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Hypothalamic BMP9 suppresses glucose production by central PI3K/Akt/mTOR pathway

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Abstract

Bone morphogenetic proteins (BMPs) are secreted ligands that belong to the transforming growth factor- β (TGF- β) superfamily. BMP7 has been reported to play a role in reversing obesity and regulating appetite in the hypothalamus. Whether BMP9 plays a central role in regulating glucose metabolism and insulin sensitivity remains unclear. Here, we investigated the impact of central BMP9 signaling and possible route of transmission. We performed intracerebroventricular (ICV) surgery and injected adenovirus expressing BMP9 (Ad-BMP9) into the cerebral ventricle of mice. Metabolic analysis, hyperinsulinemic-euglycemic clamp test, and analysis of phosphatidylinositol 3,4,5-trisphosphate (PIP3) formation were then performed. Real-time PCR and Western blotting were performed to detect gene expression and potential pathways involved. We found that hypothalamic BMP9 expression was downregulated in obese and insulin-resistant mice. Overexpression of BMP9 in the mediobasal hypothalamus reduced food intake, body weight, and blood glucose level, and elevated the energy expenditure in high-fat diet (HFD)-fed mice. Importantly, central treatment with BMP9 improved hepatic insulin resistance (IR) and inhibited hepatic glucose production in HFD-fed mice. ICV BMP9-induced increase in hepatic insulin sensitivity and related metabolic effects were blocked by ICV injection of rapamycin, an inhibitor of mammalian target of rapamycin (mTOR) signaling. In addition, ICV BMP9 promoted the ability of insulin to activate the insulin receptor/phosphoinositide 3-kinase (PI3K)/Akt pathway in the hypothalamus. Thus, this study provides insights into the potential mechanism by which central BMP9 ameliorates hepatic glucose metabolism and IR via activating the mTOR/PI3K/Akt pathway in the hypothalamus.

Key Words

- ▶ BMP9
- ▶ glucose metabolism
- ▶ hypothalamus
- ▶ insulin resistance
- ▶ mTOR

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Introduction

The global prevalence of obesity and type 2 diabetes mellitus (T2DM) is increasing. These two diseases are associated with insulin resistance (IR) and disrupted lipid and glucose metabolism (Saltiel & Kahn 2001). To understand the molecular mechanism underlying IR, a large number of studies have investigated the role of insulin signaling molecules in the liver, muscle, and fat over the past decades (Schenk *et al.* 2008). Hormones secreted from different tissues, such as adiponectin from the adipose tissue and fibroblast growth factor-21 from the liver, were found to be involved in the regulation of insulin sensitivity and energy metabolism (Kadowaki *et al.* 2006). In recent years, several reports have demonstrated that the CNS, particularly the hypothalamus, plays a key role in modulating insulin sensitivity and energy homeostasis *in vivo* (Knight *et al.* 2011, Purkayastha *et al.* 2011, Yang *et al.* 2012). Furthermore, changes in the levels of some nutrients and hormones in the hypothalamus have been found to regulate glucose and lipid homeostasis *in vivo* (Lam *et al.* 2011, Wu *et al.* 2014, Luo *et al.* 2016). Further investigation on the role of CNS, including the hypothalamus, in hormone signaling, is important for better understanding the pathogenesis of obesity and T2DM.

Bone morphogenetic proteins (BMPs) are secreted ligands that belong to the transforming growth factor- β superfamily. They not only function as developmental factors in embryonic development and growth differentiation but also play a role in adipocyte development and energy metabolism (Chen *et al.* 2004, Yamamoto & Oelgeschlager 2004, Kishigami & Mishina 2005, Tobin & Celeste 2006, Schulz & Tseng 2009). Interestingly, several BMP ligands have been found to be present in the cerebrospinal fluid and expressed in the hypothalamus (Dattatreya *et al.* 2001, Ohyama *et al.* 2008). Furthermore, different BMP receptor isoforms have been identified throughout the brain (Ebendal *et al.* 1998), suggesting that BMP signaling might regulate hypothalamic function. Townsend *et al.* (2012) conducted a study with db/db and ob/ob mice and observed that BMP7 reversed obesity and regulated appetite through a central mammalian target of rapamycin (mTOR) pathway. In addition, BMP8B was found to be expressed in brown adipose tissue (BAT) and the hypothalamus, and central treatment with BMP8B increased thermogenesis via neuronal activation of regulatory nuclei in the hypothalamus (Whittle *et al.* 2012). In the peripheral tissues, BMP8B was found to increase thermogenesis by

inducing white adipose tissue (WAT) browning (Martins *et al.* 2016, Pellegrinelli *et al.* 2018). Recently, Mahli *et al.* (2019) reported that BMP8B was related to fatty acid uptake and *de novo* lipogenesis in the liver.

BMP9 is mainly expressed in non-parenchymal hepatocytes, like endothelial and stellate cells, and can bind to a specific receptor on the hepatocytes (Song *et al.* 1995, Miller *et al.* 2000). Initially, Caperuto *et al.* (2008) showed that BMP9 might play a role in the regulation of glucose metabolism and insulin signaling. Later, Kuo *et al.* (2014) demonstrated that the administration of BMP9 antibodies-induced glucose intolerance and IR in rats. Peripheral treatment with MB109, a BMP9 analog, was shown to decrease weight gain in high-fat diet (HFD)-fed mice by reducing the size of white adipocytes and decreasing fasting blood glucose (FBG) levels without changing food intake or apparent behavioral performances. MB109 treatment also increased the expression of the browning-related genes in the subcutaneous fat of HFD-fed mice. In addition, MB109 enhanced the expression of genes related to hepatic fatty acid synthase in obese mice (Kuo *et al.* 2014). In the CNS, BMP9 was also found to be a potent inducer of the cholinergic phenotype (Lopez-Coviella *et al.* 2000). Recently, we have been reported that circulating BMP9 levels are markedly lower in newly diagnosed patients with T2DM compared with those in healthy subjects (Luo *et al.* 2017).

