

# C1q/TNF-related Protein 4 Restores Leptin Sensitivity by Downregulating NF- $\kappa$ B Signaling and Microglial Activation

**Liu Ye**

The second affiliated hospital of Chongqing medical university

**Gongwei Jia**

The second affiliated hospital of Chongqing medical university

**Yuejie Li**

The second affiliated hospital of Chongqing medical university

**Ying Wang**

The second affiliated hospital of Chongqing medical university

**Hong Chen**

The first affiliated hospital of Chongqing medical university

**Lehua Yu**

The Second Affiliated Hospital of Chongqing medical university

**Dandong Wu** (✉ [facia2010@126.com](mailto:facia2010@126.com))

The second affiliated hospital of Chongqing medical university <https://orcid.org/0000-0001-8128-0611>

---

## Research

**Keywords:** CTRP4, leptin signaling, microglial activation, NF- $\kappa$ B signaling, diet-induced obesity

**Posted Date:** January 20th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-149005/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at Journal of Neuroinflammation on July 18th, 2021. See the published version at <https://doi.org/10.1186/s12974-021-02167-2>.

# Abstract

## Objective

C1qTNF-related protein 4 (CTRP4) acts in the hypothalamus to modulate food intake in diet-induced obese mice and has been shown to exert an anti-inflammatory effect on macrophages. Since high-fat diet-induced microglial activation and hypothalamic inflammation impair leptin signaling and increase food intake, we aimed to explore the potential connection between the anorexigenic effect of CTRP4 and the suppression of hypothalamic inflammation in mice with DIO.

## Methods

Using an adenovirus-mediated hypothalamic CTRP4 overexpression model, we investigated the impact of CTRP4 on food intake and the hypothalamic leptin signaling pathway in diet-induced obese mice. Furthermore, central and plasma proinflammatory cytokines, including TNF- $\alpha$  and IL-6, were measured by Western blotting and ELISA. Changes in the hypothalamic NF- $\kappa$ B signaling cascade and microglial activation were also examined *in vivo*. In addition, NF- $\kappa$ B signaling and proinflammatory factors were investigated in BV2 cells after CTRP4 intervention.

## Results

We found that food intake was decreased, while leptin signaling was significantly improved in mice with DIO after CTRP4 overexpression. Central and peripheral TNF- $\alpha$  and IL-6 levels were reduced by central Ad-CTRP4 administration. Hypothalamic NF- $\kappa$ B signaling and microglial activation were also significantly suppressed *in vivo*. In addition, NF- $\kappa$ B signaling was inhibited in BV2 cells after CTRP4 intervention, which was consistent with the decreased production of TNF- $\alpha$  and IL-6.

## Conclusions

Our data indicate that CTRP4 reverses leptin resistance by inhibiting NF- $\kappa$ B-dependent microglial activation and hypothalamic inflammation.

## Background

In anorexigenic proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neurons and orexigenic agouti-related peptide/ neuropeptide Y (AgRP/NPY) neurons, the hypothalamic leptin signaling pathway plays a key role in the regulation of food intake and energy balance [1]. Leptin signaling is often impaired by high-fat diet (HFD)-induced hypothalamic inflammation [2–3]. Although metabolic inflammation in the medial basal hypothalamus (MBH) is characterized by the accumulation of astrocytes and microglia, the latest research showed that microglia are essential players in the

saturated fat diet-induced inflammatory process[4]. Even during the early course of HFD feeding, microglial recruitment, proliferation and size changes occurred in conjunction with altered morphology [5]. Moreover, activated microglia induce neurotoxic effects by producing inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and IL-1 $\beta$  [6–7]. In addition, several inflammatory pathways were shown to be upregulated [8], including the classic proinflammatory I $\kappa$ B $\alpha$  Kinase  $\beta$ /nuclear factor kappa B (IKK $\beta$ /NF- $\kappa$ B) signaling pathway [8]. More interestingly, studies have shown that inhibiting the NF- $\kappa$ B cascade in microglia reduces microgliosis and diet-induced weight gain [4, 9] and restores hypothalamic leptin sensitivity [10], suggesting a promising therapeutic target for HFD-induced obesity.

The CTRP (also referred to as C1qTNF-related protein) family, which features a highly conserved C-terminal complement C1q domain[11], plays important roles in multiple physiological processes, such as inflammation and metabolism [12–13]. For example, CTRP1 plays an essential role in linking dysregulated lipid metabolism and inflammatory responses in macrophages[14]. Among the CTRP family, CTRP4 is unique in possessing two C1q domains connected by a short linker and acts in the hypothalamus to modulate food intake[15]. It has been demonstrated that central injection of CTRP4 could decrease food intake in mice with diet-induced obesity (DIO); however, the underlying mechanism remains largely unknown [15]. Given that CTRP4 is able to suppress lipopolysaccharide (LPS)-induced proinflammatory cytokine production in macrophages by reducing NF- $\kappa$ B and signal transducer and activator of transcription 3 (STAT3) activation [16], there may be a connection between the anorexigenic effect of CTRP4 and hypothalamic inflammation in mice with DIO.

In the present study, we aimed to investigate whether CTRP4 regulated food intake in mice with DIO by suppressing hypothalamic inflammation. We examined whether CTRP4 exerted its anti-inflammatory effects by attenuating microglial activation and recruiting the NF- $\kappa$ B pathway, which ultimately led to improved leptin signaling and reduced food intake.

## Methods

### Construction and Amplification of Recombinant Adenoviruses

Recombinant adenoviruses were generated by using Ad-Easy technology as previously described [17]. Full fragments of murine C1qtnf4 (NM\_026161.3) were PCR-amplified and subcloned into pAdTrack-CMV. The resultant shuttle vectors were used to generate recombinant adenoviral vectors through homologous recombination with the adenoviral backbone vector in bacterial BJ5183 cells. Subsequently, the recombinant adenovirus Ad-CTR4 was generated in HEK-293 cells. The resulting Ad-CTR4 adenoviruses coexpressed GFP as a marker to track infection efficiency.

### Animals

C57BL/6 male mice weighing 18 ~ 23 g were housed in temperature-controlled polycarbonate cages (Animal Center of Chongqing Medical University, Chongqing, China) with unlimited access to food and water throughout the study period. Age-matched mice were fed a standard chow diet (SCD, 10% kcal derived from fat) or a high-fat diet (HFD, 60% kcal derived from fat, catalog no. D12492, Research Diets, USA) for 20 weeks. All animal protocols were approved by the Animal Experimentation Ethics Committee of Chongqing Medical University.

## **Stereotaxic Cannulation**

As previously described, the mice were anesthetized and fixed in a stereotaxic apparatus. A unilateral 26-gauge guide cannula was implanted into the third ventricle. The stereotaxic coordinates were 0.83 mm anterior and 0 mm right lateral to the bregma and 4.8 mm ventral to the skull. After the animals recovered from surgery, baseline food intake was measured. Then, the mice were treated i.c.v. with Ad-CTRP4 (3  $\mu$ L,  $10^{10}$  pfu), Ad-GFP (3  $\mu$ L,  $10^{10}$  pfu) or saline (3  $\mu$ L) at a flow rate of 0.1  $\mu$ L/min. Food intake and body weight were measured every day over the course of 7 days after i.c.v. injection. The i.c.v. injection was administered prior to the start of the dark cycle. For in vivo p-STAT3 measurements in the hypothalamus, after central intervention, the mice were restricted from food for 12 hours, followed by i.c.v. administration of leptin (3  $\mu$ g). After 15 minutes, the mice were anesthetized, and tissue was collected for further analysis.

## **Determination of Plasma Parameters**

Fasting blood samples were collected retro-orbitally. Plasma was separated by centrifugation and stored at -80 °C for plasma parameter analysis. Serum levels of total cholesterol (TC), triglycerides (TG), glycosylated serum protein (GSP), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) were measured with enzymatic colorimetric kits. Plasma glucose was measured by the glucose oxidase method. Plasma TNF- $\alpha$  and IL-6 were measured by ELISA kits (No. 88-7324 and No. 88-7064, Invitrogen, Carlsbad, CA, USA).

