Hypothalamic Ahi1 Mediates Feeding Behavior through Interaction with 5-HT$_{2C}$ Receptor*\textsuperscript{S}

Hao Wang\textsuperscript{1,1}, Zhenbo Huang\textsuperscript{1,1}, Liansha Huang\textsuperscript{1,1}, Shaona Niu\textsuperscript{6}, Xiurong Rao\textsuperscript{6}, Jing Xu\textsuperscript{6}, Hui Kong\textsuperscript{1}, Jianzhong Yang\textsuperscript{1,1}, Chuan Yang\textsuperscript{2,2}, Donghai Wu\textsuperscript{3,3}, Shihua Li\textsuperscript{4,4}, Xiao-Jiang Li\textsuperscript{4,4}, Tonghua Liu\textsuperscript{2,2}, and Guoqing Sheng\textsuperscript{1,1}

From the \textsuperscript{1}CAS Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China, the \textsuperscript{2}Department of Science and Technology, Beijing University of Chinese Medicine, Beijing 100029, China, the \textsuperscript{3}Department of Endocrinology, Linyi People’s Hospital, 27 Jiefang Road, Linyi 276003, China, the \textsuperscript{4}Red Cross Hospital of Yunnan Province, Kunming 650030, China, the \textsuperscript{5}Department of Endocrinology and Metabolism, The Second Affiliated Hospital of Sun Yat-sen University, Guangzhou 510020, China, and the \textsuperscript{6}Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia 30322

It is indicated that there are important molecules interacting with brain nervous systems to regulate feeding and energy balance by influencing the signaling pathways of these systems, but relatively few of the critical players have been identified. In the present study, we provide the evidence for the role of Abelson helper integration site 1 (Ahi1) protein as a mediator of feeding behavior through interaction with serotonin receptor 2C (5-HT$_{2C}$R), known for its critical role in feeding and appetite control. First, we demonstrated the co-localization and interaction between hypothalamic Ahi1 and 5-HT$_{2C}$R. Ahi1 promoted the degradation of 5-HT$_{2C}$R through the lysosomal pathway. Then, we investigated the effects of fasting on the expression of hypothalamic Ahi1 and 5-HT$_{2C}$R. Fasting resulted in an increased Ahi1 expression and a concomitant decreased expression of 5-HT$_{2C}$R. Knockdown of hypothalamic Ahi1 led to a concomitant increased expression of 5-HT$_{2C}$R and a decrease of food intake and body weight. Last, we found that Ahi1 could regulate the expression of neuropeptide Y and proopiomelanocortin. Taken together, our results indicate that Ahi1 mediates feeding behavior by interacting with 5-HT$_{2C}$R to modulate the serotonin signaling pathway.

Obesity and its associated ailments such as diabetes have become a worldwide epidemic carrying with them a heavy toll of morbidity and mortality. Over the past decades, it has become evident that neural circuits in the central nervous system play a direct and crucial role in controlling feeding and energy homeostasis (1, 2). Disruptions in the mechanisms of central nervous system energy sensing are able to alter the standard homeostatic responses and are factors that contribute to the pathophysiology of obesity and diabetes. The brain serotonin system has long been implicated in the neural regulation of food intake and energy metabolism, as highlighted by the clinical use of serotonin releasers and reuptake inhibitors as appetite suppressant and weight loss medication (3–5). Depletion of central serotonin using selective neurotoxins has been shown to result in hyperphagia and obesity, whereas the release of serotonin and the inhibition of reuptake by n-fenfluramine potently reduce feeding and body weight (6). More recently, several lines of evidence show that serotonin receptor agonists can significantly improve glucose tolerance and reduce plasma insulin in mouse models of obesity and type 2 diabetes (7–9).

Numerous serotonin receptor subtypes have been identified in which serotonin receptor 2C (5-HT$_{2C}$R)\textsuperscript{S} is recognized specifically as a mediator of serotonin-induced appetite and glucose regulation (10–13). During the past few years, both pharmacological and genetic evidence has indicated that neuropeptide Y (NPY) and melanocortin systems are the necessary mechanisms by which 5-HT$_{2C}$R agonists reduce appetite and improve diabetic conditions (14–16). Although significant progress has been made in the study of serotonin-mediated regulation of energy metabolism, we are still far from understanding the whole picture. One of the most intriguing aspects of this area that has remained mysterious is the importance of cellular and molecular interactions that regulate energy homeostasis centrally.

Abelson helper integration site 1 (Ahi1) was initially identified as a common helper provirus integration site for murine leukemia and lymphomas (17). A number of groups have identified Ahi1 mutations as a frequent cause of disease in patients with specific forms of Joubert syndrome, an autosomal recessive neurodevelopmental disorder, and its related disorders (JSRD) (18–20). The normal neural function of Ahi1, however, remains poorly defined. Both protein and mRNA studies have shown that Ahi1 is distributed in several brain areas implicated in feeding and metabolic regulation such as the hypothalamic

\*This work was supported, in whole or in part, by National Institutes of Health Grant NS036232 (to X.-J. L). This work was also supported by National Natural Science Foundation of China (NSFC) Grants 30700213 and 30811120429, Guangdong Natural Science Foundation (GDSF) Grant 07007215, and “973” Projects 2007CB947804 and 2010CB945503 (to G. S.) and by International Traditional Chinese Medicine Program for Cooperation in Science and Technology (ISC) Grant 2011DFA30920 (to T. L.).

\textsuperscript{S}This article contains supplemental Figs. 1–5.

\textsuperscript{1}These authors contributed equally to this work.

\textsuperscript{2}To whom correspondence may be addressed: Dept. of Science and Technology, Beijing University of Chinese Medicine, Beijing 100029, China. Tel.: 86-10-64286642; Fax: 86-10-64286642; E-mail: thliu@vip.163.com.

\textsuperscript{3}To whom correspondence may be addressed: 190 Kai Yuan Ave., Science Park, Guangzhou 510530, China. Tel.: 86-20-32015290; Fax: 86-20-32015299; E-mail: shenggibh@gmail.com.

