Molecular Mechanisms for Activation of the Agouti-Related Protein and Stimulation of Appetite

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OBJECTIVE—The agouti-related protein (*Agrp*) is a powerful orexigenic peptide, but little is known about its transcriptional regulation. The objective of this study was to determine molecular mechanisms for the activation of hypothalamic *Agrp* and identify compounds that stimulate appetite.

RESEARCH DESIGN AND METHODS—We used promoter analyses methods, hypothalamic cell culture and transfection, immunohistochemistry, luciferase-expressing transgenic mice, in vivo bioluminescence, anitisense RNA, mouse feeding studies, indirect calorimetry, real-time PCR, and Western blots.

RESULTS—We found that the Krüppel-like factor 4 (Klf4) is a potent activator of Agrp by binding to a specific CACCC-box in its minimal promoter. We also found that an extract of tarragon, termed PMI-5011, activated hypothalamic Klf4 and Agrp. In vivo, PMI-5011 increased *Agrp* promoter activity in luciferase-expressing transgenic mice, increased hypothalamic Klf4 and Agrp expression, increased hypothalamic *Orexin* and melanin-concentrating hormone, increased food intake, reduced circulating insulin and leptin levels, attenuated energy expenditure, and enhanced body weight but only when using a high-fat diet.

CONCLUSIONS—These data show that Klf4 augmented hypothalamic Agrp by binding to a specific CACCC-box onto its minimal promoter. In addition, the tarragon extract PMI-5011 activated Klf4 and orexigenic neuropeptides and reduced peripheral insulin and leptin levels leading to positive energy balance. *Diabetes* **60**:97–106, 2011

The orexigenic agouti-related protein (AgRP) neuropeptide is expressed in neurons that are essential in adult mice for the regulation of energy homeostasis (1,2), and its overexpression leads to increased food intake (3). We have shown that the noncoding exon of *AGRP* has significant promoter activity and is sufficient to drive expression in various tissues (4–6), whereas others have reported binding sites for FOXO1 and STAT3 (7). *AGRP* polymorphisms in humans have been associated with leanness and food preference

(8–10), but little is known about its transcriptional regulation (11).

In the present study, we set out to determine molecular mechanisms for the activation of AgRP. Bioinformatic analysis of the minimal promoter of the human *AGRP* gene identified putative binding sites for the zinc finger Krüppel-like factor 4 (*KLF4*) (aliases GKLF and EZF) that binds to canonical CACCC-box motifs (12–15). Klf4 is one of the four essential transcription factors that induce pluripotent stem cells from adult fibroblasts (16) and can act as a proto-oncogene in breast cancer (17), whereas its deletion leads to embryonic death as a result of water loss across the skin surface (18). Klf4, thus, became a candidate transcription factor for the regulation of *AGRP*.

In addition, we aimed to identify bioactive compounds that activate *AGRP* and stimulate appetite. We tested a range of botanical extracts from green tea, berries, and other botanicals and found that an extract of Russian tarragon (*Artemisia dracunculus L.*), termed PMI-5011, enhanced food intake in a complex manner that involved activation of orexigenic neuropeptides and attenuation of insulin and leptin. According to ancient folklore, tarragon was used by Greeks and Persians as an appetite stimulant and a digestive aid (19) and is still used today in culinary French cuisine. Its physiological effects, however, are multifaceted. Purified extracts of Russian tarragon, and specifically PMI-5011, have been shown to have antihyperglycemic effects and to reduce insulin and glucose levels in streptozotocin-induced diabetic mice (20), whereas others have shown that it has anticarcinogenic properties (21).

Here, we report the molecular mechanism for the activation of *Agrp* by *Klf4* and describe the wider effects of the tarragon extract PMI-5011 on Agrp, Klf4, the overall orexigenic circuitry, and hormones involved in the maintenance of energy homeostasis.

RESEARCH DESIGN AND METHODS

PMI-5011 preparation. PMI-5011 is a bioactive extract of Russian tarragon (*Artemisia dracunculus L.*) consisting of six potential components (22). It was prepared as previously described from hydroponically grown plants and purified as a resin (19,20,23).

Cell culture and reagents. The human adrenocortical carcinoma cell line NCI-h295R (American Type Culture Collection, Manassas, VA) and mouse neuronal N38 (24) and GT1-7 (25) cell lines were cultured as previously described (26). PMI-5011 was dissolved in 70% ethanol or DMSO and added to the media at the concentration of 40 μg/ml.

Whole brain primary cell cultures. Two whole mouse brains were removed and transferred immediately into 7 ml of dissection media (500 ml Hank's buffered salt solution; Invitrogen, Carlsbad, CA), 1.19 g HEPES, 2.52 g glucose, 50 μl gentamicin (Invitrogen), 1.5 g BSA (Sigma, St. Louis, MO), 1.5 g Mg_2SO_4, and 111 mg $CaCl_2$. Cells were dissociated and gradually attached to the bottoms of culture plates reaching ~90% confluency in 10–12 days.

