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Regulation of AMP-activated Protein Kinase Signaling by AFF4 Protein, Member of AF4 (ALL1-fused Gene from Chromosome 4) Family of Transcription Factors, in Hypothalamic Neurons*[S]

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Tadasuke Komori[‡], Asako Doi[§], Tetsuya Nosaka[¶], Hiroto Furuta[§], Takashi Akamizu[§], Toshio Kitamura[‖], Emiko Senba[‡], and Yoshihiro Morikawa[‡1]

From the [‡]Department of Anatomy and Neurobiology and [§]The First Department of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, the [¶]Department of Microbiology, Mie University Graduate School of Medicine, Mie 514-8507, and the [‖]Division of Cellular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Background: Transcription factors regulate expression of genes that control hypothalamic functions.
Results: AFF4, which is induced by either fasting or ghrelin, up-regulates AMP-activated protein kinase (AMPK) α2 expression in hypothalamic neurons.
Conclusion: AFF4 contributes to activation of AMPK downstream signaling via AMPKα2 expression in hypothalamic neurons.
Significance: This is the first report concerning a transcription factor regulating AMPKα2 expression in hypothalamic neurons.

In the hypothalamus, fasting induces a member of the AF4 family of transcription factors, AFF4, which was originally identified as a fusion partner of the mixed-lineage leukemia gene in infant acute lymphoblastic leukemia. However, the roles of AFF4 in the hypothalamus remain unclear. We show herein that expression of AFF4 increased upon addition of ghrelin and fasting in the growth hormone secretagogue receptor-expressing neurons of the hypothalamus. In the growth hormone secretagogue receptor-expressing hypothalamic neuronal cell line GT1-7, ghrelin markedly induced expression of AFF4 in a time- and dose-dependent manner. Overexpression of AFF4 in GT1-7 cells specifically induced expression of the AMP-activated protein kinase (AMPK) α2 subunit but failed to induce other AMPK subunits and AMPK upstream kinases. The promoter activity of the AMPKα2 gene increased upon addition of AFF4, suggesting that AFF4 regulates transcription of the AMPKα2 gene. Additionally, AFF4 also increased the phosphorylation of acetyl-CoA carboxylase α (ACCα), a downstream target of AMPK. In GT1-7 cells, ghrelin phosphorylated ACCα through AMPKα phosphorylation in the early phase (15 min) of the activation. However, ghrelin-induced expression of AMPKα2 and phosphorylation of ACCα in the late phase (2 h) of the activation were independent of AMPKα phosphorylation. Attenuation of expression of AFF4 by its siRNA in GT1-7 cells decreased ghrelin-induced AMPKα2 expression and ACCα phosphorylation in the late phase of the activation. AFF4 may therefore help to maintain activation of AMPK downstream signaling under conditions of prolonged stimulation with ghrelin, such as during fasting.

The AF4 family includes four members: AFF1 (AF4/FMR2 family member 1), AFF2, AFF3, and AFF4 (also called AF4, FMR2, LAF4, and AF5q31/MCEF, respectively). The former two are known to be involved in CNS functions. Mutations in the human *FMR2* gene cause FRAXE mental retardation, which is characterized by learning deficits, particularly speech delay (1). The mouse ortholog *Fmr2* is expressed in some regions of the brain, including the hippocampus, the piriform cortex, and the Purkinje cell layer (2), and *Fmr2*-deficient mice display learning and memory defects combined with enhanced long-term potentiation (3). A missense mutation of *Af4* in *robotic* mice causes an abnormal accumulation of AF4 proteins, leading to a jerky ataxic gait with progressive Purkinje cell death (4). Recently, it has been reported that AF4 is a critical regulator of the insulin-like growth factor-1 signaling pathway in the cerebellum (5); however, the transcriptional target genes of AF4 remain unidentified. Although it has been reported that LAF4 in mice is expressed in the developing CNS (6), the function of LAF4 in the brain is still entirely unknown.

AFF4 was initially identified as a fusion partner of the *MLL* (mixed-lineage leukemia) gene involved in infant acute lymphoblastic leukemia (7). AFF4 has been reported to interact with positive transcription elongation factor-b (8) while also repressing Tat transactivation of HIV-1 (9). In the adult human, AFF4 is expressed in the heart, placenta, skeletal muscle, and pancreas at high levels and in the brain at low levels (7). We have previously generated *Aff4*-deficient mice that show growth retardation during embryogenesis (10). Approximately 50% of the homozygous mutant embryos were unable to survive past 12.5 days postcoitus, and >70% of the homozygous mutant mice died due to having severely

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[1] To whom correspondence should be addressed. Tel.: and Fax: 81-73-441-0617; E-mail; yoshim@wakayama-med.ac.jp.

shrunken lung alveoli (10). In addition, the homozygous *Aff4*-deficient mice that survived until adulthood showed azoospermia caused by the inhibition of germ cell differentiation resulting from decreased expression of some of the spermatogenesis-related genes in the testis (10).

Food intake is regulated by multiple factors, such as hormones (ghrelin, leptin, insulin), nutrients (glucose, fatty acids), memory, stress, emotions, rewards, and hedonic feelings (11). The brain integrates information from these factors and adjusts food intake to maintain energy balance (11). As disturbances in the regulatory processes that control food intake cause obesity, anorexia, and cachexia in chronic diseases (12), there is tremendous interest in understanding the neuronal regulatory mechanisms of food intake. Although there are no reports regarding the role of AFF4 in the regulation of food intake, a cDNA microarray analysis has revealed that fasting induces the expression of AFF4 in rat hypothalamus (13), thus suggesting that AFF4 plays an important role in the hypothalamic functions that occur during fasting. In this study, we report a novel role of AFF4 in the function of hypothalamic neurons.

EXPERIMENTAL PROCEDURES

Animals—Eight-week-old male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). All mice were housed in specific pathogen-free facilities with light-controlled (12-h light/dark cycle), temperature-controlled (22–25 °C), and humidity-controlled (50–60% relative humidity) conditions. The mice were fed a standard diet (MF, Oriental Yeast, Tokyo) and given water *ad libitum*. At all times, the experiments were performed under the control of the Animal Research Control Committee in accordance with the Guidelines for Animal Experiments of Wakayama Medical University and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications 80–23). All efforts were made to minimize the number of animals used and to reduce their suffering.

Fasting Experiments—The mice were assigned to either control or fasting groups and were housed in individual cages for 3 days. At that time, there were no significant differences in body weight between the two groups. The mice were either fed *ad libitum* or fasted for 48 h (starting from 18:00 h) with free access to water.

Injection of Ghrelin in Mice—The mice were injected intraperitoneally with either saline (0.85% NaCl) or ghrelin (10 μg/mouse; Peptide Institute, Osaka, Japan) dissolved in saline at 11:00 h. As shown in a previous study (14), this dose of ghrelin is sufficient to stimulate food intake. The mice injected with either saline or ghrelin were maintained for 1–6 h with free access to water.

Tissue Preparation—For *in situ* hybridization, the mice were deeply anesthetized with diethyl ether and then transcardially perfused with ice-cold 0.85% NaCl, followed by ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were quickly removed, post-fixed in the same fixative at 4 °C for 16 h, and cryoprotected in 30% sucrose in 0.1 M PBS. To complete an immunohistochemistry analysis, the mice were perfused with ice-cold 0.85% NaCl, followed by ice-cold Zamboni's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M PBS). The brains were quickly removed, post-fixed in the same fixative at 4 °C for 3 h, and cryoprotected in 20% sucrose in 0.1 M PBS. All specimens were embedded in an optical cutting temperature medium (Sakura Finetek USA, Inc., Torrance, CA), frozen rapidly in cold *n*-hexane on dry ice, and stored at −80 °C.

Preparation of Radioisotope-labeled Probe for AFF4—A 429-bp XhoI cDNA fragment of AFF4 (coding region 1806–2234) was ligated into a pBluescript II KS(+) vector (Stratagene, La Jolla, CA), which was then linearized by cutting with EcoRV (for the antisense probe) or BamHI (for the sense probe). An *in vitro* transcription for the *in situ* hybridization histochemistry analysis using a radioisotope-labeled probe was performed using the appropriate RNA polymerases (T7 RNA polymerase for the antisense probe and T3 RNA polymerase for the sense probe) and ^{35}S-labeled dUTP (PerkinElmer Life Sciences).

