Hypothalamic Angptl4/Fiaf Is a Novel Regulator of Food Intake and Body Weight

Hyun-Kyong Kim,[1] Byung-Soo Youn,[2] Mi-Seon Shin,[3] Churl Namkoong,[1] Kyeong Han Park,[4] Ja Hyun Baik,[5] Jae Bum Kim,[6] Joong-Yeol Park,[3] Ki-up Lee,[3] Young-Bum Kim,[7] and Min-Seon Kim[3]

OBJECTIVE—The angiopoietin-like protein 4 (Angptl4)/fasting-induced adipose factor (Fiaf) is known as a regulator of peripheral lipid and glucose metabolism. In the present study, we investigated the physiological role of Angptl4 in central regulation of body weight homeostasis.

RESEARCH DESIGN AND METHODS—Hypothalamic Angptl4 expression levels were measured using immunoblot assay during feeding manipulation or after administration of leptin, insulin, and nutrients. The effects of Angptl4 on food intake, body weight, and energy expenditure were determined following intracerebroventricular (ICV) administration of Angptl4 in C57BL/6 mice. Food intake, energy metabolism, and feeding responses to leptin, insulin, and nutrients were compared between Angptl4-null mice and their wild littermates. Finally, the relationship of hypothalamic AMP-activated protein kinase (AMPK) and Angptl4 was studied.

RESULTS—Hypothalamic Angptl4 expression levels were increased upon food intake or administration of leptin, insulin, and nutrients. Furthermore, central administration of Angptl4 suppressed food intake and body weight gain but enhanced energy expenditure. These effects were mediated via suppression of hypothalamic AMPK activities. Consistently, Angptl4-null mice displayed increased body weight and hypothalamic AMPK activity but reduced energy expenditure. Food intake following a fast was significantly greater in Angptl4-null mice, which was normalized by centrally administered Angptl4. Moreover, anorectic responses to leptin, insulin, and glucose were diminished in Angptl4-null mice. In contrast, Angptl4-null mice were resistant to diet-induced obesity, indicating obesity-promoting effects of Angptl4 under the condition of fat-enriched diet.

CONCLUSIONS—We have demonstrated that hypothalamic Angptl4 is regulated by physiological appetite regulators and mediates their anorexigenic effects via inhibition of hypothalamic AMPK activity. Therefore, Angptl4 appears to have an important role in central regulation of energy metabolism. ***Diabetes*** **59:2772–2780, 2010**

From the [1]Asan Institute for Life Sciences, University of Ulsan College of Medicine, Seoul, Korea; [2]AdipoGen Inc., Incheon, Korea; the [3]Department of Internal Medicine, Asan Medical Center, Seoul, Korea; the [4]Department of Anatomy, Kangwon National University School of Medicine, Chuncheon, Korea; the [5]College of Life Sciences and Biotechnology, Korea University, Seoul, Korea; the [6]Department of Biophysics and Chemical Biology, School of Biological Sciences, Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Korea; and the [7]Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts.

Corresponding author: Min-Seon Kim, mskim@amc.seoul.kr.

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H.-K.K. and B.-S.Y. contributed equally to this work.

The past 20 years have witnessed a dramatic increase in the obese population worldwide (1). The hypothalamus is considered a key player in the regulation of body weight (2), although hypothalamic dysfunction may occur in chronic energy excess state. Information on energy intake and adiposity is relayed to hypothalamic neurons and coordinated to eventually alter feeding behavior and energy metabolism (3,4). In addition to the effectors known to be implicated in this process (5), novel factors may be involved.

Angiopoietin-like proteins (Angpts) are a family of proteins with structures similar to those of angiopoietins, NH_2-terminal coiled coil domain (CCD), and COOH-terminal fibrinogen-like domain (FLD) (6,7). Angiopoietins control angiogenesis and vascular integrity via the Tie tyrosine kinase receptors (8,9). Despite the structural similarities between angiopoietins and Angptls, the latter do not bind Tie receptors and have distinct biological functions (6,10,11). Angptl3 is an important regulator of circulating lipid concentrations (12), whereas Angptl6 is implicated in body weight metabolism (10). A recent study has shown that Angptl2 induces inflammation and insulin resistance in adipose tissues from obese animals (13). These findings collectively indicate that Angptls are active players in a variety of metabolic processes.

Angptl4/fast-induced adipose factor (Fiaf) is a ~50 kDa glycoprotein originally identified as a target gene of the nuclear receptors peroxisome proliferator–activated receptor (PPAR)-α and PPAR-γ (7,11). Like Angptl3, Angptl4 is an important regulator of triglyceride metabolism (14). Additionally, hepatic overexpression of Angptl4 improves glucose tolerance despite promotion of hepatic fat accumulation and hyperlipidemia (15). Fat-specific Angptl4 transgenic mice display a lean phenotype along with enhanced fatty acid oxidation and lipolysis (16). Furthermore, intestinal Angptl4 mediates antiobesity effects induced by germ-free conditions (17). These data suggest that Angptl4 functions as an important regulator of peripheral energy metabolism. However, the potential role of Angptl4 in central regulation of metabolism has yet to be established.

In the present study, we describe critical evidence showing that hypothalamic Angptl4 is an important player in the regulation of body weight homeostasis.

RESEARCH DESIGN AND METHODS

Peptide synthesis. Mouse Angptl4 cDNA was amplified from the mouse liver cDNA library by PCR using the following primer set: forward primer, 5′-cctagctagccagaatcatgcgctgcgctc, and reverse primer, 5′-gccgctcgagctaagag gctgctgtagcct. Amplified DNA was cloned into the pAGCF vector (AdipoGen, Incheon, Korea) and transfected into Cos7 cells using Hyperfect (AdipoGen).

