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Vitamin D/VDR regulates peripheral energy homeostasis via central renin-angiotensin system

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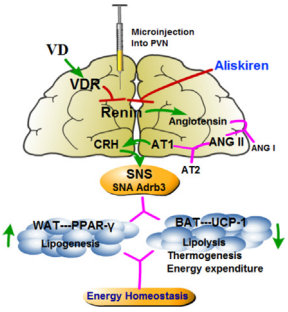
GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Some epidemiological studies have revealed that vitamin D (VD) deficiency is closely linked with the prevalence of obesity, however, the role of VD in energy homeostasis is yet to be investigated, especially in central nervous system. Given that VD negatively regulates renin in adipose tissue, we hypothesized that central VD might play a potential role in energy homeostasis.
Objectives: The present study aims to investigate the potential role of VD in energy homeostasis in the CNS and elaborate its underlying mechanisms.
Methods: This study was conducted in *Cyp27b1*$^{-/-}$ mice, VD-treated and wild-type mice. After the intraventricular injection of renin or its inhibitors, the changes of renin-angiotensin system (RAS) and its down-stream pathway as well as their effects on metabolic rate were examined.
Results: The RAS activity was enhanced in *Cyp27b1*$^{-/-}$ mice, exhibiting a increased metabolic rate. Additionally, corticotropin-releasing hormone (CRH), a RAS-mediated protein regulating energy metabolism in the hypothalamus, increased significantly in *Cyp27b1*$^{-/-}$ mice. While in VD-treated group, the RAS and sympathetic nerve activities were slightly inhibited, hence the reduced metabolic rate.
Conclusion: Collectively, the present study demonstrates that the VD/vitamin D receptor (VDR) has a significant impact on energy homeostasis through the modulation of RAS activity in the hypothalamus, subsequently altering CRH expression and sympathetic nervous activity.

Introduction

Energy balance depends on a delicately modulated homeostasis system. A disruption of energy homeostasis, such as extra calorie intake or reduced energy expenditure, may contribute to obesity and other metabolic disorders [1]. The central nervous system (CNS), particularly the hypothalamus, plays an integral role in

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maintaining energy homeostasis. Environmental and internal signals are integrated in the CNS through sensing changes in the concentrations of nutrients and hormones, then subsequent physiological actions are coordinated by the CNS [2]. Corticotropin-releasing hormone (CRH) is primarily generated in the paraventicular nucleus (PVN) of the hypothalamus. It is demonstrated that intracerebroventricular (ICV) injection of CRH inhibits food intake [3] and promotes energy expenditure [4]. Moreover, CRH is closely involved in a regulatory signaling pathway of thermogenesis in brown adipose tissue (BAT) and lypolysis in white adipose tissue (WAT) [4]. These effects are modulated by boosting CRH expression in hypothalamus, subsequently promoting sympathetic release of norepinephrine (NE) and activating sympathetic nervous system (SNS) [5,6]. The function of CRH in the mediation of energy homeostasis remains to be experimentally confirmed.

Growing incidence of obesity and its metabolic consequences have gained worldwide attraction. In addition to maintaining calcium homeostasis and skeletal health [7,8], vitamin D (VD) deficiency is closely associated with cardiovascular diseases, inflammation, and cancer [9–12]. Additionally, it is shown that vitamin D deficiency may involve in the pathogenesis of a certain number of metabolic disorders, including obesity, type 2 diabetes and metabolic syndrome [13–15]. Animal studies *in vivo* have revealed that mice lack of vitamin D receptor (VDR) display a lean phenotype with reduced body weight, decreased mass of white adipose tissue, promoted rate of β-oxidation, and enhanced energy expenditure [16]. Notably, uncoupling protein 1 (UCP1) expression in white adipose tissue (WAT) of $VDR^{-/-}$ mice is considerably elevated [17]. Conversely, over expression of VDR in WAT is associated with reduced lipolysis and β-oxidation, resulting in obesity in transgenic VDR mice [18]. It is noted that $1,25(OH)_2D_3$, an active form of vitamin D, inhibited adipogenesis by suppressing CCAAT enhancer-binding proteins (C/EBPα) and peroxisome proliferators-activated receptor γ (PPARγ) expression on certain targets genes in mouse 3T3-L1 preadipocytes *in vitro* [19]. The discrepancy of results between the *in vivo* and *in vitro studies* requires further investigation. Previous studies have mostly concentrated on the peripheral actions of vitamin D in adipose tissue. However, the impact of vitamin D on energy homeostasis in the CNS is yet to be investigated.

The renin-angiotensin system (RAS) is widely acknowledged for its multiple roles in cardiovascular physiology [20]. Accumulating evidence has showed that the sustained inappropriate RAS activity could result in hypertension, heart attack, and stroke [21,22]. Recently, the RAS has emerged as a fundamental regulator of energy metabolism [23–25]. Grobe et al. have demonstrated that triggering of the brain RAS by transgenic activation or ICV administration of angiotensin (ANG) increases the resting metabolic rate in angiotensin II type I receptor (AT1)-dependent manner [26]. ANG II has various actions on different organs, such as the brain, heart, kidney, and adrenal glands, to modulate the blood pressure, electrolyte and extracellular volume balance. The levels of CRH and sympathetic nervous activity are remarkably enhanced after the ICV administration of ANG II [27,28]. ANG II exerts its functions by binding to several receptors, including AT1 and angiotensin type II (AT2) receptors. AT1 receptor is the major target of most of the known physiologic actions of ANG II, including water consumption and vesoconstriction. Activation of AT1 in the brain triggers the energy expenditure by promoting resting metabolic rate (RMR) [24]. Zanchi et al. [29] have demonstrated that AT1 receptor blocker (ARB) telmisartan could prevent the weight gain through a decrease in food intake. In the AT1 knockout mice, promoted metabolic rate leads to a reduced weight gain and adipose deposition after high-fat feeding [30]. It is also demonstrated that AT1 stimulates CRH expression in the PVN in the hypothalamus, resulting in decreased food intake and increased energy expenditure [31].

Interactions between vitamin D and RAS activity have long been studied. Numerous studies have demonstrated the impact of vitamin D on RAS activity. Forman et al. [32] have reported that low levels of plasma 25-hydroxyvitamin D up-regulates the RAS activity in chronic kidney disease patients, resulting in elevated circulating levels of plasma renin activity and ANG II. 1,25-$(OH)_2D_3$ mitigates blood pressure and cardiac hypertrophy in patients with cardiorenal syndrome by inhibiting RAS activity [33]. Several mechanistic studies have confirmed vitamin D to be a negative endocrine regulator of the RAS [34,35]. Activation of VDR down-regulates the RAS activity and suppresses the renin synthesis [32]. VDR knockout mice exhibit enormously elevated blood pressure, renin activity and circulating plasma ANG II levels [34]. $1,25(OH)_2D_3$ down-regulates the transcription of renin gene through the blockade of cyclic adenosine monophosphate response element activity in the renin gene promoter[36]. It is demonstrated that VD deficiency may contribute to a decreased transcription of VDR and a boosted degradation of unliganded VDR, resulting in a reduction in both unliganded and liganded VDR. The decrease of liganded VDR facilitates the renin activity, while the reduction of unliganded VDR may promote the RAS activity through AT1 receptor [37]. Given that, VDRs are found to be expressed in multiple tissues, including certain neurons in the hypothalamus [38]. Therefore, we hypothesize that not just may VD/VDR play a potential role in energy homeostasis in adipose tissues, but also partially via the negative regulation of the RAS in the hypothalamus. The present study aims to investigate the potential role of VD in energy homeostasis in the CNS and elaborate its underlying mechanisms.

Methods

Animal studies

ICR mice, aged four weeks, were obtained from Changsheng Biotechnology Co., Ltd. (Liaoning, China) and housed in the Experimental and Animal Center in Shengjing Hospital of China Medical University. The mice were fed with water and chow diet on a 12-hour light/dark cycle. Global *Cyp27b1* knockout mice on an ICR background were used in our study. These mice are lack of active vitamin D, thereafter are characterized by vitamin D deficiency. All experiment protocols adopted in our present study were to minimize animal suffering. The ethic certificate was approved by the Animal Experiment Committee of Shengjing Hospital of China Medical University, Shenyang, China (2017PS267K). The genotype of *Cyp27b1* gene knockout mice and their wild-type littermates was confirmed using common PCR-based genotyping.

