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Brainstem Hap1–Ahi1 is involved in insulin-mediated feeding control

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ABSTRACT

The function of the brainstem Hap1–Ahi1 complex in the regulation of feeding behavior was investigated. When mice were fasted or treated with 2-deoxy-D-glucose (2-DG), Hap1–Ahi1 was significantly upregulated. By using streptozotocin (STZ) to decrease the circulating insulin in mice, Hap1–Ahi1 was significantly increased. Furthermore, intra-brain injection of insulin decreased the expression of Hap1–Ahi1 in the brainstem. Moreover, when we knocked down the expression of brainstem Hap1 by RNAi, the mice showed decreased food intake and lower body weights. Collectively, our results indicate that the Hap1–Ahi1 complex in the brainstem works as a sensor for insulin signals in feeding control.

Structured summary: **Ahi1** *physically interacts* with **Hap1:** shown by *anti bait coimmunoprecipitation* (view interactions 1, 2)

1. Introduction

Over the past few years, significant advances have been made regarding the neural control of feeding behavior. As a result, it has become evident that neural circuits in the central nervous system play a crucial role in controlling energy homeostasis [1]. A number of metabolic hormones, neuropeptides and signaling pathways are components of the neural circuits that regulate energy homeostasis [2–6]. Recent studies have suggested that the neuronal molecules huntingtin-associated protein 1 (Hap1) and Abelson helper integration site 1 protein (Ahi1) potentially participate in the regulation of energy homeostasis.

Hap1 was initially identified as an interacting partner for the Huntington disease protein (Htt) [7]. Hap1, as an interacting partner of Htt, has been implicated in the hypothalamic pathology of Huntington's disease. Hap1 is highly expressed in the hypothalamus [8,9] and mutant Htt binds more tightly to Hap1 than normal Htt [10]. Mice lacking Hap1 also show retarded growth and die at a very young age, possibly due to a feeding defect [11,12]. Furthermore, it was reported that Hap1 functions as a mediator of feeding behavior in the hypothalamus under normal conditions [13].

Ahi1 was initially identified as a common helper provirus integration site for murine leukemia and lymphomas [14]. However, there is some evidence suggesting that Ahi1 may also be related to energy homeostasis. It was reported that the Ahi1 gene is genetically linked with type 2 diabetes [15]. A recent study showed that Ahi1 is involved in feeding control in the hypothalamus [16].

However, whether Hap1 and Ahi1 in the extra-hypothalamic regions also participate in the control of food intake and energy homeostasis is currently unknown. A previous study showed that Hap1 and Ahi1 form a stable complex that is involved in the development of the cerebella and brainstem [17]. This finding suggests that Hap1–Ahi1 may also be involved in the regulation of feeding behavior in the brainstem. In this study, we thus investigated the changes of Hap1–Ahi1 expression in the brainstem as energy metabolism in the mice was altered. We also examined the effects of knocking down brainstem Hap1 on food intake and body weight. We provided evidence suggesting that the Hap1–Ahi1 complex in the brainstem works as a sensor for insulin signals in feeding control.

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2. Materials and methods

2.1. Animals

Male C57BL/6J mice, which were 8–10 weeks of age, were purchased from Southern Medical University in the Guangdong province of China. The animals were housed in a temperature and humidity-controlled environment with a 12 h light/dark cycle and were given access to food and water ad libitum. The animals were acclimatized to laboratory conditions for a week before testing. All efforts were made to minimize animal suffering and the number of animals used in this study. Animal care and all procedures for animal experiments conformed to the guidelines of the Animal Care and Use Committee at the Guangzhou Biomedical and Health Institute in the Chinese Academy of Sciences.

2.2. Animal models and treatments

For the fasted mouse model, C57BL/6J male mice (10–12 weeks of age) were weighed prior to the experiment and divided into two groups. They had free access to both food and water (control) or were deprived of food for 48 h. To study 2-deoxy-D-glucose (2-DG, Sigma) induced acute glucoprivation, C57BL/6J male mice (10–12 weeks) were injected with 2-DG (250 mg/kg in saline, i.p.) during the light phase and were deprived of food for 4 h. Water was available ad libitum. The mice were then euthanized and their tissues were harvested. For the mouse model of diabetes, male mice (10 weeks old) were randomly assigned to two groups that received one of the following treatments: (1) a single 200 mg/kg interperitoneal (i.p.) injection of streptozotocin (STZ, Sigma); (2) a control i.p. injection of sterile vehicle (100 mM citrate buffer, pH 4.5). At Day 3 and Day 8 post-injection, plasma glucose levels (PGLs) were measured using a Blood Glucose Test Meter (Roche ACCU-CHEK Advantage). The STZ-treated mice were considered to be diabetic once their PGLs were above 16.7 mM/l [18]. Then, the mice were euthanized and their tissues were harvested, and serum insulin levels in the animal models were measured using a commercially available Rat/Mouse Insulin ELISA kit (Millipore). The assay was performed according to the manufacturer's instructions.

