Effect of central JAZF1 on glucose production is regulated by the PI3K-Akt-AMPK pathway

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Abstract
The role of central juxtaposed with another zinc finger gene 1 (JAZF1) in glucose regulation remains unclear. Here, we activated mediobasal hypothalamus (MBH) JAZF1 in high-fat diet (HFD)-fed rats by an adenovirus expressing JAZF1 (Ad-JAZF1). We evaluated the changes in the hypothalamic insulin receptor (InsR)-PI3K-Akt-AMPK pathway and hepatic glucose production (HGP). To investigate the impact of MBH Ad-JAZF1 on HGP, we activated MBH JAZF1 in the presence or absence of ATP-dependent potassium (KATP) channel inhibition, hepatic branch vagotomy (HVG), or an AMPK activator (AICAR). In HFD-fed rats, MBH Ad-JAZF1 decreased body weight and food intake, and inhibited HGP by increasing hepatic insulin signaling. Under insulin stimulation, MBH Ad-JAZF1 increased InsR and Akt phosphorylation, and phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) formation; however, AMPK phosphorylation was decreased in the hypothalamus. The positive

Abbreviations: aCSF, artificial cerebrospinal fluid; Ad-GFP, adenovirus expressing green fluorescent protein; Ad-JAZF1, adenovirus expressing JAZF1; ARC, arcuate nucleus; AUGg, area under the curve; BAT, brown adipose tissue; BW, body weight; CNS, central nervous system; DVC, dorsal vagal complex; FAO, fatty acid oxidation; FFA, free fatty acid; HFD, high-fat diet; HGP, hepatic glucose production; HVG, hepatic branch vagotomy; GDR, glucose disappearance; GIR, rate of glucose infusion; GlcN, glucosamine; GSK3β, glycogen synthase kinase-3β; GTTs, glucose tolerance tests; GWAS, genome-wide association studies; FoxO1, forkhead box O1; InsR, insulin receptor; IR, insulin resistance; JAZF1, juxtaposed with another zinc finger protein 1; KATP, ATP-dependent potassium; LXREs, liver X receptor response elements; MBH, mediobasal hypothalamus; PEC, pancreatic-euglycemic clamp; PBNs, Primary hypothalamic neurons; PIP3, phosphatidylinositol 3, 4, 5-trisphosphate; RER, respiratory exchange; SD, Sprague Dawley; SREBP-1c, sterol regulatory element-binding protein 1c; T2DM, type 2 diabetes mellitus; UCP1, uncoupling protein 1; VO2, oxygen consumption; VCO2, carbon dioxide produced; WT, wild type; VMH, ventromedial hypothalamus.

Mengjiao Zhou and Xiaohui Xu contributed equally to this project.
1 | INTRODUCTION

The global incidence of type 2 diabetes mellitus (T2DM) is increasing rapidly, reaching an alarming level. It is estimated that the number of patients with T2DM will increase to at least 366 million by 2030.1 Although genome-wide association studies (GWAS) have confirmed that T2DM is associated with a genetic predisposition,2 we do not fully understand insulin resistance (IR), insulin secretion dysfunction, and other molecular factors related to diabetes pathophysiology.

The juxtaposed with another zinc finger protein 1 (JAZF1) gene is a transcription inhibitor, which is expressed in various tissues of both mice and humans with the highest expression found in fat cells and the testes.2 A variant of the JAZF1 gene (rs864745) has been reported to be related to an increased risk of T2DM,3 suggesting that the deletion of JAZF1 may be associated with the development of T2DM. Previously, we demonstrated that JAZF1 overexpression in 3T3-L1 cells and Hepa1-6 cells suppressed lipid synthesis and increased lipolysis.4 A subsequent study by Ming et al found that JAZF1 could regulate the expression of genes associated with lipid metabolism and inhibit lipid accumulation in adipocytes; suggesting that JAZF1 plays a role in lipid metabolism disorders.5 Recently, Jang et al and our group have found that JAZF1 transgenic mice fed a high-fat diet (HFD) exhibited reduced diet-induced weight gain, hepatic lipid accumulation, and IR compared with wild-type (WT) mice.6,7 However, the mechanism by which JAZF1 regulates glucose homeostasis is poorly understood. This may be due to the inhibition of nuclear orphan receptor TAK1/TR4 activity through protein-protein interactions to regulate PEPCK, a key enzyme involved in gluconeogenesis.8,9

More recently, we further reported that JAZF1 plays a key role in the regulation of aging and nutrition-related hepatic steatosis. Mechanistically, we found that JAZF1 suppressed sterol regulatory element-binding protein 1c (SREBP-1c) expression through inhibiting the transcriptional activity of liver X receptor response elements (LXREs) in the SREBP-1c promoter, which is mediated by AMPK.10 Taken together, these results indicate that JAZF1 regulates the energy balance and insulin sensitivity in multiple tissues in vivo, including the liver, fat, and muscle tissues. However, the role of JAZF1 in the central nervous system (CNS), especially in the metabolism-related hypothalamus, remains unknown.

Several studies have shown that neurons, especially those in the hypothalamic region, contribute to the regulation of metabolism, insulin sensitivity, and islet function in vivo.11-14 Furthermore, the level of JAZF1 mRNA expression in the hypothalamus was found to be higher than that in the liver, whereas level of its expression was lower in HFD-fed mice.15 Therefore, we hypothesize that JAZF1 may exert a key role in the regulation of glucose and energy homeostasis in the hypothalamus in addition to its role in peripheral tissues. In the present study, we performed experiments to investigate whether the direct activation of hypothalamic JAZF1 signaling can trigger a brain-liver neural transmission network to modulate glucose flux in the liver.

