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Postmenopausal osteoporosis is associated with the regulation of SP, CGRP, VIP, and NPY

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ABSTRACT

Estrogen deficiency is the main factor underlying postmenopausal osteoporosis. A large number of neuropeptides, which regulate skeletal metabolism, potentially represent a regulatory pathway for the pathogenesis of osteoporosis. The aim of this study was to explore factors involved in the regulation of bone-related neuropeptides and their association with estrogen deficiency and bone metabolism. Thirty adult female Sprague-Dawley (SD) rats were randomly divided into a control group with sham surgery (n = 15) and an ovariectomy group with bilateral oophorectomy (n = 15). After 16 weeks, serum estrogen was reduced,CTX-1 was increased and P1NP was not significantly affected in the ovariectomy group and a model of osteoporosis was established. We then investigate the gene expression and protein levels of a range of neuropeptides and their receptors, including substance P (SP) and tachykinin receptor 1 (TACR1), calcitonin gene-related peptide (CGRP) and calcitonin receptor-like (CALCRL), vasoactive intestinal polypeptide (VIP) and receptor 1 and 2 (VPAC1, 2), neuropeptide Y (NPY) and receptor Y1 and Y2, in the brain and femora. Ovariectomy reduced TACR1, CGRP, CALCRL, NPY, NPY Y2 in the brain, but increased TACR1 and decreased SP, CALCRL, VIP, VPAC2 in the bone. Collectively, our data revealed that the pathogenesis of postmenopausal osteoporosis is associated with the regulation of SP, CGRP, VIP, and NPY. These novel results are of significant importance in the development of neuropeptides as therapeutic targets.

1. Introduction

Characterized by a loss of bone mass and quality with a greater risk of fragility fractures [1], osteoporosis afflicts a significant proportion of the aged population, particularly postmenopausal women. Osteoporosis is responsible for over 1,500,000 fractures each year in the United States of America, with most cases occurring in postmenopausal women [2]. These fractures, from an individual perspective, bring about a poor quality of life, as well as a heavy personal economic burden [3,4]. Furthermore, these fractures, from a social perspective, consume substancial social resources for direct medical costs and an indirect increased need for life care [5].

Postmenopausal osteoporosis is mainly caused by an abrupt cessation of estrogen after the menopause [6]. Estrogen deficiency leads to series of metabolic alterations that break the balance between bone formation and bone reabsorption, and eventually result in a rapid reduction in bone mineral density [6]. While, many regulatory mechanisms have been proven to be involved in the complex process of postmenopausal osteoporosis, none of these have allowed us to come up with strategies that can efficaciously prevent and cure hormone-deficient osteoporosis [7]. Since the pathogenesis of postmenopausal osteoporosis has not been fully elucidated, there is a very real need to explore the development of new pharmacological targets for the treatment of postmenopausal osteoporosis.

Neuropeptides are neuronal signaling peptides involved in brain activity and other parts of the body which modulate a wide range of physiological functions, including skeletal metablism [8]. Neuropeptides regulating skeletal metabolism repreesnt a potential regulatory pathway for the pathogenesis of postmenopausal osteoporosis.

The central and peripheral regulation of neuropeptides in the skeleton is mediated by their receptors. There are four types of bone-related neuropeptides: substance P (SP) suppresses osteoclasts and promotes osteoblasts through the tachykinin receptor 1 (TACR1) in the cell membrane and cytoplasm [9,10]; calcitonin gene-related peptide

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Table 1
Details of PCR primers for real-time quantitative PCR.

Gene symbol	Product length (bp)	Forward primer (5'-3')	Reverse primer (5'-3')
SP	220	ATGAAAATCCTCGTGGCGGT	CAGCATCCCGTTTGCCCATT
TACR1	82	CCAACAGGACTTATGAGAAAGCGTA	GCGTAGCCGATCACCAGTAGAG
CGRP	134	AGTTCTCCCCTTTCCTGGTTGT	CAGTAGGCGAGCTTCTTCTTCA
CALCRL	325	GATGGGCTGTAACTACTTTTGGA	GAGATTGGATTCTGCTTGGTGT
VIP	278	CAGATAGGCTGCCGTGTTACA	TGGAAATCAAGCACTCCGTTAG
VAPC1	217	AAACTACGGCCACCCGACAT	CACCATTGAGGAAGCAGTAGAGGA
VAPC2	203	AGTACAAGAGGCTCGCCAAGT	CCTTCTTTTCAGTTCACGCTGT
NPY	232	GTGGACTGACCCTCGCTCTAT	GGGCATTTTCTGTGCTTTCTC
NPY Y1	299	CCACAATCTGCTGTTCCTGCTCT	CACAGATGTAGCCTGGGACCGTA
NPY Y2	183	AGCCTTTCCACCCTGCTAAT	GCTGACTGCAAACACCACTACC
GAPDH	96	CAACGGGAAACCCATCACCA	ACGCCAGTAGACTCCACGACAT

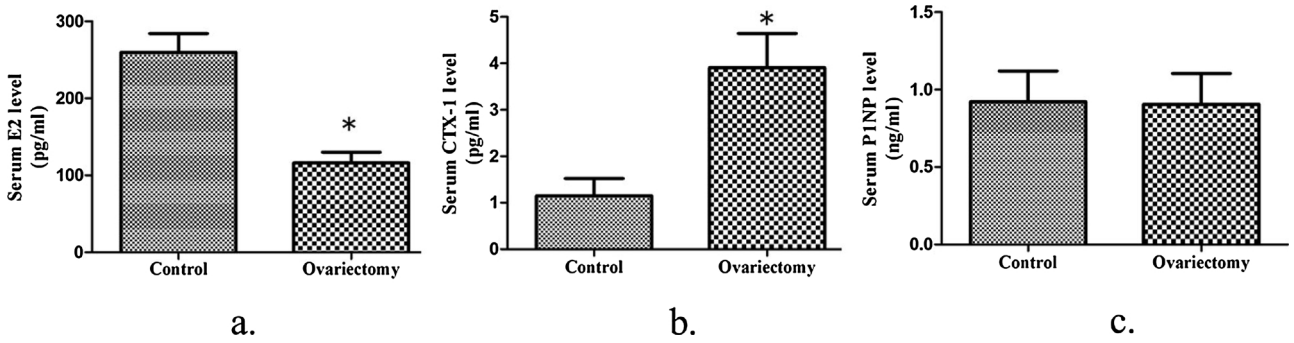

Fig. 1. Effects of ovariectomy surgery on serum estrogen, CTX-1 and P1NP levels. Serum was measured by ELISA assay. Compared with the control group, serum estrogen levels in the ovariectomy group were significantly reduced (a.). Ovariectomy also remarkably increased CTX-1 levels in serum (b.), but failed to affect serum P1NP (c.). Control group *vs.* ovariectomy group $^{*}P < 0.05$.

