HYPOTHALAMIC AHI1 MEDIATES FEEDING BEHAVIOR THROUGH INTERACTION WITH 5-HT2C RECEPTOR

Hao Wang1#, Zhenbo Huang1#, Liansha Huang2#, Shaona Niu3, Xiurong Rao1, Jing Xu1, Hui Kong1, Jianzhong Yang2, Chuan Yang3, Donghai Wu1, Shihua Li6, Xiao-Jiang Li6, Tonghua Liu2* and Guoqing Sheng1*

1CAS 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, China 510530 2Department of Science and Technology, Beijing University of Chinese Medicine, China 100029 3Department of Endocrinology, Lin Yi People's Hospital, No 27 jiefang road, LinYi 276003 4The Red Cross hospital of Yunnan Province, Kunming, China 650030 5Department of Endocrinology and Metabolism, The Second Affiliated Hospital of Sun Yat-sen University, Guangzhou, China 510020 6Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322

Running title : Hypothalamic Ahi1 in feeding control

It is indicated that there are other important molecules interacting with brain nervous systems to regulate feeding and energy balance by influencing these systems’ signaling pathways, 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 the serotonin receptor 2C (5-HT2C) known for its critical role in feeding and appetite control. First, we demonstrated the co-localization and interaction between hypothalamic Ahi1 and 5-HT2C. Ahi1 promoted the degradation of 5-HT2C through the lysosomal pathway. Then, we investigated the effects of fasting on the expression of hypothalamic Ahi1 and 5-HT2C. Fasting resulted in an increased Ahi1 expression and a concomitant decreased expression of 5-HT2C. Knockdown of hypothalamic Ahi1 led to a concomitant increased expression of 5-HT2C and a decrease of food intake and body weight. Last, we found that Ahi1 could regulate the expression of neuropeptide Y (NPY) and proopiomelanocortin (POMC). Taken together, our results indicate that Ahi1 mediates feeding behavior by interacting with 5-HT2C to modulate the serotonin signaling pathway.

Obesity and its associated ailments such as diabetes have become a worldwide epidemic and carry with it a heavy toll of morbidity and mortality. Over the past decades, it has become evident that neural circuits in the central nervous system (CNS) play a direct and crucial role in controlling feeding and energy homeostasis (1,2). Disruptions in the mechanisms of CNS 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 was shown to result in hyperphagia and obesity, whereas the release of serotonin and the inhibition of reuptake by D-fenfluramine potently reduce feeding and body weight (6). More recently, several lines of evidence show that serotonin
receptors 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 where serotonin receptor 2C (5-HT2cR) has been specifically recognized 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-HT2cR agonists reduce appetite and improve diabetes (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 more intriguing aspects of this area that has remained mysterious concerns the importance of cellular and molecular interactions that centrally regulate energy homeostasis.

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 that Ahi1 mutations are 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 highly distributed in several brain areas implicated in feeding and metabolic regulation such as the hypothalamic paraventricular nucleus (PVN), the supraoptic nucleus (SON), the arcuate nucleus (ARC), the lateral hypothalamic area (LH) and the nucleus tractus solitarius (NTS) 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 2 novel Ahi1 genetic variants linked with fasting blood glucose levels in Mexican American subjects (25). Recent studies (26,27) also showed that brain Ahi1 may play an important role in the regulation of feeding behavior.

We noticed that Ahi1 and 5-HT2cR have similar distribution in hypothalamus. This prompted us to investigate the relationship between Ahi1 and 5-HT2cR and their probable role in feeding control. The findings in the present study provide evidence that Ahi1 interact with 5-HT2cR to mediate feeding behavior. Our study reveals the normal neural function of Ahi1 in feeding control and offer insight into the understanding of how hypothalamic key molecules regulate the feeding behavior.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice of 6-10 weeks old were purchased from Southern Medical University, Guangdong province of China. The animals were housed in a temperature and humidity-controlled environment with a 12h/12h light/dark cycle and had 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 conform to the guidelines of the Animal Care and Use Committee of Guangzhou Biomedical and Health Institute, Chinese Academy of Sciences.