2.3. Immunostaining, immunoprecipitation, Western blot and quantitative RT-PCR

The antibodies against Hap1 or Ahi1 were described in previous study [17]. The methods for immunohistochemistry, immunofluorescence, immunoprecipitation, Western blot and real-time quantitative RT-PCR can be found in the Supplementary methods provided online.

2.4. RNA interference and NTS insulin injection

For the RNA interference procedure, male C57BL/6J mice (10–12 weeks of age) were anesthetized with 2.5% avertin and injected with adenovirus expressing either Hap1-siRNA with GFP or GFP alone using a stereotaxic table (David Kopf Instruments). All of the surgical procedures were performed under sterile conditions. With the head placed in the flat-skull position, 1 μl of adenoviral-GFP or adenoviral-Hap1-siRNA virus [10^8 plaque-forming units (pfu)/μl] were injected bilaterally over a period of 10 min using a Hamilton syringe and a syringe infusion pump. The titer of adenovirus was chosen according to a previous study [13]. The stereotaxic coordinates for brainstem intra-nucleus of the solitary tract (NTS) injection were as follows: anterior/posterior (AP) axis, 7.2 mm from bregma to the posterior; lateral, ±0.6 mm from midline (ML); depth, 4.0 mm from the surface of the skull (DV) as described in the atlas by Paxinos and Franklin [19]. The needle was removed after 10 min. After the injection, the mice were housed individually with ad libitum access to standard chow and water in a temperature-controlled environment with a 12 h light/dark cycle. The food intake and body weight of the mice were measured daily for 4 weeks.

For intra-brain insulin injection, bilateral thin-wall stainless steel guide cannulae (22-gauge) were aimed at the NTS (AP-7.2 mm, ML ±0.6 mm, DV −4.0 mm). Standard postoperative care, including the administration of analgesics, was provided. After seven days of recovery from surgery, insulin (Sigma, at a dose of 2 U/kg in 0.5 μl of sterile saline) or saline vehicle was bilaterally injected into the NTS (1 μl/mouse) with a 27-gauge injector extending 1 mm beyond the tip of the guide cannula. The animals were returned to their home cages. Six hours after the injections, the mice were anesthetized with a brief exposure to ether, and their brains were quickly removed after decapitation. The brainstems were dissected out and immediately frozen in liquid nitrogen for Western blot analysis.

2.5. Statistics

GraphPad Prism was utilized for data analysis. Statistical analyses were carried using Student's *t*-test (two-tail). The values $P < 0.05$ (*), $P < 0.01$ (**) were considered statistically significant.

3. Results

3.1. Hap1 and Ahi1 are expressed in the nucleus of the solitary tract (NTS) and form a protein complex

To confirm the expression and distribution of Hap1 and Ahi1 in the brainstem, we performed immunohistochemistry using Ahi1 and Hap1 polyclonal antibodies. We found that cells with strong Ahi1 and Hap1 expression were distributed in the nucleus of the solitary tract (NTS), which is a critical central region for energy homeostasis (Fig. 1A and C). As shown in Fig. 1B, the immunoreactive Ahi1 and Hap1 signals in the brainstem appear as dot-like structures. Through immunofluorescent staining, we found that Hap1 significantly colocalized with Ahi1 in the brainstem sections (Fig. 1D). Furthermore, as indicated by immunoprecipitation (Fig. 1E), Hap1 and Ahi1 were combined with each other in the brainstem lysates. These data indicate that Hap1 and Ahi1 form a protein complex in the brainstem.

3.2. Energy deficiency stimulates the expression of Hap1–Ahi1 in the brainstem

Using the fasted mouse model, we examined the expression of Hap1 and Ahi1 in the brainstem after 48 h of food deprivation. As shown in Fig. 2A, the protein levels of both Hap1 and Ahi1 in the fasted mice significantly increased ($P < 0.05$) compared with control mice (ad libitum fed). Moreover, the transcription of Hap1 and Ahi1 mRNA was also stimulated from fasting (Fig. 2B: $P < 0.01$ for Hap1 and $P < 0.05$ for Ahi1). The fasted mice showed significantly low levels of blood glucose and insulin compared to the control mice ($P < 0.05$; Fig. 2C and D).