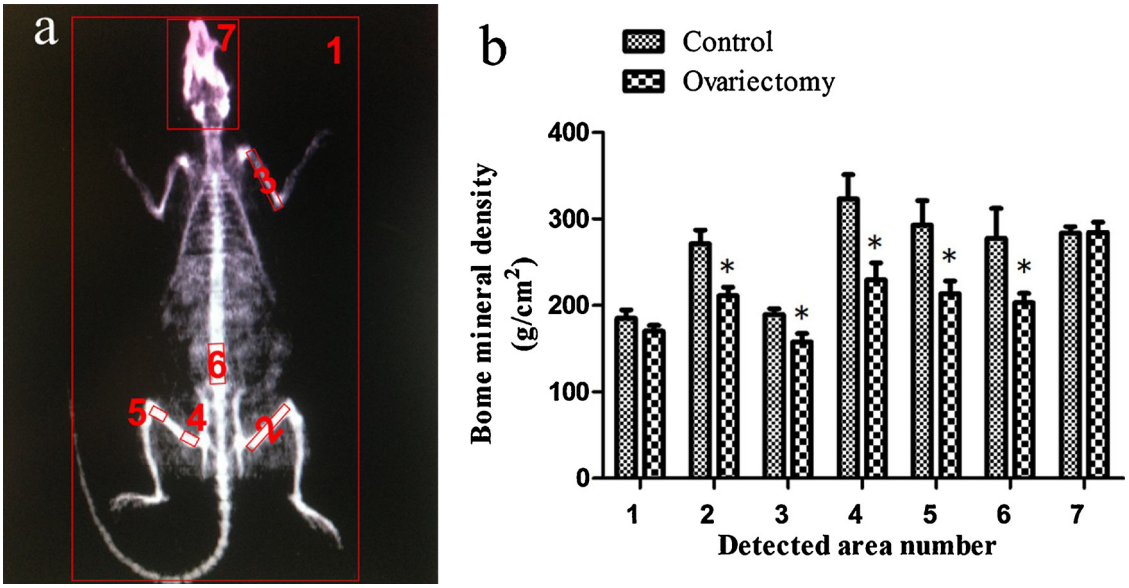

Fig. 2. Effects of ovariectomy surgery on bone mineral density. Bone mineral density (BMD) was determined by dual X-ray absorptiometry in seven areas (Fig. 2a), including (1) whole body, (2) thighbone, (3) arm bone, (4) epiphyseal of proximal thighbone, (5) epiphyseal of distal thighbone, (6) fourth and fifth vertebra lumbalis and (7) head. Data (Fig. 2b) revealed that compared with the control group, the BMD of the thighbone, arm bone, epiphyseal of proximal thighbone, epiphyseal of distal thighbone, and the fourth and fifth vertebra lumbalis were lower in the ovariectomy group. In the whole body and head area, however, there was no significant difference in terms of BMD. Control group *vs.* ovariectomy group $^{*}P < 0.05$.

(CGRP) stimulates osteogenesis and restrains bone reabsorption by the combination and modification of calcitonin receptor-like (CALCRL) and the regulated expression of runt-related transcription factor 2 (RUNX2) and the Osterix gene [11–13]; vasoactive intestinal polypeptide (VIP) regulates the activity of osteoblasts and controls the movement of osteoclasts through VIP receptor 1 (VPAC1) and 2 (VPAC2) [14] and neuropeptide Y (NPY) regulates cAMP synthesis in osteoblasts to affect osteogenesis through NPY receptor type 1 (NPY Y1) and type 2 (NPY Y2) [15,16]. Many studies also suggested that SP, CGRP, VIP and NPY not only directly affect different types of osteocytes, but also play pivotal roles in controlling bone mass centrally [17,18].

The hypothalamic-pituitary-ovarian axis is the female sex gland axis in which the nervous system and estrogen secretion interact with each other [19]. Previous research into the interplay of the nervous system

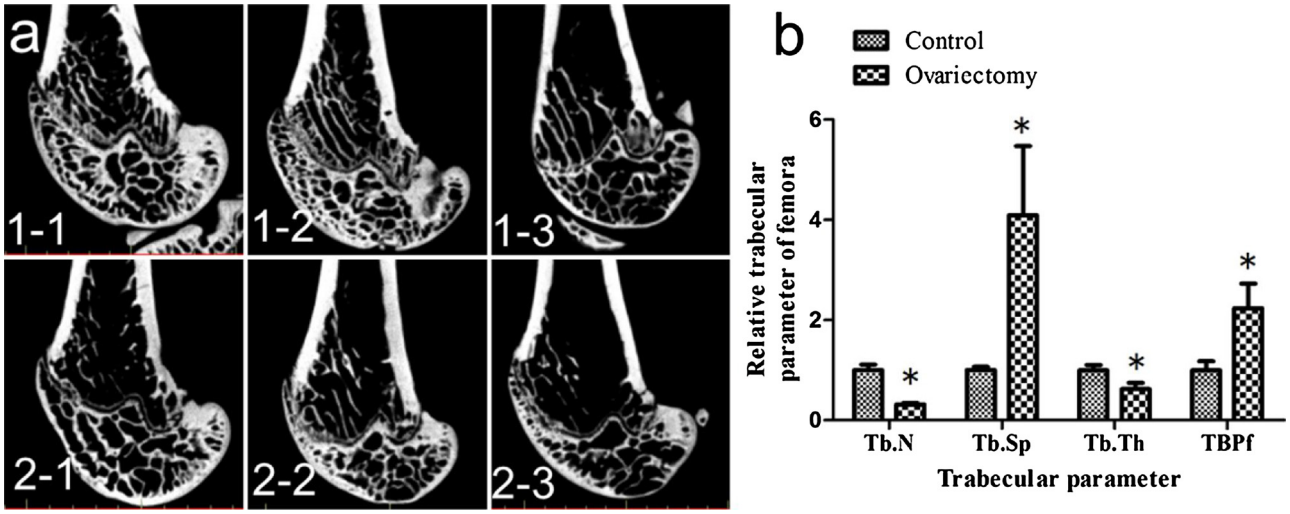

Fig. 3. Effects of ovariectomy surgery on femora microstructure. The microarchitecture of the femora was analyzed using micro-CT. Femora specimens were scanned and data analyzed to attain trabecular parameters. The microstructure of the femora showed that compared with the control group (Fig. 3a 1-1, 1-2, 1-3), cancellous and cortical bone microarchitecture in ovariectomy group (Fig. 3a 2-1, 2-2, 2-3) were markedly impaired. Trabecular parameters (Fig. 3b) showed that compared with the control group, Tb.N and Tb.Th of femora in ovariectomy decreased and Tb.Sp and TBPf increased. Control group *vs*. ovariectomy group $^{*}P < 0.05$.