In Situ Hybridization Histochemistry—An *in situ* hybridization histochemistry analysis was carried out as described previously (15). Briefly, frozen sections were cut on a cryostat at a thickness of 6 μm. After treatment with proteinase K (Roche Diagnostics), the sections were post-fixed in 4% paraformaldehyde, treated with acetic anhydride, and dehydrated with ethanol. The sections were then hybridized with either ^{35}S-labeled sense or antisense cRNA probes for AFF4 at 55 °C for 16 h. After being rinsed with 2× SSC buffer (1× SSC = 44.6 μmol/liter sodium chloride and 5 μmol/liter trisodium citrate at pH 7.0) containing 10 mM dithiothreitol, the sections were treated with ribonuclease A (10 μg/ml; Wako Pure Chemical Industries, Tokyo) at 37 °C for 30 min. High stringency washes were performed in 0.1× SSC buffer at 55 °C for 15 min. After dehydration with a graded series of ethanols, the sections were submerged in a liquid emulsion (NTB-2, Eastman Kodak), exposed for an appropriate number of days, and developed in a Kodak D-19 developer. The sections were counterstained with Mayer's hematoxylin through the emulsion and examined under dark-field lateral illumination microscopy (XF-WFL, Nikon, Tokyo).

To evaluate the expression of *Aff4* mRNA in the hypothalamic nuclei, every fifth section was chosen from a series of consecutive hypothalamic sections (6 μm), and three sections per mouse were allocated for the arcuate nucleus and the ventromedial hypothalamic nucleus (VMH).[2] For each section, the cells were considered to be positive for *Aff4* gene expression if five or more silver grains were found overlying the cell bodies.

In Situ Hybridization Combined with Immunohistochemistry—*In situ* hybridization combined with an immunohistochemistry analysis was performed with some modifications as described previously (15). Briefly, after being hybridized with the cRNA probes, the sections were incubated with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove,

[2] The abbreviations used are: VMH, ventromedial hypothalamic nucleus; GHS-R, growth hormone secretagogue receptor; AMPK, AMP-activated protein kinase; pAMPKα, phosphorylated AMPKα; pACC, phosphorylated acetyl-CoA carboxylase; CaMKK2, Ca^{2+}/calmodulin-dependent protein kinase kinase-2; ANOVA, analysis of variance.

PA) at room temperature for 1 h. Next, the sections were incubated with goat anti-growth hormone secretagogue receptor (GHS-R) antibody (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 16 h. After being washed, the sections were incubated with biotinylated donkey anti-goat IgG antibody (diluted 1:400; Jackson ImmunoResearch Laboratories) at room temperature for 1 h, followed by incubation with HRP-conjugated streptavidin (DAKO, Carpinteria, CA) at room temperature for 30 min. Thereafter, the peroxidase reaction product was visualized with 0.05% diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2. After the reaction, the sections were submerged in the liquid emulsion and counterstained through the emulsion with Mayer's hematoxylin.

To evaluate the colocalization of *Aff4* mRNA and GHS-R, every fifth section was selected from a series of consecutive hypothalamic sections (6 μm), and three sections per mouse were allocated for the arcuate nucleus and the VMH. For each section, cells were considered to be positive for *Aff4* gene expression if five or more silver grains were found overlying the cell bodies. The cells were considered to be positive for GHS-R if the cell bodies were stained brown.

Immunohistochemistry—Immunofluorescence staining was performed with some modifications as described previously (15). Briefly, frozen sections were cut on a cryostat (6-μm thickness). The sections were preincubated with 5% normal donkey serum at room temperature for 1 h, followed by incubation with goat anti-GHS-R antibody (diluted 1:400) and rabbit anti-AFF4 antibody (diluted 1:400; Abnova, Taipei, Taiwan) at 4 °C for 16 h. Later, the sections were incubated with either Cy2- or Cy3-conjugated secondary antibodies (diluted 1:800; Jackson ImmunoResearch Laboratories) at room temperature for 1 h. The sections were counterstained with DAPI. Immunofluorescence images were acquired using an epifluorescence microscope (BX50, Olympus, Tokyo) equipped with a digital CCD camera (DP71, Olympus).

The following controls were performed: (i) incubation with protein A-purified goat or rabbit IgG instead of the primary antibody and (ii) incubation without the primary antibody or without the primary and secondary antibodies. None of the controls revealed any labeling (data not shown).

Cell Culture—The mouse hypothalamic neuronal cell line GT1-7 (16) was a gift from Dr. Pamela L. Mellon (University of California, La Jolla, CA) and was grown in DMEM (Invitrogen) with 10% horse serum, 10% fetal calf serum, 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

Treatment of GT1-7 Cells with Ghrelin—The GT1-7 cells were plated in 24-well plates at a density of 1×10^5 cells/well and cultured in standard medium for 24 h. The cells were starved for 24 h to complete a Western blot analysis to detect levels of phosphorylated ERK and ERK. The cells were then treated with either saline or ghrelin (0.01–100 nM) and maintained for 0.5–4 h. In some experiments, the cells were pretreated with compound C (50 μM; Sigma), a selective AMP-activated protein kinase (AMPK) inhibitor, for 1 h before being exposed to ghrelin. Compound C was dissolved in dimethyl sulfoxide and stored at −20 °C until used. The final concentration of dimethyl sulfoxide during cell stimulation did not exceed 0.1% (v/v), and the control cells received the solvent at the same volume.

Transient Transfection—Transient transfection was carried out with some modifications as described previously (17). Briefly, GT1-7 cells were plated in 24-well plates at a density of 1×10^5 cells/well. After incubation in standard medium for 24 h, the cells were transfected with plasmids of mock or full-length mouse AFF4 (0.5 μg/well) using FuGENE 6 transfection reagent (Roche Diagnostics) and were incubated for an additional 24 h. All cells were transfected with FuGENE 6 transfection reagent (μl) and DNA (μg) in a ratio of 3:1. The plasmid of full-length mouse AFF4 was prepared as described previously (10). In some experiments, the cells were treated with compound C (50 μM) for 1 h after the transfection.

Transfection of siRNA—The transfection of siRNA was performed with some modifications as described previously (14). An siRNA duplex targeting murine AFF4 and scrambled control siRNA were obtained from Qiagen (Hilden, Germany). The sequence of siRNA targeting murine AFF4 was 5′-CCGGGAAGCCTTACAAAGAAA-3′. For the siRNA transfection, GT1-7 cells cultured in 24-well plates for 24 h were transfected with control siRNA (20 nM) and AFF4 siRNA (20 nM) using HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. Forty-eight hours later, the cells were exposed to ghrelin (100 nM) and incubated for 15 min or 2 h.

Quantitative Real-time PCR—Quantitative real-time PCR was performed with some modifications as described previously (17). Briefly, total RNA was extracted from GT1-7 cells using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). The cDNA extracted from the total RNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The following TaqMan gene expression assays (Applied Biosystems) were used: AFF4 (Mm00466683_m1), AMPKα1 (Mm01296695_m1), AMPKα2 (Mm01264788_m1), AMPKβ1 (Mm01201921_m1), AMPKβ2 (Mm01257133_m1), AMPKγ1 (Mm00450298_m1), AMPKγ2 (Mm00513977_m1), AMPKγ3 (Mm00463997_m1), and 18 S (Hs99999901_s1). Quantitative real-time PCR was performed for each gene using Rotor-Gene Q (Qiagen) and Rotor-Gene Probe PCR Master Mix (Qiagen). The PCR amplification protocol was as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The relative abundance of transcripts was normalized by the expression of 18 S ribosomal RNA and analyzed using the $\Delta\Delta C_T$ method.

Luciferase Assay—A luciferase assay was performed using a Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions with some modifications as described previously (17). Briefly, GT1-7 cells in 24-well plates were cotransfected with a plasmid of full-length mouse AFF4 (0.5 μg/well), an AMPKα2 promoter-luciferase reporter plasmid (0.1 μg/well), and a plasmid of an internal reporter (pRL-TK; 0.01 μg/well), using FuGENE 6 transfection reagent. To construct the AMPKα2 promoter-luciferase reporter plasmid, the AMPKα2 promoter (−1000 to −14) was inserted into the pGL3-Basic vector (Promega). Twenty-four hours after the transfection, the cells were washed with PBS and lysed with passive lysis buffer (Promega). The luciferase activities were defined as the ratio of *Photinus pyralis* luciferase activ-