Eight to ten week old male $Cyp27b1^{-/-}$ mice and their littermates were enrolled in our study. The wild-type (WT) mice were randomized into either the WT group or VD intervention (WTD) group (n = 10). The $Cyp27b1^{-/-}$ mice were collected in the knockout (KO) group (n = 10). The mice in WTD group were treated by the vitamin D supplements cholecalciferol cholesterol emulsion (CCE) in their drinking water (CCE: water = 10 μl: 100 ml) for two weeks. Half of the mice in each group were chose as control groups and were ICV-injected with 1 μl PBS for seven consecutive days. The other half of the mice in each group were ICV-injected with 1 μl renin (0.2×10^{-3} μg/μl) for seven consecutive days accordingly as described[39]. In addition, the WT group was further divided into two groups, one group named WTA received the ICV-injection of aliskiren (0.2 mg/ml, 1 μl/d) for seven days in a row, which was a renin inhibitor purchased from R&D system, USA, while the other group was ICV-injected with 1 μl PBS instead.

Same measures were carried out in WTD group and KO group [40]. Body weight, rectal temperature, food intake, and water intake were recorded at 3 pm (basal metabolic state). The volume of O_2 consumption, CO_2 production, and metabolic rate were examined for 24 h using a comprehensive lab animal monitoring system. Blood pressure, heart rate, and sympathetic nerve activity were recorded before sacrifice. The hypothalamus, brown adipose tissue and white adipose tissue were harvested for further analysis.

Real time PCR assay

Total RNA either from white adipose tissue or brown adipose tissue was isolated using TRIzol (Invitrogen) and reverse transcribed into cDNA by an RT reagent kit (Takara, RR047A) according to the manufacturer's instructions. Quantitative real-time PCR was conducted using SYBR Premix Ex TaqⅡ (Takara, RR8320A) on an ABI 7500 Fast sequence detector system (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method as described previously. The sequences of primers used in our study were showed in supplemental Table 1.

Western blotting

Protein was isolated from multiple tissues using a Minute total protein extraction kit (Invent, SD001) according to the manufacturer's instructions. Protein concentrations were determined using the bicinchonininc acid (BCA) method. Western blotting was carried out according to standard procedures. The following primary antibodies were purchased from Proteintech, USA: anti-AT1 (66415-1-lg, 1:1000), anti-CRH (10944-1-AP, 1:1000), anti-UCP1 (23673-1-AP, 1:1000), anti-PPAR gamma (16643-1-AP, 1:1000), and anti-GAPDH (60004-1-lg, 1:1000). The anti-PGC-1 alpha (PA5-38022, 1:1000) primary antibody was purchased from Thermo Fisher Scientific, USA. The following primary antibodies were purchased from Santa Cruz Biotechnology (CA, USA): anti-VDR (sc-1008, 1:1000), anti-angiotensin (N-10) (sc-7419, 1:500), anti-renin (N-19) (sc-22671, 1:500), anti-renin receptor (K-19) (sc-55025, 1:500), and anti-AT2 (H-143) (sc-9040, 1:500). Protein blots were developed using Immobilon Western Chemilum HRP Substrate (Millipore, WBKLS05000).

Indirect calorimetry

Mice were maintained using a comprehensive lab animal OxyletPro system (Panlab, Metabolism 3.0). After allowing the animals to adapt to the environment, oxygen consumption and carbon dioxide production were continuously measured for 24 h according to the manufacturer's instructions as described previously [41].

Immunohistochemistry staining

Immunohistochemistry staining was carried out as described previously [42]. Briefly, brain coronal sections of 20 μm were cut using a frozen microtome (Leica Microsystems, Germany) and then incubated with primary antibodies against CRH (10944-1-AP, Proteintech, USA) and renin (sc-22671, Santa Cruz, USA) as described [42]. The antibodies were diluted at a ratio of 1:200. Images were acquired using a fluorescence microscopy (Nikon, Japan).

ICV administration experiments

Initially, a sterilized stainless-steel cannula was precisely implanted into the right lateral brain vertical (−0.5 mm anterior and 1.0 mm lateral relative to bregma and 2.5 mm below the surface of the skull). After 7-day recovery, 1 μl of renin (0.2×10^{-3} μg/μl) solution or PBS was injected once daily for 7 days as described [41]. The ICV-injection of aliskiren (0.2 mg/ml, 1 μl/d) for seven days in a row was also performed.

Enzyme linked immunosorbent assay (ELISA)

Serum ANG II levels were determined using a mouse angiotensin II enzyme linked immunosorbent assay (ELISA) kit (CSB-E04495m)(CUSABIO BIOTECH CO.,LTD, USA) according to the manufacturer's instructions.

Sympathetic nerve activity

Sympathetic nerve recordings were performed as described previously [26].

Chromatin immunoprecipitation (CHIP) assay

CHIP assays were performed as described previously [43]. The SimpleCHIP® Plus Sonication Chromatin IP Kit (Cell Signaling Technology, Boston, MA, USA) was used strictly according to the manufacturer's instructions. Briefly, smashed hypothalamus tissue from control group and WTD group, which was pretreated by supplying cholecalciferol cholesterol emulsion (CCE) in their drinking water (CCE: water = 10 μl: 100 ml) for two weeks, was fixed with 1% formaldehyde for 10 min at room temperature, then cells were washed three times with PBS and quenched with 0.125 M glycine. Chromatin was digested and sonicated to yield DNA fragments of 200–500 bps. After preclearing with protein A/G argrose at 4 °C for 1 h, samples were incubated with 10 μg of anti-VDR antibody (sc-1008, Sant Cruz, USA), hystone h3 (positive control) or total IgG (negative control) with spin columns at 4 °C overnight. 15 μl of VDR anti-VDR antibody, 10 μl of hystone h3 and 1–2 μl of IgG were used in each tube for incubation. The crosslink was reversed by incubating the samples in a 5 M NaCl and proteinase K solution at 65 °C for 2 h. The enrichment of particular sequences during immunoprecipitation were determined using agarose gel electrophoresis and quantitative real-time PCR as described[43]. The agarose gel electrophoresis conditions were as follows: 110 V for 30 min. The real-time PCR reaction was programmed as follows. The Initial denaturation lasted for 30 s at 95 °C. During the second stage, the cycle was repeated 40 times at 95 °C for 3–10 s and at 60 °C for 10–30 s. At the melting curve stage, it was programmed at 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. The primer sequence used to identify renin promoter was: Forward:5′-GTTGAGGAGC CAAGCATTTG-3′; Reverse:5′-CCACACTGCCAGTTTACCCT-3′.

Assessment of blood pressure and heart rate

All mice were subjected to the intraperitoneal injection of anesthetic drugs. When vital signs became stable, the mice were placed on a operating table and connected to three electrodes: one on the left foreleg, one on the right foreleg, and one on the right hind leg. A MedLab biological signal acquisition and analysis system (MedLab-U, Beijing Zhongshidichuang Co. Ltd.) was utilized to measure and record the electric signal of the mice. A small incision was made in the neck of the mice for tracheostomy and carotid artery cannulation. The other end of the cannula was connected to a small animal ventilator (DW-3000B, Beijing Zhongshidichuang Co. Ltd.). Afterwards, the carotid artery was separated and connected to the pressure transducer (PY-2, Beijing Zhongshidichuang Co. Ltd.) of the data acquisition system. The pressure–volume (PV) catheter was placed in the left ventricle via an apical stab approach. The ventilator was then suspended during continuous recording of the left ventricular pressure-conductance volume signal to alter venous return. Heart rate was also recorded with a MedLab biological signal acquisition and analysis system.