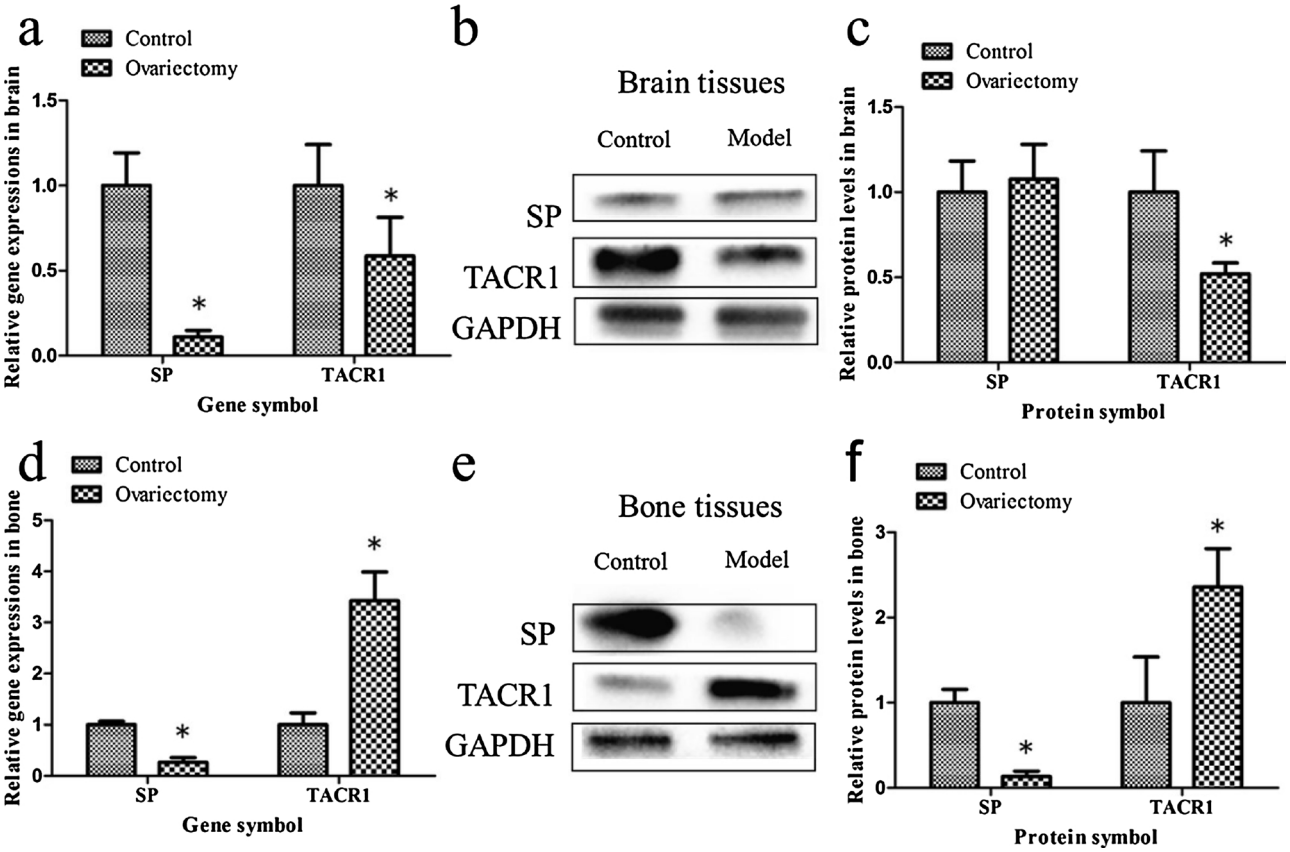

Fig. 4. Effects of ovariectomy on the expression of SP relative targets. The gene expression and protein levels of SP and TACR1 in the brain and femora were determined by real-time quantitative PCR and western blotting. The expression of the SP gene in the brain (Fig. 4a) was reduced without a corresponding reduction in SP levels in the brain (Fig. 4b, c). However, the reduced expression of the TACR1 gene in the bone, and the increased gene expression of SP and TACR1 in the bone, all occurred concomitantly with their corresponding proteins (Fig. 4b–f). Control group *vs.* ovariectomy group $^{*}P < 0.05$.

and estrogen secretion demonstrated that some neuropeptides mediate the effect of estrogen deficency on bone metabolism. For example [20], found that estrogen can increase the expression of CGRP and its receptor in the vascular endothelium, while [21] revealed that estrogen regulates SP and SP-related neuropeptides in mammals. Furthermore, SP is also known to play a crucial role in the healing course of fractures under low levels of estrogen [22]. In other research [23], showed that estrogen modulates body energy balance by interfering with NPY expression. NPY is also known to operate together with estrogen, collaborating in the control of regulatory osteogenic-adipogenic balance [24].

