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Central control of bone remodeling by neuromedin U

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Bone remodeling, the function affected in osteoporosis, the most common of bone diseases, comprises two phases: bone formation by matrix-producing osteoblasts[1] and bone resorption by osteoclasts[2]. The demonstration that the anorexigenic hormone leptin[3–5] inhibits bone formation through a hypothalamic relay[6,7] suggests that other molecules that affect energy metabolism in the hypothalamus could also modulate bone mass. Neuromedin U (NMU) is an anorexigenic neuropeptide that acts independently of leptin through poorly defined mechanisms[8,9]. Here we show that *Nmu*-deficient (*Nmu*$^{-/-}$) mice have high bone mass owing to an increase in bone formation; this is more prominent in male mice than female mice. Physiological and cell-based assays indicate that NMU acts in the central nervous system, rather than directly on bone cells, to regulate bone remodeling. Notably, leptin- or sympathetic nervous system–mediated inhibition of bone formation[6,7] was abolished in *Nmu*$^{-/-}$ mice, which show an altered bone expression of molecular clock genes (mediators of the inhibition of bone formation by leptin). Moreover, treatment of wild-type mice with a natural agonist for the NMU receptor decreased bone mass. Collectively, these results suggest that NMU may be the first central mediator of leptin-dependent regulation of bone mass identified to date. Given the existence of inhibitors and activators of NMU action[10], our results may influence the treatment of diseases involving low bone mass, such as osteoporosis.

Bone mass is maintained at a constant level between puberty and menopause by a succession of bone-resorption and bone-formation phases[11,12]. The discovery that neuronal control of bone remodeling is mediated by leptin[6] shed light on a new regulatory mechanism of bone remodeling and also suggested that bone mass may be regulated by a variety of neuropeptides[13]. In line with this observation, cannabinoids and pituitary hormones have been shown to be intimately involved in bone remodeling[14,15]. Leptin inhibits bone formation by binding to its receptors located in hypothalamus and thereby activating the sympathetic nervous system (SNS), which requires the adrenergic β2 receptors (Adrb2) expressed in osteoblasts[7,16]. Downstream of Adrb2, leptin signaling activates molecular clock genes that regulate osteoblast proliferation and hence bone formation[17]. In addition, leptin regulates bone resorption through two distinct pathways[16].

NMU is a small peptide produced by nerve cells in the submucosal and myenteric plexuses in the small intestine, and also by structures in the brain, including the dorsomedial nucleus of the hypothalamus[9]. It is generally assumed that NMU acts as a neuropeptide to regulate various aspects of physiology, including appetite, stress response and SNS activation[9]. Indeed, NMU-deficient (*Nmu*$^{-/-}$) mice develop obesity due to increased food intake and reduced locomotor activity that is believed, at least in part, to be leptin independent[8]. In addition, expression of NMU is diminished in leptin-deficient (*Lep*^{ob}) mice[18], but can be induced in these mice by leptin treatment[19]. In search of additional neuropeptides that regulate bone remodeling, we analyzed *Nmu*$^{-/-}$ mice.

When assessed at 3 and 6 months of age, both male and female *Nmu*$^{-/-}$ mice showed a high bone mass phenotype as compared to the wild type (WT), with male mice more severely affected than female mice (**Fig. 1a** and data not shown). The presence of a uniform increase in bone mineral density (BMD) along the femurs of *Nmu*$^{-/-}$ mice suggested that both trabecular and cortical bone were equally affected (**Supplementary Fig. 1** online). Microcomputed tomography analysis confirmed this observation (**Fig. 1b**,**c**). To determine whether this phenotype was secondary to the obesity of the *Nmu*$^{-/-}$ mice, we restricted their food intake for 1 month starting at 2 months of age. This manipulation normalized the body weight and serum insulin level of the *Nmu*$^{-/-}$ mice but did not affect their high bone mass phenotype (**Fig. 1d** and data not shown). Of note, when *Nmu*$^{-/-}$ mice were backcrossed to the C57BL/6J genetic background, their body weight became similar to that of their WT littermates; however, their BMD remained high (data not shown). These results suggest that NMU regulates bone mass independently of its regulation of energy metabolism, just as leptin does[7]. To better characterize the cellular nature of the bone phenotype in the *Nmu*$^{-/-}$ mice, we