Statistical analysis

Statistical analysis was carried out using Prism 6.0 software (GraphPad). All values were expressed as the mean ± SD. Differences between groups were calculated using performed by either the unpaired Student's *t* test or one-way ANOVA followed by the Student-Newman-keils (SNK) test. Difference was defined as significant when p-value is <0.05.

Results

Cyp27b1$^{-/-}$ mice exhibited a lean phenotype with increased energy expenditure

To investigate if VD/VDR exerts an impact on energy metabolism, we first compared the body size of wild-type and *Cyp27b1$^{-/-}$* mice, and found that the *Cyp27b1$^{-/-}$* mice were considerably smaller than the wild-type mice (Fig. 1A). Consistent with body size, body weight was also compared between wild-type and *Cyp27b1$^{-/-}$* mice, indicating a lean phenotype for *Cyp27b1$^{-/-}$* mice (Fig. 1B). Furthermore, *Cyp27b1$^{-/-}$* mice had significantly less body fat content and more lean mass (Fig. 1C–D). Taken together, the data mentioned above indicated that *Cyp27b1$^{-/-}$* mice exhibited a lean phenotype and less fat mass.

In order to study the potential causes of fat loss in *Cyp27b1$^{-/-}$* mice, we continuously monitored the rectal temperature of *Cyp27b1$^{-/-}$* mice and found that it was significantly higher than that of wild-type mice (Fig. 1E). Surprisingly, the food intake showed no great difference in between the *Cyp27b1$^{-/-}$* mice and wild-type mice (Fig. 1F). Additionally, consistent with our previous study [35], we also found higher water consumption volume in *Cyp27b1$^{-/-}$* mice (Fig. 1G). In accordance with previous studies, we also found that adipocyte size in *Cyp27b1$^{-/-}$* mice was much smaller than that in wild-type mice (Fig. 1F). As we expected, their energy expenditure was higher in *Cyp27b1$^{-/-}$* mice, as measured

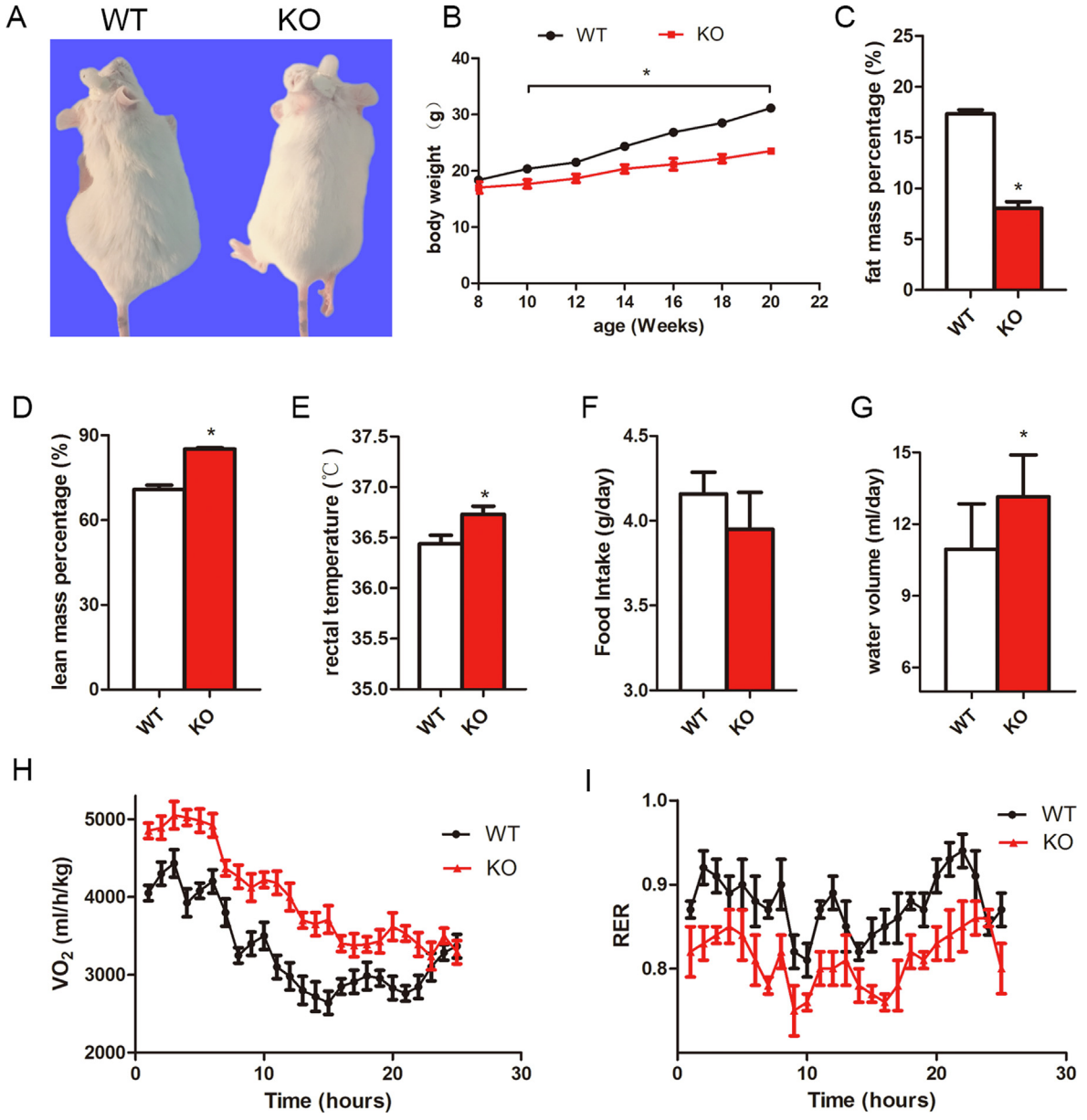

Fig. 1. *Cyp27b1$^{-/-}$* mice exhibit a lean phenotype with increased energy expenditure. (A) Gross morphology; (B) Body weight; (C) Body fat mass; (D) Lean mass; (E) Average basal rectal temperature; (F) Food intake; (G) Water intake; (H) Oxygen consumption; (I) Respiratory exchange ratio (RER). WT: wild-type mice group; KO: knockout mice group. Values are the mean ± s.e.m (n = 5–7 rats in each group), *P < 0.05.

by increased oxygen consumption and a lower respiratory exchange ratio (RER) (Fig. 1H, 1I).

Moreover, we found that the WAT cell volume was significantly smaller in *Cyp27b1*$^{-/-}$ mice (Fig. 2A). The mRNA levels of fatty acid synthase (*FASN*) were lower in the white adipose tissue of *Cyp27b1*$^{-/-}$ mice, suggesting lower levels of adipogenesis in *Cyp27b1*$^{-/-}$ mice (Fig. 2B). Conversely, mRNA levels of hormone sensitive lipase (*HSL*) were highest in the white fat of *Cyp27b1*$^{-/-}$-mice, indicating higher levels of adipolysis in *Cyp27b1*$^{-/-}$ mice (Fig. 2C). In contrast, the brown adipocyte volume in *Cyp27b1*$^{-/-}$ mice was much smaller (Fig. 2D). The levels of *Ucp1* mRNA and *Ucp1* protein, which is a well-recognized biomarker for thermogenesis in brown fat, were lower in *Cyp27b1*$^{-/-}$ mice (Fig. 2E, F).

VD-treated mice displayed an obese phenotype with decreased energy expenditure

We analyzed the metabolic parameters in mice treated by cholecalciferol cholesterol emulsion (CCE) for two weeks. Gross morphology, body weight, and fat mass were higher in VD-treated mice, while lean body mass was lower (Fig. 3A–D). Rectal temperature was decreased after the intervention of vitamin D (Fig. 3E). Although VD-treated mice exhibited a larger body size, the food consumption showed no significant difference (Fig. 3F). In addition, lower water consumption was observed in VD-treated mice (Fig. 3G). Furthermore, VD-treated mice showed lower oxygen consumption and a higher RER (Fig. 3H, I). WAT cell volume was significantly larger and the levels of *FASN* were higher, while *HSL* levels were lower in VD-treated mice (Fig. 4A–C). By contrast, BAT cell volume was smaller and *Ucp1* expression was at a lower level (Fig. 4D–E).

