Original Article

MOLECULAR METABOLISM

Overexpression of Smad7 in hypothalamic POMC neurons disrupts glucose balance by attenuating central insulin signaling

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ABSTRACT

Objective: Although the hypothalamus is crucial for peripheral metabolism control, the signals in specific neurons involved remain poorly understood. The aim of our current study was to explore the role of the hypothalamic gene mothers against decapentaplegic homolog 7 (Smad7) in peripheral glucose disorders.

Methods: We studied glucose metabolism in high-fat diet (HFD)-fed mice and middle-aged mice with Cre-mediated recombination causing 1) overexpression of Smad7 in hypothalamic proopiomelanocortin (POMC) neurons, 2) deletion of Smad7 in POMC neurons, and 3) overexpression of protein kinase B (AKT) in arcuate nucleus (ARC) in Smad7 overexpressed mice. Intracerebroventricular (ICV) cannulation of insulin was used to test the hypothalamic insulin sensitivity in the mice. Hypothalamic primary neurons were used to investigate the mechanism of Smad7 regulating hypothalamic insulin signaling.

Results: We found that Smad7 expression was increased in POMC neurons in the hypothalamic ARC of HFD-fed or middle-aged mice. Furthermore, overexpression of Smad7 in POMC neurons disrupted the glucose balance, and deletion of Smad7 in POMC neurons prevented diet- or age-induced glucose disorders, which was likely to be independent of changes in body weight or food intake. Moreover, the effect of Smad7 was reversed by overexpression of AKT in the ARC. Finally, Smad7 decreased AKT phosphorylation by activating protein phosphatase 1c in hypothalamic primary neurons.

Conclusions: Our results demonstrated that an excess of central Smad7 in POMC neurons disrupts glucose balance by attenuating hypothalamic insulin signaling. In addition, we found that this regulation was mediated by the activity of protein phosphatase 1c.

Keywords Glucose disorders; Insulin signaling; POMC neurons; Smad7

1. INTRODUCTION

Type 2 diabetes (T2D) is a group of metabolic diseases characterized by hyperglycemia resulting from insulin resistance [1]. The central nervous system (CNS) and, in particular, the hypothalamus, has substantial effects on glucose homeostasis [2–4]. The arcuate nucleus (ARC) is the part of the hypothalamus that contains various neurons, including proopiomelanocortin (POMC) neurons and agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons, which have been shown to be involved in the control of metabolism in peripheral tissues [5,6]. Recent studies show that signals in these neurons, such as transforming growth factor-β (TGF-β) and adenosine monophosphate (AMP)-activated protein kinase (AMPK), can regulate peripheral glucose metabolism [7,8]. However, the inside molecular mechanisms remain poorly understood.

The mothers against decapentaplegic homolog 7 (Smad7) is a known inhibitor of TGF-β and is normally activated by excessive TGF-β [9]. Once activated, Smad7 binds to TGF-β type 1 receptor (TβR1) and recruits ubiquitin ligase to degrade TβR1 or protein phosphatase 1c (PP1c) to dephosphorylate TβR1 [10,11]. In addition to the TGF-β-dependent regulatory mechanism, Smad7 may also regulate downstream signaling independent of TGF-β [12]. Smad7 has been shown to affect the regulation of many processes (i.e., via intensifying enteritis, promoting tumor cell growth, and inhibiting hepatic fibrosis) [13–15].

Smad7 is widely expressed throughout the body, and most of its roles are related to peripheral tissues [16]. Smad7 is also expressed in the brain [17], but the role of central Smad7 remains largely unknown, particularly regarding its role in the regulation of peripheral glucose metabolism. A previous study has shown that in lung cancer cell lines,

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Smad7 negatively regulates the phosphorylation of protein kinase B (AKT) [18], a key regulator of insulin sensitivity [19]. Because insulin sensitivity has been identified as a factor affecting the central control of glucose metabolism [2], we hypothesized that central Smad7 regulates glucose metabolism by affecting insulin signaling in the hypothalamus. The aim of our current study was therefore to investigate this hypothesis in mouse models. We explored hypothalamic Smad7 expression in high-fat diet (HFD)-fed and middle-aged mice, two mouse models that have been used to study disorders of glucose metabolism [8], and searched for new signals that are involved in glucose balance to better understand the CNS.

2. MATERIALS AND METHODS

2.1. Mice and diets

All mice were of the C57BL/6J genetic background. POMC-Cre mice were provided by Joel K. Elmquist and Tiemin Liu from the Southwestern Medical Center. Floxed Smad7 allele ($Smad7^{flox/flox}$) mice were generated as previously described [20]. Ai9 (tdTomato) reporter mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). To generate POMC neuron-specific Smad7 knockout (PSKO) mice, POMC-Cre mice were crossed with $Smad7^{flox/flox}$ mice. To visualize POMC protein-expressing neurons under a fluorescence microscope, PSKO and POMC-Cre mice were intercrossed with tdTomato reporter mice.

For the high-fat diet (HFD) study, 4-week-old PSKO or control mice were maintained on a HFD with 60% kcal of fat (Research Diets, New Brunswick, NJ, USA) for 16 weeks. Food intake and body weight were recorded daily. The mice were maintained under a 12-hour light/dark cycle (lights on at 07:00 h/lights off at 19:00 h) at 25 °C, with free access to water and chow. These experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of the Institute.

2.2. Metabolic parameter measurements

Body weight and food intake were measured weekly using a precision scale. The body composition of mice was determined using a nuclear magnetic resonance system (Bruker, DE, USA).

2.3. Cell culture and treatments

The primary culture of mouse hypothalamic neurons was performed as described previously [21]. Recombinant adenoviruses expressing green fluorescent protein (GFP) (Ad-GFP), mouse Smad7 (Ad-Smad7), scrambled short hairpin RNA (shRNA) (Ad-Scramble), and shRNA against mouse Smad7 (Ad-shSmad7) were purified and administered at the dose of 1×10^7 Pfu/well in 12-well plates for 48 h. The shRNA sequence for mouse Smad7 was 5′- TCGGACAGCTCAATTCGGACA-3′. For the in vitro insulin signaling assay, the primary cultured hypothalamic neurons were infected with Ad-GFP, Ad-Smad7, Ad-scramble, or Ad-shSmad7, as indicated, followed by stimulation with 100 nM insulin for 10 min. For protein phosphatase (PP)1c and PP2A inhibitor okadaic acid (OA; Beyotime, Shanghai, China) treatments, the cells were transfected with the indicated genes in the presence or absence of OA with the indicated concentration and stimulation time before stimulation with insulin. For PP1c and PP2A activator C2 Ceramide (C2; Santa Cruz Biotechnology, CA, USA) treatments, the cells were transfected with the indicated genes in the presence or absence of C2 Ceramide with the indicated concentration and stimulation time before stimulation with insulin.

To silence endogenous PP1c and PP2A expression, single-stranded 21-nt RNAs directed against PP1c or PP2A were chemically synthesized and purified (GenePharma, Shanghai, China). The sequence of PP1c siRNA oligonucleotide was 5′-CCAGAAGCCAACUAUCUUU-3′, and the sequence of PP2A siRNA oligonucleotide was 5′-CCAUACUCCGAGGGAAUCA-3′. The hypothalamic primary cells were transfected with siPP1c, siPP2A, or negative control siRNA (NC) for 3 days using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's recommendation.