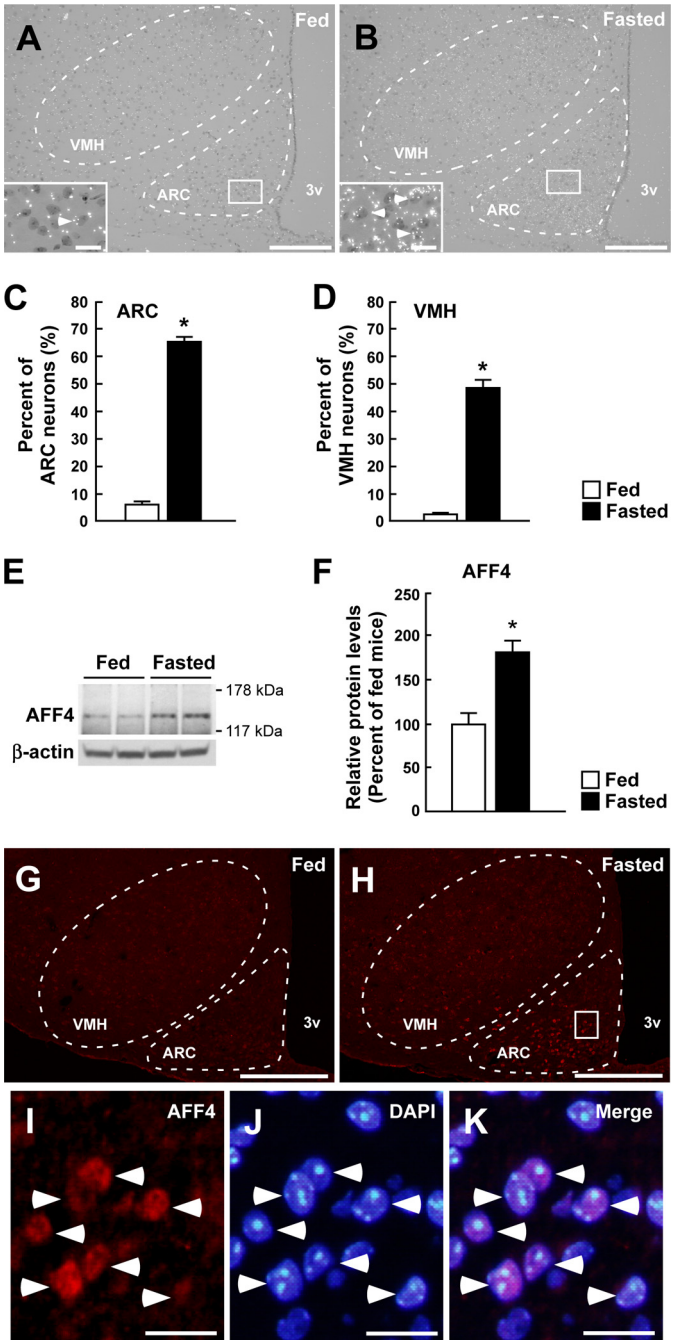

FIGURE 1. **Effects of fasting on AFF4 expression in hypothalamus.** *A* and *B*, *in situ* hybridization histochemistry analysis results for *Aff4* mRNA in the hypothalamus of mice fed *ad libitum* (*A*) or fasted for 48 h (*B*) ($n = 4$ per group). The sections were hybridized with a radiolabeled probe for AFF4. The arcuate nucleus (*ARC*) and the VMH are indicated by the *dashed lines*. The *boxed* regions are shown at a higher magnification in the *insets*. *Arrowheads* indicate the AFF4-expressing cells. *3v*, third ventricle. *Scale bars* = 200 μm (*A* and *B*) and 10 μm (*insets*). *C* and *D*, the AFF4-expressing cells were quantified as the percentage of positive neurons in the arcuate nucleus (*C*) and the VMH (*D*). *E*, Western blot analysis of AFF4 proteins in the hypothalamus of mice fed *ad libitum* or fasted for 48 h ($n = 4$ per group). The hypothalamic lysates were separated by SDS-PAGE and immunoblotted with anti-AFF4 antibodies. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. The apparent molecular masses are indicated on the right. *F*, quantitative analysis of the protein expression of AFF4. The band intensities of AFF4 were normalized to β-actin and are represented as the percentage relative to the intensities of fed mice (*white bars*) in the bar graphs. *G* and *H*, immunofluorescence staining for AFF4 in the hypothalamus of mice fed *ad libitum* (*G*) or fasted for 48 h (*H*) ($n = 4$ per group). The sections were incubated with anti-AFF4 antibodies. The arcuate nucleus and the VMH are indicated by the *dashed lines*. High magnification views of the *boxed* region in *H* are represented in *I–K*. The sections were counterstained with DAPI (*J*). The merged image is shown in *K*. *Arrowheads* in *I–K* indicate AFF4-expressing cells. *Scale bars* = 200 μm (*G* and *H*) and 10 μm (*I–K*). Data represent the means ± S.E. *, $p < 0.05$ (Student's *t* test).

ity from pGL3-Basic derivatives to *Renilla reniformis* luciferase activity from pRL-TK, which reflected the efficiency of the transfection.

Western Blot Analysis—A Western blot analysis was performed with some modifications as described previously (15). Lysates from either the cultured cells or the hypothalami of the mice were prepared using radioimmune precipitation assay buffer (Upstate Biotechnology, Lake Placid, NY) containing protease inhibitor mixture (Upstate Biotechnology), 1 mM orthovanadate, 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. The protein concentrations of the lysates were determined using the BCA protein assay kit (Pierce). Ten micrograms of protein from the cultured cells or 20 μg of protein from the hypothalami were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). The blotted membranes were incubated with mouse anti-AFF4 antibody (diluted 1:500; Abnova), rabbit anti-phosphorylated ERK antibody (diluted 1:500; Cell Signaling Technology, Beverly, MA), rabbit anti-phosphorylated AMPKα (pAMPKα) antibody (diluted 1:500; Cell Signaling Technology), rabbit anti-AMPKα antibody (diluted 1:500; Cell Signaling Technology), goat anti-AMPKα1 antibody (diluted 1:500; R&D Systems, Minneapolis, MN), goat anti-AMPKα2 antibody (diluted 1:500; R&D Systems), rabbit anti-AMPKβ1 antibody (diluted 1:500; Cell Signaling Technology), rabbit anti-AMPKγ1 antibody (diluted 1:500; Cell Signaling Technology), rabbit anti-AMPKγ2 antibody (diluted 1:500; Cell Signaling Technology), rabbit anti-phosphorylated acetyl-CoA carboxylase (pACC) antibody (diluted 1:500; Cell Signaling Technology), rabbit anti-ACC antibody (diluted 1:500; Cell Signaling Technology), goat anti-Ca^{2+}/calmodulin-dependent protein kinase kinase-2 (CaMKK2) antibody (diluted 1:500; Santa Cruz Biotechnology), and rabbit anti-LKB1 (liver kinase B1) antibody (diluted 1:500; Cell Signaling Technology). Thereafter, the membranes were incubated with HRP-conjugated donkey anti-rabbit (diluted 1:4000; GE Healthcare), donkey anti-goat (diluted 1:10,000; Jackson ImmunoResearch Laboratories), or donkey anti-mouse (diluted 1:4000; GE Healthcare) IgG antibody. Labeled proteins were detected with chemiluminescence using ECL detection reagent (GE Healthcare) according to the manufacturer's instructions. The membranes were exposed to Hyperfilm ECL (GE Healthcare) for an appropriate period. Next, the blotted membranes were stripped in 0.25 M glycine (pH 2.5) at room temperature for 10 min and incubated with rabbit anti-ERK antibody (diluted 1:500; Cell Signaling Technology) or mouse anti-β-actin antibody (diluted 1:10,000; Sigma) at 4 °C for 16 h, followed by incubation with HRP-conjugated donkey anti-rabbit (diluted 1:4000) or donkey anti-mouse (diluted 1:20,000) IgG antibody.

Statistical Analysis—The results are shown as the means ± S.E. Statistically significant differences between groups were analyzed with either Student's *t* test or an analysis of variance