2 | MATERIAL AND METHODS

2.1 | Animal preparation

Five-week-old male Sprague Dawley (SD) rats, C57BL/6J WT mice, db/db, and adiponectin knockout (Adipoq KO) mice on a C57/BL6 background were obtained from the Animal Center (Chongqing Medical University), Chongqing, China or Shanghai Biomodel and Ganismsci & Tech Develop CO., Ltd. Mice (8 weeks old) were randomly assigned to receive a normal chow diet (NCD; 60% calories of carbohydrates) or a HFD (45% calories of fat) for 12 weeks. Mice were sacrificed via isoflurane anesthesia, and the relevant tissues were extracted and stored at −160°C until further analysis. Eight-week-old male SD rats were randomly assigned to receive a normal chow diet (NCD; 60% calories of carbohydrates) or a HFD (45% calories of fat) for 12 weeks. Mice were sacrificed via isoflurane anesthesia, and the relevant tissues were extracted and stored at −160°C until further analysis. Eight-week-old male SD rats were randomly assigned to receive a normal chow diet (NCD; 60% calories of carbohydrates) or a HFD (45% calories of fat) for 12 weeks. Mice were sacrificed via isoflurane anesthesia, and the relevant tissues were extracted and stored at −160°C until further analysis. Eight-week-old male SD rats were randomly assigned to receive a normal chow diet (NCD; 60% calories of carbohydrates) or a HFD (45% calories of fat) for 12 weeks. Mice were sacrificed via isoflurane anesthesia, and the relevant tissues were extracted and stored at −160°C until further analysis.
catheter (Figure 2D). Following stereotactic surgery, rats were housed in individual cages. All experiments were supported by the Animal Experimentation Ethics Committee of Chongqing Medical University and performed in accordance with the National Health and Medical Research Council of China Guidelines on Animal Experimentation.

2.2 | Hepatic branch vagotomy

Five days before the clamping, a single group of rats was performed either Hepatic branch vagotomy (HVG) or sham (SHAM) surgery, as previously described.19 Transection of the hepatic vagus nerve interrupts the nerve connection from the brain to the liver.

2.3 | Preparation and injection of JAZF1 overexpression and control GFP adenovirus into the MBH

The AdEasy Adenoviral Vector System (Qbiogene) was used to generate adenovirus expressing JAZF1 (Ad-JAZF1). An adenovirus expressing green fluorescent protein (Ad-GFP) (Clontech, Mountain View, CA) and artificial cerebrospinal fluid (aCSF) were used as controls. One week before the pancreatic-euglycemic clamp (PEC), NCD or HFD-fed rats from littersmates housed in individual cages were randomly assigned into two groups to receive an MBH injection with either Ad-JAZF1, Ad-GFP (5 μL, 1 × 10^9 pfu/mL) or aCSF. Seven days' post-adenoviral infection, experiments were performed as previously reported.20 The peak of viral expression in the hypothalamus was observed from Day 5 to 9 as previously reported.20

2.4 | MBH infusion for signaling analysis

As mentioned above, catheters were implanted into bilateral MBH of SD rats. Five days later, insulin was infused into the MBH (2 μU per site) for 5 minutes (0.4 μ/min). Rats were sacrificed and MBH was immediately stored at −160°C for signal analysis.

2.5 | Metabolic analyses and glucose tolerance tests

Seven days' post adenovirus or aCSF injection, independent groups of rats were analyzed by indirect calorimetry in a computer-controlled open circuit calorimetry system (MM-100 CWE Inc, Pennsylvania, PA, USA). Food intake and body weight (BW) were measured daily for 2 weeks. Oxygen consumption (V_O2) and carbon dioxide produced (V_CO2) were obtained every 15 minutes. The ratio of respiratory exchange (RER) was calculated as V_CO2/V_O2. For intraperitoneal glucose tolerance test (GT Ts), the rats were fasted for 10 hours and intraperitoneally injected with 25% glucose (2.0 g/kg). Blood samples were collected at indicated time points to measure blood glucose and insulin.21

2.6 | Pancreatic-euglycemic clamp

To analyze the glucose kinetics, a Pancreatic-euglycemic clamp (PEC) (Figure 2D) was engaged in conscious rats as reported previously.22 Briefly, a continuous infusion of HPLC-purified [3-H3] glucose (6 μCi bolus, 0.2 μCi/min) was administered at 0 minutes and maintained throughout the experiments to investigate the glucose kinetics. PECs were performed within the final 2 hours (from 120 to 240 minutes) of the experiment. Somatostatin (SRIF, 3μg/kg/min) was intravenously infused together with insulin (6 mU/kg/min) for inhibiting endogenous insulin secretion, and a 25% glucose infusion was initiated and adjusted every 5-10 minutes to maintain the blood glucose at approximately 6 mM. Blood was introverted from the vein catheter at the indicated time points to measure the level of insulin, free fatty acid (FFA), and [3-H3] glucose activity.23,24 In addition, for the animals that received glibenclamide, an ATP-dependent potassium (K_ATP) channels blocker, or AICAR (an AMPK activator), an MBH infusion (0.36 μL/h) of glibenclamide (100 μM) or AICAR (50 mM) was initiated at −120 minutes and run continuously until the end of the experiment. Finally, the rats were sacrificed and tissues were stored at −160°C until further analysis of mRNA and protein expression.