Immunostaining
The antibody against Ahil was described in our previous study (23). For immunofluorescent staining, sections of brain tissue and coverslips plated with hypothalamic neurons or HEK 293 cells were blocked with 5% bovine serum albumin (BSA) in 0.02M PBS at room temperature for 1 hour, and incubated with rabbit anti-Ahi1 antibody (1:300) and goat anti-5HT2CR (1:50, SantaCruz) at 4°C overnight. Then the sections were orderly incubated with Alexa Fluor488 conjugated anti-goat second antibody and Alexa Fluor594 conjugated anti-rabbit second antibody (both from Invitrogen) at room temperature for 30 min. Tissue sections and coverslips were mounted onto glass slides. Labeled samples were imaged using 100× objective of a Leica SP2 confocal microscope.

**Immunoprecipitation**

Lysates from mouse hypothalamus or from co-transfected HEK293 cells were extracted using lysis buffer (50 mM Tris-Cl, pH8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100 and protease inhibitor cocktail) and centrifuged 12,000g 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 hour to reduce nonspecific binding. After pelleting the beads, the supernatant was then incubated with antibody to mouse Ahil (1:50), or with anti-5HT2CR monoclonal antibody (Sigma, clone HA-7, diluted 1:4000), or with anti-GFP polyclonal antibody (Abcam, ab290, diluted 1:1000), or with 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, pH8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2% Tween-20) and subjected to western blotting.

**Purification membrane fraction and western blot**

A total of 15-20 mg of whole hypothalamuses from fasted mice or Ahil 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 cocktail) on ice. The suspensions were ultracentrifuged at 59,000 g at 4°C for 15min, then the pellets (P1) were re-extracted as above mentioned in 1 ml MPB with 0.8% Triton-X 114. After 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 re-suspended in MPB.

Hypothalamus were mechanically homogenized and sonicated in homogenization buffer on ice. 20 µg of tissue protein was size-fractionated using 10% SDS-PAGE, and electro-transferred 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 Ahil or anti-5HT2CR 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 Image Quant5.2 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 CCA-3'), mouse NPY (forward primer: 5'-TAC TAC TCC GCT CTG CGA CAC-3'; reverse primer: 5'-CCA CAT GGA AGG GTC TTC AAG-3'), mouse POMC (forward primer: 5'- CGA GCC ATT AGG CTT-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'- GGA AGG CCA TGC CAG 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 a MJ 4-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 previous reports (28,29).

RNA interference

For the knockdown of Ahi1, C57BL/6J mice (8 weeks old, male) were injected bilaterally with recombinant adenovirus vector encoding Ahi1-specific siRNA (Ad-siAhi1), or scramble-siRNA (Ad-scRNA) as control. Total of \(1 \times 10^{11}\) plaque-forming units in 1 \(\mu\)l of PBS were injected bilaterally intro-hypothalamus at the stereotaxic position (anteroposterior -1.1mm, mediolateral \(\pm 0.5\)mm, dorsalventral -5.5mm). The adenoviral vector also independently expresses green fluorescent protein (GFP), which enable us to trace the vector. The injection at above coordinates allows the adenovirus to mainly infect the VMH, ARC, DMH and other nucleus in hypothalamus (Figure 4A). The mice were single-housed in regular plastic cages before and after 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 employed in glucose tolerance test (GTT) and insulin tolerance test (ITT) performed as described previously (30). Glycemia was assessed using Blood Glucose Test Meter.

Cell culture, infection, transfection and drug treatment

Co-localization of Ahi1 and 5HT2CR 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 density of \(\sim 2,000\) cells/cm². Cultures were kept in NeuroBasal medium supplemented with 2% B27, 2mM L-glutamine (all from GIBCO) for 14 days in vitro.

