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ORIGINAL ARTICLE

Neuropeptide Y knockdown in the dorsomedial hypothalamus improved basal and obesity-induced decrease in bone mass density

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Abstract

OBJECTIVE: Neuropeptide Y (NPY) has been shown to have a prominent role in the control of bone formation through the regulation of osteoblast activity. We aimed to investigate the role of hypothalamus-derived NPY in bone metabolism.
METHODS: Accordingly, adeno-associated virus (AAV)-mediated RNA interference (RNAi) was utilized to downregulate NPY gene expression in rats fed regular chow (RC) or a high-fat diet (HF). The serum concentrations of glucose, insulin, corticosterone, osteocalcin, insulin-like growth factor (IGF-1), triglycerides (TC), and cholesterol (TG) and fat mass and bone mineral density (BMD) were measured to assess the effect of NPY knockdown on basal and obesity-induced BMD. Forkhead transcription factor (FoxO1) and activating transcription factor 4 (ATF4) were measured to explore the molecular mechanism of the effect of dorsomedial nucleus (DMH) NPY knockdown on bone formation.
RESULTS: Our results showed that DMH NPY knockdown enhanced basal and the obesity-induced decrease in BMD and osteocalcin and promoted the phosphorylation of FoxO1 and reduced the expression of ATF4.
CONCLUSION: Our data suggest that DMH NPY knockdown can alter bone metabolism.

INTRODUCTION

Currently, the incidence of obesity is increasing each year, and it has become a worldwide public health problem. Obesity induces decreases in bone mineral density (BMD) and even causes osteoporosis, and both have received much attention (Yamaguchi *et al.* 2009; Nóbrega da Silva *et al.* 2014). Osteoporosis is also an extremely widespread and debilitating disease that has created major social and economic burdens on health care systems worldwide (Singer *et al.* 2015). Bone remodeling has conventionally been viewed as an endocrine- and paracrine-regulated process. How-

ever, increasing evidence indicates that neuronal factors are critical for preventing bone loss through increased mechanical load on the skeleton and enhanced cortical bone formation (Allison *et al.* 2007; Sharan & Yadav, 2014; Qi *et al.* 2016). Importantly, however, studies using cellular and animal models have highlighted that the neuropeptide Y (NPY) system is a prominent pathway in the control of bone resorption through the regulation of osteoblast activity (Shi & Baldock, 2012).

NPY is an important hypothalamic orexigenic peptide and has been proven to regulate energy homeostasis (Loh *et al.* 2015). It is notable that NPY has also been confirmed to mediate the activity of osteoblasts and osteoclasts through the signaling of central ligands: Y2 in the hypothalamus and peripheral receptors and Y1 in osteoblasts (Wong *et al.* 2008; Lee & Herzog, 2009). The specific deletion of the Y2-receptor in the hypothalamus results in a bone anabolic phenotype (Qi *et al.* 2016). Specifically, Y2-mediated changes occur consistently throughout the skeleton in mice, as studies involving the overexpression of NPY in the paraventricular nucleus (PVN) showed reduced bone formation to conserve energy under starvation conditions (Baldock *et al.* 2009). Furthermore, the ablation of Y1 receptors in mice also leads to increased bone mass (Lee *et al.* 2011; Sousa *et al.* 2012). In addition, the Y1-mediated effect on bone is not dependent on hypothalamic Y1 receptors, implying an additional direct action of the NPY system on bone formation (Lee *et al.* 2010; Kurebayashi *et al.* 2013). In addition, the overexpression of NPY in mature osteoblasts and osteocytes reduced trabecular and cortical bone volume and the expression of the Runx2 and Osterix genes (Baldock *et al.* 2009; Kurebayashi *et al.* 2013).