2.7 | JAZF1 and c-fos immunohistochemistry

On Day 7 following the injection with adenovirus, the rats were sacrificed and perfused with saline (heparin 20 U/mL) for 5 minutes followed by 4% paraformaldehyde for 15 minutes. The brain was harvested and fixed with 4% paraformaldehyde for 24 hours at 4°C. After being dehydrated in ethanol-xylene, tissues were embedded in paraffin, and coronal sections of the hypothalamus were obtained. Immunohistochemistry for the expression of JAZF1 protein and c-Fos were performed as described previously.23

2.8 | Cell culture and treatment

Human neuroblastoma cells (SH-SY5Y, CRL-2266, ATCC) were cultured and differentiated in serum-free DMEM in the absence or presence of insulin (100 nM) for 4 days.25 Primary
hypothalamic neurons (PHNs) were cultured as previously reported. To induce IR, differentiated SH-SY5Y cells and PHNs were incubated with 18 mM glucosamine (GlcN) or 1% BSA as a control in serum-free medium for 20 hours. Sixteen hours after serum starvation, differentiated SH-SY5Y cells and PHNs were infected with Ad-GFP or Ad-JAZF1 or pcDNA3.1 or pc3.1-JAZF1 for 72 hours. Cells were then treated with or without insulin (100 nM) for 30 minutes. Cell lysates were stored at −80°C.

2.9 Immunofluorescence staining

Juxtaposed with another zinc finger protein 1 immunostaining was performed as previously reported. Phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) immunostaining was performed in the arcuate nucleus (ARC) sections of fasted rats infected with either Ad-GFP or Ad-JAZF1, following insulin treatment. Rats were sacrificed 15 minutes after stimulation. PHNs were stained using an anti-PIP3 antibody co-incubated with anti-mCherry (Abbkine, California, USA), or fluorescein conjugated anti-PIP3 antibody (Z-G345; Echelon Biosciences, Salt Lake City, UT), respectively. The integrated density quantification of PIP3 in the ARC and PHNs was examined using Image J software. The PHN slides were measured including 650-800 cells/group to evaluate the PIP3 content.

3 RESULTS

3.1 JAZF1 expression and GFP distribution in the mouse hypothalamus

To explore whether the hypothalamic JAZF1 expression was changed in the IR-state, hypothalamic JAZF1 expression at the protein level was measured in db/db mice, HFD- or NCD-fed WT and Adipoq KO mice. First, we found that JAZF1 was highly expressed in the hypothalamic nuclei (eg, ARC), which is related to energy metabolism (Figure S1A). In addition, we further found that the level of JAZF1 protein expression was significantly decreased in the hypothalamus of db/db, HFD-fed WT and Adipoq KO mice, compared with NCD-fed WT mice (Figure S1B-D). These data suggest the possible involvement of hypothalamic JAZF1 in IR induced by heredity and/or diet.

To investigate the anatomical distribution of an MBH injection of Ad-GFP, we used spectral confocal microscopy to examine the GFP expression (green) in the brain. As shown in Figure S2, the results demonstrated that the GFP expression was primarily distributed within the hypothalamic region, which contains the nucleus associated with metabolic and energy regulation.

3.2 Effect of MBH Ad-JAZF1 injection on JAZF1-Expressing neurons and energy expenditure

To explore the impact of MBH Ad-JAZF1 on JAZF1 expression in neurons, immunohistochemistry was performed in the MBH of rats. The results showed that MBH Ad-JAZF1 resulted in a marked increase in the region of JAZF1 immunoreactive neurons, including the ARC and ventromedial hypothalamus (VMH), which is implicated in glucose homeostasis (Figure 1A left and middle panel). Furthermore, we found that an HFD resulted in a significant reduction of JAZF1 expression in the hypothalamus of db/db, HFD-fed WT and Adipoq KO mice, compared with NCD-fed WT mice (Figure S1B-D). These data suggest the possible involvement of hypothalamic JAZF1 in IR induced by heredity and/or diet.