VD/VDR inhibited renin expression in the hypothalamus and altered the energy expenditure

We further investigated the activity of renin angiotensin system (RAS) in the central nerve system and found that the renin protein level in the paraventricular nucleus of hypothalamus (PVN) of mice treated with vitamin D was lower than that in *Cyp27b1*$^{-/-}$ mice (Fig. 5A). Moreover, serum ANG II levels were found extremely high in *Cyp27b1*$^{-/-}$ mice (Fig. 5B), indicating renin might play a critical part in the energy homeostasis mediated by VD/VDR in the CNS. In order to further examine the possibility of vitamin D signaling system targeting the RAS in hypothalamus involved in energy consumption, we performed ICV administration of renin, and then measured the changes in energy expenditure. First, we measured VDR protein levels in the hypothalamus at baseline (Fig. 5C). After ICV-renin, body weight was dramatically reduced in all groups of mice (Fig. 5D). The enormously increased energy expenditure was validated by O_2 consumption and increased RER value as well as elevated rectal temperature (Fig. 5E–G). In addition, blood pressure and heart rate were noticeably increased (Fig. 5H, I). These results demonstrated that the ICV injection of renin can significantly enhance the energy expenditure, suggesting a likely role of RAS signaling in the regulation of energy homeostasis induced by VD/VDR.

VD signaling system in the central nervous system regulated energy homeostasis via the RAS pathways

To further explore the potential mechanism of VD signaling system regulating energy balance through renin-angiotensin system, we examined the expression levels of several key components of the renin-angiotensin system in the hypothalamus. In the hypothalamus, AT1, ANG II and renin expression were higher in *Cyp27b1*$^{-/-}$ mice, but lower in vitamin D treated mice. After the ICV injection of renin, AT1, ANG II, and renin levels increased further. However, the extent of increase was less in VD-treated mice than that in *Cyp27b1*$^{-/-}$ mice (Fig. 6A, C). Furthermore, the expression of CRH protein was similar to ANG II (Fig. 6B). Next, we examined serum levels of ANG II, which were elevated significantly after the ICV administration of renin (Fig. 6D). In addition, CHIP assays results demonstrated that the ability of VDR to bind to the renin promoter was significantly lower in the WTD group than that in the WT group (Fig. 6E). Consistently, immuno-histological staining results

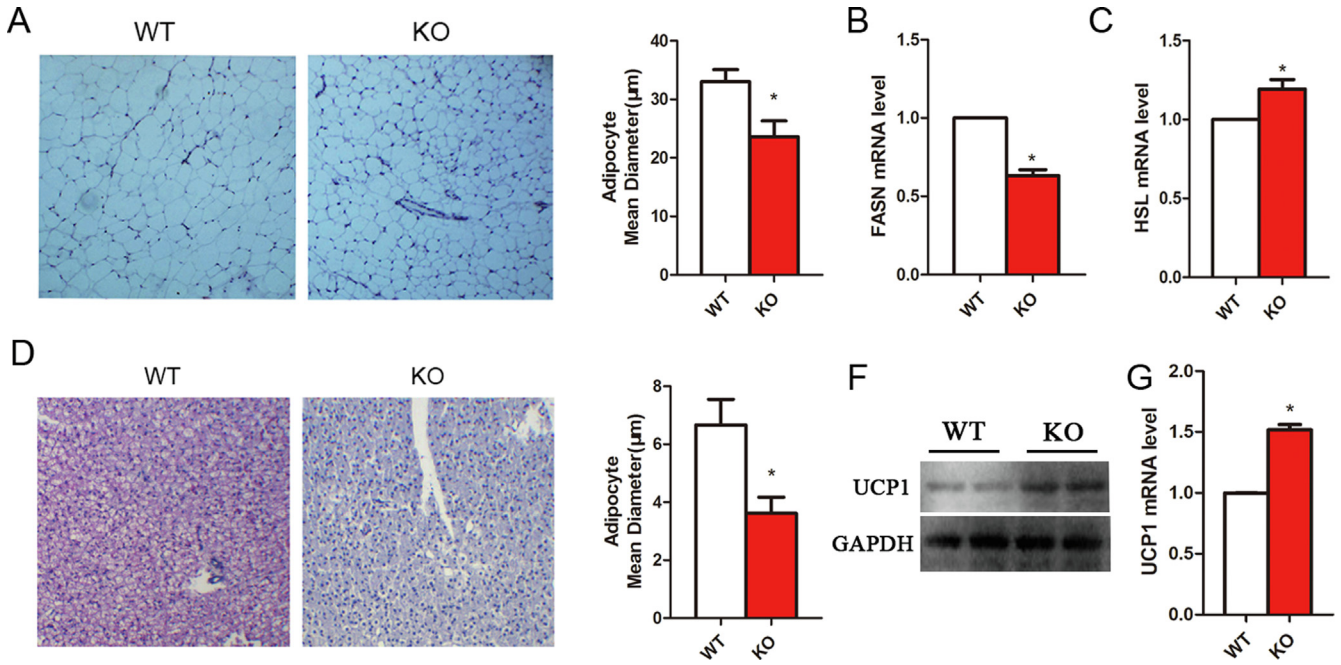

Fig. 2. The phenotype of *Cyp27b1*$^{-/-}$ mice in peripheral adipose tissue. (A) Representative images of H&E staining of abdominal WAT sections (×100); (B) *FASN* mRNA in WAT; (C) *HSL* mRNA in WAT; (D) Representative images of H&E staining of BAT sections (×100); (F) UCP1 protein in BAT; (G) Ucp1 mRNA in BAT. All studies were carried out in male control mice and *Cyp27b1*$^{-/-}$ mice fed a standard diet. WT: wild-type mice group; KO: knockout mice group; WAT: white adipose tissue; BAT: brown adipose tissue; UCP1: uncoupling protein 1; HSL: hormone sensitive lipase; FASN: fatty acid synthase. Values are the mean ± s.e.m (n = 5–7 rats in each group), *P < 0.05.