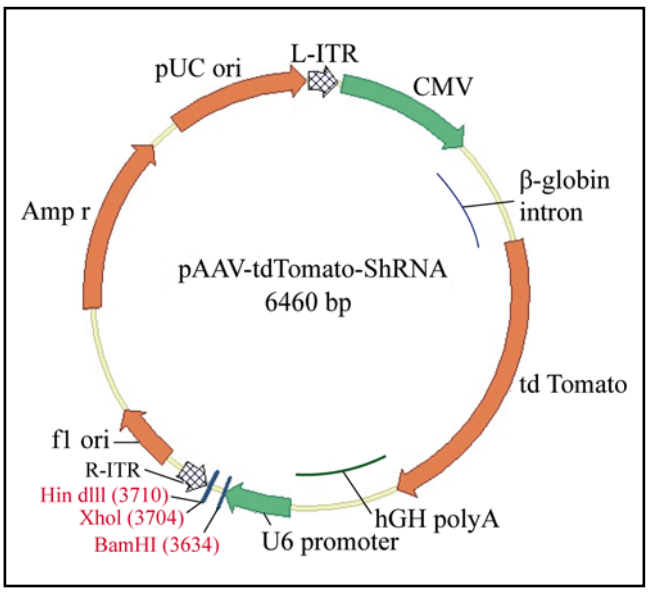

Fig. 1. pAAV-tdTomato-ShRNA-rNPY

In addition, studies in leptin-deficient mice indicated that leptin can affect bone metabolism through the activation of hypothalamic neurons, which relay via efferent sympathetic nervous output and directly regulate the activity of osteoblasts (Kellenberger *et al.* 1998; Takeda *et al.* 2002). Although NPY in the arcuate nucleus (ARC) is under the control of leptin, its regulation in the dorsomedial nucleus (DMH) is leptin independent (Bi *et al.* 2003). To date, the function of NPY in the DMH has yet to be fully determined. Although recent observations revealed that DMH NPY serves as a vital neuromodulator to modulate the energy balance, including promoting the development of brown adipocytes in inguinal white adipose tissue and improving obesity-induced insulin resistance (Chao *et al.* 2011; Mercer *et al.* 2011; Loh *et al.* 2015), the neural mechanism through which the DMH NPY acts to affect basal and obesity-induced bone homeostasis remains unclear.

To explore the effect, as well as the neural mechanism, of DMH NPY on basal and obesity-induced bone homeostasis, we use adeno-associated virus (AAV)-mediated RNAi to specifically knock down NPY expression in the DMH of rats fed regular chow (RC) or a high-fat diet (HF) diet. BMD and serum concentrations of insulin, glucose, corticosterone, triglycerides (TC), cholesterol (TG), insulin-like growth factor (IGF-1), and osteocalcin were measured to evaluate the relations between NPY knockdown and basal and obesity-induced decrease in BMD. Forkhead transcription factor (FoxO1) and activating transcription factor 4 (ATF4) were assessed to clarify the molecular mechanism involved in the effect of the knockdown of NPY on bone metabolism.

METHODS

Animals

Male Sprague-Dawley rats (provided by the Animal Laboratory Center of Henan Province, Zhengzhou, China) were individually raised (12:12 h light-dark cycle, 22–24 °C) with free access to food and water except during fasting prior to a test. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhengzhou University.

Construction of the AAV-mediated RNAi vector

Full-length rat NPY cDNA was cloned into the pAAV-tdTomato-ShRNA vector to make a recombinant NPY knockdown plasmid (Figure 1) HEK-293 cells (Stratagene) were cultured in DMEM growth medium (containing 4.5 g/L glucose, 110 mg/L sodium pyruvate, and 4 mM L-glutamine, Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum were used for viral packaging. Three plasmids of pAAVshNPY (or pAAVshCTL), pHelper (carrying adenovirus-derived genes) and pAAV-RC (carrying AAV-2 replication and capsid genes) were

co-transfected into HEK-293 cells according to the manufacturer's protocol (Stratagene). Three days after transfection, cells were harvested, and the recombinant viral vector AAVshNPY (or AAVshCTL) was purified using the AAV purification kit (Virapur, LLC) and concentrated using Centricon YM-100 (Millipore) according to the manufacturers' protocols (Takara, Japan). Virus titers were determined using quantitative PCR and ~1×10^9 particles/site were used for each virus injection.