## **Cell Culture and Treatments**

BV-2 microglial cells (Zhong Qiao Xin Zhou Biotechnology Co., Ltd., Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (D6046, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at 37 °C in a humidified 5% CO<sub>2</sub> incubator. BV-2 microglia were grown to 70% confluence before treatment. To determine the appropriate concentration of palmitate, the cells were treated by replacing the media with fresh media containing 50  $\mu$ M, 100  $\mu$ M, or 200  $\mu$ M palmitate (N0830, MCE, NJ, USA) or DMSO. Recombinant murine CTRP4 protein (2137-TN-050, Novus Biologicals, Co, USA) was diluted to 2.5  $\mu$ g/ml, 5  $\mu$ g/ml, and 10  $\mu$ g/ml in DMEM. Each cell culture plate was preincubated with different concentrations of recombinant murine CTRP4 protein for 2 h as indicated in the figure legend. After 2 h of pretreatment, the cells were treated with 100  $\mu$ M palmitate for 24 h before analysis.

## **Immunofluorescence Analysis**

The mice were anesthetized by 1 mg/kg sodium pentobarbital (i.p.) and perfused first with saline containing 20 units/mL heparin for 3 min and then with 4% paraformaldehyde in 0.1 mol/L PBS for 20 min, as previously described [17]. Then, the hypothalamus was separated, embedded in optimal cutting temperature compound, immediately frozen on dry ice, and stored at -80 °C. In vitro, BV-2 microglial cells were seeded onto glass coverslips. The samples were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with a 1:500 dilution of primary antibodies against Iba1 (Wako Pure Chemical Industries, Ltd, Japan), phospho-p65 (S536) (ab86299, Abcam, Cambridge, MA, USA), NPY (11976, Cell Signaling Technology, Beverly, MA, USA) or POMC (ab210605, Abcam, Cambridge, MA, USA). Then, the sections were incubated in fluorescent-conjugated secondary Abs (DyLight 549, Abbkine, Wuhan, China). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured with a Nikon TE2000U microscope.

## Western Blot Analysis

At 72 h after i.c.v. intervention, samples were harvested from the mice. The hypothalamus was dissected as previously described[17]. Hypothalamus tissues were homogenized, and the proteins were extracted in lysis buffer, subjected to 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed at 4 °C in the presence of primary antibodies against phospho-P65 (ser536) (3033, Cell Signaling Technology, Beverly, MA, USA), P65 (8242, Cell Signaling Technology, Beverly, MA, USA), phospho-IKK $\alpha$ / $\beta$  (2697, Cell Signaling Technology, Beverly, MA, USA), IKK $\alpha$ / $\beta$  (2697, Cell Signaling Technology, Beverly, MA, USA), phospho-I $\kappa$ B $\alpha$  (2678, Cell Signaling Technology, Beverly, MA, USA), CTRP4 (36871, Abcam, Cambridge, MA, USA), POMC (210605, Abcam, Cambridge, MA, USA), phospho-Janus kinase 2 (JAK2) (32101, Abcam, Cambridge, MA, USA), phospho-STAT3 (Tyr705) (9145, Cell Signaling Technology, Beverly, MA, USA), STAT3 (9139, Cell Signaling Technology, Beverly, MA, USA), suppressor of cytokine signaling 3 (SOCS3) (2932, Cell Signaling Technology, Beverly, MA, USA), TNF- $\alpha$  (11948, Cell Signaling Technology, Beverly, MA, USA), IL-6 (12912, Cell Signaling Technology, Beverly, MA, USA), NPY (11976, Cell Signaling Technology, Beverly, MA, USA), and CD11b (66519-1-1 g, Proteintech, Bath, United Kingdom). After being washed three times with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with secondary antibodies for 1 h at room temperature. Next, the blots were visualized with a Bio Imaging System Densitometer (Bio-Rad, Hercules, CA), and quantification of antigen-antibody complexes was performed with Quantity One analysis software (Bio-Rad).

## Statistical Analysis

The data are presented as the mean  $\pm$  SEM. Two-way ANOVA with a least significant difference post hoc test was used to compare mean values between multiple groups, and a two-tailed unpaired Student's t-test was used for two-group comparisons. A value of  $P < 0.05$  was considered significant.

## Results

# Effects of Hypothalamic CTRP4 Overexpression on Energy Homeostasis and Body Metabolism

Twenty weeks of HFD consumption significantly decreased hypothalamic CTRP4 expression. Hypothalamic CTRP4 protein levels were increased ( $P < 0.05$ ) in both SCD- and HFD-fed mice after i.c.v. Ad-CTRP4 injection compared with the controls (Fig. 1a). Then, we explored the effects of CTRP4 on energy homeostasis and revealed that central Ad-CTRP4 intervention caused significant decreases in food intake in HFD-fed mice between days 2 and 7 (Fig. 1b). While there was no significant difference in total body weight between the groups due to the short intervention period (data not shown), i.c.v. Ad-CTRP4 injection significantly slowed the daily increase in body weight (Fig. 1c). We also examined the effects of central CTRP4 on peripheral metabolism and found that the levels of fasting plasma glucose, TG, LDL-C, HDL-C, and GSP were not affected by CTRP4 intervention (Table 1). However, TC levels were significantly decreased in mice that received Ad-CTRP4 injection (Table 1).

Table 1  
General characteristics of the different groups of C57BL/6J mice

	SCS	HFS	HFG	HFC
FBG (mmol/L)	5.905 ± 0.591	6.788 ± 1.008	6.955 ± 0.391	6.828 ± 0.636
GSP (mmol/L)	5.905 ± 0.540	6.953 ± 0.536*	6.897 ± 0.457	6.809 ± 0.712
TG (mmol/L)	1.389 ± 0.094	1.834 ± 1.969**	1.773 ± 0.157	1.713 ± 0.165
TC (mmol/L)	4.187 ± 0.189	6.258 ± 0.529**	6.157 ± 0.621	5.107 ± 0.304▲▲
HDL-C(mmol/L)	0.536 ± 0.664	1.117 ± 0.109**	0.966 ± 0.327	0.995 ± 0.210
LDL-C(mmol/L)	2.044 ± 0.127	2.876 ± 0.226**	2.707 ± 0.314	2.821 ± 0.118
FBG, fasting blood glucose; GSP, glycosylated serum protein; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. The data are the mean ± SEM; n = 7. * $P < 0.05$ , ** $P < 0.01$ vs. the SCS group. ▲▲ $P < 0.01$ vs. the HFS group				

## Effects of central CTRP4 overexpression on hypothalamic leptin signaling

We investigated the mechanism by which CTRP4 suppressed food intake and found that POMC expression was obviously increased by CTRP4 intervention in the HFD-fed group (Fig. 2a), while NPY expression was significantly decreased (Fig. 2b). To determine the effect of central Ad-CTRP4 on POMC- and NPY-expressing neurons, we also performed immunofluorescence analysis in mouse hypothalamic tissue. As shown in Fig. 2c-d, CTRP4 overexpression led to a significant increase in the number of POMC-positive cells but a decrease in the number of NPY-positive cells in the ARC. Then, we examined whether central CTRP4 treatment could improve hypothalamic leptin sensitivity in mice with DIO. In standard chow diet-fed mice, there was a rapid and dramatic increase in the phosphorylation of STAT3 and JAK2 following central leptin injection. However, 20 weeks of HFD feeding significantly blunted this response, while central CTRP4 injection completely rescued leptin-induced STAT3 and JAK2 phosphorylation

(Fig. 3a-c). In addition, there was a dramatic decrease in the expression of SOCS3 (Fig. 3d-e), an inhibitor of leptin signaling, suggesting that CTRP4 restored leptin sensitivity by inhibiting SOCS3 expression.