Because 48 h of food deprivation was a long and severe starvation period, we sought to examine whether short-term energy deficiency affected the expression of brainstem Hap1–Ahi1 by using 2-deoxy-D-glucose (2-DG), an antagonist of glycolysis. Treatment of mice with 2-DG led to an increase of Hap1–Ahi1 protein in the brainstem (Fig. 3A, $P < 0.05$). For mRNA levels, an increasing but not statistically significant trend in mRNA transcription was observed in 2-DG treated mice for both Ahi1 and Hap1 (Fig. 3B).

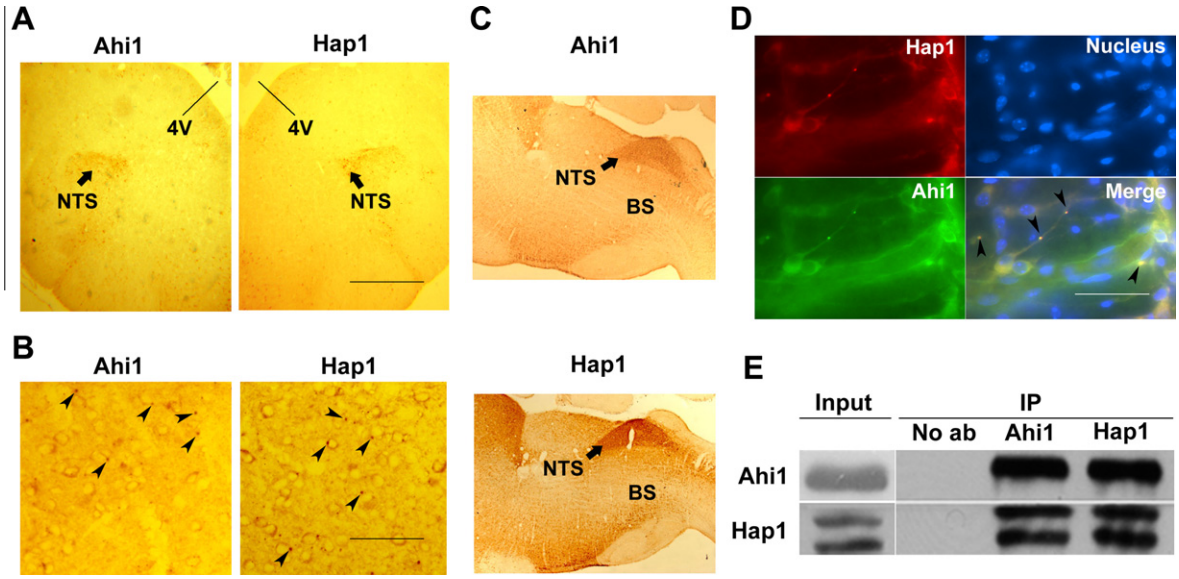

Fig. 1. The expression of Hap1 and Ahi1 in the brainstem. (A and C) Immunohistochemistry revealed that cells with strong Ahi1 and Hap1 expression were distributed in the nucleus of the solitary tract (NTS) of the brainstem. (B) The immunoreactive Ahi1 and Hap1 signals in the brainstem appeared as dot-like structures. (D) Immunofluorescence staining showed a significant colocalization of Hap1 with Ahi1 in the brainstem sections. (E) Immunoblots of immunoprecipitations (IP) from brainstem lysates using antibodies against Ahi1 and Hap1; an immunoprecipitation with no antibody was used as a control. 4 V: the fourth ventricle; NTS: nucleus of the solitary tract; BS: brainstem; No ab: no antibody. Scale bar: 200 μm in (A); 25 μm in (B and D).