AAV-mediated knockdown of NPY expression in the DMH

Fifteen rats (9 weeks old, 270–300 g) received bilateral DMH injections of AAVshNPY. Five rats received the control vector, AAVshCTL. DMH viral injections were performed as previously described (Yang *et al.* 2009). Briefly, 0.5 μL/site (1×10^9 particles/site) of recombinant AAV vector was injected into the DMH with the following coordinates and conditions: 3.1 mm caudal to the bregma, 0.4 mm lateral to the midline, and 8.6 mm ventral to the skull surface at a rate of 0.1 μL/min for 5 min, with the injector remaining in place for an additional 10 min before removal. The rats injected with AAVshNPY were euthanized (n=5) at 1, 2, and 4 weeks post-viral injection, and those injected with AAVshCTL were euthanized at 4 weeks post-injection. After euthanization, a section of the DMH was prepared to examine hrRFP expression under a DVM6 microscope (Leica Science Lab). The NPY mRNA expression level in the DMH and ARC (3.0–3.5 mm posterior to the bregma) were examined using fluorescence in situ hybridization (FISH) (Yang *et al.* 2009).

Effects of DMH NPY knockdown on body weight and food intake

After determining the effects of AAVshNPY on NPY expression, 40 rats (6 weeks old, 130–150 g) were randomly assigned to receive bilateral DMH injections of either AAVshNPY or AAVshCTL (n=20/group) as described above using the following coordinates: 2.3 mm caudal to the bregma, 0.4 mm lateral to the midline, and 7.6 mm ventral to the skull surface. Rats had access to regular chow (RC; 15.8% fat, 65.6% carbohydrates, and 18.6% protein in kcal%; 3.37 kcal/g; PMI Nutrition International, LLC). Five weeks post-viral injection, half of the rats from each group were switched to ad libitum access to a high-fat diet (HFD; 60% fat, 20% carbohydrates, and 20% protein in kcal%; 5.2 kcal/g; Research Diets). Food intake was measured weekly, and body weight was determined daily.

Tissue collection and analyses

At 16 weeks post-viral injection, BMD was measured in the rats (GE Lunar Idxa, United States). Serum samples were collected to assess hormone levels, and plasma was frozen at –20 °C for later analysis using commercial radioimmunoassay kits: insulin (RD system, USA), corticosterone (RD system, USA), IGF-1 (RD system, USA), osteocalcin (RD system, USA) TG (RD system, USA), and TC (RD system, USA), and blood glucose levels were determined with a FreeStyle glucometer (Bayer HealthCare, LLC). The hypothalamus and thighbone tissues were collected and stored at –80 °C. BMD and fat mass were measured in anesthetized rats using dual X-ray absorptiometry (DXA, GE Lunar Idxa, United States).

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from each sample using TRIzol reagent (Aidlab), and the remaining organic phase was saved for subsequent protein extractions, which were performed according to the manufacturer's protocol. Two-step quantitative real-time RT-PCR was performed to determine gene expression levels. One microgram of total RNA was reverse transcribed into first-strand cDNA using GeneCopoeia cDNA Synthesis Kits (GeneCopoeia, Inc.) with a ribonuclease inhibitor (Transgen, Inc.) added to inhibit degradation by RNase. The cDNA product was quantified using an AceQ SYBR Green Master Mix Kit (Vazyme Biotech Co., Ltd) and ABI 7900 Real-Time PCR Detection System (Applied Biosystems, ABI). β-actin was used as an internal reference for the quantification of individual mRNA levels. The following primer sets were used: FoxO1 (143 bp): forward primer: 5'-CAGCCAGGCACCTCATAACA-3' and reverse primer: 5'-TCAAGCGGTTCATGGCAGAT-3'; ATF4 (108 bp): forward primer: 5'-GTGGCATCTGTATGAGCCCTGAG-3' and reverse primer: 5'-AACCACGAGGAACACCTGGAGAA-3'; and β-actin: forward primer: 5'-CACGATGGAGGGGCCGGACTCATC-3' and reverse primer: 5'-TAAAGACCTCTATGCCAACACAGT-3'.

Western blotting

Proteins were separated using 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to an Immun-Blot PVDF membrane. The membrane was then incubated with the appropriate antibody at the recommended dilution (Abcam, United Kingdom), followed by incubation with horseradish peroxidase-labeled donkey anti-goat antibody (Boster, Biotechnology, Wuhan, China) and detection with a Luminol-based chemiluminescence reagent kit (Thermo Scientific).