## Effects of central CTRP4 overexpression on microglial activation and hypothalamic and peripheral inflammation

We then examined whether the improved leptin signaling sensitivity was associated with decreased central inflammation. We measured the expression of hypothalamic TNF- $\alpha$  and IL-6 after i.c.v. Ad-CTRP4 intervention. As shown in Fig. 4a-d, the protein levels of IL-6 and TNF- $\alpha$  decreased significantly in the hypothalamus after central Ad-CTRP4 intervention. In addition, plasma TNF- $\alpha$  and IL-6 levels also decreased significantly after central Ad-CTRP4 injection (Fig. 4e-f). Since microglia are the main source of inflammatory cytokines [18], we further evaluated microglial activation in the ARC. Iba1 immunostaining revealed that in SCD-fed mice, microglia within the ARC were primarily in the resting state, as illustrated by small somas and long branched processes (Fig. 4g-h) [19]. After twenty weeks of HFD consumption, the soma enlarged, and processes became shorter, suggesting that these microglia were activated. As shown in Fig. 4g, central CTRP4 injection blunted microglial activation, as exhibited by smaller somas and longer branched processes than those of microglia in the ARC in the controls.

## Effects of central CTRP4 treatment on NF- $\kappa$ B signaling

As NF- $\kappa$ B signaling is a crucial inflammatory pathway, we next assessed the effects of central CTRP4 on the phosphorylation of P65, inhibitor of NF- $\kappa$ B kinase subunit  $\alpha/\beta$  (IKK $\alpha/\beta$ ) and I $\kappa$ B $\alpha$ , which are proteins in the NF- $\kappa$ B signaling cascade, by Western blotting. The phosphorylation of P65 (Fig. 5a-b), IKK $\alpha/\beta$  (Fig. 5c-d) and I $\kappa$ B $\alpha$  (Fig. 5e-f) was dramatically increased by HFD consumption and was inhibited by CTRP4 intervention. Moreover, HFD consumption promoted nuclear translocation of NF- $\kappa$ B, while CTRP4 partly abolished NF- $\kappa$ B nuclear translocation (Fig. 5g-h).

## Effects of CTRP4 on NF- $\kappa$ B signaling in palmitate-activated microglia

To determine the appropriate palmitate concentration to activate microglia, we assessed CD11b expression in BV-2 cells treated with different concentrations of palmitate (50, 100, and 200  $\mu$ M). We found that CD11b protein expression and the levels of inflammatory cytokines, including TNF- $\alpha$  and IL-6, increased the most in BV-2 cells exposed to 100  $\mu$ M PA (data not shown), suggesting that 100  $\mu$ M was the most appropriate concentration to induce microglial activation. Next, BV-2 cells were pretreated with DMSO or different concentrations of CTRP4 (2.5  $\mu$ g/ml, 5  $\mu$ g/ml, and 10  $\mu$ g/ml) for 2 h, followed by DMSO or palmitate (100  $\mu$ M) treatment for an additional 24 h. Recombinant CTRP4 protein dose-dependently suppressed palmitate-induced CD11b expression (Fig. 6.a-b). Moreover, Western blotting showed that CTRP4 reduced PA-induced phosphorylation of P65, IKK $\alpha/\beta$  and I $\kappa$ B $\alpha$ , suggesting that the NF- $\kappa$ B signaling cascade was suppressed by CTRP4 intervention (Fig. 6.c-h). Next, we investigated the

nuclear translocation of P65, which is a critical step in the NF- $\kappa$ B signaling cascade. In the resting state, P65 remained mostly in the cytosol; however, following PA stimulation, P65 was significantly increased in the nucleus. CTRP4 intervention significantly decreased PA-induced P65 nuclear translocation (Fig. 6.i-j). We measured the protein expression of proinflammatory cytokines. PA treatment induced the protein expression of TNF- $\alpha$  and IL-6, whereas CTRP4 reduced PA-induced expression of these proinflammatory cytokines (Fig. 6k-n).

## Discussion

The effects of hypothalamic leptin, which mainly occur in the arcuate nucleus, is particularly important in the regulation of energy homeostasis and peripheral metabolism. However, in the context of DIO, leptin resistance develops in the hypothalamus, whereby exogenous leptin cannot phosphorylate STAT3, further disrupting global energy homeostasis. Thus, restoring leptin sensitivity is a topic of interest worldwide. The current study showed that phenotypically, hypothalamic CTRP4 overexpression could significantly decrease food intake in HFD-induced obese mice. Mechanistically, central CTRP4 intervention restored the normal response of p-STAT3<sup>tyr705</sup> to exogenous leptin stimulation in obese mice, which was blunted by a HFD, indicating the re-establishment of leptin sensitivity. Furthermore, the expression of SOCS3, a leptin signaling inhibitor, was suppressed by central CTRP4 administration. Taken together, these data suggested that hypothalamic CTRP4 overexpression could improve central leptin signaling and energy homeostasis in mice with DIO.

We further examined the effect of CTRP4 on hypothalamic inflammation, and our results demonstrated that central (hypothalamus) and peripheral (plasma) TNF- $\alpha$  and IL-6 levels were reduced by central Ad-CTRP4 intervention. However, the roles of these cytokines in central inflammation differ to some extent. TNF- $\alpha$  is a classic proinflammatory factor that causes hypothalamic insulin and leptin resistance and is believed to be a contributor to the development of obesity[20–22]. On the other hand, the pro- or anti-inflammatory role of IL-6 is thought to be context-dependent[23]. However, a recent review suggested that IL-6 could induce marked microglial activation and drive hypothalamic neuroinflammation, indicating that IL-6 is a proinflammatory cytokine in the central nervous system [24]. Peripherally, TNF- $\alpha$  and IL-6 are both thought to be proinflammatory cytokines. Previous studies have demonstrated that these factors could be downregulated in the hypothalamus via vagus nerve activation [25]. Acetylcholine, the main neurotransmitter of the vagus nerve, inhibits the production of proinflammatory cytokines through the nicotinic receptor in macrophages[26–27]. Interestingly, the excitability of the vagus nerve is decreased in diet-induced obese rodent models [27]. Thus, central CTRP4 overexpression inhibits hypothalamic inflammation and possibly exerts systemic anti-inflammatory effects through vagus nerve stimulation.

Chronic HFD feeding leads to low-grade activation of proinflammatory NF- $\kappa$ B signaling[8, 29–30]. Previously, neuronal and astrocytic NF- $\kappa$ B signaling received much attention, as deleting or inhibiting NF- $\kappa$ B in neurons or astrocytes mitigated DIO [29, 31]. However, microglial NF- $\kappa$ B signaling has been found to be critical in the onset of DIO, and selectively restraining microglial NF- $\kappa$ B signaling greatly reduced microglial activation and limited diet-induced hyperphagia and weight gain [9]. In the present study,

changes in the quantity and morphology of Iba1-labeled cells suggested that central CTRP4 overexpression inhibited HFD-induced microglial activation. Microglia are essential players responsible for increased inflammatory cytokine expression in high-fat environments and are resident immune cells in the brain [4]. Thus, the CTRP4-induced in vivo TNF- $\alpha$  and IL-6 downregulation observed in our study could be, at least in part, associated with suppressed microglial activation. On the other hand, Western blotting and immunofluorescence results showed that central CTRP4 overexpression blunted the recruitment of the NF- $\kappa$ B pathway by inhibiting the degradation and phosphorylation of I $\kappa$ B and the nuclear translocation of p65 in HFD-fed mice. By using a BV2 cell model, in vitro data further confirmed that the CTRP4 protein could effectively suppress NF- $\kappa$ B signaling cascades and microglial activation, accompanied by decreased production of TNF- $\alpha$  and IL-6, which are targets of NF- $\kappa$ B signaling. However, the current study could not determine whether the inhibition of NF- $\kappa$ B signaling only occurred in microglia or in both neuronal and glial cells.