\textsuperscript{4}The abbreviations used are: 5-HT$_{2C}$R, serotonin receptor 2C; NPY, neuropeptide Y; ARC, arcuate nucleus; POMC, proopiomelanocortin; mcPP, meta-chlorophenylpiperazine; Chlq, chloroquine; GFP, green fluorescent protein; SH3, Src homology 3; dpl, days post-injection.

© 2012 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
paraventricular nucleus, the supraoptic nucleus, the arcuate nucleus (ARC), the lateral hypothalamic area, and the nucleus tractus solitarius in the brain stem (21–23). Genetic studies also indicate that Ahi1 may be related to energy metabolism. One group has reported a significant association between variants in the Ahi1 gene and type 2 diabetes in a Dutch population (24). In addition, single nucleotide polymorphism association studies identified two novel Ahi1 genetic variants linked with fasting blood glucose levels in Mexican American subjects (25). Recent studies (26, 27) also show that brain Ahi1 may play an important role in the regulation of feeding behavior.

We noticed that Ahi1 and 5-HT2C R have similar distribution in the hypothalamus. This prompted us to investigate the relationship between Ahi1 and 5-HT2CR and their probable roles in feeding control. The findings in the present study provide evidence that Ahi1 interacts with 5-HT2CR to mediate feeding behavior. Our study reveals the normal neural function of Ahi1 in feeding control and offers insight into the understanding of how hypothalamic key molecules regulate the feeding behavior.

**MATERIALS AND METHODS**

**Animals**—Male C57BL/6J mice, 6–10 weeks old, were purchased from Southern Medical University, Guangdong Province, China. The animals were housed in a temperature- and humidity-controlled environment with a 12 h/12 h light/dark cycle with access to food and water ad libitum. Animals were acclimatized to laboratory conditions for a week before all tests. Animal care and all procedures for animal experiments conformed to the guidelines of the Animal Care and Use Committee of Guangzhou Biomedical and Health Institute, Chinese Academy of Sciences.

**Immunostaining**—The antibody against Ahi1 is described in our previous study (23). For immunofluorescent staining, sections of brain tissue and coverslips plated with hypothalamic neurons or HEK293 cells were blocked with 5% bovine serum albumin (BSA) in 0.02 M PBS at room temperature for 1 h and followed by incubating with rabbit anti-Ahi1 antibody (1:300) and goat anti-5-HT2CR (1:50, Santa Cruz Biotechnology) at 4 °C overnight. Then the sections were incubated successively with Alexa Fluor 488-conjugated anti-goat secondary antibody and Alexa Fluor 594-conjugated anti-rabbit secondary antibody (both from Invitrogen) at room temperature for 30 min. Tissue sections and coverslips were mounted onto glass slides. Labeled samples were imaged using a ×100 objective Leica SP2 confocal microscope.

**Immunoprecipitation**—Lysates from mouse hypothalami or from co-transfected HEK293 cells were extracted using lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitor mixture) and centrifuged at 12,000 × g at 4 °C for 15 min. 500 µl of supernatant (adjusted to 1 mg/ml) was first clarified by incubation with 40 µl of 50% protein A-Sepharose beads (Sigma) at 4 °C for 1 h to reduce nonspecific binding. After pelleting the beads, the supernatant was then incubated with antibody to mouse Ahi1 (1: 50), anti-hemagglutinin (HA) monoclonal antibody (Sigma, clone HA-7, diluted 1:4000), anti-GFP polyclonal antibody (Abcam, ab290, diluted 1:1000), or immunoglobulin G as control overnight at 4 °C followed by brief centrifugation. The immunoprecipitates were washed three times with low detergent buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 0.2% Tween 20) and subjected to Western blotting.

**Purification Membrane Fraction and Western Blot**—A total of 15–20 mg of whole hypothalami from fasted mice or Ahi1 knockdown mice were adequately blended in 1 ml of pre-cold membrane purification buffer (MPB: 250 mM sucrose, 30 mM KCl, 20 mM Tris-Cl, pH 7.2, 0.2 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.3% Triton X-114, and protease inhibitor mixture) on ice. The suspensions were ultracentrifugated at 59,000 × g at 4 °C for 15 min, and then the pellets (P1) were re-extracted as mentioned above in 1 ml of MPB with 0.8% Triton X-114. After a 30-min incubation on ice, the soluble materials were removed by ultracentrifugation (as above). The pellets (P2, purified membrane fraction) were washed twice with MPB and finally resuspended in MPB.

Hypothalami were mechanically homogenized and sonicated in homogenization buffer on ice. 20 µg of tissue protein was size-fractionated using 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. Cell lysates (50 µg) from treated N18TG2 cells were also subjected to SDS-PAGE and blotted. After blotting with the antibodies, anti-mouse Ahi1 or anti-5-HT2CR and anti-γ-tubulin (Sigma), anti-β-actin (ACTB, GenScript), and anti-LAMP1 detection was performed using an enhanced chemiluminescent kit (Pierce) according to the manufacturer’s instructions. Quantitations of gray density were performed using ImageQuant 5.2 software.

**Quantitative RT-PCR**—Total RNA was isolated from tissue samples or cell samples using TRIzol reagent (Ambion). cDNA was synthesized by using Moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas). Primers for mouse Ahi1 (forward primer, 5'-GAC AGG AGA ACA AGT GGC AAT G-3' ; reverse primer, 5'-ATC AGT GGT CAG CAC GAA CGA CGA-3'), mouse NPY (forward primer, 5'-TAC TAC TCC GCT CTA CGA CAC-3'; reverse primer, 5'-CCA CAT GGA AGG GTC TTC AAG-3'), mouse proopiomelanocortin (POMC) (forward primer, 5'-CGA GCG GGC ATT AGG CCT TT-3'; reverse primer, 5'-CTT GTC CTT GGG CGG GTT-3'), and reference gene GAPDH (forward primer, 5'-CTG CAC CAC CAA CTG CTT AGC-3'; reverse primer, 5'-AGG CCA TGC CAG TGA TGA-3') were optimized to an equal annealing temperature of 60 °C. Expression of Ahi1, NPY, POMC, and GAPDH was determined by real-time PCR using SYBR® Premix Ex Taq (Takara) on an MJ four-color real-time PCR system (Bio-Rad) according to the manufacturer’s instructions. The expression ratio of target genes among the experimental groups was calculated and statistically analyzed as reported previously (28, 29).