Statistical analyses

All values are presented as the mean ± standard error of the mean (SEM). Data were analyzed using SPSS17.0 software. Data on NPY mRNA expression were analyzed with one-way ANOVA. Data on body weight, food intake, and hormone levels were analyzed with two-way ANOVA with one repeated factor. Data on mRNA and protein levels were analyzed with two-

way ANOVA. All ANOVAs were followed by least-significant-difference (LSD-t) comparisons. Differences were considered statistically significant at $p<0.05$.

RESULTS

AAV-mediated knockdown of NPY expression in the DMH

The viral vectors successfully infected neurons around the region of the DMH (Figure 2A), which did not occur in other hypothalamic areas (Figure 2B–F). The significant knockdown of hrRFP expression in the DMH was detected as early as 2 weeks after viral injection. NPY mRNA expression was ablated by 25.18%, 43.22%, and 45.56% at 1, 2, and 4 weeks post-viral injection, respectively (Figure 2G). NPY levels remained reduced by 39.7% until 16 weeks post-viral injection. In contrast, NPY mRNA expression was not significantly knocked down in the ARC (Figure 2H).

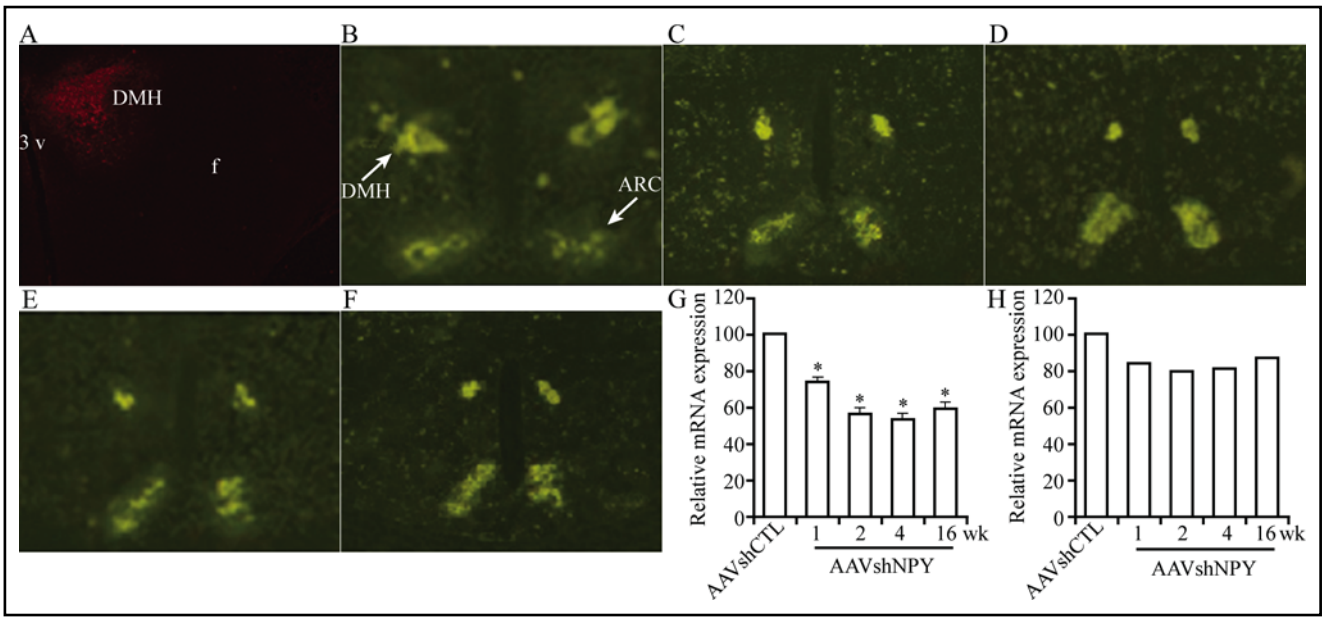

Fig. 2. Adeno-associated virus-mediated knockdown of NPY expression in the dorsomedial hypothalamus. (A) Representative micrograph shows hrRFP (humanized Renilla red fluorescent protein) expression in the DMH post-viral