Based on accumulating evidence as described previously, we hypothesized that BMP9 might be expressed in the hypothalamus and play a central role in glycoregulation and IR in obesity and T2DM. To test this hypothesis, we investigated the impact of intracerebroventricular (ICV) injection of BMP9 on glycoregulation and hepatic insulin signaling and further explored the possible underlying mechanisms.

Materials and methods

Animal preparation

Male C57BL/6J (cWT), db/db and adiponectin knockout (Adipoq^{-/-}) mice were purchased from Experimental Animal Centers of Chongqing University of Medical Sciences and Shanghai Biomodel or Ganismsci & Tech Develop Co., Ltd. Shanghai, China, respectively. All animals were adapted to feed for 7 days. In this study, 8-week-old WT and Adipoq^{-/-} mice were fed with either normal chow diet (NCD, 10% calories from fat) or HFD (45% of calories from fat; Medicine Inc. Jiangsu, China) for 12 weeks. The animals were fed in humidity and temperature controlled rooms

with free access to food and water. In the study, all kinds of treatments for mice were specifically approved and strictly carried out in accordance with the administrative animal management method of Chongqing Medical University, and at the same time, all the experiments were approved and performed according to the protocols established by Ethics Committee of Chongqing Medical University (Reference Number: 2017020).

Construction of recombinant adenovirus vectors

Adenovirus expressing BMP9 (Ad-BMP9) and encoding enhanced green fluorescence protein (Ad-GFP, as a control) were generated using the AdEasy Adenoviral Vector System (Qbiogene) as previously reported (Li *et al.* 2011). The sequence designed for Ad-BMP9 is sense: 5'-CTCGGATCCATGTGTCCTGGGGCACTGTGG-3' and antisense: 5'-CTCTCTAGACTACCTG CACCCACAC-TCTGC-3'. Large-scale amplification and purification of recombinant adenoviruses were performed with the Vira Bind Adenovirus Purification Kit, according to the manufacturer's instructions (Cell Bio Labs, San Diego, CA, USA).

Intracerebroventricular (ICV) surgery and hepatic branch vagotomy (HVG)

Seven days before the hyperinsulinemic-euglycemic clamps (HECs), mice were anesthetized and fixed in a stereotaxic apparatus. A stainless steel guide cannula (62204, RWD life science, China) was implanted into the third ventricle of the cerebrum (coordinates -1.40 mm anteroposterior, 0.00 mm lateral and 5.40 mm dorsoventral from bregma) of WT mice, then fixing the catheter with glass ionomer cement. 24 h after cannula implant, an amount of 2 μ L (5×10^9 pfu/mL) of Ad-BMP9 or Ad-GFP was injected slowly and uniformly into the third ventricle of mice within 5 min (Kooijman *et al.* 2015). Following the infusion, the guide cannula remained inserted for approximately 5 min to allow the drug to diffuse away from the cannula tip. In four subgroups of mice, the cannula was then implanted into the third ventricle of the brain for rapamycin (R0395 -1 MG, Sigma) or DMSO treatment. Three days later, a Silastic catheter (0.02 ID, Dow Corning Corporation, MI, USA) was placed in the internal jugular vein for sampling during the hyperinsulinemic-euglycemic clamp (HEC), glucose tolerance tests (GTT), and insulin tolerance tests (ITT). Another polyethylene catheter (PE50+PE10, Smiths Medical ASD, Inc, USA) was placed in the left carotid artery. To prevent blood coagulation,

catheters were flushed with 300 μ L of isotonic saline containing heparin (20 U/mL) and ampicillin (5 mg/mL) and then filled with a viscous solution of heparin (300 U/mL) and 80% polyvinylpyrrolidone (PVP-10, Fisher Scientific, Pittsburgh, PA). For the study of the mTOR signal pathway, Rapamycin or DMSO was slowly injected into ICV (20 μ g/2 μ L/5 min) 3 h before the *in vivo* experiments. For the HVG study, separate cohorts of mice were subjected to HVG or sham operation (SHAM), as previously reported (Luo *et al.* 2016). Briefly, an incision was made into the ventral midline. The hepatic branch of the ventral subdiaphragmatic vagal trunk was identified and transected by microcautery, severing and cauterizing the hepatic vagus. SHAM control mice underwent the same procedure except for the transection of the nerve.

Metabolic analyses

Mice were single-housed after adenovirus injection. Body weight and food intake were measured daily for 2 weeks. After the mice were adapted for 24 h in a computer-controlled calorimetry system (MM-100 CWE Inc., PA, USA), 24-h oxygen consumption (VO_2), the volume of carbon dioxide produced (VCO_2), and energy expenditure were measured every 10 min for 24 h on the day 7 after adenovirus injection. The respiratory exchange ratio was calculated as the $\text{RER} = \text{VO}_2/\text{VCO}_2$. The Data is represented as the means obtained in these records. Blood pressure was measured by the tail-cuff method with the BP-2010A system (Softron Biotechnology, Beijing, China).

Glucose (GTTs) and insulin tolerance tests (ITTs)

GTTs and ITTs were performed by intraperitoneal injection of 2 g/kg glucose after overnight fasting (12 h) and 0.75 units/kg insulin after 4 h fasting, respectively, as previously described (Luo *et al.* 2016). Four subgroups of mice fed with HFD were subjected to ICV injection with rapamycin (20 μ g/2 μ L) or the vehicle (DMSO) 2 h before the start of the GTT (Stevanovic *et al.* 2013). During the GTT and ITT, blood glucose was measured in indicated time points (including 0, 15, 30, 60, 90, and 120 min).