Reactive microgliosis, which is triggered by saturated fatty acids, leads to neuronal injury, and POMC neurons are especially vulnerable to this phenomenon [2, 32–34]. Recent studies have demonstrated that microglia also interfere with nutrient sensing by the hypothalamus, as restraining microglial activation by an NF- $\kappa$ B-dependent method reduces food intake, mitigates DIO and improves leptin signaling [4, 10]. Our data revealed that central CTRP4 intervention restored leptin signaling and decreased food intake, possibly by suppressing NF- $\kappa$ B-dependent microglial activation. However, little is known about the underlying mechanisms [35]. Previous studies suggest the presence of proinflammatory signals or other factors produced by activated microglia that modulate or impair neuronal responsiveness to leptin and regulate energy homeostasis [35–36]. Among these factors, TNF- $\alpha$  may mediate such microglia-neuronal cross-talk by inducing endoplasmic reticulum stress and inflammatory signaling cascades in appetite-regulating neurons [37]. Therefore, it is possible that CTRP4 acts on metabolism by suppressing microglial activation and TNF- $\alpha$  production. However, other mechanisms might also be involved in this process and contribute to the anti-inflammatory and metabolic effects of CTRP4, and should be the focus of future work.

## Conclusion

Taken together, these results indicate that CTRP4 reverses leptin resistance by inhibiting NF- $\kappa$ B-dependent microglial activation and hypothalamic inflammation. The evidence provided in our study suggests that targeting hypothalamic microglia via adipocytokines may be a promising way to mitigate diet-induced metabolic dysfunction.

## Abbreviations

POMC: proopiomelanocortin; CART: cocaine- and amphetamine-regulated transcript; AgRP: agouti-related peptide; NPY: neuropeptide Y; HFD: high-fat diet; MBH: medial basal hypothalamus; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; IL-6: interleukin-6; IKK $\beta$ : I $\kappa$ B $\alpha$  Kinase  $\beta$ ; NF- $\kappa$ B: nuclear factor kappa B; CTRP: C1qTNF-related protein; STAT3: signal transducer and activator of transcription 3; SCD: standard chow diet; TC:

total cholesterol; TG: triglycerides; GSP: glycosylated serum protein; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; JAK2: Janus kinase 2; SOCS3: suppressor of cytokine signaling 3; LPS: lipopolysaccharide.

## Declarations

**Ethics approval and consent to participate:** All animal protocols were approved by the Animal Experimentation Ethics Committee of Chongqing Medical University.

**Consent for publication:** Not applicable.

**Availability of data and materials:** The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Authors' Contributions

Liu Ye and Yuejie Li conducted the experiments. Gongwei Jia and Ying Wang analyzed the Data. Dandong Wu and Lehua Yu designed the study and drafted the article. Hong Chen revised the article. All authors critically reviewed the manuscript. All authors read and approved the final manuscript.

## Funding

This study was funded by the Natural Science Foundation of China (No. 81600615 and No. 82072543).

## Competing interests

The authors declare that they have no competing interests.

## Acknowledgements

Not applicable.

## References

- [1] Varela L, Horvath TL. Leptin and insulin pathways in POMC and AgRP neurons that modulate energy balance and glucose homeostasis. *EMBO Rep.* 2012;13:1079-1086.
- [2] Thaler JP, Yi CX, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO, et al. Obesity is associated with hypothalamic injury in rodents and humans. *J Clin Invest.* 2012;122:153–162.
- [3] Waise TM, Toshinai K, Naznin F, NamKoong C, Md Moin AS, Sakoda H, et al. One-day high-fat diet induces inflammation in the nodose ganglion and hypothalamus of mice. *Biochem Biophys Res Commun.* 2015;464:1157–1162.

- [4] Valdearcos M, Robblee MM, Benjamin DI, Nomura DK, Xu AW, Koliwad SK. Microglia dictate the impact of saturated fat consumption on hypothalamic inflammation and neuronal function. *Cell Rep*. 2014;9:2124-2138.
- [5] Berkseth KE, Guyenet SJ, Melhorn SJ, Lee D, Thaler JP, Schur EA, et al. Hypothalamic gliosis associated with high-fat diet feeding is reversible in mice: a combined immunohistochemical and magnetic resonance imaging study. *Endocrinology*. 2014;155: 2858–2867.
- [6] Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell*. 2008;132: 344–362.
- [7] Naznin F, Toshinai K, Waise TM, NamKoong C, Md Moin AS, Sakoda H, et al. Diet-induced obesity causes peripheral and central ghrelin resistance by promoting inflammation. *J Endocrinol*. 2015;226:81–92.
- [8] Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. *Cell*. 2008;135:61–73.
- [9] Valdearcos M, Douglass JD, Robblee MM, Dorfman MD, Stifler DR, Bennett ML, et al. Microglial Inflammatory Signaling Orchestrates the Hypothalamic Immune Response to Dietary Excess and Mediates Obesity Susceptibility. *Cell Metab*. 2017;26:185-197.e3.
- [10] André C, Guzman-Quevedo O, Rey C, Rémus-Borel J, Clark S, Castellanos-Jankiewicz A, et al. Inhibiting Microglia Expansion Prevents Diet-Induced Hypothalamic and Peripheral Inflammation. *Diabetes*. 2017;66:908-919.
- [11] Schäffler A, Schölmerich J, Salzberger B. Adipose tissue as an immunological organ: toll-like receptors, C1q/TNFs and CTRPs. *Trends Immunol*. 2007;28:393-399.
- [12] Wong GW, Krawczyk SA, Kitidis-Mitrokostas C, Ge G, Spooner E, Hug C, et al. Identification and characterization of CTRP4, a novel secreted glycoprotein, from adipose tissue that reduces serum glucose in mice and forms heterotrimers with adiponectin. *FASEB J*. 2009;23:241-258.
- [13] Lee W, Kim M.J, Park EJ, Choi YJ, Park SY. C1qTNF-related protein-6 mediates fatty acid oxidation via the activation of the AMP-activated protein kinase. *FEBS Lett*. 2010; 584:968-972.
- [14] Wang XQ, Liu ZH, Xue L, Lu L, Gao J, Shen Y, et al. C1q/TNF-related protein 1 links macrophage lipid metabolism to inflammation and atherosclerosis. *Atherosclerosis*. 2016;250:38-45.
- [15] Byerly MS, Petersen PS, Ramamurthy S, Seldin MM, Lei X, Provost E, et al. C1q/TNF-related protein 4 (CTRP4) is a unique secreted protein with two tandem C1q domains that functions in the hypothalamus to modulate food intake and body weight. *J Biol Chem*. 2014; 289:4055-4069.
- [16] Luo Y, Wu X, Ma Z, Tan W, Wang L, Na D, et al. Expression of the novel adipokine C1qTNF-related protein 4 (CTRP4) suppresses colitis and colitis-associated colorectal cancer in mice. *Cell Mol Immunol*.

