Steady and fluctuant methods of inhibition of acetylcholinesterase differentially regulate neurotrophic factors in the hippocampus of juvenile mice

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Abstract. The present study was designed to evaluate the effects of steady and fluctuant inhibition of acetylcholinesterase (AChE) activity on neurotrophic factors in the hippocampus of juvenile mice. Steady inhibition of AChE activity was induced by an intramuscular injection of huperizine A (HupA) sustained-release microspheres. Fluctuant inhibition of AChE activity was induced by an intragastric administration of HupA tablets. Six days after cessation of steady AChE inhibition, there was a significant increase in the levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). In contrast, fluctuant AChE inhibition had no effect on BDNF and NGF levels. Additionally, neither steady nor fluctuant inhibition of AChE activity altered the choline acetyltransferase activity or spatial learning in juvenile mice. These findings indicate that steady and fluctuant methods of inhibition of AChE have different effects on the levels of BDNF and NGF in the hippocampus. In addition, the effects of AChE inhibitors may not improve learning in normal juvenile animals.

Introduction

Previous studies have demonstrated that the major components of the cholinergic system, such as acetylcholinesterase (AChE), acetylcholine (ACh) and choline acetyltransferase (ChAT), are closely associated with important biological events, including neuronal maturation and plasticity, axon guidance, regulation of gene expression, and cell survival (1). Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are neurotrophic agents that support the growth, differentiation and survival of neurons (2). The reciprocal regulation of NGF and BDNF in hippocampal neurons by cholinergic activity and the release of ACh from presynaptic nerve terminals by neurotrophins represent a novel positive mechanism for controlling synaptic efficiency in the cholinergic system (3).

AChE plays a key role in terminating the synaptic action of ACh at cholinergic synapses (1). Inhibition of AChE can result in the potentiation of central cholinergic activity by increasing the amount of ACh available for neurotransmission. Huperzine A (HupA), a *Lycopodium* alkaloid isolated from the Chinese medicinal herb *Huperzia serrata*, is a potent, specific and reversible AChE inhibitor. It is an important regulator of cholinergic signaling in the hippocampus (4). Previous studies have reported that HupA inhibition of AChE activity in the brain lasts for approximately 6 h and that there is a wide fluctuation between peaks and troughs after oral administration of a HupA tablet (HT) (5,6). A recent study from our laboratory indicated that after an intramuscular injection of HupA sustained-release microspheres (HSMs) in mice, the plasma concentration of HupA reached a maximum on the 4th day and then slowly decreased until the drug was undetectable in plasma on the 12th day. The AChE activity continued to be significantly inhibited on the 14th day after treatment with HSM (7,8).

The changes induced by treatment of AChE inhibitors in the central cholinergic system have been widely studied in aged brain tissue. However, little research has evaluated neurobiological changes, particularly changes in the levels of neurotrophic factors associated with AChE activity, in the cholinergic system of the hippocampus in normal juvenile mice. As the brain of juvenile animals is vulnerable to neuroactive chemicals during multiple developmental roles of neurotransmitters, the immature nervous system may be more susceptible to AChE inhibitors (9). A few studies have reported that AChE inhibitors, such as donepezil or organophosphates, can activate the central cholinergic transmission and enhance the expression of neurotrophic factors (10). However, previous studies investigated the effects of AChE inhibitors that provide fluctuant inhibition, but not steady inhibition, of AChE activity.

The hippocampus receives an abundance of projections from cholinergic neurons of the medial septum. The central cholinergic pathway in the hippocampus plays a critical role in regulating numerous vital functions, including memory, learning and movement (11). The present study investigated whether steady versus fluctuant methods of AChE inhibition

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has different effects on the cholinergic system and neurotrophic factor levels in the hippocampus of juvenile mice. In addition, the effect of AChE inhibitors on learning in juvenile mice was evaluated.

Materials and methods

Animals. Juvenile (2-week-old) male Swiss mice were purchased from the Experimental Animal Center of the Shandong Engineering Research Center for Natural Drugs (Yantai, Shandong, China). Animals were housed in a climate-controlled room, maintained on a 12-h light cycle, and given food and water *ad libitum*. The Institutional Animal Care and Use Committee approved the study protocols. The animals were maintained according to the Guidelines for the Care and Use of Laboratory Animals of Yantai University. All efforts were made to minimize the number of animals used and to minimize their suffering.

