MANF in POMC Neurons Promotes Brown Adipose Tissue Thermogenesis and Protects Against Diet-Induced Obesity

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Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an emerging regulator in metabolic control. Hypothalamic proopiomelanocortin (POMC) neurons play critical roles in maintaining whole-body energy homeostasis. Whether MANF in POMC neurons is required for the proper regulation of energy balance remains unknown. Here, we showed that mice lacking MANF in POMC neurons were more prone to develop diet-induced obesity. In addition, the ablation of MANF induced endoplasmic reticulum (ER) stress and leptin resistance in the hypothalamus, reduced POMC expression and post-translational processing, and ultimately decreased sympathetic nerve activity and thermogenesis in brown adipose tissue (BAT). Conversely, MANF overexpression in hypothalamic POMC neurons attenuated ER stress, increased POMC expression and processing, and then stimulated sympathetic innervation and activity in BAT, resulting in increased BAT thermogenesis, thus protecting mice against dietary obesity. Overall, our findings provide evidence that MANF is required for POMC neurons to combat obesity.

Obesity is a major global health concern and a predominant risk factor for the development of a panel of metabolic diseases such as diabetes, fatty liver disease, and cardiovascular diseases (1–3). Current therapeutic approaches for obesity are mainly limited to recommendations regarding lifestyle modifications, such as caloric restriction or exercise; no prevention or long-term medical treatments are available (2,3). A deeper understanding of the fundamental mechanisms underlying obesity might provide new approaches for its treatment.

Dysregulation of energy homeostasis from the chronic imbalance between energy intake and energy expenditure contributes to obesity (4). The central nervous system, especially the arcuate nucleus (ARC) in the hypothalamus, plays a critical role in controlling energy balance (4,5). Among the multiple neural populations within the ARC (6), neurons that express proopiomelanocortin and cocaine- and amphetamine-regulated transcript (i.e., proopiomelanocortin [POMC] neurons) are important for metabolic control (7,8). Previous work has identified various molecules and pathways, including SIRT6 (9,10), PTP1B (11), mitofusin 2 (12), leptin signaling (13), and endoplasmic reticulum (ER) stress (7,14–16), that play a significant role for POMC neurons in maintaining energy balance. Further elucidating how POMC neurons exert negative energy balance is important for combating obesity.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is proven to be protective in multiple disease contexts, including neurodegenerative disorders (17). Recent studies have demonstrated its pivotal role in other contexts, including metabolic control. For example, MANF is essential for the maintenance of pancreatic β-cell survival and proliferation (18–23). Moreover, MANF was required in maintaining liver homeostasis through suppressing lipogenesis, inflammation, and fibrosis (24,25). In addition, we recently showed that hepatocyte-derived MANF played a critical role in protecting mice against diet-induced obesity via stimulating the browning of adipose tissues, and systemic administration of recombinant MANF protein protects mice against obesity and related metabolic disorders (26). Intriguingly, MANF appears to be abundant in several hypothalamic nuclei, including the ARC (27,28), and its overexpression in the hypothalamus

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causes severe hyperphagia and obesity (27). However, the functions and mechanisms of MANF in POMC neurons and other neural populations in the hypothalamus remain to be investigated.

In the current study, we provide strong evidence that MANF is a key mediator for POMC neurons to regulate whole-body energy balance. Specifically, we show that mice lacking MANF in POMC neurons were more susceptible to body weight gain under both a normal chow diet (CD) and high-fat diet (HFD). Conversely, mice overexpressing MANF specifically in hypothalamic POMC neurons were protected against diet-induced obesity. In addition, we found that MANF in POMC neurons is required for the thermogenesis in brown adipose tissue (BAT) via modulating its sympathetic innervation and activity. Mechanistically, our experiments suggest that MANF in POMC neurons is critical for maintaining ER homeostasis and leptin signaling, both of which might directly and/or indirectly be involved in the maintenance of proper POMC transcription and posttranslational processing.

RESEARCH DESIGN AND METHODS

Mice

Male mice on a C57BL/6J background were used in this study. *Pomc*-Cre and Ai9 (*tdTomato-reporter*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Manf*$^{loxP/loxP}$ (LoxP) mice were crossed with *Pomc*-Cre mice to generate MANF POMC neuron-specific knockout (PMKO) mice. LoxP mice were used as controls. Mice were provided ad libitum access to a CD or an HFD (Research Diets, New Brunswick, NJ). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Sichuan University.

Stereotaxic Surgery and Viral Injections

Stereotaxic surgery and viral injections were performed as previously described (9,10). Adeno-associated virus (AAV) vector containing mouse MANF (AAV-MANF) and AAV vector containing only green fluorescent protein (AAV-GFP) were obtained from OBiO Technology (Shanghai, China).

Metabolic Parameter Measurements

Indirect calorimetry was performed in a comprehensive laboratory animal-monitoring system (Sable, Las Vegas, NV) as previously described (9). Rectal temperature was measured with a rectal probe attached to a digital thermometer (KEW, Nanjing, China). Interscapular BAT temperature was determined with an infrared thermal camera (FORTRIC, Shanghai, China).

Biochemical Analysis

Serum and hepatic concentrations of total cholesterol (TC) (Biosino, Beijing, China), triglycerides (TG) (Biosino), and nonesterified fatty acid (NEFA) (FUJIFILM Wako Shibayagi Corporation, Gunma, Japan) were measured. Serum levels of glucose (Biosino), leptin, and insulin (Merck Millipore, Darmstadt, Germany) were also detected. Norepinephrine (NE) levels in BAT were measured with an ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All biochemical analyses were carried out according to the manufacturers' instructions.

Cold-Stress Experiments

For acute cold exposure, 8-week-old mice were placed in a refrigerator (4°C) for 6 h without food. For long-term cold exposure, mice were placed in 4°C conditions for 72 h with food and water. Rectal temperature was measured at indicated times. BAT removal operation was performed as previously described (29).

Measurement of NE Turnover

NE turnover (NETO) was measured with the α-methyl-p-tyrosine (AMPT) method (30,31). Briefly, half of the mice were untreated and sacrificed to obtain baseline tissue NE content. The paired other half was injected with AMPT (300 mg/kg body wt i.p.) initially and 2 h later subsequently injected with a supplemental dose of AMPT (150 mg/kg body wt i.p.) to ensure the maintenance of tyrosine hydroxylase inhibition. AMPT-treated mice were sacrificed 4 h after the initial AMPT injection, after which BAT was rapidly dissected, weighed, snap frozen in liquid nitrogen, and stored at −80°C. The NE content in BAT was measured with reverse-phase high-performance liquid chromatography with electrochemical detection (Shimadzu, Kyoto, Japan). NETO was calculated, $k = (\lg[NE]_0 - \lg[NE]_4)/(0.434 \times 4)$ and $K = k[NE]_0$, where k is the constant rate of NE efflux, $[NE]_0$ is the initial NE concentration, $[NE]_4$ is the final NE concentration, and K = NETO.

Glucose Tolerance Test and Insulin Tolerance Test

Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed as previously described (9,10). For GTT, mice were fasted for 16 h before receiving an intraperitoneal injection of D-glucose at the indicated dosage. For ITT, mice were fasted for 4 h before receiving an intraperitoneal injection of insulin at the indicated dosage. Blood glucose level was measured from the tail vein by a portable glucometer at the indicated time points.

Histological Analysis

Hematoxylin and eosin (H&E) staining of liver and adipose tissues was performed according to the manufacturer's instructions (BASO, Guangdong, China). The size of adipocytes was analyzed in H&E–stained sections with ImageJ software (National Institutes of Health). For immunohistochemistry analysis, sections were subjected to antigen retrieval and then blocked and incubated overnight at 4°C with primary antibodies (Supplementary Table 1). For oil red O staining of liver tissues, frozen sections were fixed and stained with 0.5% oil red O solution. Images were obtained with an optical microscope (Olympus, Tokyo, Japan) or a laser scanning confocal microscope (Nikon, Tokyo, Japan).

Immunofluorescence (IF) staining of brain tissues was performed as previously described (10). Briefly, free-floating sections were blocked for 1 h at room temperature, incubated with primary antibodies (Supplementary Table 1) at 4°C overnight, and then incubated with secondary antibodies at room temperature for 2 h. Images were obtained with laser scanning confocal microscope or fluorescence microscope (Nikon).

RNA Extraction and Quantitative PCR

RNA was isolated and quantitative PCR performed as previously described (9). Relative mRNA levels of genes were calculated with the comparative threshold cycle method and normalized to that of the 18S ribosomal RNA. The primer sequences used in this study are listed in Supplementary Table 2.

Protein Extraction and Western Blot Analysis

Protein extraction and Western blot analysis were performed as previously described (9), with use of the antibodies in Supplementary Table 1. Protein levels were normalized to the β-actin level of each sample.

Statistical Analysis

Data are reported as means ± SEM. Statistical significance for these data was analyzed using unpaired Student *t* test, one-way ANOVA, or two-way ANOVA. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad Prism 7.04 (San Diego, CA).

Data and Resource Availability

All data generated or analyzed during this study are included in the published article (and Supplementary Material).