AGRP promoter constructs. A 1,107-bp *AGRP* promoter fragment (−796/+312) was amplified from human genomic DNA with Platinum Pfx polymerase (Invitrogen) using the PCR primers 5′-GTCAGCTAGCGTCTTAAACCCCTGGC CTTG-3′ and 5′-CAGTCTCGAGCCTTGGGAGTCCCCTCTAGG-3′. Amplicons was
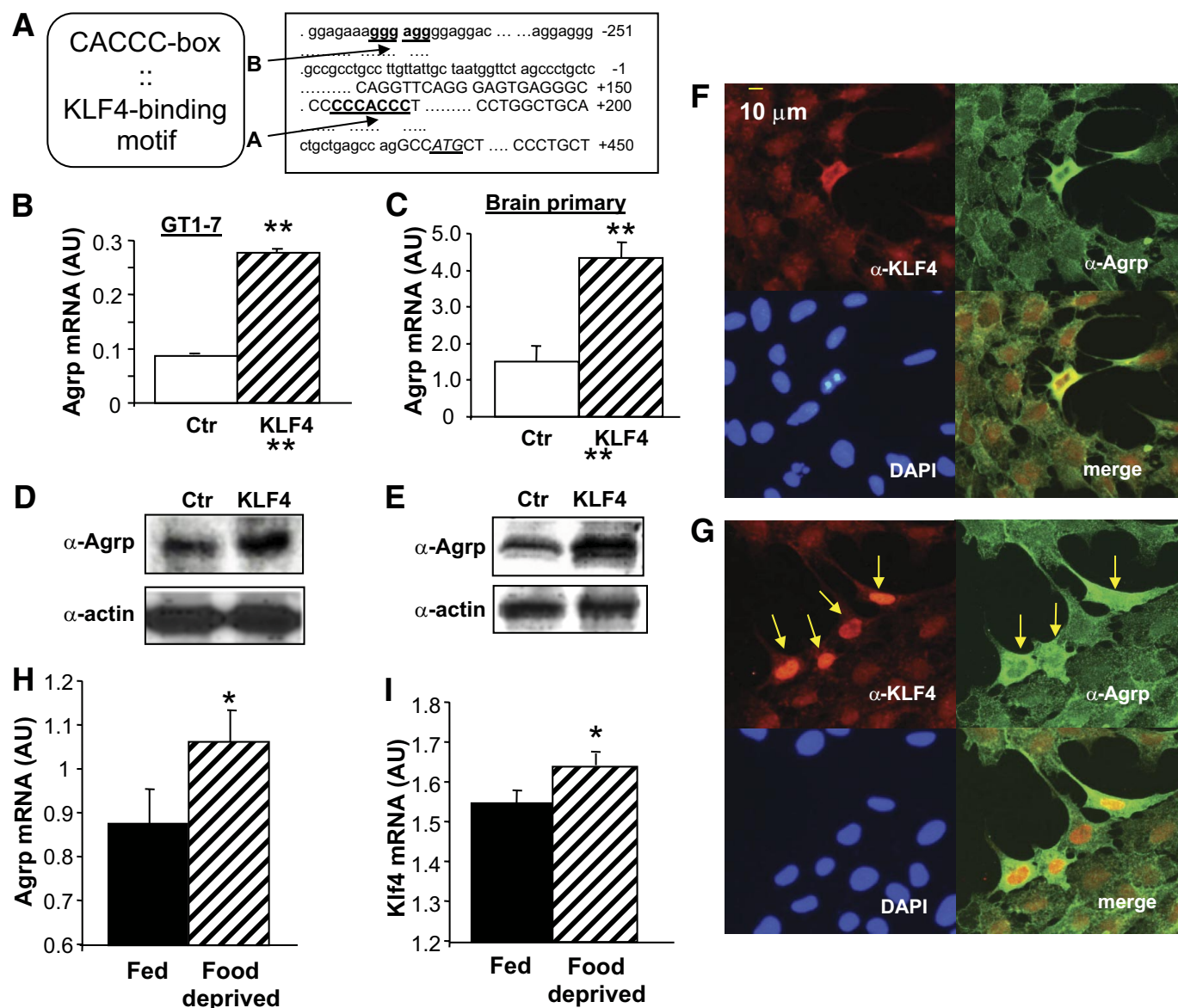
FIG. 1. Klf4 overexpression increased Agrp in GT1-7 cells, whereas overnight food deprivation augmented hypothalamic Agrp and Klf4. *A*: The minimal promoter of *AGRP* contains two CACCC-boxes (underlined) that are putative bindings sites for *KLF4* (proximal [A] and distal [B]). *B*: Transient transfection of hypothalamic GT1-7 cells with a *KLF4* expression construct increased *AGRP* mRNA and (*D*) Agrp protein levels. *C* and *E*: KLF4 overexpression also increased endogenous Agrp mRNA and protein levels in mouse whole-brain primary cultures from two male mice, respectively. *F*: Immunocytochemistry of dividing GT1-7 hypothalamic cells displaying elevated Klf4 and Agrp protein levels. *G*: Transiently transfected GT1-7 cells with a *KLF4* expression construct displayed accumulation of KLF4 in the nucleus (arrows) and overexpression of endogenous Agrp. Hypothalamic *Agrp* mRNA (*H*) and *Klf4* mRNA (*I*) were significantly increased in mice that were food deprived overnight (*n* = 6 fed mice, *n* = 7 food-deprived mice). AU, arbitrary units representing *Klf4* or *Agrp* mRNA levels adjusted by cyclophilin mRNA as endogenous control. Data are shown as means ± SE (*$P < 0.05$; **$P < 0.01$). (A high-quality digital representation of this figure is available in the online issue.)

digested with *Nhe*I/MluI and cloned into the pGL3basic luciferase vector (Promega, Madison, WI). Site-directed mutagenesis was performed as described in line n the supplementary data in the online appendix (available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0172/DC1). The numbering of DNA sequence cited in this manuscript (e.g., for the CACCC-box) was according to the sequence appearing in our previous publication (5) and GenBank accession no. AF314194.

Transient transfections. Cells were transfected with Geneporter II (Gene Therapy Systems, San Diego, CA) or Fugene 6 according to the manufacturer's protocols. Firefly and renilla luciferase activities were measured using the Dual Luciferase Kit (Promega) and a single-tube FB12 luminometer (Berthold Detection Systems, Oak Ridge, TN). More details about the constructs are provided on line in the supplementary data.

Electrophoretic mobility shift assays. Nuclear extracts from N38 cells were prepared as described elsewhere (27). A complete list of the oligonucleotides used is shown on line in the supplementary data.

Chromatin immunoprecipitation. NCI-h295R human adrenocortical cells were cultured in a 100-mm cell culture plate and transfected with *KLF4*-

expressing vector or cotransfected with *KLF4* and −796/+373 *AGRP* promoter construct for 16 h. The experimental procedure was according to the manufacturer's instructions (Upstate, Millipore, Billerica, MA). More details about the primers and conditions are provided on line in the supplementary data.

RNA extraction and real-time PCR. Total RNA was extracted from cultured cells or whole tissue using the RNeasy Mini Kit (Qiagen). Hypothalamic extracts were prepared as previously described (26). Quantitative PCR was performed using the TaqMan one-step RT-PCR core reagents kit (Applied Biosystems, Foster City, CA) as previously described (28). The primers and probes are described in detail on line in the supplementary data.