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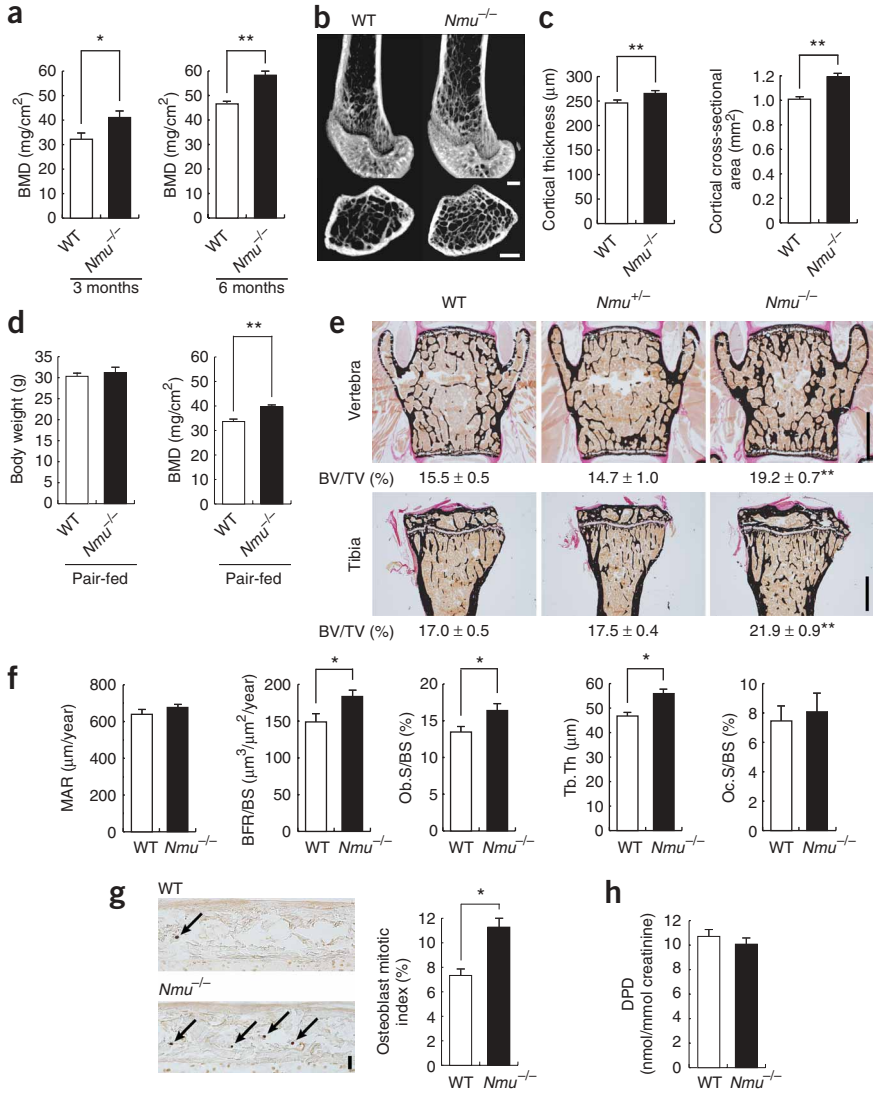

Figure 1 High bone mass in *Nmu*$^{-/-}$ mice due to increased bone formation. (**a**) Bone mineral density (BMD) of the femurs of 3 (left)- and 6 (right)-month-old male wild-type (WT) and *Nmu*$^{-/-}$ mice. (**b**) Micro-computed tomography (μCT) analysis of the distal femurs of male mice at 3 months. Scale bars, 500 μm. (**c**) Cortical thickness and cross-sectional area of the femurs of 3-month-old male mice. (**d**) Body weight and BMD of 3-month-old male mice with restricted food intake. (**e**) Histological analysis of the vertebrae and tibiae of 3-month-old male WT, *Nmu*$^{+/-}$ and *Nmu*$^{-/-}$ mice. Bone volume per tissue volume (BV/TV). Scale bars, 1 mm. (**f**) Histomorphometric analysis of the vertebrae of 3-month-old male mice. Mineral apposition rate (MAR), bone formation rate over bone surface area (BFR/BS), osteoblast surface area over bone surface area (Ob.S/BS), trabecular thickness (Tb. Th) and osteoclast surface area over bone surface area (Oc.S/BS). (**g**) Increased osteoblast proliferation in newborn *Nmu*$^{-/-}$ mice. Immunolocalization of BrdU incorporation (arrows) in the calvariae of WT and *Nmu*$^{-/-}$ mice (left). Osteoblast mitotic index (right). Scale bar, 20 μm. (**h**) Urinary elimination of deoxypyridinoline (DPD) in WT and *Nmu*$^{-/-}$ mice. **, $P < 0.01$; *, $P < 0.05$.

performed histological and histomorphometric analyses of vertebrae and tibiae in both male and female animals (**Fig. 1e** and **Supplementary Fig. 1**). At 3 and 6 months of age, *Nmu*$^{-/-}$ mice showed greater bone volume in both vertebrae and tibiae than did WT littermates, with male mice having a more pronounced phenotype (**Fig. 1e** and **Supplementary Fig. 1**). At the present time we do not have a clear explanation of the difference in phenotype severity between male and female mice. Bone formation rates (WT mice, 146.9 ± 12.3, *Nmu*$^{-/-}$ mice, 183.7 ± 10.3, $P < 0.05$) and osteoblast numbers were both significantly greater in the vertebrae and tibiae of *Nmu*$^{-/-}$ mice (**Fig. 1f** and **Supplementary Fig. 1**). The higher osteoblast numbers in the presence of a normal mineral apposition rate (**Fig. 1f** and **Supplementary Fig. 1**), which reflects the function of individual osteoblasts[20], suggested that osteoblast proliferation may be increased in *Nmu*$^{-/-}$ mice. Indeed, 5-bromo-2-deoxyuridine (BrdU)-positive proliferative osteoblast counts were significantly increased in *Nmu*$^{-/-}$ mice *in vivo* (**Fig. 1g**), demonstrating that NMU affects osteoblast proliferation. In contrast, *Nmu*$^{-/-}$ and WT mice showed comparable osteoclast numbers and osteoclast surface areas (**Fig. 1f** and **Supplementary Fig. 1**), suggesting that NMU does not affect bone resorption. This observation was further supported by the normal level of urinary elimination of deoxypyridinoline in *Nmu*$^{-/-}$ mice (**Fig. 1h**).

Taken together, these results demonstrate that NMU deficiency results in an isolated increase in bone formation leading to high bone mass. *Nmu*-heterozygote mice did not have an overt bone abnormality at any age analyzed (**Fig. 1e**).

Two cognate G protein–coupled receptors have been reported to be NMU receptors: NMUR1, which is expressed in various tissues, including the small intestine and lung (data not shown), and NMUR2, which is predominantly expressed in the hypothalamus and the small intestine (**Fig. 2a**)[18]. Both receptors and NMU itself were barely detectable in bone (**Fig. 2a**). To further exclude the possibility of a direct action of NMU on osteoblasts, we treated mouse primary osteoblasts with varying concentrations of NMU. Alkaline phosphatase activity, mineralization and expression of osteoblastic genes were all unaffected by this treatment (**Fig. 2b,c**). In addition, there were no differences between WT mice and *Nmu*$^{-/-}$ mice in the expression of osteoblastic genes *in vivo* (**Fig. 2d**). Moreover, both WT and *Nmu*$^{-/-}$ osteoblasts proliferated normally *in vitro* in response to NMU treatment (**Fig. 2e**), though *Nmu*$^{-/-}$ osteoblasts proliferated more than WT osteoblasts *in vivo* (**Fig. 1g**). Osteoclastic differentiation from bone marrow macrophages was unchanged by NMU treatment (**Fig. 2f**), as expected from the absence of a bone resorption defect *in vivo* (**Fig. 1f,h**). Taken together, these results strongly suggest that NMU's effect on bone may not come from its direct action on osteoblasts, but rather through another relay.