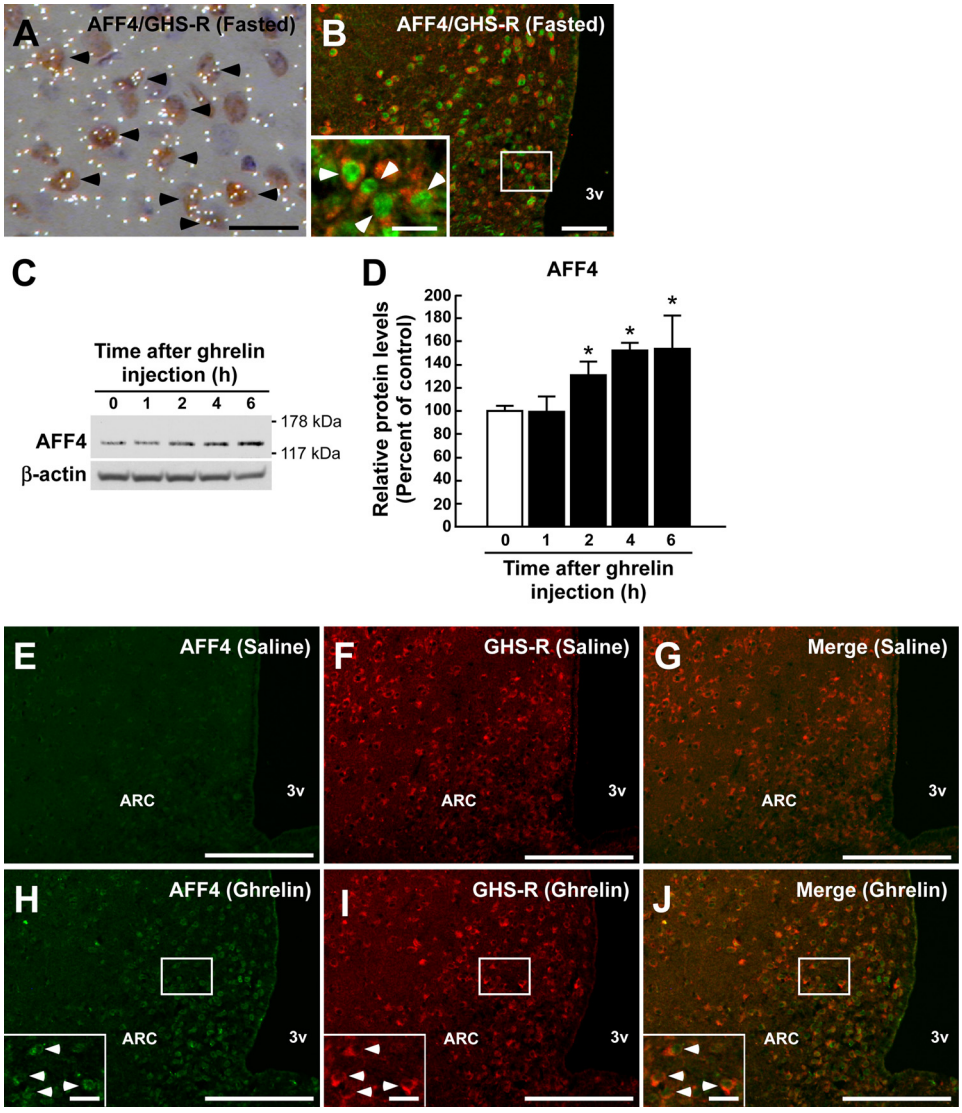

FIGURE 2. **Effects of ghrelin on expression of AFF4 in hypothalamus of mice.** *A* and *B*, colocalization of AFF4 and GHS-R in the arcuate nucleus of fasted mice. *A*, an *in situ* hybridization histochemistry analysis for *Aff4* mRNA was combined with an immunohistochemistry analysis for GHS-R in the arcuate nucleus of mice fasted for 48 h ($n = 4$). *Arrowheads* indicate GHS-R-positive neurons (*brown*) with signals for *Aff4* mRNA (*white dots*). The sections were counterstained with hematoxylin. *B*, double-immunofluorescence staining for AFF4 (*green*) and GHS-R (*red*) in the arcuate nucleus of mice fasted for 48 h ($n = 4$). The *boxed* region is shown at a higher magnification in the *inset*. *Arrowheads* indicate AFF4 and GHS-R double-positive cells. *3v*, third ventricle. *Scale bars* = 10 μm (*A* and *inset* in *B*) and 100 μm (*B*). *C* and *D*, induction of AFF4 proteins by ghrelin in the hypothalamus of mice. *C*, Western blot analysis of AFF4 in the hypothalamus of ghrelin-injected mice ($n = 4$ per group). The mice were intraperitoneally injected with either saline or ghrelin (10 μg/mouse) and maintained for 1–6 h. The hypothalamic lysates were separated by SDS-PAGE and immunoblotted with anti-AFF4 antibodies. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. The apparent molecular masses are indicated on the right. *D*, quantitative analysis of the protein expression of AFF4. The band intensities of AFF4 were normalized to β-actin and are represented as the percentage relative to the intensities of the controls (*white bar*) in the bar graph. The data represent the means ± S.E. *, $p < 0.05$ *versus* the control (ANOVA, followed by the Bonferroni post hoc test). *E–J*, colocalization of AFF4 and GHS-R in the arcuate nucleus of saline- or ghrelin-injected mice ($n = 4$ per group). Shown is the double-immunofluorescence staining for AFF4 (*E* and *H*) and GHS-R (*F* and *I*) in the arcuate nucleus of saline-injected (*E–G*) or ghrelin-injected mice (*H–J*). The merged images are shown in *G* and *J*. The *boxed* regions are shown at a higher magnification in the *insets*. *Arrowheads* indicate AFF4 and GHS-R double-positive cells. *Scale bars* = 100 μm (*E–J*) and 10 μm (*insets* in *H–J*).

(ANOVA), followed by the Bonferroni post hoc test. The criterion for statistical significance was $p < 0.05$.

RESULTS

Increased Expression of AFF4 in Hypothalamus during Fasting—To examine the localization of AFF4 in the hypothalamus, we performed an *in situ* hybridization histochemistry analysis to detect the presence of *Aff4* mRNA in hypothalamic sections obtained from wild-type mice in fed and fasted conditions. The number of neurons expressing *Aff4* mRNA in the arcuate nucleus and the VMH of mice fed *ad libitum* was estimated (Fig. 1*A*). In addition, the number of neurons expressing *Aff4* mRNA was significantly increased in the arcuate nucleus (fed, 6.0 ± 1.2%; fasted, 65.2 ± 1.6%) and the VMH (fed, 2.3 ± 0.1%; fasted, 48.4 ± 2.9%) of fasted mice compared with that in the arcuate nucleus and the VMH of mice fed *ad libitum* (Fig. 1, *A–D*).

We next examined the effects of fasting on the expression of AFF4 proteins in the hypothalamus. As shown in Fig. 1*E*, a 140-kDa band corresponding to the presence of AFF4 proteins

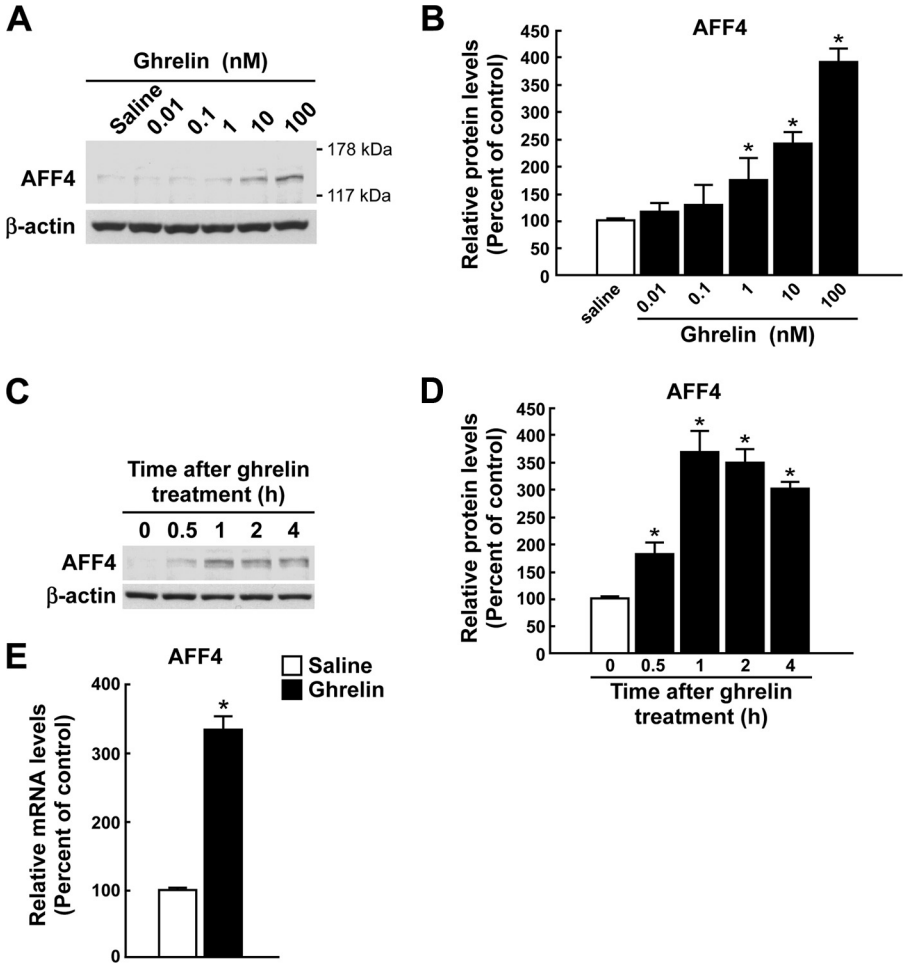

FIGURE 3. **Effect of ghrelin on AFF4 expression in GT1-7 cells.** *A–D*, effect of ghrelin on the protein expression of AFF4 in GT1-7 cells. *A*, the cells were treated with ghrelin (0–100 nM) and incubated for 2 h. *C*, the cells were treated with ghrelin (100 nM) and incubated for 0, 0.5, 1, 2, and 4 h. The whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-AFF4 antibodies. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. The apparent molecular masses are indicated on the right. *B* and *D*, quantitative analysis of the protein expression of AFF4. The band intensities of AFF4 were normalized to β-actin and are represented as the percentage relative to the intensities of the controls (*white bars*) in the bar graphs. The data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ *versus* the control (ANOVA, followed by the Bonferroni post hoc test). *E*, effect of ghrelin on the expression of *Aff4* mRNA in GT1-7 cells. Quantitative real-time PCR was performed using mRNA prepared from PBS-treated (*white bar*) or ghrelin-treated (*black bar*) GT1-7 cells. The cells were treated with PBS or ghrelin (100 nM) and incubated for 1 h. *, $p < 0.05$ (Student's *t* test).

was detected in the hypothalamus of wild-type mice fed *ad libitum*. In addition, a significant increase in the amount of AFF4 proteins expressed was observed in the hypothalamus of fasted mice (Fig. 1*F*).