N18TG2, mouse neuroblastoma cell line with endogenous expressing Ahi1 expression, were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (PAA), 1 mM non-essential amino acids (GIBCO), 2 mM L-Glutamine (GIBCO), 100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin (GIBCO). DNA constructs for expression of Ahi1 cDNA plasmids encoding full-length mouse Ahi1 (1-1047) were generated in a previous study (32). We used PCR to generate C-terminal truncated mouse Ahi1 (1-820). These Ahi1 cDNAs were sequenced and cloned to the PRK vector that links the influenza hemagglutinin (HA) epitope on the upstream of Ahi1 coding region. For transfection of foreign DNAs into N18TG2, plasmids-polyrthulenimine (PEI, Polysciences) complexes incubated with cultures for 1 hour. After 36 hours of...
transfection, N18TG2 cells were stimulated under 5 μM mCPP (Sigma) with or without 100 μM chloroquine (Chlq, Sigma) for indicated time, then immediately collected for following quantitative assays. For virus infection, the cells were infected by Ad-siAhi1 (1 × 10^7 plaque-forming units/ml media) for 8 hours and sampled at 24 hours 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. At achieved 50% confluence, the HEK293 cells were co-transfected with constructs encoding DsRed-tagged Ahi1 and GFP-tagged 5HT2CR 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-5HT2CR, were washed once and fixed for 10 min at -20 °C in 50% (v/v) acetone/methanol and air-dried. Cells were blocked by incubating for 1 hour in 5% BSA and then incubated with anti-LAMP1 primary antibody (4 µg/ml, Abcam) at 4 °C for 24 hours. Then coveslips were washed several times and incubated with Cy5-labeled anti-mouse IgG for 45 min at room temperature. After DAPI staining, 4-color slides were visualized with Leica SP2 confocal scanning microscope set up as follows: 403 nm laser (25% of power) window 410-483 nm, 488 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 were automatically measured and divided by the total cell area, which was determined by setting a lower threshold level to measure 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 5HT2CR with less than the average at 30% were excluded from analysis, because of variation in transfection efficiency. N values in the text represent the number of quantified cells.

**Statistics**

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

**RESULTS**

**The 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 (top panel and middle panel of Figure 1A). Staining of mouse brain sections also revealed an extensive overlap of the two proteins in hypothalamic nucleus such as ARC (Figure 1A bottom panel).
Co-IP study was performed in mice hypothalamus homogenates. As shown in Figure 1B, 5-HT<sub>2c</sub>R 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-HT<sub>2c</sub>R showed an extensive distribution throughout the plasma membrane and cytoplasm of the cell while Ahi1 showed a unique distribution pattern characterized as dot-like structures. Co-expression of 5-HT<sub>2c</sub>R and Ahi1 displayed apparent co-localization in punctate aggregates in the cytoplasm of HEK293 cells (Supplemental Figure 1A). Their interaction was further demonstrated by co-IP using anti-Ahi1 antibody (Supplemental Figure 1B). Then, we constructed the C-terminus truncated Ahi1. In the immunofluorescence experiments we found that the co-localization of Ahi1 and 5-HT<sub>2c</sub>R was lost in the cells transfected with C-terminus truncated Ahi1 (Figure 1D). Similarly the combination of Ahi1 and 5-HT<sub>2c</sub>R was abolished in the C-terminus truncated Ahi1 (Figure 1E). These data indicate that the C-terminus of Ahi1 is the region where it interacts with 5-HT<sub>2c</sub>R.