**RNA Interference**—For the knockdown of Ahi1, C57BL/6J mice (8-week-old males) were injected bilaterally with recombinant adenovirus vector encoding Ahi1-specific siRNA (Ad-siAhi1) or scramble-siRNA (Ad-scrRNA) as control. A total of 1 × 1011 plaque-forming units in 1 ml of PBS were injected bilaterally intrahypothalamus at the stereotaxic positions (anteroposterior −1.1 mm, mediolateral −0.5 mm, dorsalventral −5.5 mm; anteroposterior −1.1 mm, mediolateral +0.5 mm, dorsalventral −5.5 mm). The adenoviral vector also inde-
Hypothalamic Ahi1 in Feeding Control

pendently expresses green fluorescent protein (GFP), which enabled us to trace the vector. Injection at the above coordinates allowed the adenovirus to mainly infect the ventromedial hypothalamus, ARC, dorsomedial hypothalamus, and other nuclei in the hypothalamus (Fig. 4A). The mice were single-housed in regular plastic cages before and after the surgical procedure. Food intake and body weight were recorded daily for 4 weeks. After a period of 3–5 days post-surgical recovery, the mice were tested for glucose tolerance tests and insulin tolerance tests performed as described previously (31). Glycemia was assessed using a blood glucose test meter.

Cell Culture, Infection, Transfection, and Drug Treatment—Co-localization of Ahi1 and 5-HT2cR was observed on the networks of cultured mice hypothalamic neurons. The details of culture preparations have been described previously (31). In brief, hypothalamic tissue was dissected from newborn mice. Hypothalamic neural cells were planted on poly-D-lysine-coated coverslips at a density of ~2000 cells/cm². Cultures were kept in Neurobasal medium supplemented with 2% FBS and 2 mM L-glutamine (all from Invitrogen) for 14 days in vitro. N18TG2 cells, the mouse neuroblastoma cell line with endogenous Ahi1 expression, were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (PAA Laboratories), 1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). DNA constructs for expression of Ahi1 cDNA plasmids encoding full-length mouse Ahi1-(1–1047) were generated as in a previous study (32). We used PCR to generate C-terminally truncated mouse Ahi1-(1–820). These Ahi1 cDNAs were sequenced and cloned to the PRK vector that links the WD40 repeat domains and the SH3 domain found in the Ahi1 coding region. For transfection of foreign DNAs into N18TG2, plasmid-polyethylenimine (Polysciences, Inc.) complexes incubated with cultures for 1 h. After 36 h of transfection, N18TG2 cells were stimulated under 5 μM mchlorophenylpiperazine (mCPP; Sigma) with or without 100 μM chloroquine (Chlq; Sigma) for the indicated time and then immediately collected for the following quantitative assays. For virus infection, the cells were infected by Ad-siAhi1 (1 × 10⁵ plaque-forming units/ml medium) for 8 h and sampled at 24 h post-infection. HEK293 cells were cultured in DMEM containing 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. When 50% confluence had been achieved, the HEK293 cells were co-transfected with constructs encoding DsRed-tagged Ahi1 and GFP-tagged 5-HT2cR as described above.

Immunofluorescence Labeling with LAMP1 and Quantification of the Co-localization of 5-HT2cR and LAMP1—N18TG2 cells, co-transfected with PRK-mAhi1-DsRed/PRK-DsRed and GFP-5-HT2cR, were washed once, fixed for 10 min at −20 °C in 50% (v/v) acetone/methanol, and air-dried. Cells were blocked by incubating them in 1 h for 5% BSA and then with anti-LAMP1 primary antibody (4 μg/ml, Abcam) at 4 °C for 24 h. Then coverslips were washed several times and incubated with Cy5-labeled anti-mouse IgG for 45 min at room temperature. After DAPI staining, four-color slides were visualized with a Leica SP2 confocal scanning microscope set up as follows: 403 nm laser (25% of power) window, 410–483 nm; 488 nm laser (25% of power) window, 493–538 nm; 543 nm laser (90% of power) window, 548–628 nm; and 633 nm laser (25% of power), window 638–700 nm. Images were collected using the microscope in sequential mode with a frame average and a format of 1024 × 1024 pixels and were analyzed using NIH ImageJ 1.40g software. For statistical analyses, images for all conditions were analyzed using identical acquisition parameters, and untreated and treated cells from the same culture preparation were always compared with one another. The images were also collected blind to experimental condition. The total thresholded area of fluorescently labeled overlay regions was automatically measured and divided by the total cell area, which was determined by setting a lower threshold level to measure the background fluorescence produced by the fixed cells. For each experiment, the fluorescence of all cells was normalized by dividing the average fluorescence of the untreated control cells. All cells that expressed 5-HT2cR with less than the average at 30% were excluded from analysis because of the variation in transfection efficiency.

Statistics—GraphPad Prism was utilized for data analysis. Data are presented as mean ± S.E. of at least three independent experiments. Statistical analyses were carried out by one-way analysis of variance followed by Tukey’s post hoc test, and p < 0.05 was considered statistically significant.