Hyperinsulinemic-euglycemic clamp studies (HECs)

After fasting for 10 h, HECs were performed in conscious and unrestrained mice as previously reported (Li *et al.* 2009, Wu *et al.* 2014). Briefly, HPLC-purified [$3\text{-}^3\text{H}$] glucose was infused through the jugular vein catheter starting at 0 min with a bolus (5 μ Ci) followed by a

continuous infusion (0.05 μ Ci/min) for 90 min (basal period) to estimate the rate of basal glucose turnover. Following the basal period, a 2-h HEC was conducted with a primed, continuous infusion of human insulin (5 mU/kg/min) (Humulin; Eli Lilly Co., Indianapolis, IN, USA) to raise plasma insulin levels, while plasma glucose was maintained at basal concentrations with variable rates of 20% glucose infusion. The basal/clamped periods were 90/120 min (Fig. 3C). At the end of the HEC, mice were killed, and tissues were freeze-clamped *in situ* and stored at -160°C for subsequent analysis.

In vivo glucose uptake

Glucose uptakes in fat and muscle tissues were examined as previously reported (Boini *et al.* 2006). Briefly, mice for fasting 12 h were injected intraperitoneally with 2-deoxy- ^3H glucose (2-DG, 150 μ Ci/kg; Amersham, Los Angeles, CA) mixed with 20% glucose (2g/kg). Blood samples were extracted at 0, 15, 30, 60 min to measure blood glucose and glucose-specific activity. After 120 min, the mice were killed, and 100 mg of tissues were homogenized in 1 mL of water. Seven percent ice-cold perchloric acid (800 μ L) was added to homogenate (800 μ L). The sample was then cleared by centrifugation, and 1 mL of the supernatant was neutralized for 30 min with 2.2 mol/L KHCO_3 . The precipitate was removed by centrifugation, and 500 μ L of the supernatant was used to determine total ^3H radioactivity.

C-fos and BMP9 immunohistochemistry (IHC)

On day 7 of ICV Ad-BMP9 or Ad-GFP injection, animals were anesthetized and perfused with saline/heparin (20 U/mL) for 3 min and then with 4% paraformaldehyde/PBS (0.1 mol/L) for 20 min. The hypothalamus was excised and transferred to 4% paraformaldehyde, finally, fixed at 4°C for 24 h. After being dehydrated with ethanol and xylene, the specimens were embedded in paraffin, and coronal sections of the hypothalamus were obtained. IHC for c-Fos (1:200, Abcam Cat# ab71809, RRID: AB_2737414) and BMP9 (Abcam Cat# ab71809, RRID: AB_1267808) were performed as previously reported (Wu *et al.* 2014).

Analysis of phosphatidylinositol 3,4,5-trisphosphate (PIP3) formation *in situ*

PIP3 formation was carried out using a fluorescein-conjugated Anti-PIP3 (Echelon Biosciences Cat# Z-G345, RRID: AB_427218) as reported previously (Könner *et al.*

2007). For the quantitative analysis of PIP3, three coronal sections of the arcuate nucleus (ARC) in each mouse at bregma 1.3, 1.4 and 1.5 mm were examined including 600–800 cells. The percentage of positively stained ARC cells was presented as PIP3 levels. Image J software was used to score the positively stained area, which was presented as PIP3 formation.

Analysis of real-time quantitative PCR and Western blot

Mice were anesthetized using CO_2 and were decapitated. Brains were removed immediately and the hypothalamus was isolated, frozen in liquid nitrogen and kept at -160°C until processed for total RNA and protein extraction (Sahu *et al.* 2017). RT-PCR was performed as described previously (Wu *et al.* 2014). The primer pairs used for RT-PCR experiments were listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article). Primary antibodies used for Western blot detection included anti-insulin receptor (InsR) (Cell Signaling Technology Cat# 3025, RRID:AB_2280448)/anti-phospho-InsR (Cell Signaling Technology Cat# 3024, RRID:AB_331253), anti-AKT kinase (AKT) (Cell Signaling Technology Cat# 9272, RRID:AB_329827)/anti-phospho-AKT (Cell Signaling Technology Cat# 9271, RRID:AB_329825), anti-mammalian target of rapamycin (mTOR) (Cell Signaling Technology Cat# 2972, RRID:AB_330978)/anti-phospho-mTOR (Cell Signaling Technology Cat# 2971, RRID:AB_330970), and anti-p70 ribosomal S6 kinase (p70S6k) (Cell Signaling Technology Cat# 9202, RRID:AB_331676)/anti-phospho-p70S6k (Cell Signaling Technology Cat# 9204, RRID:AB_2265913), anti-glucose-6-phosphatase (G6Pase) (Abcam Cat# ab83690, RRID:AB_1860503), anti-BMP9 (Abcam Cat# ab71809, RRID:AB_1267808), anti-phosphoenolpyruvatecarboxykinase (PEPCK) (Santa Cruz Biotechnology Cat# sc-32879, RRID:AB_2160168) and β -actin (Cell Signaling Technology Cat# 4970, RRID:AB_2223172). In addition, anti-BMP9 was identified (Supplementary Fig. 1).

Analytical procedures

[3- ^3H] Glucose and 2-DG radioactivities were determined by the scintillation counter. Serum-free fatty acid (FFA) was measured spectrophotometrically using a commercial kit. An ELISA kit was used to measure serum insulin levels. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured by an auto-analyzer (Hitachi High-Technologies Corp.).

