Huperzine A ameliorates obesity-related cognitive performance impairments involving neuronal insulin signaling pathway in mice

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INTRODUCTION

Type 2 diabetes (T2D) and Alzheimer’s disease (AD) share several common pathophysiological features. Huperzine A (Hup A), a Lycopodium alkaloid extracted from the Chinese herb moss Huperzia serrata, is a specific and reversible inhibitor of acetylcholinesterase, which is clinically used for the treatment of AD. In this study, we investigated whether Hup A improved the metabolic and cognitive functions in the high fat-induced (HFD) obese mice and genetic ob/ob mice. HFD and ob/ob mice were treated with Hup A (0.1, 0.3 mg · kg−1 · d−1, ig) for 3 months. Body weight was monitored and glucose tolerance tests were performed. Novel object recognition test and Morris water maze assay were conducted to evaluate the cognitive functions. We found that the Hup A treatment had no significant effect on peripheral metabolism of obese mice, whereas Hup A (0.1, mg · kg−1 · d−1) improved both the abilities of object recognition and spatial memory in HFD-fed mice, but not in ob/ob mice. Furthermore, Hup A treatment significantly upregulated the insulin and phosphorylated Akt levels in the cortex of HFD-fed mice, but not in ob/ob mice. In addition, Hup A (0.3 mg · kg−1 · d−1) significantly decreased cortical β-secretase (BACE1) expression. In conclusion, these results demonstrate that treatment with Hup A (0.1, mg · kg−1 · d−1) can effectively improve the cognitive functions, at least in diet-induced obese mice.

Keywords: obesity; huperzine A; cognitive dysfunction; neuronal insulin signaling

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were allowed to explore two identical objects (A1 and A2) for 5 min. (Stoelting, USA), which can record the time spent on the objects. Object exploration was tracked by the Any-maze tracking system. On the third day, to examine long-term memory, mice were allowed to explore the apparatus for 5 min in the presence of two objects (the familiar object A1 and the new object B). Two hours later, mice underwent a short-term memory test, during which they were allowed to adapt to the pool for 20 s with free swimming. Then, mice were trained in the spatial learning session for six trials per day for 5 consecutive days. In each trial, mice were placed in the water at one of four positions (NE, NW, SW, and SE) facing the pool wall. Then, the mice were required to swim to find a hidden platform (13 cm in diameter, located in the SW quadrant) submerged 1 cm below the water. During each trial, mice were allowed to swim until they found the hidden platform and stayed on the platform for 20 s before being returned to a holding cage. Mice that failed to find the hidden platform in the 120 s were guided to the platform and allowed to remain there for 20 s. Twenty-four hours after the last trial, the probe test was performed. Mice were returned to the pool without the platform at a new point for 120 s, and their swimming paths were recorded.

**RESULTS**

Huperzine A did not alter the metabolic characteristics of HFD-fed mice or those of ob/ob mice. To generate diet-induced obesity, mice were fed a HFD, and body weight changes were measured weekly. As expected, compared with LFD-fed mice, mice fed a HFD for 18 weeks showed...
significant body weight gain (increased by 25% vs. LFD, Fig. 1a, b) and impaired glucose tolerance indicated by high glucose levels at 30 min and 60 min after glucose loading and a high area under the curve (Fig. 1c, d).

During Hup A treatment, we continued to monitor the body weight of mice in each group. However, Hup A was unable to decrease weight (Fig. 1e, g) or improve glucose intolerance (Fig. 1f) in HFD-fed or ob/ob mice. Hup A treatment was also incapable of reducing hyperglycemia in ob/ob mice (Fig. 1h). These results suggested that Hup A might not improve peripheral glucose metabolism in obese mice.

Hup A enhanced the recognition memory of HFD-fed mice but not ob/ob mice
To investigate whether Hup A reduces cognitive impairment, mice were submitted to the novel object recognition test for an assessment of their short- and long-term memory. In this test, the interaction time used for the recognition index was defined specifically by “sniffing or touching the object with the nose”. Although there was no significant difference in terms of the time spent on the old and novel objects (Fig. 2d), HFD-fed mice treated with 0.1 mg/kg every day Hup A showed a higher recognition index in the short-term memory test than HFD-fed mice treated with vehicle (Fig. 2b), indicating that short-term memory was enhanced. Although there was no statistically significant difference in the long-term memory test, there were increased trends in both the recognition index and time spent on the old and novel objects in HFD-fed mice treated with 0.1 mg/kg every day Hup A (Fig. 2c, e). Most of the ob/ob mice did not exhibit a tendency to explore objects during training; there, we could not obtain relevant data. During the acclimatization period, the movement distance of all the mice was measured in the probe test. According to the results, the obese mice were less active than LFD-fed mice or WT mice, but Hup A increased the activity level of ob/ob mice (Fig. 2f).

