ORIGINAL ARTICLE

Thyroid Transcription Factor-1 Regulates Feeding Behavior via Melanocortin Pathway in the Hypothalamus

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OBJECTIVE—α-Melanocyte–stimulating hormone (α-MSH) and agouti-related peptide (AgRP) control energy homeostasis by their opposing actions on melanocortin receptors (MC3/4R) in the hypothalamus. We previously reported that thyroid transcription factor-1 (TTF-1) controls feeding behavior in the hypothalamus. This study aims to identify the function of TTF-1 in the transcriptional regulation of AgRP and α-MSH synthesis for the control of feeding behavior.

RESEARCH DESIGN AND METHODS—TTF-1 activity in AgRP and pro-opiomelanocortin (POMC) transcription was examined using gel-shift and promoter assays and an in vivo model of TTF-1 synthesis inhibition by intracerebroventricular injection of an antisense (AS) oligodeoxynucleotide (ODN). Double immunohistochemistry was performed to colocalize TTF-1 and AgRP or α-MSH in the hypothalamic arcuate nucleus (ARC). To determine whether TTF-1 action on food intake is mediated through MC3/4R, we measured changes in food intake upon intracerebroventricular injection of MC3/4R antagonists (SHU9119 and AgRP) into rat brain preinjected with the AS ODN.

RESULTS—TTF-1 stimulated AgRP but inhibited POMC transcription by binding to the promoters of these genes. TTF-1 was widely distributed in the hypothalamus, but we identified some cells coexpressing TTF-1 and AgRP or α-MSH in the ARC. In addition, intracerebroventricular administration of leptin decreased TTF-1 expression in the hypothalamus, and AS ODN-induced inhibition of TTF-1 expression decreased food intake and AgRP expression but increased α-MSH expression. Anorexia induced by the AS ODN was attenuated by the administration of MC3/4R antagonists.

CONCLUSIONS—TTF-1 transcriptionally regulates synthesis of AgRP and α-MSH in the ARC and affects feeding behavior via the melanocortin pathway. ***Diabetes* 60:710–719, 2011**

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The central nervous system plays a critical role in maintaining energy homeostasis by regulating both energy intake and expenditure, and the hypothalamus is a core site that integrates the peripheral and central signals.

Neuropeptidergic, monoaminergic, and endocannabinoid systems are involved in the central control of appetite. A key system for regulation of energy homeostasis includes functionally opposing neuronal populations in the arcuate nucleus (ARC) that express neuropeptide Y (NPY) and agouti-related peptide (AgRP) to stimulate food intake and proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) to induce anorexia (1).

The melanocortin (MC) signaling pathway in the hypothalamus is important for energy homeostasis (2). Regulation through this pathway is exerted by two opposing neuronal components: AgRP and α-melanocyte–stimulating hormone (α-MSH), which is produced from a POMC precursor protein. α-MSH activates target neurons expressing MC-3 (MC3R) and MC-4 (MC4R) receptors, increases energy expenditure, and decreases food intake, whereas AgRP counteracts the anorexic effect of α-MSH as an MC3/4R antagonist (2). Thus a characteristic obese phenotype, typified with hyperphagia, increased linear growth, and metabolic defects, appears in mice bearing *POMC*- and *MC4R*-null mutations and in transgenic mice overexpressing AgRP (3–5). Likewise, humans with an *MC4R* mutation also show severe early-onset obesity (6). Together, these studies suggest the importance of the central MC signaling pathway in the regulation of food intake and energy expenditure.

Thyroid transcription factor-1 (TTF-1), also known as Nkx2.1 and T/ebp, is a transcription factor that was first identified in the thyroid gland (7). TTF-1 is also expressed in discrete regions of the postnatal rat brain, where it regulates the synthesis of neuropeptides and proteins involved in the control of body homeostasis (8–13). The ARC is one of the hypothalamic nuclei that exhibits strong TTF-1 expression in the postnatal rat brain (9,14). We have previously reported that TTF-1 synthesis blockade in the hypothalamus results in decreased food intake and body weight (14). However, the mechanism by which TTF-1 regulates food intake is largely unknown. Here we show that TTF-1 plays a critical role in the control of the MC pathway by regulating *AgRP* and *POMC* gene transcription in the ARC.

RESEARCH DESIGN AND METHODS

Cell culture and promoter assays. Rat neuroblastoma B35 cells and mouse pituitary adenoma AtT-20 cells were grown in Dulbecco's modified Eagle's medium supplemented with high glucose (4.5 g/L) and 10% fetal bovine serum

at 37°C in a humidified atmosphere with 5% CO_2. Cells were transiently transfected with AgRP (AGRP-pGL3 in B35 cells) or POMC (POMC-pGL3 in AtT-20 cells) promoter-luciferase reporter constructs and various concentrations (100–500 ng/well) of the TTF-1 expression vector TTF-1-pcDNA. Lipofectamine/PLUS (Invitrogen Life Technologies, Gaithersburg, MD) was used to improve the transfection efficacy, which was normalized by cotransfecting the β-galactosidase reporter plasmid pCMV-β-gal (Clontech, Palo Alto, CA) at 20 ng/well. The transfection efficiency of this method, as measured by the green fluorescent protein (GFP) expression ratio after transfecting GFP-conjugated TTF-1, was ~70% (data not shown). Cells were harvested 24 h after transfection for luciferase and β-galactosidase assays.

DNA constructs. The human *AgRP* promoter (15) (NCBI GenBank database accession no. AF314194) inserted into a luciferase reporter plasmid (16) was provided by Dr. M.S. Kim (College of Medicine, University of Ulsan, Seoul, Korea). The proximal promoter of the rat *POMC* gene (−706 to +3 bp) was cloned by PCR from rat genomic DNA, based on sequence information (17) (NCBI GenBank database accession no. X03171; sense primer, 5′-GCT TCC ACT TCC CTC CAC AGA-3′; antisense primer, 5′-TTT GGT CCC TGT CAC TCT TCT-3′), and was inserted into a pGL-3 basic vector (Promega, Madison, WI). Mutant *AgRP* and *POMC* promoter constructs carrying deletions of the core TTF-1 binding motifs were generated using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To inhibit endogenous TTF-1 synthesis in B35 and AtT-20 cells, cells were transfected with the pLKO.1-Puro vector (Sigma-Aldrich) carrying a small hairpin RNA (shRNA) that targeted the 30th to 50th nucleotides (5′-GTT CTC AGT GTC TGA CAT CTT-3′) in the TTF-1 mRNA sequence (18).

Electrophoretic mobility shift assay. The expression and purification of the TTF-1 homeodomain (TTF-1 HD) protein has been described previously (19). Double-stranded oligodeoxynucleotide (ODN) probes, containing putative TTF-1 core binding motifs and their flanking sequences from the *AgRP* and *POMC* promoter regions (Supplementary Figs. 1 and 2), were synthesized as complementary pairs, end-labeled with [γ-^{32}P]ATP in a reaction with T4 DNA polynucleotide kinase, and purified over a NICK column (Amersham Pharmacia). ODNs C and Cβ were used as a positive and negative control, respectively (20). Electrophoretic mobility shift assay (EMSA) was performed as previously described (10,11) using the TTF-1 HD protein at 75 and 150 nmol/L with ODNs at 5 mmol/L. To determine the binding activity of hypothalamic nuclear extracts to probes containing TTF-1 binding domains, nuclear protein from rat hypothalamus was prepared using a nuclear protein extraction kit (Pierce, Rockford, IL). The binding assay was performed using 15 μg of protein and 20,000 counts per minute of probe (−133 in the *AGRP* promoter and −566 in the *POMC* promoter). To confirm the presence of immunoreactive TTF-1 in nuclear extracts, proteins were incubated with 1 μl of undiluted TTF-1 antiserum (NeoMarkers, Fremont, CA) for 30 min at room temperature before the binding reactions.