DMH injection as examined under fluorescence microscopy. (B–F) Fluorescence in situ hybridization (FISH) shows suppressed Npy expression in the DMH and ARC. B: Rats receiving bilateral DMH injections of AAVshCTL at 4 weeks post-injection. AAVshNPY (16 w). C–F: Rats receiving bilateral DMH injections of AAVshNPY at 1,2,4,16weeks post-injection. C: AAVshNPY (4 w); D: AAVshNPY (2 w); E: AAVshNPY (1 w); F: AAVshCTL (4 w). (G, H) Mean±SEM NPY mRNA levels significantly decreased in the DMH of AAVshNPY rats at 1, 2, 4 and 16 weeks after viral injection compared with AAVshCTL rats, but NPY mRNA levels in the arcuate nucleus (ARC) did not differ between AAVshCTL and AAVshNPY rats at any time points. n=5 per group. *$p<0.05$ compared with AAVshCTL rats. f: fornix; 3v: the third ventricle.

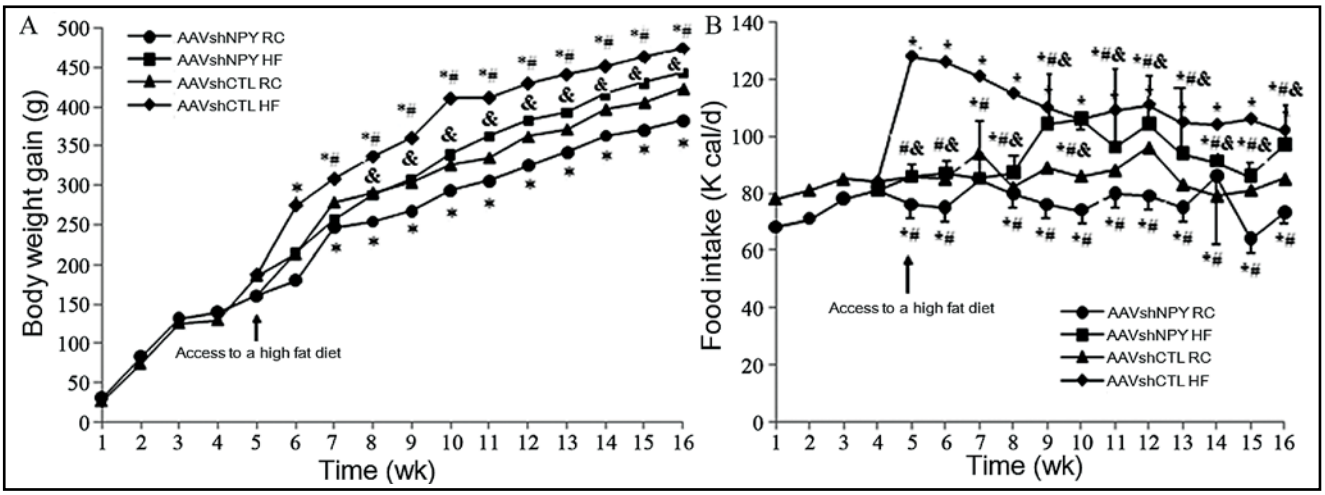

Fig. 3. Effects of DMH NPY knockdown on body weight and food intake. (A) Body weight gain in AAVshCTL and AAVshNPY rats with access to a regular chow (RC) or high-fat (HF) diet; (B) Daily food intake in the four groups of rats. Values are means ± SEM n=6 rats per group. *$p<0.05$ compared with AAVshCTL·RC rats, #$p<0.05$ compared with AAVshNPY·HF rats, & $p<0.05$ compared with AAVshNPY·RC rat.

DMH NPY knockdown reduced body weight and energy intake

Our results agree with a study that the NPY knockdown decrease the weight and energy intake (Chao *et al.* 2011), (Figure 3).