Hup A enhanced the spatial memory of HFD-fed mice but not ob/ob mice
The Morris water maze was used to confirm whether Hup A improves the spatial learning and memory of obese mice. As shown in Fig. 3a, on the first training day, mice that were treated with Hup A and mice fed a LFD spent less time searching for the hidden platform than the vehicle control mice fed a HFD. However, there was no significant difference on the other training days. There was no difference in escape latency among the ob/ob mice during the training days (Fig. 3b). As expected, in the probe test, mice fed a HFD spent less time in the platform-located quadrant than mice fed a LFD (Fig. 3e). After the HFD-fed mice were treated with 0.1 mg/kg every day Hup A for 3 months, the time spent in the platform-located quadrant was significantly increased to a level almost the same as that of LFD mice (Fig. 3e). Hup A-treated ob/ob mice showed a trend toward an improvement in spatial memory (Fig. 3e). There was no significant difference in the number of hidden platform location crossings between groups (Fig. 3f). Furthermore, ob/ob mice treated with 0.3 mg/kg Hup A showed faster swimming speed on the first 2 training days, and ob/ob mice treated with 0.1 mg/kg Hup A showed the fastest swimming speed on the fourth training day than those treated with vehicle, whereas the HFD mice treated with either 0.1 mg/kg or 0.3 mg/kg Hup A did not show significant changes (Fig. 3c, d). However, there was no significant difference in swimming speed between the HFD-fed groups or ob/ob groups during the probe test (Fig. 3g).

Hup A enhanced spatial memory involved improvement of cerebral insulin signaling
Insulin signaling has been reported to play an important role in cognitive function [39, 40]. To further investigate the changes in insulin signaling after Hup A treatment, we tested the phosphorylation level of Akt at serine 473 (p-Akt), which is a necessary residue for Akt activity [41]. Compared with obese mice treated...
with vehicle, obese mice treated with Hup A exhibited remarkably greater Akt-serine 473 phosphorylation in the hippocampus (Fig. 4a, c). Moreover, in the cortex, HFD-fed mice treated with Hup A also showed significantly greater Akt-serine 473 phosphorylation than vehicle-treated HFD-fed mice, whereas Hup A had no significant effect on Akt-serine 473 phosphorylation in 

Because the insulin concentration in the brain decreases with aging [20] and the high density of insulin in the brain enhances spatial learning and memory [42], the insulin level in the cortex was tested. The HFD-fed and ob/ob mice treated with Hup A showed notably higher insulin concentrations in the cortex than those mice treated with vehicle (Fig. 4d). Therefore, Hup A may improve spatial memory in obese mice by enhancing insulin signaling and increasing cortical insulin levels.

High-dose Hup A inhibited cerebral BACE1 expression and reduced Aβ levels

Studies have shown that BACE1 has a pivotal role in dementia [43, 44] and correlates with metabolic disorders caused by obesity or T2D [6, 9, 45]. Moreover, Hup A prevents increases in the membrane distribution of BACE1 in the cortex [34]. The ob/ob mice treated with 0.3 mg/kg every day Hup A showed significantly lower hippocampal BACE1 expression than vehicle-treated ob/ob mice, whereas Hup A did not exhibit a significant difference with Hup A treatment (Fig. 5a, c). Obese mice treated with a high dose of Hup A showed remarkably lower cortical BACE1 expression than those treated with vehicle (Fig. 5b, c). HFD mice treated with the low dose of Hup A showed a trend toward a decrease in cortical BACE1 expression compared with vehicle-treated HFD mice (Fig. 5b, c).

To investigate whether decreased BACE1 expression affects the Aβ42 concentration, we examined the concentration of Aβ42 in the cortex. Here, ob/ob mice administered 0.3 mg/kg every day Hup A had a lower concentration of Aβ42 than vehicle-treated ob/ob mice (Fig. 5d). These results suggested that decreased expression of BACE1 may contribute to the protection of Hup A against cognitive dysfunctions caused by obesity.