Animals. Two-month-old male Sprague-Dawley rats (Daehan Animal Breeding Company, Chungwon, Korea) were used, and animal experiments were conducted as required by the Regulations of the University of Ulsan for the Care and Use of Laboratory Animals. Rats were housed in a room with a conditioned photoperiod (12-h light/12-h dark cycle; lights on from 0600–1800 h) and temperature (23–25°C) and allowed ad libitum access to tap water and pelleted rat chow.

Micropunch dissection. Rats were killed by decapitation, and brains were rapidly frozen in 2-methylbutane on dry ice for 5 min. Brains were sectioned (500-μm thickness) in a cryostat at −15°C and then mounted on glass slides. Using anatomical landmarks from a rat brain atlas, the ARCs were punched out using a micropunching set (Stoelting, Wood Dale, IL) and were kept in microcentrifuge tubes at −80°C.

Real-time PCR. RNA was isolated from the ARC using Trizol reagent (Sigma-Aldrich) and then reverse-transcribed and amplified by real-time PCR using the following primer sets: AgRP sense primer, 5′-TCC CAG AGT TCT CAG GTC TA -3′; AgRP antisense primer, 5′-CTT GAA GAA GCG GCA GTA G -3′; POMC sense primer, 5′-GCT AGG TAA CAA ACG AAT GG-3′; POMC antisense primer, 5′-GCA TTT TCT GTG CTT TCT CT-3′; GAPDH sense primer, 5′-TGT GAA CGG ATT TGG CCG TA-3′; and GAPDH antisense primer, 5′-ACT TGC CGT GGG TAG AGT CA-3′. Real-time PCR was performed using a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA) for ~40 cycles.

Western blot analysis. ARC fragments were homogenized in tissue protein extract reagent (Pierce) containing protease inhibitor cocktail (1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 3 mmol/L aprotinin) and 1 mmol/L sodium orthovanadate (pH 6.8). Electrophoretically separated polypeptides were transferred onto a nitrocellulose membrane at 20 mA for 16 h. After incubation with monoclonal mouse TTF-1 (1:2,000; NeoMarkers) or phosphorylated STAT3 (pSTAT3) (1:1,000; Cell Signaling, Beverly, MA) antibody for 1 h, bound antibody was detected using an enhanced chemiluminescence kit (Pierce).

Immunohistochemistry. Rats were anesthetized with tribromoethanol (250 mg/kg body weight; Sigma-Aldrich) and transcardially perfused with 100 mL phosphate buffer (PB; pH 7.4), followed by 100 mL 4% paraformaldehyde. Slide-mounted sections were prepared with a cryostat. We followed the immunohistochemistry (IHC) protocol described previously (14), using primary antibodies (anti-TTF-1 [1:1,000; NeoMarkers], anti-AgRP [1:100; Santa Cruz Biotechnology, Santa Cruz, CA], anti-α-MSH [1:10,000; Millipore, Billerica, MA], and anti-STAT3 [1:200; Cell Signaling]) and secondary antibodies (anti-mouse IgG [1:500; Vector Laboratories, Burlingame, CA] for TTF-1 and anti-goat IgG [1:500; Vector Laboratories] for AgRP, α-MSH, and STAT3). Immunofluorescence was visualized using the TSA System (NEN Life Science, Boston, MA). For double immunofluorescence detection, after generation of the first signals, sections were incubated in 0.3 mol/L H_2O_2 for 30 min at 25°C, followed by three 10-min washes in PB, and then developed. To determine the percentage of cells that coexpress TTF-1 and AgRP or α-MSH in the ARC, double-labeled cells were counted under microscopy (three sections per rat).Two observers independently counted the cells and defined colocalization as overlapping of double immunoreactivities (TTF-1 and AgRP or α-MSH) with counter nuclear staining (Hoeschst).

Combined IHC fluorescence in situ hybridization. The ObRb (a long-form leptin receptor) cDNA template was cloned by PCR based on sequence information deposited in the NCBI GenBank database (accession no. AF281268) and linearized by *Spe*I and *Sac*2 digestion to produce either sense or antisense RNA probes, respectively. In vitro transcription of biotin-labeled ObRb cRNA probes was performed using an RNA labeling mix (Roche, Mannheim, Germany) and SP6 and T7 RNA polymerases (Promega). We performed fluorescence in situ hybridization (FISH) experiments according to a previous report (13) by incubating brain sections with an antisense (or sense for negative control) RNA probe for ObRb overnight at 63°C. The mRNA signals of ObRb were visualized using a fluorescein isothiocyanate-conjugated TSA system (NEN Life Sciences). To colocalize ObRb mRNA with TTF-1, IHC with TTF-1 antibody was performed as described above.

Administration of materials. A polyethylene cannula (outer diameter, 1.05 mm; inner diameter, 0.35 mm) was stereotactically implanted into the lateral ventricle (coordinates: AP = 1.0 mm caudal to the bregma; V = 3.6 mm from the dura mater; L = 0.16 mm from the midline). After 1 week of recovery, test materials were injected through the cannula.

To determine whether leptin regulates hypothalamic TTF-1 expression, rats were fasted overnight and then injected intracerebroventricularly or intraperitoneally with recombinant rat leptin (4 μg i.c.v. and 2 mg/kg i.p. injection; R&D Systems, Minneapolis, MN). Rats were killed 1 h after leptin injection.

To block endogenous TTF-1 expression in the hypothalamus, a phosphorothioate antisense (AS) ODN (GenoTech, Daejeon, Korea) was delivered into the lateral ventricle of adult male rats. The sequence of the AS ODN used to disrupt TTF-1 synthesis and its scrambled (SCR) control sequence were designed as described previously (10). ODNs were diluted to a final concentration of 0.5 nmol/μL in artificial cerebrospinal fluid (10). To determine its effect, AS ODN (2 nmol) was injected daily with an infusion syringe pump (KDS 100; KD Scientific, Holliston, MA) up to 7 days. The animals were killed 12 h after ODN injection, and total RNA samples were prepared.

To determine whether MC3/4R mediates anorexia that was induced by blocking hypothalamic TTF-1 synthesis, rats fasted overnight were intracerebroventricularly injected daily with SHU9119 (Phoenix Pharmaceuticals, Burlingame, CA) and AgRP (Phoenix Pharmaceuticals) 4 h after AS TTF-1 ODN injection for 3 days. Immediately thereafter, rats were allowed access to food ad libitum, and their cumulative food intake was measured 6 and 24 h or 3 days after the injection.

Statistics. Student *t* test was used for comparison of two groups. Differences among more than three groups were analyzed by one-way ANOVA with Dunnett multiple comparison post hoc tests. For analysis of daily food intake, two-way ANOVA was used with Bonferroni post hoc tests.