Because the anorexigenic effect of NMU requires a hypothalamic relay[8,19] and because hypothalamic neurons have been shown to regulate bone mass, we tested whether NMU's regulation of bone formation could involve a central relay. Continuous intracerebroventricular (i.c.v.) infusion of NMU into *Nmu*$^{-/-}$ mice decreased their fat mass and fat pad weight significantly, although body weight was not

affected (**Fig. 2g** and **Supplementary Fig. 2** online). In addition, NMU i.c.v. infusion eliminated the high bone mass phenotype in *Nmu*$^{-/-}$ mice (**Fig. 2g** and **Supplementary Fig. 2**), suggesting that NMU inhibits bone formation through the central nervous system.

The central nature of bone remodeling regulation by NMU, along with the notion that the anorexigenic effect of NMU may be independent of leptin[8], prompted us to examine whether leptin could be involved in the regulation of bone formation by NMU. To address this question, we performed i.c.v. infusion of NMU or leptin in *Lep*^{ob} mice. NMU decreased fat pad weight significantly, albeit to a milder extent than that achieved by leptin (**Fig. 3a** and **Supplementary Fig. 3** online). Body weight was not significantly changed by the NMU infusion, indicating that this treatment had only a mild effect on energy metabolism (data not shown). In contrast, NMU decreased bone mass in *Lep*^{ob} mice as efficiently as leptin did (**Fig. 3a**). These results indicate that NMU inhibits bone formation in a leptin-independent manner. Next, we asked whether leptin could correct the high bone mass phenotype of *Nmu*$^{-/-}$ mice. Leptin i.c.v. infusion decreased bone volume and bone formation in WT mice, as previously reported (**Fig. 3b** and **Supplementary Fig. 3**)[6]. However, the leptin paradoxically increased bone volume and osteoblast number in *Nmu*$^{-/-}$ mice (**Fig. 3b,c** and **Supplementary Fig. 3**). The fact that leptin decreased fat mass and fat pad weight in *Nmu*$^{-/-}$ mice and increased urinary elimination of normetanephrine, a metabolite of noradrenaline[17], verified that the administration of leptin was properly performed (**Fig. 3b,d** and **Supplementary Fig. 3**). Therefore, taken together, these results suggest that NMU acts downstream of leptin to regulate bone formation.