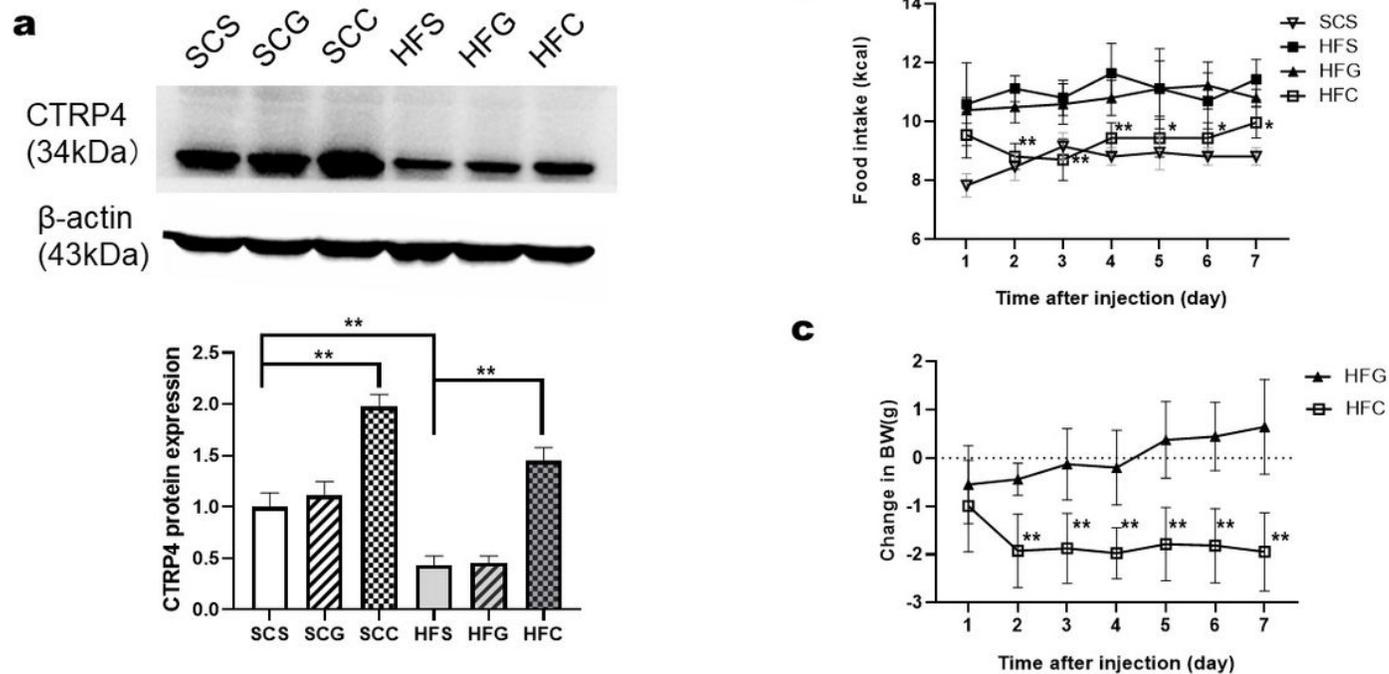
2016;13:688-699.

- [17] Li Y, Ye L, Jia G, Chen H, Yu L, Wu D. C1q/TNF-related Protein 4 Induces Signal Transducer and Activator of Transcription 3 Pathway and Modulates Food Intake. *Neuroscience*. 2020; 429:1-9.
- [18] Hanisch UK. Microglia as a source and target of cytokines. *Glia*. 2002;40:140-155.
- [19] Shi P, Grobe JL, Desland FA, Zhou G, Shen XZ, Shan Z, et al. Direct pro-inflammatory effects of prorenin on microglia. *PLoS One*. 2014;9:e92937.
- [20] Miyazaki Y, Pipek R, Mandarino LJ, DeFronzo RA. Tumor necrosis factor alpha and insulin resistance in obese type 2 diabetic patients. *Int J Obes Relat Metab Disord*. 2003;27:88 –94.
- [21] Picardi PK, Caricilli AM, de Abreu LL, Carvalheira JB, Velloso LA, Saad MJ. Modulation of hypothalamic PTP1B in the TNF-alpha-induced insulin and leptin resistance. *FEBS Lett*. 2010;584:3179–3184.
- [22] Gao Y, Han C, Huang H, Xin Y, Xu Y, Luo L, et al. Heat shock protein 70 together with its co-chaperone CHIP inhibits TNF-alpha induced apoptosis by promoting proteasomal degradation of apoptosis signal-regulating kinase1. *Apoptosis*. 2010;15:822–833.
- [23] Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. *Nature Immunology*. 2015;16: 448– 457.
- [24] Bobbo VCD, Jara CP, Mendes NF, Morari J, Velloso LA, Araújo EP. Interleukin-6 Expression by Hypothalamic Microglia in Multiple Inflammatory Contexts: A Systematic Review. *Biomed Res Int*. 2019;22:1365210.
- [25] Guijarro A, Laviano A, Meguid MM. Hypothalamic integration of immune function and metabolism. *Prog Brain Res*. 2006;153:367-405.
- [26] Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature*. 2003;421:384–388.
- [27] Ulloa L. The vagus nerve and the nicotinic anti-inflammatory pathway. *Nat Rev Drug Discov*. 2005; 4:673–684.
- [28] Browning KN, Fortna SR, Hajnal A. Roux-en-Y gastric bypass reverses the effects of diet-induced obesity to inhibit the responsiveness of central vagal motoneurons. *J Physiol*. 2013; 591:2357–2372.
- [29] Benzler J, Ganjam GK, Pretz D, Oelkrug R, Koch CE, Legler K, et al. Central inhibition of IKKb/NF-kB signaling attenuates high-fat diet-induced obesity and glucose intolerance. *Diabetes*. 2015;64:2015–2027.

- [30] Tsaousidou E, Paeger L, Belgardt BF, Pal M, Wunderlich CM, Brönneke H, et al. Distinct roles for JNK and IKK activation in agouti-related peptide neurons in the development of obesity and insulin resistance. *Cell Rep.* 2014;9:1495–1506.
- [31] Douglass JD, Dorfman MD, Fasnacht R, Shaffer LD, Thaler JP. Astrocyte IKKb/NF-kB signaling is required for diet-induced obesity and hypothalamic inflammation. *Mol Metab.* 2017; 6:366-373.
- [32] Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, et al. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. *J Neurosci.* 2009; 29:359–370.
- [33] Block ML, Hong JS. Chronic microglial activation and progressive dopaminergic neurotoxicity. *Biochem Soc Trans.* 2007;35:1127–32.
- [34] Yi CX, Walter M, Gao Y, Pitra S, Legutko B, Kälin S, et al. TNF $\alpha$  drives mitochondrial stress in POMC neurons in obesity. *Nat Commun.* 2017;8:15143.
- [35] Douglass JD, Dorfman MD, Thaler JP. Glia: silent partners in energy homeostasis and obesity pathogenesis. *Diabetologia.* 2017; 60:226-236.
- [36] Reis WL, Yi CX, Gao Y, Tschöp MH, Stern JE, et al. Brain innate immunity regulates hypothalamic arcuate neuronal activity and feeding behavior. *Endocrinology.* 2015;156:1303–1315.
- [37] Dalvi PS, Chalmers JA, Luo V, Han DY, Wellhauser L, Liu Y, et al. High fat induces acute and chronic inflammation in the hypothalamus: effect of high-fat diet, palmitate and TNF- $\alpha$  on appetite-regulating NPY neurons. *Int J Obes (Lond).* 2016; 41:149-158.