RESULTS

TTF-1 regulates *AgRP* and *POMC* promoter activities. Examination of the 5′-flanking region of the *AgRP* and *POMC* genes revealed that both regions contain several consensus core motifs for TTF-1 binding (19; Supplementary Figs. 1 and 2). Thus we performed assays for these promoter activities affected by TTF-1. Cotransfection with increasing concentrations of the TTF-1 expression vector resulted in a dose-dependent increase in *AgRP* promoter activity (Fig. 1*A* and Supplementary Fig. 3*A*) but a dose-dependent decrease in *POMC* promoter activity (Fig. 1*B*

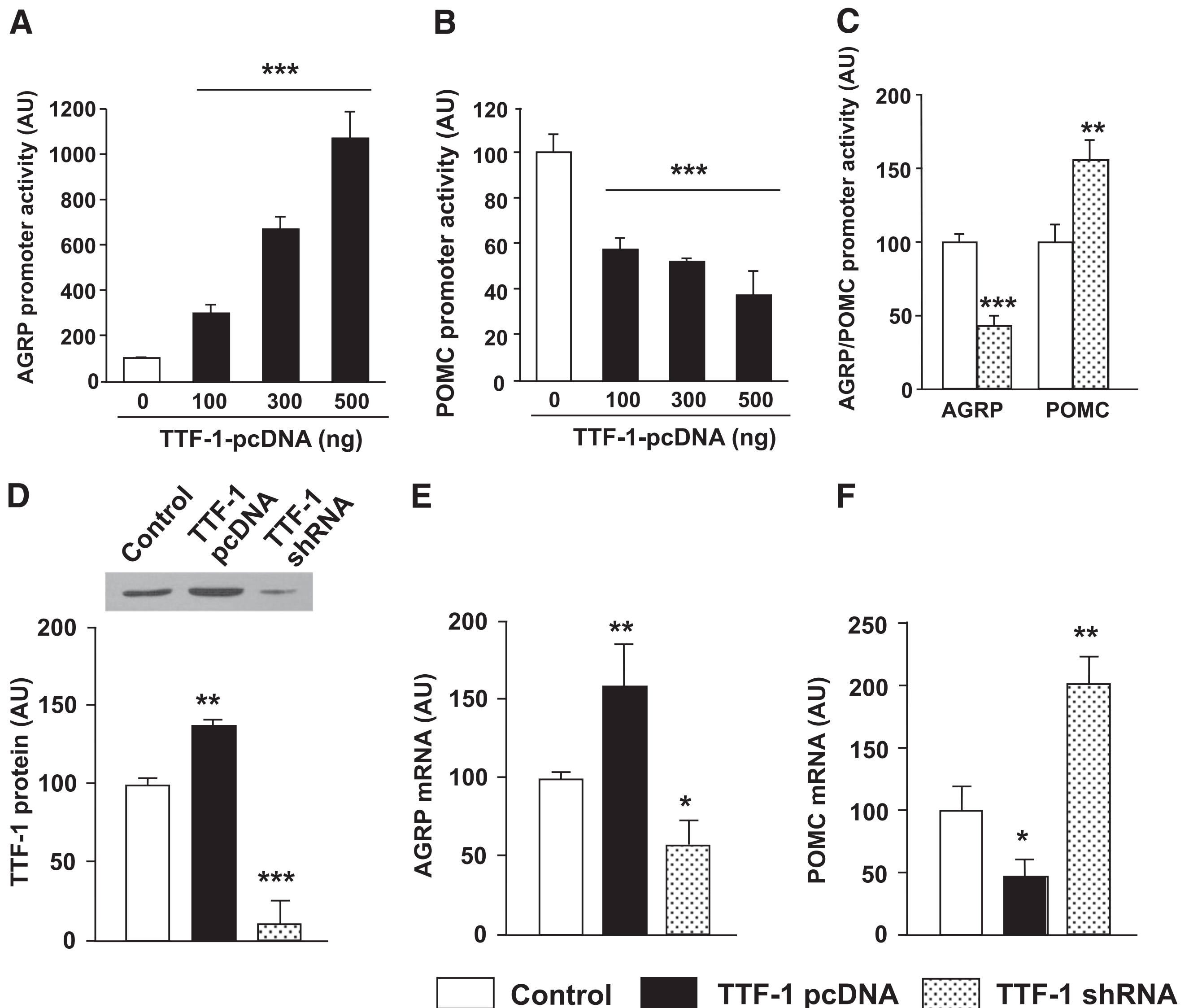

FIG. 1. Effect of TTF-1 on the transcriptional activity of the *AgRP* and *POMC* genes. Luciferase reporter constructs (pGL3) containing the 5′-flanking region of the human *AgRP* (AGRP-pGL3) and rat *POMC* (POMC-pGL3) genes were cotransfected into B35 and AtT-20 cells, respectively, with an expression vector carrying the rat TTF-1–coding region (TTF-1-pcDNA) or TTF-1 shRNA, at the final concentration indicated. *A*: Transactivation of the *AgRP* promoter by different concentrations of TTF-1-pcDNA. *B*: Inhibitory effect of TTF-1 on the activity of the *POMC* promoter. *C*: Inhibitory and stimulatory effect of TTF-1 shRNA on the *AgRP* and *POMC* promoter activities, respectively. *D–F:* TTF-1-pcDNA or TTF-1 shRNA was transfected into B35 cells, and cells were harvested for Western blotting and real-time PCR assays 24 h after transfection. *D:* Western blotting showing changes in the TTF-1 protein level after TTF-1-pcDNA or TTF-1 shRNA tranfection. *E* and *F:* Real-time PCR analysis revealing changes in AgRP (*E*) and POMC mRNA (*F*) by overexpression and inhibition of TTF-1 synthesis. Data are represented as means ± SEM. Each value represents the mean of at least six repeated measurements. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control. AU, arbitrary units.

and Supplementary Fig. 3*B*). To confirm these effects of TTF-1 on *AgRP* and *POMC* promoter activities in B35 and AtT-20 cells, respectively, promoter assays were performed after inhibiting TTF-1 synthesis. Blockade of endogenous TTF-1 synthesis in these cell lines using TTF-1 shRNA (18) decreased *AgRP* promoter activity and increased *POMC* promoter activity (Fig. 1*C*).

To further confirm TTF-1 function in regulation of rat *AgRP* and *POMC* gene expression, real-time PCR analysis of mRNA was performed using RNA samples from rat neuroblastoma B35 cells. Cells were transfected with the TTF-1 expression vector or TTF-1 shRNA to increase or decrease the level of TTF-1 protein (Fig. 1*D*), respectively. The AgRP mRNA level exhibited changes similar to that of the TTF-1 protein level (Fig. 1*E*), whereas the change in POMC mRNA level contrasted that of the TTF-1 protein level (Fig. 1*F*), suggesting that TTF-1 activates *AgRP* expression but inhibits *POMC* expression.

TTF-1 binds in the 5′-flanking region of the *AgRP* and *POMC* genes. EMSAs were performed to determine the ability of the TTF-1 HD protein to physically interact with putative TTF-1 binding domains in the *AgRP* and *POMC* promoters (Fig. 2*A*). Double-stranded ODN probes containing TTF-1 binding motifs (Supplementary Figs. 1 and 2) were used. Of six and nine putative TTF-1 binding domains in the *AgRP* and *POMC* promoters, four and six probes, respectively, generated positive signals with TTF-1 HD (Fig. 2*B*).