RESULTS

Deletion of MANF in POMC Neurons Causes Obesity

Firstly, we found significantly lower *Manf* levels in the hypothalamus of mice with diet-induced obesity in comparison with mice fed a CD (Supplementary Fig. 1*A*). For labeling of the POMC neurons in vivo, *Pomc*-Cre mice were intercrossed with Ai9 mice, generating POMC/Ai9 mice. IF staining showed that MANF was highly expressed in POMC neurons, and its level was decreased significantly in hypothalamic POMC neurons of high-fat diet (HFD)-fed mice relative to that in control mice (Supplementary Fig. 1*B* and *C*), which indicates that MANF in hypothalamic POMC neurons is implicated in maintenance of whole-body energy balance. To investigate the metabolic effects of MANF in hypothalamic POMC neurons, we genetically deleted MANF specifically in POMC neurons, generating PMKO mice. *Manf* mRNA levels were ∼50% lower in the mediobasal hypothalamus of PMKO mice than in LoxP mice but remained unchanged in other brain areas and tissues (Supplementary Fig. 1*D*). For determination of the specificity and efficiency of MANF deletion, PMKO mice were intercrossed with Ai9 mice, generating PMKO/Ai9 mice. IF staining showed that MANF was colocalized with POMC neurons in ARC of POMC/Ai9 mice, but this colocalization was largely reduced in PMKO/Ai9 mice (Fig. 1*A–C*). Anatomical evaluation of POMC neurons throughout the ARC of POMC/Ai9 and PMKO/Ai9 mice showed similar distributions and numbers (Supplementary Fig. 1*E–G*), which indicates that MANF deletion did not alter POMC neuron differentiation and survival.

To determine whether the deletion of MANF in POMC neurons affects whole-body energy balance, LoxP and PMKO mice were fed either a CD or an HFD. When fed with a CD, PMKO mice showed significantly greater body weight gain than LoxP mice from 12 weeks of age onward (Supplementary Fig. 2*A*). PMKO mice also showed greater weights of epididymal white adipose tissue (eWAT), inguinal WAT (iWAT), and BAT, as well as larger adipocytes in eWAT and iWAT (Supplementary Fig. 2*B–G*). Under an HFD, PMKO mice showed a more significant increase in body weight, which was associated with increased total fat mass but unchanged lean mass (Fig. 1*D–F*). MRI and micro-computed tomography scans further confirmed that PMKO mice exhibited a significant accumulation of fat as compared with the LoxP mice (Supplementary Fig. 3*A* and *B*). Furthermore, the weights of eWAT, iWAT, and BAT, as well as the adipocyte volume in eWAT and iWAT, were all increased in PMKO mice compared with that in LoxP mice (Fig. 1*G–K*), whereas the weights of other organs remained unchanged (Supplementary Fig. 3*C*). These results suggest that MANF deficiency in POMC neurons increases fat storage and body weight.

Deletion of MANF in POMC Neurons Impairs Glucose and Lipid Homeostasis and Promotes Adipose Tissue Inflammation

Obesity is usually accompanied by several other metabolic disorders (1,2). GTT showed comparable glucose tolerance between LoxP and PMKO mice fed with HFD (Fig. 2*A*). However, both the area under the curve and the glucose decay rate in ITT (kITT) (32,33) revealed that HFD-fed PMKO mice developed more severe insulin resistance than LoxP mice (Fig. 2*B* and *C*). PMKO mice also showed comparable serum glucose levels, whereas serum insulin levels were significantly increased (Fig. 2*D* and *E*). Serum TC levels were comparable between LoxP and PMKO mice, while serum TG and NEFA levels were significantly reduced in PMKO mice (Fig. 2*F–H*). Moreover, PMKO mice showed a significant increase in liver weight and hepatic lipid accumulation (Fig. 2*I–M*). Consistently, *Cd36* (a marker of NEFA uptake) and lipogenic genes were significantly upregulated in the liver of PMKO mice, while the expression of genes related to fatty acid oxidation or secretion of very VLDL remained unchanged (Supplementary Fig. 4*A–D*). PMKO mice also showed a significant increase in the inflammatory factors *F4/80*, *Mcp-1*, and *Il-1β* in the liver compared with LoxP mice (Supplementary Fig. 4*E*). In addition, PMKO mice showed a significant increase in

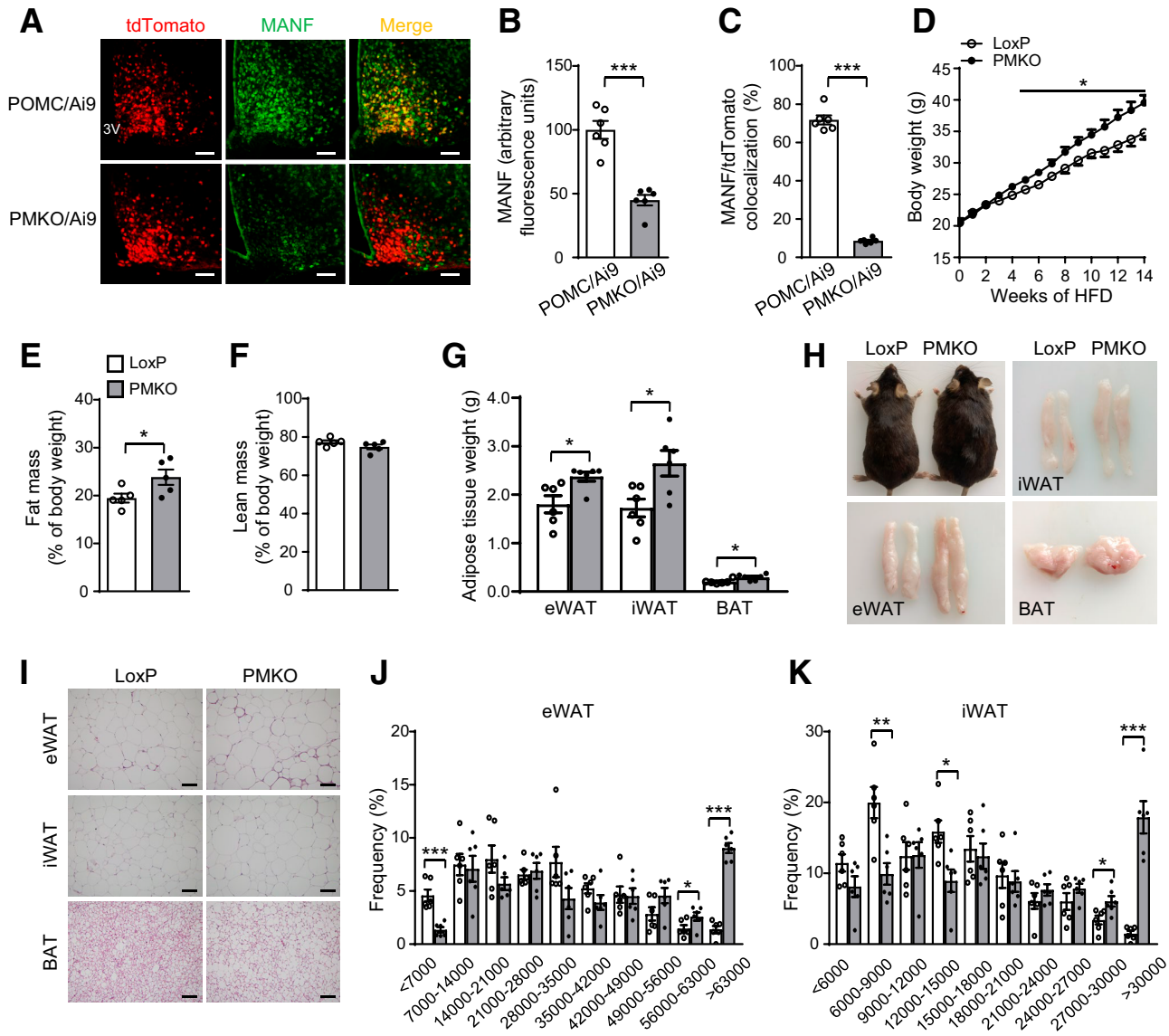

Figure 1—Deletion of MANF in POMC neurons sensitizes mice to obesity under an HFD. *A*: Representative images showing IF staining of MANF in POMC neurons of POMC/Ai9 and PMKO/Ai9 mice, POMC neurons (red), MANF (green), and merge (yellow). 3V, third ventricle. Scale bar, 100 μm. *B* and *C*: Integrated density quantification ($n = 6$). *D*: Body weight curve of LoxP ($n = 6$) and PMKO ($n = 7$) mice. *E* and *F*: Body fat mass and lean mass of LoxP and PMKO mice ($n = 5$). *G* and *H*: Weight and representative photographs of eWAT, iWAT, and BAT from 22-week-old LoxP and PMKO ($n = 6$) mice. *I*: Representative H&E–stained sections of eWAT, iWAT, and BAT from LoxP and PMKO mice. Scale bar, 100 μm. *J* and *K*: Frequency distribution of adipocyte cell size in eWAT and iWAT from LoxP and PMKO mice. Data are means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the number of F4/80-positive cells in the eWAT (Fig. 2*N*). Correspondingly, the gene expression of inflammatory factors such as *Mcp-1* and *Tnf-α*, and the macrophage markers such as *F4/80*, *Cd68*, *Cd11b*, and *Cd11c*, was also significantly upregulated in the eWAT from PMKO mice (Fig. 2*O*).