To determine the localization of AFF4 proteins in the hypothalamus during fasting, we performed immunofluorescence staining using anti-AFF4 antibody. Consistent with the *in situ* hybridization histochemistry data (Fig. 1, *A–D*), the expression of AFF4 proteins was observed in some neurons in the arcuate nucleus and the VMH (Fig. 1*G*) of mice fed *ad libitum*. After fasting, AFF4-positive neurons increased in the arcuate nucleus and the VMH (Fig. 1*H*). In addition, AFF4 was colocalized with DAPI staining (Fig. 1, *I–K*), thus indicating that AFF4 proteins were subcellularly localized in the nucleus.

Induction of Expression of AFF4 by Ghrelin in Hypothalamus—To elucidate the relationship between AFF4 expression and the activities of GHS-R-positive neurons in the arcuate nucleus and the VMH during fasting, we examined the colocalization of AFF4 and GHS-R using an *in situ* hybridization histochemistry analysis combined with an immunohistochemistry analysis and double-immunofluorescence staining. Most of the GHS-R-positive neurons expressed *Aff4* mRNA in the arcuate nucleus (80.9 ± 2.3%) (Fig. 2*A*) and the VMH (76.3 ± 3.5%) (data not shown) after fasting. In addition, GHS-R-positive neurons also contained AFF4 proteins in the arcuate nucleus (Fig. 2*B*) and the VMH (data not shown). These results suggest that AFF4 is related to ghrelin signaling in the hypothalamus.

To investigate the effects of ghrelin on AFF4 expression in the hypothalamus, we injected ghrelin intraperitoneally into wild-type mice. Fifteen minutes after the ghrelin injection, ERK was markedly phosphorylated only in the GHS-R-positive neurons of the arcuate nucleus (supplemental Fig. S1, *A–F*), suggesting that intraperitoneal injection of ghrelin directly acts on GHS-R-positive neurons in the hypothalamus. A Western blot analysis revealed that the expression of AFF4 proteins in the hypothalamus of wild-type mice began to increase at 2 h, peaked at 4 h, and was still maintained at high levels 6 h after the ghrelin injection was administered (Fig. 2, *C* and *D*).

To examine the localization of AFF4 proteins in the hypothalamus after ghrelin injection, we performed double-im-

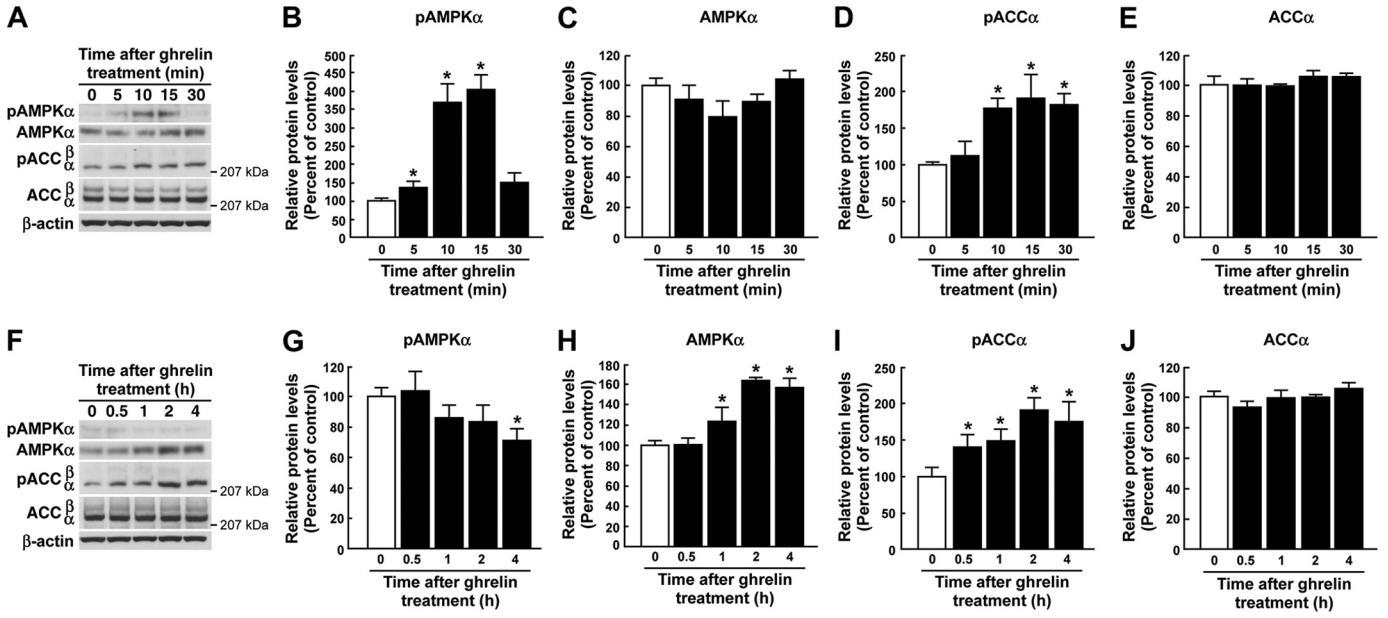

FIGURE 4. **Effect of ghrelin on activation of AMPK signaling in GT1-7 cells.** *A* and *F*, the cells were treated with ghrelin (100 nM) and incubated for 5, 10, 15, and 30 min (*A*) or for 0.5, 1, 2, and 4 h (*F*). The whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-pAMPKα, anti-AMPKα, anti-pACC, or anti-ACC antibody. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. The apparent molecular masses are indicated on the right. *B–E* and *G–J*, quantitative analysis of pAMPKα (*B* and *G*), AMPKα (*C* and *H*), pACCα (*D* and *I*), and ACCα (*E* and *J*). The band intensities of pAMPKα, AMPKα, pACCα, and ACCα were normalized to β-actin. All band intensities are represented as the percentage of the controls (*white bars*) in the bar graphs. The data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ *versus* the control (ANOVA, followed by the Bonferroni post hoc test).

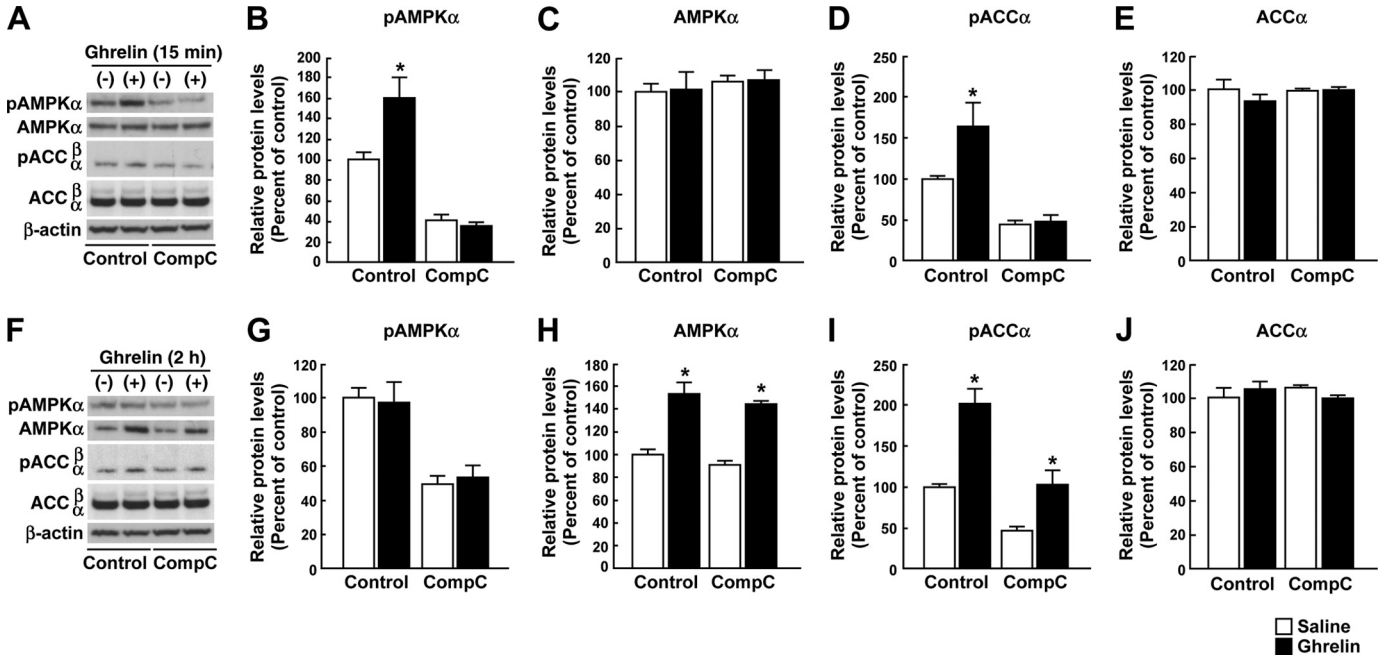

FIGURE 5. **Effect of ghrelin-mediated AMPK phosphorylation on ACCα phosphorylation in GT1-7 cells.** *A* and *F*, the cells were pretreated with either Me_2SO (*Control*) or compound C (*CompC*; 50 μM) for 1 h, followed by incubation with ghrelin for 15 min (*A*) or 2 h (*F*). The whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-pAMPKα, anti-AMPKα, anti-pACC, or anti-ACC antibody. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. *B–E* and *G–J*, quantitative analysis of pAMPKα (*B* and *G*), AMPKα (*C* and *H*), pACCα (*D* and *I*), and ACCα (*E* and *J*). The band intensities of pAMPKα, AMPKα, pACCα, and ACCα were normalized to β-actin. All band intensities are represented as the percentage of the controls (*white bars*) in the bar graphs. The data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ *versus* the control (ANOVA, followed by the Bonferroni post hoc test).

