RANK deletion in NPY neurons attenuates estrogen deficiency related bone loss

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Abstract

The RANKL pathway is known to be an important aspect of the pathogenesis of estrogen-deficiency induced bone loss. RANK deletion specifically in NPY neurons has been shown to enhance the ability of the skeleton to match increases in body weight caused by high fat diet feeding, likely via the modulation of NPY levels. Here, we use ovariectomy in female mice to show that RANK deletion in NPY neurons attenuates bone loss caused by long-term estrogen deficiency, particularly in the vertebral compartment. Ovariectomy led to a reduction in NPY expression levels in the arcuate nucleus of $NPY^{cre/+};RANK^{lox/lox}$ mice compared to $NPY^{cre/+};RANK^{lox/+}$ controls. As NPY deficient mice also displayed a similar protection against ovariectomy induced bone loss, modulation of hypothalamic NPY signalling is the likely mechanism behind the protection from bone loss in the $NPY^{cre/+};RANK^{lox/lox}$ mice.

Introduction

Estrogen deficiency as a result of menopause in humans is associated with weight gain, increased adiposity, reduced lean mass and bone loss. The RANKL/RANK/OPG signalling pathway is one of the most important downstream mediators of the effects of estrogen-deficiency on bone. RANKL is an essential mediator of osteoclast formation, activation and survival acting through its receptor, RANK, and is inhibited by OPG, a soluble decoy receptor. Ovariectomy, a surgical model of estrogen-deficiency, leads to an increase in RANKL resulting in increased bone resorption and subsequent bone loss. Indeed, the bone loss caused by ovariectomy can be prevented using antibodies directed against RANKL, illustrating the importance of the RANKL system in this process. However, RANKL signalling is not restricted to the bone environment. RANKL exists in both a membrane-bound form and a soluble form and is also produced from cells such as immune cells. RANKL and its receptor RANK are also expressed in the brain [1] and neuronal specific deletion of RANK has been implicated in the control of thermoregulation in female mice [1]. In addition, we have shown that the deletion of RANK specifically from neuropeptide Y (NPY) neurons in male mice regulates bone mass and aids the skeleton in matching bone mass to changes in body weight caused by high fat diet feeding [2].

NPY is a major regulator of food intake, energy expenditure and bone mass and a known target for sex hormones. The arcuate nucleus (ARC) of the hypothalamus contains at least two neuronal populations: the AgRP/NPY neurons that produce NPY and the Agouti-related protein (AgRP) in close proximity to proopiomelanocortin (POMC)-producing neurons. POMC neurons exert their catabolic and anorectic actions by releasing α-melanocyte-stimulating hormone (α-MSH), while AgRP/NPY neurons oppose this action by exerting tonic GABAergic inhibition of POMC neurons and releasing the melanocortin receptor inverse agonist AgRP. Importantly, these neurons also release NPY, which acts inhibitory on POMC and other secondary target neurons. Both NPY and AgRP have been implicated in the control of bone formation [3, 4]. Analysis of brain specific NPY overexpressing and Y receptor knockout models has revealed a powerful anabolic pathway involving hypothalamic Y2 receptors and osteoblastic Y1 receptors in the control of bone mass [5]. The gut-derived satiety peptide Peptide YY (PYY), another ligand of the NPY family, has also been shown to

be critical for the control of bone mass [6]. The role of the NPY family in the regulation of bone mass has been shown to be particularly important under conditions of stress and altered energy balance [6-8] and has been shown to facilitate the matching of bone acquisition to alterations in energy status [9]. Levels of NPY in the hypothalamus are increased in post-menopausal women [10] and transiently increased by ovariectomy in rodents [11-13]. The temporary increase in NPY levels (seen at 2-3 weeks but no longer evident at 7 weeks post-ovariectomy) has been shown to contribute to the accretion of fat mass as well as mediating metabolic changes induced by the early stages of estrogen deficiency, such as changes in oxygen consumption and energy expenditure [14]. However, NPY ablation in mice does not prevent the long-term development of obesity following ovariectomy [14].