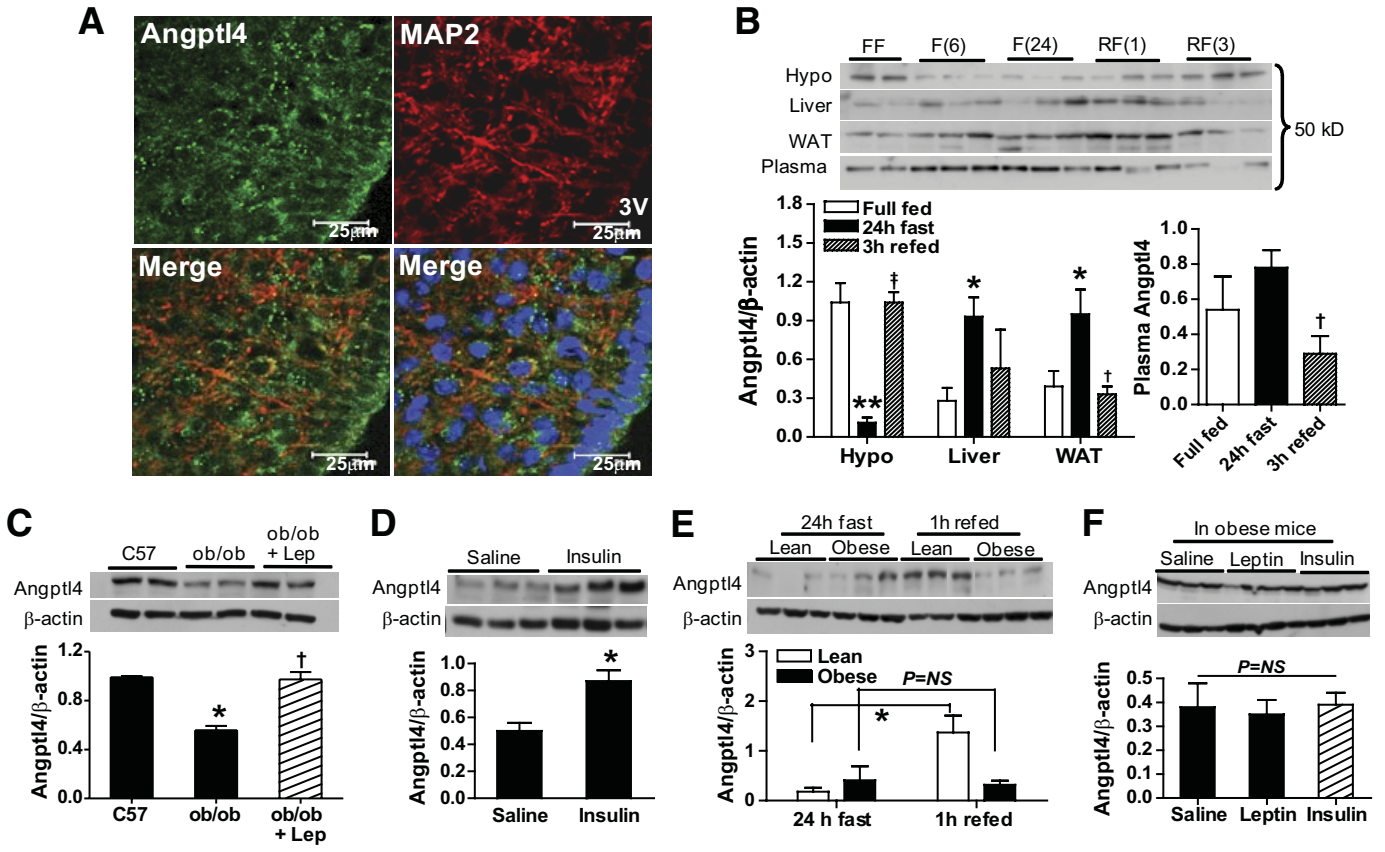

FIG. 1. Expression and regulation of hypothalamic Angptl4. *A*: Double immunofluorescence staining of Angptl4 and neuron marker MAP2 in the hypothalamic arcuate nucleus from normal mice. *B*: Changes in protein expression levels of full-length Angptl4 in the hypothalamus (Hypo), liver, white adipose tissue (WAT), and plasma during the fasting (F) and refeeding (RF) periods (n = 5–6). *P < 0.05 and **P < 0.005 vs. the full-fed (FF) group. †P < 0.05 and ‡P < 0.005 vs. the 24-h fasted group. *C*: Hypothalamic Angptl4 expression in leptin-deficient *ob/ob* mice with or without leptin treatment (n = 5–6). *P < 0.01 vs. C57BL/6 mice. †P < 0.05 vs. *ob/ob* mice with no leptin treatment. *D*: Effect of intracerebroventricular administration of insulin on hypothalamic Angptl4 expression (n = 4). *P < 0.01 vs. saline-injected control. *E* and *F*: Changes in hypothalamic Angptl4 expression following food intake or ICV administration of 3 μg leptin and 3 mU insulin in HFD-fed obese mice. *P < 0.01 between indicated groups. Data are presented as means ± SEM. NS, not significant. (A high-quality digital representation of this figure is available in the online issue.)

Based upon the SMART search, each CCD and FLD of Angptl4 were assigned, amplified, and cloned into expression vectors described as the above. For the amplification of CCD and FLD, the following primer sets were used: for CCD amplification, forward primer 5′-cctagctagccagaatcatgcgctgcgctc and reverse primer 5′-cccgctcgagcttgtctactccattgtccag, and for FLD amplification, forward primer 5′-ctagctagcaagaagctttccaagatgacc and reverse primer 5′-cgcggatc cctaagaggctgctgtagcct. Culture supernatant fractions were collected and concentrated through ultrafiltration. FLAG-tagged mouse Angptl4 was purified using anti-FLAG affinity column chromatography. Endotoxin was removed with two consecutive column chromatography cycles using Detoxigel (Pierce, Rockford, IL).

Animals. Adult male C57BL/6 and *ob/ob* mice were obtained from Japan SLC (Hamamatsu, Japan). *Angptl4*$^{-/-}$ mice on a C57BL/6J genetic background were generated as previously described (17) and kindly presented by Dr. Andras Nagy. Mice were fed a standard chow diet (Samyang, Seoul, Korea) ad libitum unless otherwise indicated. For feeding manipulation, mice were starved overnight and refed for 4 h (fully fed state), followed by fasting and refeeding for the indicated times. To generate diet-induced obesity (DIO), mice were fed a high-fat diet (HFD) (60% fat, Research Diet) for 8 weeks. Lean control mice were fed a low-fat diet (16% fat) over the same period. Animals were housed under controlled temperature (23 ± 1°C) conditions and subjected to a 12-h light-dark cycle, with light from 0700 to 1900 h. All procedures were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences.

Cell culture. Primary hypothalamic neuron cultures were prepared from fetal rat brains obtained on embryonic day 18 as previously described (18). Differentiation was induced by culturing cells in neurobasal medium (Invitrogen) supplemented with 5 μg/ml insulin, 100 μ/ml iron-free human transferrin, 100 μmol/l putrescin, 30 nmol/l sodium selenite, and 20 nmol/l progesterone for 7–8 days.

Cannulation and injection. Stainless steel cannulae (26 gauge; Plastics One) were implanted into the third cerebral ventricle (ICV) of mice as previously described (19). Following a 7-day recovery period, the correct positioning of each cannula was confirmed by a positive dipsogenic response to angiotensin-2 (50 ng/mouse). Angptl4, insulin, glucose, leptin, and 5′-aminoimidazole-4-carboxyamide ribonucleoside (AICAR) were dissolved in 0.9% saline, as well as 5-tetradecyloxy-2-furoic acid (TOFA) (Yuhan Research Institute, Seoul, Korea) dissolved in 100% DMSO, and administered via ICV injection in a total volume of 2.5 μl, respectively. The majority of feeding studies were performed in the early light phase in animals subjected to overnight fasting. Food intake and body weight were monitored for 24 h postinjection.