When fed with a CD, PMKO mice showed impaired glucose tolerance but normal insulin sensitivity (Supplementary Fig. 5*A–C*). LoxP and PMKO mice did not differ in serum glucose, insulin, TC, TG, or NEFA levels or hepatic lipid content (Supplementary Fig. 5*D–M*). Nevertheless, the expression of macrophage markers, such as *F4/80* and *Cd11b*, was significantly upregulated in the eWAT from PMKO mice compared with that of LoxP controls (Supplementary Fig. 5*N*).

Deletion of MANF in POMC Neurons Reduces Energy Expenditure by Suppressing Thermogenesis in BAT

To determine the mechanisms underlying the above phenotypes, we assessed food intake and energy expenditure. Under an HFD, daily food intake was comparable between LoxP and PMKO mice, but O_2 consumption, CO_2 production, and energy expenditure were lower in PMKO mice during both the dark and light phases (Fig. 3*A–G*). No difference in total physical activity was observed between LoxP and PMKO mice (Supplementary Fig. 6*A* and *B*).

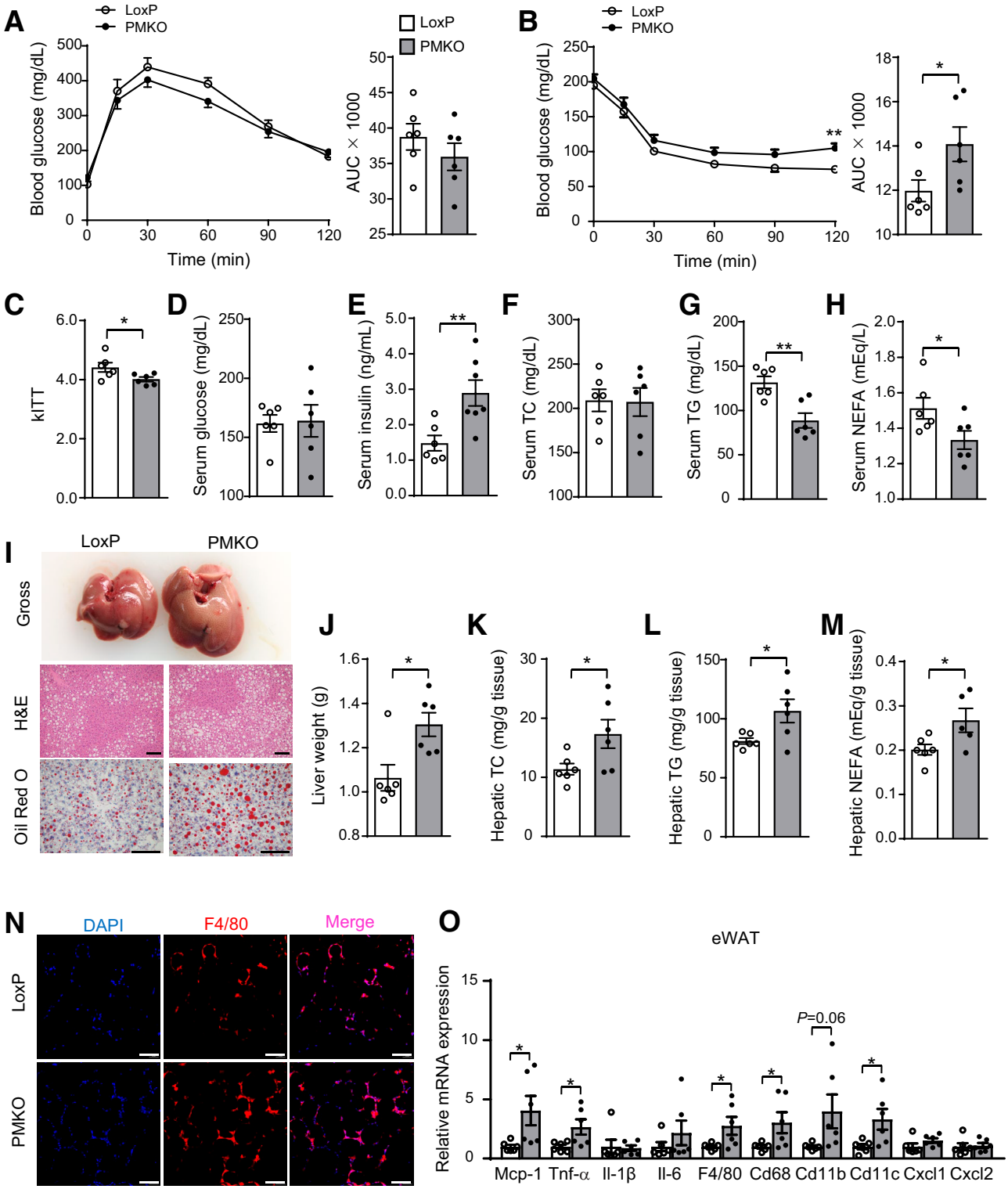

Figure 2— Deletion of MANF in POMC neurons promotes insulin resistance, liver steatosis, and adipose tissue inflammation in mice on an HFD. *A*: GTT in LoxP and PMKO mice (n = 6) at 20 weeks of age. AUC, area under the curve. *B*: ITT in LoxP and PMKO mice (n = 6). *C*: kITT (%/min). kITT is calculated using the formula $0.693/t_{1/2} \times$ % 100 (%/min), and the blood glucose half-time ($t_{1/2}$) was calculated from the slope of the least squares regression of the blood glucose concentration during the linear phase of decline. *D–H*: Serum glucose, insulin, total TC, TG, and NEFA levels in overnight-fasted LoxP (n = 6) and PMKO (n = 7) mice. *I*: Representative images of H&E– and oil red O–stained sections of liver from LoxP and PMKO mice at 22 weeks of age. Scale bar, 100 μm. *J*: Liver weight of LoxP and PMKO mice (n = 6). *K–M*: Hepatic TC, TG, and NEFA levels of LoxP and PMKO mice (n = 6). *N*: IF staining of F4/80 (red) in eWAT from LoxP and PMKO mice. Scale bar, 100 μm. *O*: mRNA levels of macrophage markers and immune cell markers in eWAT of 22-week-old LoxP and PMKO mice (n = 6). Data are means ± SEM. *$P < 0.05$; **$P < 0.01$.