DISCUSSION

A substantial number of studies have shown the classic effects of Hup A in inhibiting acetylcholinesterase, increasing acetylcholine, and activating cholinergic receptors, eventually improving the cognitive decline for AD patients and animals [46–48]. In this study, we observed increased cortical insulin levels in the brains of obese (HFD or ob/ob) mice after Hup A treatment. Cortical insulin levels in the vehicle HFD and ob/ob group were higher than those in the vehicle LFD and WT group, which together with the GTT results indicated that the obese mice exhibited insulin resistance. Hup A further increased the insulin concentration accordingly to meet the needs of the brain for neuroprotection and to help to maintain higher cognitive processes, including learning, memory, executive function, and attention [21, 49]. Brain insulin levels have been reported to be slightly higher in ob/ob mice at 8–10 weeks of age than in their lean littermates [50]. Similarly, our data showed that brain cortical insulin levels were higher in HFD-fed and ob/ob mice at 44–45 weeks of age than in their respective controls. Treatment with Hup A further elevates insulin levels in the brain in obese mice, but how higher insulin levels are beneficial to the memory deficits in these animals is still unclear. Excessive insulin likely facilitates the insulin signaling pathway despite insulin

Fig. 2 Novel object recognition test in 11-month-old mice. a Schematic diagram of the novel object recognition test. b, c The cognition index of short- and long-term memory assessed in 11-month-old mice using a new object recognition test (n = 5 in each group). d, e Time spent on the old and novel objects during short-term and long-term memory tests. f Traveled distance determined during the acclimatization period. Data are presented as the mean ± SEM. Data are compared using two-way ANOVA with Bonferroni post hoc test d, e and one-way ANOVA with Tukey post hoc test a–c, f. *P < 0.05; ***P < 0.001

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resistance, allowing insulin to be effective in controlling glucose homeostasis under conditions of hyperinsulinemia. In addition, intranasally administered insulin has been reported to penetrate directly from the nose to the brain to improve the functions of the central nervous system [51, 52], and more specifically, intracerebroventricular (ICV) STZ-induced memory impairments in rats were prevented by treatment with intranasal insulin [52, 53]. Previous studies have reported that impairments in insulin or insulin-like growth factor signaling are observed in the brains of AD patients, and these abnormalities are involved in the decreased levels of phospho-Akt, the insulin receptor substrate (IRS), and the IRS-associated phosphatidylinositol 3-kinase and the increased levels of glycogen synthase kinase-3β activity and APP [54]. Insulin is well known to promote neurite outgrowth, modulate activity-dependent synaptic plasticity via PI3K-AKT signaling, and have a key role in the development and maintenance of excitatory synapses [54]. Although epidemiologic studies have indicated that long-term hyperinsulinemia is a risk factor for dementia, insulin administered to the AD brain maintains glucose homeostasis and improves memory function [55, 56]. In addition, HFD has been shown to impair brain insulin signaling and synaptic plasticity [57, 58]. In our study, HFD or ob/ob mice treated with Hup A for 3 months exhibited an increase in the insulin concentration in the cortex, resulting in improved cognitive function.

In our current study, obese mice exhibited lower levels of phospho-Akt in the brain than WT mice, which suggests impaired neuronal insulin signaling. After Hup A treatment, the expression of p-Akt was fully restored. Hup A has also been shown to ameliorate oxidative glutamate toxicity in immortalized hippocampal HT22 cells, and this protection might be involved in regulating the BDNF/TrkB-dependent PI3K/Akt/mTOR signaling pathway [32]. Therefore, we speculated that the improvement in HFD-induced obesity-related cognitive dysfunctions by a low dose of Hup A (0.1 mg/kg) is attributable to the increased insulin concentration and expression of p-Akt in the brain.

In addition, a previous study demonstrated that Hup A could reduce body weight and improve blood glucose levels in STZ-induced diabetic rats [33]. However, our study did not...
Fig. 4  The effects of Hup A on the brain insulin signaling pathway. a Western blot of Akt and phosphorylated Akt (p-Akt) protein levels in the hippocampus of HFD and ob/ob mice. b Western blot of Akt and phosphorylated Akt (p-Akt) protein levels in the cortex of HFD and ob/ob mice. c Quantification of the ratio of p-Akt/Akt protein expression according to A and B (n = 5 in each group). d The insulin levels in the cortex by ELISA (n = 4 in each group). Data are presented as the mean ± SEM. Data are compared using one-way ANOVA with Tukey’s post hoc test. *P < 0.05; **P < 0.01; ***P < 0.001