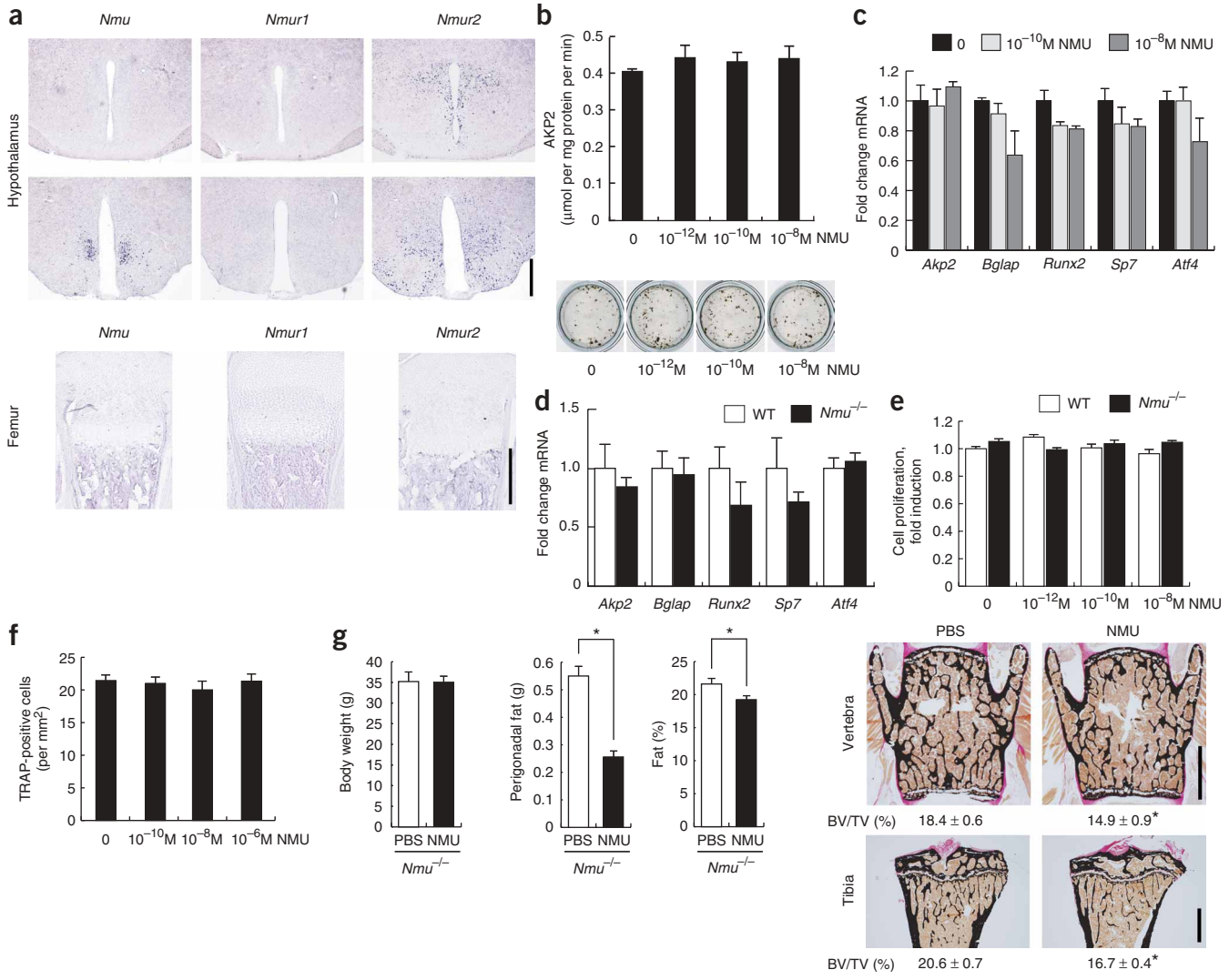

Figure 2 Absence of NMU's direct effect on osteoblasts; decrease in bone mass by NMU i.c.v. infusion. (**a**) Expression of *Nmu*, *Nmur1* and *Nmur2* in the hypothalamus at the atlas-levels of 38 (top) and 43 (bottom) and in the femur as shown by *in situ* hybridization. Note the expression of *Nmu* in the dorsomedial nucleus of the hypothalamus (DMH) (bottom) and *Nmur2* in paraventricular nucleus (top), arcuate nucleus and DMH (bottom). Scale bars, 500 μm. (**b**–**d**) Effect of NMU on osteoblast differentiation. (**b**,**c**) WT osteoblasts treated with NMU. (**b**) Alkaline phosphatase (AKP2) activity (top), mineralized nodule formation (bottom). (**c**) Expression of osteoblastic genes (*Akp2*, *Bglap*, *Runx2*, *Sp7* and *Atf4*), depicted as fold change over WT expression. (**d**) Expression of osteoblastic genes in WT and *Nmu*$^{-/-}$ femurs. (**e**) Effect of NMU on osteoblast proliferation. WT or *Nmu*$^{-/-}$ osteoblasts treated with NMU. (**f**) Effect of NMU on osteoclast differentiation. Bone marrow–derived osteoclasts treated with NMU. (**g**) Effect of NMU i.c.v. infusion on body weight, fat pad weight (perigonadal fat) and fat mass (left). Histological analysis of the vertebrae (top right) and tibiae (bottom right). Male mice at 3 months of age were used. Scale bars, 1 mm. *, $P < 0.05$.