Western blotting. For Western blot analysis, total protein lysates (30–50 μg/lane) were separated on 15% or 10% SDS-PAGE and blotted to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Antibodies for Agrp were from Alpha Diagnostic International (San Antonio, TX) and Klf4 from Santa Cruz Biotechnology (Santa Cruz, CA). For loading control anti–glyceraldehyde-3-phosphate dehydrogenase (AM4300; Ambion) or β-actin (Abcam, Cambridge, MA) antibody were used. The signal was detected with
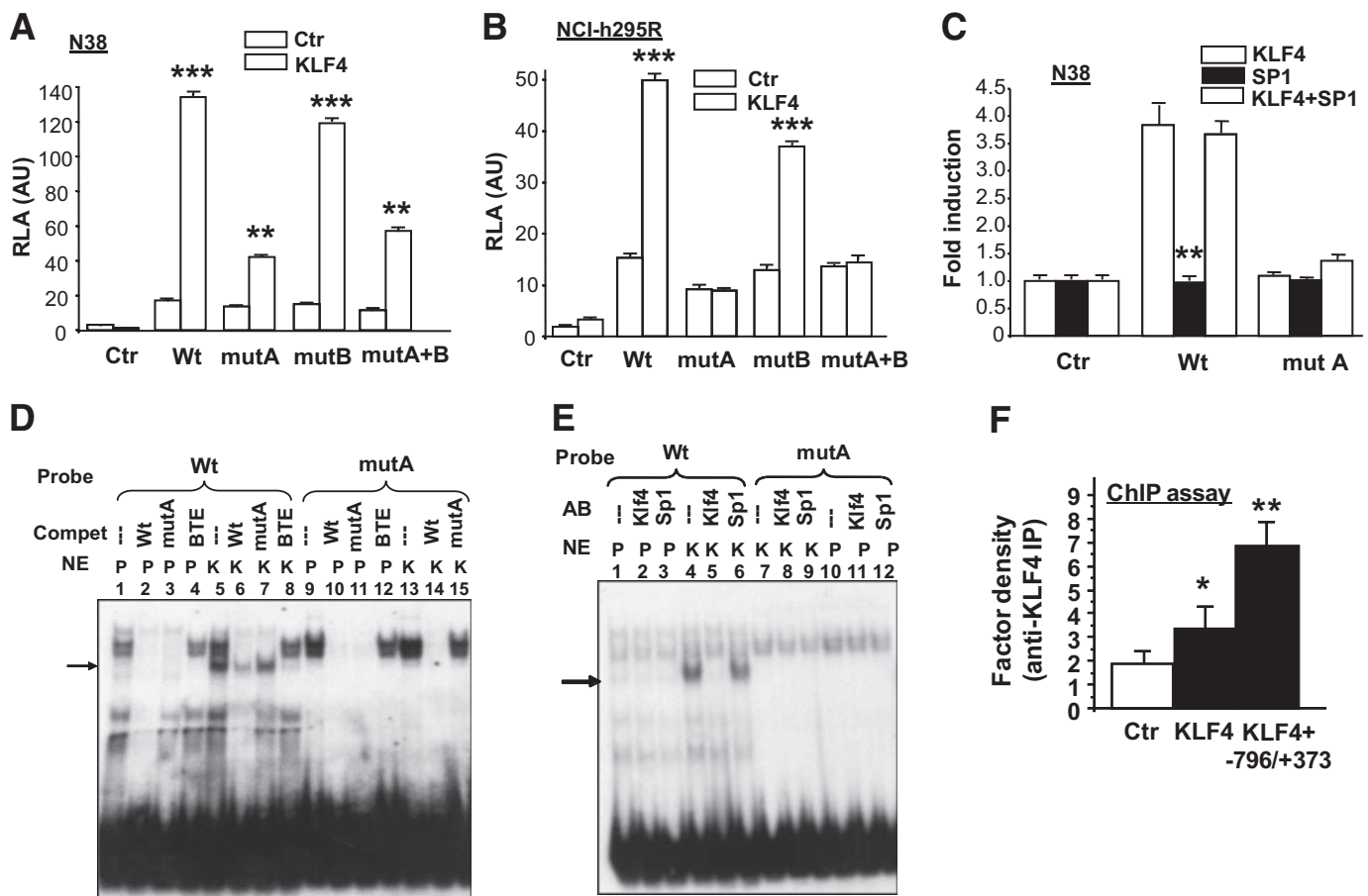
FIG. 2. *AGRP* promoter analysis showed *KLF4* specificity for the proximal CACCC-box. *A*: Mutagenesis of the proximal (mutA) but not the distal (mutB) CACCC-box significantly diminished the effect of KLF4 on *AGRP* promoter activity in transiently transfected clonal hypothalamic N38 cells. *B*: Mutagenesis of the proximal (mutA) but not the distal (mutB) CACCC-box completely abrogated the effect of *KLF4* on *AGRP* promoter activity in transiently transfected human adrenocarcinoma NCI-h295R cells. *C*: *Sp1* did not increase *AGRP* promoter activity on its own or when cotransfected with *KLF4*, whereas mutagenesis of the proximal CACCC-box (mutA) abrogated the effect of *KLF4* on its own or in combination with *Sp1*. *D*: EMSA experiments using wild-type (Wt) and mutant (mutA) competition (Compet) primers. *E*: Antibody-supershift EMSA confirmed that Klf4 directly bound the promoter of *AGRP* at the proximal CACCC-box (mutA). Nuclear extracts (NE) were obtained from clonal hypothalamic N38 cells transfected either with the empty-vector pcDNA3.1 (P) or the *KLF4* expression vector (K). The position of the KLF4-containing protein complex is marked by an arrow. Basic transcription element (BTE) corresponds to a known KLF4 binding site as the BTE of the CYP1A1 gene (30), as a positive control. *F*: Chromatin immunoprecipitation (ChIP) assays, using lysates from *KLF4*-transfected cells (second bar) or cells cotransfected with a combination of *KLF4−* and *AGRP−796/+373* promoter. Increased immunoprecipitates were found in the cotransfected cells because of enrichment for the CACCC-box (RLA, relative luciferase activity adjusted by renilla). Data are shown as means ± SE (*P < 0.05; **P < 0.01; ***P < 0.001).

Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, U.K.). Western blot images were quantified using Quantity One software (Bio-Rad).

Immunofluorescence. GT1-7 cells were plated on 12-mm glass coverslips coated with poly-D-lysine. Cells were washed in PBS and fixed in 4% paraformaldehyde. Immunocytochemistry was performed using Klf4 and Agrp antibodies (dilution 1:200) in a cocktail. This was followed by three washes in the presence of a secondary Alexa Fluor 488 goat anti-rat antibody and Alexa Fluor 594 goat anti-rabbit antibody (Invitrogen, Eugene, OR). Finally, slides were mounted in Prolong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (Invitrogen). Microphotographs of stained cells were taken with a Axioscop 2 microscope (Zeiss) at 40× magnification. Color images were captured with a Zeiss Axiocam HRc CCD camera at 1,300 × 1,030 resolution.

In vivo bioluminescence. In vivo bioluminescence imaging was conducted on an IVIS 100 system (Xenogen, Alameda, CA) using LivingImaging acquisition and analysis software (Xenogen). The rationale for the production of these mice and a full description of the constructs used is provided elsewhere (6). The mice used in this experiment contained the *Klf4*-binding CACCC-box and additional enhancer elements from downstream regions. Mice were anesthetized with isoflurane and received an intraperitoneal injection of the substrate D-luciferin (100 mg/kg). An integration time of 3 min with a binning of 100 pixels was used for luminescence image acquisition. Signal intensity was quantified as the sum of all detected photon counts within the region of interest after subtraction of background luminescence. Mice were fed chow diet (Chow 5001, 12.5% Kcal, Purina lab diet; Framingham, MA) ad libitum and

imaged the day before the start of feeding with PMI-5011 and imaged again 1 week later, at the end of the experiment.

Indirect calorimetry. The study protocols for all mouse feeding studies were approved by the institutional animal care and use committee of the Pennington Biomedical Research Center (Louisiana State University System, Baton Rouge, LA). Mice were maintained on a 12-h/12-h day/light cycle in a pathogen-free animal facility with lights coming on at 6:00 A.M. Indirect calorimetry was performed as we have previously described (29). More details are provided on line in the supplementary online data.

Body composition. Fat-free body fluid and fat-free mass were measured with a nuclear magnetic resonance instrument (Bruker Optics, The Woodlands, TX) as we have previously reported (29). Fat-free mass (i.e., muscle mass) was calculated by subtracting the fat mass and fluid mass from total body weight. Data are shown as means of grams of fat or grams of lean mass.

Mouse feeding studies with PMI-5011. Several mouse-feeding studies were performed using chow and high-fat diets (HFDs) with and without PMI-5011. Details about each study are provided on line in the supplementary data.

Statistical analyses. Statistical significance was evaluated using one-way ANOVA and the Student t test. The data were expressed as means ± SE and calculated using variance analysis and the Newman-Keuls test for multiple comparisons among groups. Bonferroni adjustments were made for multitesting. P values <0.05 were considered to be statistically significant. The same methods were used for testing for differences in circulating hormone levels. Data analysis was carried out on SAS (SAS version 9.1).

For the meta-analysis, the data from the three HFD studies were combined

to examine whether there were statistically significant differences in body weight between the HFD- and PMI-5011–fed groups. The random effects ANOVA meta-analysis model was used. The results represent the unweighted ANOVA analysis. The ANOVA analysis in a bootstrap fashion was also used, and again the *P* value was nearly identical. Data analysis was carried out on SAS (SAS version 9.1).

Nomenclature. The gene names used were according to standard nomenclature (http://www.genenames.org/ and http://www.informatics.lax.org/mglhome/nomen/gene.shtml).

RESULTS

Overexpression of Klf4 activated endogenous Agrp in hypothalamic cells. Algorithmic analysis of the human *AGRP* promoter (Tess/Transfac and Alibaba2) revealed the presence of two conserved CACCC-boxes at position $+163/+169$ (position A or proximal position), and position $-277/-283$ that is a reverse CACCC-box/G-rich stretch (position B or distal position) (Fig. 1*A*). CACCC-boxes are typical binding motifs for the transcription factor KLF4 (12–15), which became a candidate effector of *AGRP*. The direct effect of *KLF4* on *Agrp* was tested by transiently transfecting GT1-7 cells with a *KLF4* expression construct, which resulted in an increase of endogenous Agrp mRNA and protein levels (Fig. 1*B* and *D*, respectively). This effect was recapitulated in mouse whole-brain primary cultures from two male mice (Fig. 1*C* and *E*).