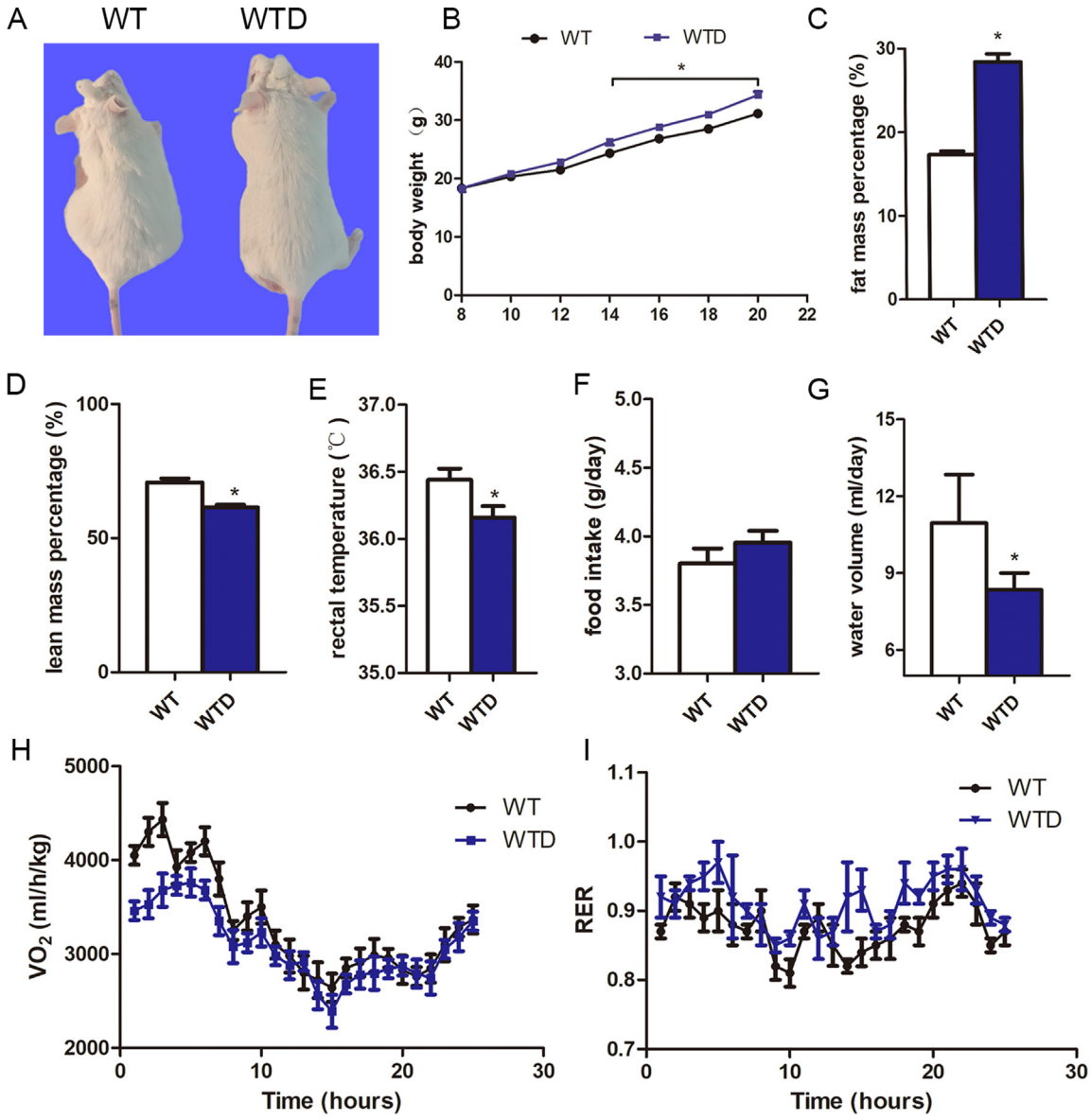

Fig. 3. VD-treated mice exhibit an obese phenotype with decreased energy expenditure. (A) Gross morphology; (B) Body weight; (C) Body fat mass; (D)Lean mass; (E) Average basal rectal temperature; (F) Food intake; (G) Water intake; (H) Oxygen consumption; (I) Respiratory exchange ratio (RER); WT: wild-type mice group. WTD group was treated by supplying cholecalciferol cholesterol emulsion (CCE) in the drinking water (CCE: water = 10 μl: 100 ml) for 2 weeks. KO group was the *Cyp27b1*$^{-/-}$ mice. VD: vitamin D. VO_2: oxygen consumption. Values are the mean ± s.e.m (n = 5–7 rats in each group), *P < 0.05.

showed similar changes, CRH was promoted most in *Cyp27b1*$^{-/-}$ mice after the ICV injection of renin. Collectively, these data demonstrated that VD/VDR may influence energy homeostasis by negatively regulating RAS activities and altering the levels of CRH.

Sympathetic nerve activity (SNA) is able to regulate adaptive thermogenesis, thereby affecting energy metabolism. To assess the SNA changes, we recorded sympathetic nerve activity as previously described [26], sympathetic nerve activity was enhanced following ICV administration of renin and was partially neutralized in VD-treated mice (Fig. 7A). As a result, the mRNA levels of *Adrb3*, indicating sympathetic nerve activity in muscle and fat tissue, were enhanced most in WAT and BAT of *Cyp27b1*$^{-/-}$ mice and were partially blocked by the treatment of VD (Fig. 7B, C). After ICV injection of renin, increased heat production was observed in BAT of *Cyp27b1*$^{-/-}$ mice and vitamin D treatment normalizes this effect in a sense (Fig. 7D). Similar changes were also found in white adipose tissue in *Cyp27b1*$^{-/-}$ mice (Fig. 7E).

To further investigate the effect of vitamin D on RAS activity, aliskiren, a renin inhibitor, was ICV injected into WT, vitamin D treated and *Cyp27b1*$^{-/-}$ mice respectively. Initially, the energy expenditure was markedly decreased, while the respiratory exchange ratio was noticeably elevated following the ICV injection of aliskiren in *Cyp27b1*$^{-/-}$ mice (Fig. 7F–G). Furthermore, the key components of RAS system and CRH were examined in WT, VD-treated and *Cyp27b1*$^{-/-}$ mice the levels of ANG II, AT1 and CRH were reduced following administration of aliskiren (Fig. 7I), indicating targeted regulation of RAS system in the central nervous system plays an important role in regulating energy homeostasis.

Discussion

It is well established that VD is closely associated with energy homeostasis. Numerous studies have attempted to investigate

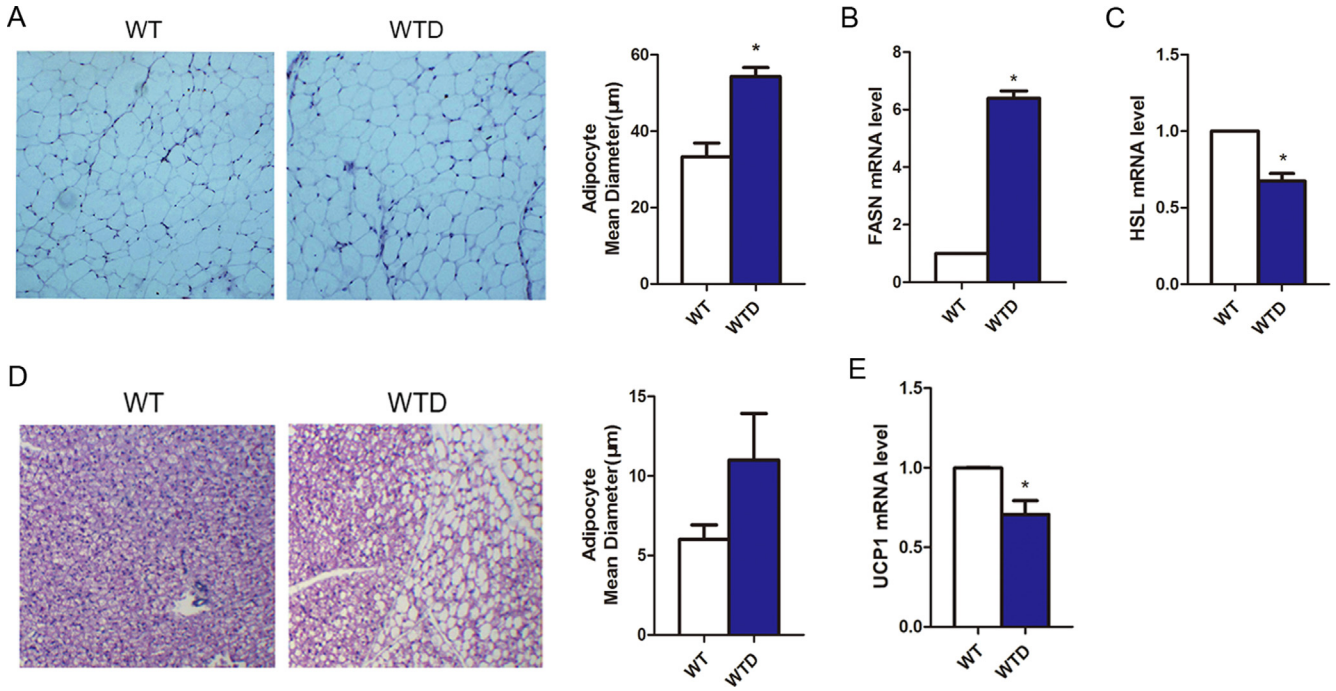

Fig. 4. The phenotype of VD-treated mice in peripheral adipose tissue. (A) Representative images of H&E staining of abdominal WAT sections (×100); (B) *FASN* mRNA in WAT; (C) *HSL* mRNA in WAT; (D) Representative images of H&E staining of BAT sections (×100); (E) UCP1 mRNA in BAT; All studies were carried out in male control mice and VD-treated mice fed a standard diet. WTD group was treated by supplying cholecalciferol cholesterol emulsion (CCE) in the drinking water (CCE: water = 10 μl: 100 ml) for 2 weeks. WT: wild-type mice group; KO: knockout mice group; VD: vitamin D; WAT: white adipose tissue; BAT: brown adipose tissue; UCP1: uncoupling protein 1; HSL: hormone sensitive lipase; FASN: fatty acid synthase. Values are the mean ± s.e.m (n = 5–7 rats in each group), *P < 0.05.