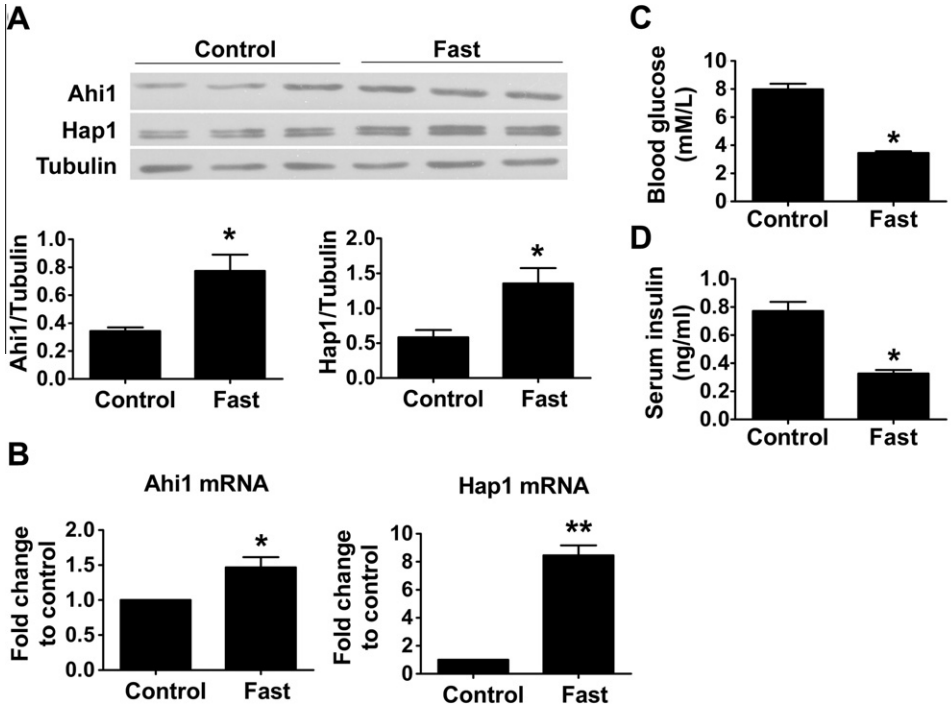

Fig. 2. Fasting stimulated the expression of brainstem Hap1–Ahi1. (A) Western blot showed that the protein levels of Hap1 and Ahi1 in the fasted mice significantly increased ($P < 0.05$) compared with control mice (ad libitum-fed). (B) Real-time PCR showed that the transcription of Hap1 and Ahi1 mRNA was also stimulated by fasting ($P < 0.01$ for Hap1, $P < 0.05$ for Ahi1). Blood glucose levels (C) and insulin levels (D) were also measured in the mice. The histograms are shown as means ± S.E.M. of three independent experiments ($n = 3$ per group) for each experiment; $^{*}P < 0.05$, $^{**}P < 0.01$.

The blood glucose and insulin levels tested 4 h after 2-DG treatment were not significantly different between the two groups (Fig. 3C and D).

3.3. Diabetes affects the expression of Hap1–Ahi1 in the brain stem

To test the effects of diabetes on Hap1–Ahi1 expression, streptozotocin (STZ) was used to induce a model of diabetes in mice. Non-fasting blood glucose measurements were taken from the saphenous vein. Once the measurement levels were greater than 16.7 mM/l, the animals were considered to be diabetic [18]. The Western blot results showed that the brainstem Hap1–Ahi1 protein level was higher in diabetic mice compared to control mice (Fig. 4A, $P < 0.05$). Moreover, STZ treatment induced a significant increase of Hap1 and Ahi1 mRNA expression in the brainstem (Fig. 4B, $P < 0.01$).