Immunocytochemistry was performed to determine the specificity of the effect of KLF4 on Agrp expression. Endogenous Klf4 protein was upregulated in dividing GT1-7 cells, which coincided with significant upregulation of Agrp (Fig. 1*F*). Cells that had been successfully transfected with a *KLF4* expression construct displayed significant accumulation of KLF4 in the nucleus and robust upregulation of Agrp (Fig. 1*G*), suggesting that KLF4 directly enhances endogenous Agrp expression in hypothalamic cells.

Overnight food deprivation increased hypothalamic Agrp and Klf4 expression. We assessed the effects of overnight food deprivation on mouse hypothalamic Klf4 and Agrp and found that both were upregulated in mRNA preparations (Fig. 1*H* and *I*).

KLF4 binds to a specific CACCC-box on the AGRP promoter. The effect of *KLF4* on the *AGRP* promoter was evaluated by site directed mutagenesis of the two candidate CACCC-boxes at positions A and B. The mouse clonal hypothalamic cell line (N38) and the human adrenocortical carcinoma cell line NCI-h295R were used for these experiments because they both express endogenous *Agrp* whereas the NCI-h295R cell line also represents a "human" environment because we are using the human *AGRP* promoter. Mutagenesis of the proximal (position A or mutA) but not the distal CACCC-box (mutB) diminished significantly the effect of *KLF4* on *AGRP* promoter in the mouse N38 (Fig. 2*A*) and completely abolished promoter activity in the human environment of the NCI-h295R cells (Fig. 2*B*). Because KLF4 had been suggested to interact with the Sp1 family of transcription factors (30), we used a *Sp1* expression construct but found that it had no effect on *AGRP* promoter activity, and neither did it enhance the effect of *KLF4* (Fig. 2*C*).

Electrophoretic mobility shift assays (EMSAs) revealed a complex binding pattern for vector control (P) and a specific band (arrow) in *KLF4* (K)-transfected cells (Fig. 2*D*). Preincubation with excess unlabeled wild-type probe competed out this band in the *KLF4*-transfected cells (*lane 6*) but not with the mutant probe (*lane 7*), further emphasizing the significance of the intact CACCC-box at
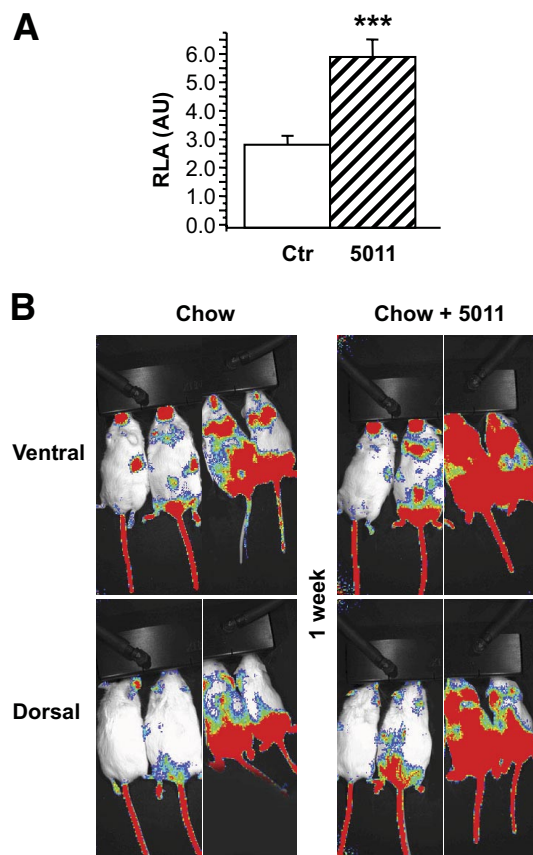
A

B

FIG. 3. PMI-5011 activated the *AGRP* promoter in vitro and in vivo. *A*: PMI-5011 treatment of GT1-7 cells that had been transiently transfected with a construct containing the minimal promoter of AgRP PMI-5011, displayed significant increase of promoter activity. The data shown are the mean of three independent experiments, each experiment represented by three replicates (±SE). *B*: Feeding luciferase-expressing transgenic mice with diet supplemented with PMI-5011 for 1 week, led to a sixfold increase of luciferase that was driven by the minimal promoter of AgRP. The *left panel* in all images shows two mice from founder A and the *right panel* shows two mice from founder B. Luciferase expression was measured as the total number of pixels using the IVIS 100 software (***P < 0.001). (A high-quality digital representation of this figure is available in the online issue.)

position A. Importantly, the complex of interest could be completely competed away with a known *KLF4* binding oligonucleotide from the cytochrome *CYP1A1* gene (30) referred to in this figure as "BTE" (Fig. 2*D*, *lane 8*). The radiolabeled mutant probe (mutA) did not result in the appearance of the additional band after incubation with the *KLF4*-transfected nuclear extracts (Fig. 2*D*, *lanes 9–15*).

In an EMSA supershift experiment, specific antibodies were used to identify the band of interest (Fig. 2*E*). Again, only *KLF4* cotransfected extracts (K) resulted in the formation of an additional band (Fig. 2*E*, *lane 4*). Binding was completely blocked with an anti-Klf4 antibody (Fig. 2*E*, *lane 5*) but not with the negative control antibody directed against Sp1 (Fig. 2*E*, *lane 6*). The mutant probe again failed to form any Klf4-specific DNA:Protein complexes (Fig. 2*E*, *lanes 7–12*).