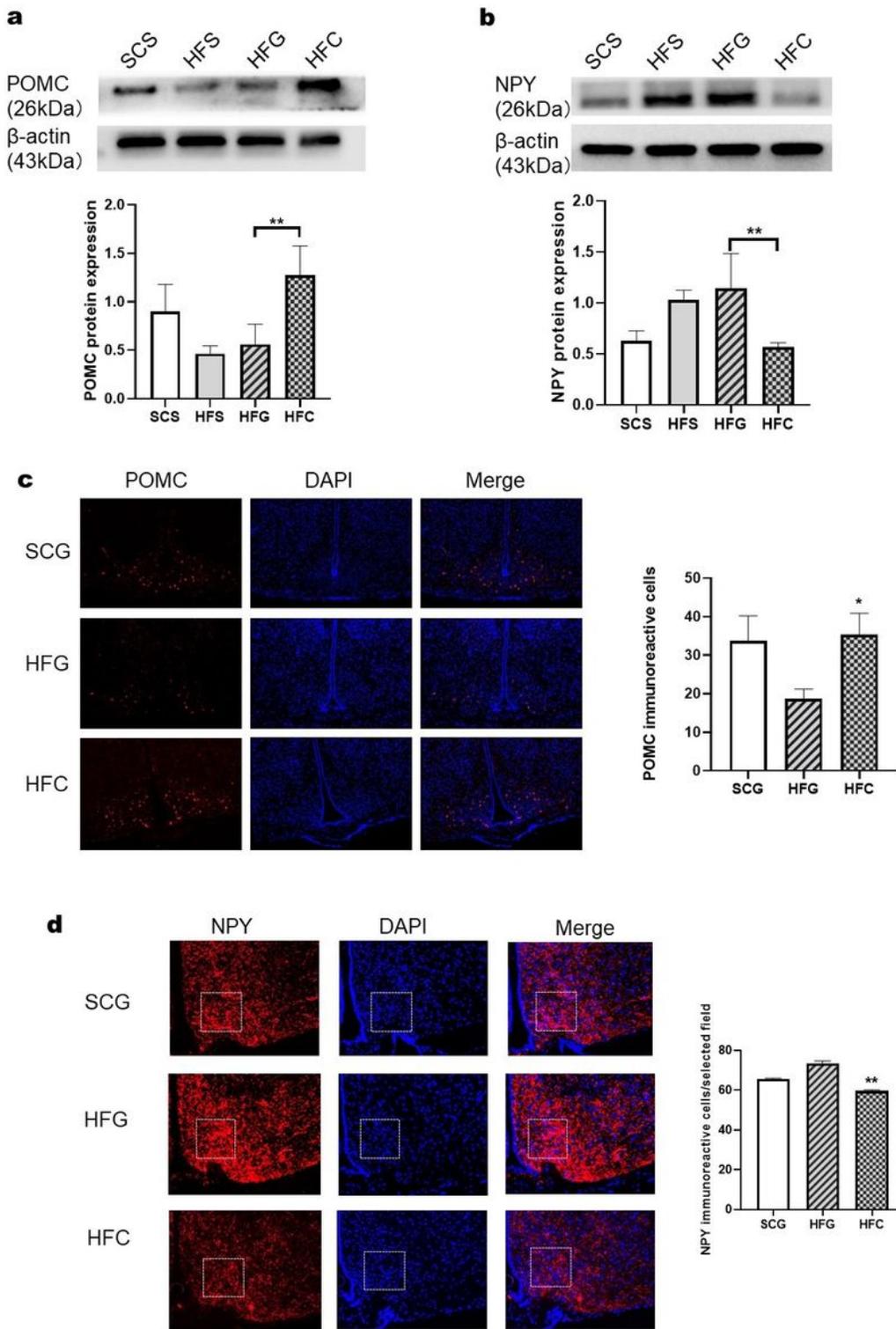
## Figures

**Fig. 1**



**Figure 1**

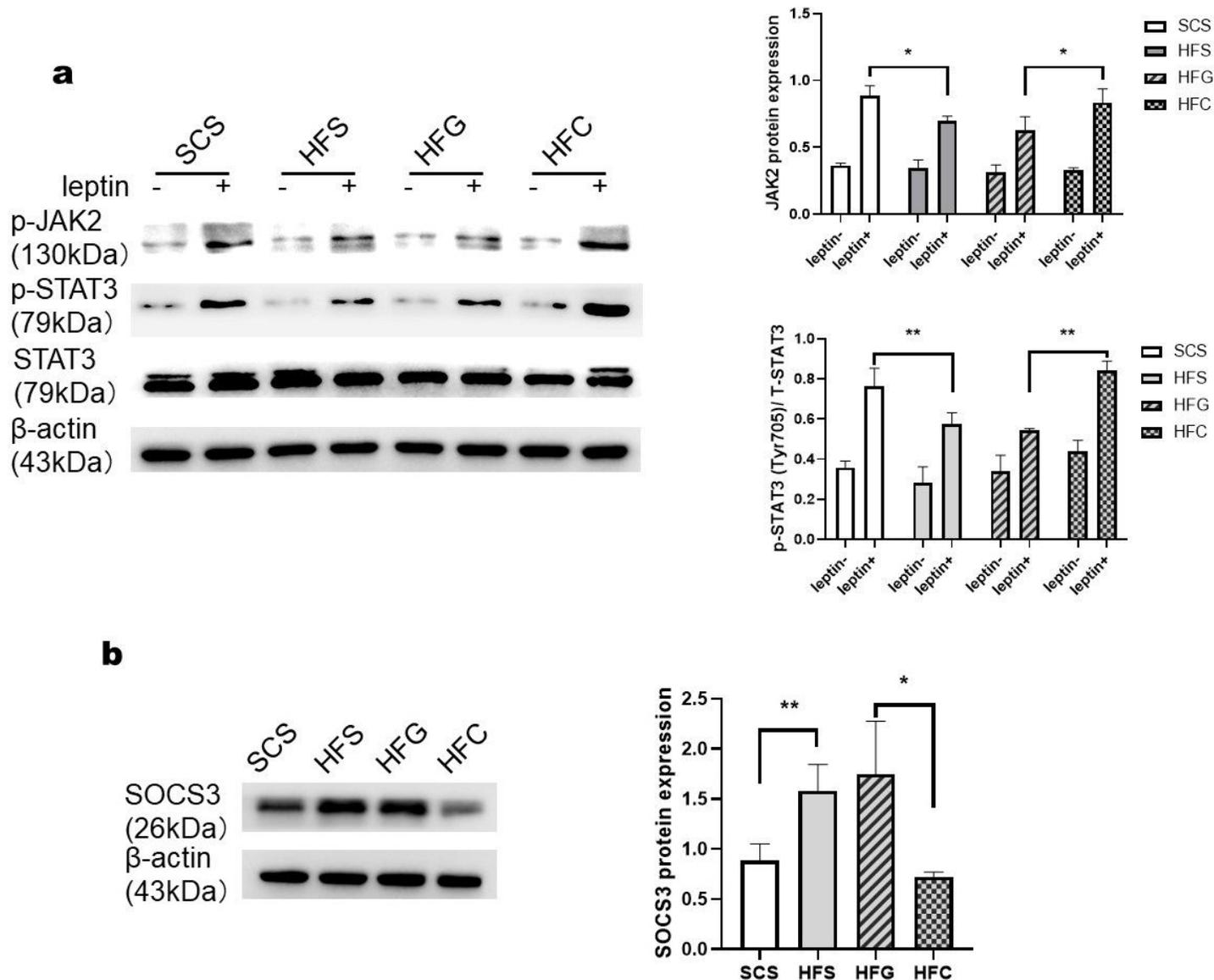
Effects of central Ad-CTRP4 injection on hypothalamic CTRP4 protein expression, food intake and body weight in mice. a. At 72 hours after i.c.v. injection, hypothalami were collected from mice fed a standard chow diet (SCD) or a high-fat diet (HFD). Representative Western blot analysis and densitometric analysis of CTRP4 expression in the hypothalamus in mice that received saline (SCS and HFS), Ad-GFP (SCG and HFG) and Ad-CTRP4 (SCC and HFC). b. Daily food intake after central intervention. c. Changes in body weight after central intervention. The data are the mean  $\pm$  SEM.  $n=5$ . Statistical analyses were performed by two-way ANOVA with Bonferroni post hoc tests (b) or two-tailed unpaired Student's t-tests (c-d). \* $P<0.05$ , \*\* $P<0.01$ .