2.4. Stereotaxic surgery and viral injections

As the adenoviruses may induce significant inflammatory responses and gliosis that last for a few days [22], whereas the adeno-associated viruses (AAVs) are considered to be relatively safe [23], we used the AAVs to inject mice and conducted experiments after an at least 4-week recovery from the surgery. Stereotaxic surgeries were performed on the mice as described previously [24]. Surgery was performed with a stereotaxic frame (Steolting, IL, USA). For overexpressing Smad7 in hypothalamic POMC neurons, POMC-Cre mice were bilaterally injected either with a Cre-dependent AAV vector containing Smad7 in the opposite orientation flanked by two inverted loxP sites (AAV9-Syn-DIO-Smad7-mCherry, 1.2×10^{12} Pfu/ml, HANBIO, Shanghai, China) at a volume of 200 nL/side into the ARC (1.5 mm posterior to the bregma, ± 0.27 mm lateral to the midline, and 5.7 mm below the surface of the skull) or with an AAV vector containing only mCherry in the opposite orientation flanked by two inverted loxP sites (AAV9- Syn -DIO- mCherry, 1.2×10^{12} Pfu/mL, HANBIO, Shanghai, China) as a control.

To assess the reversal effect of AKT activation on Smad7 overexpression in POMC neurons, we injected the ARC of POMC-Cre mice with AAVs expressing AKT (AAV9-Syn-AKT-EGFP, OBiO Technology, Shanghai, China) or Smad7 (AAV9-Syn-DIO-Smad7-mCherry, OBiO Technology, Shanghai, China), as well as with control AAVs expressing mCherry (AAV9-Syn-DIO-mCherry) or EGFP (AAV9-Syn-EGFP), as indicated. The aforementioned AAVs were all diluted to a concentration of 1.2×10^{12} Pfu/mL, and the two indicated AAVs were mixed at a 1:1 ratio to yield a total volume of 200 nL/side.

2.5. Intracerebroventricular (ICV) cannulation and treatments

ICV cannulation experiments were conducted as previously described [25]. The cannula was implanted into the third ventricle at the midline coordinates of 1.5 mm posterior to the bregma and 5.0 mm below the surface of skull. *In vivo* central insulin signaling assays were performed as described previously [26]; mice were fasted for 4 h and then injected with insulin (2 mU, Novonordisk, Denmark) or artificial cerebrospinal fluid (aCSF, Tocris Bioscience, Bristol, UK) via a pre-implanted cannula into the third ventricle of the hypothalamus. Twenty minutes later, the hypothalamus was isolated and snap-frozen for insulin signaling analysis.

2.6. Blood glucose and serum insulin measurement, glucose tolerance test (GTT), insulin tolerance test (ITT), pyruvate tolerance test (PTT), and the homeostasis model assessment of insulin resistance (HOMA-IR) index

The level of blood glucose was measured with a Glucometer Elite monitor (Abbott Diabetes Care Ltd, Oxon, UK) with blood obtained from the tail tip. Blood samples were obtained from tail vein bleeding and then centrifuged to collect the serum. Serum insulin was measured using Mercodia Ultrasensitive Rat Insulin ELISA kit (ALPCO Diagnostic, Salem, NH, USA). The GTT, ITT, and PTT were performed by intraperitoneal (IP) injection of 2 g/kg glucose after overnight fasting, 0.75 U/kg insulin after 4 h of fasting, and 2 g/kg pyruvate after overnight fasting, respectively. The HOMA-IR index was calculated according to

the following formula: [fasting glucose levels (mmol/L)] × [fasting serum insulin (μU/mL)]/22.5.

2.7. Immunofluorescence (IF) staining

IF staining was performed as previously described [24] with the following primary antibody: anti-Smad7 (1:500, sc-101152, Santa Cruz Biotechnology, CA, USA).

2.8. RNA isolation and relative quantitative real-time polymerase chain reaction (RT-PCR)

RNA was isolated and RT-PCR performed as previously described [27]. The sequences of primers used in this study are available in Supplementary Table 1.

2.9. Western blotting analysis

Western blotting was performed as previously described [27] with the following primary antibodies: anti-Smad7 (1:500, MAB2029, R&D systems, NM, USA), anti-PI3K [1:1000, 4292s, Cell Signaling Technology (CST), MA, USA], anti-p-PDK1 (1:1000, 3061s, CST), anti-t-PDK1 (1:1000, 3062s, CST), anti-p-IR (1:1000, 3024s, CST), anti-t-IR (1:1000, 3025s, CST), anti-p-AKT (1:1000, 9271s, CST), anti-t-AKT (1:1000, 9272s, CST), anti-p-GSK3β (1:1000, 9336s, CST), anti-t-GSK3β (1:1000, 9315s, CST), and anti-GAPDH (1:3000, ab0037, Abways Biotechnology, Shanghai, China).

2.10. Statistical analyses

Statistical analyses were performed in GraphPad Prism, version 8.0 (GraphPad Software, San Diego, CA, USA). All values are presented as the mean ± the standard error of the mean (SEM). When two groups were compared, the data were analyzed for statistical significance using a two-tailed unpaired Student *t* test. For experiments with multiple comparisons, the data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by the Student—Newman—Keuls (SNK) test. For GTTs, ITTs, and PTTs, the data were analyzed for statistical significance using the Student *t* test or one-way ANOVA to compare the differences between or among different groups of mice at each time point examined; testing multiple time points of the curve was analyzed using two-way repeated measures ANOVA. For other experiments with two independent interventions, the data were analyzed for statistical significance using two-way but not repeated ANOVA. In addition, the individual datapoints on every histogram are shown with the individual variability of their measures represented. Statistical significance was defined as $P < 0.05$.

3. RESULTS

3.1. Hypothalamic Smad7 is increased in HFD-fed and middle-aged mice

To study the function of central Smad7, we conducted IF staining and found that Smad7 was abundant in the hypothalamus compared with other regions of the brain. Moreover, Smad7 was particularly rich in the ARC compared with other hypothalamic nuclei, including the dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), and ventromedial hypothalamus (VMH) (Supplementary Figure 1). Interestingly, Smad7 expression in the hypothalamic ARC was markedly increased both in HFD-fed and middle-aged mice relative to that in control mice (Figure 1A,C).

As POMC neurons in the ARC play indispensable roles in regulating obesity and diabetes [5], we next investigated whether Smad7 expression levels in POMC neurons also increased under insulin resistance-promoting conditions by examining Smad7 staining in POMC-Cre-Ai9 mice. As predicted, IF staining of tdTomato (indicating the presence of POMC neurons) and Smad7 showed that Smad7 levels were increased in the hypothalamic POMC neurons of HFD-fed and middle-aged mice (Figure 1B,D).