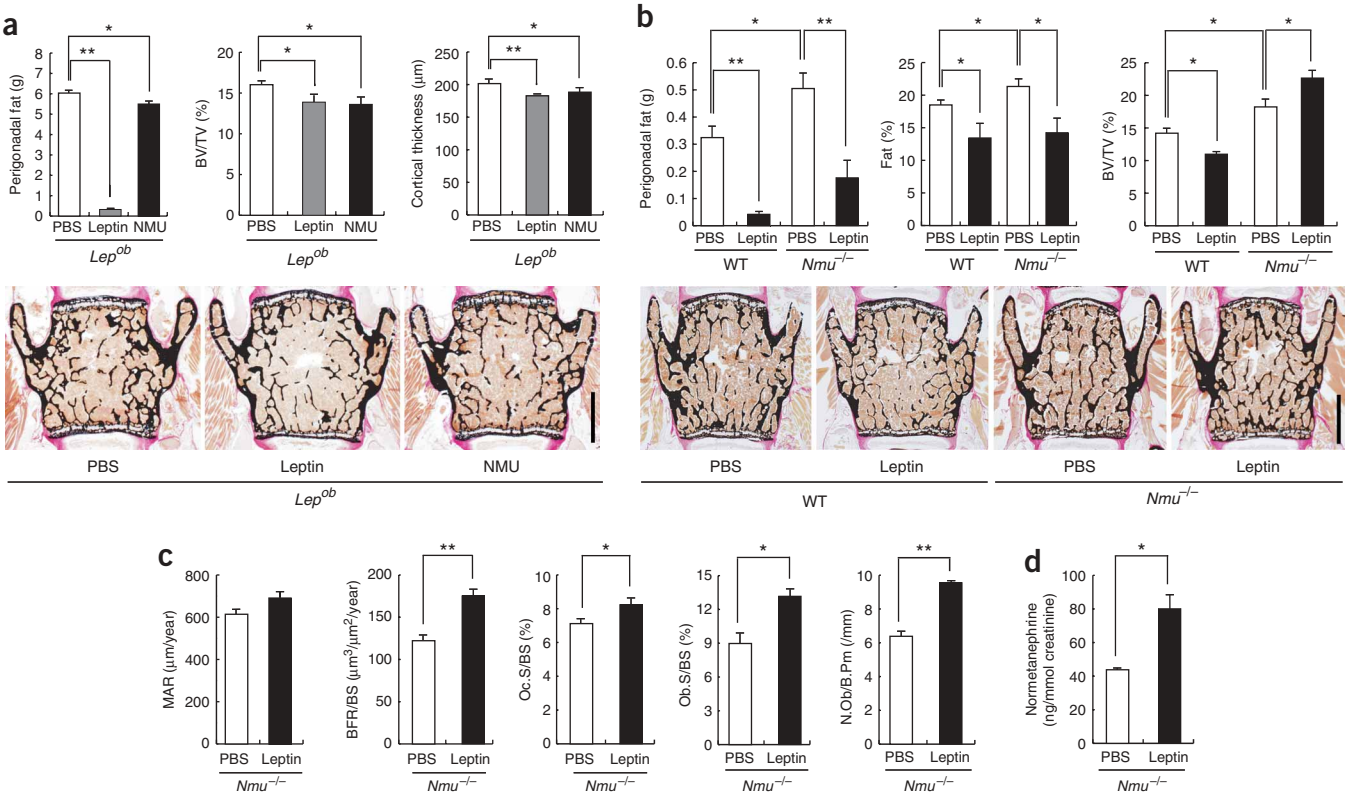

Figure 3 Leptin does not eliminate high bone mass in *Nmu*$^{-/-}$ mice. (**a**) Effect of NMU or leptin i.c.v. infusion in *Lep*^{ob} mice (3-month-old males). Fat pad weight and bone mass were determined by histology and cortical thickness by μCT analysis. (**b**–**d**) Effect of leptin i.c.v. infusion on *Nmu*$^{-/-}$ mice (3-month-old males). (**b**) Fat pad weight, fat mass and bone mass shown by histology. (**c**) Histomorphometric analysis. N. Ob/B.Pm indicates the number of osteoblasts per bone perimeter. (**d**) Urinary elimination of normetanephrine. Scale bars, 1 mm. **, $P < 0.01$; *, $P < 0.05$.

The SNS is a major mediator of leptin's antiosteogenic action[7]. NMUR2 is expressed in paraventricular nuclei, whose neurons directly project to the sympathetic preganglionic neurons, and NMU stimulates sympathetic outflow[9,21]. These observations, along with the fact that *Nmu*$^{-/-}$ mice have osteoblastic defects similar to the one observed in *Adrb2*-deficient mice[16], prompted us to explore whether NMU and sympathetic tone are in the same pathway regulating bone formation. Indeed, *Nmu/Adrb2* double heterozygote mice had higher bone mass than *Adrb2* single heterozygote mice (**Fig. 4a**), although *Nmu* single heterozygote mice had normal bone mass (**Fig. 1e** and **Supplementary Fig. 1**). Given that *Nmu* expression in the hypothalamus was reduced in *Nmu* single heterozygote mice (data not shown), compound heterozygosity of *Nmu* and *Adrb2* may have resulted in higher bone mass. Furthermore, this result suggests that these two pathways share a common molecule. Of note, *Nmu*$^{-/-}$ mice had a higher degree of urinary elimination of normetanephrine than WT littermates (**Fig. 4b**), which would decrease bone mass, yet they had high bone mass. This suggests that their high bone mass phenotype is not caused by decreased SNS activity, but is instead the result of resistance to the antiosteogenic activity of the SNS. This is in agreement with the observation that i.c.v. infusion of leptin, a potent stimulator of SNS activity, did not decrease bone mass in *Nmu*$^{-/-}$ mice (**Fig. 3b** and **Supplementary Fig. 3**). Furthermore, injection of isoproterenol, a sympathomimetic, reduced bone mass in WT mice[7] but not in *Nmu*$^{-/-}$ mice (**Fig. 4c** and **Supplementary Fig. 4** online). Thus, *Nmu*$^{-/-}$ mice are resistant to the antiosteogenic effects of both leptin and the SNS.

We present six experimental arguments to strongly suggest that the failure of leptin or isoproterenol to decrease bone mass in *Nmu*$^{-/-}$ mice is not due to leptin-SNS signaling defects. First, leptin infusion decreased fat pad weight equally well in WT and in *Nmu*$^{-/-}$ mice and could increase normetanephrine abundance in *Nmu*$^{-/-}$ mice (**Fig. 3b**,**d** and **Supplementary Fig. 3**). Second, the expression of *Adrb2* was not different in WT and *Nmu*$^{-/-}$ bones (**Fig. 4d**). Third, treatment with NMU did not affect *Adrb2* expression in osteoblasts (**Supplementary Fig. 5** online). Fourth, isoproterenol induced expression of *Tnfsf11* (encoding tumor necrosis factor superfamily, member 11) and decreased expression of *Tnfrsf11b* (encoding tumor necrosis factor superfamily, member 11b, also known as osteoprotegerin), *Runx2* (encoding runt-related transcription factor-2) and *Col1a1* (encoding collagen type I), molecular markers for the effect of SNS activation on osteoblasts, in both WT and *Nmu*$^{-/-}$ osteoblasts (**Fig. 4d**). Fifth, isoproterenol induced cAMP production equally well in WT and *Nmu*$^{-/-}$ osteoblasts (**Fig. 4e**). Sixth, and most notably, leptin increased bone resorption to a similar extent in WT and *Nmu*$^{-/-}$ mice (**Fig. 3c** and **Supplementary Fig. 3**).

The fact that the leptin-SNS pathway is intact in *Nmu*$^{-/-}$ mice, together with the paradoxical increase in osteoblast number induced by leptin i.c.v. infusion in *Nmu*$^{-/-}$ mice (**Fig. 3c**), suggests that NMU affects only the negative regulator of bone remodeling by leptin, that is, the molecular clock. Indeed, the expression of *Per1* and *Per2* (encoding period homolog-1 and -2, respectively) was downregulated in *Nmu*$^{-/-}$ bones as compared to WT bones (**Fig. 4f** and **Supplementary Fig. 6** online). Thus, NMU, acting through the central nervous system, affects the molecular clock in bone.