DMH NPY knockdown enhanced BMD

To determine whether knockdown of NPY in the DMH could enhance bone mass, we first measured BMD by DXA, which was previously used for the analysis of bone phenotype in germline Y1–/– mice (Lee *et al.* 2011). DXA analysis revealed that DMH NPY knockdown resulted in a small but significant increase in the whole body and thighbone BMD and in serum osteocalcin after 16 weeks when rats were maintained on RC relative to the controls (Table 1, $p<0.05$). In addition, we found no significant effect on serum concentrations of insulin, glucose, corticosterone, TC, TG, and IGF-1 and whole and belly fat mass in the above groups (Table 1). Because a high-fat diet normally reduces BMD and serum osteocalcin and increases serum concentrations of insulin, glucose, corticosterone, TC, and TG and whole and belly fat mass, we concluded that NPY knockdown significantly ameliorated these effects (Table 1, $p<0.05$).

We next determined whether the BMD improvement was due to alterations in the activity of osteoblasts. Genetic experiments have shown that FoxO1 and ATF4 cooperate to reduce insulin secretion and increase insulin resistance, which also result from the synergistic effect of the two transcription factors that suppresses the activity of osteocalcin (Kode *et al.* 2012). We further examined the gene and protein expression of FoxO1 and ATF4. Compared to control rats, DMH NPY knockdown rats showed a significant reduc-

Tab. 1. Body composition, serum parameters and BMD of rats with AAVshCTL or AAVshNPY.

	AAVshCTL+RC	AAVshNPY+RC	AAVshCTL+HF	AAVshNPY+HF
Whole fat mass (g)	35.94±0.003	33.25±0.002	52.00±0.001^{ab}	43.29±0.003^{abc}
Belly fat mass (g)	23.36±0.002	21.04±0.003	37.44±0.002^{ab}	29.01±0.001^{abc}
Belly/whole (%)	64.99	63.30	72.02	67.01
Whole BMD (g/cm^2)	0.276±0.002	0.295±0.001^{a}	0.231±0.001^{ab}	0.259±0.001^{abc}
Thighbone BMD (g/cm^2)	0.277±0.001	0.292±0.002^{a}	0.234±0.002^{ab}	0.255±0.002^{abc}
Osteocalcin (μg/L)	0.98±0.32	1.52±0.14^{a}	0.59±0.27^{ab}	0.86±0.18^{bc}
Glucose (mg/dL)	85.05±0.221	74.70±0.191	84.60±0.258	81.00±0.081
Insulin (ng/mL)	1.777±0.022^{c}	1.150±0.018^{c}	3.337±0.060	1.495±0.017^{c}
IGF-1 (ng/mL)	219.34±18.58	210.67±15.30	247.37±17.49^{ab}	233.95±13.16^{ab}
Corticosterone (ng/mL)	93.39±0.48	81.12±0.39	137.43±0.45^{ab}	113.46±0.41^{abc}
TC (mmol/L)	1.63±0.087	1.57±0.099	3.45±0.137^{ab}	2.43±0.093^{abc}
TG (mmol/L)	0.93±0.093	0.87±0.103	1.91±0.735^{ab}	1.37±0.134^{abc}

Note: Mean±SEM, n=5. $^{a}p<0.05$ compared with AAVshCTL+RC rats; $^{b}p<0.05$ compared with AAVshNPY+RC rats; $^{c}p<0.05$ compared with AAVshCTL+HF rats.