To explore the impact of MBH Ad-JAZF1 on JAZF1 expression in neurons, immunohistochemistry was performed in the MBH of rats. The results showed that MBH Ad-JAZF1 resulted in a marked increase in the region of JAZF1 immunoreactive neurons, including the ARC and ventromedial hypothalamus (VMH), which is implicated in glucose homeostasis (Figure S1B and 1A, right panel). As expected, an Ad-JAZF1 injection in the MBH significantly increased the level of JAZF1 expression in the hypothalamus (Figure 1A, right panel). The effects of MBH Ad-JAZF1 on the biochemical parameters in NCD- or HFD-fed rats are presented in Table S3. In HFD-fed rats, Ad-JAZF1 treatment resulted in reduced BW and insulin levels, whereas in NCD-fed rats, Ad-JAZF1 did not result in any changes to these parameters.
We subsequently investigated the effects of MBH Ad-JAZF1 on energy expenditure. When fed a NCD, the food intake of MBH Ad-JAZF1 rats and that of the control rats was similar throughout the 14 days after MBH administration (Figure 1B). However, when fed an HFD, Ad-JAZF1-treated rats exhibited a decrease in food intake compared with Ad-GFP- or aCSF-treated animals (Figure 1B). We investigated the alteration in BW on Days 3-17 post-adenoviral injection, and no significant difference was observed among the NCD animals that received MBH Ad-JAZF1, Ad-GFP, or aCSF treatment (Figure 1C). However, MBH Ad-JAZF1 resulted in a significant reduction in BW on Days 7-17 in HFD-fed rats.
FIGURE 2  MBH injection of Ad-JAZF1 increases insulin sensitivity in HFD-fed rats. Rats were fed a NCD or HFD for 12 weeks and received the MBH injection of Ad-GFP, Ad-JAZF1 or aCSF. A, Blood glucose (left and middle panel) and AUC_{glucose} (right panel) during GTT. B, Insulin levels (left and middle panel) and AUC_{insulin} (right panel) during GTT. C, Schematic representation of the working hypothesis. D, Experimental procedure and clamp protocol. E, GIR curves in NCD-fed rats. F, GIR curves in HFD-fed rats. G, Average GIR. H, GDR. I, HGP. J, Percentage of suppression of HGP. K, Hepatic JAZF1 protein expression. L, Hepatic PEPCK and G6Pase mRNA expression. M, Hepatic PEPCK and G-6-Pase protein expression. N, Phosphorylation of InsR, Akt and IRS-1 in the liver. aCSF, artificial cerebrospinal fluid; AUC, the area under the curve for glucose or insulin; GDR, the rate of glucose disappearance; GIR, glucose infusion rate; HFD, high-fat diet; HGP, hepatic glucose production; MBH, mediobasal hypothalamus; NCD, normal chow diet; SRIF, somatostatin. Values are shown as mean ± SD. n = 5-6 rats per group. *P < .05 or **P < .01 vs aCSF- or Ad-GFP-treated rats fed with HFD.
ZHOU et al. (Figure 1C), whereas the control vectors had no discernible effects on this parameter. The effects of a single injection of MBH Ad-JAZF1 on food intake and BW were sustained for at least 10 days (Figure 1B,C). Thus, JAZF1 overexpression within the MBH via transfection with Ad-JAZF1 was sufficient to decrease the daily caloric intake and BW in rats fed an HFD. To test whether the effect of MBH Ad-JAZF1 on BW was also mediated by decreased thermogenesis, we performed indirect calorimetry experiments. The volume of VO2 and VCO2 of MBH Ad-JAZF1 rats fed an HFD were significantly elevated over that of the control rats (Figure 1D). Since the elevation of VO2 was greater than that of VCO2, the RER (VCO2/VO2) was lower for MBH Ad-JAZF1 rats than for the control rats fed an HFD (Figure 1D), indicating increased fatty acid oxidation following MBH Ad-JAZF1 administration. In addition, the Kcal/h/kg of the MBH Ad-JAZF1-treated rats was significantly higher than those of MBH Ad-GFP or aCSF rats (Figure 1D). Consistent with these findings, the expression of uncoupling protein 1 (UCP1) in brown adipose tissue (BAT) was significantly increased in MBH Ad-JAZF1 treated rats, suggesting the involvement of BAT activity (Figure S3). These data suggest that MBH Ad-JAZF1 increased BAT thermogenesis and energy expenditure in HFD-fed rats.

3.3 | JAZF1 overexpression in the hypothalamus heightens glucose handling in HFD-fed rats

We next examined the glucose handling of rats treated with MBH Ad-JAZF1 or Ad-GFP. When fed a NCD, the blood glucose levels and the area under the curve (AUGg) were increased to a similar extent in MBH Ad-JAZF1 and control rats during GTTs (Figure 2A, left and right panel). However, when fed an HFD, MBH Ad-JAZF1 rats exhibited markedly decreased glucose and AUGg compared with the control animals (Figure 2A, middle and right panel). Accordingly, MBH Ad-JAZF1 rats on an HFD exhibited lower insulin levels and AUGi (Figure 2B). These data demonstrated
heightened glucose handling in HFD-fed animals treated with MBH Ad-JAZF1.

3.4 | MBH Ad-JAZF1 improves hepatic insulin resistance in rats fed an HFD

Next, we assessed the impact of MBH Ad-JAZF1 on glucose kinetics using insulin clamp in NCD- or HFD-fed rats (Figure 2C,D). JAZF1 overexpression in the MBH of NCD-fed rats did not affect the ability of insulin to elevate the rate of glucose infusion (GIR) (Figure 2E,G) and suppress hepatic glucose production (HGP) (Figure 2LI) compared with the control animals. However, under an HFD, MBH Ad-JAZF1 significantly increased the GIR (Figure 2F,G) and lowered glucose production (Figure 2LI) during the clamping compared with control rats. In addition, the rate of glucose disappearance (GDR) was significantly increased in the MBH Ad-JAZF1 rats (Figure 2H). However, MBH JAZF1 did not affect the level of JAZF1 protein expression in the liver (Figure 2K). These results suggest that activation of MBH JAZF1 was sufficient to bypass IR to decrease GHP in HFD-fed rats.

3.5 | MBH JAZF1 decreases PEPCK and G6Pase expression and enhances insulin signaling in the liver of HFD-fed rats

To explore the molecular mechanism by which hypothalamic JAZF1 regulates energy metabolism, we evaluated the impact of MBH JAZF1 on the level of PEPCK and G-6-Pase protein in the livers. MBH JAZF1 did not alter the level of hepatic G-6-Pase and PEPCK mRNA and protein in NCD-fed rats (Figure 2LM). However, under an HFD, MBH JAZF1 resulted in a marked reduction in the level of G-6-Pase and PEPCK mRNA and protein in the liver (Figure 2LM). Furthermore, we investigated the phosphorylation of insulin signaling molecules by Western blot. As expected, MBH JAZF1 markedly enhanced the phosphorylation of hepatic insulin receptor (InsR Tyr\(^{1150/1151}\)), Akt (Ser\(^{473}\)), and IRS-1 (Tyr\(^{612}\)) in HFD rats (Figure 2N). These data demonstrated that MBH JAZF1 modulates glucose homeostasis and enhanced hepatic insulin signaling.

3.6 | MBH-JAZF1 enhances the central insulin sensitivity via an insulin receptor-PI3K-Akt-dependent pathway

To characterize the signaling pathway activated by hypothalamic JAZF1, we first examined the effects of JAZF1 on the phosphorylation of insulin signaling molecules in the hypothalamus. We found that under insulin stimulation, MBH Ad-JAZF1 treatment significantly increased Akt (Ser\(^{473}\)), InsR (Tyr\(^{1150/1151}\)) and IRS-1 (Tyr\(^{612}\)) phosphorylation in the hypothalamus of HFD rats, but not in the hypothalamus of NCD-fed rats (Figure 3A).