Due to the evidence that SP, CGRP, VIP and NPY can affect bone

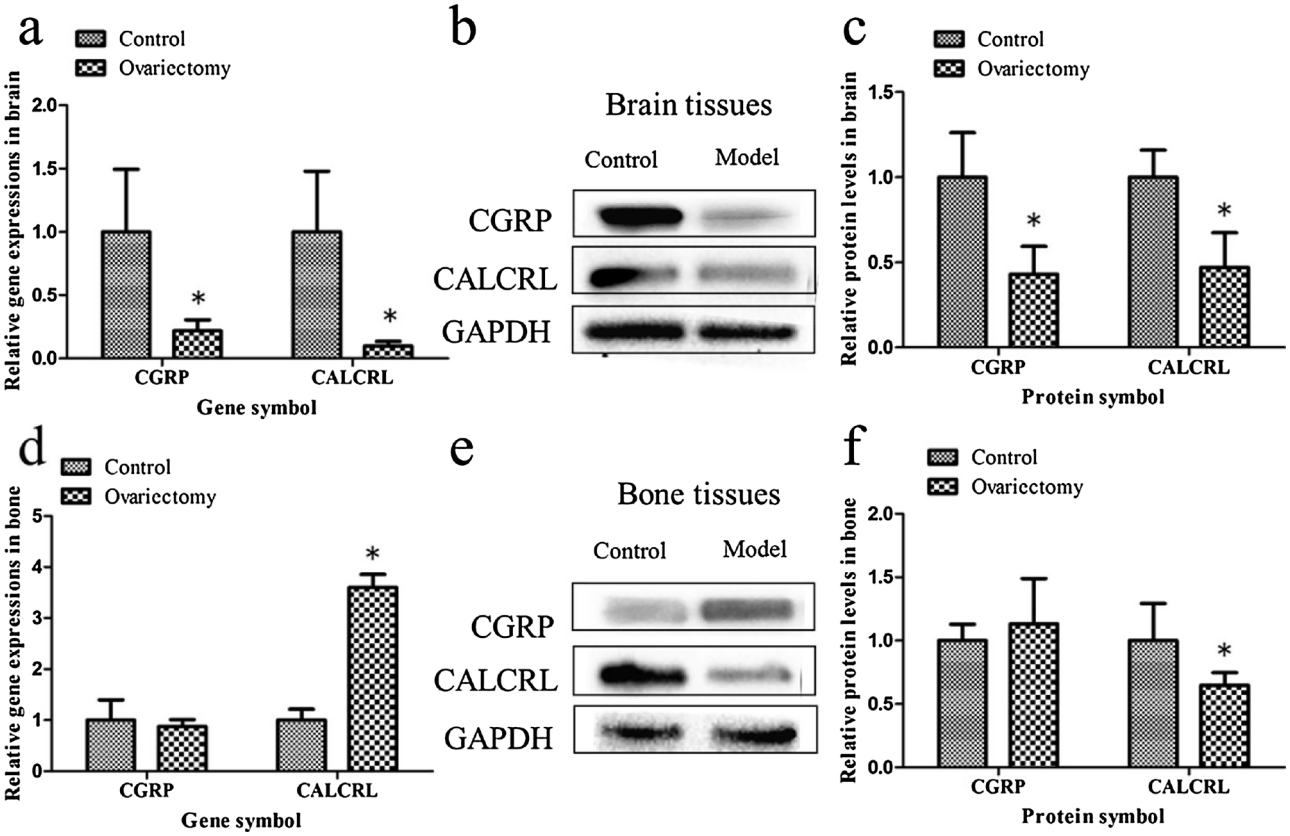

Fig. 5. Effects of ovariectomy on the expression of CGRP-related targets. The gene expression and protein levels of CGRP and CALCRL in the brain and femora were detected by real-time quantitative PCR and western blotting. The gene expression of CGRP and CALCRL in both the brain and bone decreased with their corresponding proteins after ovariectomy (Fig. 5a–c). The expression of CGRP in bone showed no significant difference when compared between the two groups. Interestingly, the gene expression of CALCRL in the bone increased, while the level of CALCRL protein decreased (Fig. 5d, c, f). Control group *vs.* ovariectomy group $^{*}P < 0.05$.

metabolism both centrally and peripherally, as well as their interrelationship with estrogen, there are strong grounds for believing that the pathogenesis of postmenopausal osteoporosis is associated with the regulation of SP, CGRP, VIP and NPY neuropeptides. This study will help other researchers to further understand the mechanisms of postmenopausal osteoporosis.

2. Materials & methods

2.1. Reagents & materials

The following reagents were used in the study: Estrogen ELISA kits (Cloud-clone Corp., Katy, TX, USA); CTX-1 and P1NP ELISA kits (Jianglai Corp., Shanghai, China); GAPDH, NPY and CGRP primary antibodies, goat anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA); NK1R primary antibody (Merck Millipore Corp., Darmstadt, Germany); CRLR primary antibody (OriGene Technologies, Inc., Rockville, MD, USA); NPY1R, NPY2R, VIP and VIP1R primary antibodies (Abcam Co., Cambridge, UK); SP and VIP2R primary antibodies (Arigo Biolaboratories Corp., Hamburg, Germany); synthesized PCR primers (Guge Biotech Corp., Ltd, Wuhan, China); PMSF, Protein Loading Buffer, RIPA Lysis buffer (Beyotime Biotechnology Ltd., Shanghai, China); TE buffer (Generay Biotech Corp. Ltd., Shanghai, China); bull serum albumin (BSA), RT-PCR SYBR® kit (F. Hoffmann-La Roche Ltd., Basel, Switzerland); Reverse transcription kit, TRIzol (TaKaRa Biotechnology Corp., Shiga, Japan); Chloroform, isopropyl alcohol (Guangzhou Chemical Agent Ltd., Guangzhou, China); Prestained protein markers and BCA kit (Thermo Fisher Scientific Inc. Waltham, MA, USA); ECL chemiluminescence kit (DOCLAB Ltd., Guangzhou, China); Precast gels (10%) for Western blots (Fude Biotechnology Ltd., Hangzhou, China); polyvinylidene difluoride membranes (0.2 μm) (Merck Millipore Ltd.).

2.2. Instrumentation & software

The following equipment or software were used in the study: A dual X-ray scanner (Lunar Prodigy, GE Co., USA); U-CT80 micro CT system (SCANCO Medical AG, Brüttisellen, Switzerland); a microplate absorbance reader (Model 680, Bio-Rad Co., Hercules, CA, USA); a G-Storm Gradient PCR thermal cycler (Veriti 96-Well, Applied Biosystems Co., Foster City, CA, USA); a quantitative fluorescence PCR system (Light Cycler® 480, Roche Diagnostics Company Ltd., Basel Switzerland); a gel imaging system (Bio-Rad Co.); and a microplate reader (Bio-Rad 680, Bio-Rad Co.); SPSS statistical analyses system version 22.0 (SPSS Inc., Chicago, IL, USA); Quantity One® analysis software version 4.3.0. (Bio-Rad Co.).