**Ahi1 promotes the lysosomal degradation of 5-HT<sub>2c</sub>R**

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 vesicles trafficking and is co-localized with microtubules and microtubule organizing centre (MTOC) (33,34). We postulated that Ahi1 could participate in 5HT<sub>2c</sub>R-vesicles sorting to degradation after endocytosis. To test this hypothesis, we treated cells with meta-chlorophenylpiperazine (mCPP), an agonist of 5-HT<sub>2c</sub>R (13). As shown in Figure 2A, the 5-HT<sub>2c</sub>R decreased in a time-dependent way in the cells transfected with full length Ahi1, while in the cells transfected with truncated Ahi1 this decrease was inhibited. Then we addressed the pathway of 5-HT<sub>2c</sub>R degradation. We first investigated the possible involvement of the lysosomal pathway with the treatment of chloroquine (Chlq), a lysosomal enzyme inhibitor. Analysis by western blot revealed that the treatment of Chlq increased the level of 5-HT<sub>2c</sub>R in mCPP treated N18TG2 cells (lane 3 vs. lane 2 in Figure 2B), thereby indicating that the lysosomal pathway mediates internalized 5-HT<sub>2c</sub>R 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-HT<sub>2c</sub>R was significantly increased as compared to that of an empty vector (lane 5 vs. lane 2 in Figure 2B) and this increased 5-HT<sub>2c</sub>R degradation could be blocked by adding Chlq to the cells (lane 6 in Figure 2B). As shown in Supplemental Figure 2, the addition of lactacystin (LAC, a proteasome inhibitor) could not block the degradation of 5HT<sub>2c</sub>R, which indicates that the proteasome pathway is not involved in the 5-HT<sub>2c</sub>R degradation. In the cells expressing GFP-5HT<sub>2c</sub>R alone, the receptor was distributed throughout the plasma membrane as well as the cytoplasm when visualized under the fluorescence microscope (bottom panel of Figure 2C). Co-expression of Ahi1 dramatically changed the distribution pattern of 5-HT<sub>2c</sub>R as expected and strong 5-HT<sub>2c</sub>R signals
were found on the lysosome (LAMP1 positive dots) where 5-HT$_{2C}$R co-localized with Ahil (top panel of Figure 2C). Quantification of 5-HT$_{2C}$R and LAMP1 co-localization (Figure 2D) showed significant overlapping regions between the 5-HT$_{2C}$R and LAMP1-labeled pixels when Ahil was expressed (563.49% of mCPP-untreated cells, n=34). However, the value was much lower when Ahil was absent (109.90% of mCPP-untreated cells, n=32). Taken together, these results strongly support the idea that Ahil interacts with 5-HT$_{2C}$R and enhances the trafficking of internalized 5-HT$_{2C}$R to lysosomes, thereby promoting its degradation.

Fasting alters the expression of hypothalamic Ahil and 5-HT$_{2C}$R

Then we examined the role of hypothalamic Ahil and 5-HT$_{2C}$R in the regulation of feeding behavior. As shown in Figure 3A, hypothalamic Ahil protein was significantly increased after a fasting period of 24 or 48 hours. Similarly, the transcription of Ahil mRNA was significantly up-regulated (Figure 3B). In order to investigate the involvement of 5-HT$_{2C}$R in this process, we isolated the member fraction of hypothalamus lysates and assessed 5-HT$_{2C}$R located on the neuronal membrane under the condition of fasting. As shown in Figure 3C, the down-regulation of membrane 5-HT$_{2C}$R levels was concomitant with the up-regulation of Ahil in the time course study. To further confirm the correlation between 5-HT$_{2C}$R and Ahil, We employed pharmacological modulators of 5-HT$_{2C}$R. First, we pre-treated (i.p.) fasted mice with mCPP. The up-regulation of hypothalamic Ahil in fasted mice was significantly inhibited by mCPP pre-treatment (Figure 3D). Then, we used olanzapine (OLP), an antagonist of 5-HT$_{2C}$R (35). The expression of Ahil protein in N18TG2 cell line, as shown in Supplemental Figure 3A, was increased by 70% after 72 hours of exposure to OLP. Similarly, as compared to the control group, 4 weeks treatment of OLP significantly increased the levels of Ahil expression in the mice hypothalamus (Supplemental Figure 3B). These data suggest that 5-HT$_{2C}$R is a necessary component of the Ahil-dependent signaling pathway that controls feeding behavior.

Knockdown of hypothalamic Ahil suppresses food intake

To further establish a role for endogenous Ahil in feeding, we used RNA interference (RNAi) to suppress the expression of Ahil in the hypothalamus. We generated a mouse Ahil-specific siRNA that effectively inhibits the expression of endogenous Ahil in N18TG2 cells (Supplemental Figure 4). This siRNA was expressed from an adenoviral vector that also independently expressed green fluorescent protein (GFP). Thus, cells labeled with GFP should also express Ahil-specific siRNA. An adenoviral vector expressing scramble-siRNA (Ad-scRNA) served as a control. As shown in Figure 4A, a high-level of expression of GFP-fused Ad-siAhil 4 days post-injection (dpi) was observed on stereotactic coronal sections. Immunohistochemistry (Figure 4B) and western blot (Figure 4C) showed that Ad-siRNA produced a significant knockdown of Ahil 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 Figure 4D, food intake in the Ad-siAhil mice and the control mice were minimal immediately following surgery. After 24 hours, both groups started to increase their food intake. However, the
Ad-siAhi1 mice consumed significantly less food compared to the control mice, and this difference remained relatively constant throughout the first two weeks. The food intake of Ad-siAhi1 mice gradually returned to a normal level after 2 weeks, possibly because the expression of Ad-siRNA decreased 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 (Figure 4E).