Energy expenditure and locomotor activity. Energy expenditure was measured using an Oxymax apparatus (Columbus Instruments) over 24 h after a 3-day acclimatization period. The behavioral test was performed between 1400 and 1800 h. Mice were individually housed and allowed to acclimatize for 1 h in individual transfer cages to the behavioral testing room. Locomotor activity was evaluated using an Activity Monitor (MED Associates), comprising an open-field chamber (43.2 × 43.2 × 30.5 cm) containing 16 × 16 photocells for measuring horizontal movements. Locomotor activity was measured as the total distance traveled for 60 min.

Immunoblotting. Animals were killed by decapitation 2 h following the administration of Angptl4, leptin, insulin, and nutrients. After injection, mice were fasted until they were killed. The medial part of the hypothalamus was dissected in the anterior border of optic chiasm, posterior border of the mammillary body, upper border of anterior commissure, and lateral border halfway from the lateral sulcus in the ventral side of brain. Immunoblot analysis was conducted as described earlier (20). Tissue lysates (30- to 50-μg samples) were separated by 8% SDS-PAGE and transferred to a polyvinylidine fluoride membrane. For immunoblot assay of plasma Angptl4, plasma was diluted with lysate buffer (1:500) and 5 ul of diluted plasma was loaded.

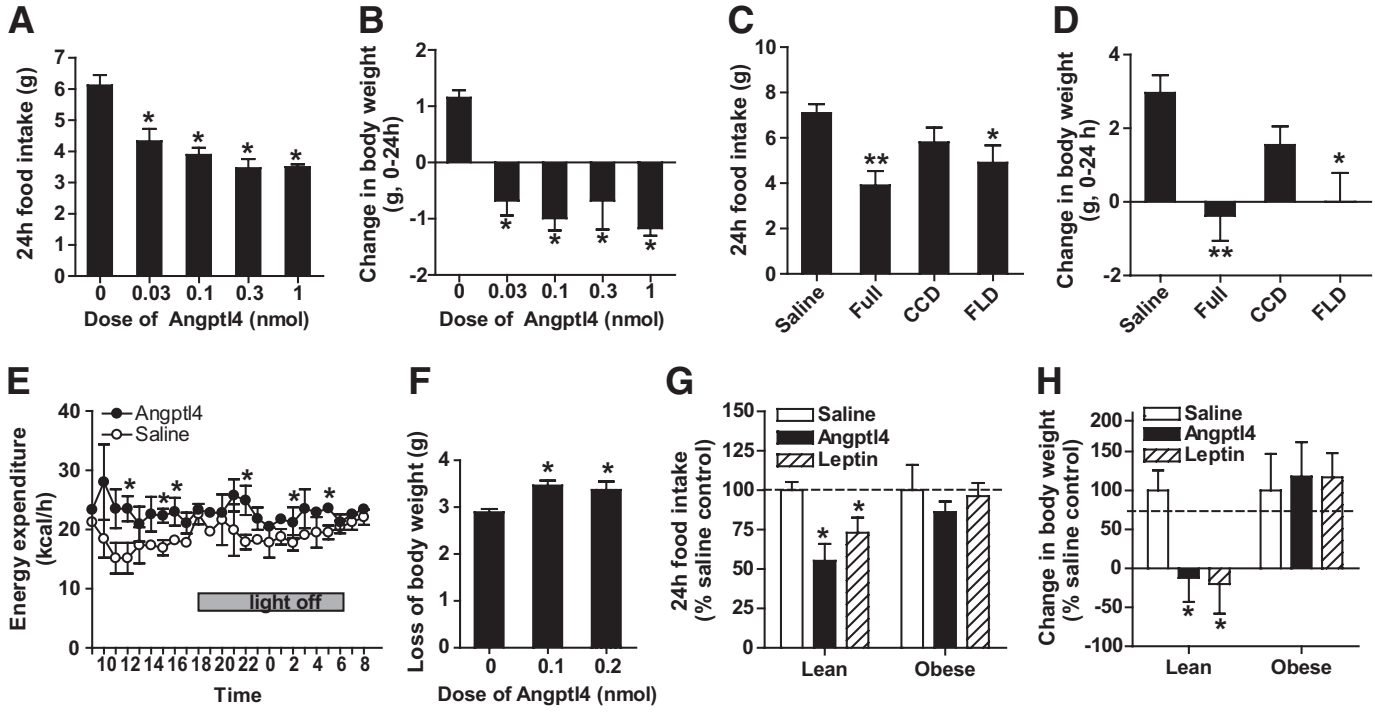

FIG. 2. Effects of Angptl4 on food intake, body weight, and energy expenditure. *A* and *B*: ICV injection of full-length mouse Angptl4 led to decreased food intake and body weight for 24 h postinjection (n = 6–7). *P < 0.005 vs. saline-injected controls. *C* and *D*: Comparison of the effects of Angptl4 full-length, FLD, and CCD fragments on food intake and body weight (n = 5–6). *P < 0.05 and **P < 0.01 vs. saline-injected controls. *E*: Energy expenditure over a 24-h period following ICV injection of saline or 0.1 nmol Angptl4. *P < 0.05 vs. saline-injected controls. *F*: ICV injection of 0.1 nmol Angptl4 caused a greater weight loss even in the fasted condition (n = 8–9). *P < 0.05 vs. saline-injected controls. *G* and *H*: The inhibitory effects of leptin and Angptl4 on food intake and body weight were blunted in obese mice (n = 5–6). *P < 0.05 vs. saline-injected controls. Data are presented as means ± SEM.

Following incubation in blocking buffer, membranes were incubated overnight at 4°C with antibodies against Angptl4 (1:1,500, rabbit; AdipoGen), phosphorylated (Thr 172), and total forms of AMP-activated protein kinase (AMPK) or phosphorylated (Ser 79) and total acetyl CoA carboxylase (ACC) (Cell Signaling). Blots were developed using horseradish peroxidase–linked anti-rabbit secondary antibody and the chemiluminescent detection system (Perkin Elmer). Band density was measured with a densitometer (VersaDoc Multi Imaging Analyzer System; BIO-RAD) and corrected based on the density of β-actin or total forms of AMPK and ACC.

Immunofluorescence. C57BL/6 mice were perfused with 4% paraformaldehyde through the heart under anesthesia for 15 min following a 5-h fast. Following postfixation and the dehydration process, coronal brain sections (15 μm thick) were obtained using cryostat (Leica,Wetzlar, Germany) and incubated with primary antibodies against Angptl4 (1:200, goat; Santa Cruz) and microtubule-associated protein 2 (MAP2) (1:800, mouse; Sigma) or glial fibrillary acid protein (GFAP) (1:300, mouse; Sigma) at 4°C for 48 h. After washing, slides were incubated with Alexa-Fluor 546–conjugated donkey anti-goat antibody and Alexa-Fluor 488–conjugated donkey anti-mouse antibodies (Invitrogen) at room temperature for 1 h. For nuclear staining, slides were treated with DAPI (1:20,000; Invitrogen) for 10 min before mounting. Triple immunofluorescence was examined using confocal microscopy (Leica). The specificity of Angptl4 antibody was tested in *Angptl4*$^{-/-}$ mice (data not shown).