In chromatin immunoprecipitation (ChIP) experiments, the human NCI-h295R cells were transiently transfected first with the *KLF4* expression construct, and in a second experiment they were cotransfected with the *KLF4* expression construct plus the *AGRP* $-796/+312$ promoter construct. The second experiment represented a positive
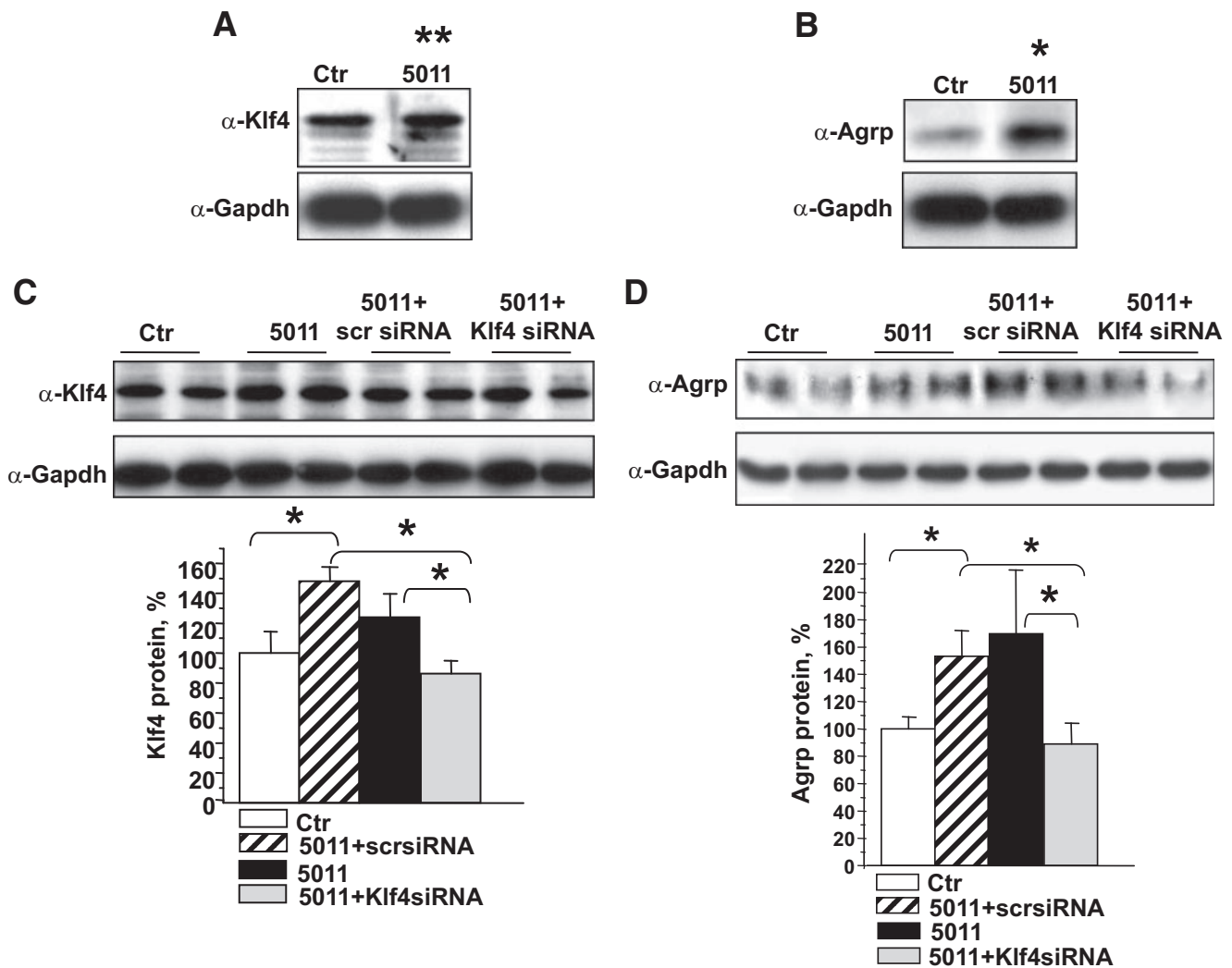
FIG. 4. Silencing of *Klf4* abrogated endogenous activation of Agrp by PMI-5011. *A*: PMI-5011 increased endogenous Klf4 protein levels in hypothalamic GT1-7 cells. *B*: PMI-5011 increased endogenous Agrp protein levels in hypothalamic GT1-7 cells. *C*: Antisense (siRNA) primers against *Klf4* blocked overexpression of *Klf4* by PMI-5011. *D*: Antisense siRNA primers against *Klf4* also abrogated the activation of Agrp by PMI-5011 (scr siRNA, scrambled siRNA, negative, control primers). Bars represent percent mean values of the control (from three independent experiments and three replicates per experiment). Data are shown as means ± SE (*$P < 0.05$; **$P < 0.01$).

control because this promoter construct contains the *KLF4*-binding CACCC-box. The coimmunoprecipitate from the first experiment was higher in the *KLF4*-transfected cells and this effect was further enhanced in the promoter cotransfected cells from the second experiment that had been enriched for the CACCC-box (Fig. 2*F*).

PMI-5011 activated the AGRP promoter in vitro and in vivo. We tested the hypothesis that a botanical extract of tarragon, PMI-5011, could activate Agrp because tarragon has traditionally been used as an appetite stimulant. First, we tested the hypothesis that PMI-5011 activates *Agrp* at the promoter level in vitro. GT1-7 cells were transient transfection with a promoter construct containing the minimal promoter of *AGRP* and cells were treated overnight with PMI-5011. The activity of the *AGRP* promoter ($-796/+373$) was significantly increased by PMI-5011 (Fig. 3*A*). We then tested the hypothesis that PMI-5011 also activates the *AGRP* promoter in vivo. We used luciferase-expressing transgenic mice whereby luciferase was driven by the minimal promoter of *AGRP* that encompasses the proximal CACCC-box at position $+163/+169$, as we have described elsewhere (6). Two mice from two

different founders were imaged while consuming a chow diet (Fig. 3*B*). Their diet was subsequently supplemented with PMI-5011 for 1 week, and the same mice were imaged again. Luciferase expression was increased sixfold by PMI-5011 in mice from both founders and at all sites of expression as illustrated by the ventral and dorsal views (Fig. 3*B*).

Silencing of Klf4 abrogated the activation of Agrp by PMI-5011. In this experiment, we tested the hypothesis that Klf4 mediates the activation of Agrp by PMI-5011. First, we showed that PMI-5011 activated both endogenous Agrp and Klf4 in GT1-7 cells (Fig. 4*A* and *B*, respectively). The specific requirement of *Klf4* for the activation of *Agrp* by PMI-5011 was confirmed using antisense siRNA probes against *Klf4*. First, the efficacy of siRNA primers against Klf4 was confirmed in GT1-7 cells by blocking the upregulation of Klf4 in cells treated with PMI-5011 (Fig. 4*C*), whereas the control (scrambled) primers had minimal effect. siRNA against *Klf4* also abrogated the upregulation of Agrp by PMI-5011 (Fig. 4*D*).

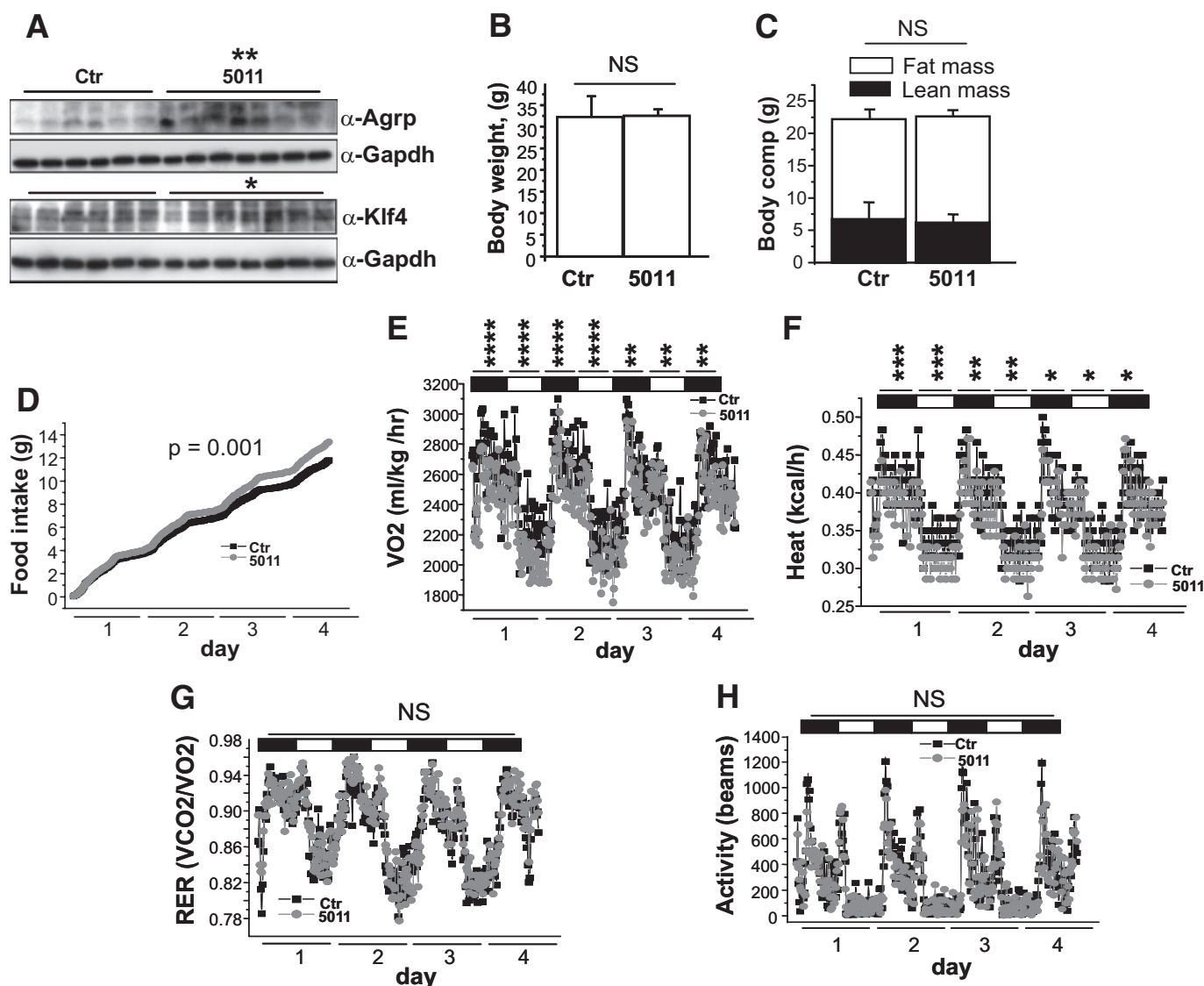**PMI-5011 increased endogenous Klf4 and Agrp, stimulated food intake, and reduced energy expenditure.**