Statistical analyses

Data were presented as mean $\pm$ s.e. A two-way ANOVA with a least significant difference *post hoc* test was used to compare mean values between multiple groups, and a two-sample paired, or unpaired Student's *t*-test was used for comparison between two groups. Energy expenditure was analyzed by ANCOVA (Tschöp *et al.* 2011). $P < 0.05$ was considered significant.

Results

BMP9 expression and distribution of GFP in the hypothalamus of mice

First, we analyzed BMP9 mRNA expression using RT-PCR and found that BMP9 was highly expressed in the hypothalamus, but hardly in the kidney (Supplementary Fig. 2). To explore whether hypothalamic BMP9 expression was altered under IR state, its expression level was determined in db/db mice, and NCD- or HFD-fed WT and Adipoq^{-/-} mice. We found that the levels of BMP9 protein were markedly decreased in the hypothalamic tissues of db/db mice (Fig. 1A), HFD-fed WT and Adipoq^{-/-} mice (Fig. 1B and Supplementary Fig. 3A) compared to those in NCD-fed WT mice. In addition, the levels of BMP9 protein in HFD-fed Adipoq^{-/-} mice were also markedly lower than those in NCD-fed WT and Adipoq^{-/-} mice (Fig. 1B). These data indicated a possible involvement of hypothalamic BMP9 in genetic- and environmental-induced IR. To examine the anatomical distribution of the viral vector post-ICV injection, we used spectral confocal microscopy to evaluate GFP expression (green) in the brain. As shown in Fig. 1C, the results demonstrated that GFP was mainly expressed in the hypothalamic region. In addition, IHC staining of BMP-9 protein expression was shown in Fig. 1D.

Effect of ICV Ad-BMP9 on hypothalamic BMP9 expression and energy expenditure

To assess the impact of ICV injection of Ad-BMP9 on hypothalamic BMP9 expression in mice, we performed Western blot analyses. As shown in Fig. 2A and Supplementary Fig. 3B, treatment with central Ad-BMP9 resulted in increased BMP9 protein levels in the hypothalamus of both NCD- and HFD-fed mice. In addition, ICV Ad-BMP9 treatment of HFD-fed mice resulted in an increase in the number of Fos-positive cells in the ARC, paraventricular (PVN), and dorsomedial

nucleus (DMN), which are involved in energy balance (Fig. 2B). The serum levels of total cholesterol, triglyceride, FBG, insulin, and FFA were similar between Ad-BMP9- and Ad-GFP-treated mice fed an NCD (Supplementary Table 2). In HFD mice, however, ICV Ad-BMP9 treatment led to a significant reduction in insulin, total cholesterol, triglyceride, and FFA levels compared with Ad-GFP treatment (Supplementary Table 2). Subsequently, we investigated the impact of ICV Ad-BMP9 treatment on energy expenditure. The results showed that, under HFD feeding, Ad-BMP9-treated mice displayed an increase in rectal temperature (Fig. 2C), and decrease in body weight (Fig. 2D and E), fat mass (Supplementary Fig. 4A), food intake (Fig. 2F), feeding blood glucose (Fig. 2G), and blood pressure (Fig. 2H) compared with Ad-GFP-treated mice. These results also suggest that the effects of BMP9 overexpression on food intake and body weight may not be due to virus delivery, as shown in NCD-fed mice. Furthermore, we used indirect calorimetry to measure energy expenditure in NCD- and HFD-fed mice treated with Ad-BMP9 or Ad-GFP. We found that under HFD conditions, ICV Ad-BMP9-treated mice exhibited markedly increased VO₂ (Fig. 2I), CO₂ production (Fig. 2J), and heat (Fig. 2L), and decreased RER (Fig. 2K) compared with Ad-GFP-treated mice. Furthermore, the relationship between energy expenditure and body weight were analyzed by ANCOVA in these mice (Fig. 2M). These results demonstrated that ICV Ad-BMP9 reduced body weight in HFD-fed mice by elevating energy expenditure.

Overexpression of BMP9 in the mediobasal hypothalamus enhances glucose metabolism in HFD-fed mice

To investigate the glycoregulatory effects of ICV administration of Ad-BMP9, we first examined whether overexpression of hypothalamic BMP9 is sufficient to alter glucose kinetics *in vivo*. Ad-BMP9-treated HFD-fed mice showed significantly increased glucose tolerance and clearance, as indicated by the results of GTT and ITT, respectively, compared with Ad-GFP-treated mice (Fig. 3A and B). In addition, GTT in HFD fed mice was similar on the third day of Ad-GFP and Ad-BMP9 treatment (Supplementary Fig. 4B).

Next, we assessed the impact of Ad-BMP9 treatment on glucose turnover by HEC in NCD- or HFD-fed mice (Fig. 3C). During HEC, the serum parameters used are shown in Supplementary Table 3. Blood glucose was clamped at 4.5–6 mM and insulin levels were raised to ~four-fold the basal value. The time courses of blood glucose and

Figure 1

BMP9 expression and distribution of GFP in the hypothalamus of mice. (A) BMP9 protein expression in the hypothalamus of C57BL/6J and db/db mice. (B) BMP9 protein expression in the hypothalamus of NCD- or HFD-fed C57BL/6J and Adipoq^{-/-} mice. (C) Frontal sections of the brain for expression region of the fluorescent reporter GFP (green). (D) Immunohistochemical staining for BMP-9 expression in the hypothalamus. Data are expressed as mean $\pm$ s.e. NCD, normal chow diet; HFD, high-fat diet; Adipoq, adiponectin. The data are expressed as the mean $\pm$ s.e. ($n = 3/\text{group}$). ** $P < 0.01$ compared with C57BL/6J mice.