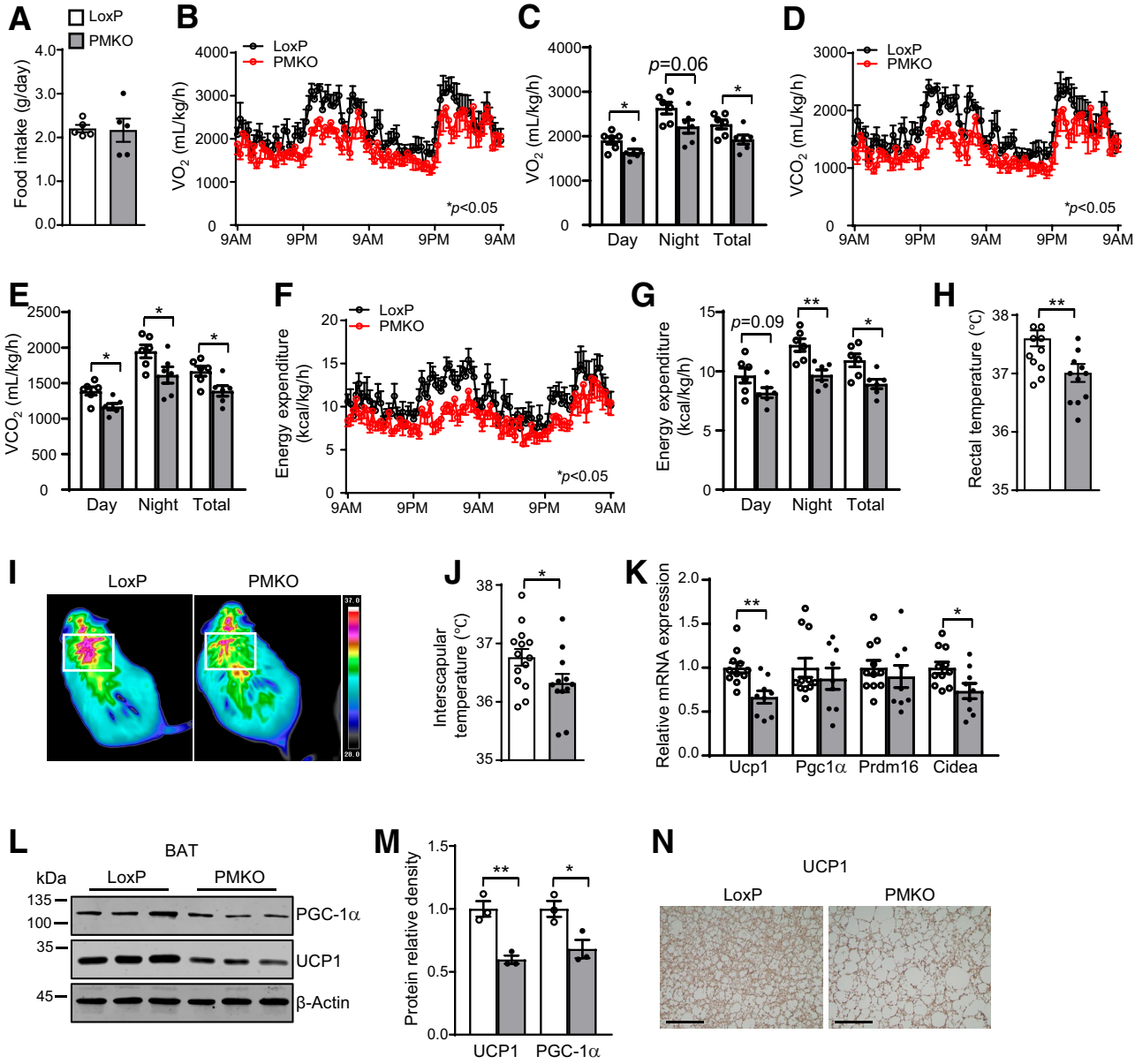

Figure 3—Deletion of MANF in POMC neurons reduces energy expenditure by suppressing thermogenesis in BAT of mice fed with HFD. *A*: Daily food intake of LoxP and PMKO mice (n = 5). *B–G*: O_2 consumption, CO_2 production, and energy expenditure of LoxP and PMKO mice (n = 6). *H*: Rectal temperature of overnight-fasted LoxP (n = 14) and PMKO (n = 12) mice. *I* and *J*: Representative thermal images and surface temperature of the interscapular region (n = 12–14). *K*: mRNA expressions of thermogenic genes in BAT from LoxP (n = 11) and PMKO (n = 9) mice. *L* and *M*: Western blot analysis and quantification of UCP1 and PGC-1α protein levels in BAT. *N*: Representative immunohistochemistry images of UCP1 in BAT. Scale bar, 100 μm. Data are means ± SEM. *$P < 0.05$; **$P < 0.01$.

Rectal temperature was significantly lower in PMKO mice than that of LoxP mice (Fig. 3*H*). PMKO mice also showed a significant lower interscapular BAT temperature (Fig. 3*I* and *J*), which implicates impaired BAT thermogenesis in the decreased energy expenditure observed in PMKO mice. In support of this, we found a significant downregulation of the thermogenic markers, including UCP1 and PGC-1α, in the BAT of PMKO mice (Fig. 3*K–N*). However, no differences were observed in terms of the browning-related parameters examined in iWAT between LoxP and PMKO mice (Supplementary Fig. 6*C–E*). Levels of lipolytic proteins such as phosphorylated (p)-HSL, ATGL, and p-Plin1 in eWAT and iWAT were all significantly lower in PMKO mice than in LoxP mice (Supplementary Fig. 6*F–I*). Similar results were observed in mice fed a CD (Supplementary Fig. 7*A–H*).

Deletion of MANF in POMC Neurons Impairs Sympathetic Nervous System–Dependent Thermogenesis in BAT

Next, we investigated how the signals in the brain were delivered to BAT. Previous studies have shown that activation

of sympathetic nervous system (SNS) increases BAT thermogenesis (34–36). Here, we found that the expression of tyrosine hydroxylase (TH), an enzyme required for the synthesis of catecholamines, and the NE receptor Adrβ3 was remarkably lower in BAT from PMKO mice than that of LoxP mice when fed with a CD or an HFD (Fig. 4*A*–*D* and Supplementary Fig. 8*A* and *B*), while several other genes implicated in sympathetic regulation were comparable between LoxP and PMKO mice (Supplementary Fig. 8*C*). The NE content in BAT was also greatly reduced in PMKO mice (Fig. 4*E*). In addition, we measured NETO based on the content of NE in BAT before and after the injection of

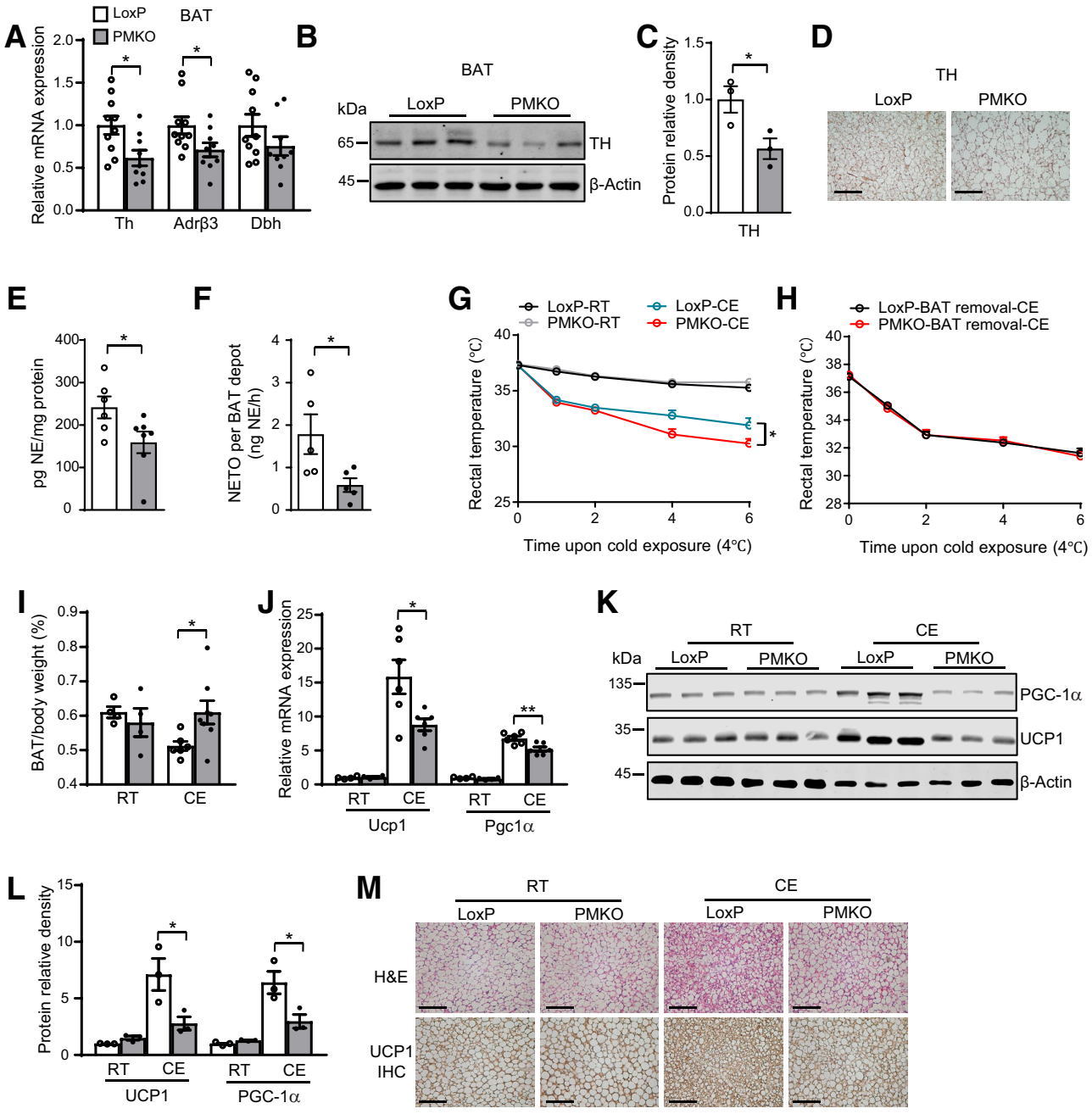

Figure 4—Deletion of MANF in POMC neurons impairs SNS-dependent thermogenesis in BAT. *A*: mRNA levels of *Th*, *Adrβ3*, and *Dbh* in BAT from LoxP (n = 10) and PMKO (n = 9) mice. *B* and *C*: Western blot analysis and quantification of TH protein levels in BAT. *D*: Representative immunohistochemistry images of TH in BAT. Scale bar, 100 μm. *E*: ELISA analysis of NE content in BAT from LoxP (n = 6) and PMKO (n = 7) mice. *F*: NETO in BAT of LoxP and PMKO mice (n = 5). *G*: Rectal temperature on room temperature (RT) or cold exposure (CE) for 6 h of LoxP and PMKO mice (n = 4–8). *H*: Rectal temperature on cold exposure for 6 h of LoxP and PMKO mice after BAT-removal operation (n = 4). *I*: BAT weight of LoxP and PMKO mice on room temperature or cold exposure for 6 h (n = 4–8). *J*: Expression levels of thermogenic genes in BAT of LoxP and PMKO mice (n = 4–6). *K* and *L*: Western blot analysis and quantification of UCP1 and PGC-1α in BAT. *M*: Representative images of H&E and UCP1-stained sections of BAT. Scale bar, 100 μm. Data are means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

AMPT, an inhibitor of TH that is a rate-limiting enzyme for NE synthesis, with known usage as a potential surrogate for sympathetic nerve activity (30,37). NETO in BAT was significantly lower in PMKO mice (Fig. 4*F*), which indicates that sympathetic nerve activity related to BAT was suppressed in PMKO mice.

Cold can induce BAT thermogenesis by activating SNS tone. We next investigated whether PMKO mice exhibited impaired thermogenesis under cold exposure. After acute exposure to an ambient temperature of 4°C for 6 h, PMKO mice showed a faster decrease in rectal temperature compared with LoxP mice (Fig. 4*G*), while this difference disappeared when interscapular BAT was removed (Fig. 4*H*). After cold exposure, the weight of BAT was significantly reduced in LoxP mice, but this effect was attenuated in PMKO mice (Fig. 4*I*). Furthermore, the mRNA and protein levels of UCP1 and PGC-1α were induced in LoxP mice on cold exposure, but these inductions were also attenuated in PMKO mice (Fig. 4*J–M*). However, during the long-term cold exposure, LoxP and PMKO mice exhibited comparable rectal temperature, and the expression levels of browning-related markers were similar between LoxP and PMKO mice (Supplementary Fig. 8*D–G*).