Because bone resorption in *Nmu*$^{-/-}$ mice was comparable to that in the wild type, despite the high SNS activity in these mice, we also

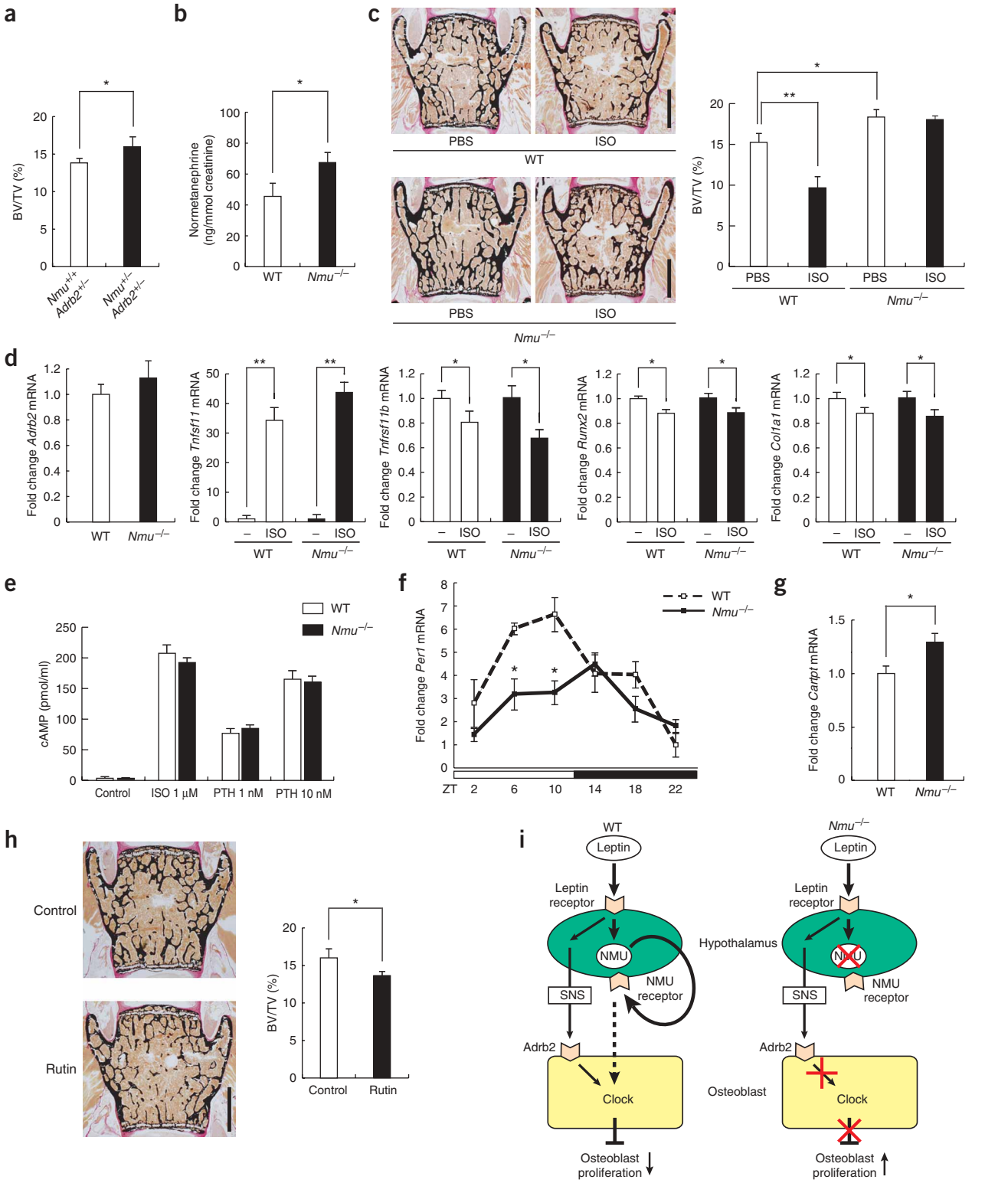

Figure 4 Sympathetic activation does not rescue high bone mass in *Nmu*$^{-/-}$ mice. (**a**) Bone mass in *Adrb2*$^{+/-}$/*Nmu*$^{+/-}$and *Adrb2*$^{+/-}$/*Nmu*$^{+/+}$ mice as determined by histology (3-month-old males). (**b**) Increased urinary elimination of normetanephrine in *Nmu*$^{-/-}$ mice. (**c**) Effect of sympathetic activation by isoproterenol (ISO) injection in *Nmu*$^{-/-}$ mice (3-month-old males). Shown is the bone mass of vertebrae as determined by histology. (**d**) Expression of *Adrb2* in the femurs of WT and *Nmu*$^{-/-}$ mice (left). Gene expression changes induced by isoproterenol (ISO) treatment of WT and Nmu$^{-/-}$ osteoblasts (four rightmost graphs). (**e**) cAMP concentration in the culture medium of WT and *Nmu*$^{-/-}$ osteoblasts after ISO treatment. Parathyroid hormone (PTH) was used as a control. (**f**) Expression of *Per1* in the femurs of WT and *Nmu*$^{-/-}$ mice. Zeitberger time (ZT) is indicated on the *x*-axis. (**g**) Expression of *Cartpt* in the hypothalamus of WT and *Nmu*$^{-/-}$ mice. (**h**) Rutin decreases bone mass in WT mice as determined by histological analysis of vertebrae (left) and quantitative histomorphometric analysis (right) (3-month-old males). Scale bar, 1 mm. **, $P < 0.01$; *, $P < 0.05$. (**i**) Model of leptin, sympathetic nervous system (SNS) and NMU signaling for the regulation of bone formation in WT mice (left) and *Nmu*$^{-/-}$ mice (right).

tested whether the expression of *Cartpt* (encoding cocaine- and amphetamine-regulated transcript propeptide), a central mediator of leptin's action on bone resorption[16], was altered in these mice. Indeed, *Cartpt* expression was increased in *Nmu*$^{-/-}$ mice as compared to WT littermates (**Fig. 4g** and **Supplementary Fig. 7** online). These results suggest that the protective activity of Cart on bone resorption compensates for the bone-resorbing activity induced by the SNS in *Nmu*$^{-/-}$ mice. The effect of other leptin-regulated neuropeptides, such as NPY (neuropeptide Y), AgRP (agouti-related protein) and α-MSH (α-melanotropin), will be limited, because the expression of *Npy* and *Agrp* was unchanged in *Nmu*$^{-/-}$ mice[8] and melanocortin 4 receptor, a major receptor for α-MSH, has been shown to have little effect on bone remodeling by itself[22].