the potential mechanism of VD/VDR in regulating energy homeostasis. In human cells, VD promotes the adipogenesis process in adipocytes, while *in vitro* VD suppresses the same process in 3T3-L1 preadipocytes through regulation of C/EBPα and PPARγ expression [44]. In addition, VD inhibits the process of brown adipocyte differentiation [45]. VDR plays a critical role in the regulation of energy balance via modulating the remodeling of adipose tissue [46]. A number of studies have illustrated that vitamin D elicits an anti-inflammatory impact on adipocytes, resulting in reduced chemokine and cytokine release by adipocytes. However, the effects of vitamin D on energy homeostasis may not only exist in the adipose tissue but also in the CNS. The hypothalamus is a major area in the CNS to regulate the energy homeostasis. Therefore, the focus of our present study is to explore the impact of vitamin D on energy homeostasis in the hypothalamus. The present study, to the best of our knowledge, is the first to shed a light on a novel role of VD/VDR in the regulation of energy homeostasis through the RAS pathway in the hypothalamus.

In the present study, we provide evidence suggesting *Cyb27b1*$^{-/-}$ mice lack of active VD exhibited a lean phenotype compared with wild-type mice. In line with the reduced fat mass, higher rectal temperature and more water consumption were found in *Cyb27b1*$^{-/-}$ mice than that in wild-type mice. However, there was no significant difference in the quantity of food intake. Similar with *Cyb27b1*$^{-/-}$ mice, a lower body weight and reduced white adipose tissue were also observed in *VDR*$^{-/-}$ mice [16,34]. The *Cyb27b1*$^{-/-}$ mice showed increased energy expenditure with higher oxygen consumption. Consistently, the adipocytes in *Cyb27b1*$^{-/-}$ mice were smaller than in wild-type mice, suggesting a reduced lipid accumulation. In addition, compared with the wild-type mice, the expression of *Ucp1*, a hallmark of the thermogenesis in brown adipose tissue, increased considerably in *Cyb27b1*$^{-/-}$ mice, indicating higher levels of thermogenesis and increased metabolic rate in mice. By contrast, VD-treated mice displayed an obese phenotype with decreased energy expenditure. Similar with the phenotype of VD transgenic mice [18], Vitamin D treated mice displayed lower oxygen consumption and a higher RER. Based on the similar amount of food consumption, the increased metabolic rate might result in the decrease of fat mass in *Cyb27b1*$^{-/-}$ mice, whereas the decreased metabolic rate in VD-treated mice might promote the increase of fat mass storage. These results were consistent with previous studies [17,18,46].

VDR, a member of nuclear receptor family, expresses almost ubiquitously. After being activated by $1,25(OH)_2D_3$, VDR exert diverse biological actions [44]. VDR is found to be expressed in multiple tissues, including certain neurons in hypothalamus [38]. High levels of VDR expressions were observed in hypothalamus in *Cyb27b1*$^{-/-}$ mice. Accumulating evidence suggests an essential role of the RAS in the regulation of energy homeostasis [47]. Immunofluorescence staining results also demonstrated that lower renin protein levels were observed in the PVN of the hypothalamus in VD-treated mice. These results reminded us that there might be a cross talk between vitamin D and RAS activity in the central nervous system.

In recent years, the interplay between vitamin D and RAS activity have been investigated extensively. Accumulating evidence has elucidated the impact of vitamin D on RAS activity. Initially, clinical studies have demonstrated that the blood pressure was attenuated in patients with primary hypertension by vitamin D supplement intake. The reduced serum levels of renin and ANG II were observed. Recently, a cohort study revealed that low levels of plasma 25-hydroxyvitamin D was associated with the RAS activity in patients with chronic kidney disease, accompanied by increased circulating levels of plasma renin activity and ANG II [32]. Recently, the RAS has also emerged as a key regulator of energy homeostasis [23–25]. RAS components, including renin, angiotensinogen, ANG II, and AT1 are all present in the brain. Previous studies have illustrated that pharmacological blockade or genetic deletion of any

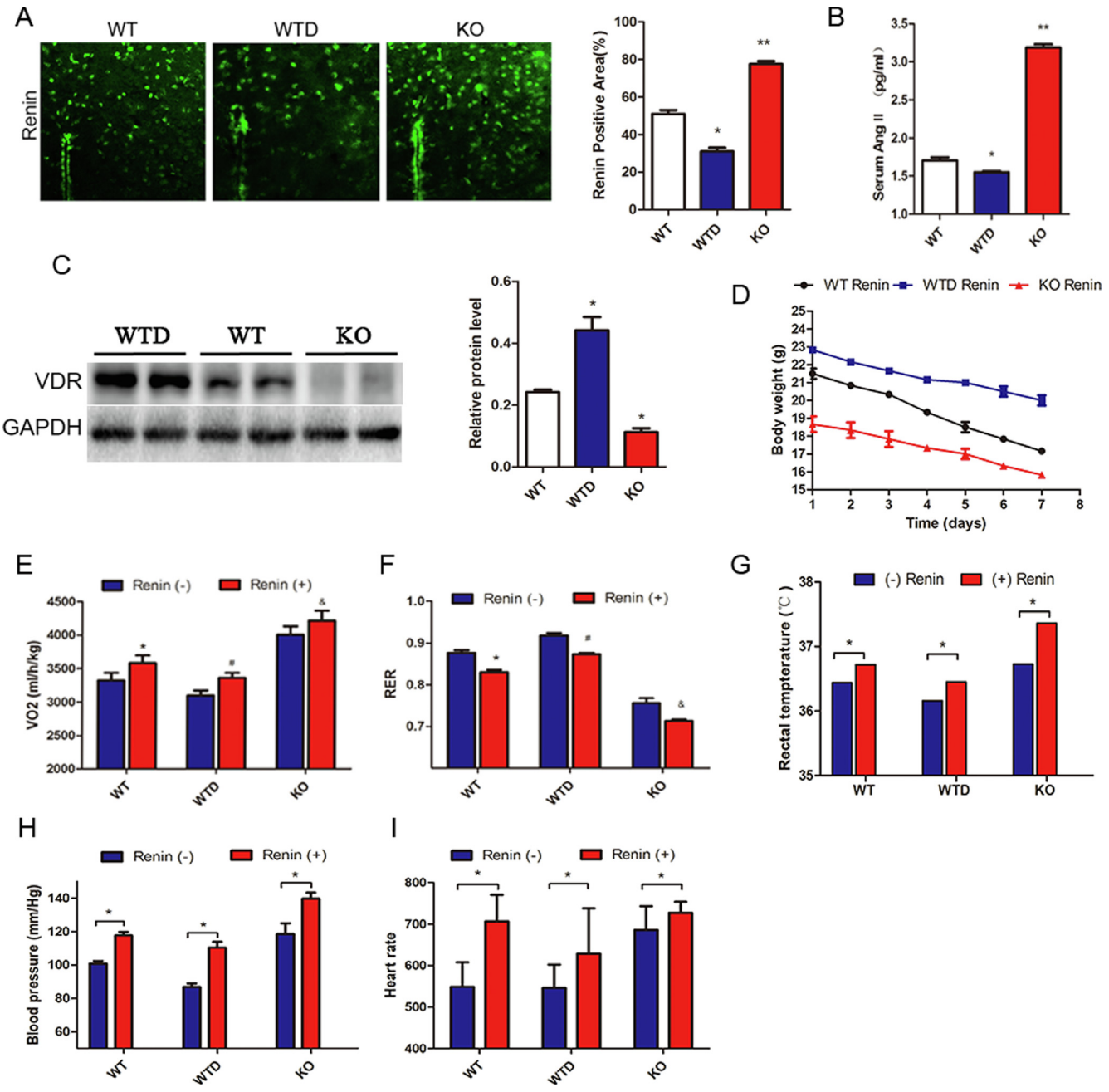

Fig. 5. Intracerebroventricular administration of renin significantly enhanced the ablation of VD signaling system-stimulated energy expenditure. (A) Representative images of immunofluorescence staining of renin of frozen hypothalamus sections (×200). (B) Serum ANG II levels were determined by enzyme linked immunosorbent assay (ELISA); (C) VDR protein in hypothalamus; (D) Body weight; (E) Oxygen consumption; (F) Respiratory exchange ratio (RER); (G) Average basal rectal temperature; (H) Blood pressure; (I) Heart rate. WTD group was treated by supplying cholecalciferol cholesterol emulsion (CCE) in the drinking water (CCE: water = 10 μl: 100 ml) for 2 weeks. WT: wild-type mice group; KO: knockout mice group; VD: vitamin D; VDR: vitamin D receptor; VO_2: oxygen consumption; ANG: angiotensin. Values are the mean ± s.e.m (n = 5–7 rats in each group), $^{*}P < 0.05$; $^{**}P < 0.01$.