To further confirm the role of SNS in MANF regulation of BAT thermogenesis, we intraperitoneally injected the β3-adrenergic agonist CL316243 (38,39) or saline into LoxP and PMKO mice. If the decreased SNS activity is responsible for the suppression of BAT thermogenesis by MANF deletion in hypothalamic POMC neurons, activation of SNS activity would be expected to reverse this inhibition. As expected, treatment with CL316243 reversed the increase in BAT weight and the decrease in expression levels of thermogenic parameters in BAT of PMKO mice (Supplementary Fig. 8*H–L*). These findings support that the defective thermogenesis in BAT of PMKO mice is due to reduced sympathetic nervous activity.

Deletion of MANF in POMC Neurons Induces ER Stress and Leptin Resistance in the Hypothalamus

To investigate the mechanisms underlying decreased energy expenditure in PMKO mice, we first assessed neuropeptides expression in the hypothalamus. Under an HFD, the mRNA levels of *Agrp* and *Npy* were comparable between LoxP and PMKO mice, but the expression levels of POMC was downregulated in the ARC of PMKO mice (Fig. 5*A–C*). The levels of critical enzymes involved in the posttranslational processing of POMC, particularly prohormone convertase 2 (PC2), were decreased in the hypothalamus of PMKO mice compared with LoxP mice (Fig. 5*D* and *E*). Furthermore, expression of α-melanocyte stimulating hormone (α-MSH), an important bioactive neuropeptide generated from POMC processing, was significantly decreased in the paraventricular nucleus of PMKO mice (Fig. 5*F*).

Previous studies have shown that MANF plays a critical role in the maintenance of ER homeostasis (18,21–23,40–42). We therefore asked whether disturbed ER homeostasis (i.e., ER stress) was developed in PMKO mice. We found that PMKO mice exhibited upregulation in the expression levels of ER stress markers, including GRP78, p-IRE1α, XBP1s, and p-PERK (Fig. 5*G* and *H* and Supplementary Fig. 9*A*). Transmission electron microscope analysis also showed swollen ERs in the ARC of PMKO mice compared with that of LoxP mice (Fig. 5*I*). These results suggest that deletion of MANF in POMC neurons triggers unresolved ER stress and activated unfolded protein response (UPR) signaling in the hypothalamus.

ER stress in the hypothalamus is associated with the induction of leptin resistance (7). Indeed, the serum leptin level was higher in PMKO mice, and the expression of negative regulators of leptin receptor signaling, including *Ptp1b* and *Socs3*, was upregulated in the hypothalamus of PMKO mice (Fig. 5*J* and *K*). In addition, leptin signaling pathway activity, as indicated by the level of phosphorylation of signal transducer and activator of transcription 3 (p-STAT3, Tyr705), was significantly reduced in the ARC of PMKO mice (Fig. 5*L* and *M*). Notably, the blunted leptin sensitivity seems not to be associated with the induction of hypothalamic inflammation (Supplementary Fig. 9*B*).

MANF Overexpression in Hypothalamic POMC Neurons Protects Against Diet-Induced Obesity

Next, we asked whether overexpression of MANF in hypothalamic POMC neurons would result in a phenotype opposite that observed in PMKO mice. For this purpose, we administered, via bilateral stereotaxic injection, AAV-MANF or AAV-GFP into the ARC of male *Pomc*-Cre mice with diet-induced obesity. These viruses were exclusively expressed in the ARC region of the hypothalamus, not in other areas (Supplementary Fig. 10*A*), and the virus was specifically expressed in POMC-expressing neurons (Supplementary Fig. 10*B* and *C*). Overexpression of MANF was confirmed by IF staining and quantitative PCR analysis (Fig. 6*A–C*).

We then investigated the effects of the overexpression of MANF in hypothalamic POMC neurons on whole-body energy homeostasis. We found a significant retarded body weight gain in PMOE mice (Fig. 6*D* and *E*). Consistently, the weights of eWAT, iWAT, and BAT, as well as the size of adipocytes in the eWAT and iWAT from PMOE mice, were all reduced (Fig. 6*E–I*). These data suggest that overexpression of MANF specifically in hypothalamic POMC neurons can attenuate diet-induced obesity.

MANF Overexpression in Hypothalamic POMC Neurons Improves Obesity-Associated Metabolic Disorders

We next assessed whether the improved obesity observed in PMOE mice was accompanied by improvements in whole-body metabolism. PMOE mice showed no significant alteration in glucose tolerance but remarkably improved insulin sensitivity (Fig. 7*A–C*). Serum glucose levels were comparable between control and PMOE mice, whereas serum insulin levels were significantly decreased

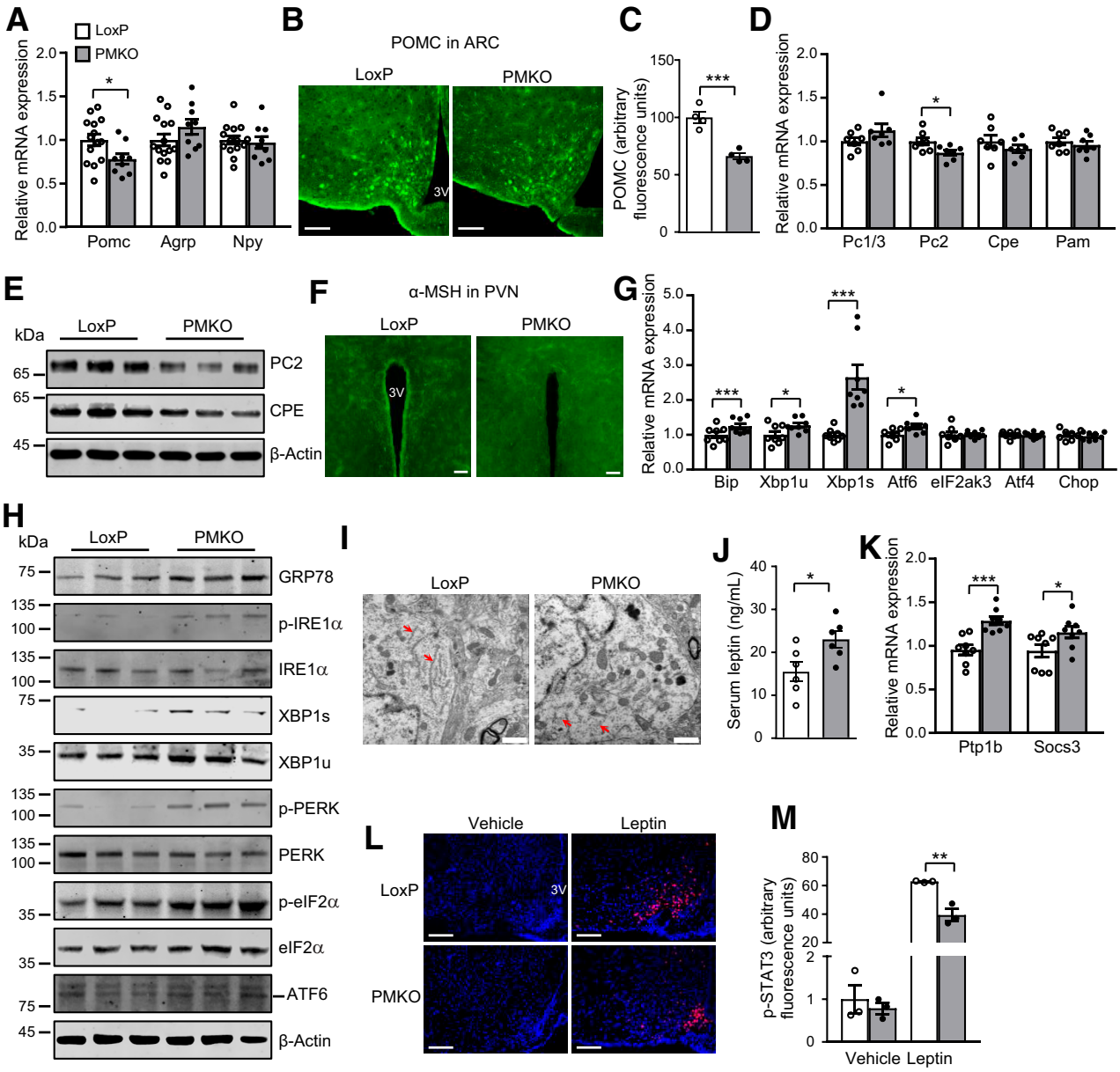

Figure 5—Deletion of MANF in POMC neurons impairs POMC transcription and posttranslational processing and induces ER stress and leptin resistance in the hypothalamus. *A*: Quantitative PCR analysis of the expression of neuropeptides in the hypothalamus of LoxP (n = 14) or PMKO (n = 9) mice. *B* and *C*: IF staining and integrated density quantification of POMC in the ARC of the LoxP and PMKO mice. 3V, third ventricle. Scale bar, 100 μm. *D*: Quantitative PCR analysis of the expressions of key enzymes involved in POMC processing in the hypothalamus of LoxP or PMKO mice (n = 7). *E*: Western blot analysis of protein levels of prohormone convertase 2 (PC2) and carboxypeptidase E (CPE) in the hypothalamus. *F*: Representative images of the α-melanocyte stimulating hormone (α-MSH) immunoreactive fibers in the paraventricular nucleus (PVN) of LoxP or PMKO mice. Scale bar, 100 μm. *G*: Quantitative PCR analysis of the expression of ER stress–responsive genes in the hypothalamus from LoxP and PMKO mice (n = 8). *H*: Western blot analysis of ER stress–related markers in the hypothalamus from LoxP and PMKO mice. *I*: Representative transmission electron microscope images of ER changes in the ARC of LoxP and PMKO mice. Scale bar, 1 μm. Red arrows indicate ER. *J*: Serum leptin levels of overnight-fasted LoxP and PMKO mice (n = 6). *K*: Quantitative PCR analysis of the expressions of negative regulators of leptin signaling in the hypothalamus of LoxP or PMKO mice (n = 8). *L* and *M*: Representative IF images and integrated density quantification of p-STAT3 (Tyr705) in ARC from 8-week-old LoxP and PMKO mice 45 min after a single injection of 0.1% BSA or leptin (1 μg/g i.p.). Scale bar, 100 μm. Data are means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