RESULTS

Co-localization and Interaction between Hypothalamic Ahi1 and 5-HT2cR—First, we cultured hypothalamic neurons in vitro. In double-labeled immunofluorescence staining assays of cultured hypothalamic neurons, Ahi1 was observed to co-localize significantly with 5-HT2cR (Fig. 1A, top and middle rows). Staining of mouse brain sections also revealed an extensive overlap of the two proteins in hypothalamic nucleus such as ARC (Fig. 1A, bottom row). A co-immunoprecipitation study was performed in mice hypothalimus homogenates. As shown in Fig. 1B, 5-HT2cR selectively bound to endogenous Ahi1, whereas no signal was detected in immunoprecipitates from non-immune rabbit serum control. To confirm this physical interaction, we subsequently expressed them in transfected HEK293 cells; GFP-5-HT2cR showed an extensive distribution throughout the plasma membrane and cytoplasm of the cell, whereas Ahi1 showed a unique distribution pattern characterized as dot-like structures. Co-expression of 5-HT2cR and Ahi1 displayed apparent co-localization in punctate aggregates in the cytoplasm of HEK293 cells (supplemental Fig. 1A). Their interaction was further demonstrated by co-immunoprecipitation using anti-Ahi1 antibody (supplemental Fig. 1B). Then, we constructed the C-terminal-truncated Ahi1. In the immunofluorescence experiments, we found that the co-localization of Ahi1 and 5-HT2cR was lost in the cells transfected with C terminal-truncated Ahi1 (Fig. 1D). Similarly the combination of Ahi1 and 5-HT2cR was abolished in the C terminal-truncated Ahi1 (Fig. 1E). These data indicate that the C terminus of Ahi1 is the region where it interacts with 5-HT2cR.

Ahi1 Promotes Lysosomal Degradation of 5-HT2cR—Given the WD40 repeat domains and the SH3 domain found in the Ahi1 protein (32), we hypothesized that Ahi1 may play a role in neurotransmitter receptor trafficking as an adaptor between
cytoskeleton and membrane protein. It has been reported that Ahi1 participates in the process of intracellular vesicle trafficking and is co-localized with microtubules and the microtubule-organizing center (33, 34). We postulated that Ahi1 could participate in 5-HT2CR vesicles sorting to degradation after endocytosis. To test this hypothesis, we treated cells with mCPP, an agonist of 5-HT2CR (13). As shown in Fig. 2A, the 5-HT2CR decreased in a time-dependent way in the cells transfected with full-length Ahi1, whereas in the cells transfected with truncated Ahi1 this decrease was inhibited. Then we addressed the pathway of 5-HT2CR degradation. We first investigated the possible involvement of the lysosomal pathway by treatment with Chlq, a lysosomal enzyme inhibitor. Analysis by Western blot revealed that treatment with Chlq increased the level of 5-HT2CR in mCPP treated N18TG2 cells (Fig. 2A, lane 3 versus lane 2), thereby indicating that the lysosomal pathway mediates internalized 5-HT2CR degradation. To address the possible function of Ahi1 in lysosomal sorting, we investigated whether the expression of Ahi1 affects the degradation of internalized serotonin receptors. When we co-transfected with a RFP-Ahi1 vector, the degradation of activated 5-HT2CR was significantly increased as compared with that of an empty vector (Fig. 2B, lane 5 versus lane 2), and this increased 5-HT2CR degradation could be blocked by adding Chlq to the cells (Fig. 2B, lane 6). As shown in supplemental Fig. 2, the addition of lactacystin (a proteasome inhibitor) could not block the degradation of 5-HT2CR, which indicates that the proteasome pathway is not involved in the 5-HT2CR degradation. In the cells expressing GFP-5-HT2CR alone, the receptor was distributed throughout the plasma membrane as well as the cytoplasm when visualized under the fluorescence microscope (Fig. 2C, bottom row). Co-expression of Ahi1 dramatically changed the distribution pattern of 5-HT2CR as expected, and strong 5-HT2CR signals were overlapped with LAMP1 (as lysosome marker) positive dots, where 5-HT2CR co-localized with Ahi1 (Fig. 2C, top row). Quantification of 5-HT2CR and LAMP1 co-localization (Fig. 2D, bottom row) showed significant overlapping regions between the 5-HT2CR and LAMP1-labeled pixels when Ahi1 was expressed (563.49% of mCPP-untreated cells, a total of 34 cells were quantified). However, the value was much lower when Ahi1 was absent (109.90% of mCPP-untreated cells, a total of 32 cells were quantified). Taken together, these results strongly support the idea that Ahi1 interacts with 5-HT2CR and enhances the trafficking of internalized 5-HT2CR to lysosomes, thereby promoting its degradation.

Fasting Alters Expression of Hypothalamic Ahi1 and 5-HT2CR —We then examined the role of hypothalamic Ahi1 and 5-HT2CR in the regulation of feeding behavior. As shown in
Fig. 3A, hypothalamic Ahi1 protein was significantly increased after a fasting period of 24 or 48 h. Similarly, the transcription of Ahi1 mRNA was significantly up-regulated (Fig. 3B). To investigate the involvement of 5-HT2CR in this process, we isolated the membrane fraction of hypothalamus lysates and assessed 5-HT2CR located on the neuronal membrane under the condition of fasting. As shown in Fig. 3C, the down-regulation of 5-HT2CR levels was concomitant with the up-regulation of Ahi1 in the time course study. To further confirm the correlation between 5-HT2CR and Ahi1, we employed pharmacological modulators of 5-HT2CR (mCPP and olanzapine). First, we pretreated (intraperitoneally) fasted mice with mCPP, an agonist of 5-HT2CR. The up-regulation of hypothalamic Ahi1 in fasted mice was significantly inhibited by mCPP pre-treatment (Fig. 3D). Then, we used olanzapine, an antagonist of 5-HT2CR (35). The expression of Ahi1 protein in the N18TG2 cell line, as shown in supplemental Fig. 3A, was increased by 70% after 72 h of exposure to olanzapine. Similarly, as compared with the control group, 4 weeks of treatment with olanzapine significantly increased the levels of Ahi1 expression in the mice hypothalamus (supplemental Fig. 3B). These data suggest that 5-HT2CR is a necessary component of the Ahi1-dependent signaling pathway that controls feeding behavior.