components of RAS in mice gave rise to dramatic alternations in body weight, body composition, food intake, and metabolic rate [48], whereas transgenic expression of renin in mice could cause obesity [26]. It was demonstrated that showed that in *VDR*$^{-/-}$ mice, the levels of renin and ANG II expression were dramatically increased, leading to increased blood pressure and water intake [34]. Mechanistic study suggested that liganded VDR could bind with renin through the cyclic adenosine monophosphate-response element-binding protein (CRECREB-CBP) complexes in the promoter region, hence diminishing renin expression [36]. Furthermore, VD deficiency may reduce the transcription of VDR and promote a degradation of unliganded VDR, leading to a reduction in both unliganded and liganded VDR. The decrease of liganded VDR facilitates the renin activity, while the reduction of unliganded VDR may promote the RAS activity through AT1 receptor [37]. In our present study, intracerebroventricular administration of renin significantly enhanced the energy expenditure. Consistently, de Kloet et al. [49] pointed out that ICV injection of ANG II led to decreased food intake and promoted the expression of anorexigenic corticotrophin in the hypothalamus in rats. Furthermore, in the present study, we found that in *Cyb27b1*$^{-/-}$ mice, brain RAS activity was stimulated to catalyze the energy home-

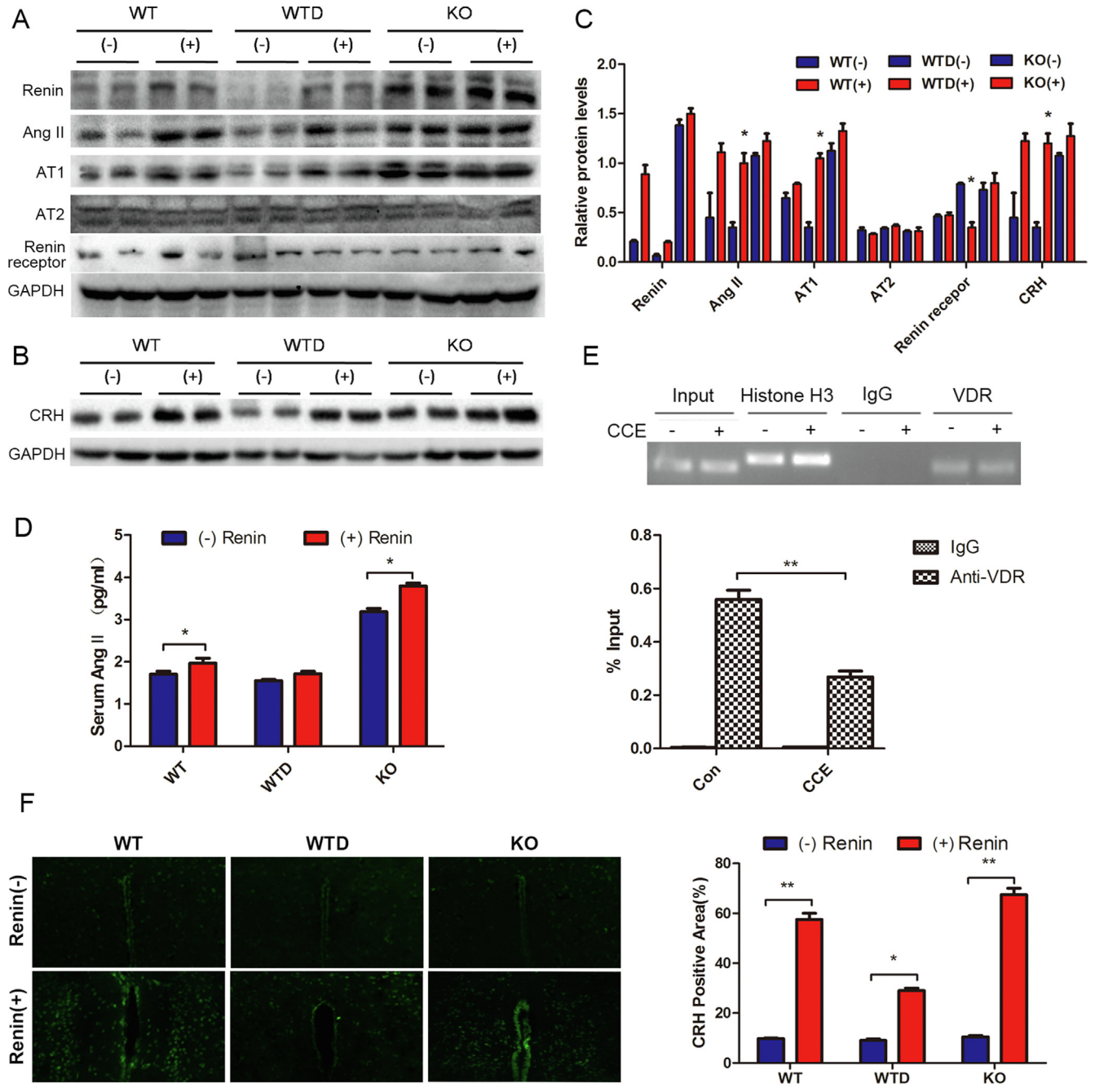

Fig. 6. VD system in the central nervous system regulates energy homeostasis via RAS pathways. (A) Summary data of relative protein levels; (B) showed CRH protein expressions in hypothalamus using western blot analysis. (C) The relative protein levels are shown as relative to GAPDH protein levels determined using Graphpad Prism 5. (D) Serum ANG Ⅱ levels were determined by enzyme linked immunosorbent assay (ELISA); (E) Functional interaction between VDR and renin promoter was evaluated by Chromatin Immunoprecipitation (CHIP) assay; (F) Representative images of immunofluorescence staining of CRH of frozen hypothalamus sections (×200). WTD group was treated by supplying cholecalciferol cholesterol emulsion (CCE) in the drinking water (CCE: water = 10 μl: 100 ml) for 2 weeks. WT: wild-type mice group; KO: knockout mice group; VD: vitamin D; VDR: vitamin D receptor; RAS: renin-angiotensin system (RAS); ANG II: angiotensin II; CRH: corticotropin-releasing hormone. (+): intracerebroventricular (ICV) injection of 1 μl renin (0.2×10^{-3} μg/μl), (−): ICV injection of 1 μl PBS. Values are the mean ± s.e.m (n = 5–7 rats in each group), $^{*}P < 0.05$; $^{**}P < 0.01$.