To further validate the effect of JAZF1 on hypothalamic insulin signaling in vitro, SH-SY5Y cells and PHNs were incubated with glucosamine, a glucokinase inhibitor, to induce IR through activation of the hexosamine biosynthetic pathway. Consistent with our in vivo observations, Ad-JAZF1 treatment in insulin-resistant SH-SY5Y cells and PHNs resulted in a significant elevation of InsR (Tyr\(^{1150/1151}\)), Akt (Ser\(^{473}\)) and IRS-1 (Tyr\(^{612}\)) phosphorylation following insulin treatment, but not in non-IR cells (Figure 3B,C). Furthermore, we measured the levels of forkhead box O1 (FoxO1 (Ser\(^{256}\)) and glycogen synthase kinase-3β (GSK3β) (Ser\(^{9}\)) phosphorylation, two downstream targets of Akt, in insulin-resistant SH-SY5Y cells. In Ad-JAZF1-treated cells, the levels of FoxO1 (Ser\(^{256}\)) and GSK3β (Ser\(^{9}\)) phosphorylation were significantly increased (Figure S5), further suggesting activation of the Akt pathway. We next determined the functional effect of MBH JAZF1 on the ability of insulin to activate phosphatidylinositol 3-kinase (PI3K) signaling in the hypothalamic ARC of HFD-fed rats and PHNs. Under insulin stimulation, PIP3 formation was markedly elevated in the ARC of MBH Ad-JAZF1 rats, compared with the Ad-GFP rats (Figure 3D). Under basal conditions, immunoreactive PIP3 formation was lower in both pcDNA3.1 and pc3.1-JAZF1-treated PHNs. Insulin stimulation resulted in increased PIP3 formation in both PHNs. However, insulin-stimulated immunodetectable PIP3 levels were higher in both pcDNA3.1 and pc3.1-JAZF1-treated PHNs (Figure 3E, right panel). Quantitative analysis of PIP3 formation exhibited a markedly higher percentage of PIP3-positive cells in pc3.1-JAZF1 PHNs (Figure 3E, left panel).

3.7 | Activation of hypothalamic AMPK blocks the ability of MBH JAZF1 to regulate glucose metabolism

Consistent with increasing glucose production, AMPK phosphorylation on threonine 172 (p-AMPK Thr\(^{172}\)), indicating AMPK activation, in the hypothalamus of HFD rats was significantly elevated compared with that of NCD rats (Figure 4A). However, MBH JAZF1 significantly reduced p-AMPK (Thr\(^ {172}\)) in the hypothalamus of HFD rats (Figure 4A,E), p-AMPK (Ser\(^ {485/491}\)) was markedly increased, and p-AMPK (Ser\(^ {496}\)) remained unchanged, indicating inhibition of AMPK activity (Figure S6). In addition, p-AMPK (Thr\(^ {172}\)) was also decreased in IR-SH-SY5Y cells treated with Ad-JAZF1 compared with Ad-GFP cells (Figure 4B). To explore the effect of hypothalamic AMPK on MBH
JAZF1 activity, we next investigated whether the activation of hypothalamic AMPK would block the ability of MBH JAZF1 to decrease HGP using the pharmacological activator, AICAR, during clamping (Figure 4C,D). First, we observed that MBH JAZF1 resulted in a reduction of p-AMPK (Thr172) in the hypothalamus of HFD rats (Figure 4E), and an increase of p-AMPK (Thr172) in the liver (Figure S7). However, MBH Ad-JAZF1 + AICAR treatment eliminated the effect of MBH Ad-JAZF1 on p-AMPK (Thr172) in the both hypothalamus and liver (Figures 4E and S7). Importantly, the co-administration of Ad-JAZF1 with AICAR in the MBH blocked the effects of MBH Ad-JAZF1 alone on the GIR (Figure 4F,G), GDR and HGP (Figure 4H-J). Consistent with the changes, the co-administration of Ad-JAZF1 with AICAR also attenuated the effect of MBH JAZF1 on the levels of G-6-Pase and PEPCK expression in the liver (Figure 4K,L). Our data indicate that inactivation of hypothalamic AMPK pathway is required for JAZF1 to suppress HGP.