Lastly, we treated WT mice with rutin, a natural NMUR2 agonist found in daily foods such as buckwheat[23]. Consistent with the high bone mass phenotype of the *Nmu*$^{-/-}$ mice, rutin decreased bone mass significantly in WT mice (**Fig. 4h**). This result, together with the predominant expression of *Nmur2* in the hypothalamus (**Fig. 2a**), suggests that NMU regulates bone remodeling through NMUR2.

Collectively, these results suggest that NMU, through a central relay and via an unidentified pathway, acts as a modulator of leptin-SNS-Adrb2 regulation of bone formation (**Fig. 4i**). However, one concern still remains: because leptin affects several pathways originating in the hypothalamus and elsewhere in the brain, i.c.v. infusion of leptin may have resulted in an uncoordinated change in leptin-regulated bone remodeling that does not reflect a physiological role of leptin. To rigorously address that question, an analysis of a mouse model in which a specific nucleus of the hypothalamus is activated by leptin will be necessary. From a therapeutic point of view, given the lack of an obesity phenotype in *Nmur2*-deficient mice[24], an NMU antagonist may be a candidate for the treatment of bone-loss disorders without inducing unwanted body weight gain.

METHODS

Animals. *Nmu*$^{-/-}$ and *Adrb2*$^{-/-}$ mice were previously described[8,16]. We purchased C57BL/6J mice and C57BL/6J *Lep*^{ob} from the Jackson Laboratory. We maintained all of the mice under a 12 hr light-dark cycle with *ad libitum* access to regular food and water, unless specified. For pair-fed experiments, we caged *Nmu*$^{-/-}$and WT mice individually for 12 weeks as described[8]. In brief, *Nmu*$^{-/-}$ mice were given access to water *ad libitum* and fed the amount of chow eaten on the previous day by a WT littermate. We determined mouse genotypes by PCR as previously described[8,16]. We injected isoproterenol (10 mg/kg, Sigma) intraperitoneally (i.p.) once daily for 4 weeks. Rutin (Sigma) was administered orally 300 mg per kg body weight per day for 4 weeks. All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

Dual X-ray absorptiometry and microcomputed tomography analysis. We measured bone mineral density (BMD) of the femurs and fat pad composition by DCS–600 (Aloka). We obtained two-dimensional images of the distal femurs by microcomputed tomography (μCT, Comscan). We measured cortical thickness and cross-sectional area at the center of the femur. We examined at least eight mice for each group.

Histological and histomorphometric analysis. We injected calcein (25 mg/kg, Sigma) i.p. 5 and 2 d before sacrifice. We stained undecalcified sections of the third and fourth lumbar vertebrae and tibiae with von Kossa staining. We performed static and dynamic histomorphometric analyses using the Osteomeasure Analysis System (Osteometrics). We analyzed 8–10 mice for each group.

***In situ* hybridization analysis.** We performed *in situ* hybridization analysis according to the established protocol[25]. Antisense cRNA probe for *Cartpt* was previously described[26]. We used fragments of cDNA for *Nmu* (105 base pairs upstream to 647 base pairs downstream of the initiation codon), *Nmur1* (13–1242 base pairs downstream of the initiation codon) and *Nmur2* (16–1252 base pairs downstream of the initiation codon) to generate antisense probes. We stained sections hybridized with ^{35}S-labeled probes with Hoechst 33528 and quantitatively analyzed the expression of *Cartpt* with a phosphorimager (Bass–2500, Fuji). The atlas-level of designations corresponds to those described previously[27]. We analyzed six mice for each group.

Measurement of deoxypyridinoline cross-links and normetanephrine. We measured urinary deoxypyridinoline cross-links (DPD) and normetanephrine with the METRA DPD-EIA kit (Quidel) and the Normetanephrine-ELISA kit (ALPCO), respectively, according to the manufacturer's instructions. We used creatinine values to standardize between samples (Creatinine Assay Kit, Cayman). We examined eight samples for each group.

Cell culture. *In vitro* primary osteoblast cultures were established as previously described[6]. Briefly, we cultured primary osteoblasts from calvariae of 4-d-old mice in α-MEM (Sigma) containing ascorbic acid (0.1 mg/ml, Sigma). We added NMU to the medium twice daily. After 14 d, we measured alkaline phosphatase activity with the ALP kit (Wako). For the mineralization assay, we supplemented the medium with β-glycerophosphate (5 mM, Sigma). We assessed mineralized nodule formation by von Kossa staining. We performed the cell proliferation and cAMP assays with the Cell Proliferation Assay (Promega) and cAMP EIA kit (Cayman Chemical), respectively. *In vitro* osteoclast differentiation has been described previously[16]. Briefly, bone marrow cells of 2-month-old mice were cultured in the presence of human macrophage colony-stimulating factor (10 ng/ml, R&D Systems) for 2 d and then differentiated into osteoclasts with human RANKL (50 ng/ml, Peprotech) and human macrophage colony-stimulating factor (10 ng/ml) for 3 d. We counted tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (more than 3 nuclei). We performed all the cell cultures in triplicate or quadruplicate wells and repeated more than 3 times.

BrdU immunohistochemistry. For BrdU labeling, we injected 100 μg BrdU i.p. into 3-d-old mice 1 h before sacrifice. We embedded calvariae in paraffin and cut coronally. We detected BrdU-incorporated osteoblasts with the BrdU Immunohistochemistry Kit (Exalpha Biologicals). We calculated the number of BrdU-positive osteoblasts over the total number of osteoblasts (osteoblast mitotic index) at three different locations (+3.0, 3.5 and 4.0 AP (0 point: bregma)) per mouse. We analyzed six mice per group.