We further investigated the effects of hypothalamic Ahi1 knockdown on glucose metabolism. Mice on 5 dpi, 10 dpi and 15 dpi underwent glucose tolerance tests (GTT) and insulin tolerance tests (ITT) separately. GTT revealed that on 5 and 10 dpi, but not on 15 dpi, Ad-siAhi1 treated mice exhibited a significant improvement of glucose tolerance as compared to control mice (Figure 4F). In ITT, Ad-siAhi1 mice exhibited an apparent improvement in insulin sensitivity (Figure 4G). The Ad-siAhi1 mice became significantly more hypoglycemic at 30 min on 5 dpi and at 30 min and 60 min on 10 dpi after insulin injection than control mice. We also assessed 5-HT2cR located on the neuronal membrane under the condition of artificial Ahi1 knockdown (Supplemental Figure 5). When Ahi1 expression was knocked down by RNAi (on 7 days 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 the expression of NPY and POMC**

Last, we explored the downstream targets of hypothalamic Ahi1 signaling pathway in feeding control. Previous reports (15,16,36) have revealed that serotonin receptors activation inhibits the expression of orexigenic neuropeptide Y (NPY) and yet stimulates the expression of anorexigenic proopiomelanocortin (POMC) in the hypothalamus. Since Ahi1 can inhibit the activity of 5-HT2c-R, 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 decreased in the mRNA levels of NPY and an increased expression of POMC in neuronal cell line N18TG2. In contrast, over-expression of Ahi1 resulted in higher NPY expression and lower POMC production (Figure 5A). Moreover, Ad-siAhi1 intra-hypothalamic injection reduced the mRNA level of NPY by 35% and increased the mRNA level of POMC by 49% in the mice hypothalamus on day 3 post-injection. Similar changes of POMC and NPY by Ad-siAhi1 treatment were also found on day 9 post-injection (Figure 5B). These changes of feeding-related neuropeptides are consistent with the decreased appetite as illustrated in Figure 4D.

**DISCUSSION**

Although much progress has been made in the understanding of how neural circuits control feeding behavior and energy metabolism, we are far from get the whole picture. There are 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 structure...
characteristics make Ahi1 an ideal candidate for signal transduction in the CNS. 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 on 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 in intracellular trafficking (40), but also leads to abnormal development of neuronal interactions and its associated networks. The dysfunction of Ahi1 causes development disorders. Abnormal cerebellar and cortical development as well as lack of axonal decussation was caused by Ahi1 mutation in JSRD (18,22). Moreover, our previous study has proved that dysfunctional interaction of Hap1-Ahi1 causes defects in axonal guidance and alters brain connections in the postnatal brainstem and cerebellum (23).