in PMOE mice in comparisons with control mice (Fig. 7*D* and *E*). Moreover, PMOE mice showed decreased serum TC levels, increased serum TG levels, and comparable serum NEFA levels (Fig. 7*F–H*). PMOE mice also showed less hepatic lipid accumulation and lower liver weight than control mice (Fig. 7*I–M*). In addition, the expressions

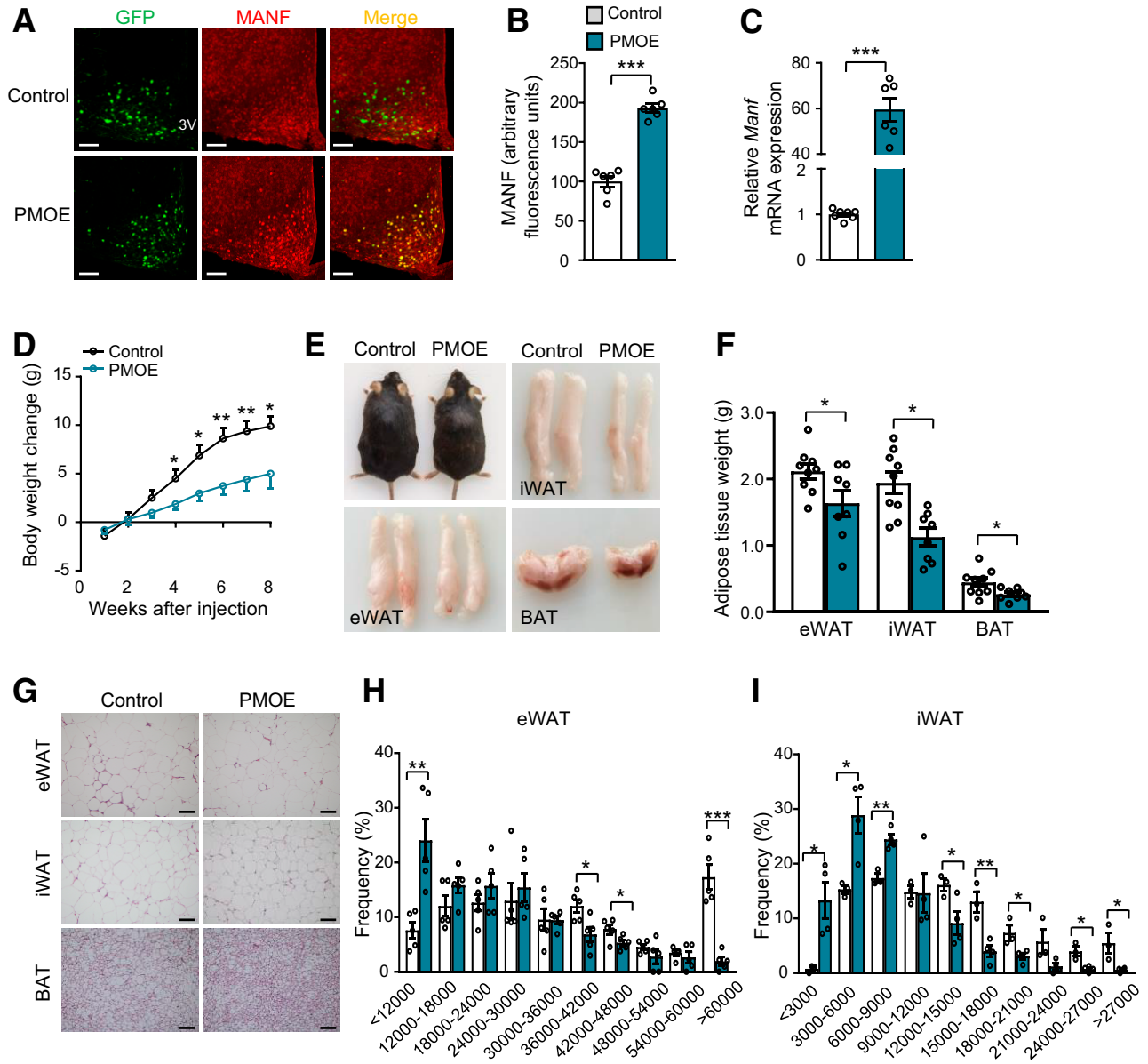

Figure 6—Overexpression of MANF in hypothalamic POMC neurons protects mice against diet-induced obesity. *A*: Representative images showing IF staining for GFP (green), MANF (red), and merge (yellow) in ARC sections. 3V, third ventricle. Scale bar, 100 μm. *B*: Quantification of integrated density of MANF in ARC (n = 6). *C*: *Manf* mRNA expression in the hypothalamus from control (n = 7) and PMOE (n = 6) mice. *D*: Body weight change of control (n = 9) and PMOE (n = 8) mice. *E* and *F*: Weight and representative photographs of eWAT, iWAT, and BAT from control (n = 9) and PMOE (n = 8) mice. *G*: Representative H&E–stained sections of eWAT, iWAT, and BAT from control and PMOE mice. Scale bar, 100 μm. *H* and *I*: Frequency distribution of adipocyte cell size in eWAT and iWAT from control and PMOE mice. Data are means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

of inflammatory cytokines and macrophage markers including *Mcp-1*, *Tnf-α*, *F4/80*, *Cd68*, *Cd11b*, *Cd11c*, and *Cxcl2*, were significantly reduced in the eWAT from PMOE mice (Fig. 7*N* and *O*).

MANF Overexpression in Hypothalamic POMC Neurons Increases BAT Thermogenesis, Possibly via Attenuation of ER Stress in the Hypothalamus

We then investigated the mechanisms underlying the improved obesity in PMOE mice. No alteration was observed in daily food intake between control and PMOE mice (Fig. 8*A*), whereas energy expenditure was significantly upregulated in PMOE mice, as both the rectal and BAT temperature showed a significant increase (Fig. 8*B–D*). Consistently, the mRNA levels of the thermogenic genes, including *Ucp1*, *Pgc1α*, *Prdm16*, and *Cidea*, were all upregulated in the BAT from PMOE mice (Fig. 8*E*). PGC-1α and UCP1 protein levels were also upregulated (Fig. 8*F* and *G* and Supplementary Fig. 11*A*). However, the expression levels of browning-related parameters in iWAT were comparable between control and PMOE mice (Supplementary Fig. 11*B–D*). In contrast, the protein levels of the key lipolytic enzymes