**Knockdown of Hypothalamic Ahi1 Suppresses Food Intake—**
To further establish a role for endogenous Ahi1 in feeding, we used RNA interference (RNAi) to suppress the expression of Ahi1 in fasted mice. We used a lentiviral vector expressing shRNA specific for Ahi1 to knockdown Ahi1 expression. As shown in Fig. 4A, the expression of Ahi1 was significantly reduced by shRNA treatment compared to the control group. Similar results were obtained using a small interfering RNA (siRNA) approach (Fig. 4B). These data suggest that the suppression of Ahi1 expression significantly reduced food intake in fasted mice.
Ahi1 in the hypothalamus. We generated a mouse Ahi1-specific siRNA that effectively inhibited the expression of endogenous Ahi1 in N18TG2 cells (supplemental Fig. 4). This siRNA was expressed from an adenoviral vector that also independently expressed GFP. Thus, cells labeled with GFP should also express Ahi1-specific siRNA. An adenoviral vector expressing scramble-siRNA (Ad-scRNA) served as a control. As shown in Fig. 4A, a high level of expression of GFP-fused Ad-siAhi1 4 days post-injection (dpi) was observed on stereotactic coronal sections. Immunohistochemistry (Fig. 4B) and Western blot (Fig. 4C) showed that Ad-siRNA produced a significant knockdown of Ahi1 protein in the hypothalamus. After surgery, the animals were housed individually. Food intake and body weight were measured daily for 4 weeks. As shown in Fig. 4D, food intake in the Ad-siAhi1 mice and the control mice was minimal immediately following surgery. After 24 h, both groups started to increase their food intake. However, the Ad-siAhi1 mice consumed significantly less food compared with the control mice, and thus the difference remained relatively constant throughout the first 2 weeks. The food intake of Ad-siAhi1 mice gradually returned to a normal level after 2 weeks, possibly because of the invalidating of Ad-siRNA over time. Correspondingly, body weight also showed a dramatic decrease in both groups in the beginning. Subsequently, body weight gradually increased in the control mice, but it decreased until the fifth day in the Ad-siAhi1 mice. The average body weight of the Ad-siAhi1 mice was significantly lower than the control group throughout the testing period (Fig. 4E). We further investigated the effects of hypothalamic Ahi1 knockdown on glucose metabolism. Mice on 5, 10, and 15 dpi underwent glucose tolerance tests and insulin tolerance tests separately. The glucose tolerance tests revealed that on 5 and 10 dpi, Ad-siAhi1-treated mice exhibited a significant improvement of glucose tolerance as compared with control mice (Fig. 4F). In insulin tolerance tests, Ad-siAhi1 mice exhibited an apparent improvement in insulin sensitivity (Fig. 4G). The Ad-siAhi1 mice became significantly more hypoglycemic at 30 min on 5 dpi and at 30 and 60 min on 10 dpi after insulin injection than did the control mice. We also assessed 5-HT2CR located on the neuronal membrane under the condition of artificial Ahi1 knockdown (supplemental Fig. 5). When Ahi1 expression was knocked down by RNAi (at 7 or 14 days post-surgery), membrane 5-HT2CR was significantly increased, after which a regression followed that was due to Ahi1 expression recovery.

Ahi1 Regulates Expression of NPY and POMC—Lastly, we explored the downstream targets of the hypothalamic Ahi1 signaling pathway in feeding control. Previous reports (15, 16, 36) have revealed that serotonin receptors activation inhibits the expression of orexigenic NPY and yet stimulates the expression of anorexigenic POMC in the hypothalamus. Because Ahi1 inhibits the activity of 5-HT2CR, we hypothesized that Ahi1 may also regulate the expression balance of NPY and POMC. The abundance of NPY and POMC mRNA was measured using real-time quantitative PCR. Notably, knockdown expression of Ahi1 led to a decrease in the mRNA levels of NPY and an increased expression of POMC in neuronal cell line N18TG2. In contrast, overexpression of Ahi1 resulted in higher NPY expression and lower POMC production (Fig. 5A). Moreover, Ad-siAhi1 intrahypothalamic injection reduced the mRNA level of NPY by 35% and increased the mRNA level of POMC by 49% in the mice hypothalami on day 3 post-injection. Similar changes in the levels of POMC and NPY by Ad-siAhi1 treatment
were also found on day 9 post-injection (Fig. 5B). These changes in feeding-related neuropeptides are consistent with decreased appetite as illustrated in Fig. 4D.

**DISCUSSION**

Although much progress has been made in understanding how neural circuits control feeding behavior and energy metabolism, we are far from getting the whole picture. Many other important cellular and molecular interactions within the circuits remain unknown. Our study indicates that hypothalamic Ahi1 could be one of the important interaction molecules within the circuits. Its high expression in the central nervous system and its structural characteristics make Ahi1 an ideal candidate for signal transduction in the central nervous system. Ahi1 contains several distinct protein domains, including six WD40 repeats, one SH3 domain, potential SH3 binding sites, and an N-terminal coiled-coiled domain (32). WD40 domains have been found in proteins involved in a variety of functions including signal transduction, RNA processing, transcriptional regulation, cytoskeleton assembly, vesicle trafficking, and cell division (37). Similarly, SH3 domains are a common feature of signaling molecules involved in numerous pathways (38, 39). Multiple lines of evidence have shown Ahi1 as a key regulator of neuronal development. Ahi1 mutation not only causes defects...
Ahi1 contains neurons are not co-expressed with 5-HT2CR, 5-HT2CR to the lysosome for degradation. Considering that the

we speculate that Ahi1 inhibits POMC expression by trafficking

(5-HT2CR is expressed in POMC-containing neurons (52), and

the lysosomes for degradation. A previous study has shown that

interacted by Ahi1 through the transportation of the receptors to

endocytic sorting of 5-HT2CR. After endocytosis, Ahi1 facili-

tates the trafficking of 5-HT2CR to lysosomal compartmen-

t. As such, the anorectic effect of the 5-HT2CR pathway is coun-

tered by Ahi1 through the transportation of the receptors to

the lysosomes for degradation. A previous study has shown that

5-HT2CR is expressed in POMC-containing neurons (52), and

we speculate that Ahi1 inhibits POMC expression by trafficking

5-HT2CR to the lysosome for degradation. Considering that the

NPY-containing neurons are not co-expressed with 5-HT2CR,

we hypothesize that the effect of Ahi1 on stimulating NPY

expression is most likely mediated indirectly by other molecu-

lar pathways. On the other hand, blockade of Ahi1-dependant

lyosomal sorting will increase the functional expression of

5-HT2CR (supplemental Fig. 5). Knockdown of Ahi1 expression

leads to less 5-HT2CR trafficking into lysosomes, consequently

resulting in the stimulation of POMC expression and the inhi-
bition of NPY expression (Fig. 5B) that subsequently reduce

food intake (Fig. 4D) and decrease body weight (Fig. 4E).