The present study showed that under normal conditions hypothalamic Ahi1 interacts with 5-HT2CR to regulate food intake. 5-hydroxytryptamine (5-HT; serotonin) system has long been associated with food intake and body weight regulation. A variety of studies have demonstrated that hypothalamic NPY and POMC are important intermediaries in the serotonin system induced-feeding response (15,16,41-43). Serotonin receptor activation inhibits the expression of orexigenic NPY and stimulates the expression of anorexigenic POMC in the hypothalamus (15,16,36). Two sets of neurons, with opposite effects on feeding, have been identified in the hypothalamic arcuate nucleus—the POMC and NPY neurons. The neurons that express POMC are anorexigenic, due to the release of the cleavage products of POMC precursor—α- and β-melanocyte stimulating hormones (α- and β-MSH) (44,45), which, in turn, reduce food intake and body weight as well as increase energy expenditure in animals and humans (46-48) by acting on melanocortin receptor subtypes 3 and 4 (MC3 and 4R) (49). In contrast, the neurons containing NPY are orexigenic (50). NPY potently stimulates food intake and reduces energy expenditure (51). In this study, we demonstrate the important role that Ahi1 plays in the endocytic sorting of 5-HT2CR. After endocytosis, Ahi1 facilitates the trafficking of 5-HT2CR to lysosomal compartments. As such, the anorectic effect of 5-HT2CR pathway is counteracted by Ahi1 through the transportation of the receptors to the lysosomes for degradation. Previous study has shown that 5HT2CR is expressed in POMC-containing neurons (52), and we speculate that Ahi1 inhibits POMC expression by trafficking 5HT2CR to the lysosome for degradation. Considering that the NPY containing neurons are not co-expressed with 5HT2CR, we hypothesize that the effect of Ahi1 on stimulating NPY expression is most likely mediated indirectly by other molecular pathways. On the other hand, blockade of Ahi1-dependant lysosomal sorting will increase the functional expression of 5-HT2CR (Supplemental Figure 5). Knockdown of Ahi1 expression leads to less 5HT2CR trafficking into lysosomes, and consequently results in the stimulation of POMC expression and the inhibition of NPY expression (Figure 5B) that
subsequently reduces food intake (Figure 4D) and decreases body weight (Figure 4E).

Interestingly, we observed an increased level of Ahi1 expression in olanzapine treated mice (Supplemental Figure 3). There is a growing concern about the increased risk of diabetes in patients with schizophrenia where normal health risks are elevated by the obesogenic and diabetogenic side effects of antipsychotics (53-55). It has been found that the risk is greater with the atypical antipsychotics such as olanzapine, which has serotonin receptors antagonist properties (56). It has been demonstrated that olanzapine is associated with antipsychotic-induced significant 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 receptors binding profile of atypical antipsychotics, 5-HT2C receptor has been implicated to mediate orexigenic effect and diabetogenic side effect. The agonists of 5-HT2C, such as fenfluramine and mCPP, have been shown to be anorexigenic (60,61). There is a correlation between the drug affinity for 5-HT2C and the morbidity rate of type 2 diabetes mellitus (62). We speculate that Ahi1 may be involved in the metabolic side effects of antipsychotic drugs. 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-HT2C-R, elevated Ahi1 expression will impair glucose metabolism which will contribute the morbidity rate of type 2 diabetes mellitus. Improved understanding 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 5HT2C-R as a critical receptor mediator of serotonin’s effects on feeding behavior (12,52,63,64). Recent animal studies using knockout mice have shown the 5HT2C gene to be involved in the control of appetite and feeding behavior. 5HT2C 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 5HT2C mutant mice are chronically hyperphagic since 5 weeks of age, it would be interesting to investigate whether the elevation of Ahi1 function caused by the dissociation between Ahi1 and 5HT2C-R may contribute to this phenomenon. Conditional knockout 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 5HT2C-R, our findings provide a further understanding of how hypothalamic key molecules regulate the feeding behavior.
REFERENCES

FIGURE LEGENDS

Figure 1 Hypothalamic Ahil co-localized and interacted with 5-HT2CR. (A) Immunofluorescence analysis of the co-localization between Ahil and 5-HT2CR in cultured hypothalamic neuron and in hypothalamus ARC section. Ahil significantly co-localized with the 5-HT2CR in the soma of hypothalamic neurons (2-dimentional photo in top panel and 3-dimentional photo in medial, arrows indicate the co-localization of Ahil and 5HT2CR) and hypothalamus nucleus such as ARC (bottom panel). (B) 5- HT2CR was co-precipitated by the Ahil antibody in the hypothalamus of mice. (C) Schematic of full length Ahil and C-terminus truncated Ahil. (D) Full length or truncated Ahil was co-transfected with 5HT2CR into HEK293 cells and then immunofluorescence was performed. Note that in the cells transfected with truncated Ahil the co-localization of Ahil and 5HT2CR was lost. (E) Co-immunoprecipitation showed that the combination of Ahil and 5HT2CR was abolished in the C-terminus truncated Ahil. Scale bar: 20 μm. Abbreviation: ARC, arcuate nucleus; C-ter, C-terminus; Ahil-F, Ahil-full length; Ahil-T, Ahil-truncated; IgG, immunoglobulin G; HA, hemagglutinin; GFP, green fluorescent protein; IP, immunoprecipitation; IB, immunoblotting.