Drug dosing schedule. Sixty-three mice were randomly divided into the following three groups (n=21 in each group): vehicle group (VEH), HT group (0.2 mg/kg/day), and HSM group (3 mg/kg/15 days). The mice in the VEH group were given sodium carboxymethyl cellulose. HTs (Henan Tailong Pharmaceutical Co., Ltd., Zhengzhou, Henan, China) were dissolved in 2.5% (w/v) sodium carboxymethyl cellulose and were given intragastrically in a volume of 0.2 ml/10 g body weight daily. HSMs (Luye Pharmaceutical Co., Yantai, Shandong, China) were dissolved in 2.5% (w/v) sodium carboxymethyl cellulose and injected intramuscularly in a volume of 0.1 ml/10 g body weight (HSM group). To induce steady inhibition of AChE, the HSM group was dosed on the 1st, 16th and 31st day. To induce fluctuant inhibition of AChE, the HT group was dosed daily for 45 days. The dosages of HupA were determined by previous studies of Tang *et al* (5). In addition, the dosages of HSM were designed according to the pharmacokinetics and pharmacodynamics of HSM reported by Chu *et al* (7).

Morris water maze task. After a 45-day course of drug treatment, the spatial learning ability of the mice was measured using the Morris water maze for 6 days (Institute of Materia Medica, Academy of Medical Science, China). Before initiating the Morris water maze test, the mice were allowed to swim freely in a pool of water (diameter, 90 cm; depth, 19 cm; temperature, 26±1˚C) for 60 sec without an escape platform. Afterward, a platform (diameter, 5 cm) was placed 1 cm below the surface of the water. Learning consisted of 4 trials/day for 5 consecutive days. In each trial, the starting location was randomized to 1 of 4 starting positions (north, east, south or west), and the latency to escape onto the platform was recorded. Mice that were unable to find the platform within 60 sec were placed on the platform for 20 sec, and their escape latency was recorded as 60 sec. An automated tracking system analyzed the swim path of each subject, and the mean escape latency was calculated (the time between being placed in the water and finding the hidden platform).

Biochemical analyses. The mice were sacrificed by decapitation 6 days (the duration of the Morris water maze test) after the 45-day course of treatment. The hippocampus was separated on ice and homogenized with ice-cold saline to yield a 10% (w/v) homogenate. The homogenate was centrifuged at 3,500 x g for 10 min at 4˚C, after which the supernatant was stored at -80˚C until subsequent analyses. The total protein concentration was estimated by a previously described method (12).

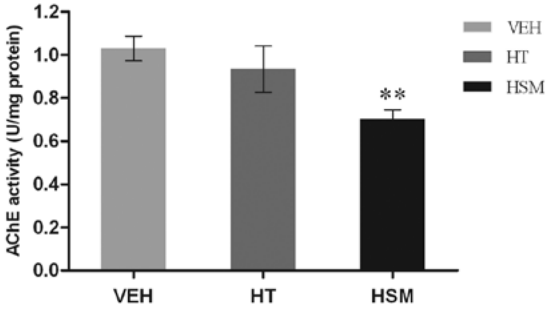

Figure 1. Effects of HT and HSM on AChE activity in the mouse hippocampus. HT treatment had no effect on AChE activity when compared with vehicle treatment. However, AChE activity in the mice treated with HSM significantly decreased when compared with mice treated with vehicle. Data are presented as the mean ± SEM, n=7 mice/group. **P<0.01 compared to the vehicle-treated group.

AChE activity was determined according to the methods of Ellman *et al* (13). ChAT activity was determined using the spectrometric method of Chao and Wolfgram (14) with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorption of the final solution was measured via an automated ELISA reader (Synergy HT, USA). The levels of BDNF and NGF in the hippocampus were measured using ELISA assay kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The minimum detection limits of the kits are 3 ng/ml for BDNF and 15 pg/ml for NGF. Each sample was analyzed in duplicate.

Statistical analyses. Data were analyzed using statistical product and service solutions (SPSS 13.0) computer software (SPSS, Inc., Chicago, IL, USA). The main treatment effect on the escape latency was analyzed using analysis of variance (ANOVA) with repeated measures. Fisher's least significant difference Post-hoc test was used to test the differences between groups. One-way ANOVA was used to analyze the biochemical data. A P-value <0.05 was considered to represent a statistically significant difference. All values are presented as the mean ± SEM.