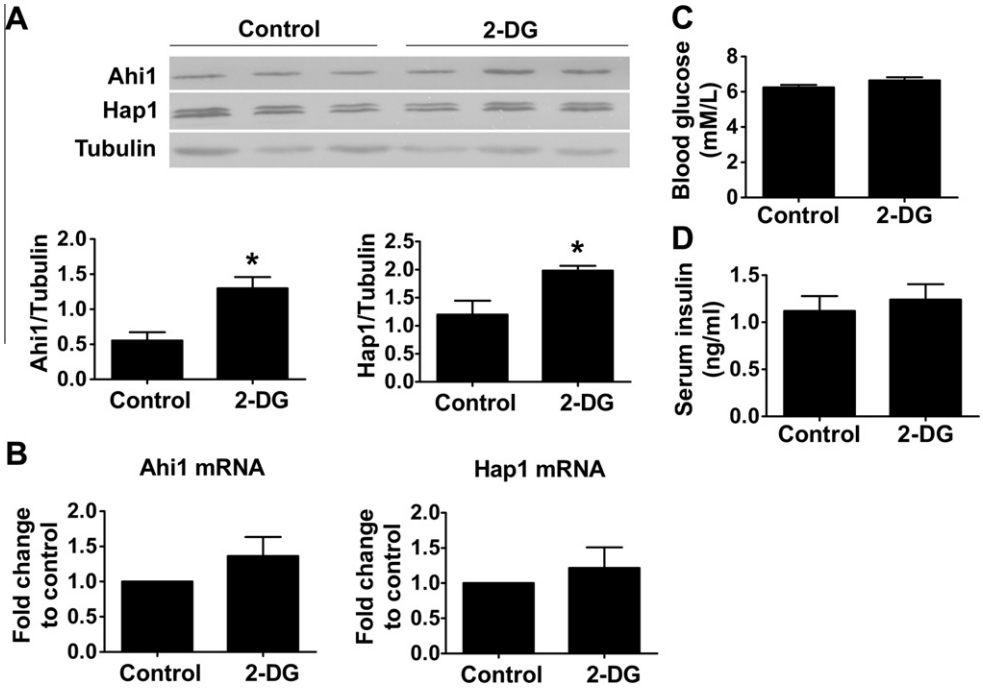

Fig. 3. Hap1–Ahi1 increased in the 2-deoxy-D-glucose (2-DG) mouse model. (A) 2-DG (250 mg/kg in saline, i.p.), an antagonist of glycolysis, was injected into mice. Western blot analysis showed that brainstem Hap1–Ahi1 levels increased after treating the animals with 2-DG for 4 h ($P < 0.05$). (B) Real-time PCR showed that there was an increasing, but not significant, trend in the expression of Ahi1 and Hap1 in 2-DG-treated mice. Blood glucose levels (C) and insulin levels (D) were also measured in the mice. The histograms are shown as means ± S.E.M. of three independent experiments ($n = 3$ per group) for each experiment; $^{*}P < 0.05$.

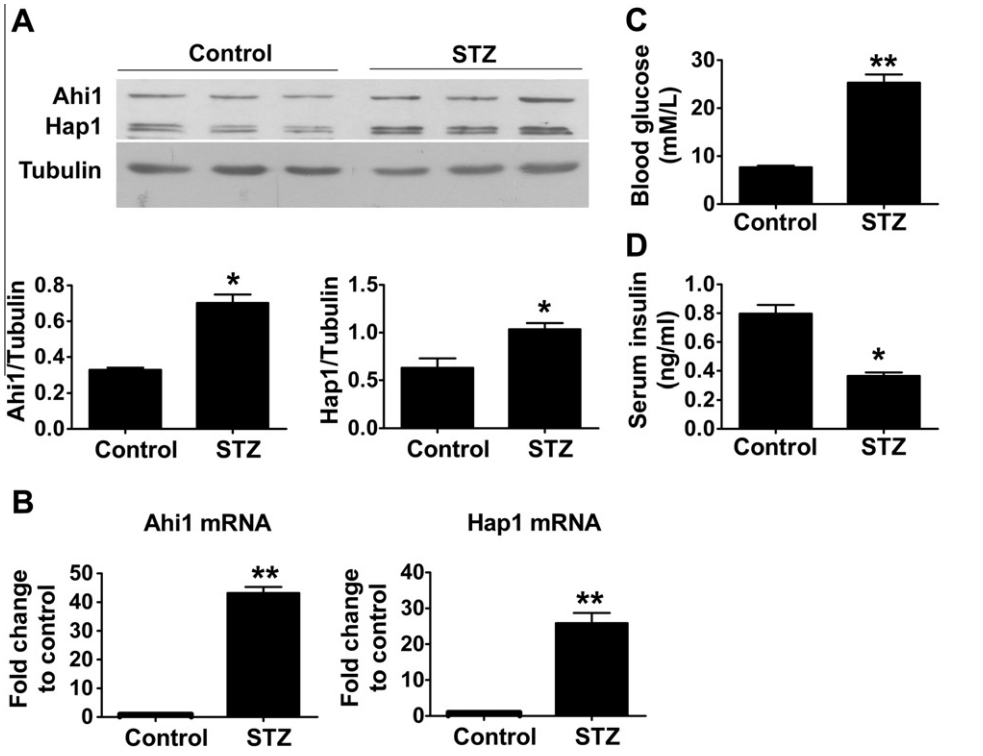

Fig. 4. Diabetes affected the expression of brainstem Hap1–Ahi1. (A) Western blot showed that brainstem Hap1–Ahi1 significantly increased in the STZ-induced diabetic mice ($P < 0.05$). (B) Real-time PCR showed that Ahi1 and Hap1 mRNA levels were significantly higher in the STZ-induced diabetic mice ($P < 0.01$). Blood glucose levels (C) and insulin levels (D) were also measured in the mice. The histograms are shown as means ± S.E.M. of three independent experiments ($n = 3$ per group) for each experiment; $^{*}P < 0.05$, $^{**}P < 0.01$.