3.2. Smad7 overexpression in POMC neurons disrupts glucose balance

Because the hypothalamic POMC neuronal Smad7 levels were increased in the HFD-fed mice and middle-aged mice, demonstrating glucose disorders, we investigated whether overexpression of Smad7 in POMC neurons would lead to a glucose imbalanced phenotype similar to those observed in HFD-fed and middle-aged mice. To this end, Cre-dependent AAVs encoding Smad7 (AAV-DIO-Smad7-mCherry) or mCherry (AAV-DIO-mCherry) were injected into the ARC of POMC-Cre mice. Overexpression of Smad7 in the ARC, but not in other parts of the brain (e.g., the cortex), was confirmed by RT-PCR and western blotting analysis (Figure 2A,B, Supplementary Figure 2A-C).

The body weight, food intake, and body composition were similar between the two groups of mice (Supplementary Figure 2D-G). Although there was no difference in the fed blood glucose or the fed and fasting serum insulin, the fasting blood glucose and the HOMA-IR index were increased in mice with Smad7 overexpression in POMC neurons (Figure 2C—E). To perform a general study of glucose homeostasis, we conducted a GTT and found that increasing levels of Smad7 expression in POMC neurons impaired glucose sensitivity (Figure 2F).

Following this observation, we further studied whether glucose tolerance was impaired by conducting an ITT, which showed that the ability of insulin to lower blood glucose was significantly decreased in mice with overexpression of Smad7 compared with that in control mice (Figure 2G). Moreover, the PTT exhibited an increase of gluconeogenesis in mice with Smad7 overexpression in POMC neurons (Figure 2H).

3.3. Smad7 knockout in POMC neurons ameliorates glucose disorders in HFD-fed and middle-aged mice

Next, we investigated whether Smad7 deletion in POMC neurons could improve the condition of HFD-fed mice with glucose disorders. To this end, we genetically deleted Smad7 in POMC neurons, producing PSKO mice. *Smad7* mRNA levels were significantly decreased in the ARC but not in other brain areas (e.g., the cortex) of PSKO mice than in those of control mice (Figure 3A). The deletion efficiency was not a huge decrease because there were many other types of cells in the ARC [28] that may also express Smad7. In addition, IF staining of tdTomato (indicating the presence of POMC neurons) and Smad7 showed that Smad7 was co-localized with POMC neurons in control mice but was absent in POMC neurons of PSKO mice (Figure 3B). Except for fasting glucose, the HOMA-IR index, and the pyruvate tolerance, which were improved in PSKO mice, other phenotypes including body weight, food intake, body composition, and glucose metabolism-related parameters were not changed compared with those of control mice (Supplementary Figure 3).

We next investigated whether Smad7 deletion in POMC neurons could produce resistance to glycometabolic disturbances in HFD-fed mice. After 3 months of HFD feeding, both the fed and fasting blood glucose were lower in PSKO mice than in control mice (Figure 3C). Fed and fasting insulin levels were unchanged, but the HOMA-IR index was decreased in PSKO mice (Figure 3D,E). Furthermore, glucose tolerance, insulin sensitivity, and pyruvate tolerance were markedly higher

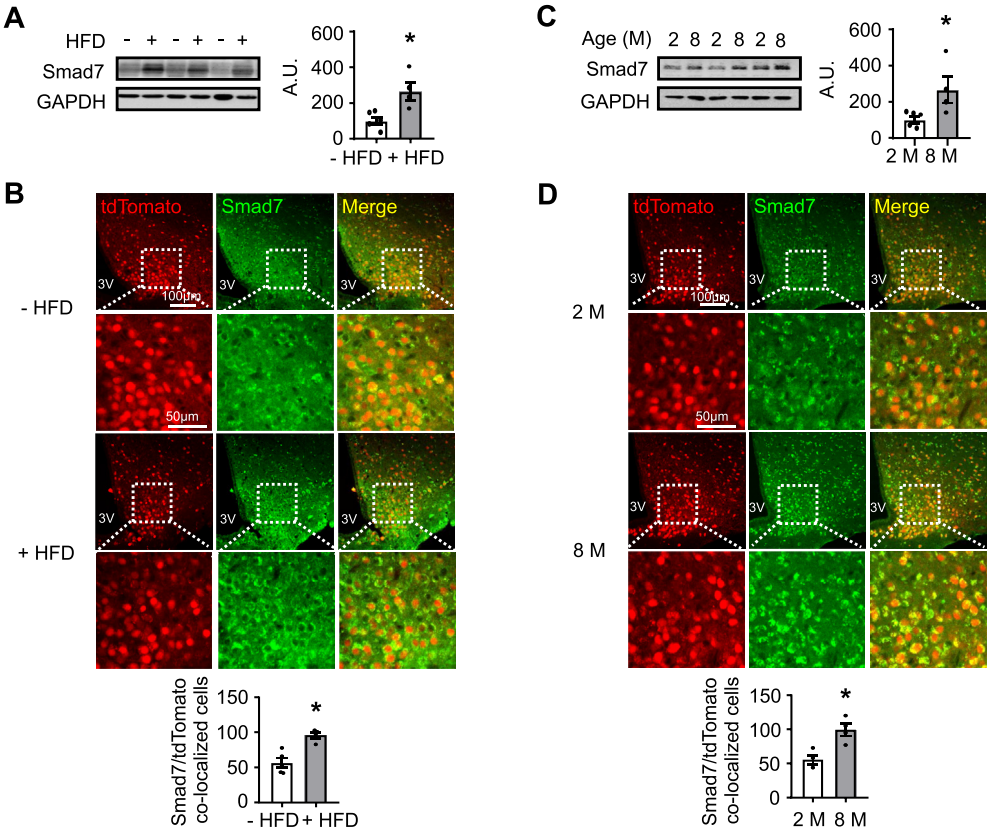

Figure 1: High-fat diet and middle age increase Smad7 expression in hypothalamic POMC neurons. (A, C) Smad7 protein in the hypothalamic arcuate nucleus (ARC) by western blotting (left) and quantified by densitometric analysis (right); A.U.: arbitrary units. (B, D) Immunofluorescence (IF) staining for tdTomato (red), Smad7 (green), and merge (yellow) in ARC sections (top), and quantification of Smad7 and tdTomato co-localized cell numbers (bottom); 3V, third ventricle. Studies for A were conducted using 18- to 20-week-old male wild-type mice fed a normal chow diet (-HFD) or a high-fat diet (+HFD) for 12 weeks; studies for B were conducted using 18- to 20-week-old male POMC-Cre-Ai9 mice fed a normal chow diet (-HFD) or high-fat diet (+HFD) for 12 weeks; studies for C were conducted using 8-week-old (2 M) or 32-week-old (8 M) male wild-type mice fed a normal chow diet; studies for D were conducted using 8-week-old (2 M) or 32-week-old (8 M) male POMC-Cre-Ai9 mice fed a normal chow diet. Data are expressed as the mean ± SEM (n = 4–5 per group, as indicated), with individual datapoints. $^*P < 0.05$ for the effect of HFD vs. the control group, or the effect of the 8 M vs. the 2 M group.

in PSKO mice than in control mice (Figure 3F–H). Although the body weight, the absolute fat mass, and the lean mass of PSKO mice decreased, food intake and percentage of body composition were not different between the two strains of mice (Supplementary Figure 4).