**Figure 2**

Effects of central CTRP4 overexpression on hypothalamic neuropeptides by Western blot and immunofluorescence analysis. At 72 hours after i.c.v. injection, SCD- and HFD-fed mice were fasted for 12 hours, and then the hypothalami were dissected. a-b. Levels of POMC (a) and NPY (b) were measured by Western blotting and densitometric analysis in mice that received saline (SCS and HFS), Ad-GFP (HFG) and Ad-CTR4 (HFC). c. Representative images of POMC immunoreactivity (red) and DAPI nuclear

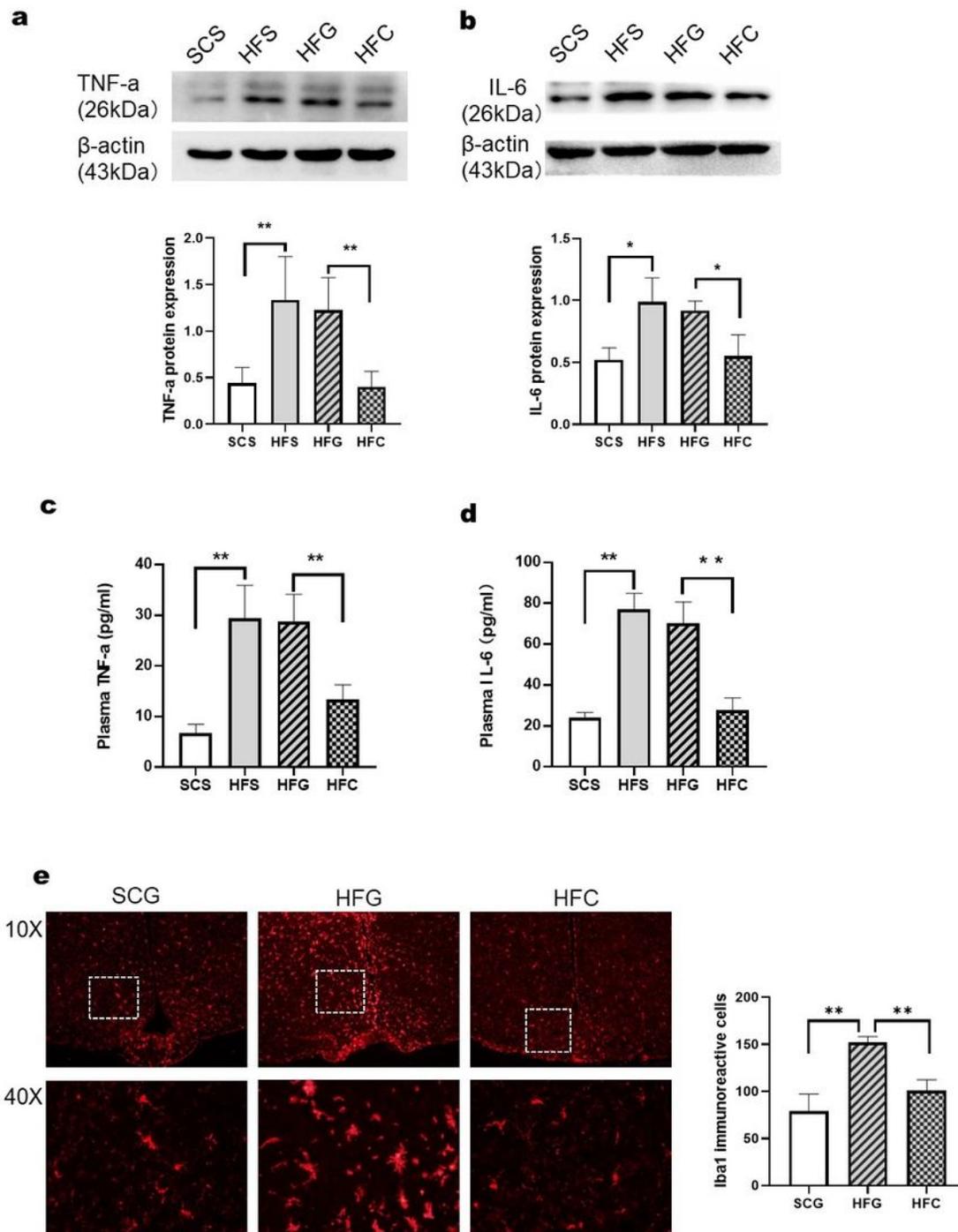
staining (blue) in the ARCs of mice after Ad-GFP (SCG and HFG) or Ad-CTRP4 intervention (HFC). d. Representative images of POMC immunoreactivity (red) and DAPI nuclear staining (blue) in the ARCs of mice after Ad-GFP (SCG and HFG) or Ad-CTRP4 intervention (HFC). n=5. Statistical analyses were performed by two-tailed unpaired Student's t-tests. \*P<0.05, \*\*P<0.01.



**Figure 3**

Effects of central CTRP4 overexpression on hypothalamic leptin signaling. At 72 hours after i.c.v. injection, the mice were fasted for 12 hours and then i.c.v. injected with leptin (3 $\mu$ g). After 15 minutes, the mice were anesthetized, and hypothalamic tissue was collected for Western blot analysis (a). a. Immunoblots showing p-STAT3Tyr705, STAT3, p-JAK2 and  $\beta$ -actin levels in saline- and leptin-stimulated mice fed an SCD (SCS) and HFD (HFS, HFG and HFC) after central intervention. At 72 hours after i.c.v. injection, the mice were fasted for 12 hours, and then hypothalamic tissue was collected for SOCS3 protein analysis (b). b. Immunoblots showing hypothalamic SOCS3 in SCD- and HFD-fed mice that

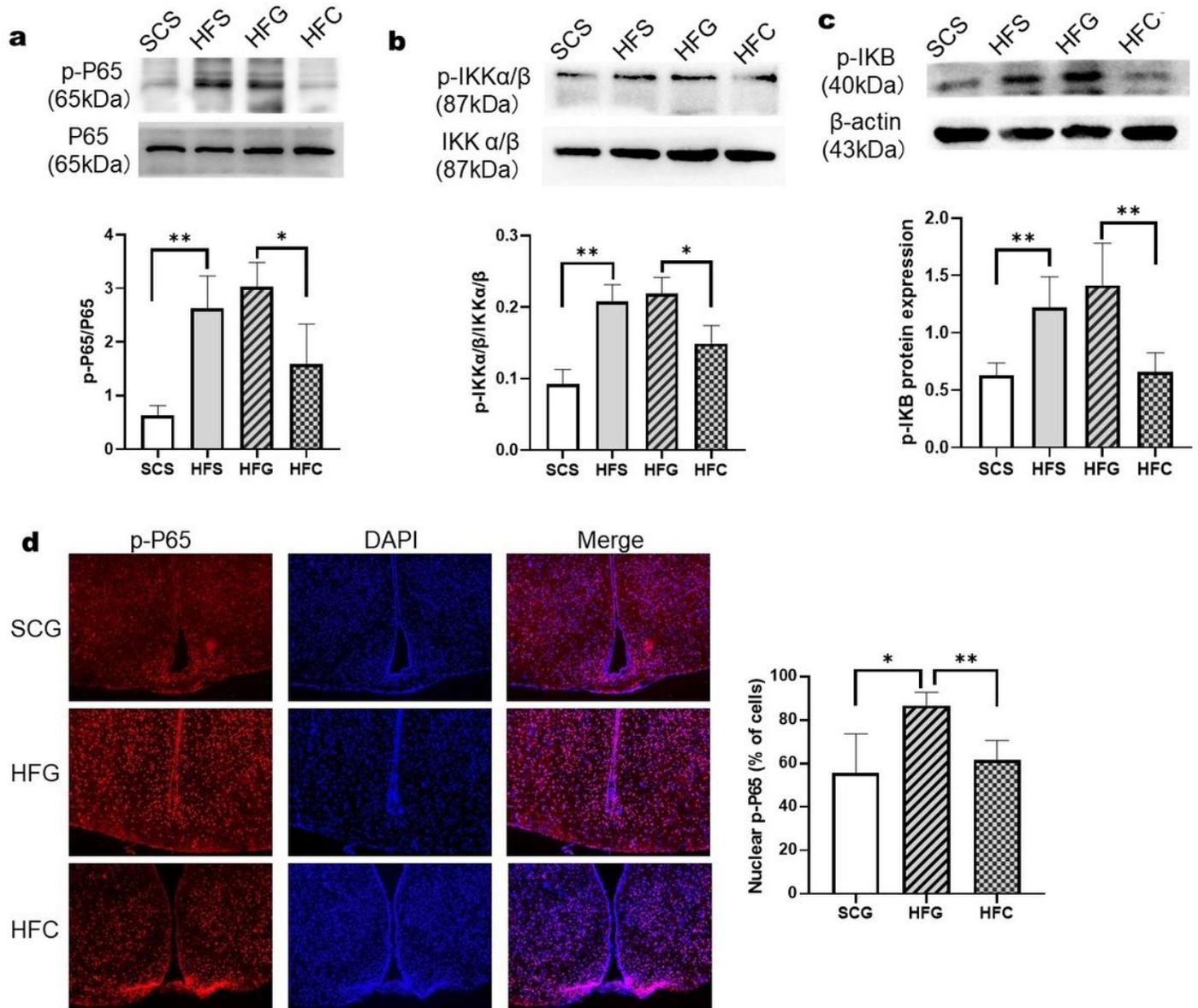
received saline (SCS and HFS), Ad-GFP (HFG) and Ad-CTRP4 (HFC). n=5. Statistical analyses were performed by two-tailed unpaired Student's t-tests. \*P<0.05, \*\*P<0.01.