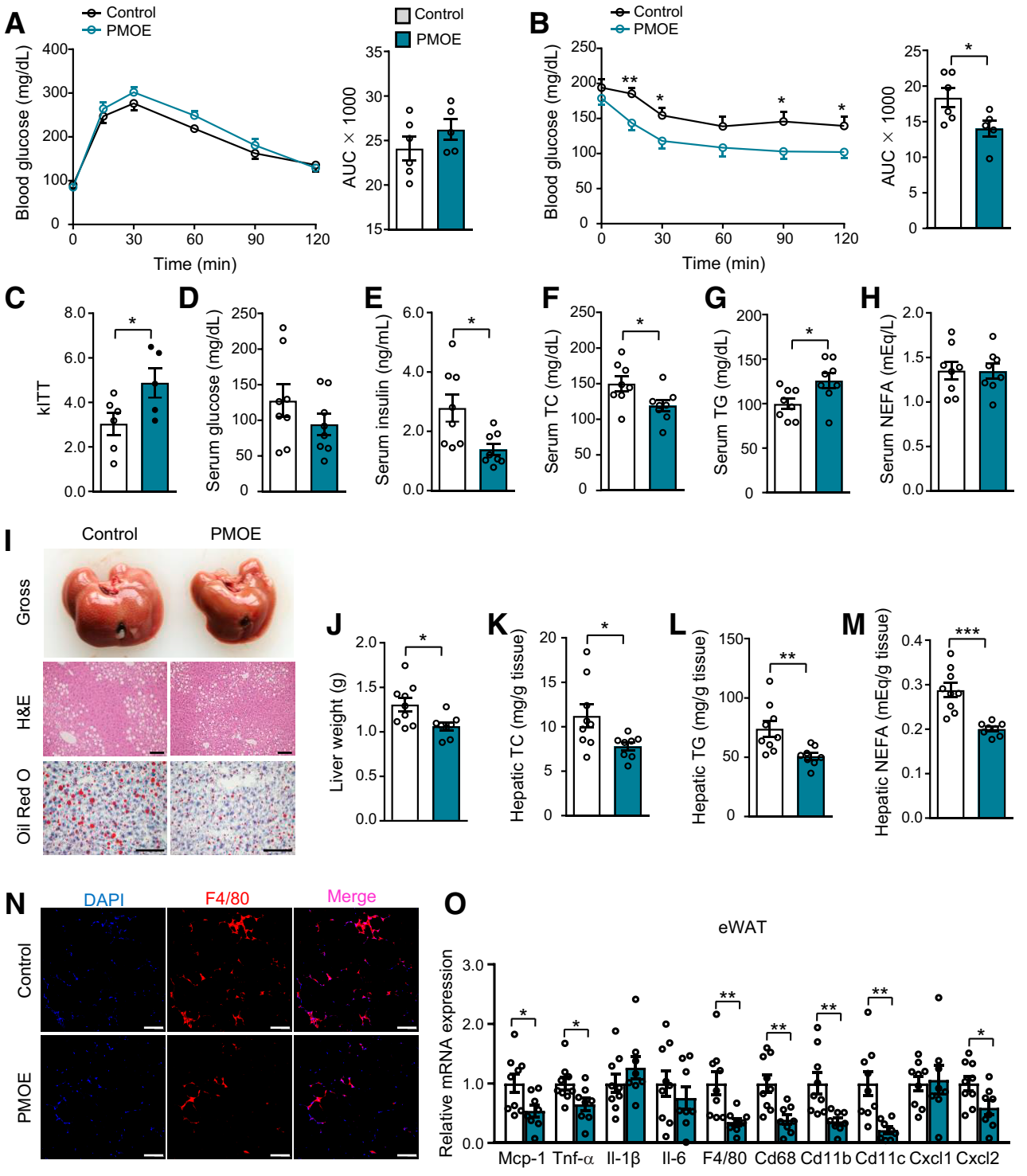

Figure 7—Overexpression of MANF in hypothalamic POMC neurons improves insulin sensitivity, reduces hepatic lipid accumulation, and decreases adipose tissue inflammation in mice. *A*: GTT in control ($n = 6$) and PMOE ($n = 5$) mice. AUC, area under the curve. *B*: ITT in control ($n = 6$) and PMOE ($n = 5$) mice. *C*: kITT (%/min). *D–H*: Serum glucose, insulin, TC, TG, and NEFA levels in overnight-fasted control and PMOE mice ($n = 8$). *I*: Representative photographs, images of H&E– and oil red O–stained sections of control and PMOE mice. Scale bar, 100 μm. *J*: Liver weight of control ($n = 9$) and PMOE ($n = 8$) mice. *K–M*: Hepatic TC, TG, and NEFA levels of control ($n = 9$) and PMOE ($n = 8$) mice. *N*: IF staining of F4/80 in eWAT from control and PMOE mice. Scale bar, 100 μm. *O*: Quantitative PCR analysis of the expression of macrophage markers and immune cell markers in eWAT from control ($n = 9$) and PMOE ($n = 8$) mice. Data are means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

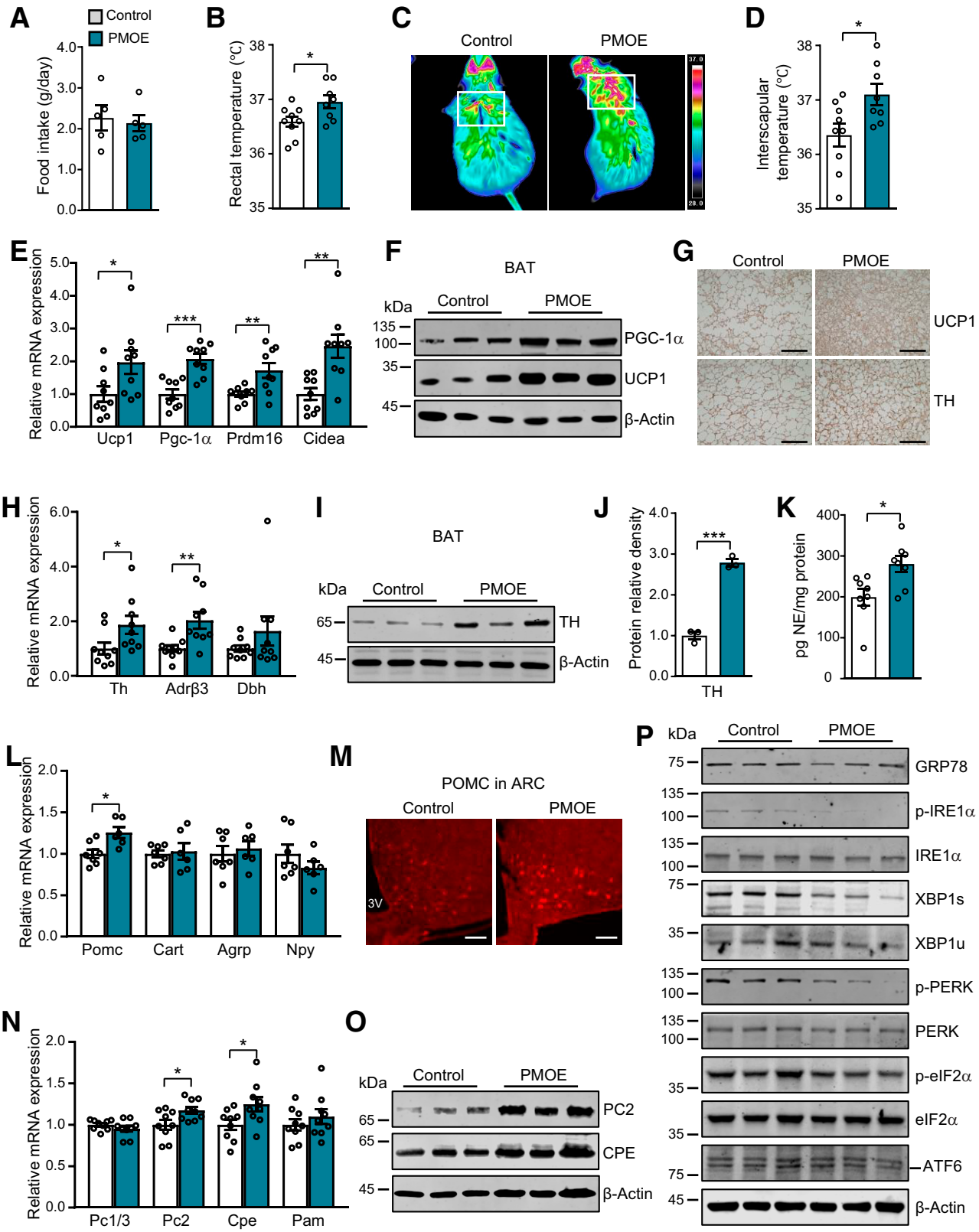

Figure 8—Overexpression of MANF in hypothalamic POMC neurons increases BAT thermogenesis and attenuates ER stress in hypothalamus. *A*: Daily food intake of control and PMOE mice (n = 5). *B*: Rectal temperature of overnight-fasted control (n = 9) and PMOE (n = 8) mice. *C* and *D*: Representative thermal images and surface temperature of the interscapular region of control (n = 9) and PMOE (n = 8) mice. *E*: Quantitative PCR analysis of the expressions of thermogenic genes in the BAT from control and PMOE mice (n = 9). *F*: Western blot analysis of UCP1 and PGC-1α protein levels in BAT. *G*: Representative immunohistochemistry images of UCP1 and TH in BAT from control and PMOE mice. Scale bar, 100 μm. *H*: Quantitative PCR analysis of the expressions of *Th*, *Adrβ3*, and *Dbh* in BAT from control

such as p-HSL and ATGL were significantly upregulated in the eWAT and iWAT from PMOE mice (Supplementary Fig. 11*E–H*).

Next, we investigated whether the induced thermogenesis of BAT in PMOE mice was mediated by the hypothalamus-SNS axis. We found that MANF overexpression in hypothalamic POMC neurons activated SNS in BAT, as shown by the markers reflecting activated SNS including the increased levels of *Th* and *Adrβ3* mRNA, TH protein, and NE content in BAT, without obvious alterations in several other sympathetic regulation–related genes (Fig. 8*G–K* and Supplementary Fig. 12*A*). In addition, both the mRNA and protein levels of POMC and PC2 were significantly upregulated in the hypothalamus of PMOE mice (Fig. 8*L–O* and Supplementary Fig. 12*B*). Moreover, the protein levels of ER stress markers, including p-IRE1α, XBP1s, p-PERK, and p-eIF2α, were significantly downregulated in the hypothalamus of PMOE mice (Fig. 8*P* and Supplementary Fig. 12*C*), which suggests that overexpression of MANF in hypothalamic POMC neurons ameliorates unresolved ER stress. Furthermore, serum leptin levels were significantly decreased in PMOE mice compared with those in control mice, and in vitro studies showed that MANF overexpression increased leptin-induced *Pomc* expression (Supplementary Fig. 12*D* and *E*).