munofluorescence staining to detect AFF4 and GHS-R in hypothalamic sections of mice that received injections of either saline or ghrelin. Consistent with the data obtained with mice fed *ad libitum* (Fig. 1*G*), the expression of AFF4 was observed in some neurons in the arcuate nucleus (Fig. 2*E*) and the VMH (data not shown) of mice that received saline injections. In the ghrelin-injected mice, AFF4 was markedly increased in the arcuate nucleus (Fig. 2*H*), and all of the AFF4-positive neurons expressed GHS-R (Fig. 2, *H–J*). In the VMH of the mice that received ghrelin injections,

AFF4-positive neurons were also increased and contained GHS-R (data not shown).

Induction of Expression of AFF4 by Ghrelin in Hypothalamic Cell Line GT1-7—An immortalized neuronal cell line derived from mouse hypothalamus (GT1-7) expresses GHS-R (17). In GT1-7 cells, phosphorylated ERK started to increase at 5 min, reached a peak at 10 min, and then gradually decreased until 30 min after the treatment was administered (supplemental Fig. S1, *G* and *H*). The expression of AFF4 was induced by ghrelin treatment in a dose-dependent manner (Fig. 3, *A* and *B*). In addition, the expression of AFF4 began to increase at 0.5 h, peaked at 1 h, and was gradually down-regulated 4 h after ghrelin treatment was administered (Fig. 3, *C* and *D*). Quantitative real-time PCR revealed that *Aff4* mRNA in GT1-7 cells was also induced by ghrelin 1 h after the treatment was administered (Fig. 3*E*).

Activation of AMPK Signaling Pathway by Ghrelin in GT1-7 Cells—In the hypothalamus, AMPK has recently been proposed to be a target of ghrelin (18). To examine the role of AFF4 in the hypothalamus, we investigated the effects of ghrelin on the activation of the AMPK signaling pathway in GT1-7 cells. Phosphorylation of AMPKα in GT1-7 cells began to increase at 5 min, reached a peak at 15 min, returned to the control level at 1 h, and then gradually decreased until 4 h after ghrelin treatment was administered (Fig. 4, *A*, *B*, *F*, and *G*). The expression of AMPKα was slightly decreased until 10 min after ghrelin treatment was administered and returned to the control level 30 min after ghrelin treatment was administered (Fig. 4, *A* and *C*). However, a significant increase in the expression of AMPKα in GT1-7 cells was observed 2 h after ghrelin treatment was administered (Fig. 4, *F* and *H*). The fact that these changes in AFF4 and AMPKα expression occurred in the same time frame raises the possibility that AFF4 regulates the expression of AMPKα. In addition, phosphorylation of ACCα began to increase at 5 min, reached a peak at 2 h, and was maintained at high levels until 4 h after ghrelin treatment was administered (Fig. 4, *A*, *D*, *F*, and *I*). In contrast to the increased expression of AMPKα, no significant difference in the expression of ACCα was observed after ghrelin treatment was administered (Fig. 4, *A*, *E*, *F*, and *J*).

To investigate the effects of ghrelin-mediated AMPKα phosphorylation on the regulation of ACCα phosphorylation in GT1-7 cells in both the early (15 min) and late (2 h) phases of the activation, we inhibited phosphorylation of AMPKα with compound C, a selective inhibitor of AMPK phosphorylation. Treatment of the GT1-7 cells with compound C abolished the ghrelin-induced phosphorylation of AMPKα and ACCα 15 min after ghrelin treatment was administered (Fig. 5, *A*, *B*, and *D*). However, compound C had no effect on the ghrelin-induced expression of AMPKα and phosphorylation of ACCα 2 h after ghrelin treatment was administered (Fig. 5, *F*, *H*, and *I*). The expression of ACCα was not affected by compound C either 15 min or 2 h after ghrelin treatment was administered (Fig. 5, *E* and *J*). These results suggest that ghrelin-induced ACCα phosphorylation in the late phase (2 h after ghrelin treatment was administered) was independent of AMPKα phosphorylation.

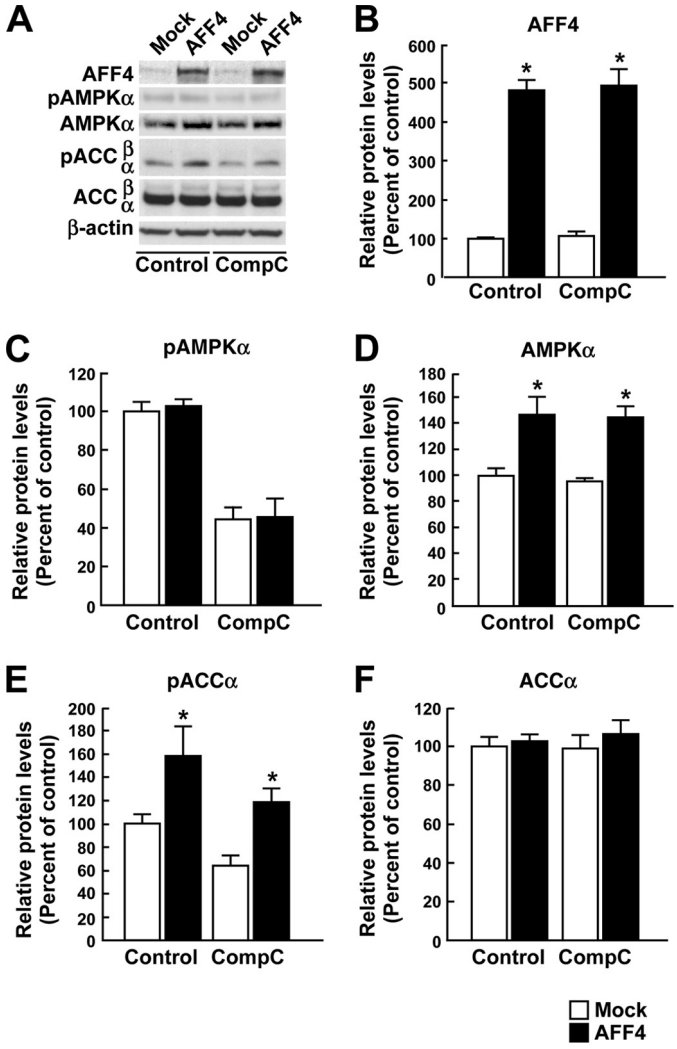

FIGURE 6. **Effects of AFF4 on activation of AMPK signaling in GT1-7 cells.** *A*, the cells were either mock-transfected or transfected with AFF4 (0.5 μg) for 24 h, followed by incubation with either Me_2SO (*Control*) or compound C (*CompC*; 50 μM) for 1 h. The whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-AFF4, anti-pAMPKα, anti-AMPKα, anti-pACC, or anti-ACC antibody. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. *B–F*, quantitative analysis of AFF4 (*B*), pAMPKα (*C*), AMPKα (*D*), pACCα (*E*), or ACCα (*F*). The band intensities of AFF4, pAMPKα, AMPKα, pACCα, and ACCα were normalized to β-actin. All band intensities are represented as the percentage relative to the intensities of mock-transfected cells (*white bars*) in the bar graphs. The data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ (Student's *t* test).