2.3. Experimental design

All animal protocols were approved by the local Institutional Animal Care and Use Committee. Thirty Sprague-Dawley (SD) rats (female, 3-months of age, No.: SCXK 2013-0034) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Upon receipt, rats were housed with *ad libitum* access to water and a standard diet on a 12/12-h-light/dark cycle inside a Chinese national approved animal vivarium (No.: SYXK 2012-0117). The rats were randomly divided into two groups and treated with surgery [25]: a control group receiving sham surgery (n = 15) and an ovariectomy

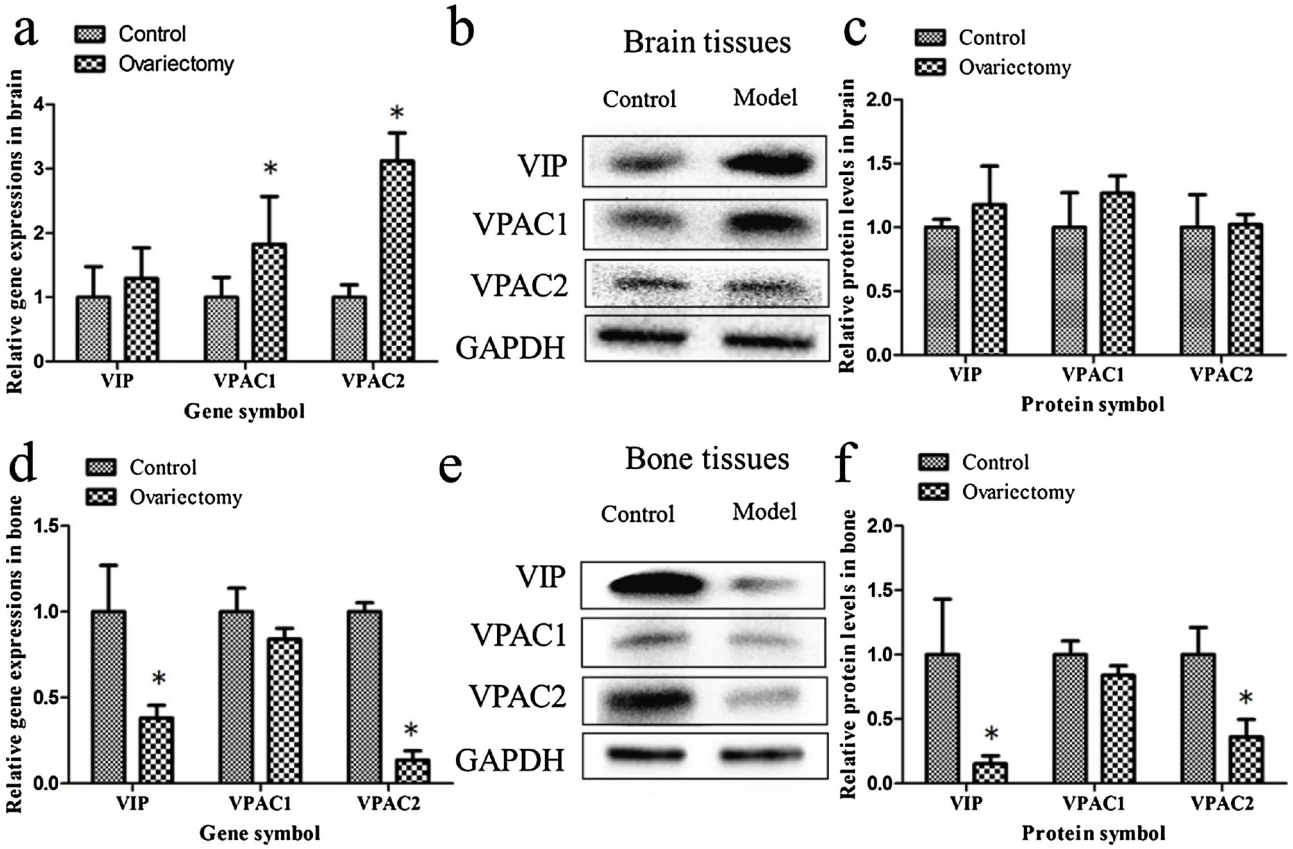

Fig. 6. Effects of ovariectomy on the expression of VIP-related targets. The gene expression and protein levels of VIP, VPAC1 and VPAC2 in the brain and femora were determined by real-time quantitative PCR and western blotting. Gene expression and protein levels of VIP in the brain were not significantly different between the two groups, while the gene expression of APAC1 and APAC2 in the brain increased in the ovariectomy group but without change in their corresponding proteins (Fig. 6a–c). In bone tissue, VIP and VPAC2 protein levels decreased as their gene expression also decreased, while VPAC1 protein levels and gene expression showed no significant difference (Fig. 6d, c, f). Control group *vs.* ovariectomy group $^*P < 0.05$.

group receiving bilateral oophorectomy (n = 15). Rats had access *ad libitum* to water and food for the next 16 weeks of the study. Then blood was collected and serum used to determine estrogen levels. Bone mineral density was also determined. At the end of the study, all rats were euthanized in an inhalation chamber by CO_2 overdose. Then, the brain and femora were dissected from each animal and preserved in liquid nitrogen or utilized immediately. A micro-CT scan was carried out on the femora and trabecular analysis was conducted. The gene expression and protein levels of SP, TACR1, CGRP, CALCRL, VIP, VPAC1, VPAC2, NPY, NPY Y1, and NPY Y2 were determined in the brain and femora by real-time quantitative PCR and western blotting, respectively. Statistical analyses to identify significant differences in the data were performed as previously reported [26]. The t-test was used for all statistical tests with a significance level of 0.05.

2.4. Enzyme-linked immunosorbent assay (ELISA) for estrogen, CTX-1 and P1NP

An ELISA assay was used to detect estrogen, carboxy-terminal cross-linking telopeptide of type I collagen (CTX-1) and procollagen I N-terminal propeptide (P1NP) levels in serum samples and was deployed in accordance with the manufacturer's instructions. One hundred microliters of standard, or samples, were added into each well of the ELISA plate and incubated for 1 h at 37 °C. Liquid was then removed from the wells and 100 μL of pre-prepared detection reagent A was added; this mixture was then incubated for 1 h at 37 °C. The liquid was then removed and the wells washed three times with washing buffer. Then, 100 μL of pre-prepared detection reagent B was added and incubated for 30 min at 37 °C. The liquid was then removed again and wells washed five times with washing buffer. Next, 90 μL of substrate solution was added and incubated for 20 min at 37 °C, followed by 50 μL of stop solution. The optical density of each well was then read at 450 nm using a microplate reader.