To confirm whether TTF-1 physically interacts with its binding domains in the *AgRP* and *POMC* promoters, EMSAs were performed with nuclear proteins extracted from rat hypothalamus. The nuclear proteins strongly

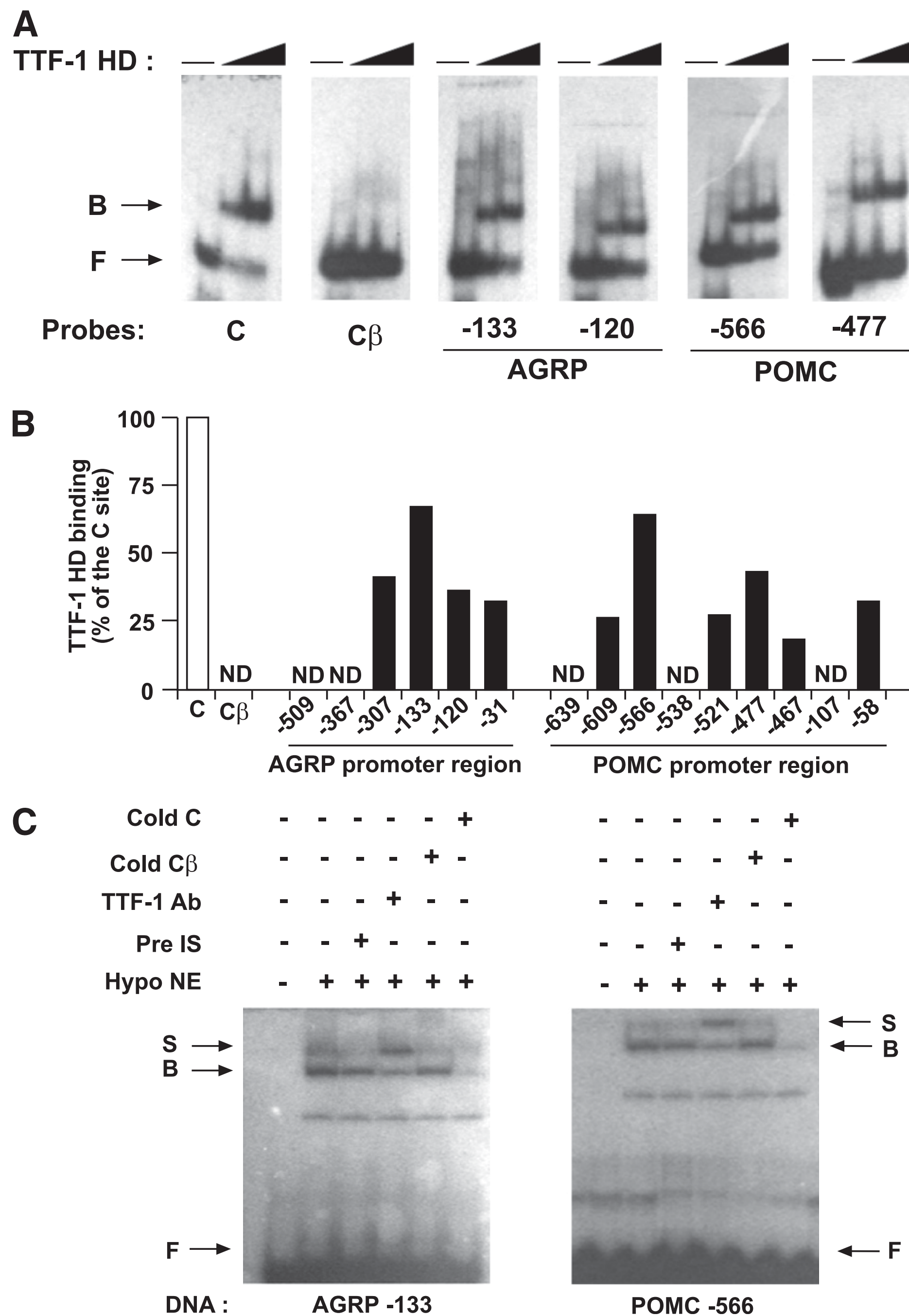

FIG. 2. EMSAs. EMSAs were performed with double-stranded oligomer probes (5 nmol/L) containing the putative TTF-1 binding core motifs shown in Supplementary Figs. 1 and 2 and with two different concentrations of synthetic TTF-1 HD protein (75 and 150 nmol/L). *A*: Representative autoradiograms showing shifted bands generated by protein-DNA complexes. C, positive control probe. Cβ, negative control probe carrying mutations in the TTF-1 binding domain. *B*: Relative binding activities calculated as a percentage of TTF-1 HD binding to the positive control probe C. ND, not detectable. *C*: Presence of bioactive TTF-1 in hypothalamic nuclear extracts. Hypothalamic nuclear extracts (Hypo NE) were incubated with oligonucleotide probes indicated below the autoradiograms, in the presence (+) or absence (−) of a 50-fold excess of cold oligonucleotide C and Cβ and TTF-1 antibody (TTF-1 Ab) or preimmune serum (Pre IS). Incubation of nuclear proteins with a TTF-1 antibody before the protein-DNA binding reaction (S, supershift) delays the migration of the protein-DNA complex. B, protein-bound DNA; F, free DNA; S, supershifted protein-DNA complex.

bound probes containing TTF-1 binding domains in *AgRP* (−133) and *POMC* promoters (−566) (Fig. 2*C*). The interaction between labeled probes and hypothalamic nuclear proteins was reduced by the addition of a 50-fold excess of unlabeled oligonucleotide C, whereas an oligonucleotide carrying a mutated core TTF-1 binding sequence (Cβ) was ineffective. Preincubation of nuclear proteins with TTF-1 antibody resulted in a supershifted band from the protein-DNA complexes generated by each of the AgRP and POMC probes, indicating that TTF-1 is part of these complexes.

Deletion of TTF-1 binding motifs blocks the TTF-1–induced change in *AgRP* and *POMC* promoter activities. We next performed promoter assays with TTF-1 expression vectors after deleting each core binding motif (5′-CAAG-3′ or 5′-CTTG-3′) from the promoters by site-directed mutagenesis.

TTF-1–dependent activation of the *AgRP* promoter significantly decreased with each single deletion of the binding domains (Fig. 3*A*), whereas TTF-1–dependent inhibition of *POMC* promoter activity was significantly reversed by a single deletion of each of the six TTF-1 binding motifs (Fig. 3*B*). Thus the binding motifs identified by EMSAs are required for TTF-1–dependent regulation of *AgRP* and *POMC* promoter activities.

TTF-1 is coexpressed with AgRP and α-MSH in the ARC. The above data strongly suggested a functional hierarchy of TTF-1 with AgRP and α-MSH. Thus we examined the anatomical relationship of TTF-1 with AgRP and α-MSH in the ARC. As previously reported (12,13,18), TTF-1 immunoreactivity (green; Supplementary Fig. 4 and Fig. 4*A* and *C*) was widely distributed in the nucleus of cells stained with Hoechst (blue; Supplementary Fig. 4) throughout the ventromedial hypothalamic region. Conversely, AgRP and α-MSH immunoreactivities (red; Fig. 4*A* and *C*, respectively) were localized primarily in cells of the ARC. Higher magnification images revealed that the locations of AgRP and α-MSH immunoreactivities (red; Fig. 4*B* and *D*, respectively) were closely related to the nuclear TTF-1 signals. Most of the AgRP immunoreactivities were observed close to TTF-1–immunopositive signals (Fig. 4*B*). α-MSH immunoreactivities appeared in the cytoplasm surrounding nuclei containing TTF-1 immunoreactivities (Fig. 4*D*). Double staining of TTF-1 and AgRP or α-MSH demonstrated that 80.7 ± 5.78% and 94.7 ± 1.86% (n = 5 rats) of AgRP- and α-MSH–immunopositive cells were also positive for TTF-1 immunoreactivity, respectively.