Interestingly, we observed an increased level of Ahi1 expres-
sion in olanzapine-treated mice (supplemental Fig. 3). There is

a growing concern about the increased risk of diabetes in

patients with schizophrenia, where normal health risks are ele-
vated by the obesogenic and diabetogenic side effects of antipsy-
chotics (53–55). It has been found that the risk is greater with

the atypical antipsychotics such as olanzapine, which has sero-

tonin receptor antagonist properties (56). It has been demon-

strated that olanzapine is associated with significant antipsy-

chotic-induced weight gain (57) and impaired glucose

effectiveness (58). However, the mechanisms responsible for

the diabetes mellitus associated with some antipsychotics are

still not fully understood. It has been proposed that the specific

binding profile of different antipsychotic agents may help

explain the occurrence of particular side effects associated with

each drug (59). Among the multiple receptor binding profiles of

atypical antipsychotics, 5-HT2CR has been implicated as medi-

ating an orexigenic effect and diabetogenic side effect. The ago-

nists of 5-HT2CR, such as fenfluramine and mCPP, have been

shown to be anorexigenic (60, 61). There is a correlation

between the drug affinity for 5-HT2CR and the morbidity rate of
type 2 diabetes mellitus (62). We speculate that Ahi1 may be

involved in the metabolic side effects of atypical antipsychotics.

The use of olanzapine will result in increased hypothalamic

Ahi1 expression. In the short term, Ahi1 will promote food

intake, which will lead to weight gain. In the long term, through

its interaction with 5-HT2CR, elevated Ahi1 expression will

impair glucose metabolism, which will contribute to the mort-

bidity rate of type 2 diabetes mellitus. An improved under-

standing of the role of Ahi1 in the metabolic side effects of

antipsychotic drugs may help provide the potential target to

alleviate these adverse effects.
Both pharmacological and genetic evidence implicates 5-HT$_{2c}$R as a critical receptor mediator of the effect of serotonin on feeding behavior (12, 52, 63, 64). Recent animal studies using knock-out mice have shown the 5-HT$_{2c}$R gene to be involved in the control of appetite and feeding behavior. 5-HT$_{2c}$R null mice display hyperphagia, reduced sensitivity to the anorectic effects of mCPP and dexfenfluramine, enhanced susceptibility to type 2 diabetes, and late-onset obesity syndrome (11, 12). Because 5-HT$_{2c}$R mutant mice are chronically hyperphagic from 5 weeks of age, it would be interesting to investigate whether the elevation of Ahi1 function caused by the dissociation between Ahi1 and 5-HT$_{2c}$R contributes to this phenomenon. Conditional knock-out mice strategy will help us to explore how Ahi1 influences the serotonin receptor-neuropeptide system and, subsequently, feeding and energy metabolism. Although additional studies are needed to fully characterize the neural network in which Ahi1 associates and modulates 5-HT$_{2c}$R, our findings provide a further understanding of how hypothalamic key molecules regulate feeding behavior.

Acknowledgment—We thank Prof. Marc G. Caron, Duke University Medical Center, for providing SHT$_{2c}$R-GFP and SHT$_{2c}$R-FLAG vectors.