Figure 2 Ahil promoted the degradation of 5-HT2CR through the lysosomal pathway. (A) Full length or truncated Ahil was co-transfected with 5HT2CR into N18TG2 cells and then immunoblotting was performed. In the cells transfected with full length Ahil the 5HT2CR decreased in a time-dependent way after mCPP treatment, while in the cells transfected with truncated Ahil this decrease was inhibited. (B) Co-expression of 5HT2CR with Ahil in N18TG2 cells led to increased degradation of mCPP-activated 5HT2CR compared with the expression of 5HT2CR alone (lane 5 compared with lane 2), which could be blocked under the application of Chlq (lane 6). (C) 5HT2CR located in the lysosomes was increased with the presence of Ahil. In cells expressing GFP-5-HT2CR and RFP-Ahil, the 5HT2CR mainly overlapped with the lysosomes (LAMP1 as marker) and co-localized with Ahil (arrows...
indicating, top panel). In the absence of Ahi1, 5HT2CR is distributed throughout the plasma membrane as well as the cytoplasm (bottom panel). (D) Quantifying the co-localization of 5-HT2CR with the lysosomes in N18TG2 cells by calculating the area containing 5-HT2CR and lysosome-labeled pixels with or without Ahi1. Scale bar: 10 μm. Data are expressed as mean ± SEM. *Statistically different from control group. Abbreviation: p-Ahi1, plasmid-Ahi1; p-5HT2CR, plasmid-5HT2CR; mCPP, meta-chlorophenylpiperazine; chlq, chloroquine; LAMP1, lysosomal-associated membrane protein 1; RFP, red fluorescent protein.

**Figure 3** The expression of hypothalamic Ahi1 and 5-HT2CR was changed by fasting. (A) Western blot showed that the protein levels of Ahi1 were significantly increased after fasting for 24 or 48 hours in mice. (B) Real-Time PCR demonstrated that the transcription of hypothalamic Ahi1 mRNA was also stimulated by fasting. (C) 5-HT2CR located on the neuronal membrane under the conditions of fasting. When up-regulated the expression of hypothalamic Ahi1 by fasting, 5HT2CR distributing on the membrane of hypothalamic neurons was significantly reduced. (D) Up-regulation of hypothalamic Ahi1 induced by fasting could be significantly inhibited by mCPP pre-treatment. Data are expressed as mean ± SEM. *Statistically different from control group. Abbreviation: ACTB, actin-beta; mCPP, meta-chlorophenylpiperazine.

**Figure 4** Knockdown of hypothalamic Ahi1 led to a decrease of food intake and body weight and an improvement of glucose tolerance and insulin sensitivity. (A) Ad-siAhi1 (GFP-labeling) was expressed in the majority of the hypothalamus, including DMH, VMH, and ARC. Fluorescent microscopic image shows the expression of adenoviral vectors with stereotaxic position. Ad-siAhi1 or Ad-scRNA (as control) vectors were bilaterally delivered into the mice hypothalamus. (B) Immunohistochemistry and (C) western blot showed that Ad-siRNA produced a significant knockdown of Ahi1 protein in the hypothalamus. Animals were fed with a regular chow diet for 4 weeks post-injection. (D) Accumulative food intake and (E) body weight during the 4 weeks post-injection were measured (red arrow indicating the injection day). (F) GTT was performed by measuring blood glucose concentration at 30, 60, 90, 120 min after glucose injection (i.p.) in Ad-siAhi1 mice and Ad-scRNA control mice on day 5, 10, 15. (G) ITT was performed by measuring blood glucose abundance in Ad-siAhi1 mice and Ad-scRNA control mice at the indicated times after intraperitoneal injection of insulin. Scale bar: 200 μm in (A); 100 μm in (B). Data are expressed as mean ± SEM. *Statistically different from Ad-scRNA group. Abbreviation: Ad-siAhi1, adenoviral vector with Ahi1-specific siRNA; Ad-scRNA, adenoviral vector with scramble-siRNA; GTT, glucose tolerance test; ITT, insulin tolerance test; dpi, days post-injection.