The blood glucose levels were significantly higher in STZ-treated mice than control mice (Fig. 4C, $P < 0.01$), and blood insulin levels were significantly lower in the STZ-treated mice (Fig. 4D, $P < 0.05$).

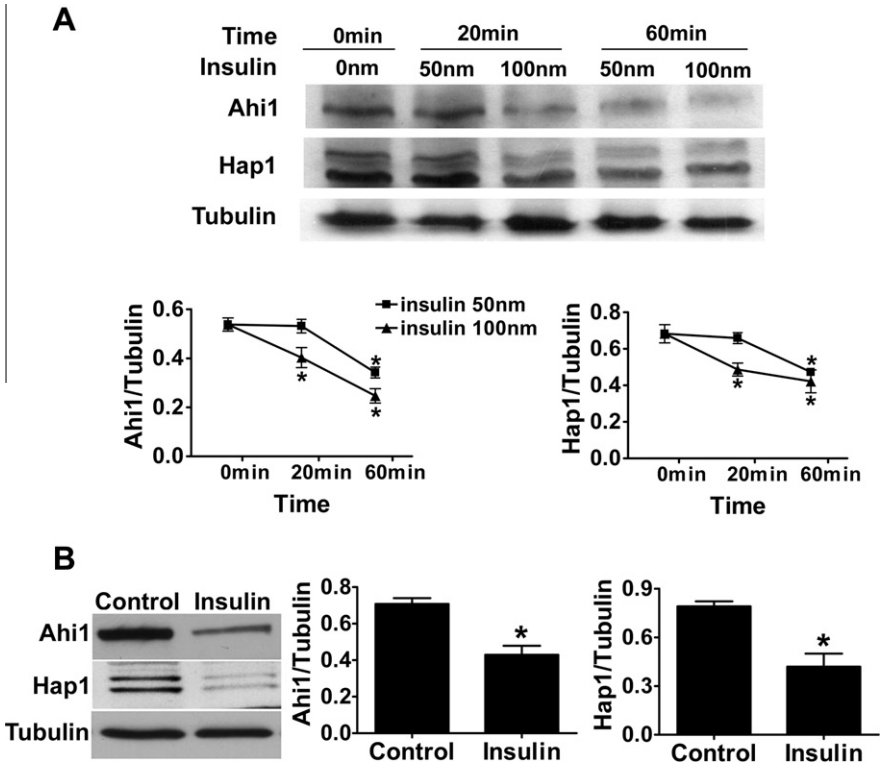

Fig. 5. The expression of Hap1–Ahi1 was regulated by insulin. (A) The expression of Hap1–Ahi1 in the N18TG2 neuronal cell line was decreased by insulin treatment. The cells were treated with 50 or 100 nM insulin, harvested at the indicated time, and subjected to Western blot analysis. (B) Bilateral injection of insulin to the nucleus of the solitary tract (NTS) in the brainstem decreased the expression of Hap1–Ahi1. The data are shown as means ± S.E.M. of three independent experiments; $^{*}P < 0.05$.

3.4. Insulin regulates the expression of Hap1–Ahi1

The low levels of blood insulin in fasted mice and STZ-treated mice led us to speculate that decreased insulin levels may cause the upregulation of brainstem Hap1–Ahi1. A previous study showed that circulating insulin can regulate the expression of hypothalamus Hap1 through ubiquitination in order to control feeding behavior [13]. We hypothesized that insulin may also regulate the expression of brainstem Hap1–Ahi1. To determine whether insulin directly affects Hap1–Ahi1 expression, the neuronal cell line N18TG2 was stimulated with insulin. As shown in Fig. 5A, the expression of Hap1–Ahi1 was decreased by insulin in a time- and dose-dependent manner. Then, we injected insulin directly into the NTS in the brainstem to test whether insulin can decrease the expression of Hap1–Ahi1 in vivo. As shown in Fig. 5B, the level of Hap1–Ahi1 protein in the brainstem was significantly decreased by insulin ($P < 0.05$).