Fig. 5  The effects of Hup A on BACE1 and Aβ42 levels in the hippocampus or cortex. a Western blot of BACE1 protein expression in the hippocampus of HFD and ob/ob mice. b Western blot of BACE1 protein expression in the cortex of HFD and ob/ob mice. c Quantification of BACE1/β-actin protein expression according to A and B (n = 5 in each group). d The level of Aβ42 in the cortex by ELISA (n = 4 in each group). Data are presented as the mean ± SEM. Data are compared using one-way ANOVA with Tukey’s post hoc test. *P < 0.05; **P < 0.01
show similar results in HFD-fed or \(ob/ob\) mice. Neither body weight nor glucose homeostasis was changed by Hup A treatment. This discrepancy may result from the difference between the animal species and diabetic models. Mao et al. [33] used Wistar rats treated with STZ, which are generally recognized as a model of type 1 diabetes. In contrast, the HFD and \(ob/ob\) mice used in our study, which showed obvious obesity and insulin resistance, are accepted as models of T2D. HFD-fed rodents gradually develop significant weight gain, obesity and insulin resistance. These features are more relevant to human T2D. However, \(ob/ob\) mice are severely obese owing to a genetic leptin deficiency, which is an important anorexigenic hormone that controls food intake. The metabolic and behavioral differences in these two models strongly influence the degree of obesity.

We also demonstrated for the first time that Hup A decreased BACE1 expression in the hippocampus and cortex of \(ob/ob\) mice and in the cortex of HFD-fed mice. BACE1, a proamyloidogenic enzyme, is known to cleave APP, which has a critical role in the generation of the amyloid-\(\beta\) peptides implicated in the pathogenesis of AD. There is a positive correlation between BACE1 levels and plaque numbers in AD brains [59, 60]. Although highly expressed in the brain, BACE1 is also expressed in nonneuronal tissues, including the pancreas, skeletal muscle, and liver. BACE1 activity in these tissues may be increased under stress conditions [61, 62]. Deletion or reduction of BACE1 decreases mouse body weight and enhances insulin sensitivity when fed a regular chow diet but also partially prevents HFD-induced obesity. These actions may be involved in the increase in the uncoupling of proteins in skeletal muscle and brown fat tissues. Therefore, decreased BACE1 activity and its level may ameliorate metabolic abnormalities caused by HFD [9]. Moreover, a recent study showed that neuronal BACE1-overexpressing mice exhibit systemic diabetes. Essentially, BACE1 knock-in causes systemic glucose intolerance, fatty liver and impaired hepatic glycogen storage in mice. These phenomena are also associated with hypothalamic pathology, such as a dysregulated melanocortin system and advanced endoplasmic reticulum stress [6]. Studies have shown that the expression of both hippocampal and cortical BACE1 increases considerably in obese and diabetic mice, and inhibiting BACE1 may serve as a new strategy to intervene in T2D and AD [63–65]. Consequently, neuronal BACE1 is a key factor for metabolic homeostasis, thus affording a potential mechanism for metabolic disturbance in the AD brain. Apart from its reversible, potent, and selective inhibitory effect on AChE, Hup A treatment regulates APP processing by activating ADAM 10 and modulating extracellular signal-regulated kinase 1/2 pathways and protein kinase C, resulting in elevated sAPPα expression and decreased Aβ production in HEK293swe cells [34, 66].

We used two doses of Hup A, 0.1 and 0.3 mg/kg every day, according to previous reports [22, 28, 33, 66, 67]. Obviously, low-dose Hup A is most commonly used and shows the best effect, which is reflected in the enhanced short-term memory and spatial learning and memory and corresponds to the increase in insulin in the cortex and phospho-Akt expression in the hippocampus and cortex. Although low-dose Hup A did not affect the levels of BACE1 and Aβ42, studies have indicated that Aβ itself is not the primary neurotoxin causing AD but is the initiator of the pathologic changes in the brain that finally cause neurodegeneration years later [68]. In any case, cognitive performance can be improved independent of Aβ [69, 70]. We also gave mice 0.3 mg/kg Hup A to determine whether the higher dose would have stronger effects than 0.1 mg/kg in our mouse models. Nevertheless, 0.3 mg/kg Hup A did not further improve cognitive function.

In summary, our results have demonstrated for the first time that Hup A protects against the obesity-associated cognitive impairment in HFD-fed mice but not in \(ob/ob\) mice. Although Hup A had no effect on body weight change and peripheral glucose metabolism regulation, it significantly enhanced the activity of the neuronal insulin signaling pathway, thereby contributing to its improvement in cognitive dysfunctions associated with T2DM. The novel findings in this study provide important information for a potential new application of Hup A in addition to its classical treatment of AD.

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**AUTHOR CONTRIBUTIONS**

HYW and XQX designed the research; HYW, MW, and JLD performed the research; HYW and MW analyzed the data; JBL and YXS contributed the materials; and HYW and XQX wrote the paper.

**ADDITIONAL INFORMATION**

**Competing interests:** The authors declare no competing interests.

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