Measurement of AMPK activity. Medial hypothalamus and soleus muscle was lysed with digitonin buffer. Tissue lysate (40 μg) was immunoprecipitated by incubation with specific antibodies against the α1- and α2-AMPK catalytic subunits (Cell signaling) and 20 μl of 25% (wt/vol) protein G-sepharose beads (Santa Cruz Biotechnology) overnight at 4°C. AMPK activity was measured using a modified method of Davies et al. *(21)*.

Statistical analysis. Data are presented as means ± SEM. Statistical analysis was performed using SPSS-PC14. The statistical significance among the groups was tested using one-way ANOVA followed by a post hoc LSD test or an unpaired Student's t test where appropriate. Repeated ANOVA was employed for analysis of body weight data from *Angptl4*$^{-/-}$ mice experiments. Two-way ANOVA was used to analyze the effects of leptin, insulin, and glucose in *Angptl4*$^{-/-}$ mice and ICV Angptl4 in DIO mice. Significance was defined as $P < 0.05$.

RESULTS

Hypothalamic Angptl4 expression is regulated by hormones and nutrients. We firstly investigated Angptl4 expression in the brain from C57BL/6 normal mice. Angptl4 was ubiquitously expressed in hypothalamus, cerebrum, and cerebellum (supplementary Fig. 1, available in the online appendix [http://diabetes.diabetesjournals.org/cgi/content/full/db10-0145/DC1]). Double immunofluorescence staining revealed that Angptl4 was coexpressed with neuronal marker MAP2 but not with glial marker glial fibrillary acid protein in the hypothalamic arcuate nucleus (Fig. 1*A* and supplementary Fig. 2). Moreover, Angptl4 was secreted by primary cultured hypothalamic neurons and SH-SY5Y neuron cells (supplementary Fig. 3). Taken together, Angptl4 secreted from the hypothalamic neurons may function in an autocrine and paracrine manner, although Angptl4 receptors remained yet to be cloned.

We next investigated whether Angptl4 expression levels in the medial hypothalamus were affected by nutritional status. Several studies have shown that fasting and food restriction lead to elevated Angptl4 expression levels in the plasma, adipose tissue, and liver (7,11,22). Consistently, full-length Angptl4 protein levels in the liver, white adipose tissue, and plasma were increased upon starvation of normal mice and decreased following food intake (Fig. 1*B*). In contrast, hypothalamic Angptl4 expression was decreased during a fast and increased following food intake and oral ingestion of glucose, protein, and lipid (Fig. 1*B* and supplementary Fig. 4). Thus, Angptl4 expression in central and peripheral tissues was differentially regulated by fuel supply. On the other hand, we observed no remarkable differences in truncated Angptl4 expression

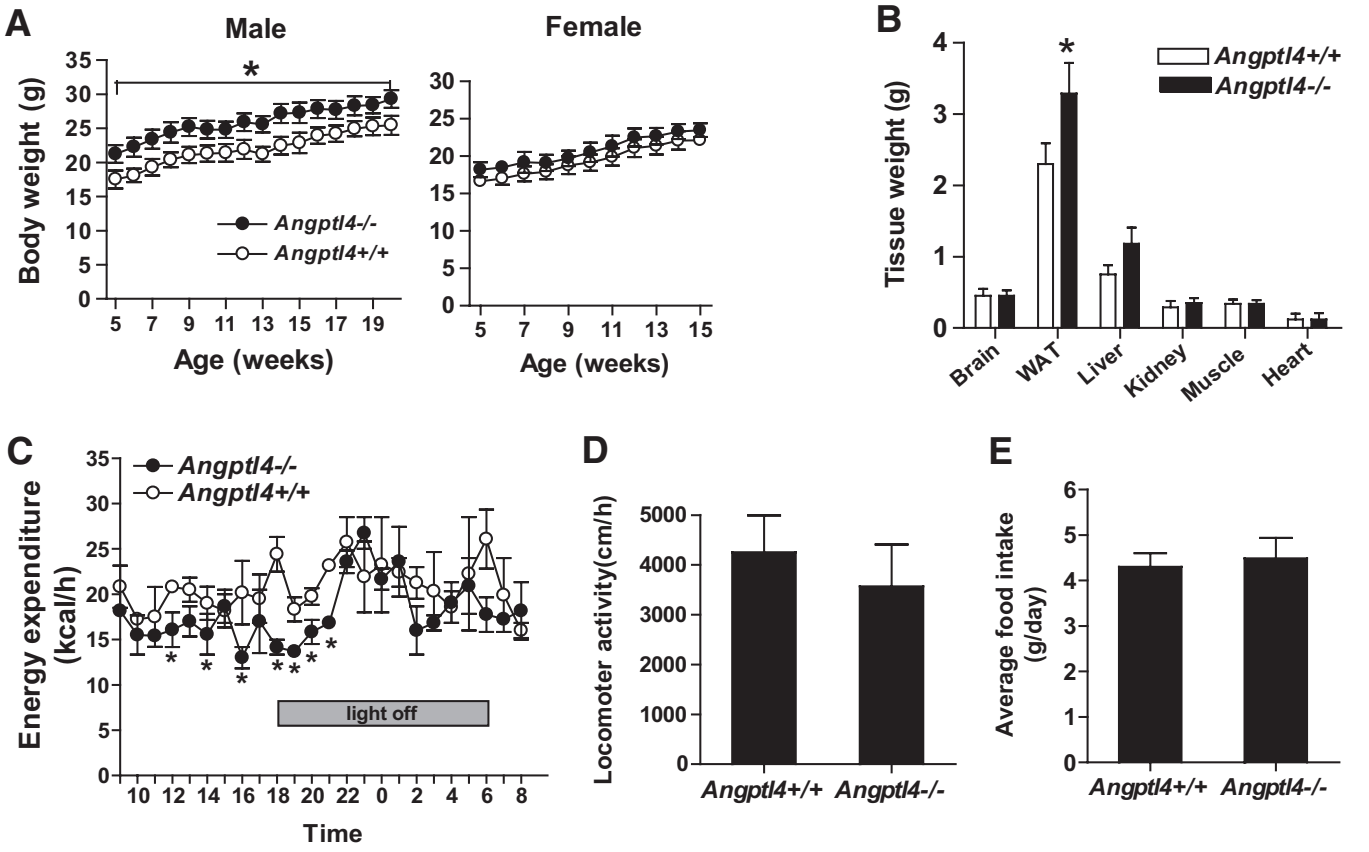

FIG. 3. Metabolic phenotypes of Angptl4-deficient mice. *A* and *B*: Increased body weight and fat mass in *Angptl4*$^{-/-}$ male mice ($n = 5$–6). *A*: *left panel*, males; *right panel*, females. *$P < 0.05$ vs. wild-type littermates. *C* and *D*: Reduced energy expenditure and locomotor activity in *Angptl4*$^{-/-}$ male mice aged 20 weeks ($n = 4$). *$P < 0.05$ vs. *Angptl4*$^{+/+}$ mice. *E*: Food intakes in the freely fed state in *Angptl4*$^{-/-}$ mice.

levels in response to fasting and refeeding in the hypothalamus (data not shown).