2.5. Dual-energy X-ray absorptiometry

Bone mineral density (BMD) was determined by dual X-ray absorptiometry. Animals were scanned using a dual X-ray absorptiometry machine and data was collected by a computer. We analyzed the following areas: whole body, thighbone, arm bone, epiphyseal of the proximal thighbone, the epiphyseal of the distal thighbone, the fourth and fifth vertebra lumbalis, and the head.

2.6. Micro-CT analysis

The microarchitecture of the femora was analyzed by micro-CT. Femora specimens were scanned and the data was analyzed to determine a range of trabecular parameters, including trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th) and trabecular bone pattern factor (TBPf).

2.7. Real-time quantitative PCR

The expression of SP, TACR1, CGRP, CALCRL, VIP, VPAC1, VPAC2, NPY, NPY Y1, and NPY Y2 in the brain and femora were determined by reverse transcription real-time quantitative PCR.

Briefly, femora or brain samples were triturated in liquid nitrogen to create a tissue homogenate, which was then reacted with TRIzol.

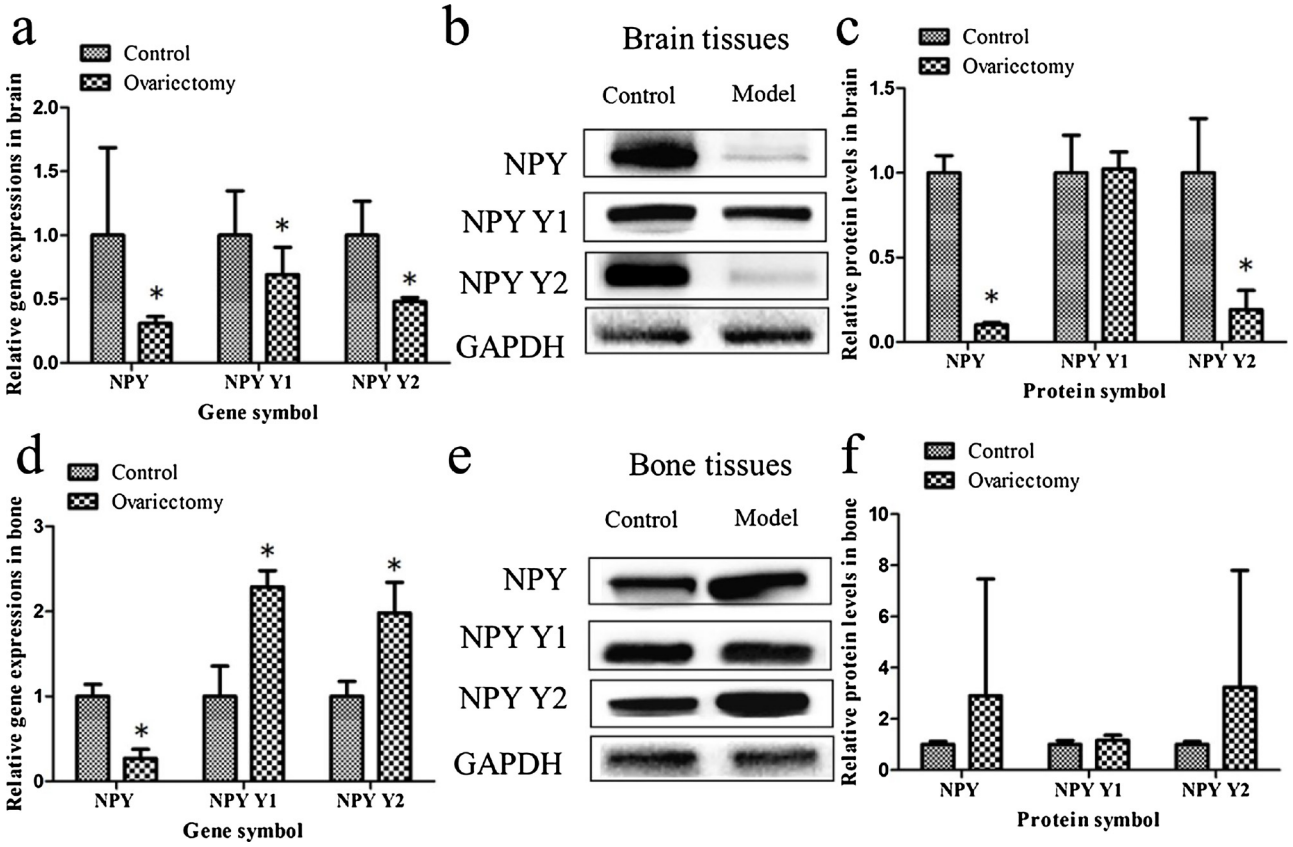

Fig. 7. Effects of ovariectomy on the expression of NPY-related targets. The gene expression and protein levels of NPY, NPY Y1 and NPY Y2 in the brain and femora were detected by real-time quantitative PCR and western blotting. The gene expression of NPY and NPY Y2 in the brain decreased with their corresponding protein levels after ovariectomy. The gene expression of NPY Y1 decreased in the brain of the ovariectomy group but showed no significant difference when compared between the two groups (Fig. 7a–c). For bone tissue, the gene expression of NPY, NPY Y1, and NPY Y2 all increased in the ovariectomy group, while their corresponding protein levels showed no significant difference (Fig. 7d, c, f). Control group *vs.* ovariectomy group $^*P < 0.05$.

Table 2
The effect of ovariectomy on expression of SP-related targets.

Target	Gene expression	Protein level
Brain SP	Decrease	Not significant
Brain TACR1	Decrease	Decrease
Bone SP	Decrease	Decrease
Bone TACR1	Increase	Increase

Table 3
The effect of ovariectomy on the expression of CGRP-related targets.

Target	Gene expression	Protein level
Brain CGRP	Decrease	Decrease
Brain CALCRL	Decrease	Decrease
Bone CGRP	Not significant	Not significant
Bone CALCRL	Increase	Decrease

Table 4
The effect of ovariectomy on the expression of VIP-related targets.

Target	Gene expression	Protein level
Brain VIP	Not significant	Not significant
Brain VPAC1	Increase	Not significant
Brain VPAC2	Increase	Not significant
Bone VIP	Decrease	Decrease
Bone VPAC1	Not significant	Not significant
Bone VPAC2	Decrease	Decrease

Table 5
The effect of ovariectomy on the expression of VIP-related targets.