3.5. Knockdown of brainstem Hap1 results in decreased food intake and body weight

To determine the effects of brainstem Hap1 knockdown on feeding and body weight, we bilaterally injected Ad-*Hap1*-siRNA or adenovirus vectors into the brainstem in mice. As shown in Fig. 6A with immunofluorescence and in Fig. 6B with a Western blot, Ad-*Hap1*-siRNA produced a significant knockdown of Hap1 protein in the brainstem. After surgery, the animals were housed individually. Food intake and body weight were measured daily for 4 weeks. As shown in Fig. 6C, food intake in the siHap1 mice and the control mice were minimal immediately following surgery. After 24 h, both groups started to increase their food intake. However, the siHap1 mice consumed significantly less food compared to the control mice, and this difference remained relatively constant throughout the first two weeks. Consequently, body weight also showed an initial dramatic decrease in the siHap1 mice and control mice during the first 2 days. Subsequently, body weight gradually increased in the control mice, but it decreased at the sixth day in the siHap1 mice. The average body weight of the siHap1 mice was significantly lower than the control group throughout the testing period (Fig. 6D).

4. Discussion

Feeding and energy metabolism are basic and vital life processes that are essential for animals and human beings to survive. The brainstem has been strongly implicated in the neural regulation of food intake and energy balance [20,21]. Regions of brain stem such as the nucleus of the solitary tract (NTS), area postrema, dorsal motor nucleus of the vagus, and the locus coeruleus are critical regulators of the neural circuits in energy homeostasis [22]. Additionally, the caudal brainstem contains neurons and circuits that are involved in the autonomic control of ingestion, digestion, and absorption of food [23]. More important, the brainstem can achieve autonomic control even without forebrain influence [24], just as in respiration and circulation, functions essential for survival. Previous studies in a chronic decerebrate rat preparation found that taste concentrations, gastric preloads and cholecystokinin (CCK; a peptide released from intestinal endocrine cells during feeding) similarly affected meal size compared to intact rats [25]. These results indicated that when the brainstem was isolated from its forebrain connection, the basic behavior of satiety was preserved. In addition to meal size control, decerebrate rats also showed a fully formed sympathoadrenal response to systemic 2-DG administration [26], which suggested that the brainstem houses a system that is responsive to glucoprivation. The brainstem may also have a basic system for the automatic control of