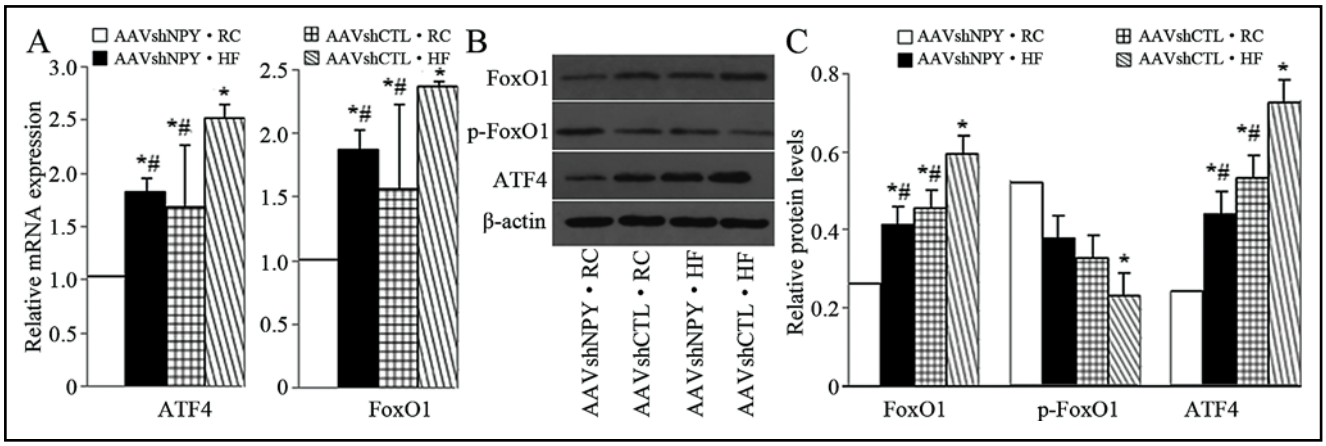

Fig. 4. Effects of DMH NPY knockdown on bone metabolism. (A) ATF4 and FoxO1 mRNA were expressed in the bone tissue of AAVshCTL and AAVshNPY rats on RC or HF as determined by RT-PCR (n=3); (B) ATF4, FoxO1, and p- FoxO1 protein were produced in the bone tissue of AAVshCTL and AAVshNPY rats on RC or HF as determined by Western blot. Means ± SEM, n=3. *$p<0.05$ compared with AAVshNPY·RC rats, #$p<0.05$ compared with AAVshNPY HF rats.

tion in FoxO1 and ATF4 (Figure 4A and 4B). We also found that NPY knockdown noticeably improved the HF-induced decrease in the phosphorylation of FoxO1 (Figures 4B and 4C, increased by 42.6%, p=0.043). Therefore, DMH NPY knockdown improves bone mass and enhances the activity of osteoblasts via the activation of FoxO1 and ATF4.

DISCUSSION

The present study demonstrated that NPY knockdown in the DMH plays a powerful role in bone formation and enhances the obesity-induced decrease in BMD. The overexpression of NPY in the hypothalamus has previously been shown to have an effect on osteoblast- and osteocyte-specific expression (Baldock *et al.* 2005; Allison *et al.* 2009; Shi & Baldock, 2012). The overexpression of NPY was shown to induce the loss of bone mass, including cortical and trabecular bone, in vitro (Baldock *et al.* 2009). However, this study did not reveal whether NPY overexpression in the hypothalamus alters the obesity-induced decrease in BMD and osteoporosis. Our study evaluated the impact of NPY knockdown in the DMH on osteoblasts and bone mass in rats fed RC and an HFD.

In the present study, we show that the AAV can be transfected stably and chronically in the DMH with stereotaxic technology. Up to 16 weeks post-viral injection, NPY was reduced by 39.7%. The FISH results revealed that the injection did not affect other areas. After euthanization, anatomy and histology were analyzed in the brain slices prepared from the rats. It is worth noting that the rats weighed 300 g, following the recommendation in the book by Paxinos and Franklin (2004); otherwise, we could not perform the injection correctly. In this book, the coordinates and conditions of NPY of rats weighted 130–150 g is 3.1 mm caudal to the bregma, 0.4 mm lateral to the midline, and 7.6 mm ventral to the skull surface.The rats weighted 270–300 g is 3.1 mm caudal to the bregma, 0.4 mm lateral to the midline, and 7.6 mm ventral to the skull surface.The area of NPY is not a spot. At the $1^{th,}$ 2^{th}, 4^{th} and 16^{th} week, we showed the knockdown effect of NPY (Figures 2B–F).

We observed that DMH NPY knockdown in rats enhanced both osteoblast activity and bone mass. Although a high-fat diet normally results in increases in weight and food intake, knockdown of NPY in the DMH altered this trend. In addition, DMH NPY knockdown in rats fed RC resulted in increased BMD associated with increased osteocalcin, which were observed in the rats fed RC without any changes in belly fat mass and serum concentrations of insulin, glucose, corticosterone, TC, TG, and IGF-1. This central action is consistent with signaling via Y2 receptors expressed on neurons of the ARC, as is evidenced by the opposing response reported following the specific deletion of Y2 receptors in this region of the hypothalamus (King *et al.* 2000; Shi *et al.* 2010). Therefore, the knockdown of NPY in the DMH can promote bone formation through signals transduced by Y2 receptors.