FIGURE 3 MBH JAZF1 enhances central insulin sensitivity via insulin receptor-PI3K-Akt-dependent pathway. Rats were fed a NCD or a HFD for 12 weeks and received the MBH injection of Ad-GFP or Ad-JAZF1. Insulin (0.4 µU/min) was infused bilaterally into the MBH for 5 minutes as indicated in the Methods. A, The phosphorylation of the InsR, IRS1, and Akt in the MBH (left panel). The phosphorylation levels were quantified by densitometry and normalized for the total protein (right panel). B, SH-SY5Y cells were treated as indicated in the Methods. Following solubilization, cell lysates were subjected to Western blotting. The phosphorylation of the InsR, IRS1, and Akt was analyzed (upper panels) and quantified by densitometry and normalized for the total protein (below panels). C, PHNs were treated as in the Methods. Cell lysates were subjected to Western blotting. The phosphorylation of the InsR, IRS1, and Akt was analyzed (upper panels) and quantified by densitometry and normalized for the total protein (below panels). D, PIP3 formation in the ARC neurons. Rats were fed a HFD for 12 weeks and received the MBH injection of Ad-GFP or Ad-JAZF1. Immunohistochemistry of ARC sections was performed in overnight-fasted rats, which were injected with either aCSF or insulin into the MBH and sacrificed 10 minutes after stimulation. Blue, DAPI; Red, PIP3 (n = 3). E, PIP3 formation in PHNs. The PHNs were cultured and treated as indicated in the Methods. The amount of PIP3 was classified as high (arrows) and moderate (arrowheads) (left panels). Quantification of PIP3 cells displaying from moderate to high PIP3 levels in the DMEM or insulin treatment (right panel). IR, the cells were incubated with glucosamine; Blue, DAPI; Green, PIP3. 800-1000 ARC cells per group were examined. Data are means ± SD. *P < .05 or **P < .01 vs Ad-GFP, lane 7 or pcDNA3.1
Hypothalamic administration of AICAR activates hypothalamic AMPK and abolishes the HGP lowering effect induced by MBH Ad-JAZF1 in HFD-fed rats. A, The level of AMPK phosphorylation in the MBH of NCD- or HFD-fed rats treated with Ad-JAZF1 or Ad-GFP. B, The level of AMPK phosphorylation in IR or no-IR SH-SY5Y cells infected with or without Ad-JAZF1. C, Schematic representation of working hypothesis. D, Experimental procedure and clamp protocol. HFD-fed rats were injected Ad-GFP or Ad-JAZF1 into the MBH and received the MBH infusion of AICAR during clamps. E, The level of AMPK phosphorylation in the MBH. F, GIR curves. G, Average GIR. H, GDR. J, HGP. J, Percentage of the suppression of HGP. K, Hepatic PEPCK and G6Pase mRNA expression. L, Hepatic PEPCK and G6Pase protein expression. IR, the cells were incubated with glucosamine; MBH, mediobasal hypothalamus; SRIF, somatostatin. Values are shown as mean ± SD. n = 4-6 rats per group. *P < .05 or **P < .01 vs Ad-GFP, or all other groups.
3.8 | MBH JAZF1 activates the K\textsubscript{ATP} channels to inhibit HGP

We next investigated whether MBH JAZF1 leads to specific neuronal activation in hypothalamic ARC regions. As shown in Figure 5A, MBH Ad-JAZF1 resulted in a marked increase in c-fos positive cells in the hypothalamic ARC and the data confirmed that MBH Ad-JAZF1 activated glucose metabolism-related neurons in the hypothalamic nuclei. Thus, the modulating activity on hypothalamic neurons may contribute to the glucose-related role of MBH JAZF1.

To explore the downstream effectors of central JAZF1 in the dorsal vagal complex (DVC), we further suppressed K\textsubscript{ATP} channels in the MBH using a K\textsubscript{ATP} channel blocker (glibenclamide) in the presence or absence of MBH Ad-JAZF1 to investigate whether the role of MBH Ad-JAZF1 is abolished (Figure 5B,C). Indeed, glibenclamide infusion in HFD-fed rats weakened the role of MBH Ad-JAZF1 to increase the GIR (Figure 5D,E) and lower HGP (Figure 5G,H). The effect of MBH Ad-JAZF1 on GDR was also attenuated by glibenclamide (Figure 5F). Furthermore, we observed that MBH glibenclamide also blocked the ability of MBH JAZF1 to alter the expression levels of G-6-Pase and PEPCK in the liver of HFD rats (Figure 5I,J). These data suggest that the effect of MBH JAZF1 on HGP is dependent on the activation of MBH K\textsubscript{ATP} channels.

3.9 | Hepatic vagus nerve is required for the impact of MBH JAZF1 on HGP

To evaluate the downstream pathway that mediates the impact of MBH JAZF1 on HGP, we repeated the PECs with MBH Ad-JAZF1 in HFD-fed rats that received hepatic HVG or SHAM (Figure 6A,B). No significant difference was found regarding BW 5 days before the clamping between the HVG and SHAM groups (Figure S4B). However, MBH Ad-JAZF1 administration in HFD-fed rats resulted in a significant elevation in GIR and a significant suppression in HGP. HVG blocked the role of MBH Ad-JAZF1 on GIR (Figure 6C,D) and HGP (Figure 6F,G). The effect of MBH Ad-JAZF1 on GDR was also modified by HVG (Figure 6E). In addition, the ability of MBH Ad-JAZF1 to reduce hepatic PEPCK and G-6-Pase expression was also abolished by HVG (Figure 6H,I). These results indicate that the role of MBH JAZF1 on hepatic glucose flux and insulin signaling was mediated by the descending pathway of the hepatic vagus nerve.

4 | DISCUSSION

The hypothalamus plays a pivotal role in the central regulation of energy homeostasis in vivo.\textsuperscript{17} A previous study found that the JAZF1 mRNA expression in the hypothalamus was higher than that in the liver.\textsuperscript{15} Here we, thus, focused on the contribution of central JAZF1 signaling on peripheral metabolism and insulin signaling. We found that in obese and IR phenotypes, JAZF1 expression in the hypothalamus was significantly reduced. This was consistent with the previous finding that the JAZF1 expression in the brain was 60% lower in HFD-fed rats.\textsuperscript{15} Our results indicate that hypothalamic JAZF1 may be involved in the pathogenesis of IR and obesity.

In this study, the overexpression of JAZF1 in the hypothalamus was found to reduce BW and food intake, and increase energy expenditure in HFD-fed rats, which protected the rats from IR and obesity. Indeed, it has been well established that short-term excess energy can induce IR, while short-term insufficient energy can improve insulin sensitivity in both mice and humans.\textsuperscript{28,29} Thus, it is important to explore how MBH JAZF1 mediates its effects on food intake and BW.