glucose infusion rate (GIR) during HEC were shown in Supplementary Fig. 5A and B. During the steady-state of HEC, compared to Ad-GFP, Ad-BMP9 treatment did not affect the ability of insulin to increase GIR (Fig. 3D) and inhibit hepatic glucose production (HGP) in NCD mice (Fig. 3E and F). However, in HFD-fed mice, Ad-BMP9 treatment significantly elevated the GIR required to prevent hypoglycemia (Fig. 3D) and lowered HGP (Fig. 3E and F) during the clamps, as compared to Ad-GFP treatment. Under the HFD condition, the rate of glucose disappearance (GRd) was also elevated in Ad-BMP9-treated mice compared with that in Ad-GFP-treated mice (Fig. 3G). To determine the impact of ICV Ad-BMP9 on

insulin-induced peripheral glucose utilization, tissue-specific sensitivity to insulin was determined *in vivo* by measuring 2-DG uptake in the WAT and skeletal muscle. Glucose uptake in the WAT of NCD- and HFD-fed mice was comparable between the Ad-BMP9 and Ad-GFP treatment groups (Fig. 3H). However, in the skeletal muscle of HFD-fed mice, Ad-BMP9 treatment induced a 20% increase in glucose uptake compared to Ad-GFP treatment (Fig. 3I). These results indicate that central BMP9 overexpression in HFD-fed IR mice improves hepatic and peripheral insulin action. However, we cannot exclude the BMP9-mediated effects on glucose metabolism may be secondary to weight loss.

Figure 2

Overexpression of hypothalamic BMP9 increases energy expenditure in HFD-induced obesity and insulin resistance. Mice received intracerebroventricular (ICV) injection of adenovirus expressing BMP9 (Ad-BMP9) or green fluorescent protein (Ad-GFP) prior to all measurements on day 7 post-adenoviral injection. (A) BMP9 protein expression in the hypothalamus. (B) Fos immunoreactivity (ir) in hypothalamic regions, including the arcuate (ARC), paraventricular, and dorsomedial nucleus, in ICV Ad-BMP9 or vehicle-treated mice. Left panel: photomicrographs of coronal brain sections; right panel: number of Fos-ir cells per section. (C) Rectal temperature. (D) Body weight for 2 weeks. (E) Body weight changing. (F) Food intake for 2 weeks. (G) Blood glucose levels. (H) Blood pressure. (I) 24-h oxygen consumption. (J) 24-h CO_2 production (VO_2). (K) Respiratory exchange ratio (RER: V_{CO_2}/V_{O_2}). (L) Energy expenditure. (M) Energy expenditure per mouse was plotted against body weight (left panel) and the adjusted means of energy expenditure (right panel) analyzed by ANCOVA. The data are expressed as the mean $\pm$ s.e. (Western blot: $n = 3$ /group; animal experiments: $n = 5-8$ /group). NCD, normal chow diet; HFD, high-fat diet. * $P < 0.05$; ** $P < 0.01$ vs Ad-GFP.

Figure 3

Activation of hypothalamic BMP9 by ICV injection of Ad-BMP9 ameliorates hepatic insulin resistance. Mice received ICV injection of adenovirus expressing BMP9 (Ad-BMP9) or green fluorescent protein (Ad-GFP) prior to all experiments on day 7 post-adenoviral injection. (A) Intraperitoneal glucose tolerance tests (GTT). (B) Insulin tolerance tests (ITT). (C) Experimental procedure and clamp protocol. (D) Glucose infusion rates (GIR). (E) Hepatic glucose production (HGP). (F) Percentage of suppression of endogenous glucose production. (G) Glucose disposal rate (GRd). (H) Rates of 2DG uptake in visceral adipose tissue. (I) Rates of 2DG uptake in skeletal muscle. (J) PEPCK and G6Pase mRNA expression in the liver. (K) PEPCK and G6Pase protein levels in the liver. (L) Immunoblot analysis of phospho-InsR/total InsR ratio and phospho-Akt/total Akt ratio in the liver. Data are means $\pm$ s.e. (Western blot: $n = 3$ /group; animal experiments: $n = 5$ –6/group). NCD, normal chow diet; HFD, high-fat diet. * $P < 0.05$; ** $P < 0.01$ vs Ad-GFP.

ICV Ad-BMP9 improves hepatic IR in HFD-fed mice

Based on the changes in glucose turnover, we examined the mRNA and protein levels of PEPCK and G6Pase, two key gluconeogenic enzymes, in the liver. In NCD-fed mice, ICV Ad-BMP9 treatment did not alter the mRNA and protein levels of hepatic G6Pase and PEPCK (Fig. 3J and K). As expected, HFD feeding increased the mRNA and protein levels of hepatic G6Pase and PEPCK compared to NCD feeding. However, in HFD-fed mice, ICV Ad-BMP9 treatment significantly decreased the mRNA and protein levels of the two gluconeogenic genes in the liver, as compared with Ad-GFP treatment (Fig. 3J and K). Next, we investigated the impact of ICV injection of Ad-BMP9 on hepatic insulin signaling. As shown in Fig. 3L, HFD feeding decreased InsR and AKT phosphorylation in the liver. However, overexpression of hypothalamic BMP9 under HFD condition significantly enhanced insulin-stimulated phosphorylation of InsR and AKT in the liver, as compared with the control group (Fig. 3L).