Leptin, a potent anorexigenic hormone, suppressed Angptl4 expression in the adipose tissue (7). By contrast, hypothalamic Angptl4 expression was reduced in leptin-deficient (*ob/ob*) mice and was increased by leptin treatment (1 mg · kg^{-1} · day^{-1} for 3 days via intraperitoneal injection) (Fig. 1*C*). In normal mice, insulin (3 mU) and leptin (3 μg) elevated hypothalamic Angptl4 expression levels when administered intracerebroventricularly following an overnight fast (Fig. 1*D* and supplementary Fig. 5). Thus, it appeared that hypothalamic Angptl4 expression was upregulated by the appetite suppressing-hormones leptin and insulin. Feeding-, leptin-, and insulin-induced increases in hypothalamic Angptl4 levels were significantly blunted in HFD-fed obese mice (Fig. 1*E* and *F*), suggesting that the normal regulatory processes affecting hypothalamic Angptl4 levels were disrupted in obese animals.

Central administration of Angptl4 causes anorexia and weight loss. To gather insight into the role for hypothalamic Angptl4 in body energy homeostasis, we administered full-length mouse Angptl4 (0.03–1 nmol) via ICV cannulae to overnight-fasted mice. A single ICV administration of Angptl4 led to potent suppression of food intake and body weight gain during the 24-h postinjection period (Fig. 2*A* and *B*). Intraperitoneal administration of large amounts of Angptl4 (1 and 3 mg/kg) caused a modest and short-lasting reduction in food intake and body weight (supplementary Fig. 6). Thus, Angptl4 seems to regulate body weight metabolism mainly through central mechanisms.

To identify the domain of Angptl4 responsible for the central metabolic actions, we generated the NH_2-terminal CCD and COOH-terminal FLD fragments of mouse Angptl4. Angptl4 full length, CCD, and FLD (0.1 nmol each) were administered in fasted mice to compare their effects on food intake and body weight. ICV administration of FLD, but not CCD, caused a significant reduction in food intake and body weight (Fig. 2*C* and *D*), indicating that Angprl4-induced anorexia and weight loss may be mediated via FLD.

We additionally tested whether hypothalamic Angptl4 was involved in central regulation of energy metabolism. For this, we monitored energy expenditure for a 24-h period following ICV injection of Angptl4 (0.1 nmol). Notably, energy expenditure was increased by ICV injection of Angptl4; the effect lasted over 24 h postinjection (Fig. 2*E*). In addition, ICV Angptl4 caused a greater weight loss compared with ICV saline even in the fasted condition (Fig. 2*F*), suggesting that enhanced energy expenditure may contribute to Angptl4-induced reduction in body weight. These findings collectively imply that central administration of Angptl4 triggers a negative energy balance by suppressing energy intake and also by stimulating energy expenditure.

Obese animals have defects in production of satiety signals in the hypothalamus. To test whether abnormal response to Angptl4 may be associated with this defect, we compared the feeding responses to Angptl4 and leptin

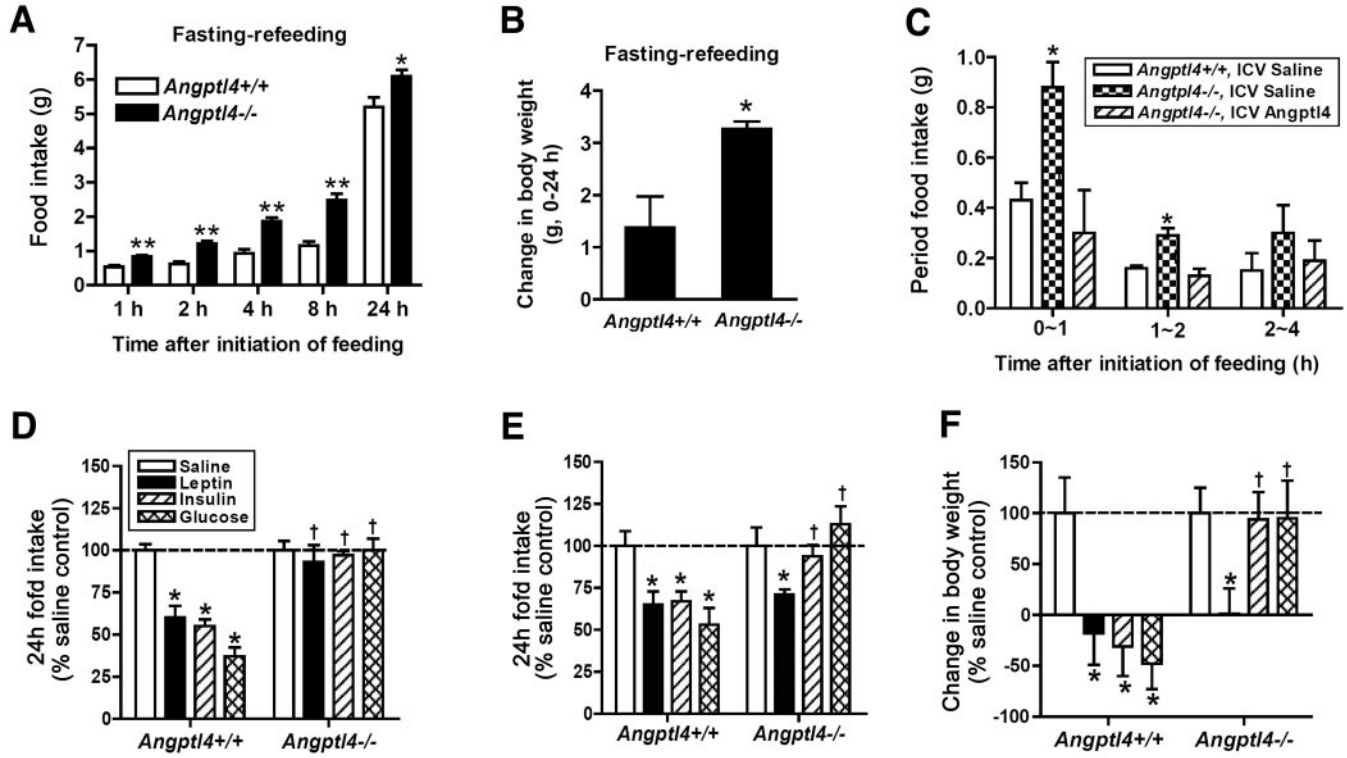

FIG. 4. Decreased satiety signals in Angptl4-deficient mice. *A* and *B*: Food intake and body weight gain were greater in *Angptl4*$^{-/-}$ mice over a 24-h refeeding period. *$P < 0.05$ and **$P < 0.005$ vs. *Angptl4*$^{+/+}$ littermates. *C*: ICV injection of Angptl4 before reestablishment of food intake suppressed fast-induced hyperphagia in *Angptl4*$^{-/-}$ mice. *$P < 0.05$ vs. saline-injected *Angptl4*$^{+/+}$ mice. †$P < 0.05$ vs. saline-injected *Angptl*$^{-/-}$mice. *D–F*: Effects of ICV administration of leptin, insulin, and glucose on food intake and body weight in *Angptl4*$^{-/-}$ mice aged 8–12 weeks (n = 5–6). *$P < 0.05$ vs. saline-injected groups. †$P < 0.05$ vs. *Angptl4*$^{+/+}$ mice injected with the same agents. Data are presented as means ± SEM.