Many studies have shown that adipose tissue is negatively correlated to BMD. Obesity is a risk factor for osteoporosis and not a protective factor. Our results showed that an HFD led to hyperinsulinemia, hypertriglyceridemia, hypercholesterolemia, and increases in corticosterone and belly fat mass, whereas DMH NPY knockdown altered these trends and increased the HF diet-induced decrease in BMD and osteocalcin. In contrast, hyperinsulinemia or insulin resistance stimulates the production of IGF-1 and restrains the production of IGF binding protein (IGFBP), causing a higher level of free IGF-1 (Crossey *et al.* 2000). However, IGF-1 is a double-edged sword in terms of osteoporosis. On the one hand, IGF-1 promotes osteoblast differentiation and the maturation of osteoblasts and marrow stromal cells. On the other hand, IGF-1 weakens the inhibition of osteoclasts and promotes bone absorption by inhibiting the expression of osteoprotegerin (OPG) and promoting the expression of nuclear factor (NF-κB) ligand (RANKL) (Rubin *et al.* 2002; Zhao *et al.* 2008). Therefore, the effect of IGF-1 on BMD depends on the balance between these two aspects.

Recently, a study showed that NPY signaling via osteoblastic Y1 receptors not only is important for the control of glucose homeostasis but also significantly regulates bone mass (Lee *et al.* 2015). Our results also showed that knockdown of DMH NPY expression resulted in increased BMD and osteocalcin. Numerous reports have indicated that osteocalcin has a major role in bone deposition and glucose metabolism: osteocalcin levels are inversely correlated with the risk of type 2 diabetes (Reyes-Garcia *et al.* 2012; Liatis *et al.* 2014). Osteocalcin expression is suppressed by two transcription factors, FoxO1 and ATF4 (Vijayan *et al.* 2013), and enhanced by relieving the suppression of Runt-related transcription factor 2 (Runx2) (Aisha *et al.* 2014). FoxO1 antagonizes oncogene expression by interacting with the oncogene-promoting transcription factor Runx2. FoxO1 downregulates the expression of Esp, with the ultimate effect of promoting osteocalcin decarboxylation and osteocalcin metabolic bioactivity (Mirdamadi *et al.* 2015). Our results showed that FoxO1 mRNA and protein levels were reduced compared to the controls. In addition, we also showed that FoxO1 phosphorylation was significantly increased in NPY knockdown rats fed RC, suggesting that p-FoxO1 leads to its nuclear exclusion and inhibition of Esp expression. Consistently, osteoblast-specific FoxO1 deletion has been shown to reduce Esp expression, enhance osteocalcin, and protect against obesity and diabetes (Rached *et al.* 2010). ATF4 is upregulated by sympathetic nervous system activation and induces Esp and Ocn gene expression, promoting insulin resistance and impairing the activity of osteocalcin. Consistent with these lines of evidence, our results also showed that

ATF4 was reduced in the NPY knockdown rats relative to the controls, suggesting that NPY may regulate bone mass via the sympathetic nervous system (Takeda *et al.* 2002). Accordingly, osteoblast-specific ATF4 deletion enhanced bone mass and insulin sensitivity (Kode *et al.* 2012). These results provide additional evidence indicating that NPY knockdown regulates osteocalcin and bone mass in part though FoxO1 and ATF4 and the sympathetic nervous system.

In summary, DMH NPY knockdown ameliorated the basal and obesity-induced decrease in bone mass, serum osteocalcin, and FoxO1 phosphorylation and reduced the obesity-induced increase in belly fat mass. These results indicate that NPY in the DMH maintains bone mass in part though the FoxO1 signaling pathway and in part though the sympathetic nervous system. In addition, these results indicate that DMH NPY is a potential target site for therapies aimed at combating obesity-induced osteoporosis.

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Conflicts of interest: *All of the authors declare that they have no conflicts of interest regarding this paper.*

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