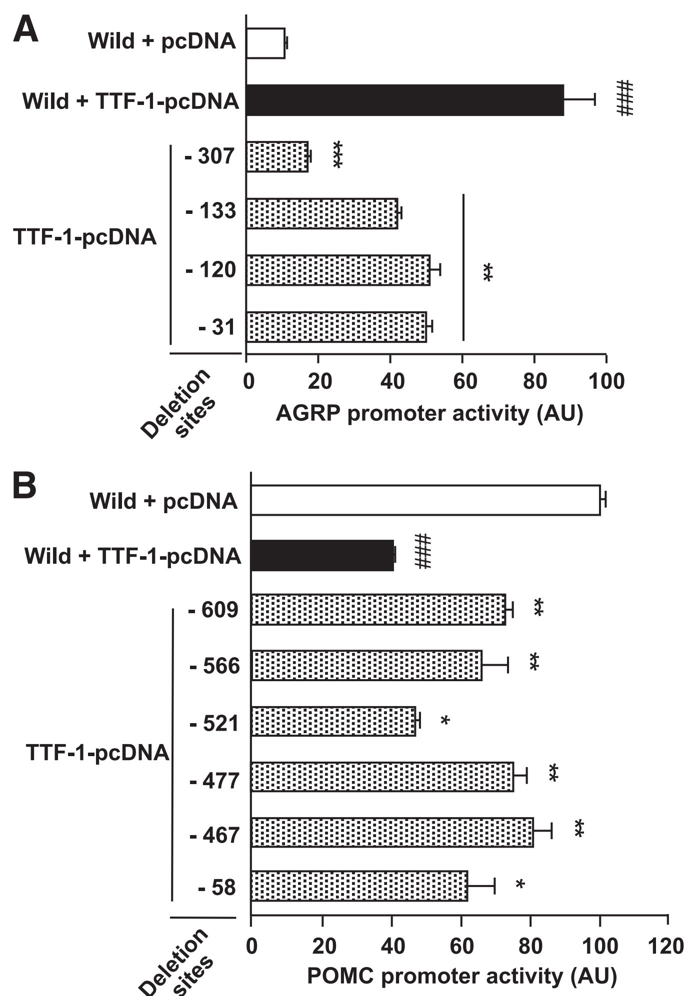

FIG. 3. Effect of deleting core TTF-1 binding motifs on the regulation of AgRP and POMC transcription by TTF-1. Single mutants (200 ng) with core TTF-1 binding motifs deleted from the *AgRP* (*A*) and *POMC* (*B*) promoters were cotransfected with 500 ng of the TTF-1 expression vector TTF-1-pcDNA. The positions of the deleted binding sites are indicated. Data are represented as means ± SEM of 12 wells per construct. ###P < 0.001 vs. the wild-type promoter transfected with control pcDNA; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. change in the wild-type promoter activity induced by TTF-1-pcDNA. AU, arbitrary units.

Effect of in vivo TTF-1 synthesis blockade on *AgRP* and *POMC* expression in the ARC. To determine whether TTF-1 is required for transcriptional regulation of the *AgRP* and *POMC* genes in the ARC, two analyses were performed on the ARC of rats that were administered the TTF-1 AS ODN: histological determination of AgRP and α-MSH and real-time PCR analysis of AgRP and POMC mRNA. The in vivo decrease in TTF-1 availability induced opposite effects on the immunoreactivities of AgRP (decrease) and α-MSH (increase) in the ARC (Fig. 5*A*). This decrease in AgRP immunoreactivity was very similar to that induced by the AgRP AS ODN (Supplementary Fig. 5). Real-time PCR analysis using RNA samples from the ARC revealed essentially the same results as the histological data: the AS ODN decreased AgRP mRNA but increased POMC mRNA (Fig. 5*B*). Thus endogenously synthesized TTF-1 may play an essential role in maintaining AgRP and α-MSH synthesis in the ARC.

Effect of leptin on the hypothalamic expression of TTF-1. Leptin is a well-known adiposity hormone that regulates the MC signaling pathway (21,22). Thus we examined the effect of leptin on TTF-1 expression. First, to establish an anatomical background for leptin action on TTF-1, leptin receptor ObRb mRNA as well as STAT3 and TTF-1 proteins were localized in the rat ARC. Combined FISH and IHC demonstrated that TTF-1 proteins are present in some ObRb mRNA-containing cells of the ARC (Fig. 6*A*–*C*). The donut-shaped green fluorescence revealed cytoplasmic localization of ObRb mRNA (Fig. 6*A* and *C*), whereas round-shaped TTF-1 immunoreactivities indicated their nuclear localization (Fig. 6*B* and *C*). The sense RNA probe did not generate a positive ObRb mRNA signal (data not shown). Double IHC revealed that STAT3 and TTF-1 proteins are coexpressed in some cells of the ARC (Fig. 6*D*–*F*).

Leptin, administered by intracerebroventricular or intraperitoneal injection, significantly decreased TTF-1 expression in the hypothalamus (Fig. 7*A* and *B*), whereas it increased pSTAT3 (Fig. 7*C*). The intracerebroventricularly injected leptin also induced a decrease in AgRP mRNA (Fig. 7*D*) and an increase in POMC mRNA (Fig. 7*E*) in the rat hypothalamus.

MC3/4R function in TTF-1–induced anorexia. To test the in vivo function of TTF-1, we first determined the effect of the AS TTF-1 ODN on the animals' food intake. After daily administration of the AS ODN for 7 days, food intake was maintained at a low level throughout the entire observation period (Fig. 8*A*). We next asked whether TTF-1 action on food intake is mediated by MC3/4R. To determine this, we studied the effect of daily intracerebroventricular administration of SHU9119, a synthetic MC3/4R antagonist, and AgRP, an endogenous antagonist, on TTF-1 AS ODN-induced change in food intake of rats whose food was deprived overnight. SHU9119 increased food intake for 6 and 24 h and 3 days in SCR ODN-injected animals

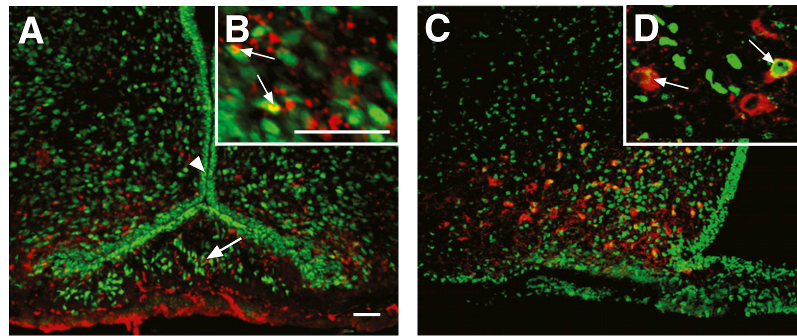

FIG. 4. Detection of TTF-1 immunoreactive materials in AgRP- and α-MSH–immunopositive cells in the rat hypothalamic ARC. Double immunohistochemistry was performed on brain sections derived from normally fed 2-month-old male rats. TTF-1 immunoreactivities (green) showed a relatively wide distribution in the section, whereas immunopositive AgRP (red; *A* and *B*) and α-MSH (red; *C* and *D*) were present only in the ARC. *A*: TTF-1 immunoreactivity near the AgRP immunoreactivity in the ARC as well as in the ependymal/subependymal cells lining the third ventricle (arrowhead) and astrocytes in the median eminence (arrow). *B*: A higher magnification image showing colocalization of the immunoreactivity of TTF-1 and AgRP in the ARC. AgRP immunoreactivity appeared close to TTF-1–positive nuclei with some overlapping (arrows). *C*: Presence of TTF-1 immunoreactivity in α-MSH–immunopositive cells in the ARC. *D*: A higher magnification image highlighting colocalization of both TTF-1 and α-MSH immunoreactivities in some cells (arrows). Scale bar = 50 μm. (A high-quality digital representation of this figure is available in the online issue.)