Activation of AMPK Signaling Pathway by AFF4 in GT1-7 Cells—To examine the direct effects of AFF4 on the expression of AMPKα, GT1-7 cells were transfected with the full-length *Aff4* gene. The expression of AFF4 was significantly increased in the AFF4-transfected cells compared with the mock-transfected cells (Fig. 6, *A* and *B*). The expression of AMPKα was significantly elevated by AFF4 (Fig. 6, *A* and *D*), whereas no significant difference in the expression of ACCα between the mock- and AFF4-transfected cells was observed (Fig. 6, *A* and *F*). However, a significant increase in ACCα phosphorylation was observed in the AFF4-transfected cells compared with the mock-transfected cells (Fig. 6, *A* and *E*). However, no changes were observed in the phosphorylation levels of AMPKα in the AFF4- or mock-transfected cells (Fig. 6, *A* and *C*). In addition,

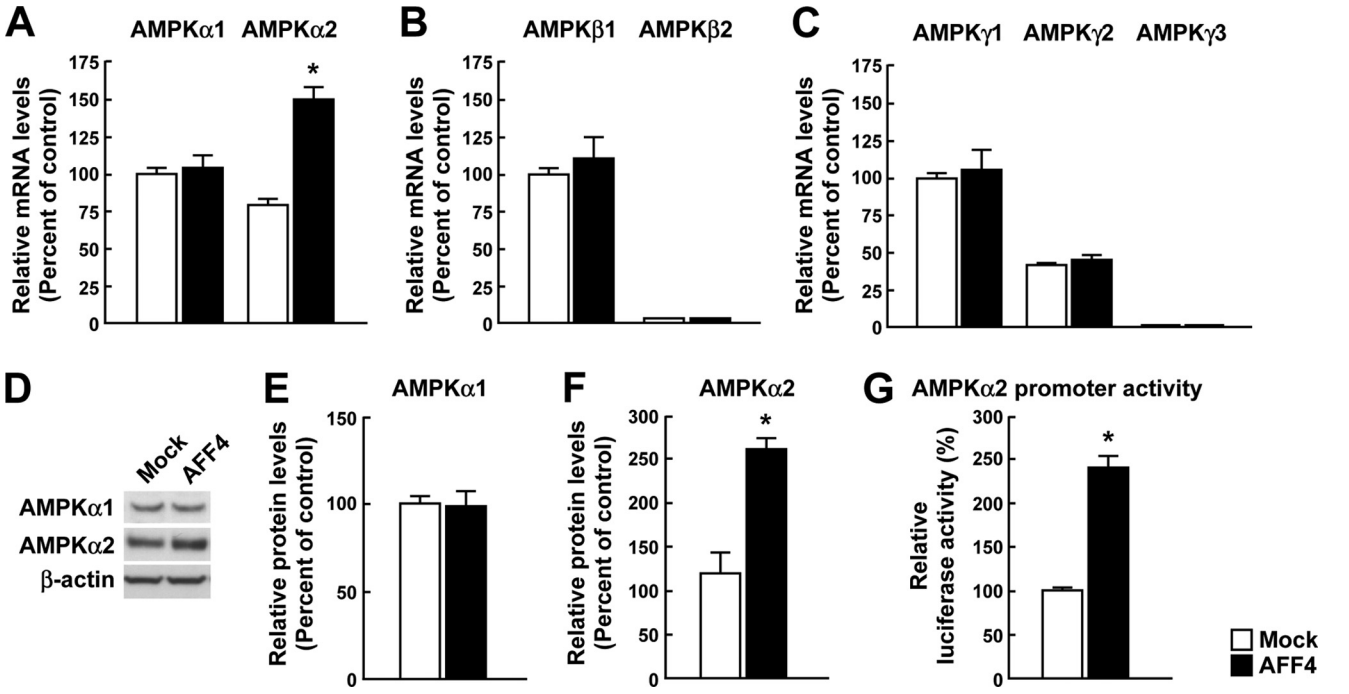

FIGURE 7. **Effects of AFF4 on transcription of AMPKα2 in GT1-7 cells.** *A–C*, effect of AFF4 on the expression of AMPK subunits in GT1-7 cells. Quantitative real-time PCR of AMPKα1 and AMPKα2 (*A*), AMPKβ1 and AMPKβ2 (*B*), and AMPKγ1, AMPKγ2, and AMPKγ3 (*C*) was performed using mRNA prepared from either mock-transfected (*white bars*) or AFF4-transfected (*black bars*) GT1-7 cells. The cells were either mock-transfected or transfected with AFF4 (0.5 μg) for 24 h. *D*, Western blot analysis of AMPKα1 and AMPKα2 in mock- or AFF4-transfected GT1-7 cells. GT1-7 cells were either mock-transfected or transfected with AFF4 (0.5 μg) for 24 h. The whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-AMPKα1 or anti-AMPKα2 antibody. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. *E* and *F*, quantitative analysis of the expression of AMPKα1 (*E*) and AMPKα2 (*F*). The band intensities of AMPKα1 and AMPKα2 were normalized to β-actin. All band intensities are represented as the percentage relative to the intensities of mock-transfected cells (*white bars*) in the bar graphs. *G*, effects of AFF4 on the promoter activities of AMPKα2 in GT1-7 cells. Either mock or AFF4 (0.5 μg) was cotransfected with the AMPKα2 promoter-luciferase fusion construct (0.1 μg) and the *Renilla* luciferase control reporter plasmid pRL-TK (0.01 μg) in GT1-7 cells, and the cells were incubated for 24 h. The promoter activities of AMPKα2 in mock-transfected (*white bar*) or AFF4-transfected (*black bar*) cells were normalized to *Renilla* luciferase activity and are shown as a percentage relative to the activities of the mock-transfected cells. The data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ (Student's *t* test).

the expression of AMPKα and phosphorylation of ACCα induced by AFF4 were not affected by compound C (Fig. 6, *A*, *D*, and *E*), although AMPK phosphorylation was at a very low level when cells were treated with compound C (Fig. 6, *A* and *C*).

Induction of AMPKα2 Expression by AFF4 in GT1-7 Cells—In mammals, AMPK is a heterotrimer with α, β, and γ subunits, each of which is encoded by two or three genes (α1, α2, β1, β2, γ1, γ2, and γ3) (19). We next examined which types of AMPK subunits are increased by AFF4. Quantitative RT-PCR revealed that AMPKα2 mRNA was increased by AFF4 (Fig. 7*A*), whereas no difference was observed in the expression of AMPKα1 mRNA between the mock- and AFF4-transfected cells (Fig. 7*A*). Consistent with these data, AFF4 induced the expression of AMPKα2 proteins and did not induce the expression of AMPKα1 proteins (Fig. 7, *D–F*). Other AMPK subunits expressed in GT1-7 cells (β1, γ1, and γ2) were not increased by AFF4 at either the mRNA (Fig. 7, *B* and *C*) or protein (supplemental Fig. S2, *A–D*) level. AMPKβ2 and AMPKγ3 were expressed in small amounts in GT1-7 cells (Fig. 7, *B* and *C*). In addition, two upstream kinases of AMPK, CaMKK2 and LKB1, were not increased in GT1-7 cells by overexpression of AFF4 (supplemental Fig. S2, *E–G*). To investigate whether AFF4 directly activates transcription of AMPKα2, we performed a luciferase assay using the promoter region of AMPKα2. The promoter activity of AMPKα2 was significantly increased in the AFF4-transfected GT1-7 cells compared with the mock-transfected GT1-7 cells (Fig. 7*G*). These results suggest that AFF4 regulates AMPKα2 transcription.

Inhibition of Ghrelin-induced AMPK Signaling by AFF4 siRNA in GT1-7 Cells—To examine the involvement of AFF4 in ghrelin-activated AMPK signaling, we blocked AFF4 expression with siRNA in GT1-7 cells. In the early phase of the activation (15 min), silencing AFF4 with siRNA had no effect on ghrelin-induced phosphorylation of AMPKα or ACCα (Fig. 8, *A*, *C*, and *E*). The expression of AMPKα2 and ACCα was not changed by silencing AFF4 with siRNA in the early phase (Fig. 8, *A*, *D*, and *F*). On the other hand, silencing AFF4 significantly attenuated the ghrelin-induced expression of AMPKα2 and phosphorylation of ACCα in the late phase (2 h) of the activation (Fig. 8, *G*, *J*, and *K*). There were no changes in AMPKα phosphorylation or ACCα expression in the late phase (Fig. 8, *G*, *I*, and *L*).