The short-term effects of estrogen deficiency on NPY levels are likely direct effects through estrogen receptors on NPY neurons for which the brain eventually compensates. Given the new knowledge of the ability for RANKL signalling through central RANK receptors to modulate NPY levels and subsequently alter bone mass [2], we sought to investigate whether RANKL signalling through NPY neurons plays a role in the control of bone mass following estrogen deficiency. Therefore, we generated mice where RANK receptors were deleted specifically in NPY neurons ($NPY^{cre/+};RANK^{lox/lox}$) and investigated the effect of ovariectomy on body composition and bone mass in a long-term study in order to avoid the transient changes in NPY levels seen at the early stages of estrogen deficiency. In addition, neuronal deletion of RANK has been shown to be involved in thermoregulation in female but not male mice [1], but it is unknown exactly which neurons are involved in this process. Therefore, we also used our female $NPY^{cre/+};RANK^{lox/lox}$ mice to investigate whether NPY neurons contribute to the control of thermoregulation by RANK.

Materials and methods

Mice

All animal experiments were approved by the Garvan Institute / St Vincent's Hospital Animal Experimentation Ethics Committee and conducted in accordance with relevant guidelines and regulations. Mice were housed under conditions of controlled temperature (22ºC) with a 12h light, 12h dark cycle (lights on at 0700 h). Mice had *ad libitum* access to either a standard chow diet or a high fat diet (43% calories from fat, 17% calories from protein and 20 MJ/kg) from 7 weeks of age. Body weight was monitored weekly. All data presented are from female mice.

$RANK^{lox/lox}$ mice were crossed with an $NPY^{cre/+}$ transgenic line resulting in germline $NPY^{cre/+};RANK^{lox/lox}$ mice as published previously [2]. To account for any potential influences caused by the presence of the Cre-recombinase transgene, $NPY^{cre/+};RANK^{lox/+}$ heterozygous littermates were used as controls. OVX and sham operations were performed on female germline $NPY^{cre/+};RANK^{lox/lox}$ and control mice at 8 weeks of age. Gonadal (uterus plus ovaries where present) weights were measured at the end of the study to confirm the success of OVX procedures. $NPY^{cre/+};RANK^{lox/lox}$ and control mice were culled at 18 weeks of age for tissue analysis.

The generation of germline $NPY^{-/-}$ mice has been described previously [15]. OVX and sham operations were performed on female $NPY^{-/-}$ mice at 8 weeks of age. $NPY^{-/-}$ mice were culled at 16 weeks of age.

Metabolic phenotyping of mice

Temperature of interscapular BAT and lumbar body temperature were measured and analysed in 14 week old mice by non-invasive high-sensitivity infrared camera (ThermoCAM T640, FLIR) as described previously [16].

Whole body lean mass, fat mass, bone mineral content (BMC) and bone mineral density (BMD) were measured in $NPY^{cre/+};RANK^{lox/lox}$ and control mice anesthetised

with isoflurane using a dual X-ray absorptiometry (DXA) (Lunar Piximus II, GE Medical Systems, Madison WI) 2 days prior to cull.

Tissue Collection

Mice were culled between 13.00 and 16.00 hours by cervical dislocation and decapitation for collection of trunk blood. Serum was separated, immediately frozen and stored at -20ºC. Brains were collected and frozen flat on dry ice, then stored at -80 °C. The interscapular brown adipose tissue (BAT) and white adipose tissue (WAT) from the inguinal, perigonadal, retroperitoneal and mesenteric deposits were excised and weighed. The organs, including gonads (ovaries plus uterus), spleen, pancreas, kidney, liver and heart, were also excised and weighed. Femurs, tibias and caudal vertebrae were excised, fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4ºC and then stored in 70% ethanol at 4ºC before undergoing processing.

In-situ hybridisation

Fresh frozen brains were sectioned at 25 μm thickness and thaw-mounted on Superfrost Plus® glass microscope slides (Lomb Scientific Pty Ltd., NSW 2229, Australia). *In situ* hybridisation was performed, as previously described in detail [17]. Briefly, matching hypothalamic sections of $NPY^{cre/+};RANK^{lox/lox}$ and control mice subjected to either sham or OVX were hybridised with DNA oligonucleotides complementary to mouse NPY mRNA (5'-GAGGGTCAGTCCACACAGCCCCATTCGCTTGTTACCTAGCAT-3'), which were labelled with [^{35}S] thio-dATP (Perkin Elmer, Waltham, MA, USA) using terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany). Hybridization signals on sections were visualized by exposure to BioMax MR film (Kodak, Rochester, NY, USA). Digitalized images from the scanned autoradiograms were acquired for quantitative analysis. Quantification was performed by measuring the relative optical densities (RODs) within the brain areas of interest outlined with consistent defined dimensions across corresponding sections on the photomicrographs using ImageJ software (US National Institutes of Health). Background labelling was

subtracted from the resultant signal density. Data are presented as percentage of ROD averaged from nine sections per animal, n = 5 animals per group.