ostasis, whereas the exogenous administration of $1,25(OH)_2D_3$ inhibited the central RAS activity to reduce the energy expenditure. The CHIP assay results showed there was a negative functional interaction between VDR and renin promoter. It was in line with previous study [36]. In addition, the expressions of ANG II and AT1, which were key members of RAS components, were enhanced in *Cyb27b1*$^{-/-}$ mice. After the ICV injection of renin, the levels of ANG II and AT1 were further stimulated. On the contrary, the treatment of vitamin D was able to reduce the expressions of ANG II and AT1. After the ICV injection of renin, vitamin D normalized the stimulated effect of RAS activity. However, no significant change of the levels of AT2 expression was observed. In line with our results, elevated levels of ANG II in the brain promote energy expenditure, as well as the sympathetic activation of brown and white adipose tissue, which may give rise to elevated thermogenesis and lipolysis [27,28,49,50]. Additionally, stimulation of AT1 in the brain promotes the energy expenditure via enhanced RMR [24]. In the AT1 knockout mice, enhanced metabolic rate facilitates a

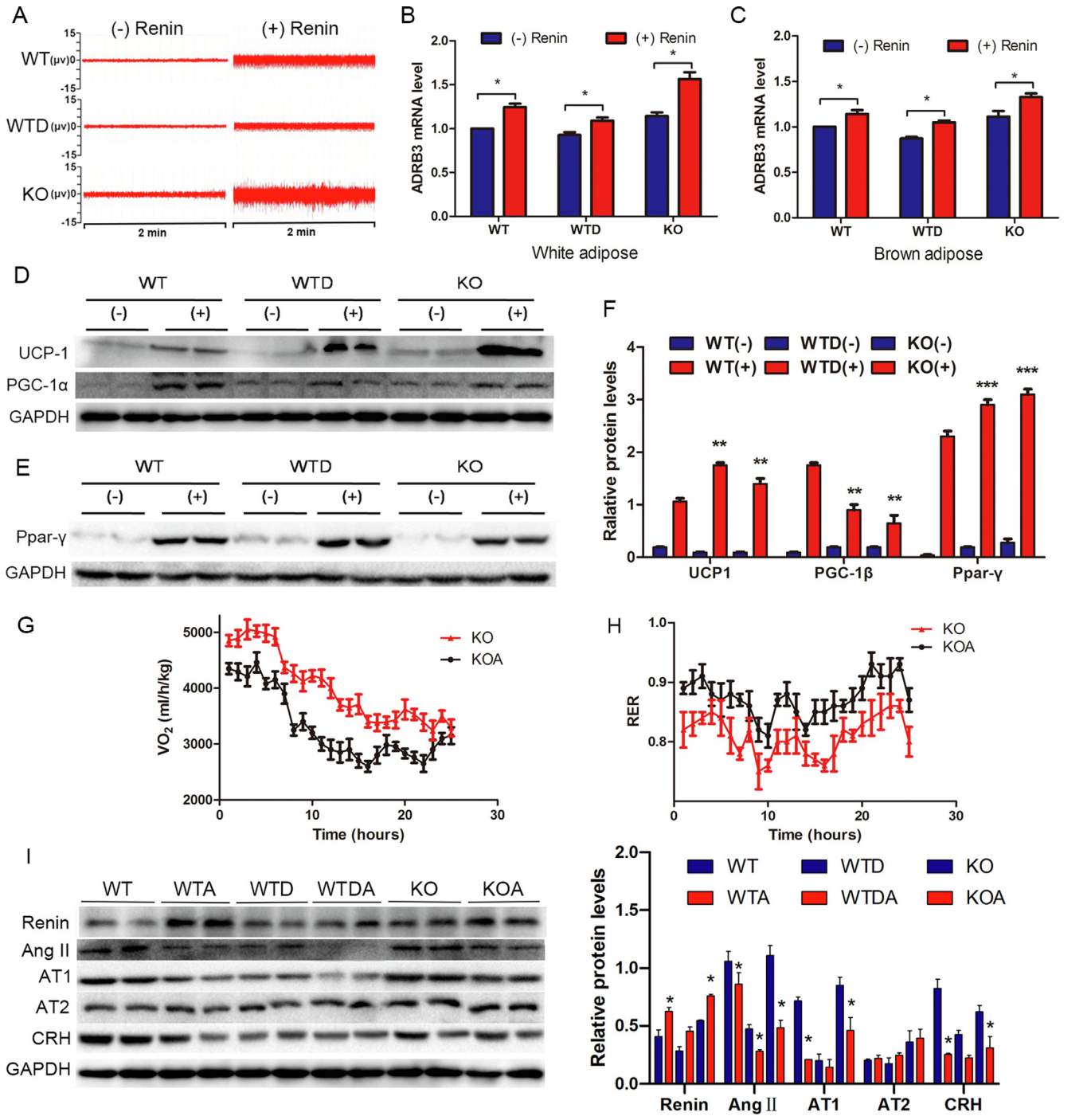

Fig. 7. The VD signaling system regulates energy homeostasis via altered sympathetic nerve activity. (A) Representative electrograms from sympathetic nerves; (B) *Adrb3* mRNA in WAT. (C) *Adrb3* mRNA in BAT; (D) showed UCP1 and PGC1α proteins in BAT; (E) demonstrated PPARγ proteins in WAT; (F) The relative protein levels are shown as relative to GAPDH protein levels determined using Graphpad Prism 5. (G) Oxygen consumption; (H) Respiratory exchange ratio (RER); (I) Summary data of relative protein levels were determined by western blot. WT group was the control group of wild-type mice. WTD group was treated by supplying cholecalciferol cholesterol emulsion (CCE) in the drinking water (CCE: water = 10 μl: 100 ml) for 2 weeks. KO group is the *Cyp27b1*$^{-/-}$ mice. WTA group, WTDA group, KOA group were treated by Aliskiren respectively (0.2 mg/ml, 1 μl/d) (Renin inhibitor). VD: vitamin D; WAT: white adipose tissue; BAT: brown adipose tissue; UCP1: uncoupling protein 1; PPARγ: peroxisome proliferators-activated receptor γ; VO_2: oxygen consumption; ANG II: angiotensin II; CRH: corticotropin-releasing hormone; ADRB3:Beta 3 adrenergic receptor; PGC1α: peroxlsome proliferator-activated receptor-γ coactlvator-1α; AT1: angiotensin II type I receptor; AT2: angiotensin II type II receptor. (+): intracerebroventricular (ICV) injection of alisiken (0.2 mg/ml, 1 μl/d) for seven days, (−): ICV injection of 1 μl PBS; Values are the mean ± s.e.m (n = 5–7 rats in each group), $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

reduced weight gain and adipose deposition after high-fat feeding [30]. Interestingly, AT2 receptor was mainly associated with nutrient appetite [51]. Collectively, vitamin D/VDR may exert an impact on energy homeostasis via RAS activity.

Furthermore, AT1 triggers CRH expression in the PVN in the hypothalamus, leading to reduced food intake and stimulated energy expenditure [31]. Interestingly, after ICV injection of renin, we found a significant increase in levels of CRH expression in *Cyb27b1*$^{-/-}$ mice. In addition, our present study also showed enhanced levels of CRH expression in the PVN of hypothalamus in *Cyp27b1*$^{-/-}$ mice, which is consistent with previous study [31]. Intraventricular injection of aliskiren, a renin inhibitor, signifi-

cantly reduced oxygen consumption, decreased the levels of ANG II and AT1 expressions, further proving that targeted renin can inhibit metabolic rate. It was noted that the renin expressions were enhanced, which might be explained by the compensatory local renin rise, also termed as aldosterone escape [52]. $1,25(OH)_2D_3$ was suggested to be used to antagonize this surge of renin [53].

In summary, the present study has demonstrated that VD signaling system may modulate the actions of the renin and AT1 as well as ANG II, further lead to the altered activity of CRH and sympathetic nerve, ultimately to mediate the whole body energy balance. These insights clarify the essential role of VD signaling system in the SNS control of energy homeostasis. To our knowledge, these results proved insight into a novel role for VD in the regulation of energy homeostasis via the RAS in the hypothalamus.

Compliance with Ethics Requirements

All Institutional and National Guidelines for the care and use of animals were followed.

CRediT authorship contribution statement

Han Su: Investigation, Data curation, Formal analysis, Writing - original draft. **Ning Liu:** Data curation, Investigation. **Yalin Zhang:** Data curation, Investigation. **Juan Kong:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.01.011.

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