DISCUSSION

In this study, we demonstrated that the expression of AFF4 in GHS-R-expressing neurons in the hypothalamus was increased by both fasting and ghrelin. Ghrelin directly induced the expression of AFF4 in a hypothalamic neuronal cell line (GT1-7). Ghrelin also increased the expression of AMPKα and phosphorylation of ACCα following induction of AFF4. In addition, AFF4 directly induced the expression of AMPKα2 at the transcriptional level. Furthermore, down-regulation of AFF4 in

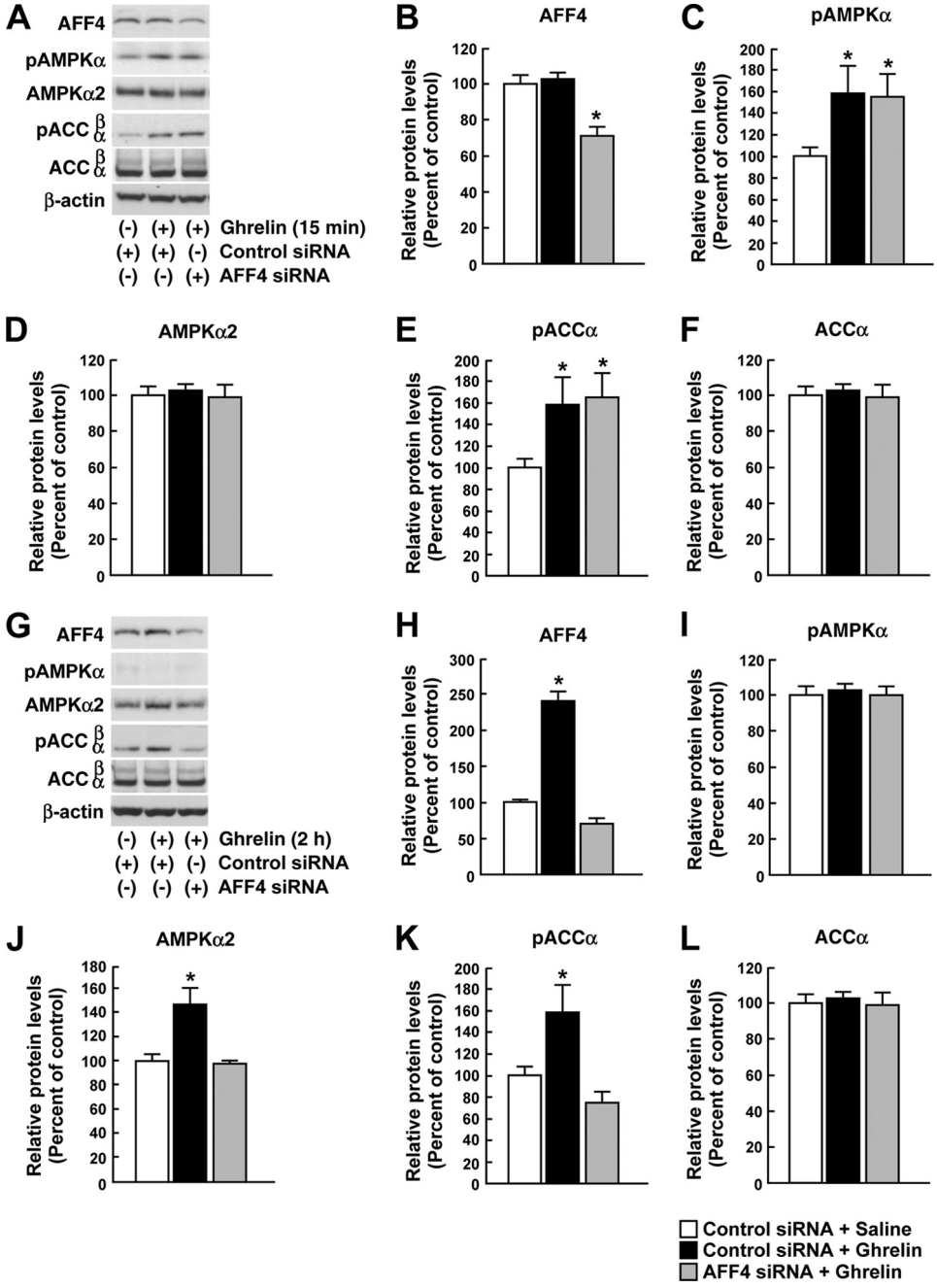

FIGURE 8. **Effects of AFF4 on ghrelin-activated AMPK signaling.** *A* and *G*, the cells were transfected with either control or AFF4 siRNA for 48 h, followed by incubation with either saline or ghrelin (100 nM) for 15 min (*A*) and 2 h (*G*). The whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-AFF4, anti-pAMPKα, anti-AMPKα2, anti-pACC, or anti-ACC antibody. The blots were stripped and reprobed with anti-β-actin antibodies to ensure equal loading of proteins. *B–F* and *H–L*, quantitative analysis of the expression of AFF4 (*B* and *H*), pAMPKα (*C* and *I*), AMPKα2 (*D* and *J*), pACCα (*E* and *K*), and ACCα (*F* and *L*). The band intensities of AFF4, pAMPKα, AMPKα2, pACCα, and ACCα were normalized to β-actin. All band intensities are represented as the percentage relative to the intensities of the controls (*white bars*) in the bar graphs. *, $p < 0.05$ (Student's *t* test).

GT1-7 cells led to decreases in ghrelin-induced expression of AMPKα and phosphorylation of ACCα in the late phase of the activation.

Orexigenic factors produced in peripheral tissues, such as ghrelin and adiponectin, are increased in the blood by fasting and enter the brain by crossing the blood-brain barrier (20–22). It is well known that GHS-R, a receptor for ghrelin, is strongly expressed in the arcuate nucleus and the VMH (23, 24), whereas the receptors for adiponectin, AdipoR1 and AdipoR2, are localized in the arcuate nucleus and the paraventricular hypothalamic nucleus (25). In addition, AdipoR1 and AdipoR2 are expressed in the astrocytes of the arcuate nucleus (25). In this study, the expression of AFF4 was increased in the neurons of the arcuate nucleus and the VMH by fasting. These results suggest that AFF4 is induced by ghrelin, rather than by adiponectin, in the hypothalamus.

Ghrelin is an acylated peptide produced predominantly in the stomach (26). Several investigators have demonstrated that both central and peripheral injections of ghrelin can stimulate food intake in rodents (27–30). Peripheral ghrelin transmits the

orexigenic signal to the hypothalamus by two pathways. In the first pathway, circulating ghrelin enters the brain by crossing the blood-brain barrier (21) and directly binds to GHS-R expressed in hypothalamic neurons. In the second pathway, ghrelin binds to GHS-R expressed in gastric vagal afferents and transmits a signal to the hypothalamus via the brainstem (31). In this study, ERK was activated in the hypothalamus 15 min after a ghrelin injection was administered, suggesting that ghrelin injected intraperitoneally can directly act on the hypothalamus. In addition, the ghrelin-induced expression of AFF4 was observed exclusively in GHS-R-positive neurons of the arcuate nucleus and the VMH. Furthermore, ghrelin induced the expression of AFF4 in hypothalamic neuronal GT1-7 cells. These results suggest that peripheral ghrelin induces the expression of AFF4 in the arcuate nucleus and the VMH, at least in part, through the humoral pathway.

Minokoshi *et al.* (19) have reported that phosphorylation of AMPKα is important in activating AMPK among three subunits: a catalytic subunit (α subunit) and two regulatory subunits (β and γ subunits). The peripheral hormones ghrelin and adiponectin increase phosphorylation of AMPKα, whereas leptin and insulin decrease phosphorylation of AMPKα in the hypothalamus (18, 22, 32). Although CaMKK2 has been recently reported to phosphorylate AMPKα in the hypothalamus (33), the expression mechanisms of AMPKα are still largely uncharacterized. In this study, overexpression of AFF4 in GT1-7 cells induced the expression of AMPKα2 mRNA and proteins. In addition, AFF4 also increased the activity of the AMPKα2 promoter. AFF4 proteins are localized in the nucleus and bind to positive transcription elongation factor-b, which activates transcription by RNA polymerase II (8). These results indicate that the expression of AMPKα2 is regulated by AFF4 at the transcriptional level. This is the first report identifying the molecule that directly induces transcription of AMPKα2.

In this study, phosphorylation of ACCα was increased in the early phase of the activation (15 min after ghrelin treatment was administered), which depends on ghrelin-induced AMPKα phosphorylation. Interestingly, in the late phase of the activation (after 2 h of ghrelin treatment), phosphorylation of ACCα was observed together with an absence of AMPKα phosphorylation. Some investigators have reported that ACCα is phosphorylated with no changes in the phosphorylation levels of AMPKα (34, 35). In addition, Horike *et al.* (36) demonstrated that overexpression of AMPKα increases phosphorylation of ACC, thus suggesting that AMPKα can increase phosphorylation of ACC without inducing phosphorylation of AMPKα. We also demonstrated that overexpression of AFF4 induced phosphorylation of ACCα without inducing phosphorylation of AMPKα. In this study, AMPKα2 expression increased, whereas no changes in the expression of other AMPK subunits (α1, β1, β2, γ1, γ2, and γ3), CaMKK2, or LKB1 was observed. Conversely, decreases in AFF4 caused by siRNA inhibited the ghrelin-induced expression of AMPKα2 and phosphorylation of ACCα only in the late phase of the activation (2 h after treatment was administered). From these findings, it follows that the increased expression of AMPKα2 caused by AFF4 may play a functionally important role in maintaining phosphorylation of ACCα under conditions with prolonged stimulation, such as fasting.

In conclusion, we demonstrated that fasting/ghrelin-induced AFF4 plays an important role in the expression of AMPKα2 and phosphorylation of ACCα in hypothalamic neurons. Our study provides strong evidence for the interplay between AFF4 and AMPK downstream signaling and thereby sheds new light on the central transcriptional mechanisms involved in the regulation of food intake.

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