Results

Effects of HTs and HSMs on AChE activity in the hippocampus. Compared with vehicle treatment, AChE activity in HT-treated mice did not change significantly (P>0.05), while HSM-treated mice had a significant decrease in AChE activity (P<0.01) (Fig. 1).

Effects of HTs and HSMs on BDNF and NGF levels in the hippocampus. Compared with vehicle-treated mice, there were no differences found in BDNF and NGF levels in the HT-treated mice. However, treatment with HSMs resulted in a significant increase in BDNF and NGF levels (P<0.05) (Fig. 2).

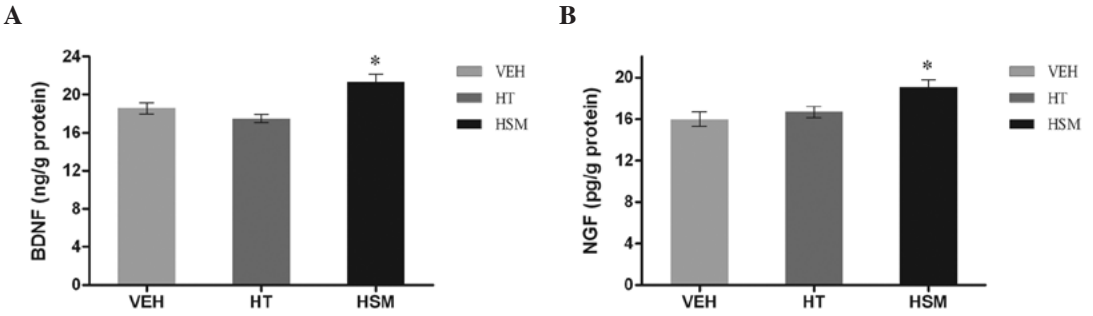

Figure 2. Effects of HTs and HSMs on BDNF and NGF levels in the hippocampus. (A) There was no difference in BDNF levels in the HT-treated mice compared with vehicle-treated mice. However, treatment with HSMs resulted in a significant increase in BDNF levels. Data are presented as the mean ± SEM, n=7 mice/group. *P<0.05 compared with the vehicle-treated group. (B) No difference was found in NGF levels in the HT-treated mice compared with vehicle-treated mice, but treatment with HSM resulted in a significant increase in NGF levels. Data are presented as the mean ± SEM; n=7 mice/group. *P<0.05 compared with vehicle-treated group.

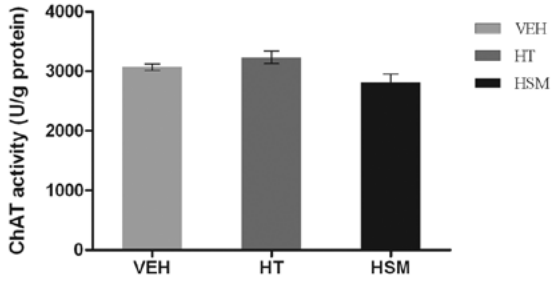

Figure 3. Effects of HTs and HSMs on ChAT activity in the mouse hippocampus. Neither HT nor HSM had a significant effect on ChAT activity when compared with vehicle treatment. Data are presented as the mean ± SEM; n=7 mice/group.

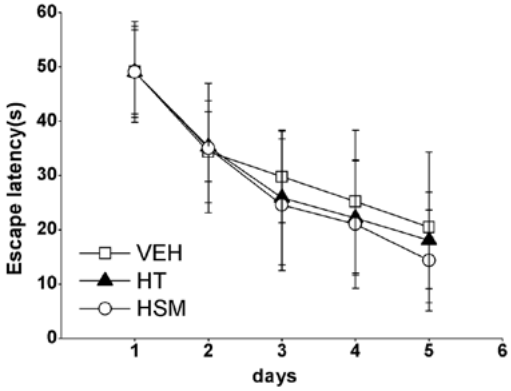

Figure 4. Effects of HTs and HSMs on escape latency in the Morris water maze test. The HSM group had the steepest learning curve, followed by the HT group, while the VEH group took the longest to learn how to escape. However, both the HT group and the HSM group exhibited no significant difference in finding the hidden platform when compared with the vehicle group. Data are presented as the mean ± SEM; n=21 mice/group.