Intracerebroventricular infusion. Intracerebroventricular infusion was performed as previously described[6]. Briefly, we exposed the calvaria of an anesthetized mouse, implanted a 28-gauge cannula (Plastics ONE) into the third ventricle and then connected the cannula to an osmotic pump (Durect) placed in the dorsal subcutaneous space of the mouse. We infused rat Neuromedin U-23 (Peptide Institute) or human leptin (Sigma) at 0.125 nmol/hr or 8 ng/hr, respectively, for 28 d.

Quantitative RT–PCR analysis. After flushing mouse bone marrow out of the bone with PBS, we extracted bone RNA with Trizol (Invitrogen) and performed reverse transcription for cDNA synthesis. We performed quantitative analysis of gene expression with the Mx3000P real-time PCR system (Stratagene). Primer sequences are available upon request. We used GAPDH expression as an internal control.

Statistical analysis. All data are represented as mean ± s.d. ($n = 8$ or more). We performed statistical analysis by Student's *t*-test. Values were considered statistically significant at $P < 0.05$. Results are representative of more than four individual experiments.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

S. Sato conducted most of the experiments. K. Kangawa and M. Kojima generated *Nmu*$^{-/-}$ mice. R. Hanada and T. Ida conducted *in vitro* experiments. S. Fukumoto, Y. Takeuchi and T. Fujita contributed by conducting dual X-ray absorptiometry analyses and providing suggestions on the project. M. Iwasaki prepared the constructs. A. Kimura performed i.c.v. infusion experiments. H. Inose conducted μCT analyses. T. Matsumoto and S. Kato conducted histological analyses for brain tissue. T. Abe and M. Mieda performed *in situ* hybridization analysis. S. Takeda and K. Shinomiya designed the project. S. Takeda supervised the project and wrote most of the manuscript.

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1. Rodan, G.A. & Martin, T.J. Therapeutic approaches to bone diseases. *Science* **289**, 1508–1514 (2000).
2. Teitelbaum, S.L. & Ross, F.P. Genetic regulation of osteoclast development and function. *Nat. Rev. Genet.* **4**, 638–649 (2003).
3. Saper, C.B., Chou, T.C. & Elmquist, J.K. The need to feed: homeostatic and hedonic control of eating. *Neuron* **36**, 199–211 (2002).
4. Ahima, R.S. & Flier, J.S. Leptin. *Annu. Rev. Physiol.* **62**, 413–437 (2000).
5. Spiegelman, B.M. & Flier, J.S. Obesity and the regulation of energy balance. *Cell* **104**, 531–543 (2001).
6. Ducy, P. *et al.* Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100**, 197–207 (2000).
7. Takeda, S. *et al.* Leptin regulates bone formation via the sympathetic nervous system. *Cell* **111**, 305–317 (2002).
8. Hanada, R. *et al.* Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway. *Nat. Med.* **10**, 1067–1073 (2004).
9. Brighton, P.J., Szekeres, P.G. & Willars, G.B. Neuromedin U and its receptors: structure, function, and physiological roles. *Pharmacol. Rev.* **56**, 231–248 (2004).
10. Fang, L., Zhang, M., Li, C., Dong, S. & Hu, Y. Chemical genetic analysis reveals the effects of NMU2R on the expression of peptide hormones. *Neurosci. Lett.* **404**, 148–153 (2006).
11. Riggs, B.L., Khosla, S. & Melton, L.J. 3rd. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *J. Bone Miner. Res.* **13**, 763–773 (1998).
12. Karsenty, G. & Wagner, E.F. Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* **2**, 389–406 (2002).
13. Harada, S. & Rodan, G.A. Control of osteoblast function and regulation of bone mass. *Nature* **423**, 349–355 (2003).
14. Idris, A.I. *et al.* Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors. *Nat. Med.* **11**, 774–779 (2005).
15. Abe, E. *et al.* TSH is a negative regulator of skeletal remodeling. *Cell* **115**, 151–162 (2003).
16. Elefteriou, F. *et al.* Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature* **434**, 514–520 (2005).
17. Fu, L., Patel, M.S., Bradley, A., Wagner, E.F. & Karsenty, G. The molecular clock mediates leptin-regulated bone formation. *Cell* **122**, 803–815 (2005).
18. Howard, A.D. *et al.* Identification of receptors for neuromedin U and its role in feeding. *Nature* **406**, 70–74 (2000).
19. Wren, A.M. *et al.* Hypothalamic actions of neuromedin U. *Endocrinology* **143**, 4227–4234 (2002).
20. Parfitt, A.M. The physiological and clinical significance of bone histomorphometric data. in *Bone Histomorphometry* (ed. Recker, R.R.) 143–223 (CRC Press, Boca Raton, FL, 1983).
21. Chu, C. *et al.* Cardiovascular actions of central neuromedin U in conscious rats. *Regul. Pept.* **105**, 29–34 (2002).
22. Ahn, J.D., Dubern, B., Lubrano-Berthelier, C., Clement, K. & Karsenty, G. Cart overexpression is the only identifiable cause of high bone mass in melanocortin 4 receptor deficiency. *Endocrinology* **147**, 3196–3202 (2006).
23. Kalinova, J., Triska, J. & Vrchotova, N. Distribution of vitamin E, squalene, epicatechin, and rutin in common buckwheat plants (*Fagopyrum esculentum* Moench). *J. Agric. Food Chem.* **54**, 5330–5335 (2006).
24. Zeng, H. *et al.* Neuromedin U receptor 2–deficient mice display differential responses in sensory perception, stress, and feeding. *Mol. Cell. Biol.* **26**, 9352–9363 (2006).
25. Elias, C.F. *et al.* Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* **23**, 775–786 (1999).
26. Graham, E.S. *et al.* Neuromedin U and Neuromedin U receptor-2 expression in the mouse and rat hypothalamus: effects of nutritional status. *J. Neurochem.* **87**, 1165–1173 (2003).
27. Paxinos, G. & Franklin, K. *The Mouse Brain in Stereotaxic Coordinates* 2nd edn. (Academic Press, San Diego, 2001).