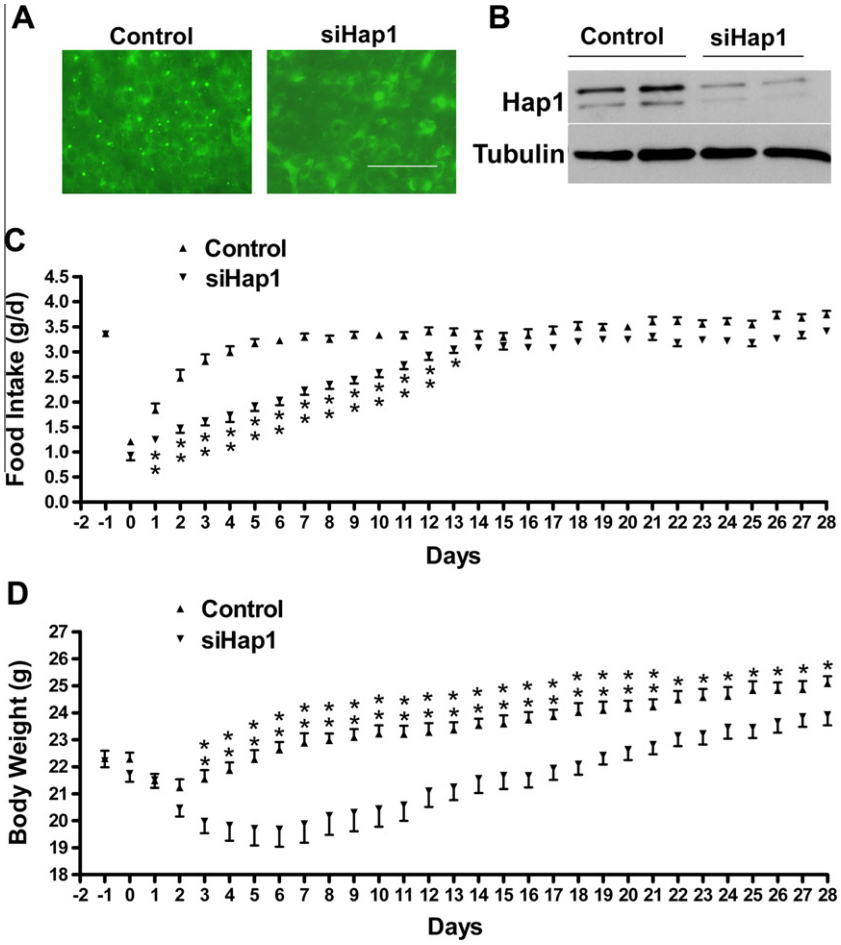

Fig. 6. Ad-*Hap1*-siRNA administration decreased food intake and reduced body weight. (A) Immunofluorescent staining showed a significant fluorescence intensity decrease in Hap1 in the brainstem section of Ad-*Hap1*-siRNA-treated mice at Day 7 post-injection. Scale bar: 25 μm. (B) Western blot revealed that the protein level of Hap1 in the brainstem was significantly decreased by Ad-*Hap1*-siRNA at Day 7 post-injection. (C) Food intake and (D) body weight were measured daily for 4 weeks post-injection. Note that the Ad-*Hap1*-siRNA-treated mice showed significantly less food intake for approximately 2 weeks after surgery and displayed lower body weights throughout the testing period compared to control mice. The data are shown as means ± S.D.; $n = 12$ for control group, $n = 13$ for siHap1 group; $^{*}P < 0.05$, $^{**}P < 0.01$.

feeding and energy metabolism. Our study indicated that brainstem Hap1–Ahi1 could be a complex that is responsive to energy deficiency. When we treated mice with 2-DG, which caused a short-term energy deficiency, the expression of Hap1–Ahi1 increased in the brainstem. When mice were fasted for 48 h, which resulted in severe energy deficiency, not only did the expression of brainstem Hap1–Ahi1 protein levels increase, but the mRNA levels also increased. The increased expression of brainstem Hap1–Ahi1 may promote food intake to supply energy. When we decreased the expression of Hap1 in the brainstem, the mice showed decreased food intake and lower body weights.

The brain was once considered to be an insulin-insensitive tissue because insulin is not a major regulator of glucose in the brain [27] and due to the widespread belief that the insulin peptide is too large to cross the blood–brain barrier. However, recent observations have revealed that insulin does enter the brain, combines with specific insulin receptors on brain neurons, and triggers diverse events such as reducing food intake or reducing body weight [28,29]. Serum insulin in the cerebrospinal fluid has been detected in animals and humans [30,31], and the proportion of insulin in the fluid to its plasma level also has to be calculated [32]. Food intake, fasting, and refeeding can all influence the entry of insulin into the brain [33,34]. This process is adaptive because when the available energy is low, circulating insulin has less access to the brain, which promotes food intake; and when the available energy is high, insulin more readily enters the brain and limits food intake. Insulin receptors have been identified in the dorsal vagal complex and a number of other caudal brainstem sites [35,36]. The brainstem neurons bearing insulin receptors are also endowed with intracellular signaling substrates including insulin receptor substrate-1 and phosphatidylinositol 3-kinase [37]. A prior study reported that circulating insulin can regulate the expression of hypothalamus Hap1 through ubiquitination in order to control feeding behavior [13]. We hypothesized that insulin may also regulate the expression of brainstem Hap1–Ahi1. When we decreased circulating insulin with STZ in mice, we observed a high expression of brainstem Hap1–Ahi1. In the fasted mice with low circulating insulin levels, we also found an upregulation of brainstem Hap1–Ahi1. However, in the 2-DG model, we did not observe a decrease in the circulating insulin level. Instead, there was an intial increase in blood glucose soon after the injection of 2-DG and a consequent increase in insulin. However, even though the circulating insulin level increased, the 2-DG-treated mice still exhibited energy deficiency in the brain. This situation is different from the situation, where there is an increase in blood glucose after eating and a consequent increase in insulin, which provides the animals' brains with energy sufficiency information. It has been shown that refeeding the fasted mice for 4 h, which would supply energy for the fasted mice and generate a subsequent increase in circulating insulin, decreased the upregulation of Ahi1 in the hypothalamus [16]. Our results also showed that insulin can decrease the expression of Hap1–Ahi1 both in a neuronal cell line (in vitro) and in the brainstem (in vivo). In

conclusion, the increased expression of brainstem Hap1–Ahi1 may not be solely dependent on the circulating insulin level. In other words, the regulatory mechanism of brainstem Hap1–Ahi1 expression is more complex than previously thought. The mechanism could be a combination of regulatory processes between central neural information and peripheral insulin signaling.

Even though the specific regulatory mechanism of brainstem Hap1–Ahi1 expression needs to be further addressed, our study showed that the Hap1–Ahi1 complex in the brainstem is involved in energy metabolism and can be regulated by insulin.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.11.059.

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