As age is a big risk factor for diabetes [8], we next investigated whether the increased Smad7 levels contributed to age-dependent glucose disorders in middle-aged (8–12 months old) PSKO mice. After Smad7 deletion, body weight, food intake, and body composition were not changed (Supplementary Figure 5), but glucose and insulin levels in both the fed and fasting states were decreased in PSKO mice compared with control mice (Figure 4A,B). As a result, the HOMA-IR index was also decreased in PSKO mice (Figure 4C). Similarly, the GTT, ITT, and PTT were all improved in PSKO mice (Figure 4D–F).

3.4. Increased Smad7 in POMC neurons attenuates hypothalamic insulin sensitivity

Next, we explored how the Smad7 signals in POMC neurons influence the peripheral glucose metabolism. Previous studies have indicated that hypothalamic insulin sensitivity, which is affected by POMC neurons [29], has an important role in the peripheral glucose metabolism [19], suggesting that Smad7 in POMC neurons might regulate peripheral glucose metabolism in a similar manner. To test the hypothesis, we examined hypothalamic insulin signaling by delivering insulin via a cannula into the hypothalamus of control or POMC neuronal Smad7 overexpressed mice.

Smad7 overexpression in hypothalamic POMC neurons impaired the insulin sensitivity, which was evaluated by examining insulin-stimulated phosphorylation of insulin receptor (IR) on Tyr 1150/1151 (p-IR), protein kinase B on Ser473 (p-AKT), and glycogen synthase kinase 3β on Ser 9 (p-GSK-3β) (Figure 5A). Consistently, the insulin signaling was enhanced by deleting Smad7 in POMC neurons, as was demonstrated by the increased levels of p-IR, p-AKT, and p-GSK3β compared with those of control mice (Figure 5B). Similar results were obtained in the primary cultured hypothalamic neurons with either Smad7 overexpression or knockdown (Supplementary Figure 6).

3.5. Stimulating AKT in the ARC reverses the glucose disorder in POMC-Smad7 overexpressing mice

To test whether the decreased hypothalamic insulin signaling was the cause of the impairment of peripheral glucose metabolism in POMC-Smad7 overexpressing mice, we investigated the disorder reversal effect of the overexpression of AKT, the key component of insulin signaling [30], by injecting AAVs expressing AKT (AAV-AKT-EFGP) and/or Smad7 (AAV-DIO-Smad7-mCherry) into the ARC of the POMC-Cre mice (Supplementary Figure 7A and 7B). *Smad7* and *Akt* mRNA levels were significantly increased in the ARC, but not in other brain

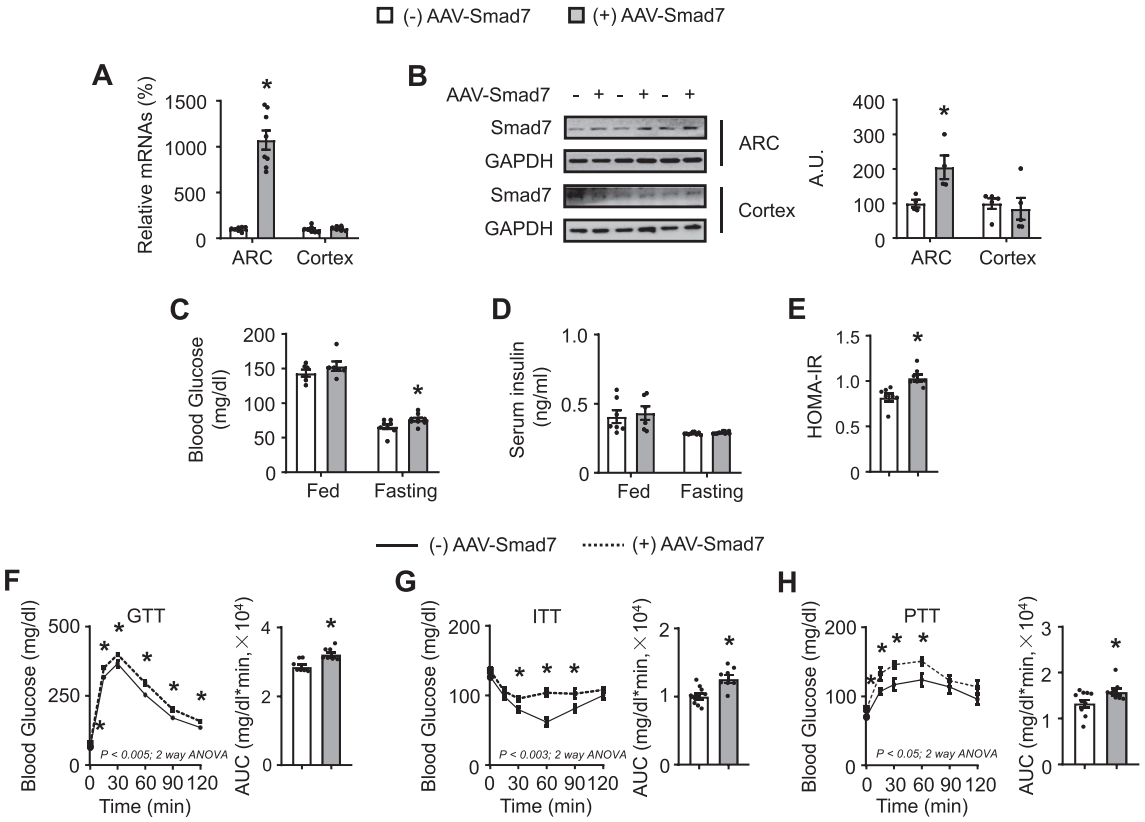

Figure 2: Smad7 overexpression in hypothalamic POMC neurons induces glucose disorder. (A) Gene expression of *Smad7* in hypothalamic arcuate nucleus (ARC) and cortex by RT-PCR. (B) Smad7 protein in ARC and cortex by western blotting (left) and quantified by densitometric analysis (right); A.U.: arbitrary units. (C) Fed and fasting blood glucose levels. (D) Fed and fasting serum insulin levels. (E) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (F) Glucose tolerance tests (GTTs). (G) Insulin tolerance tests (ITTs). (H) Pyruvate tolerance tests (PTTs). Studies were conducted using 13- to 14-week-old male POMC-Cre mice receiving AAVs expressing mCherry [(−) AAV-Smad7] or Smad7 [(+) AAV-Smad7]. Data are expressed as the mean ± SEM (n = 4–10 per group, as indicated), with individual datapoints. $^*P < 0.05$ for the effect of (+) AAV-Smad7 mice vs. (−) AAV-Smad7 mice.

areas (e.g., the cortex), of AAV-AKT or AAV-Smad7-injected mice (Supplementary Figure 7C). Similar results were found in the western blots (Supplementary Figure 7D). The insulin sensitivity was impaired in Smad7 overexpressing mice, but this inhibition was reversed by AKT overexpression in the ARC (Supplementary Figure 7E). Although some of the parameters (i.e., body weight, food intake, body composition, fed blood glucose, and serum insulin) were not affected, the increased fasting blood glucose, impaired HOMA-IR, glucose tolerance, insulin sensitivity, and pyruvate tolerance were all improved by activation of AKT in the ARC in POMC-Smad7 overexpressing mice (Figure 5C–H and Supplementary Figure 7F-I).