DISCUSSION

In this study, we reveal that MANF is required for POMC neurons to modulate whole-body energy balance by loss- and gain-of function strategies. We found that MANF deficiency in POMC neurons accelerated body weight gain under both a CD and an HFD due to impaired BAT thermogenesis. In these mice, sympathetic nerve innervation and activity in BAT were decreased. In contrast, MANF overexpression in hypothalamic POMC neurons stimulated BAT thermogenesis via increasing its sympathetic innervation and activity, thus protecting the mice against diet-induced obesity and associated metabolic disorders. Mechanistically, we found that MANF is required for maintaining ER homeostasis as well as proper POMC expression and processing in the hypothalamus.

MANF is abundantly expressed in the brain, especially several hypothalamic nuclei, including the ARC (27,28,43). Previous studies showed that the expression of MANF in the hypothalamus was upregulated on fasting, and MANF overexpression in the hypothalamus caused hyperphagia and obesity, whereas MANF knockdown led to hypophagia and reduced body weight gain (27). In the current study, we showed that the *Manf* mRNA expression in the hypothalamus was significantly suppressed in diet-induced obese mice, further supporting its involvement in whole-body energy balance. However, the hypothalamus, including the ARC, contains multiple types of neural populations (6). Because the functions of different types of cells in the hypothalamus in controlling energy homeostasis are diverse and complex, revealing the specific function and corresponding mechanism of MANF in these cell populations is necessary and helpful to expand our knowledge of the onset and development of obesity. In the current study, we focus on POMC neurons and reveal the crucial roles of MANF for POMC neurons in controlling energy homeostasis. Further studies are required to reveal the effects and mechanisms of MANF in other neural populations for metabolic control.

Energy homeostasis is maintained by the balance of energy intake and expenditure. Long-term increase in food intake and/or reduction of energy expenditure can contribute to obesity and related metabolic disorders (4,44,45). In the current study, we found that deleting or overexpressing MANF in POMC neurons did not significantly alter food intake in mice, regardless of whether they were fed a CD or an HFD. In contrast, MANF deficiency reduced, whereas MANF overexpression increased, rectal and interscapular BAT temperatures. These results are in disagreement with previous observations showing that overexpression or knockdown of MANF in the hypothalamus caused hyperphagia or hypophagia, respectively, while energy expenditure remained unaffected. It is speculated that other cell types in the hypothalamus, rather than POMC neurons, are mediating the changes of energy intake by the overexpression and/or deletion of MANF in the hypothalamus, which requires further investigation.

In this study, we found that MANF deficiency in POMC neurons significantly decreased the expression levels of POMC. Interestingly, the metabolic phenotypes observed in our study are not entirely consistent with *Pomc* deficiency, the latter of which resulted in hypophagia and obesity in both humans and rodents (46,47). In contrast, we found that MANF ablation in POMC neurons reduced energy expenditure via suppressing thermogenesis in BAT, with no effect on food intake. Consideration of the possible causes of this inconsistency is complex. It is speculated that the changes in neural circuits caused by MANF deficiency in POMC neurons are not exactly the same as in *Pomc* deficiency and may, to a large extent, be only a part

and PMKO mice (n = 9). *I* and *J*: Western blot analysis and quantification of TH protein levels in BAT. *K*: ELISA analysis of NE content in BAT from control and PMOE mice (n = 8). *L*: Quantitative PCR analysis of hypothalamic neuropeptide expression in the hypothalamus of control (n = 7) and PMOE (n = 6) mice. *M*: Representative IF images of POMC in the ARC of control and PMOE mice. 3V, third ventricle. Scale bar, 100 μm. *N*: Quantitative PCR analysis of the expressions of key enzymes involved in POMC processing in the hypothalamus from control and PMOE mice (n = 9). *O*: Western blot analysis of protein levels of PC2 and CPE in the hypothalamus of mice. *P*: Western blot analysis of ER stress–responsive markers in the hypothalamus from control and PMOE mice. Data are means ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of the changes caused by *Pomc* deficiency. In addition, recent studies have unraveled that POMC neurons are heterogeneous in nature in terms of the neurotransmitters and receptors they express, and distinct POMC neuronal populations in the ARC may influence diverging downstream pathways that ultimately will affect food intake and energy expenditure (48). In our study, the ablation of MANF may have divergent effects on different subgroups of POMC neurons, which might also contribute to this inconsistency.

MANF is an ER-resident protein, and extensive studies have demonstrated its crucial role in maintaining ER homeostasis (18,21–23,40–42,49). Not surprisingly, our data showed that MANF deficiency in POMC neurons induced UPR signaling, while MANF overexpression ameliorated ER stress in the hypothalamus. Previous studies have associated ER stress with leptin resistance in the hypothalamus. For example, chemically inducing ER stress increases the expression of the negative regulators of leptin receptor signaling including *Ptp1b* and *Socs3* (50,51). Central infusion of thapsigargin, a blocker of the ER calcium importer SERCA, blocks leptin signaling in the hypothalamus (50,52). Conversely, treatment with tauroursodeoxycholic acid, a compound with chemical chaperone properties, reverses ER stress–induced blockage of leptin receptor signaling (50,52). In the current study, we showed that MANF deletion in POMC neurons induced leptin resistance, while MANF overexpression increased leptin-induced *Pomc* transcription and expression. These results implicate leptin signaling in the effects of MANF knockout or overexpression in our mouse models. Noteworthy, previous studies also linked impaired ER homeostasis with abnormal POMC posttranslational processing via decreasing PC2 (one of the critical enzymes) protein levels, whereas its mRNA expression remained unaltered (50). In the current study, we found that both the mRNA and protein levels of PC2 were regulated by the deletion or overexpression of MANF in POMC neurons, which indicates that ER stress may indirectly regulate PC2 expression. In fact, it might be an effect of regulated leptin signaling because it is reported that PC2 is regulated by leptin receptor signaling at the transcriptional level (53). Thus, our studies suggest that MANF in POMC neurons regulates ER homeostasis and leptin signaling, both of which might directly and/or indirectly be involved in the maintenance of proper POMC transcription and posttranslational processing.

MANF is known to act as both an intracellular ER response protein and an extracellular factor, but the molecular details of when and how it performs each function remain unclear. MANF overexpression in the hypothalamus was shown to trigger insulin resistance via enhancing the ER localization and activity of PIP4k2b, a negative regulator of insulin signaling (27). The same study showed that MANF is more likely to function intracellularly in the hypothalamus to regulate energy homeostasis, as no significant difference in food intake was observed when recombinant MANF was administered either into the third ventricle or directly into the hypothalamus (27). Similarly, the induction of ER stress in the hypothalamus of PMKO mice in the current study also revealed that it is more likely for MANF to act intracellularly in hypothalamic POMC neurons for controlling energy balance. However, the extracellular effects of MANF in this process could not be fully excluded, as the secreted MANF in outer space of cells could also promote ER homeostasis via undefined receptors or mechanisms (22,40). In addition, whether MANF requires additional cofactors and/or other cellular signaling pathways to control energy balance also remained unclear and should be investigated.

One limitation of our study relates to the use of mice expressing constitutive *Pomc*-Cre to selectively delete the *Manf* gene in POMC neurons under the control of the POMC promoter. Previous studies have shown that prenatal and postnatal ablation of certain neurons results in disparate metabolic phenotypes, which suggests that phenotypes caused by prenatal ablation may be influenced by developmental compensation (54). Moreover, *Pomc*-Cre induces DNA recombination during early development, so we could not exclude the potential contributions of altered POMC neuron development to the metabolic changes (55,56). Furthermore, it is noteworthy that embryonic expression of Cre recombinase may result in deletion of *Manf* gene in peripheral tissues and multiple lineages of hypothalamic neurons that also express POMC during embryonic stage but do not necessarily differentiate to POMC neurons postnatally (56). Therefore, we also cannot rule out the potential contributions of MANF deletion in other tissues or cells. To circumvent some of these issues, Cre-inducible adeno-associated viral vector or tamoxifen-inducible *Pomc*-Cre mouse models are required to achieve adult-onset deletion (54,57). These strategies contribute to a possible temporal assessment of the influence of genetic manipulations of a number of genes in POMC neurons at different developmental and adult ages.

In summary, our findings indicate that MANF in hypothalamic POMC neurons is critically involved in controlling energy balance. MANF deletion in POMC neurons promoted obesity in mice. Conversely, MANF overexpression in POMC neurons protected mice against diet-induced obesity and metabolic disorders. The mechanistic studies suggested that MANF deficiency in POMC neurons induced ER stress in the hypothalamus, impairing leptin signaling and thereby inhibiting POMC expression and processing. This ultimately decreases thermogenesis in BAT via suppressing sympathetic innervation and activity; consistently, MANF overexpression increased BAT thermogenesis via improving leptin signaling in the hypothalamus and thus increasing sympathetic innervation and activity in the BAT. Thus, our findings further support that MANF is an important molecular regulator in

metabolic control and provide insight into an additional mechanism for POMC neurons control of energy metabolism.

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Author Contributions. Q.T. and J.H. developed the study rationale and wrote the manuscript. Q.T. and Q.L. designed and performed most of the experiments. J.L., J.Y., X.J., J.Z., Y.Xi., Y.Xu., and Y.L. performed the experiments and assisted with data analysis. J.H. is 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.

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