in lean and DIO mice. Angptl4 (0.1 nmol ICV) and leptin (3 μg ICV) induced anorexia and weight loss in lean mice, which was significantly attenuated in DIO mice (Fig. 2*G* and *H*). Based on these findings, we propose that impaired hypothalamic Angptl4 activity may contribute to the pathogenesis of obesity. We additionally tested the relationship between leptin and Angptl4 in feeding regulation. The anorexigenic effects of Angptl4 (0.1 nmol) were well preserved in leptin-deficient (*ob/ob*) and leptin receptor–deficient (*db/db*) mice (supplementary Fig. 7). These findings suggest that leptin and leptin receptors are not critical for Angptl4-caused anorexia.

Angptl4-null mice develop obese phenotypes. To further explore the function of Angptl4 in body weight homeostasis, we determined body weight changes in *Angptl4*$^{-/-}$ mice. In general, *Angptl4*$^{-/-}$ male mice were modestly heavier than their wild (*Angptl4*$^{+/+}$) littermates (Fig. 3*A*). Increased body weight was not due to accelerated growth because body lengths did not differ between wild and knockout mice (supplementary Fig. 8). Consistently, epididymal fat pad weight was greater in *Angptl4*$^{-/-}$ male mice at 20 weeks of age (Fig. 3*B*). However, limited differences in body weight were observed between female *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ mice (Fig. 3*A*), suggesting that Angptl4-null phenotypes were affected by sex.

To establish the mechanisms underlying increased body weight of *Angptl4*$^{-/-}$ mice, we assessed energy metabolism, locomotor activity, and food intake. *Angptl4*$^{-/-}$ male mice displayed lower energy expenditure, especially during the light-on period, and a tendency toward decreased locomotor activities (Fig. 3*C* and *D*). Food consumption when food was freely available was not significantly altered in *Angptl4*$^{-/-}$ mice (Fig. 3*E*). However, marked hyperphagia and greater weight gain were observed when *Angptl4*$^{-/-}$ mice resumed food intake following an overnight fast (Fig. 4*A* and *B*). These findings raise the possibility that lack of hypothalamic Angptl4 causes aberrant hypothalamic signaling of satiety. This hypothesis is supported by the finding that central administration of Angptl4 (0.1 nmol) completely reversed fast-induced hyperphagia in *Angptl4*$^{-/-}$ mice (Fig. 4*C*).

We further determined whether feeding responses to leptin, insulin, or glucose were impaired in *Angptl4*$^{-/-}$ mice. The early anorexigenic effects of leptin (3 μg), insulin (three mU), and glucose (0.75 mg) were attenuated in *Angptl4*$^{-/-}$ mice (Fig. 4*D*). The late anorexigenic and weight-reducing effects of insulin and glucose were also diminished in *Angptl4*$^{-/-}$ mice, whereas leptin-induced delayed anorexia and weight loss were preserved (Fig. 4*E* and *F*). These results, in conjunction with hypothalamic Angptl4 expression data, indicate that hypothalamic Angptl4 mediates the effects of insulin, leptin, and glucose on food intake and body weight. However, the delayed effects of leptin occur via an Angptl4-independent mechanism.

Angptl4 inhibits hypothalamic AMPK activity. AMPK is a serine-threonine kinase that plays a key role in the recovery from energy-depleted conditions (23). AMPK is expressed in hypothalamic neurons and functions as an important signaling molecule mediating the feeding effects of leptin, insulin, glucose, melanocortins, and α-lipoic acid (24–26). Accordingly, we examined whether hypothalamic AMPK may serve as a downstream signaling pathway of Angptl4.