Bone Micro-Computed Tomography (micro-CT) and Histomorphometry

Micro-CT analysis of femora was carried out as previously described [2]. Briefly, following fixation, left femora were cleaned of muscle and analyses of the bone were carried out by micro-CT with a Skyscan 1172 scanner and associated analysis software (Skyscan, Aartselaar, Belgium). Analyses of the cortical bone were carried out in 229 slices (1.0 mm) selected 572 slices (2.5 mm) proximally from the distal growth plate resulting in calculations of the following parameters: total tissue area, bone area, marrow area, periosteal perimeter, cortical thickness, and polar moment of inertia (an index of strength). Analyses of the trabecular bone were carried out in 229 slices (1.0 mm) selected 114 slices (0.5 mm) proximally from the distal growth plate resulting in calculations of the following parameters: total tissue volume, bone volume, trabecular number, trabecular thickness and trabecular separation.

The 3rd lumbar vertebrae were also scanned with a Skyscan 1172 scanner and analysed with the associated Skyscan software. An elliptical region of interest was fitted through the trabecular bone offset from the growth plates at each end by 10 slices (0.044 mm). Analyses within the region of interest resulted in calculations of the following parameters: total tissue volume, bone volume, trabecular number, trabecular thickness and trabecular separation.

Statistical analyses

All data are expressed as means ± SEM. Differences between groups were assessed using Student's *t* test, ANOVA followed by Bonferroni's multiple comparison post hoc test or Tukey honestly significant difference test, or repeated measures ANOVA where appropriate. Statistical analyses were performed with Prism, version 7 (GraphPad Software Inc, La Jolla, CA, USA). $P<0.05$ was taken to be statistically significant.

Results

The generation of *NPY$^{cre/+}$;RANK$^{lox/lox}$* mice and confirmation of the successful deletion of RANK from NPY neurons has been reported previously. Whilst we have shown that germline deletion of RANK from NPY neurons had no overt effects on body weight or body composition in male mice [2], germline *NPY$^{cre/+}$;RANK$^{lox/lox}$* female mice had a significantly lower body weight than control mice at 6 weeks of age and this difference persisted as the mice aged (Figure 1A) although it was no longer statistically significant at the time of cull (Control: 22.3 ± 0.5, *NPY$^{cre/+}$;RANK$^{lox/lox}$* mice: 21.4 ± 0.4 g, $p = 0.12$). DXA analysis prior to cull at 16 weeks of age revealed that the lower body weight was due to a significant reduction in fat mass (Figure 1B) with no difference in lean mass, bone mineral content or bone mineral density compared to controls (Figure 1C-E). However, there were no statistically significant differences in the weights of individual white adipose tissue depots at cull (Supplementary Figure 1A). In addition, no differences were observed in the weights of internal organs (Supplementary Figure 1B).

OVX leads to increased body weight and lean mass in the absence of RANK in NPY neurons

In order to investigate whether central RANKL signalling through RANK receptors on NPY neurons plays a role in the response to estrogen deficiency, we measured body weight and body composition in *NPY$^{cre/+}$;RANK$^{lox/lox}$* and control mice that had been subjected to either OVX or a sham operation at 8 weeks of age. Interestingly, a significant increase in body weight over time was observed in *NPY$^{cre/+}$;RANK$^{lox/lox}$* mice subjected to OVX compared to sham which was not apparent in control mice (Figure 1F-G). This increase in body weight was associated with a significant increase in lean mass as measured by DXA at 18 weeks of age (Figure 1H). As expected given the known obesogenic effect of ovariectomy in rodents, OVX led to a significant increase in fat mass in both *NPY$^{cre/+}$;RANK$^{lox/lox}$* and control mice, however there was no difference between genotypes (Figure 1I). As expected, OVX led to a significant decrease in both bone mineral content and bone mineral density in control mice (Figure 1J-K). Interestingly however, RANK deletion in NPY neurons markedly protected against the OVX-induced reduction in bone mineral content (Figure 1J) and

NPY$^{cre/+}$*;RANK*$^{lox/lox}$ mice subjected to OVX also displayed a less pronounced drop in bone mineral density compared to control mice (Figure 1K). Spleen weight was significantly increased by OVX in *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ female mice compared to sham mice (OVX: 0.082 ± 0.003 g; Sham: 0.062 ± 0.002 g; p=0.014), a difference not evident in control mice (OVX: 0.078 ± 0.005 g; Sham: 0.073 ± 0.005 g; p=0.82). No other differences were observed between genotypes in the weights of internal organs (Supplementary Figure 1C).