ICV Ad-BMP9 increases anorexigenic neuropeptide expression and enhances insulin sensitivity via the InsR-PI3K-Akt dependent pathway in HFD-fed mice

Next, we used quantitative RT-PCR to analyze the expression of hypothalamic proopiomelanocortin (POMC), an anorexigenic neuropeptide, and agouti-related peptide (AgRP) and neuropeptide Y (NPY), two orexigenic peptides. In NCD-fed mice, Ad-BMP9 treatment did not alter the expression of NPY, POMC, and AgRP mRNA in the hypothalamus of mice (Fig. 4A). However, under the HFD condition, Ad-BMP9 treatment led to a reduction of NPY expression and an increase of POMC expression in the hypothalamus as compared with Ad-GFP treatment (Fig. 4A). We further investigated whether Ad-BMP9 treatment affected insulin signaling in the hypothalamus of mice. Under NCD condition, the levels of hypothalamic InsR and AKT phosphorylation, in response to insulin, were not different between Ad-BMP9- and vehicle-treated mice (Fig. 4B). However, under the

Figure 4

Activation of hypothalamic BMP9 ameliorates central insulin sensitivity. Mice received ICV injection of adenovirus expressing BMP9 (Ad-BMP9) or green fluorescent protein (Ad-GFP) prior to all experiments on day 7 post-adenoviral injection. (A) The mRNA expressions of NPY, agouti-related peptide, and POMC in the hypothalamus. (B) Immunoblot analysis of phospho-InsR/total InsR ratio and phospho-Akt/total Akt ratio in the hypothalamus. (C) Immunofluorescence for PIP3 formation (left panels) and quantification of PIP3 levels (right panels) in ARC sections. Short arrows indicate PIP3 formation. (D) Phosphorylation of mTOR/7S6K1 in the hypothalamus (left, Western blot; right, quantitative measurement of protein). Data are means $\pm$ s.e. (Western blot: $n = 3$ /group; animal experiments: $n = 5$ – 6 /group). NCD, normal chow diet; HFD, high-fat diet. * $P < 0.05$; ** $P < 0.01$ vs Ad-GFP.

HFD condition, InsR and AKT phosphorylation in the hypothalamus was lower than that in the NCD condition. Importantly, ICV Ad-BMP9 treatment in HFD-fed mice led to an increase in InsR and AKT phosphorylation in the hypothalamus compared to vehicle treatment (Fig. 4B).

To investigate whether the major downstream pathway of InsR was activated by ICV injection of Ad-BMP9, we assessed the impact of hypothalamic Ad-BMP9 on the ability of insulin to promote PIP3 formation in the hypothalamic neurons of HFD-fed mice. Immunofluorescence staining showed that PIP3 formation in the ARC was increased in Ad-BMP9-treated mice (Fig. 4C). These data suggest that Ad-BMP9 treatment increases the ability of insulin to activate the PIP3 kinase pathway.

Inhibition of hypothalamic mTOR/S6K pathway reverses the ability of ICV Ad-BMP9 to regulate HGP and insulin signaling

To map the signaling pathways by which BMP9 regulates peripheral metabolic events, we performed

ICV injection of Ad-BMP9 and evaluated the activation of potential signaling candidates by western blot analysis. Consistent with previous reports (Ono *et al.* 2008), HFD-feeding significantly reduced mTOR and p70S6k phosphorylation in the hypothalamus of mice (Fig. 4D). However, ICV Ad-BMP9 treatment markedly increased the phosphorylation of mTOR and S6K1 in the hypothalamus of HFD-fed mice compared with Ad-GFP treatment (Fig. 4D). Finally, to elucidate the functional significance of BMP9-mediated mTOR activation in the regulation of glucose metabolism, Ad-BMP9- or Ad-GFP-treated HFD-fed mice were subjected to ICV injection of rapamycin, an inhibitor of mTOR complex 1 (p70S6K) signaling. The results showed that rapamycin treatment almost completely abolished BMP9-induced mTOR and p70S6K phosphorylation in the hypothalamus (Fig. 5A). Consistent with results obtained in the hypothalamus, rapamycin treatment largely reversed Ad-BMP9-induced improvement in glucose tolerance and clearance (Fig. 5B and C). Accordingly, rapamycin treatment also abolished the effects of ICV Ad-BMP9 on the mRNA (Fig. 5D) and

Figure 5

Inhibition of hypothalamic mTOR by ICV injection of rapamycin reverses the increase of hepatic insulin sensitivity induced by hypothalamic BMP9. HFD-fed mice received ICV injection of adenovirus expressing BMP9 (Ad-BMP9) or green fluorescent protein (Ad-GFP) prior to all experiments on day 7 post-adenoviral injection. Rapamycin or DMSO was injected into ICV 3 h before the experiment. (A) Phosphorylation of mTOR/70S6K1 in the hypothalamus (left, Western blot; right, quantitative measurement of protein). (B) Intraperitoneal glucose tolerance tests (GTT). (C) Insulin tolerance tests (ITT). (D) PEPCK and G6Pase mRNA expression in the liver. (E) PEPCK and G6Pase protein levels in the liver. (F) Immunoblot analysis of phospho-InsR/total InsR and phospho-Akt/total Akt ratio in the liver. Data are means $\pm$ s.e. (Western blot: $n = 3$ /group; animal experiments: $n = 5-6$ /group). * $P < 0.05$; ** $P < 0.01$ vs DMSO.

protein (Fig. 5E) expression of gluconeogenic genes and insulin signaling (Fig. 5F) in the liver.

Hepatic innervation is required to mediate the effect of ICV Ad-BMP9 on glucose metabolism

To further explore the pathway that mediates the impact of BMP9 on glucose metabolism, we performed HVG or sham operation in Ad-GFP- or Ad-BMP9-treated HFD-fed mice. As showed in Supplementary Fig. 6A, there were no significant changes in body weight and food intake 6 days before virus injection in the HVG and SHAM groups. However, HVG completely eliminated the effects of ICV Ad-BMP9 on body weight and food intake (Supplementary Fig. 6A). Results of GTT and ITT showed that, in sham mice, ICV Ad-BMP9 treatment significantly lowered blood glucose level and the glucose area under the curve (AUC) compared to Ad-GFP treatment. However, in HVG mice, the effects of Ad-BMP9 on blood glucose were attenuated, as shown by GTT and ITT (Supplementary Fig. 6B and C). These results suggest that vagal innervation is required for mediating the effect of BMP9 on glucose metabolism.