FIG. 5. PMI-5011 increased Agrp/Klf4 and food intake and reduced energy expenditure (indirect calorimetry experiment). *A*: Hypothalamic Agrp and Klf4 protein levels were higher in the PMI-5011–fed group ($n = 6$ in control group and $n = 7$ in PMI-5011 group) in metabolic chambers. Starts above Westerns indicate statistical significance at $P < 0.05$. *B*: Body weight did not differ between Control (Ctr) and PMI-5011–fed mice. *C*: Body composition did not differ between control and PMI-5011–fed mice. *D*: Diurnal food intake was significantly higher in the PMI-5011 group (*P* value by ANOVA analysis). Error bars are depressed because of the high density of measurements (every 15 min) by 8.3% in control group and 4.6% in the PMI-5011 group. *E*: Oxygen consumption was significantly lower in the PMI-5011–fed group. Error bars are less than symbol size by 6.5% in control group and 7.0% in the PMI-5011 group. *F*: Heat production was significantly lower in the PMI-5011–fed group. Error bars are less than symbol size by 5.3% in control group and 7.8% in the PMI-5011 group. *G*: Respiratory exchange ratio (RER) did not differ between control and PMI-5011–fed mice. Error bars are lower by 1.1% in control group and 1.8% in the PMI-5011 group. *H*: Total activity did not differ between control and PMI-5011–fed mice. Light and dark periods in *E–H* are shown above each graph by open and filled bars, respectively, along with the *P* value between pairs of groups. Data are shown as means ± SE (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

The in vivo effects of PMI-5011 on energy expenditure and body composition were evaluated by indirect calorimetry in metabolic chambers. Two groups of mice of equal mean body weights received chow or chow plus PMI-5011 diets, and metabolic parameters were measured for 4 days. Hypothalamic Klf4 and Agrp protein levels were significantly higher in the PMI-5011 group (Fig. 5*A*). Body weight and body composition (fat and fat-free mass) were not affected by PMI-5011 (Fig. 5*B* and *C*), but food intake was significantly higher (Fig. 5*D*). Oxygen consumption and energy expenditure (heat production) were significantly lower in the PMI-5011–fed group (Fig. 5*E* and *F*). The Respiratory exchange ratio (RER) and total activity were not affected by PMI-5011 (Fig. 5*G* and *H*).

PMI-5011 increased Agrp, food intake, and body weight under a HFD. Because body weight was not increased as it would be expected given the increased food intake and reduced energy expenditure, another study was performed to examine the long-term (24-day) effects of PMI-5011 on food intake still under a chow diet. Hypothalamic Klf4 and Agrp protein levels, as well as total food intake, were significantly higher in the PMI-5011–fed group of mice (Fig. 6*A* and *B*), but body weight was again unaffected (data not shown). We then examined the hypothesis that a higher fat content may be required to be present in the diet for effects of PMI-5011 to become evident on body weight. We examined the data from three previously performed studies that had used PMI-5011 in a
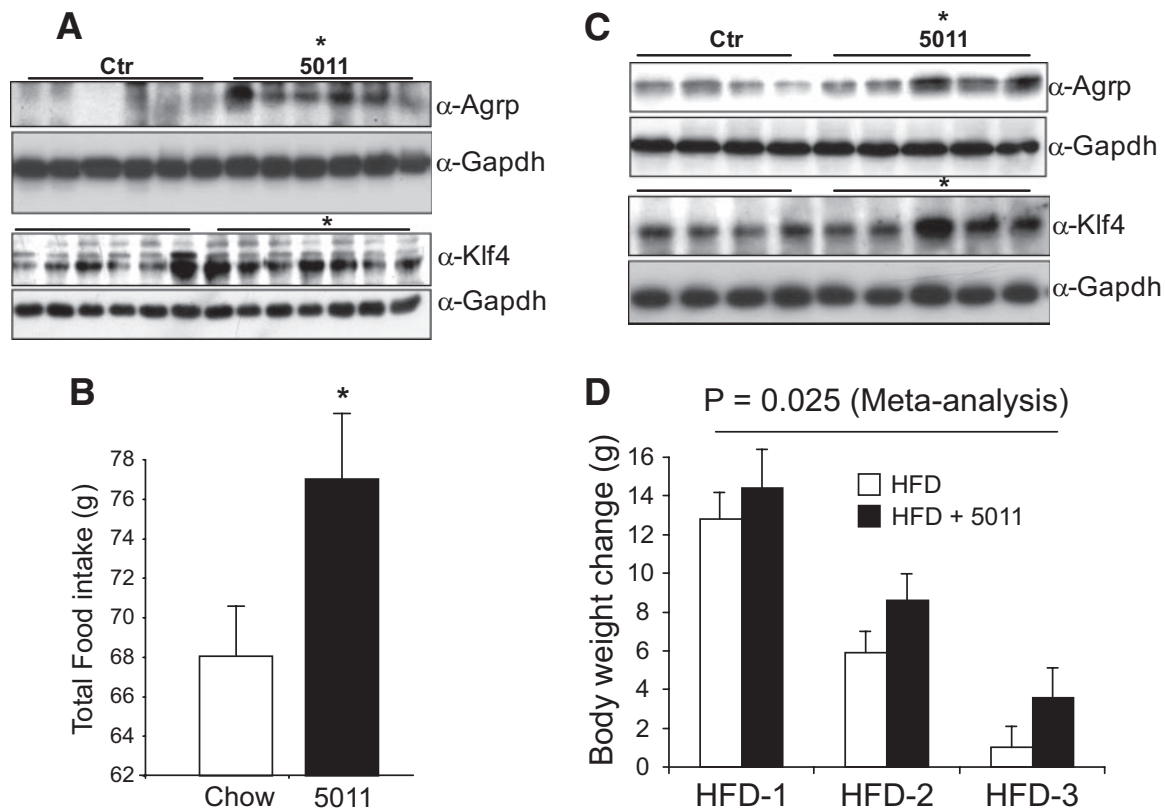
FIG. 6. PMI-5011 increased Agrp/Klf4 and food intake, but body weight was increased only under an HFD. *A*: Agrp and Klf4 protein levels were significantly higher in the group receiving PMI-5011 in a chow diet for 24 days. $n = 6$ in the chow diet group and $n = 7$ in the chow diet plus PMI-5011 group. *B*: Total food intake was significantly higher in the PMI-5011–fed group. *C*: Agrp and Klf4 protein levels were significantly higher in the mice receiving PMI-5011 in HFD (data shown for HFD-1). *D*: Meta-analysis of the combined HFD studies showed significantly higher body weights for the PMI-5011–fed mice. Data are shown as means ± SE (*$P < 0.05$).