Since BW is maintained by a balance between food intake and energy expenditure, we considered that these two aspects may contribute to weight loss. The decrease in food intake may be due to activation of the PI3K/Akt pathway to inhibit the expression of orexigenic neurons, as we and others have previously shown.\textsuperscript{30-32} Moreover, the hypothalamus has been shown to modulate energy balance and BAT thermogenesis by controlling sympathetic outflow.\textsuperscript{33} Therefore, increased oxygen consumption, including fatty acid oxidation (FAO) and energy expenditure, in MBH Ad-JAZF1-treated rats indicates an important link between MBH JAZF1 projections and the sympathetic regulation of BAT thermogenesis and energy balance.\textsuperscript{34} Thereby, the effects of central JAZF1 signaling on BW and glucose metabolism may be multifaceted.

It remains unclear why MBH JAZF1 affects BW and food intake only in HFD-fed rats. We speculate that the increased JAZF1 signaling in hypothalamus only plays a role under pathophysiological conditions. Indeed, it has been reported that hypothalamic nutrient-sensing mechanisms are impaired in diet-induced obesity.\textsuperscript{35,36} Therefore, we consider that in diet-induced obesity, the inability of hypothalamic nutrient-sensing mechanisms may lead to the increased sensitivity of anorexic neurons to JAZF1 signaling. Such effects would then result in reduced food intake, weight loss, and changes in hepatic insulin signaling.

To analyze the association of central JAZF1 with glucose metabolism in vivo, we showed that viral-mediated JAZF1 overexpression in the MBH of HFD-fed rats decreased the rate of hepatic gluconeogenesis, which was consistent with the decrease in HGP. These results were accompanied by reduced BW and food intake, reduced hepatic PEPCK and G-6-Pase expression, and increased hepatic insulin activity. Consistent with a previous peripheral observation,\textsuperscript{7} the activation of hypothalamic JAZF1 was sufficient to recapitulate the systemic effects of JAZF1 regarding both HGP and the...
FIGURE 5 Activation of K\textsubscript{ATP} channels within the MBH is required for MBH JAZF1 action in HFD-fed rats. A, Photomicrographs of coronal brain sections showing c-fos immunostaining in ARC and VMH after MBH Ad-JAZF1 or Ad-GFP treatment. B, Schematic representation of the working hypothesis. C, Experimental procedures and clamp protocol. D, GIR curves. E, Average GIR. F, GDR. G, HGP. H, Percentage of the suppression of HGP. I, Hepatic PEPCK and G6Pase mRNA expression. J, Hepatic PEPCK and G6Pase protein levels. ARC, arcuate nuclei; VMH, ventromedial hypothalamus; MBH, mediobasal hypothalamus; SRIF, somatostatin. GLI, glibenclamide. Values are shown as mean ± SD. n = 5-7 rats per group. *P < .05 or **P < .01 vs all other groups.
insulin signaling. Therefore, we believe that MBH may be an important anatomical site mediating the role of central JAZF1.

The effects of MBH JAZF1 on HGP and insulin signaling raised two questions regarding how insulin signaling in the hypothalamus was activated and how the signaling was conveyed to the liver. It has been previously revealed that the activation of the hypothalamic InsR-PI3K pathway and KATP channels provides a potential confluence point for the modulation of hormones in energy metabolism. However, it is unknown whether JAZF1 also regulates glucose metabolism through this signaling pathway as a transcription factor. Thus, we examined the role of JAZF1 on regulating the insulin-stimulated activation of the InsR/PI3 kinase/Akt pathway and K_{ATP} channel in the hypothalamus. Our results confirmed that MBH Ad-JAZF1 promoted the insulin-mediated activation of InsR/PI3 kinase as well as subsequent PIP3 formation, and Akt activation in hypothalamic neurons. These data indicate that there is a strong evidence for the importance of the InsR-PI3 kinase-Akt pathway in the hypothalamic regulation of energy homeostasis. However, the underlying mechanism by which JAZF1 promotes insulin signaling in the hypothalamus is unclear. It has been reported that JAZF1 is a selective co-factor for TAK1, which is involved in the regulation of glucose metabolism and insulin signaling. Therefore, we speculate that the interaction between JAZF1 and TAK1 might contribute to changes in insulin signaling in the hypothalamus. However, the direct effect of JAZF1 acting...
on insulin signaling molecules (e.g., InsR and Akt) cannot be excluded as a potential mechanism. Thus, further studies are required to confirm these possibilities.

Both genetic and electrophysiological studies have revealed the presence of $K_{\text{ATP}}$ channels in the hypothalamus.\textsuperscript{39} It has also been reported that $K_{\text{ATP}}$ channels are expressed in the hypothalamus\textsuperscript{37} and activated by some hormones and nutritional substrates.\textsuperscript{14,36-40} The altered activity of the $K_{\text{ATP}}$ channels in the hypothalamus regulated blood glucose by changing the autonomic input to the liver.\textsuperscript{41} Therefore, $K_{\text{ATP}}$ channel might be necessary for the regulation of HGP by JAZF1. However, it is unclear as to whether JAZF1 as a transcription factor in the MBH regulates HGP in association with the $K_{\text{ATP}}$ channel. We addressed this issue in the current study. As expected, our results revealed that the co-treatment of the glibenclamide, a $K_{\text{ATP}}$ channel blocker, with Ad-JAZF1 in the MBH inhibited the role of MBH JAZF1 to increase GIR and inhibit HGP in HFD-fed rats. Therefore, we believe that the metabolic role of MBH JAZF1 is mediated by the $K_{\text{ATP}}$ channel. Although the mechanism of MBH JAZF1 on the activation of the $K_{\text{ATP}}$ channel remains to be clarified, we speculate that JAZF1 may regulate the Kir 6.2 subunit of $K_{\text{ATP}}$ channel in MBH by post-translational modification of AMPK, which is a substrate for AMPK.\textsuperscript{42} Further studies to test this hypothesis have been taken into our consideration.