**Figure 4**

Effects of central CTRP4 overexpression on inflammatory factors and microglial activation. At 72 hours after i.c.v. injection, the mice were fasted for 12 hours, hypothalamic tissue was collected, and fasting blood samples were collected retro-orbitally. a-b. Immunoblots showing hypothalamic TNF-α (a) and IL-6

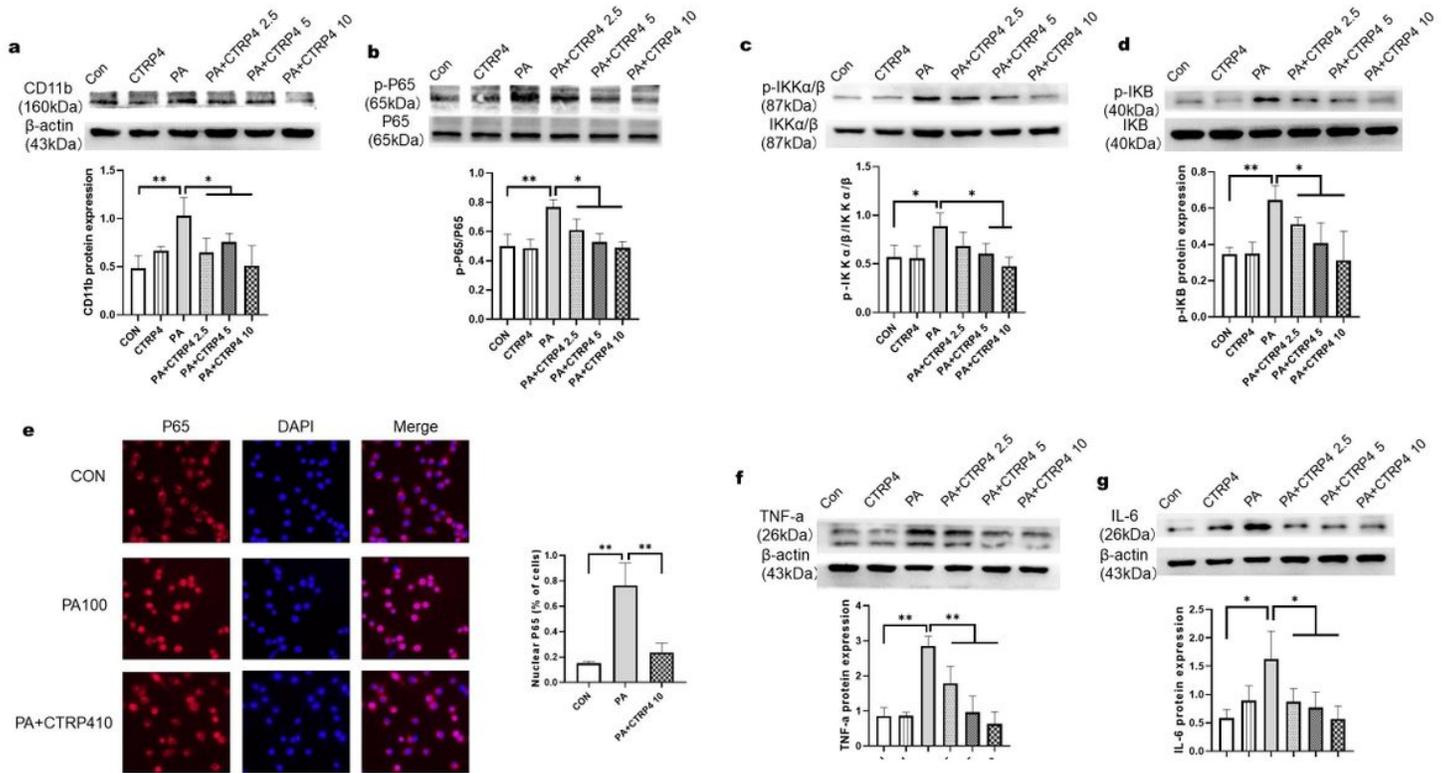
(b) in SCD- and HFD-fed mice that received saline (SCS and HFS), Ad-GFP (HFG) and Ad-CTRP4 (HFC). c. d. Plasma TNF- $\alpha$  (c) and IL-6 (d) levels were measured by ELISA after central intervention. e. Representative images of Iba1 immunoreactivity in the ARCs of mice after Ad-GFP (SCG and HFG) or Ad-CTRP4 intervention (HFC). n=5. Statistical analyses were performed by two-tailed unpaired Student's t-tests. \*P<0.05, \*\*P<0.01.



**Figure 5**

Effects of central CTRP4 overexpression on hypothalamic NF- $\kappa$ B signaling. At 72 hours after i.c.v. injection, the mice were fasted for 12 hours, and then hypothalamic tissue was collected. Immunoblots showing hypothalamic p-P65 and P65 (a), p-IKK $\alpha/\beta$  and IKK $\alpha/\beta$  (b), and p-I $\kappa$ B in SCD- and HFD-fed mice that received saline (SCS and HFS), Ad-GFP (HFG) and Ad-CTRP4 (HFC). d. Representative images of p-P65 immunoreactivity (red) and DAPI nuclear staining (blue) in the ARCs of mice after Ad-GFP (SCG and

HFG) or Ad-CTRP4 intervention (HFC). n=5. Statistical analyses were performed by two-tailed unpaired Student's t-tests. \*P<0.05, \*\*P<0.01.



**Figure 6**

Effects of central recombinant CTRP4 on NF-κB signaling, proinflammatory factors and microglial activation (BV-2 cells). BV-2 cells were pretreated with DMEM or 2.5 μg/ml, 5 μg/ml, or 10 μg/ml recombinant murine CTRP4 protein for 2 h, followed by 100 μM palmitate or DMSO for 24 h before Western blot analysis (a-d, f-g). Immunoblots showing CD11b (a), p-P65 and P65 (b), p-IKKα/β and IKKα/β (c), p-IκB (d), TNF-α (f), and IL-6 (g) in BV2 cells. BV-2 cells were pretreated with 10 μg/ml recombinant murine CTRP4 protein or DMEM for 2 h, followed by 100 μM palmitate for 24 h before immunofluorescence analysis (e). e. Representative images of p-P65 immunoreactivity (red) and DAPI nuclear staining (blue) in BV2 cells. n=5. Statistical analyses were performed by two-tailed unpaired Student's t-tests. \*P<0.05, \*\*P<0.01.