(Fig. 8*B–D*), as previously reported (23). When SHU9119 was administered to AS ODN-injected animals, it partially antagonized the anorexic effect of the AS ODN (Fig. 8*B–D*). Thus the decrease in food intake induced by the TTF-1 AS ODN may, at least partially, occur through MC3/4R. AgRP did not alter the food intake of SCR ODN-injected rats (Fig. 8*E* and *F*). Conversely, it dramatically recovered anorexia induced by the AS ODN, and moreover it induced a relatively greater attenuation of the anorexia caused by AS TTF-1 ODN than the SHU9119. Together, these data suggest that endogenously synthesized TTF-1 regulates the levels of α-MSH (decrease) and AgRP (increase) that converge and act on MC3/4R to regulate food intake.

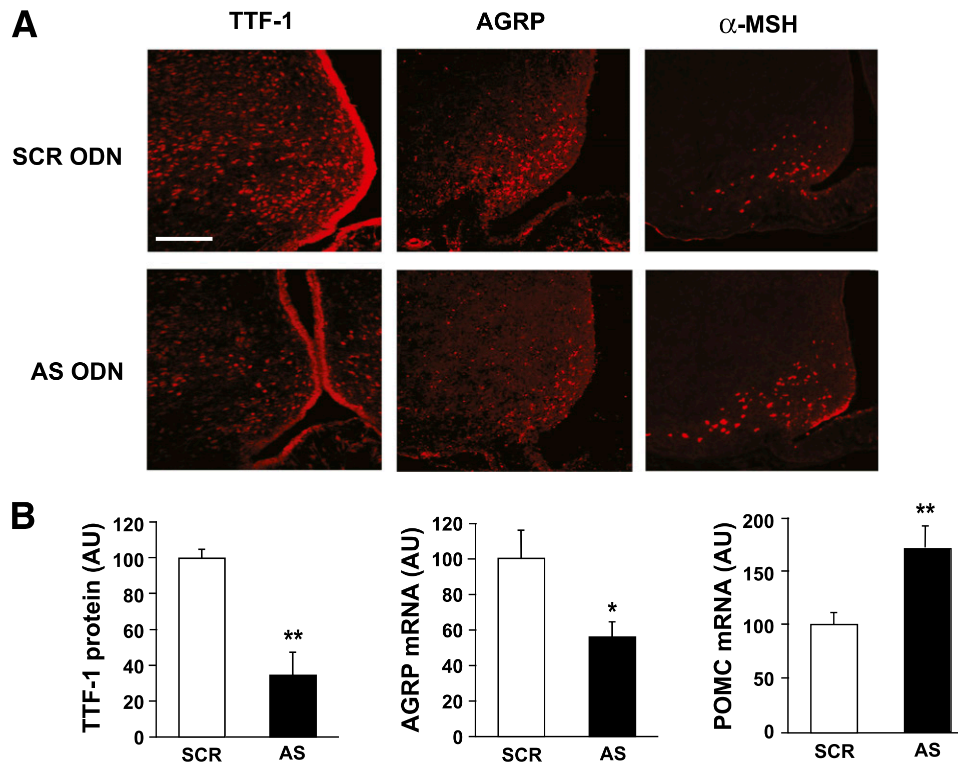

FIG. 5. Effect of TTF-1 synthesis blockade by AS TTF-1 ODN on AgRP and α-MSH synthesis in the ARC. The TTF-1 AS ODN or SCR ODN was injected into the lateral ventricle of 2-month-old male rats. Twelve hours after the ODN injection, brains were fixed by transcardiac perfusion for IHC or were sliced for excising the ARC using a micropunch. Hypothalamic expression of TTF-1, AgRP, and α-MSH (or POMC) was determined by IHC and real-time PCR. *A*: Representative microphotographs showing a decrease in immunoreactive signals of TTF-1 and AgRP and increased α-MSH immunoreactivity in the ARC caused by the AS TTF-1 ODN. *B*: Data from real-time PCR analysis showing decreased AgRP mRNA and increased POMC mRNA resulting from decreased TTF-1 content in the ARC, which is induced by intracerebroventricular administration of the TTF-1 AS ODN (AS) compared with the SCR ODN (SCR). Data are represented as means ± SEM (n = 6). *P < 0.05 and **P < 0.01 vs. SCR. AU, arbitrary units. (A high-quality color representation of this figure is available in the online issue.)

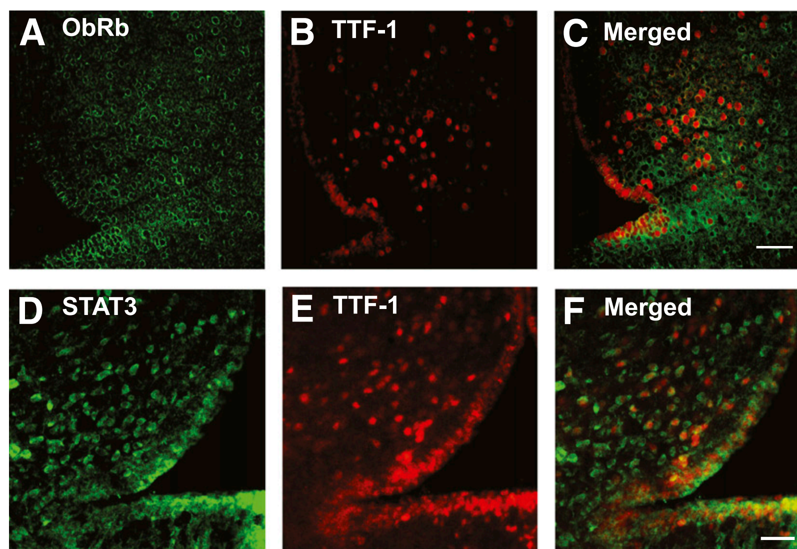

FIG. 6. Identification of TTF-1 immunoreactivity in cells expressing ObRb and STAT3 in the rat ARC. Combined FISH and IHC were performed to determine ObRb mRNA and TTF-1 protein (*A–C*), and double IHC was performed for colocalization of STAT3 and TTF-1 (*D–F*). *A:* Green fluorescence signals representing ObRb mRNA in the cytoplasm of the ARC cells. *B:* Red signals revealing TTF-1 immunoreactivity in the nucleus of cells in the ARC and some ependymal cells of the third ventricle. *C:* Merged image of *A* and *B* showing some cells double positive for ObRb mRNA and TTF-1. *D* and *E:* STAT3 (green; *D*) and TTF-1 (red; *E*) immunoreactivities in cells of the rat ARC and ependymal cells of the third ventricle. *F:* Merged image of *D* and *E* showing some cells with colocalization of TTF-1 and STAT3 proteins. Scale bar = 50 μm. (A high-quality digital representation of this figure is available in the online issue.)

DISCUSSION

Our results demonstrate an important role for TTF-1 in the control of food intake via regulation of the synthesis of AgRP and α-MSH in the ARC.

Based on well-conserved TTF-1 binding core motifs previously found in the target gene promoters (8,10,11,13,19), we identified six and nine putative TTF-1 binding motifs in the 5′-flanking regions of the *AgRP* and *POMC* genes, respectively. EMSAs revealed that TTF-1 interacts with four and six sites, respectively, from the presumptive binding domains in the *AgRP* and *POMC* promoters. Promoter assays, using mutant promoters bearing a single deletion of each of the four (AgRP) and six (POMC) core binding motifs, revealed a significantly reduced TTF-1 dependency in the activities of these promoters, suggesting that TTF-1 directly regulates AgRP and POMC transcription by binding to recognition sites in the promoters of these genes.