Discussion

In the current study, our data demonstrate that: (1) hypothalamic BMP9 expression is downregulated in insulin-resistant animals, (2) overexpression of hypothalamic BMP9 increases energy expenditure, decreases body weight, and food intake, (3) ICV administration of Ad-BMP9 diminishes HGP and reinforces hepatic insulin action, (4) Ad-BMP9 enhances insulin sensitivity via the InsR/PI3k/Akt pathway in the hypothalamus, and (5) mTOR/S6K signaling is essential for mediating the effect of hypothalamic BMP9 on glycoregulation and insulin action. These findings reveal the central role of BMP9 in the regulation of energy equilibrium and insulin sensitivity *in vivo*.

As in our previous study and other publications (Contreras *et al.* 2014, Wu *et al.* 2014, Quaresma *et al.* 2017), in this study, we performed stereotaxic injection of a viral vector for gene manipulation in the ICV to build an adult-onset overexpression of BMP9 in the hypothalamus. In recent years, stereotaxic injection of a viral vector has become an indispensable tool that allows scientists to functionally dissect the roles of hormones and neural circuits *in vivo* (Nectow *et al.* 2017). However, one limitation of this study is that an adenoviral infection

may cause brain inflammation, thereby complicating data interpretation. We used a GFP-expressing adenovirus as a control to address this problem. Another limitation of this study is that ICV injection was performed, and therefore, the nucleus specificity was lost. However, similar to previous studies (Inquimbert *et al.* 2013, de Moura *et al.* 2016, Barahona *et al.* 2018), we found that after ICV injection with Ad-GFP or Ad-BMP9, GFP was mainly distributed in the hypothalamus, while IHC staining showed that BMP-9 was expressed in hypothalamic nuclei related to metabolisms, such as the VMH and ARC. Thus, we believe that these results can enhance our understanding of BMP9 signaling in the CNS.

Notably, a previous study has reported that the ICV administration of BMP7 in mice leads to an acute decrease in food intake and an increase in energy expenditure (Townsend *et al.* 2012). In addition, another study also found that hypothalamic BMP8B increased thermogenesis, food intake, and weight loss via regulation of the hypothalamic AMP-activated protein kinase (AMPK) pathway (Whittle *et al.* 2012). Therefore, these reports are reminiscent of the mechanism of action of BMP underlying the regulation of energy equilibrium in the CNS. In the present study, we not only observed that BMP9 expression in the hypothalamus was downregulated in IR state, such as in db/db, Adipoq^{-/-}, and HFD-fed mice, but also demonstrated that ICV injection of Ad-BMP9 decreased appetite and body weight, and increased energy expenditure in HFD-fed insulin-resistant mice. Thus, data from the current study suggest that BMP9 may be a target for the hypothalamic regulation of energy homeostasis and that BMP9 is a cytokine with pleiotropic functions.

Furthermore, previous reports have shown that sex affects the impact of BMPs, such as BMP8B, whose thermogenic effect is only observed in females (Whittle *et al.* 2012, Martins *et al.* 2016). In previous studies in humans, no gender differences in circulating BMP9 levels were observed (Luo *et al.* 2017). Thus, we used male mice for the current study. However, whether the effect of BMP9 is sexually dimorphic requires further investigation.

In recent years, several studies by our group and other groups have highlighted that the CNS plays a crucial role in the modulation of whole-body energy homeostasis (Lam *et al.* 2011, Zhang *et al.* 2013, Wu *et al.* 2014, Yang *et al.* 2017). Indeed, some hormonal and cytokine signals within the hypothalamus can regulate HGP and insulin sensitivity. In the current study, overexpression of BMP9 in the hypothalamus of insulin-resistant mice enhanced insulin-mediated inhibition of HGP via inhibition of gluconeogenic enzymes, promoted glucose uptake in

the skeletal muscle, and increased the phosphorylation of insulin signaling molecules in the liver. Therefore, we consider that the effects of the central BMP9 signal on HGP might be multifaceted, including reduced food intake, weight loss, increasing energy consumption, and direct effects on the HGP. In addition, we found that the metabolic effects of ICV Ad-BMP9 were only seen in obese mice with IR. Therefore, it is possible that the regulatory roles of BMP9 in metabolism and insulin sensitivity are limited to obesity and IR states when BMP9 activity is impaired in the hypothalamus of mice with genetic- and diet-induced IR.

These findings further indicated that hormones and cytokines, such as BMP9 and adiponectin act in the hypothalamus to ameliorate peripheral IR, thereby proposing the need for further studies on the role of BMPs in HGP regulation. However, the molecular targets of these hormones or cytokines remain unknown. Our group and other groups have reported that hormone and cytokine signals in the hypothalamus activate K_{ATP} channels and trigger the hepatic vagus nerves to reinforce hepatic insulin signaling and regulate hepatic glucose fluxes *in vivo* (Knight *et al.* 2011, Lam *et al.* 2011). However, the transmission pathways of these signals within the hypothalamus remain elusive. In addition, the effects of different hormones and cytokines on insulin sensitivity may be both temporally and mechanistically distinct. Therefore, it is crucial to investigate the pathways that mediate BMP9 signaling in the brain.