3.6. Smad7 attenuates hypothalamic insulin signaling by stimulating dephosphorylation of AKT via PP1c

The results showed that Smad7 could attenuate the phosphorylation of AKT in the hypothalamus; however, the mechanisms underlying this regulation remain unknown. Because AKT phosphorylation can be promoted by its upstream regulator phosphatidylinositol 3-kinase (PI3K) and by pyruvate dehydrogenase kinase isozyme 1 (PDK1) [31,32], we examined the levels of PI3K in the hypothalamic primary neurons with overexpression or knockdown of Smad7. However, no difference was detected in the levels of PI3K and PDK1 in either case (Figure 6A,B).

As the levels of AKT phosphorylation can also be influenced by protein phosphatase (PP) 2A [31] and Smad7 can recruit PP1c to regulate dephosphorylation process [11], we next investigated whether the impaired AKT phosphorylation caused by Smad7 was due to the stimulated dephosphorylation of AKT. To test this possibility, we examined the effect of OA, the phosphatase inhibitor of PP1c and PP2A [11], on insulin signaling in Smad7 overexpressing hypothalamic primary neurons. Smad7-reduced p-AKT and p-GSK3β, following insulin stimulation, were reversed by OA treatment (Figure 6C,D). In addition, stimulation of PP1c/PP2A by the agonist C2 Ceramide [33] could inhibit Smad7 knockdown-induced AKT/GSK3β activation (Figure 6E,F). To explore which phosphatase played the key role in the regulation, we designed the siRNA for PP1c and PP2A, respectively, and found that only PP1c knockdown improved the Smad7-atteunated insulin signaling (Figure 6G,H, Supplementary Figure 8A and B).

4. DISCUSSION

The hypothalamus plays a pivotal role in whole-body metabolism, and it has been shown that hypothalamic POMC neurons are critical for the regulation of peripheral glucose metabolism [34]. However, their central mechanisms remain poorly understood. Obesity and older age are high-risk factors for developing diabetes [1], and over-production of TGF-β in the hypothalamic POMC neurons promotes glucose disorders under these conditions [8]. As an inhibitor of TGF-β signaling [16], the role of central Smad7 in glucose metabolism has not been investigated, although many functions of Smad7 in peripheral tissues

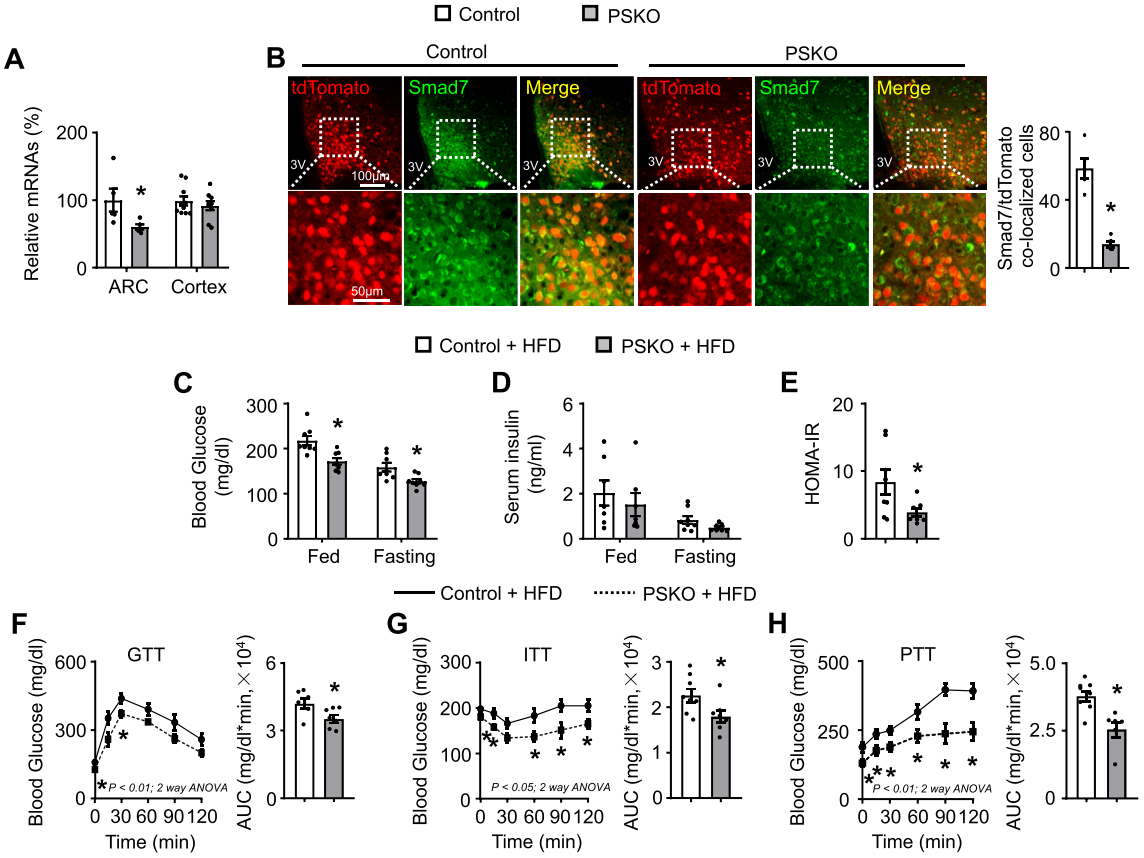

Figure 3: Deletion of Smad7 in POMC neurons improves high-fat diet (HFD)-induced glucose disorder. (A) Gene expression of *Smad7* in ARC and cortex by RT-PCR. (B) Immunofluorescence (IF) staining for tdTomato (red), Smad7 (green) and merge (yellow) in hypothalamic arcuate nucleus sections (left), and quantification of Smad7 and tdTomato co-localized cell numbers (right); 3V, third ventricle. (C) Fed and fasting blood glucose levels. (D) Fed and fasting serum insulin levels. (E) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (F) Glucose tolerance tests (GTTs). (G) Insulin tolerance tests (ITTs). (H) Pyruvate tolerance tests (PTTs). Studies for A were conducted using 8- to 12-week-old male control mice (Control) or mice with Smad7 deletion in POMC neurons (PSKO); studies for B were conducted using 8- to 12-week-old male POMC-Ai9 mice (Control) or Smad7 deletion in POMC neurons-Ai9 (PSKO) mice; studies for C–H were conducted using 20- to 22-week-old male control mice (Control) or PSKO (PSKO) mice fed a HFD. Data are expressed as the mean ± SEM (n = 4–10 per group, as indicated), with individual datapoints. $^{*}P < 0.05$ for the effect of PSKO mice vs. control mice.

have been reported [12]. Based on the well-known relationship between TGF-β and Smad7 [16], we speculated that hypothalamic Smad7 expression levels might be high in response to the overproduction of TGF-β in HFD-fed or middle-aged mice. As expected, we found that Smad7 expression levels were high in hypothalamic POMC neurons in these mice. Interestingly, the increased Smad7 levels in hypothalamic POMC neurons disrupted the whole-body glucose balance, based on the results of GTT, ITT, and PTT. As ARC contains many types of cells [28,35] and Smad7 also increased in the non-POMC neurons (Figure 1B,D), we suspect that the Smad7 in non-POMC neurons may also have a role in regulating glucose balance, which requires future study.