Overexpression of TTF-1 increased *AgRP* promoter activity and mRNA, whereas it decreased *POMC* promoter activity and mRNA. Transfection with shRNA resulted in decreased availability of TTF-1, producing results contrary with those observed with TTF-1 overexpression: a decrease and an increase in *AgRP* and *POMC* promoter activities and mRNAs, respectively. Thus TTF-1 transcriptionally regulates the endogenous agonist (α-MSH) and antagonist (AgRP) of the MC pathway. The opposing actions of TTF-1 in regulating closely related target genes have been reported for genes encoding growth hormone (inhibited by TTF-1) and prolactin (activated) in anterior pituitary cell lines (18) and luteinizing hormone-releasing hormone (activated) and preproenkephalin (inhibited) in the rat hypothalamus (8).

In vivo inhibition of TTF-1 synthesis in the rat ARC exerted the same effect as that observed in the in vitro cell studies: it decreased AgRP mRNA and protein levels and increased POMC mRNA and α-MSH content in the ARC. It was also associated with decreased food intake, which was reversed by MC3/4R antagonists (SHU9119 and AgRP). To inhibit TTF-1 synthesis in vivo, we used an AS TTF-1 ODN (10,11,13,14). Although intracerebroventricular administration of the AS ODN may affect areas adjacent to the ventricular region, we demonstrated, using IHC, that injection of the AS ODN into the lateral ventricle led to a marked decrease in TTF-1 availability in the ARC. We confirmed this by immunoblot analysis of TTF-1 in the ARC, which revealed a decreased TTF-1 signal induced by the AS ODN.

TTF-1 is expressed in the embryonic diencephalon and lung (24). In brains of mice carrying a TTF-1–null mutation, extensive abnormalities are found in the preoptic and hypothalamic areas, and the ARC is absent (25), suggesting that TTF-1 is important for the formation of these areas. TTF-1 is expressed in discrete regions of the postnatal rat brain and can regulate the synthesis of several neuropeptides and proteins in adult animals (8–13). Although previous studies have reported expression of TTF-1 mRNA and/or protein in the ARC (8,9,14), this study is the first to directly show histochemically that TTF-1 functionally associates with the leptin receptor and AgRP and α-MSH in the ARC, the center of neural control of energy balance. Notably, cells in the ARC that are positive for AgRP and POMC coexpress TTF-1, providing anatomical evidence for the functional hierarchy between them.

The regional domain of TTF-1 expression in the hypothalamus is broader than that of AgRP and POMC expression. Moreover, only some TTF-1–positive cells in the ARC were also positive for AgRP or POMC, but most cells expressing either of the two neuropeptides were also immunoreactive to TTF-1. This finding is consistent with previous studies showing multiple functions of TTF-1 in the transregulation of several other target genes in the hypothalamus (8,12,14).

The hypothalamic MC signaling pathway is important in the regulation of energy homeostasis through integration of peripheral hormonal signals such as leptin, cholecystokinin, and ghrelin (26). We showed that leptin, which is

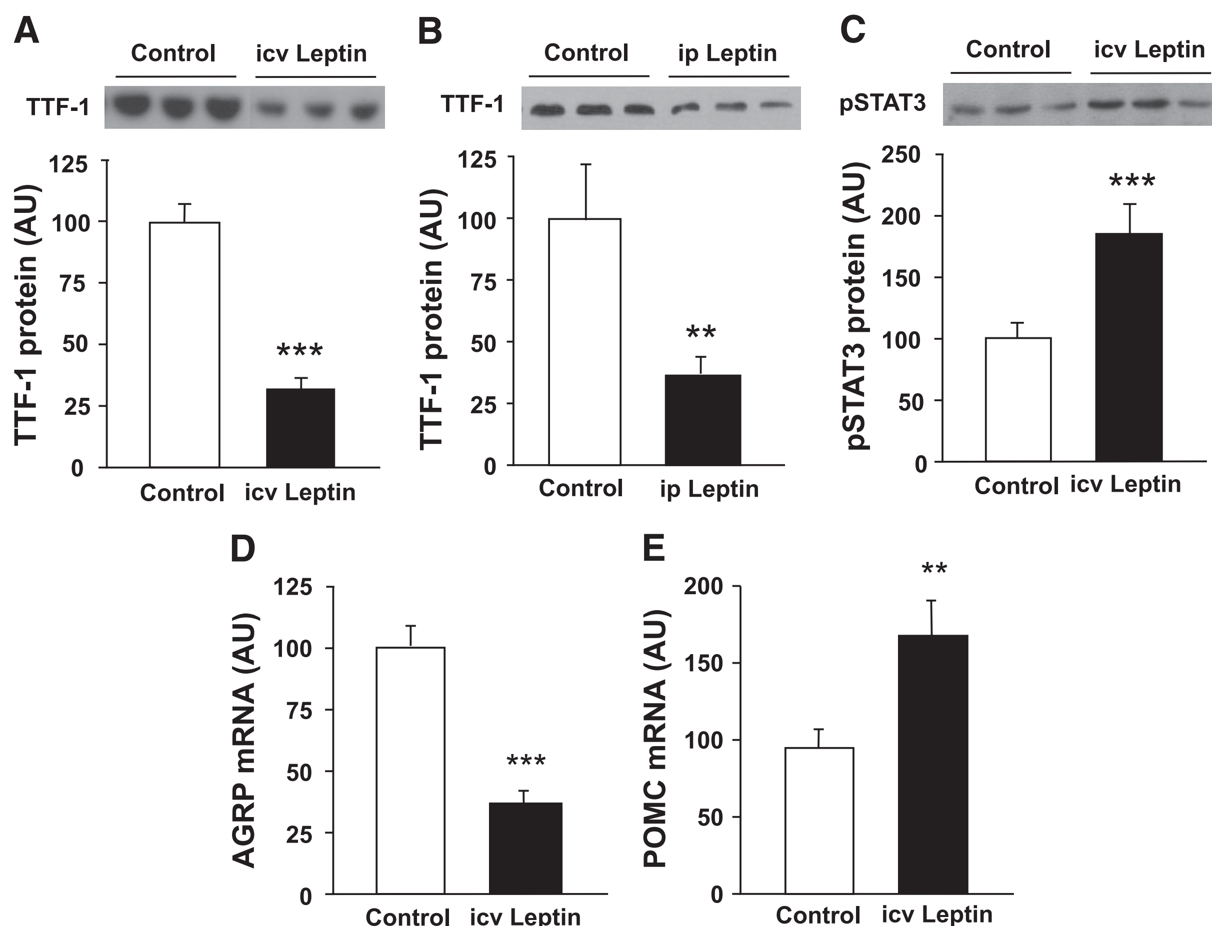

FIG. 7. Effect of leptin on the hypothalamic expression of TTF-1. Two-month-old male rats were administered recombinant rat leptin (4 μg for i.c.v. injection, or 2 mg/kg for i.p. injection), and the hypothalamic content of TTF-1 was determined. *A* and *B:* Western blot analysis showing decreased TTF-1 content by intracerebroventricular (*A*) and intraperitoneal (*B*) administration of leptin. *C:* Western blot data revealing the effect of intracerebroventricular leptin injection on the pSTAT3 level in the hypothalamus. *D* and *E:* Real-time PCR analyses showing that intracerebroventricular administration of leptin induced a decrease in AgRP mRNA (*D*) and an increase in POMC mRNA (*E*). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

known to regulate the MC signaling pathway by stimulating α-MSH and inhibiting AgRP (22), decreased hypothalamic TTF-1 expression. This was further supported by histochemical data demonstrating that expression domains of ObRb and STAT3 overlap with that of TTF-1 in some ARC cells. Thus TTF-1 may mediate regulatory inputs such as leptin to control feeding behavior.