HFD. We found that body weight of the mice receiving the PMI-5011 was enhanced in all three studies and both Agrp and Klf4 were significantly elevated (Fig. 6*C*). A meta-analysis was then performed using the data from all three studies that showed a statistically significant increase for the overall body weight of mice consuming HFD supplemented with PMI-5011 (Fig. 6*D*).

PMI-5011 reduced circulating levels of leptin and insulin. We measured the effects of PMI-5011 on the circulating levels of hormones known to affect Agrp expression and energy balance: insulin, leptin, ghrelin (active), peptide tyrosine tyrosine (PYY) (total), and gastric inhibitory polypeptide or glucose-dependent insulinotropic peptide (GIP) (total). In this experiment, C57BL/6 male mice were fed ad libitum HFD or HFD plus PMI-5011 for 11 days. PMI-5011 was provided by gavage. Insulin and leptin were significantly lower in the PMI-5011 group (Fig. 7*A* and *B*) but ghrelin, PYY, and GIP were not significantly affected (Fig. 7*C–E*).

PMI-5011 stimulated Mch and Orexin expression. In addition to *Agrp*, the expression levels of other orexigenic and anorexigenic neuropeptides were measured in the hypothalami of control and PMI-5011 chow-fed mice that had been used in the metabolic chambers. Overall, hypothalamic *Agrp* mRNA levels were increased in the PMI-5011 group (Fig. 8*A*), and this effect was more pronounced in four of seven mice (i.e., high responders $P < 0.05$, not shown here). Neuropeptide Y (*Npy*) mRNA levels were not significantly affected by PMI-5011 (Fig. 8*B*), but the mRNA levels of the other orexigenic peptides, *Orexin* and *Mch*, were significantly higher in the PMI-5011 group (Fig. 8*C*

and *D*). The mRNA levels of the anorexigenic peptides *Pomc* and *Cart* were not significantly affected by PMI-5011 (Fig. 8*E* and *F*).

DISCUSSION

In the present study, we investigated the effects of a bioactive extract of tarragon, PMI-5011, on appetite stimulation. Agrp was our primary target because of it is a well-characterized appetite effector (10,11,31). We found that PMI-5011 significantly upregulated the promoter of the human *AGRP* gene and increased its expression levels in neuroblastoma cells as well as in mouse hypothalamic primary cell cultures. A landmark feature of hypothalamic Agrp is its upregulation by food deprivation (3,26,32). We found that Klf4 was also upregulated by overnight food deprivation. This suggests that the two genes may be coregulated. We do not know if Klf4 upregulation precedes that of Agrp, which would enhance the notion that it may be involved in the upregulation of Agrp by food deprivation in vivo. Further experiments would be required to confirm this possibility.

Using bioinformatics tools, *KLF4* was identified as a candidate transcription factor for the activation of *AGRP* because of the presence of conserved CACCC-boxes along its promoter, which are typical binding motifs for *KLF4* (33,34). PMI-5011 was found to augment both Klf4 and Agrp protein levels, whereas transient transfections of cells with a *KLF4* expression construct confirmed the direct stimulation of Agrp. Further experiments using
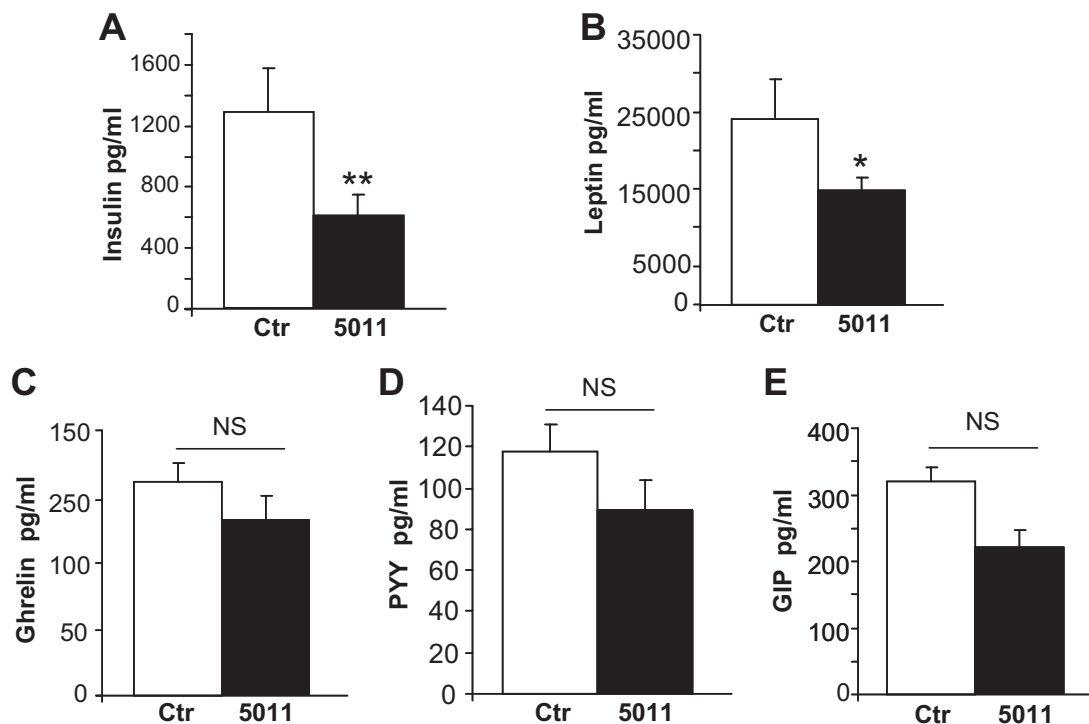
FIG. 7. PMI-5011 reduced the circulating levels of insulin and leptin. PMI-5011 was provided by gavage to mice fed a HFD ad libitum. $n = 8$ in control group and $n = 7$ in PMI-5011 group). Insulin (*A*) and leptin (*B*) were significantly reduced by PMI-5011. Ghrelin (*C*), PYY (*D*), and GIP (*E*) were lower in the PMI-5011 group but at marginal levels. Statistical differences were determined by ANOVA and Bonferroni post hoc corrections. Data are shown as means ± SE (*$P < 0.05$; **$P < 0.01$).

siRNA showed that Klf4 is required for the upregulation of Agrp by PMI-5011.

Two candidate CACCC-boxes at positions +163/+169 and −277/−283 on the *AGRP* promoter were studied, but only the proximal box was found to be a functional binding site for *KLF4*. Subsequent EMSA, supershifts, and ChIP experiments established the significance of the proximal CACCC-box for the binding of *Klf4*. Luciferase-expressing transgenic mice with luciferase driven by the *AGRP* promoter encompassing the KLF4-binding CACCC-box at position +163/+169 confirmed in vivo the activation of the *AGRP* promoter by PMI-5011.