Consistent with the deleterious effect of Smad7 found in our study, overexpression of Smad7 induced by tail vein injection has been shown to aggravate HFD-induced obesity and inflammation [36]. Moreover, the effect of Smad7 in our study was probably independent of the change in body weight or fat content, because more of the experiments with manipulation of Smad7 expression in POMC neurons had no effect on fat content, except for in PSKO mice under a HFD. These results suggest that the expression levels of central Smad7 had a direct effect on peripheral glucose metabolism. Consistent with our study, the central control of obesity and glucose regulation is separated under some conditions [8,37].

Insulin secreted from the pancreas also has receptors in the hypothalamus [2]. Studies have shown that knockdown of the insulin receptor in AgRP neurons increases hepatic glucose production and that injecting insulin into the hypothalamus decreases body weight [19,34], suggesting that insulin signaling plays an important role in the brain's control of whole-body metabolism. It has been shown that insulin signaling is impaired in the peripheral tissues of HFD-fed and aged mice [1,38], as well as in the brain [3,39]. In addition, a previous study has shown that in lung cancer cell lines, Smad7 negatively regulates the phosphorylation of AKT [18], a key regulator of insulin sensitivity [19]. We hypothesized that insulin signaling may be involved in central Smad7 regulation of glucose metabolism.

As expected, we found that Smad7 was significantly associated with an attenuated insulin sensitivity in the brain. We then confirmed the importance of the impaired insulin sensitivity in the central Smad7 regulation of metabolism by examining the reversal effect of AKT overexpression in vitro and in vivo in hypothalamic neurons. However, AAVs used to over-express AKT in ARC could cause additional AKT over-expression in non-POMC neurons, which may also contribute to

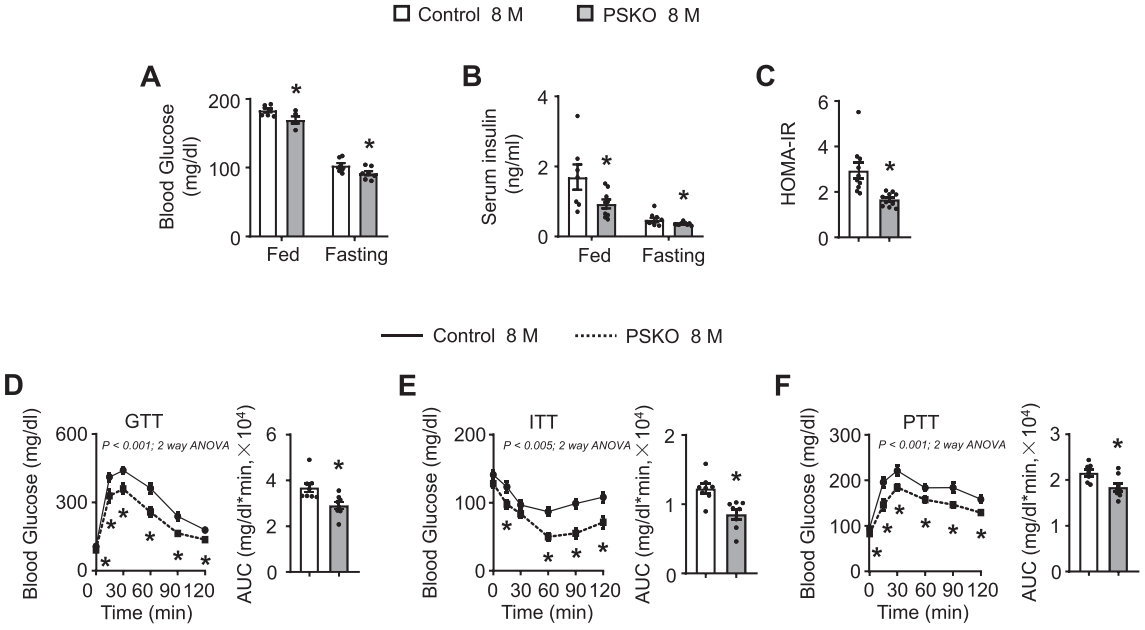

Figure 4: Deletion of Smad7 in hypothalamic POMC neurons improves glucose metabolism in middle-aged mice. (A) Fed and fasting blood glucose levels. (B) Fed and fasting serum insulin levels. (C) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (D) Glucose tolerance tests (GTTs). (E) Insulin tolerance tests (ITTs). (F) Pyruvate tolerance tests (PTTs). Studies were conducted using 32- to 36-week-old (8 M) male control mice (Control) or mice with Smad7 deletion in POMC neurons (PSKO) fed a normal chow diet. Data are expressed as the mean ± SEM (n = 5–10 per group, as indicated), with individual datapoints. $^{*}P < 0.05$ for the effect of PSKO mice vs. control mice under 8 M.

the effect of Smad7 in POMC neurons. Their contribution should not be ignored and requires future exploration. Despite of these facts, our results not only improve the understanding of the pathway that controls insulin signaling but also provide important evidence that Smad7 is relevant to the regulation of insulin sensitivity.

We then further explored how the increased Smad7 inhibited AKT phosphorylation. AKT phosphorylation is induced by increased PI3K and PDK1 [40] and/or decreased dephosphorylation by PP2A [31]. We found that Smad7 did not reduce AKT phosphorylation by PI3K signaling, but rather stimulated its dephosphorylation, as shown by the blocking effect of protein phosphatase inhibitor OA [11] and activating effect of C2 Ceramide [33]. Ser/Thr phosphatases mainly include PP1c and PP2A [41]. Previous research found that Smad7 could promote the dephosphorylation of TβR1 by recruiting PP1c [11]. It has also been shown that AKT phosphorylation can be regulated by PP2A [31]. However, whether Smad7 affects PP2A or the known Smad7 interacting protein PP1c affects AKT phosphorylation has not been tested. Our results showed that Smad7 was likely to affect AKT phosphorylation by PP1c, but not PP2A. These results also implied a new phosphatase regulating AKT phosphorylation, although the detailed mechanisms remain unclear. Understanding these proteins and phosphatases is important for understanding the mechanisms of AKT signaling regulation as well as those of signaling downstream from Smad7.

However, several questions remained unanswered. One of the important issues is about interpreting the data obtained from POMC-Cre mice, because it is shown that POMC-Cre mice efficiently mark not only POMC neurons, but also mark a subpopulation of non-POMC neurons during the embryonic phase [42,43]. Therefore, we could not conclude that the phenotypes observed in POMC-Smad7 KO mice resulted from deletion of Smad7 only in the hypothalamic POMC neurons; the contribution of Smad7 deleted in non-POMC neurons needs further investigation.

Central insulin signaling has many effects on the peripheral tissue, including regulation of food intake, lipid metabolism, glucose balance, and thermogenesis [2,34]. In our study, we found that POMC neuronal Smad7 had a consistent effect on glucose balance in all the mice models with manipulation of Smad7 expression in POMC neurons; whereas, there was almost no effect on food intake or body weight, except in PSKO mice under a HFD. Similar phenomena were observed in other studies [8,27,37]. A possible mechanism is that Smad7-AKT signaling influences the vagus nerve but not the sympathetic nerve, which mainly regulates feeding and regulating adipose tissue [2]; in addition, Smad7 may activate STAT3 signaling [12], suggesting that another mechanism may exist that controls feeding or regulating adipose tissue. These possibilities are topics for future study.