The hypothalamic nucleus regulates target tissues and organs through the autonomic nervous system. Moreover, the hepatic vagus nerve transmits the communication between the brain and the liver. Previous reports have also demonstrated that hepatic nerve innervation is important for the modulation of HGP and insulin signaling via a central mechanism.\textsuperscript{43-46} As previously reported, the inhibition of HGP by the central treatment of insulin or fatty acids is eliminated by selective hepatic vagotomy.\textsuperscript{45} Therefore, we can infer that MBH JAZF1 may regulate insulin sensitivity through a neuronal pathway from the brain to the liver, and we further verified this hypothesis in an additional in vivo experiment with HVG. The results showed that HVG ablated the effects of MBH JAZF1 on HGP, GDR and insulin signaling in HFD rats. Therefore, the effects of MBH JAZF1 on HGP were dependent on hepatic vagus nerve input to the liver. Currently, the mechanism by which central JAZF1 is blocked by HVG on GDR is not completely understood. We speculate that HVG may not only affect glucose clearance of the liver itself, but also affect the synthesis and release of enzymes involved in glucose metabolism in the liver, thereby impacting the GDR. In addition, HGV may also affect the GDR of other tissues and organs (e.g., muscles) through neuroendocrine regulation. Although a brain-liver circuit may be important for restraining HGP and enhancing insulin signaling by central JAZF1, changes in BW, food intake and energy expenditure may also contribute to the regulation of HGP and insulin signaling.

AMPK is a pivotal modulator of energy equilibrium and its activity is decreased by anorexigenic signals and stimulated by orexigenic signals in the hypothalamus.\textsuperscript{47,48} It has been reported that in POMC or AgRP neurons, the specific knockout of AMPK results in the destruction of energy balance, and the inhibition of AMPK in the hypothalamus lowers HGP and blood glucose in diabetes and obesity,\textsuperscript{40} emphasizing that hypothalamic AMPK is an important
important issue of concern. Similar to previous reports, Ad-JAZF1 resulted in the inhibition of hypothalamic AMPK phosphorylation (Thr172). This effect was accompanied by an inhibition of HGP, as well as G-6-Pase and PEPCK activity and increased hepatic insulin signaling. Furthermore, hepatic AMPK phosphorylation (Thr172) was increased. We consider that it is mainly due to JAZF1 enhances the insulin signal and inhibits the AMPK phosphorylation (Thr172) in the hypothalamus, which leads to the increase of insulin sensitivity in the liver, as previously reported. However, the pharmacological activation of AMPK by AICAR in the hypothalamus eliminated the role of MBH JAZF1 to inhibit HGP and enhance insulin signaling. Notably, the central AICAR treatment did not lead to the inhibition of AMPK phosphorylation (Thr172) in the liver and an increase in HGP. These results are similar to other publications. We speculate that it may be due to the short-term infusion of AICAR (120 minutes) in the hypothalamus did not lead to changes in hepatic AMPK signaling and HGP. Furthermore, the effects of central AICAR on the liver may be a dose-dependent manner. However, the precise reason for this phenomenon requires further investigation. Collectively, these findings indicate that MBH Ad-JAZF1 altered hypothalamic AMPK activity, and AMPK is required for MBH JAZF1-mediated regulation of HGP and insulin signaling in the liver. In addition, it has been reported that crosstalk exists between Akt and AMPK signaling. Therefore, we believe that MBH JAZF1 activates the InsR/PI3 kinase/Akt pathway and inhibits AMPK signaling, which results in the activation of the K_{ATP} channels to reduce HGP.

Recently, the stereotaxic injection of a viral vector has become an indispensable tool which allows scientists to functionally dissect the role of hormone and neural circuits in vivo. Similar to previous reports, we performed a stereotaxic injection of a viral vector for gene manipulation in the MBH to induce adult-onset JAZF1 overexpression. However, an adenoviral injection in the MBH may cause inflammation which could complicate data interpretation. According to the convention, we used Ad-GFP as a control to address this potential issue. In addition, the molecular localization of JAZF1 signaling by MBH Ad-JAZF1 is another important issue of concern. Similar to previous reports, we demonstrated that the injection of Ad-GFP into the MBH resulted in an effect that was anatomically restricted to the hypothalamic region related to energy balance. It should also be noted that in addition to the association between the liver and the brain, changes in food intake, BW, and other metabolic tissues (eg, fat and muscle) can also alter the degree of insulin sensitivity in the entire body and liver. However, in the short term, these factors are unlikely to play a major role in the effect of MBH JAZF1 on liver insulin sensitivity.

In conclusion, our data indicate that the JAZF1 overexpression leads to the activation of InsR/PI3 kinase/Akt pathway and the inhibition of AMPK in the hypothalamus of HFD-fed rats, which in turn opens K_{ATP} channels. This results in the activation of autonomic projections from the hypothalamus and generates an effenter vagal impulse to the liver to regulate HGP and insulin signaling (Figure 7). Therefore, our study provides a novel perspective to enhance our understanding of the modulation of energy equilibrium through transcriptional factors in the CNS.

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CONFLICT OF INTEREST

The authors declare no competing interests.

ETHICS APPROVAL

All applicable institutional and/or national guidelines for the care and use of animals were followed.

AUTHOR CONTRIBUTIONS

M. Zhou, M. Yang, H. Wang, H. Guo, and X. Zhao performed experiments and acquired data; X. Xu and Z. Zhu contributed to discussion and edited the manuscript; Z. Zhu and J. Song contributed to discussion and provided materials; L. Li and G. Yang contributed to the study concept and design and wrote the manuscript; L. Li and G. Yang are the guarantor of this work and, as such, had 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.

DATA AVAILABILITY STATEMENT

The data analyzed during this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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