Leptin and NPY have been reported to modulate food intake and energy metabolism via activation of the InsR/PI3K/Akt signaling cascade in the hypothalamus (Kim *et al.* 2000, Niswender *et al.* 2003). Therefore, we investigated whether BMP9 action in the hypothalamus was mediated by the InsR/PI3K/Akt signaling pathway to regulate hepatic glucose fluxes.

We hypothesized that BMP9 exerts its roles in hypothalamic neurons through the InsR/PI3K/Akt signaling pathway. As expected, we found that insulin-stimulated InsR and Akt phosphorylation was markedly elevated in the hypothalamus of HFD-fed mice treated with Ad-BMP9 compared to that in vehicle-treated mice. In addition, Ad-BMP9 treatment markedly increased PIP3 formation in ARC neurons compared to Ad-GFP treatment. These results suggest that BMP9 increases the ability of insulin to activate the InsR/PI3K/Akt pathway in the CNS. In an *in vitro* study, PIP3 was shown to directly activate K_{ATP} channels (Kim *et al.* 2000). Therefore, we speculate that BMP9 may promote insulin-stimulated phosphorylation of InsR and Akt in the hypothalamus and then activate

PI3 kinase in obese mice. Subsequently, increasing PIP3 formation leads to K_{ATP} -channel activation. Finally, the hepatic vagus is triggered, and this signal is relayed to the liver to regulate hepatic glucose fluxes.

The hypothalamic mTOR/S6K pathway has been considered to be a major pathway in the hypothalamic regulation of energy homeostasis. The importance of this signaling pathway in the modulation of food intake and glucose and lipid metabolism has been highlighted in previous reports (Cota *et al.* 2006, Blouet *et al.* 2008, Mori *et al.* 2009). However, previous studies have reported conflicting findings regarding the metabolic effects of the hypothalamic mTOR/S6K pathway (Cota *et al.* 2008, Hu *et al.* 2016). Therefore, the precise mechanisms underlying the regulatory roles of the mTOR/S6K pathway remain largely unknown and require further investigation. In agreement with a previous report (Ono *et al.* 2008), but in contrast to two other reports (Blouet *et al.* 2008, Cota *et al.* 2008), we found that HFD-feeding led to a decrease in mTOR/S6K signaling in the hypothalamus, suggesting a role of hypothalamic mTOR/S6K signaling in HFD-induced IR and obesity. However, Ad-BMP9 treatment reversed this effect, accompanied by an increase in peripheral insulin sensitivity. Importantly, the ICV injection of rapamycin, an inhibitor of mTOR, largely attenuated the effects of Ad-BMP9.

Interestingly, two previous studies have reported that the effect of BMP8B on BAT thermogenesis is mediated by hypothalamic AMPK (Whittle *et al.* 2012, Martins *et al.* 2016). In the current study, we found that the effect of BMP9 on energy homeostasis in the hypothalamus was mediated by the mTOR pathway. Notably, previous studies have shown that AMPK inhibits mTORC1 both indirectly, through the phosphorylation and activation of the tuberous sclerosis complex 2, and directly, through the phosphorylation of Raptor. Therefore, AMPK may be an upstream regulator of mTOR signaling (Saxton & Sabatini 2017). We speculate that since the thermogenic effect of BMP8B is mediated by inhibition of hypothalamic AMPK, the effect of BMP9 on energy homeostasis may also be mediated by inhibition of AMPK, facilitating the activation of the hypothalamic mTOR pathway. However, further studies are needed to determine the association between BMP9 and these signaling pathways.

The hepatic vagus nerve is important for relaying CNS signals to the liver to regulate HGP (Luo *et al.* 2016). In this study, we found that the effect of hypothalamic BMP9 on glucose metabolism can be abrogated by HVG, suggesting that hypothalamic BMP9 signals are also relayed to the liver through vagal efferent nerve fibers. Our findings

Figure 6

A model illustrating the working hypothesis of hypothalamic BMP9 signaling.

indicate a potential pathway for the transmission of BMP9 signals from the CNS to the liver and suggest that hypothalamic mTOR/S6K signaling and vagal innervation are essential for mediating the metabolic effects of hypothalamic BMP9. Thus, BMP9 may be considered as a neuro-regulated cytokine that acts through the canonical mTOR/S6K pathway in the hypothalamus, and this signal is finally relayed to the liver by vagal efferent for regulating HGP.

There were also some limitations in the current study, such as we did not use genetic animal models with BMP9 deficiency. Therefore, the interference of endogenous BMP9 cannot be excluded. Furthermore, we also cannot rule out that the effect of BMP9 on glucose metabolism may be secondary to weight loss.

Conclusion

The current study revealed that BMP9 is expressed in the hypothalamus and is downregulated under conditions of genetic- or HFD-induced IR. We showed that BMP9 overexpression in the hypothalamus of obese animals improves IR by promoting the ability of insulin to activate the InsR/PI3K/Akt/mTOR pathway. The InsR/PI3K/mTOR signaling cascade has been reported to induce K_{ATP} channel activation and membrane hyperpolarization to decrease the firing rate of hypothalamic neurons. Finally, the vagal efferent signals are triggered and relayed to the liver to regulate hepatic glucose fluxes and insulin signaling.

Thus, we illustrated a possible brain–liver axis, as shown in Fig. 6. This neuronal circuitry will help increase our understanding of the central regulation of BMP9 in energy homeostasis, as it pertains to obesity and IR.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0591>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data statement

All data points were included in our reported analysis.

Author contribution statement

Y H, C Z, M L and J C researched and analyzed the data. H F G reviewed and edited the manuscript. Y L contributed to the writing of the manuscript and helpful discussion. H F G reviewed and edited the manuscript. L L contributed to write, reviewed, and edited the manuscript. Y G and X Z are the guarantor of this work and have full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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