Moreover, we have noticed that there seems to be a discrepancy in the findings between ours and those by Yan et al. [8]. In their study, the levels of TGF-β1 are increased in the hypothalamus of HFD-fed and middle-aged mice. But, the increased TGF-β1 levels do not always indicate an activation of TGF-β-dependent downstream signaling. For example, in the condition of colitis, the TGF-β1 levels are increased, whereas the TGF-β1 signaling (reflected by p-Smad2/3) is decreased [44–46]. If TGF-β signaling is also decreased in the study conducted by Yan et al. [8], our study has no discrepancy with this research. If TGF-β signaling is activated in their study, the discrepancy between ours and their work further supports a TGF-β-independent mechanism mediating the effect of Smad7. These possibilities require future study. In addition, we were unable to determine the mechanisms underlying the increased expression of hypothalamic Smad7 in HFD-fed or middle-aged mice. This may have resulted from the overproduction of TGF-β. In addition, other factors have also been shown to induce Smad7 expression, including interferon-gamma and nuclear factor kappa-B [16,47], which are both increased in correlation with HFD and aging [48,49]. Therefore, how the hypothalamic Smad7 was induced under these conditions requires more investigation.

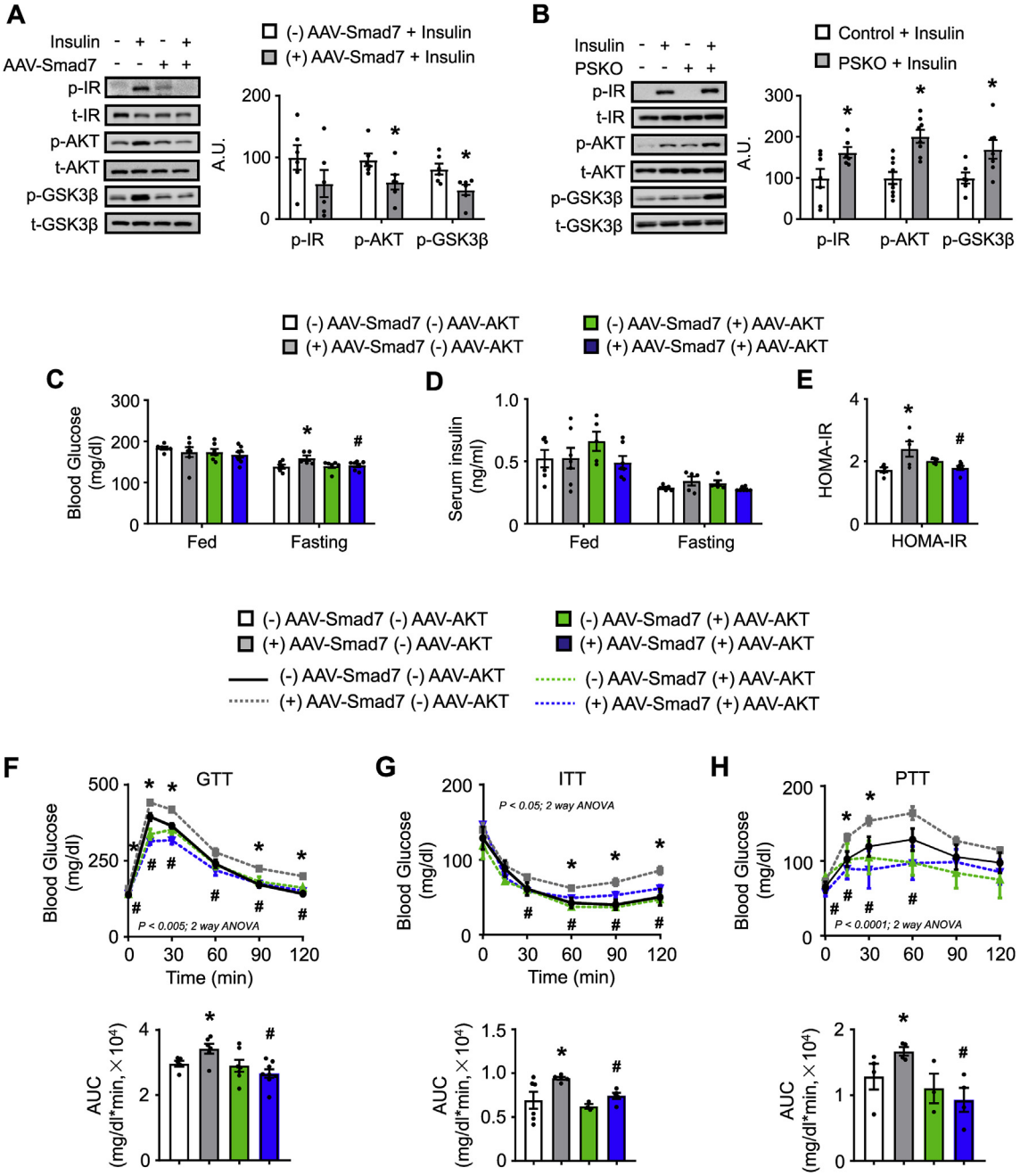

Figure 5: Smad7 in POMC neurons impairs hypothalamic insulin sensitivity. (A, B) P-IR, t-IR, p-AKT, t-AKT, p-GSK3β, and t-GSK3β proteins in hypothalamus by western blotting (top) and quantified by densitometric analysis (bottom); A.U.: arbitrary units. (C) Fed and fasting blood glucose levels. (D) Fed and fasting serum insulin levels. (E) Homeostasis model assessment of insulin resistance (HOMA-IR) index. (F) Glucose tolerance tests (GTTs). (G) Insulin tolerance tests (ITTs). (H) Pyruvate tolerance tests (PTTs). Studies for A were conducted using 13- to 14-week-old male POMC-Cre mice receiving AAVs expressing mCherry [(−)AAV-Smad7] or Smad7 [(+)AAV-Smad7] with (+insulin) or without insulin (-insulin) injection into third ventricle; studies for B were conducted using 28- to 30-week-old male control mice (Control) or mice with Smad7 deletion in POMC neurons (PSKO) with (+insulin) or without (-insulin) insulin injection into third ventricle; studies for C–H were conducted using 12- to 14-week-old male POMC-Cre mice receiving AAVs expressing GFP and mCherry [(−) AAV-Smad7 (−) AAV-AKT], Smad7 and GFP [(+) AAV-Smad7 (−) AAV-AKT], mCherry and AKT [(−) AAV-Smad7 (+) AAV-AKT], or Smad7 and AKT [(+) AAV-Smad7 (+) AAV-AKT] injection into hypothalamic arcuate nucleus. Data are expressed as the mean ± SEM (n = 3–9 per group, as indicated), with individual datapoints. $^{*}P < 0.05$ for the effect of any group vs. control group; $^{\#}P < 0.05$ for the effect of (+) AAV-Smad7 (+) AAV-AKT mice vs. (+) AAV-Smad7 (−) AAV-AKT mice for C.