RANK deletion in NPY neurons led to a significant reduction in NPY mRNA expression levels in the arcuate nucleus (Arc) of OVX mice, a change that was not evident in control mice (Figure 1L). Reduced NPY levels have been shown to result in increased brown adipose tissue thermogenesis and, consistent with this, brown adipose tissue mass was significantly increased in ovariectomised *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ female mice compared to sham controls (Figure 1M).

NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ *mice are protected again OVX-induced bone loss

Detailed μCT analysis was used to further investigate the effect of OVX on the bone phenotype of female *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice. Similar to data shown previously on male mice, female germline *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice under normal control conditions had no alterations in either femoral cancellous or cortical bone parameters compared to control mice (Figure 2A-H). However, consistent with their increased body weight and lean mass, ovariectomised *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice had a significant increase in femur length compared to sham *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice (Figure 3A). Furthermore, *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice were partially protected from the reduction in cortical bone parameters induced by OVX and evident in control mice (Figure 3B-C). Although OVX led to a reduction in cortical thickness in both control and *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice (Figure 3C), cortical bone volume and polar moment of inertia, an index of bone strength, were significantly reduced in control but not *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice (Figure 3B, D). Interestingly, whilst OVX induced a similar reduction in trabecular bone volume, trabecular thickness and trabecular number in the femora of both control and *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice (Figure 3E-H), RANK deletion in NPY neurons conferred protection against trabecular bone loss in the vertebral compartment. In the vertebrae, OVX led to a significant reduction in

trabecular bone volume and trabecular number as well as an increase in trabecular separation in control mice whilst none of these parameters were significantly altered by OVX in *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice (Figure 3I-L).

As hypothalamic NPY signalling has been shown to be anti-osteogenic, the reduction in hypothalamic NPY expression levels seen in ovariectomised *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice is consistent with the attenuated bone loss observed in these mice. However, although it has been shown that NPY ablation did not prevent the development of obesity in ovariectomised mice, the effect of NPY signalling on bone loss due to estrogen deficiency has not been investigated. Thus, we used μCT to determine the impact of NPY ablation on OVX induced bone loss using germline NPY deficient (*NPY*$^{-/-}$) mice. As shown in Figure 4A-C, OVX tended to reduce trabecular bone volume and trabecular number in the femora of *NPY*$^{-/-}$ mice. However, both femoral cortical bone parameters (Figure 4D-F) and vertebral trabecular bone parameters (Figure 4G-J) were unaltered by OVX in *NPY*$^{-/-}$ mice. Together these data show that NPY deficiency partially protects against bone loss caused by estrogen loss in female mice and thus the reduction in hypothalamic NPY expression seen in *NPY*$^{cre/+}$*;RANK*$^{lox/lox}$ mice is the likely cause of their reduced bone loss.

RANK signalling in NPY neurons is not involved in female thermoregulation

One of the main functions discovered for central RANK signalling is its influence on body temperature in female mice [1]. Since NPY is also critically involved in the control of energy expenditure and thermogenesis, we investigated whether RANKL signalling in NPY neurons is involved in the control of thermoregulation in female mice. For this we employed high sensitive infra-red thermal imaging in freely moving mice focusing on both brown adipose tissue temperature and lumbar temperature as a representative measure of body temperature. Interestingly however, both brown adipose tissue and lumbar temperature were unaltered by deletion of RANK in NPY neurons (Table 1) suggesting that direct signalling of RANK on NPY neurons is not responsible for the previously shown effect of neuronal RANK signalling on body temperature and thermogenesis, however, some indirect activation of NPY neuronal pathways through RANK that control thermogenesis can not be excluded.

Discussion

Estrogen deficiency results in elevated circulating RANKL levels associated with bone loss. Here we show that by blocking RANKL signalling through RANK receptors on NPY neurons, this bone loss can be attenuated particularly in the vertebral compartment via the down-regulation of hypothalamic NPY signalling. Furthermore, germline deletion of NPY confers more pronounced protection from ovariectomy-induced bone loss. In fact, female NPY deficient mice subjected to OVX at 8 weeks of age showed similar femoral cortical bone parameters and vertebral trabecular bone parameters to sham operated mice 8 weeks following surgery. The reduction in femoral trabecular bone volume suggests that this protection is not complete but nevertheless bone loss is significantly delayed which could have major consequences for the treatment of bone loss following menopause in humans.