ICV injection of 0.1 nmol Angptl4 decreased hypotha-

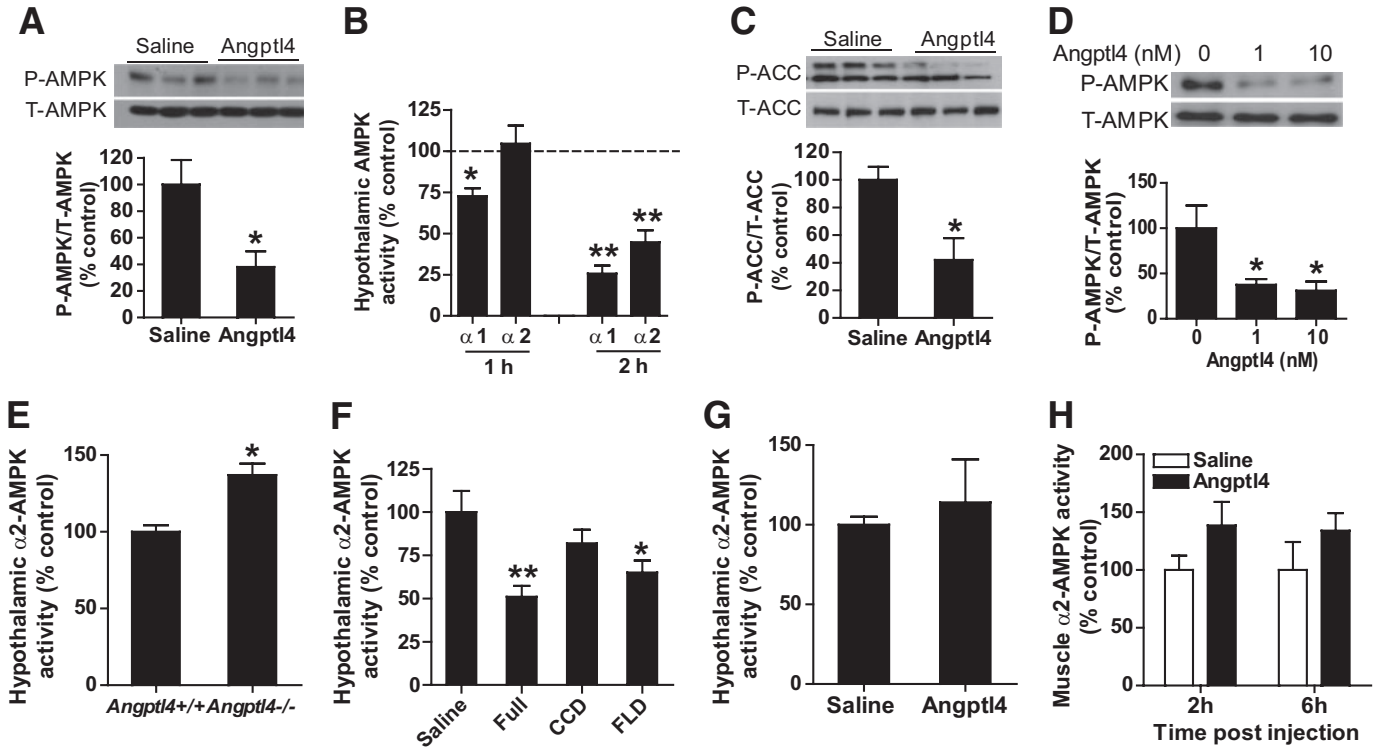

FIG. 5. Suppressive effects of Angptl4 on hypothalamic AMPK activity. *A–C*: ICV administration of 0.1 nmol Angptl4 decreased AMPK, ACC phosphorylation, and α1- and α2-specific AMPK activities in the medial hypothalamus at 2 h postinjection ($n = 5$). *$P < 0.05$ and **$P < 0.005$ vs. saline-injected controls. *D*: Effect of Angptl4 on AMPK phosphorylation in primary cultured hypothalamic neurons. $P < 0.05$ vs. untreated controls. *E*: Hypothalamic α2-AMPK activities in 24-week-old *Angptl4*$^{-/-}$ mice ($n = 4$). *$P < 0.05$ vs. *Angptl4*$^{+/+}$ littermates. *F*: Effects of full-length, CCD, and FLD fragments of Angptl4 on hypothalamic AMPK activities. Hypothalamus was collected at 2 h postinjection ($n = 5–6$). *$P < 0.05$ and **$P < 0.01$ vs. saline. *G*: ICV injection of 0.1 nmol Angptl4 did not alter hypothalamic AMPK activity in DIO mice ($n = 4–5$). *(H)* Effect of ICV injection of 0.1 nmol Angptl4 on α2-AMPK activities in soleus muscle. Muscle was collected 2 h and 6 h post–ICV injection ($n = 4–5$).

lamic AMPK phosphorylation, an index of AMPK activation, in overnight-fasted mice (Fig. 5*A*). Hypothalamic α1 and α2 isoform-specific AMPK activities were also reduced by 74 and 55%, respectively, following Angptl4 administration (Fig. 5*B*). In parallel, ICV Angptl4 suppressed hypothalamic levels of phosphorylated ACC, a well-known downstream target of AMPK (Fig. 5*C*), which would result in increased ACC activity. In cultured hypothalamic neurons, treatment with Angptl4 also suppressed AMPK phosphorylation (Fig. 5*D*). Consistently, *Angptl4*$^{-/-}$ mice had higher hypothalamic AMPK activity compared with their wild littermates (Fig. 5*E*), confirming the inhibitory actions of Angptl4 on hypothalamic AMPK.

Similarly to feeding effects, FLD fragment had a suppressive effect on hypothalamic AMPK activity, while CCD had no effect (Fig. 5*F*). Moreover, Angptl4-induced reduction in hypothalamic AMPK activity was attenuated in DIO mice (Fig. 5*G*). This may provide a molecular mechanism via which DIO mice were resistant to exogenous Angptl4 (Fig. 2*H*). Interestingly, central administration of Angptl4 (0.1 nmol) tended to increase α2-AMPK activity in skeletal muscle at 2 h and 6 h postinjection (Fig. 5*H*). Thus, central administration of Angptl4 appears to have an opposing effect on hypothalamic and muscle AMPK. Angptl4-derived increase in skeletal muscle AMPK activity may stimulate fatty acid oxidation (27), leading to increased energy expenditure as seen in Fig. 2*E*.

Hypothalamic AMPK mediates the anorexigenic effect of Angptl4. Finally, we investigated whether Angptl4-induced AMPK inhibition is essential for the anorexigenic effects of Angptl4. For this, we injected the AMPK activator AICAR (30 pmol) 1 h prior to 0.1 nmol Angptl4 injection via ICV cannulae in overnight-fasted mice. The AICAR dose was selected based on our preliminary study showing no significant increase in food intake or body weight at this concentration. As expected, ICV AICAR alone did not alter food intake or body weight. However, coadministration of AICAR prevented Angptl4-induced anorexia and weight loss, confirming an important role for hypothalamic AMPK as a downstream signaling molecule for Angptl4 (Fig. 6*A* and *B*).

In hypothalamic neurons, decreased AMPK activity leads to elevated malonyl CoA levels through ACC activation (28). Accumulating evidence has shown that increased hypothalamic malonyl CoA levels are associated with anorexia (29,30). In our study, the ICV administration of ACC inhibitor TOFA (2 μg), which would reduce malonyl CoA levels, blocked the anorexigenic and weight-reducing effects of Angptl4 (Fig. 4*C* and *D*). These data confirm the hypothesis that elevation in hypothalamic malonyl CoA levels is important for the anorexigenic actions of many appetite regulators including Angptl4 (Fig. 4*E*).

DISCUSSION

Our findings firstly demonstrate that hypothalamic Angptl4 is engaged in the regulation of feeding behavior and body weight metabolism. The role for hypothalamic Angptl4 in feeding regulation is further supported by the finding that ICV administration of Angptl4 corrects the impaired satiety generation in Angptl4-deficient mice. Previous studies