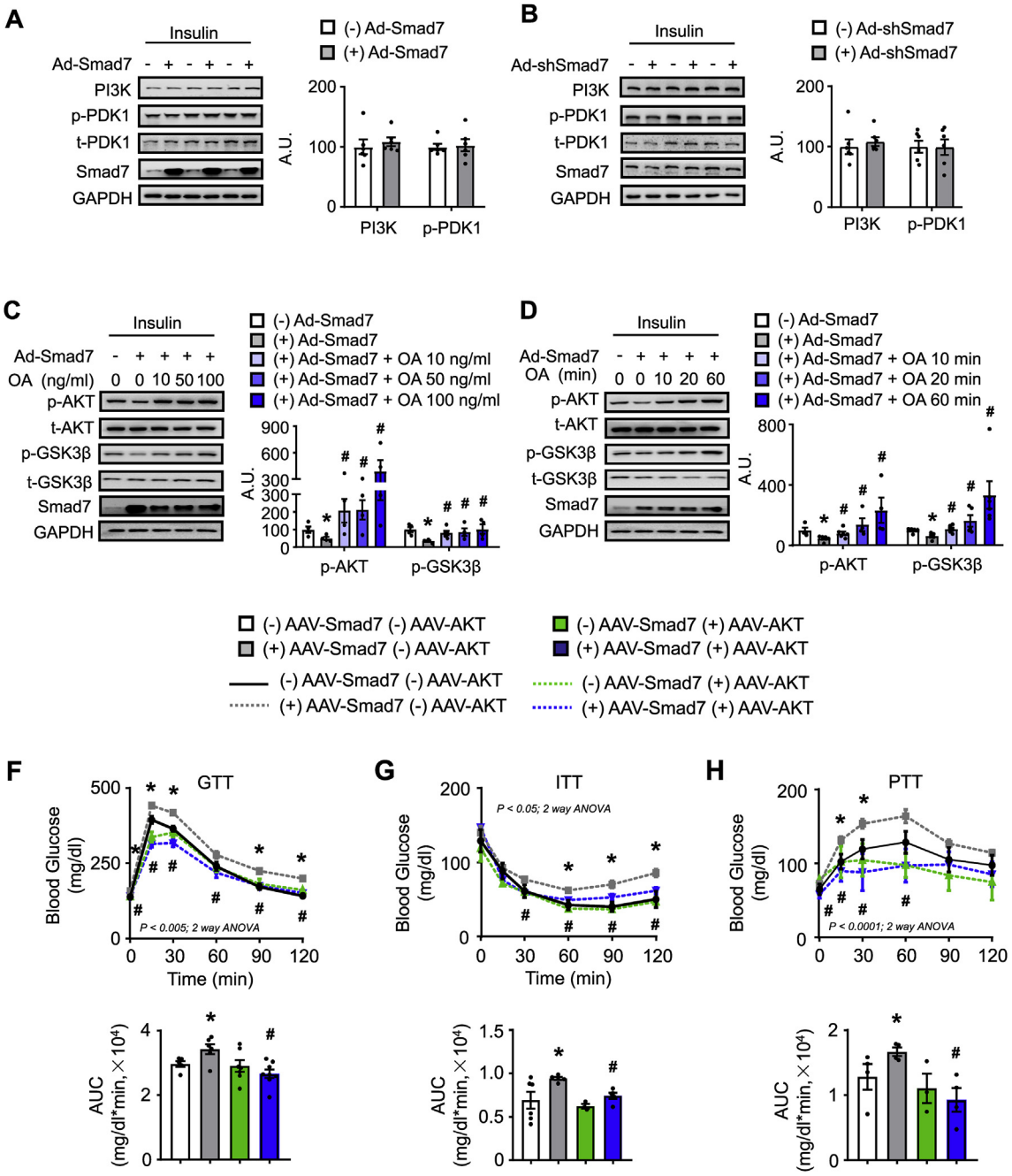

Figure 6: Smad7 dephosphorylates AKT via protein phosphatase 1/2. (A, B) PI3K, p-PDK1, t-PDK1, Smad7, and GAPDH proteins in hypothalamic primary cells by western blotting (left) and quantified by densitometric analysis (right); A.U.: arbitrary units. (C, D) P-AKT, t-AKT, p-GSK3β, t-GSK3β, Smad7, and GAPDH proteins in hypothalamic primary cells by western blotting (left) and quantified by densitometric analysis (right). (E, F) P-AKT, t-AKT, p-GSK3β, t-GSK3β, and PP1/PP2 proteins in hypothalamic primary cells by western blotting (left) and quantified by densitometric analysis (right). (G, H) P-AKT, t-AKT, p-GSK3β, t-GSK3β, and GAPDH proteins in hypothalamic primary cells by western blotting (left) and quantified by densitometric analysis (right). Studies were conducted in hypothalamic primary cells treated with Ad-GFP [(−) Ad-Smad7], Ad-Smad7 [(+) Ad-Smad7], Ad-scramble [(−) Ad-shSmad7] or Ad-shSmad7 [(+) Ad-shSmad7] for A and B; Ad-GFP [(−) Ad-Smad7] or Ad-Smad7 [(+) Ad-Smad7] with concentration gradient of okadaic acid (OA) for C or time gradient of OA for D; Ad-Scramble [(−) Ad-shSmad7] or Ad-shSmad7 [(+) Ad-shSmad7] with concentration gradient of C2 Ceramide (C2) for E or time gradient of C2 for F; Ad-GFP [(−) Ad-Smad7], Ad-Smad7 [(+) Ad-Smad7], control siRNA [(−) siPP1c], and/or siPP1c [(+) siPP1c] for G; or Ad-GFP [(−) Ad-Smad7], Ad-Smad7 [(+) Ad-Smad7], control siRNA [(−) siPP2A], and/or siPP2A [(+) siPP2A] for H, all followed by stimulation with 100 nM insulin for 10 min. Data are expressed as the mean ± SEM (n = 4–10 per group, as indicated), with individual datapoints. $^{*}P < 0.05$ for the effect of any group vs. control group; $^{\#}P < 0.05$ for the effect of any group vs. (+) Ad-Smad7 group for C and D, the effect of any group vs. (+) Ad-shSmad7 group for E and F, or the effect of [(+) Ad-Smad7 (+) siPP1c] group vs. [(+) Ad-Smad7 (−) siPP1c] group for G.

5. CONCLUSION

Our results demonstrated a new function of central POMC neuronal Smad7 in the regulation of global glucose metabolism in obese and aging subjects. Moreover, we found Smad7 decreased hypothalamic insulin signaling via PP1c and influenced the whole-body glucose balance (Supplementary Figure 8C). Our findings provided a new signal in specific neurons in regulation glucose balance in response to nutrient excess and aging.

AUTHORS' CONTRIBUTIONS

F.G., F.Y. and Y.C. planned and supervised the experimental work and data analysis; F.Y. and H.Y. performed the experiments and wrote the manuscript; H.J., F.J. and Y.N. researched data and provided technical support; S.C., H.Y., Q.Z. and Y.C. provided research material; F.G. directed the project, contributed to discussion and wrote, reviewed, and edited the manuscript. The manuscript was revised and approved by all authors.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101084.

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