Feeding studies were performed to evaluate the effects of PMI-5011 on Klf4/Agrp and other appetite-regulating neuropeptides and to assess its effects on overall metabolic and physiological parameters. In a study using indirect calorimetry, PMI-5011 increased hypothalamic Klf4/Agrp and stimulated food intake. The respiratory exchange ratio and total locomotor activity were not affected, but oxygen consumption and energy expenditure were significantly lower in the PMI-5011–fed group. Based on these outcomes, one would expect the PMI-5011–fed mice to gain body weight, but this was not the case probably because of the short period of feeding and/or because of the type of diet used (chow). Thus, a 24-day feeding experiment was performed to test the hypothesis that PMI-5011 may require additional time to confer body-weight gain. Food intake and hypothalamic Klf4/Agrp protein levels were again significantly higher in the PMI-5011–fed group, but body weight was again not affected. We therefore examined the effects of PMI-5011 in three previous studies that had used HFD to determine whether a high fat content was required for PMI-5011 to affect body weight. In all three studies, body weight was increased consistently and data meta-analysis combining the three

studies showed statistical significance. Food intake had not been measured in these mice because these studies had been set up to measure the effects of PMI-5011 on insulin sensitivity under severe obesigenic conditions (35). These data show that hypothalamic increases in Klf4 and Agrp by PMI-5011 can lead to increased food intake but body weight is affected only under HFD.

We also found that PMI-5011 reduced the circulating levels of insulin and leptin that are known inhibitors of hypothalamic Agrp (36). The gut-secreted glucagon-like peptide 1 (GLP-1) is also affected by tarragon (20), whereas PMI-5011 has antidiabetic effects (20), and, importantly, it attenuates expression of protein tyrosine phosphatase 1B (PTP-1B) (35). Attenuation or deletion of PTP-1B confers resistance to body-weight gain (37), and we speculate that our mice did not gain significantly in body weight by PMI-5011 because of peripheral reduction in PTP-1B (that was not measured in our experiments). These multifaceted effects of PMI-5011 could be because of one or more of its six components (22). Each component (or several of them in concert) could thus have specific or pleiotropic effects on metabolic processes, perhaps by crossing the blood brain barrier, and/or by modulating peripheral hormones like insulin and leptin.

The complex effects of PMI-5011 are further underscored by the fact that *Orexin* and *Mch* were also upregulated, whereas the anorexigenic *Pomc* and *Cart* were not significantly affected. It merits further investigation to determine whether Klf4 is the mediating transcription factor that activates *Orexin* and *Mch* that express in different neurons. By algorithmic analysis, we found that *Orexin* has a CACCC-box in its minimal promoter, but we do not know if it is functional. We did not find a putative CACCC-box on the *Mch* promoter, but Klf4 interacts with SP1 (30), and it could use SP1 binding sites on these two
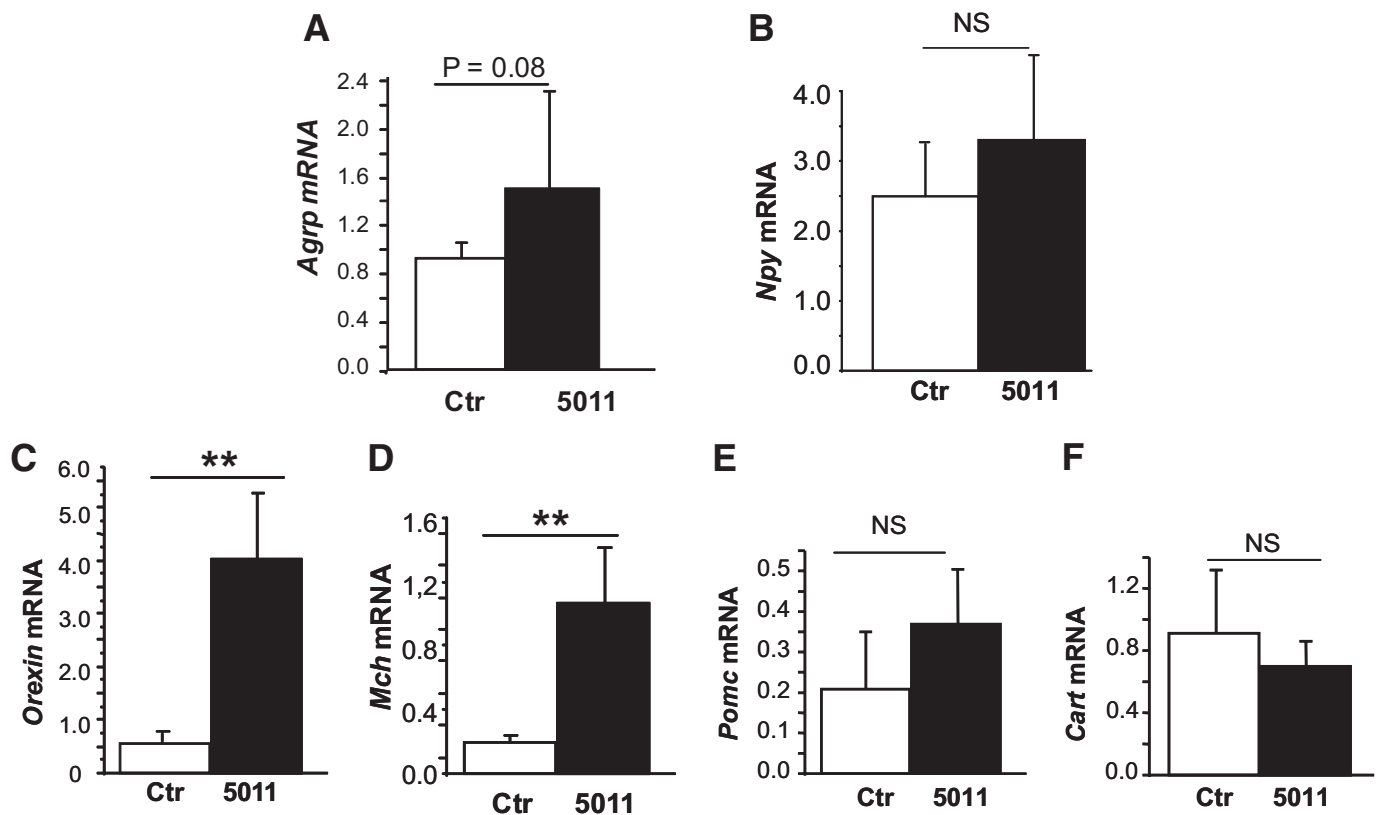
FIG. 8. PMI-5011 increased *Agrp*, *Mch*, and *Orexin* mRNA. *A*: Hypothalamic *Agrp* mRNA mRNA levels were higher in the PMI-5011–fed group in metabolic chambers (*P* = 0.08) (mice were the same as in Fig. 6). *B*: *Npy* mRNA levels were not affected by PMI-5011. *Orexin* (*C*) and melanin-concentrating hormone (*Mch*) (*D*) mRNA levels were significantly higher in the PMI-5011 group. Proopiomelanocortin (*Pomc*) mRNA (*E*) and cocaine and amphetamine-regulated transcript (*Cart*) mRNA (*F*) levels were not significantly affected by PMI-5011. *n* = 6 in control group and *n* = 7 in the PMI-5011 group. Data are shown as means ± SE (**P < 0.01).

orexigenic peptides. Importantly, these data show that PMI-5011 affects the expression levels of multiple orexigenic neuropeptides in a complex fashion that may extend beyond Klf4 and involve other transcription factors and perhaps the hypothalamic receptors of peripheral hormones that are also affected by PMI-5011 (e.g., insulin, leptin).

The data presented here identify Klf4 as a major transactivator of Agrp. In addition, we report that the tarragon extract PMI-5011 has complex metabolic effects that include an increase in hypothalamic Klf4 and food intake, activation of multiple components of the orexigenic circuitry (i.e., Agrp, Mch, Orexin), and reduction of insulin and leptin circulating levels. Klf4 is typically downregulated in colorectal cancers (13,17,38,39) and has anticarcinogenic properties when normally expressed (21). PMI-5011 could thus be used for enhancing Klf4 and appetite in conditions like anorexia and cancer cachexia.

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