Similar to previous investigations using male mice [2], female $NPY^{cre/+};RANK^{lox/lox}$ mice did not have an overt phenotype under basal conditions. Despite a slight reduction in body weight and fat mass, they had no differences in lean mass or bone parameters compared to control mice suggesting that central RANK signalling does not play a major role in the regulation of body composition under standard conditions. In addition, although previous work has shown that RANK deletion in NPY neurons led to a trend towards reduced arcuate NPY mRNA levels in male mice, no significant difference in arcuate NPY mRNA levels was observed in intact, sham-operated female $NPY^{cre/+};RANK^{lox/lox}$ mice compared to controls. However, previous work in male mice has shown that it was only under high fat diet conditions that the systemic effects of RANK/NPY signalling were manifest, with increased energy expenditure and markedly greater bone mass, data consistent with reduced NPY levels [2]. Again here, when mice were subjected to OVX and the associated effects of estrogen deficiency, a significant reduction in arcuate NPY expression was observed which was associated with an attenuation of bone loss and increased brown adipose tissue mass. This suggests that in the basal state, estrogen signalling through NPY neurons may compensate for the lack of RANK signalling, maintaining NPY expression levels. Hypothalamic NPY expression has been shown to be increased in post-menopausal women [10] and transiently increased by OVX in rodents [11-13]. Consistent with this transient increase, NPY ablation has been shown to prevent the early changes in

energy expenditure observed with OVX [14]. In addition, $NPY^{-/-}$ mice subjected to OVX exhibited lower body weight and fat mass relative to OVX controls 2 weeks following OVX [14]. However, in the long-term, NPY deficiency did not prevent OVX-induced weight and fat gain [14].

Previous studies have identified the NPY receptors responsible for transmission of the NPY-ergic signals to bone. In the hypothalamus, Y2 receptors signal to bone [18, 19], while in the periphery [20] and directly in bone cells [21, 22] Y1 receptors control bone mass, both prominently through the control of osteoblast activity. Y1 receptor antagonism achieved through the oral administration of the selective Y1 receptor antagonist BIBO3304 has been shown to increase bone mass in mice and was associated with a significant increase in the anabolic activity of osteoblasts [23]. Dual deletion of Y1 and Y2 receptors does not confer additive effects on bone, suggesting they may act in parallel [24]. The effect of these receptors on metabolic and skeletal responses to gonadectomy has also been examined and has shed some light on the mechanism whereby RANK signalling may modulate these processes. Fat accrual post ovariectomy was markedly increased in Y1 receptor null mice [25], a response not evident in Y2 receptor null mice [26], nor in $NPY^{cre/+};RANK^{lox/lox}$ mice, suggesting that this axis may involve central NPY-ergic neurons. The skeletal response to ovariectomy was attenuated in both Y1 receptor null [25] and Y2 receptor null mice [26], through increased bone anabolism, with no effect upon bone resorption. This suggests that both central and peripheral NPY circuits may contribute to the suppression of estrogen deficient bone loss. However in male mice, this attenuated bone loss in response to orchiectomy, was not evident in Y1 receptor null mice, suggesting that $NPY^{cre/+};RANK^{lox/lox}$ signalling may involve Y2 receptor circuits, as evident for metabolic effects. Further reinforcing the role of central Y2 receptors in post-ovariectomy signalling through RANK positive NPY-ergic cells, conditional deletion of Y2 receptors from hypothalamic neurons was sufficient to recapitulate the germline Y2 receptor null effects of attenuated post gonadectomy changes in body composition and bone mass. Thus the RANK mediated modulation of NPY signalling post-ovariectomy is most likely occurring in hypothalamic neurons, consistent with the altered NPY expression evident under this condition, however we can not exclude the possible additional involvement of altered peripheral and/or central NPY circuits.

Interestingly, RANK deletion specifically in NPY neurons was not able to replicate the alterations in body temperature previously shown in mice with neuronal-wide deletion of RANK suggesting that direct signalling of RANK on NPY neurons does not fulfil this function. However, considering the critical role of hypothalamic NPY neurons in the control of thermogenesis it cannot be ruled out that RANK targets these NPY neurons through an indirect pathway which is not affected by this selective deletion of RANK in NPY neurons.