MC3/4R is found in brain areas involved in the control of energy balance, including the ARC, and the paraventricular, lateral, and ventromedial nuclei of the hypothalamus (27). Central administration of MC3/4R agonists α-MSH and melanotan-II (MTII) reduced food intake and body weight (28). Conversely, treatment with MC3/4R antagonists AgRP and SHU9119 resulted in hyperphagia and increased body weight (28–30). Because hypothalamic TTF-1 simultaneously regulates AgRP and α-MSH synthesis oppositely, TTF-1 in the ARC may exert its orexigenic effect through the MC3/4R signaling pathway. This was supported by experiments showing that SHU9119 and AgRP diminished anorexia induced by in vivo TTF-1 synthesis blockade in the ARC. However, the sustained anorexic effect of TTF-1 AS ODN suggests that TTF-1 may act on feeding behavior partly through pathways other than MC3/4R, because exogenous infusion of α-MSH or MTII did not cause sustained reduction in food intake (31–33). Moreover, anorexia induced by AS TTF-1 ODN was not completely abolished by blockade of MC3/4R with SHU9119 or AgRP. Therefore, TTF-1 may also contribute to the regulation of appetite through other pathway(s) in addition to the MC pathway. For example, our previous studies have shown that TTF-1 regulates the transcription of genes encoding pituitary adenylate cyclase-activating polypeptide (10), proenkephalin (8), and more importantly NPY (14), which are known to be involved in the regulation of food intake.

Although this study demonstrated TTF-1 action on food intake, how upstream signaling inputs such as leptin alter TTF-1 expression, and what normal physiological changes require TTF-1 for feeding regulation are largely unknown. Although our previous finding showed that fasting significantly induced an increase in TTF-1 expression (14), further studies are required to answer these questions.

In summary, this study revealed that TTF-1 regulates AgRP and POMC expression in the hypothalamus under a signaling input from leptin, thus affecting food intake.

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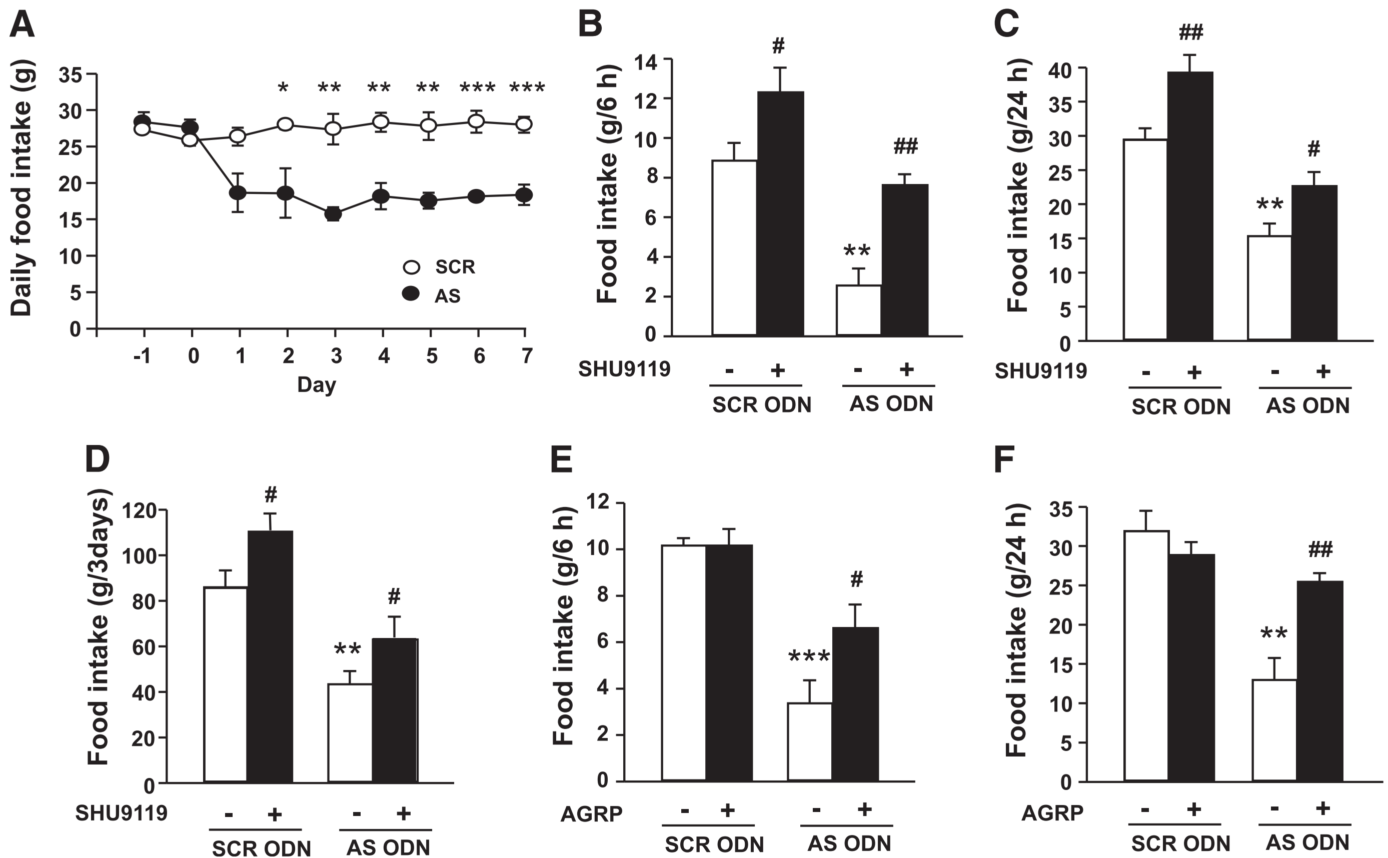

FIG. 8. Effect of SHU9119 and AgRP on anorexia resulting from the inhibition of TTF-1 synthesis. Two-month-old male rats were fasted overnight (16 h) and intracerebroventricular-infused with ODNs 4 h before intracerebroventricular injection of SHU9119 or AgRP. Rats were allowed access to food immediately after the final treatment. *A:* Daily food intake by individual animals was measured for 1 week, after daily administration of the AS TTF-1 ODN (from day 0 to day 6). *B–F:* Cumulative food intake was measured for 6 h (*B* and *E*), 24 h (*C* and *F*), and 3 days (D) after the injection of SHU9119 (*B–D*) or AgRP (*E* and *F*). Data are represented as means ± SEM ($n = 4$ for *A*; $n = 6$ for others). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. SCR ODN-injected rats; #$P < 0.05$, ##$P < 0.01$ vs. control rats that received artificial cerebrospinal fluid (−).

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No potential conflicts of interest relevant to this article were reported.

J.G.K., B.S.P., C.H.Y., and H.J.K. researched data. S.S.K. contributed to discussion. A.V.D. researched data. J.G.K. and G.D. wrote the article. K.-U.L. reviewed and edited the article. J.W.P., E.S.K., and I.S.N contributed to discussion. Y.I.K. reviewed and edited the article. B.J.L. wrote the article.

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