Target	Gene expression	Protein level
Brain NPY	Decrease	Decrease
Brain NPY Y1	Decrease	Not significant
Brain NPY Y2	Decrease	Decrease
Bone NPY	Decrease	Not significant
Bone NPY Y1	Increase	Not significant
Bone NPY Y2	Increase	Not significant

Chloroform (20% v/v) was then added to the solution for 5 min and then centrifuged (12,000 g) for 5 min at 4 °C. Then, 0.5 ml of the supernatant liquid was added to a new EP tube and isopropyl alcohol (0.25 ml) added. The solution was then mixed for 10 min and centrifuged (12,000 g) for 15 min at 4 °C to precipitate total RNA. Precipitated RNA was then washed twice with 75% ethanol and dissolved in DEPC-treated water (20 μl). The samples were always kept on ice during experiments.

cDNA was obtained by reverse transcription of the total RNA. In brief, reverse transcription was performed in a 96-well plate and the reverse transcription enzymatic reaction was carried out as follows: total RNA (10 μl), PrimeScriptRT Enzyme Mix I (1 μl), RT Primer Mix (4 μl), 5 × PrimeScript Buffer II (4 μl), and RNase Free dH2O (1 μl). Temperature cycling conditions were as follows: 42 °C for 15 min, 85 °C for 5 s, and 4 °C for preservation.

Subsequently, the cDNA was used in real-time quantitative PCR to determine the gene expression of SP, TACR1, CGRP, CALCRL, VIP, VPAC1, VPAC2, NPY, NPY Y1, NPY Y2, and GAPDH. Primer sequences

are shown in Table 1. In brief, real-time quantitative PCR reaction was performed in a 96-well plate using the following reactants: 2 × SYBR green master-mix diluted gene primers (10 μl), cDNA (2 μl, 30 ng), forward primer (1 μl, 0.5 μM), reverse primer (1 μl, 0.5 μM), and DEPC-treated water (6 μl). The quantitative analysis procedure was as follows: 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 30 s, with a total of 40 cycles. The fluorescence signal was detected at the end of the second step of each cycle.

Samples were analyzed in triplicate and gene expression was normalized against the GAPDH housekeeping gene, which was used as a control. Gene expression was calculated using the △△Ct quantification approach.

2.8. Western blotting

The protein levels of SP, TACR1, CGRP, CALCRL, VIP, VPAC1, VPAC2, NPY, NPY Y1, and NPY Y2 were determined in the brain and femora by western blotting.

In brief, femora or brain samples were triturated in liquid nitrogen to create a tissue homogenate and were then reacted with RIPA lysis buffer. Total protein concentrations were then determined using the BCA assay. Samples were then diluted to an appropriate concentration using RIPA lysis buffer.

Protein samples (30 μg) were separated by 12% dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto 0.2 μm polyvinylidene difluoride membranes, which were then blocked with blocking buffer (5% skimmed milk powder diluted in TBST). Membranes were incubated sequentially with primary antibodies overnight (1:1000 dilution in BSA) and secondary antibodies (1:3000 dilution in BSA) for 1–2 h and were subsequently washed and rinsed with ECL detection reagents. Chemiluminiscence bands were captured by a gel imaging system and the images quantified by Quantity One® analysis software.

Samples were detected in triplicate and protein expression was normalized against the GAPDH housekeeping protein as a control.

3. Results

3.1. Serum estrogen, CTX-1 and P1NP levels

Sixteen weeks after bilateral oophorectomy, serum estrogen levels in rats from the ovariectomy group were significantly lower than that of rats undergoing sham surgery, indicating that ovariectomy effectively reduced the levels of estrogen in the body and mimicked menopause. Ovariectomy also remarkably increased CTX-1 levels in serum, but failed to affect serum P1NP. (Fig. 1).

3.2. Bone mineral density

BMD in the thighbone, arm bone, the epiphyseal of the proximal thighbone, the epiphyseal of the distal thighbone, and the fourth and fifth vertebra lumbalis area fell dramatically after bilateral oophorectomy, indicating that ovariectomy effectively reduced bone mass and mimicked postmenopausal osteoporosis. BMD in the whole body and head area showed no significant difference between the two groups, indicating that areas where the skeleton metabolized more actively lost bone mass quicker (Fig. 2).

3.3. Trabecular parameters

Trabecular number and thickness decreased significantly in the ovariectomy group, while separation increased. This indicated that ovariectomy lead to an impairment of the cancellous and cortical bone microarchitecture. An increase of TBPf, indicating the translation of trabecular from clintheriform to rhabditiform bone, and thus osteoporosis, was also demonstrated in rats from the ovariectomy group (Fig. 3).

3.4. The expression of Sp-related targets

The expression of SP in the brain decreased but without a corresponding decrease of SP protein in the brain. However, the reduced gene expression of TACR1 in the bone, along with the increased expression of SP and TACR1 in the bone, were all seen concomitant with their corresponding protein, indicating that ovariectomy affected the regulation of brain TACR1, bone SP, and bone TACR1 by affecting their expression (Fig. 4).

3.5. The expression of CGRP-related targets

The expression of CGRP and CALCRL genes in both the brain and bone decreased along with their corresponding proteins after ovariectomy. The expression of CGRP in the bone showed no significant difference when compared between the two groups. Interestingly, the expression of the CALCRL gene in bone increased while the level of CALCRL protein decreased. This result revealed that ovariectomy is probably associated with the regulation of CGRP in the brain (Fig. 5).

3.6. The expression of VIP-related targets

The gene expression and protein levels of VIP in the brain showed no significant difference when compared between the two groups, while the gene expression of APAC1 and APAC2 in the brain increased in the ovariectomy group but without any difference in their corresponding proteins. In bone tissue, VIP and VPAC2 proteins decreased as their gene expression decreased, while VPAC1 protein levels and gene expression showed no significant difference. Data revealed that ovariectomy is probably associated with the regulation of bone VIP and VPAC2 (Fig. 6).

3.7. The expression of NPY-related targets

The gene expression of NPY and NPY Y2 in the brain decreased with their corresponding protein levels after ovariectomy. The gene expression of NPY Y1 decreased in the brain in the ovariectomy group but showed no significant difference when compared between the two groups. For bone tissue, the gene expression of NPY, NPY Y1, and NPY Y2 all increased in the ovariectomy group, while their corresponding protein levels showed no significant difference. Data revealed that ovariectomy is potentially associated with the regulation of brain NPY and NPY Y2 (Fig. 7).