In summary, this study is the first to show that NPY deficiency attenuates bone loss associated with estrogen deficiency up to 8 weeks following OVX. Identifying the mechanisms contributing to menopause-induced bone loss could lead to improved means of attenuating menopause-associated osteoporosis.

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Figure Legends

Figure 1. (A) Body weight over time of $NPY^{cre/+}RANK^{lox/lox}$ (KO) and heterozygous $NPY^{cre/+}RANK^{lox/+}$ (control) mice. (B) Fat mass, (C) lean mass, (D) bone mineral content (BMC), and (E) bone mineral density (BMD) as measured by DXA at 15 weeks of age. Body weight (F) over time and (G) at cull of both $NPY^{cre/+}RANK^{lox/lox}$ (KO) and control mice subjected to either a sham operation or ovariectomy (OVX) at 8 weeks of age. (H) Lean mass, (I) fat mass, (J) bone mineral content (BMC), and (K) bone mineral density (BMD) as measured by DXA at 19 weeks of age. (L) Quantification of NPY mRNA expression in the Arcuate as determined by in-situ hybridisation. (M) Weight of isolated brown adipose tissue (BAT) at cull in $NPY^{cre/+}RANK^{lox/lox}$ (KO) compared to control mice subjected to either sham or OVX. Data are means ± SEM of at least 5 per group. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ as indicated.

Figure 2. (A) Femur length of $NPY^{cre/+}RANK^{lox/lox}$ (KO) and heterozygous $NPY^{cre/+}RANK^{lox/+}$ (control) mice. µCT analysis showing (B) cancellous bone volume (BV/TV), (C) trabecular thickness, (D) trabecular number, (E) cortical bone volume, (F) cortical thickness, (G) periosteal perimeter, and (H) mean polar moment of inertia (MMI) in $NPY^{cre/+}RANK^{lox/lox}$ (KO) and control mice. (I) Representative sagittal images from femora of $NPY^{cre/+}RANK^{lox/lox}$ (KO) and control mice. Data are means ± SEM of at least 8 per group.

Figure 3. (A) Femur length of $NPY^{cre/+}RANK^{lox/lox}$ (KO) and heterozygous $NPY^{cre/+}RANK^{lox/+}$ (control) mice subjected to either sham or OVX. µCT analysis showing (B) cortical bone volume, (C) cortical thickness, (D) mean polar moment of inertia (MMI), (E) cancellous bone volume (BV/TV), (F) trabecular thickness, and (G) trabecular number in the femora of $NPY^{cre/+}RANK^{lox/lox}$ (KO) and control mice subjected to either sham or OVX. (H) Representative sagittal images. µCT analysis showing (I) cancellous bone volume (BV/TV), (J) trabecular thickness, (K) trabecular number, and (L) trabecular separation in the third lumbar vertebrae of $NPY^{cre/+}RANK^{lox/lox}$ (KO) and control mice subjected to either sham or OVX. Data are means ± SEM of at least 5 per group. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ as indicated.

Figure 4. μCT analysis showing (A) cancellous bone volume (BV/TV), (B) trabecular thickness, (C) trabecular number, (D) cortical bone volume, (E) cortical thickness, and (F) mean polar moment of inertia (MMI) in the femora of germline *NPY*$^{-/-}$ mice subjected to either sham or OVX. μCT analysis showing (G) cancellous bone volume (BV/TV), (H) trabecular thickness, (I) trabecular number, and (J) trabecular separation in the third lumbar vertebrae of germline *NPY*$^{-/-}$ mice subjected to either sham or OVX. Data are means ± SEM of at least 6 per group. *p* as indicated.

Supplementary Figure 1. (A) Weight of isolated inguinal (WATi), perigonadal (WATp), mesenteric (WATm) and retroperitoneal (WATr) white adipose tissue depots and (B) internal organs at cull in NPY$^{cre/+}$RANK$^{lox/lox}$ compared to control mice. (C) Weight of internal organs at cull in NPY$^{cre/+}$RANK$^{lox/lox}$ compared to control mice subjected to either sham or OVX. Data are means ± SEM of at least 5 per group. *=$p<0.05$, ***=$p<0.001$ as indicated.

Table 1: Brown adipose tissue (BAT) and lumbar temperature in $NPY^{cre/+};RANK^{lox/lox}$ and heterozygous $NPY^{cre/+};RANK^{lox/+}$ control mice.

	Control	RANK deletion
BAT	35.7 ºC ± 0.13	35.6 ºC ± 0.18
Lumbar	33.8 ºC ± 0.14	33.4 ºC ± 0.16