**Figure 5** Ahi1 modulated the expression of NPY and POMC. (A) Ahi1 regulated the transcription of POMC and NPY in N18TG2 cells. Quantitative RT-PCR indicated that down-regulation of Ahi1 by siRNA decreased NPY and increased POMC expression, while over-expression of Ahi1 resulted in higher NPY expression and lower POMC production. (B) Knockdown of Ahi1 in mice hypothalamus led to an increase of POMC mRNA and a decrease of NPY mRNA on day 3 and day 9 post-injection. (C) Proposed model of
hypothalamic Ah1 in feeding control. Data are expressed as mean ± SEM. *Statistically
different from control group. Abbreviation: NPY, neuropeptide Y; POMC,
proopiomelanocortin; KD, knockdown; OE, over-expression; dpi, days post-injection.

FIGURE 1
FIGURE 4

A

B

C

D

E

F

G

FIGURE 5

A

B

C

Fasting → Ahi1 ↑ → 5HT2cR ↓ → NPY ↑ → POMC ↓ → Food intake
SUPPLEMENTAL DATA

Supplemental Figure 1 DsRed-Ahi1 and GFP-5-HT2C-R were transfected into HEK293 cells, and immunofluorescence and co-IP were performed on these cells. (A) Immunofluorescence showed clear overlap of Ahi1 and 5-HT2CR in HEK293 cells. (B) Co-IP of 5-HT2CR with Ahi1 in transfected HEK293 cells. Scale bar: 10 μm. Abbreviation: IP, immunoprecipitation; IB, immunoblotting.

Supplemental Figure 2 The degradation of 5-HT2CR mediated by Ahi1 was through lysosomal pathway rather than proteasome pathway. When we treated cells co-transfected with Ahi1 and 5-HT2C-R by chloroquine (Chlq, a lysosomal inhibitor), the degradation of 5-HT2C-R was reversed; whereas, the treatment of lactacystin (LAC, a proteasome inhibitor) did not reverse the degradation of 5-HT2C-R. Abbreviation: mCPP, meta-chlorophenylpiperazine.
Supplemental Figure 3 Olanzapine increased the expression of Ahi1. (A) Ahi1 levels were measured in N18TG2 cells treated by OLP (Sigma) for 72 hours. OLP induced a significant increase of Ahi1 protein. (B) Mice were injected (i.p.) with OLP (Sigma, 2 mg/kg) for 4 weeks and the hypothalamic Ahi1 was measured by western blot. OLP treatment significantly increased the expression of Ahi1 in hypothalamus. Data are expressed as mean ± SEM. *Statistically different from control group. Abbreviation: OLP, olanzapine.

Supplemental Figure 4 Ahi1-specific siRNA effectively inhibited the expression of endogenous Ahi1 in N18TG2 cells. Western blot showed the concentration of $10^7$ plaque-forming units of Ad-siAhi1-2 was effective in knocking down the expression of Ahi1. Data are expressed as mean ± SEM. *Statistically different from control group. Abbreviation: Ad-siAhi1, adenoviral vector with Ahi1-specific siRNA; Ad-scRNA, adenoviral vector with scramble-siRNA.
Supplemental Figure 5 5-HT\(_2\)CR located on the neuronal membrane under the condition of artificial knockdown by RNA interference. As shown by western blot, the membrane distribution of 5-HT\(_2\)CR significantly increased when the expression of Ahi1 was down-regulated, after which a regression followed that was due to Ahi1 expression recovery. Data are expressed as mean ± SEM. *Statistically different from control group. Abbreviation: KD, knockdown; ACTB, actin-beta.
Hypothalamic Ahi1 mediates feeding behavior through interaction with 5-HT2C receptor

Hao Wang, Zhenbo Huang, Liansha Huang, Shaona Niu, Xiurong Rao, Jing Xu, Hui Kong, Jianzhong Yang, Chuan Yang, Donghai Wu, Shihua Li, Xiao-Jiang Li, Tonghua Liu and Guoqing Sheng

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