4. Discussion

The menopause transition and aging are directly or indirectly associated with an increase in a wide range of health risk factors including diseases such as osteoporosis. In the present study, we used the ovariectomized rat as a pathological model to mimic postmenopausal woman with osteoporosis.

Our data revealed that ovariectomy clearly suppressed estrogen levels in the circulatory system. The deficiency of estrogen led to a series of disorders in bone metabolism. Serum detection also showed ovariectomy significantly increased CTX-1, a biomarker of bone resorption [27], but failed to affect in PNIP, a biomarker of bone formation [28], indicating that the deficiency of estrogen impacted bone chiefly by promoting osteoclast. Following surgery, the BMD fell dramatically in areas in which the skeleton metabolism was relatively active. Micro-CT analyses reconfirmed that, in the ovariectomy group, the femora was losing bone mass because the trabecular in the femora suffered from a considerable decline in quantity and thickness with a translation from a clintheriform to a rhabditiform type. We considered that the change in skeletal type was triggered by estrogen deficiency

and that the specific approach of estrogen deficiency was associated with neuropeptide regulation. The influence of certain regulatory peptides on bone metabolism can be predicted but have not been studied in detail. In the present study, we quantified four bone-related neuropeptides and their correlative targets in order to explore their roles in postmenopausal osteoporosis.

The first neuropeptide we considered was SP. The effect of SP on bone is generated by combination with its receptor, TACR1. SP nerves are widely distributed in brain and bone and SP is a critical element in bone pain perception and inflammation [29]. In the present study, ovariectomy reduced the levels of brain TACR1, probably because of the negative modulation caused by osteoporotic inflammation. TACR1 exists in bone marrow mesenchymal stem cells, from which osteoblasts originate from, and in bone marrow macrophage cells, from which osteoclasts stems originate from. A previous study found that an SP signal cannot only stimulate the proliferation of pre-osteoblasts but can also promote osteoclastogenesis by receptor activation of the nuclear factor kappa B ligand (RANKL), which is activated by combination with SP-TACR1 [30]. We considered that estrogen deficiency increased the rate of bone turnover by boosting the SP signal and that the increase in bone TACR1 in our study was caused by feedback related to changes in bone SP (Table 2).

The second neuropeptide we considered was CGRP. The CGRP signal is mediated by CALCRL, which is found on the surface of osteocytes. CGRP can promote osteogenesis *via* the cAMP/protein kinase A pathway [31] and inhibit osteoblast apoptosis by activating the Wnt/β-catenin signaling pathway [13]. On the other hand, CGRP can suppress osteoclasts by inhibiting the RANK-mediated downregulation of osteoclast gene expression [32]. CGRP has been proven to be beneficial for the repair of impaired bone. As a result, CGRP signals can directly influence bone cells and exert a positive effect on bone mass. We found that ovariectomy damaged the skeleton by reducing CGRP signals. Interestingly, the gene expression of CALCRL in bone increased, while the level of CALCRL protein decreased, thus indicating there must be other forms of regulation involved in the translation of CALCRL in bone. Similar to SP, CGRP is also related to pain perception [33]. We considered that bone pain caused by osteoporosis was also associated with a reduction in CGRP (Table 3).

The third neuropeptide we considered was VIP. This neuropeptide is a polypeptide that belongs to the glucagon/secretin superfamily [34] and is expressed in some internal organs and the neurons of the central nervous system [35,36]. VIP plays essential roles in a broad spectrum of biological functions including bone metabolism [35]. VIP can modulate the skeleton by promoting neuroendocrine-immune communication and influencing the capillaries in bone [37]. VPAC1 and VPAC2 are receptors for VIP and exist in both the central and peripheral nervous system [38]. VIP can also directly affect bone cells. For osteoblastic activity, VIP stimulates ALP and increases calcium accumulation in bone nodules [39,40]; for osteoclastic activity, VIP inhibits osteoclast formation [41,42]. The present study showed that ovariectomy did not make a difference in terms of brain VIP and its receptors, but suppressed VIP and VPAC2 in bone, which impaired the skeleton, thus indicating that ovariectomy affected bone metabolism through local VIP signals (Table 4).

The final neuropeptide we considered was NPY. This is one of the most abundant neuropeptides in the mammalian brain and exerts a variety of physiological processes *via* its receptor [43]. NPY Y1 and NPY Y2 are the two most common receptors for NPY. In particular, NPY Y2 is the most abundant Y subtype receptor in the central nervous system and is widely distributed in many areas of the brain including the hypothalamus, hippocampus, brain stem, amygdala and lateral septum [44–46]. At the level of the central nervous system, NPY exerts its function in bone homeostasis through the hypothalamic Y2 receptor [47]. Our present study demonstrated that ovariectomy suppresses NPY and NPY Y2 in the brain to attain negative regulation of the skeleton. Conversely, although a previous study found a regulatory effect of NPY signals upon bone cells [48], the present study observed no significant difference between the control group and the ovariectomy group, indicating that estrogen deficiency will not affect local bone NPY regulation (Table 5).

In conclusion, ovariectomy led to a reduction of TACR1, CGRP, CALCRL, NPY, NPY Y2 in the brain, and increased TACR1 but decreased SP, CALCRL, VIP, VPAC2 in the bone. The effect of estrogen deficiency caused by ovariectomy on bone is associated with the regulation of SP, CGRP, VIP, and NPY. This study, ranging from central control to local bone regulation, with a relatively higher number of detected targets, revealed a relatively holistic modulation mechanism of neuropeptides in the bone regulation system. Our novel results are of great significance in the development of neuropeptide targets as medicines for osteoporosis.

However, although the regulatory function of SP, CGRP, VIP, and NPY in bone metabolism has been proven, many aspects of the mechanisms involved remain unclear. Crosstalk and collateral connections of different signaling mechanisms make the regulatory system even more complicated and may obscure the single effects of a specific neuropeptide. It will therefore be critical to gain a better understanding of how these neuropeptide signals interact or correlate with each other in the regulation of bone metabolism and how hormones impact upon the development and function of these modulation circuits, such as feedback loops.

Author contributions

Ronghua Zhang directed the experiment; Xiaofeng Zhu conceived and designed this study; Li Yang managed and conducted the laboratory work; Hengrui Liu, Yingquan Xiong, Chaopeng Wang fed the animals and conducted the surgery, Xiaoguang Liu performed the gene and protein quantification; Xiaoguang Liu and Hengrui Liu also analyzed the data and drafted the manuscript; all the authors revised the paper.

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Conflicts of interest

The authors declare that this research was undertaken in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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