REFERENCES

1. Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S., and Schwartz, M. W. (2006) Central nervous system control of food intake and body weight. Nature 443, 289–295
2. Sandoval, D., Cota, D., and Seeley, R. J. (2008) The integrative role of CNS fuel-sensing mechanisms in energy balance and glucose regulation. Anna. Rev. Physiol. 70, 513–535
3. Jespersen, S., and Scheel-Krüger, J. (1973) Evidence for a difference in mechanism of action between fenfluramine- and amphetamine-induced anorexia. J. Pharm. Pharmacol. 25, 49–54
4. Shor-Posner, G., Grinker, J. A., Marinucci, C., Brown, O., and Leibowitz, S. F. (1986) Hypothalamic serotonin in the control of meal patterns and macronutrient selection. Brain Res. Bull. 17, 663–671
5. Vickers, S. P., Benwell, K. R., Porter, R. H., Bickerdike, M. J., Bennett, G. A., and Dourish, C. T. (2000) Comparative effects of continuous infusion of mCPP, Ro 60-0175, and d-fenfluramine on food intake, water intake, body weight, and locomotor activity in rats. Br. J. Pharmacol. 130, 1305–1314
6. Pinder, R. M., Brogden, R. N., Sawyer, P. R., Speight, T. M., and Avery, G. S. (1975) Fenfluramine: a review of its pharmacological properties and therapeutic efficacy in obesity. Drugs 10, 241–323
7. Vezzosi, D., Cartier, D., Régner, C., Otal, P., Bennet, A., Parmentier, F., Plantavid, M., Lacroix, A., Lefebvre, H., and Caron, P. (2007) Familial adrenocorticotropin-independent macronodular adrenal hyperplasia with aberrant serotonin and vasopressin adrenal receptors. Eur. J. Endo-crinom. 156, 21–31
8. Kring, S. I., Werge, T., Holst, C., Toubro, S., Astrup, A., Hansen, T., Persdson, O., and Sorensen, T. I. (2009) Polymorphisms of serotonin receptor 2A and 2C genes and COMT in relation to obesity and type 2 diabetes. PLoS One 4, e6696
9. Zhou, L., Sutton, G. M., Rochford, J. J., Semple, R. K., Lam, D. D., Oksanen, G. M., Bellacchio, E., Battini, R., Cruse, R. P., Dobyns, W. B., Krishnamoorthy, K. S., Lagier-Tourenne, C., Magee, A., Pascual-Castroviejo, I., Salpietro, C. D., Sarco, D., Dallapiccola, B., and Gieson, J. G. (2004) Mutations in the AHI1 gene, encoding Joubert syndrome cause specific forms of Joubert syndrome-related disorders. Ann. Neurol. 59, 527–534
10. Doering, J. E., Kane, K., Hsiao, Y. C., Yao, C., Shi, B., Slowik, A. D., Dhagat, B., Scott, D. D., Ault, J. G., Page-McCaw, P. S., and Ferland, R. J. (2008) Species differences in the expression of Ahi1, a protein implicated in the neurodevelopmental disorder Joubert syndrome, with preferential accumulation to the brainstem. J. Comp. Neurol. 511, 238–256
11. Ferland, R. J., Eyaid, W., Collura, R. V., Tully, L. D., Hill, R. S., Al-Nouri, D., Al-Rumayyan, A., Topcu, M., Gascon, G., Bodell, A., Shugart, Y. Y., Ruvo, M. M., and Walsh, C. A. (2004) Abnormal cerebellar development and axonal decussation due to mutations in AHI1 in Joubert syndrome. Nat. Genet. 36, 1008–1013
12. Sheng, G., Xu, X., Lin, Y. F., Wang, C. E., Rong, J., Cheng, D., Peng, J., Jiang, X., Li, S. H., and Li, J. X. (2008) Huntington-associated protein 1 interacts with Ahi1 to regulate cerebellar and brainstem development in mice. J. Clin. Invest. 118, 2785–2795
13. Salonen, J. T., Uimari, P., Aalto, J. M., Pirskanen, M., Kaikkonen, J., Todoro- rova, B., Hyypönen, J., Korhonen, V. P., Asikainen, J., Devine, C., Tuomainen, T. P., Luedemann, J., Nauck, M., Kerner, W., Stephens, R. H., New, J. P., Oller, W. E., Gibson, J. M., Payton, A., Horan, M. A., Pendleton, N., Mahoney, W., Meyre, D., Delplanque, J., Froogel, P., Luzzatto, O., Yakir, B., and Darvasi, A. (2007) Type 2 diabetes whole-genome association study in four populations: the DiaGen consortium. Am. J. Hum. Genet. 81, 338–345
14. Prior, M. J., Foletta, V. C., Jowett, J. B., Segal, D. H., Carless, M. A., Curran, J. E., Dyer, T. D., Moses, E. K., McAinche, A. J., Konstantopoulos, N., Bo- zago, G., Collier, G. R., Cameron-Smith, D., Blangero, J., and Wald, K. R. (2010) The characterization of Abelson helper integration site-1 in skeletal muscle and its links to the metabolic syndrome. Metabolism 59, 1057–1064
15. Han, C. M., and Cosicina, D. V. (1995) Ineffectiveness of hypothalamic serotonin to block neuropeptide Y-induced feeding. Pharmacol. Biochem. Behav. 51, 641–646
16. Heisler, L. K., Cowley, M. A., Tecott, L. H., Fan, W., Low, M. J., Smart, J. L., Rubinstein, M., Tatro, J. B., Marcus, J. N., Holstege, H., Lee, C. E., Cone, R. D., and Elmqist, J. K. (2002) Activation of central melanocortin pathways by fenfluramine. Science 297, 609–611
17. Rogers, P., McKibbin, P. E., and Williams, G. (1991) Acute fenfluramine administration reduces neuropeptide Y concentrations in specific hypothalamic regions of the rat: possible implications for the anorectic effect of fenfluramine. Peptides 12, 251–255
18. Poirier, Y., Kozak, C., and Jolicoeur, P. (1988) Identification of a common helper provirus integration site in Abelson murine leukemia virus-induced lymphoma DNA. J. Virol. 62, 3985–3992
19. Dixon-Salazar, T., Silhavy, J. L., Marsh, S. E., Barna, G., Bertini, E., Boltschauser, E., Zaki, M. S., Abdel-Aleem, A., Abdel-Salam, G. M., Bellacchio, E., Battini, R., Cruse, R. P., Dobyns, W. B., Krishnamoorthy, K. S., Lagier-Tourenne, C., Magee, A., Pascual-Castroviejo, I., Salpietro, C. D., Sarco, D., Dallapiccola, B., and Gieson, J. G. (2006) AHI1 gene mutations cause specific forms of Joubert syndrome-related disorders. Ann. Neurol. 59, 527–534
20. Valente, E. M., Brancati, F., Silhavy, J. L., Castori, M., Marsh, S. E., Barna, G., Bertini, E., Boltschauser, E., Zaki, M. S., Abdel-Aleem, A., Abdel-Salam, G. M., Bellacchio, E., Battini, R., Cruse, R. P., Dobyns, W. B., Krishnamoorthy, K. S., Lagier-Tourenne, C., Magee, A., Pascual-Castroviejo, I., Salpietro, C. D., Sarco, D., Dallapiccola, B., and Gieson, J. G. (2006) AHI1 gene mutations cause specific forms of Joubert syndrome-related disorders. Ann. Neurol. 59, 527–534
21. Prior, M. J., Foletta, V. C., Jowett, J. B., Segal, D. H., Carless, M. A., Curran, J. E., Dyer, T. D., Moses, E. K., McAinche, A. J., Konstantopoulos, N., Bozago, G., Collier, G. R., Cameron-Smith, D., Blangero, J., and Wald, K. R. (2010) The characterization of Abelson helper integration site-1 in skeletal muscle and its links to the metabolic syndrome. Metabolism 59, 1057–1064
22. Han, S. B., Choi, B. I., Lee, D., Kee, S. H., Kim, H. S., Sun, W., and Kim, H.
Hypothalamic Ahi1 in Feeding Control

(2009) Regulation of AHI1 expression in adult rat brain: Implication in hypothalamic feeding control. Biochem. Biophys. Res. Commun. 390, 535–540

27. Niu, S. N., Huang, Z. B., Wang, H., Rao, X. R., Kong, H., Xu, J., Li, X. J., Yang, C., and Sheng, G. Q. (2011) Brainstem Hap1-Ahi1 is involved in insulin-mediated feeding control. FEBS Lett. 585, 85–91

28. Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45

29. Yuan, J. S., Reed, A., Chen, F., and Stewart, C. N., Jr. (2006) Statistical analysis of real-time PCR data. BMC Bioinformatics 7, 85

30. Sutton, G. M., Trevaskis, J. L., Hulver, M. W., McMillan, R. P., Markward, N. J., Babin, M. J., Meyer, E. A., and Butler, A. A. (2006) Diet-genotype interactions in the development of the obese, insulin-resistant phenotype of C57BL/6 mice lacking melanocortin-3 or -4 receptors. Endocrinology 147, 2183–2196

31. Swandulla, D., and Misgeld, U. (1990) Development and properties of a synaptic network in a network of rat hypothalamic neurons grown in vitro. J. Neurophysiol. 64, 715–726

32. Jiang, X., Hanna, Z., Kaouass, M., Girard, L., and Jolicoeur, P. (2002) Ahi-1, a novel gene encoding a modular protein with WD40-repeat and SH3 domains, is targeted by the Ahi-1 and Mis-2 provirus integrations. J. Virol. 76, 9046–9059

33. Keller, L. C., Geimer, S., Romijn, E., Yates, J., 3rd, Zamora, I., and Marshall, W. F. (2009) Molecular architecture of the centriole proteome: the conserved WD40 domain protein POC1 is required for centriole duplication and length control. Mol. Biol. Cell 20, 1150–1166

34. Louie, C. M., Caridi, G., Lopes, V. S., Brancati, F., Kispert, A., Lancaster, M. T., and Feron, R. J. (2009) Ahi1, whose human ortholog is mutated in Joubert syndrome and is a modifier for retinal degeneration in nephronophthisis. Mol. Biol. Cell 20, 3926–3941

35. Scheepers, F. E., Gespen de Wied, C. C., and Kahn, R. S. (2001) The effect of long-term olanzapine treatment: weight change and weight-related dysregulation: evaluating the risk/benefit equation and improving the standard of care. J. Clin. Psychopharmacol. 21, 57–64

36. Heisler, L. K., Cowley, M. A., Kishi, T., Tecott, L. H., Fan, W., Low, M. J., Smart, J. L., Rubinstein, M., Tatro, J. B., Zigman, J. M., Cone, R. D., and Barsh, G. S. (1997) Antagonism of central melanocortin receptors promotes hyperphagia via downstream activation of melanocortin 4 receptors. Endocrinology 149, 1323–1328

37. Lam, D. D., Przydzial, M. J., Rice, S. H., Yeo, G. S., Rochford, J. J., O’Rahilly, S., and Heisler, L. K. (2008) Serotonin 5-HT2C receptor agonist promotes hyperphagia in mice lacking melanocortin receptors. J. Clin. Psychiatry 69, 208–213

38. Currie, P. J., Saxena, N., and Tu, A. Y. (1999) 5-HT1A(2A/2C) receptor antagonists in the paraventricular nucleus attenuate the action of DOI on NPY-stimulated eating. Neuropeptides 10, 3033–3036

39. Hsiao, S. H., Chung, H. H., Inui, A., Tong, Y. C., and Cheng, J. T. (2006) Inhibitory effect of 5-hydroxytryptamine on hyperphagia in mice with genetic overexpression of neuropeptide Y. Neurosci. Lett. 394, 256–258

40. Boston, B. A., Blaydon, K. M., Varnerin, J., and Cone, R. D. (1997) Independent and additive effects of central POMC and leptin pathways on hypothalamic feeding control. J. Neurosci. 17, 256–258

41. Lee, Y. S., Challis, B. G., Thompson, D. A., Yeo, G. S., Keogh, J. M., Modonna, M. E., Wraith, V., Sims, M., Vatin, Y., Meyre, D., Shield, J., Burren, C., Ibrahim, Z., Cheetham, T., Swift, P., Blackwood, A., Hung, C. C., Wareham, N. J., Froguel, P., Millhauser, G. L., O’Rahilly, S., and Farooqi, I. S. (2006) A POMC variant implicates beta-melanocyte-stimulating hormone in the control of human energy balance. Cell Metab. 3, 135–140

42. Bierbmann, H., Castaneda, T. R., van Landeghem, F., van Deimling, A., Escher, F., Brabant, G., Hebebrand, J., Hinney, A., Tschop, M. H., Grüters, A., and Krude, H. (2006) A role for beta-melanocyte-stimulating hormone in human body-weight regulation. Cell Metab. 3, 141–146

43. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385, 165–168

44. Asan, R. A., Cone, R. D., Burbach, J. P., and Gispen, W. H. (1994) Differential effects of melanocortin peptides on neural melanocortin receptors. Mol. Pharmacol. 46, 1182–1190

45. Ollmann, M. M., Wilson, B. D., Yang, Y. K., Kerns, J. A., Chen, Y., Gantz, L., and Barsh, G. S. (1997) Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 278, 135–138

46. Stanley, B. G., and Leibowitz, S. F. (1984) Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. Life Sci. 35, 2635–2642

47. Lam, D. D., Przydzial, M. J., Ridley, S. H., Yeo, G. S., Rochford, J. J., O’Rahilly, S., and Heisler, L. K. (2008) Serotonin 5-HT2C receptor agonist promotes hyperphagia in mice lacking melanocortin receptors. J. Physiol. 585, 85–91

48. Iordanidou, M., Tavridou, A., Vasiladis, M. V., Arvanitidis, K. I., Petridis, J., Christakidis, D., Vargemezis, V., Bougioukas, G., and Manolopoulos, V. G. (2008) The -759 C/T polymorphism of the 5-HT2C receptor is associated with type 2 diabetes in male and female Caucasians. Pharmacogenet. Genomics 18, 159–169

49. Adan, R. A., Cone, R. D., Burbach, J. P., and Gispen, W. H. (1994) Fasting and secretion of melanocortins in neural melanocortin receptors. Mol. Pharmacol. 46, 1182–1190