ob/ob) mouse. Endocrinology 132:1939–1944

Wortley KE, Garcia K, Okamoto H et al (2007) Peptide YY regulates bone turnover in rodents. Gastroenterology 133:1534–1543

Zhang X, Shi T, Holmberg K et al (1997) Expression and regulation of the neuropeptide Y Y2 receptor in sensory and autonomic ganglia. Proc Natl Acad Sci USA 94:729–734