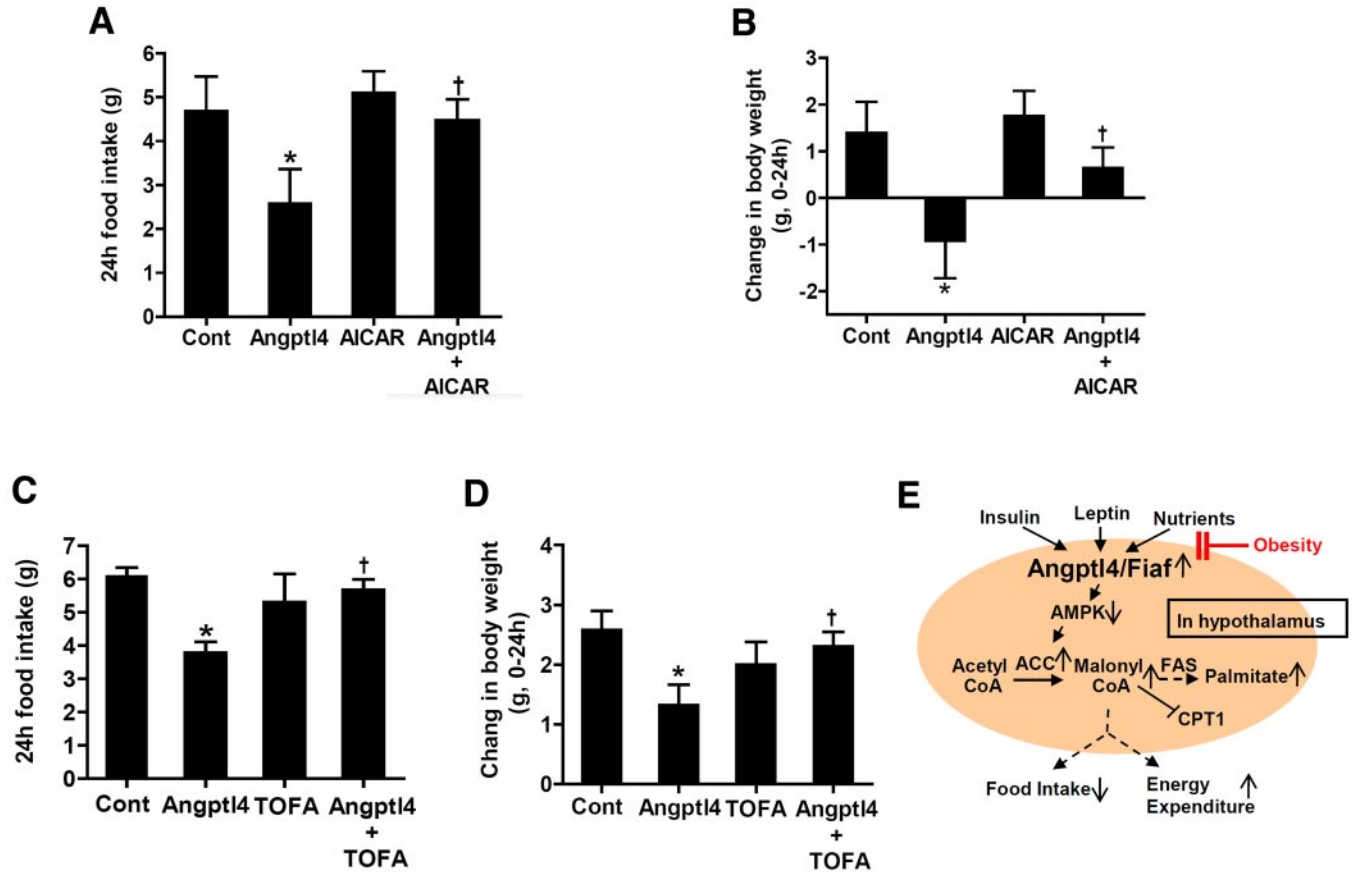

FIG. 6. Hypothalamic AMPK mediates the central actions of Angptl4. *A-D*: Prior ICV administration of the AMPK activator, AICAR, and ACC inhibitor TOFA blocked the effects of Angptl4 on food intake and body weight ($n = 6$). *$P < 0.05$ vs. saline-injected controls. †$P < 0.05$ vs. Angptl4 alone–treated group. Data are presented as means ± SEM. *E*: Diagram summarizing the findings of the study. Hypothalamus Angptl4 is upregulated by leptin, insulin, and nutrients, which is impaired in obese mice. Increased hypothalamic Angptl4 causes anorexia and weight loss through regulation of hypothalamic AMPK and ACC activities. (A high-quality color representation of this figure is available in the online issue.)

showed that Angptl4 regulates adiposity through peripheral lipid metabolism– and gut microbiota–associated mechanisms (16,17). Thus, we propose that Angptl4 governs adiposity through both central and peripheral mechanisms.

In the hypothalamus, Angptl4 appears to act as a common downstream mediator of physiological anorexigenic factors such as insulin, leptin, and glucose. This notion is evidenced by the fact that administration of insulin, leptin, or glucose increased hypothalamic Angptl4 expression levels. Furthermore, anorectic responses to insulin, leptin, and glucose were reduced in Angptl4-deficient mice. However, the delayed effects of leptin on food intake and body weight were well preserved in Angptl4-null mice, suggesting that Angptl4 is not involved in the late actions of leptin.

Notably, hypothalamic Angptl4 expression patterns regulated to food availability were reciprocal to those in peripheral tissues. Similarly, Angptl4 expression levels in hypothalamic and adipose tissues were differentially regulated by leptin (7). Nutrient-dependent alterations in hypothalamic Angptl4 protein content were not in line with changes in Angptl4 mRNA levels (supplementary Fig. 9). On the other hand, leptin and insulin administration significantly increased the relative amounts of full-length versus truncated Angptl4 (supplementary Fig. 5). Therefore, metabolism-related regulation of hypothalamic Angptl4 activity may occur in the process of degradation to less effective truncated Angptl4.

The appetite regulators such as leptin, insulin, and ghrelin exert their actions through regulation of hypothalamic AMPK activity (25,31). In the present study, hypothalamic AMPK activity was suppressed upon Angptl4 administration but enhanced in Angptl4-null mice. Moreover, forced activation of hypothalamic AMPK prevented Angptl4-induced anorexia and weight loss. Thus, central metabolic effects of Angptl4 occur, at least in part, through suppression of hypothalamic AMPK activity. As depicted in Fig. 6*E*, an Angptl4-induced decrease in AMPK activity promotes a de novo fatty acid synthesis pathway in the hypothalamus by increasing hypothalamic ACC activities. Indeed, administration of ACC inhibitors blocked the effects of Angptl4 on food intake and body weight. These findings further support the important role of hypothalamic fatty acid metabolism pathway in appetite regulation.

Given that hypothalamic Angptl4 is an important regulator of feeding behavior, obesity may be associated with abnormalities in hypothalamic Angptl4. In our study, DIO mice were unable to elevate anorexigenic Angptl4 in the hypothalamus in response to food intake, leptin, and insulin. Furthermore, the effects of exogenous Angptl4 on food intake and hypothalamic AMPK activity were significantly reduced in obese mice. Thus, dysregulation of hypothalamic Angptl4 may contribute to the pathogenesis

of obesity, and manipulations improving hypothalamic Angptl4 regulation and actions may have a therapeutic potential for obesity.

Because Angptl4 inhibits the lipoprotein lipase activity (32), overexpression of Angptl4 was expected to suppress fat storage in the adipose tissues. Consistently, Angptl4 transgenic mice generated using AP2 promoter had reduced body fat mass coupled with elevated plasma lipid levels (16). The effects of Angptl4 overexpression were amplified by an HFD, resulting in markedly elevated plasma lipid levels and impaired glucose tolerance in Angptl4 transgenic mice fed a HFD. However, there was not a report on whether Angptl4-transgenic mice were susceptible or resistant to DIO. We unexpectedly found that Angptl4-null mice were resistant to DIO, whereas on chow they displayed increased body weight (supplementary Fig. 10). The anti-obesity phenotype of Angptl4-null mice upon a fat-rich meal is hardly understandable at present and needs further investigations to unveil the metabolic action of Angptl4 in fat-abundant nutritional environment.

In summary, we identified hypothalamic Angptl4 as a new physiological regulator of body weight metabolism. However, to gain more insight into the central role of Angptl4, it would be of interest to examine the phenotype of neuronal Angptl4-deficient mice in the future.

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