Effects of HTs and HSMs on ChAT activity in the hippocampus. The effects of HTs and HSMs on ChAT activity in the hippocampus of juvenile mice are shown in Fig. 3. The data revealed that neither HT nor HSM had a significant effect on ChAT activity (P>0.05).

Effects of HTs and HSMs on escape latency in juvenile mice. ANOVA revealed a significant effect of the testing day on escape latency within the groups (F=548.86; P<0.01), suggesting that all mice effectively improved their spatial learning across the 5-day training period. However, no significant main treatment effect on escape latency was found (F (2,40) =0.315; P>0.05), demonstrating that neither fluctuant nor steady inhibition of AChE had any effect on spatial learning in juvenile mice (P>0.05) (Fig. 4).

Discussion

AChE is found in presynaptic (cholinergic) and postsynaptic (cholinoceptive) components of the central cholinergic pathways, where it terminates the synaptic action of ACh (1). The AChE inhibition provided by AChE inhibitors should strengthen the ACh effect by allowing more ACh molecules to bind to the nicotinic and muscarinic ACh receptors, leading to the activation of downstream signaling pathways.

As an AChE inhibitor, HupA was rapidly absorbed and eliminated *in vivo* after an oral administration of HT; thus, the plasma concentration of HupA was fluctuant. However, previous experiments *in vivo* demonstrated that HSM in mice can maintain a steady-state HupA concentration for 14 days after a single intramuscular administration of HSM (7). Thus, HT and HSM treatments can provide steady and fluctuant cholinergic stimulation, respectively.

The present study revealed that AChE activity was still significantly inhibited 6 days after HSM treatment. Furthermore, no marked changes were found in the HT group compared with the VEH group. The results indicate that the steady versus fluctuant AChE inhibition exerted different effects on AChE activity in the hippocampus.

BDNF and NGF provide trophic support to cholinergic neurons that project mainly to the hippocampus and the cortex. It has been demonstrated that NGF levels critically regulate cholinergic neuron size, cholinergic hippocampal innervation, and neurochemical differentiation during development (15,16). In addition, BDNF can increase the size of cholinergic neurons and the maturation of cholinergic projections to the hippocampal formation (17). Most importantly, it has been reported that in the hippocampus, there is positive feedback between cholinergic activity and neurotrophins, such

as NGF and BDNF (17,18). In the central cholinergic system, studies of conventional AChE inhibitors have demonstrated that fluctuant inhibition of AChE can upregulate the expression of BDNF and NGF in the hippocampus (19,20). In the present study, 6 days after withdrawal of steady and fluctuant AChE inhibition, the levels of BDNF and NGF in the hippocampus were investigated. Both BDNF and NGF levels increased after the steady inhibition of AChE in HSM-treated mice. In contrast, BDNF and NGF levels did not show any significant changes after the fluctuant inhibition of AChE in HT-treated mice. These results differ from previous studies, which concluded that AChE inhibitors increase neurotrophic factors levels (18,20-22). Regarding the contradictory results, it must be noted that the neurotrophic factor levels in the previous studies were measured immediately (4 to 24 h) after administration of AChE inhibitors. Our findings demonstrated that steady AChE inhibition caused a long-term increase in the levels neurotrophic factors in the hippocampus. The present study also indicated a consistent correlation between low AChE activity and the levels of BDNF and NGF.

ChAT, an enzyme that catalyzes the biosynthesis of ACh, has been used as a specific marker for cholinergic neurons (23). The chronic administration of AChE inhibitors may modify the ACh recycling pathway. However, neither the HSM group nor the HT group showed any alterations in ChAT activity in the hippocampus. The absence of changes in ChAT activity may be attributed to other components of the cholinergic system, which need to be investigated further.

AChE inhibitors can improve cognitive function in dementia animals with cholinergic dysfunction. However, it is unknown whether AChE inhibitors affect learning and memory in normal juvenile animals. Using the Morris water maze test, the present study demonstrated that compared with the VEH group, mice in the HT and HSM groups did not show any improvement in learning. These results suggest that the actions of AChE inhibitors on learning differ between normal and dementia model mice.

In conclusion, the present study indicated that steady AChE inhibition leads to a decrease in AChE activity and an increase in BDNF and NGF levels in the hippocampus. However, fluctuant AChE inhibition did not increase the BDNF or NGF levels. The present findings also suggest that AChE inhibitors do not improve learning in normal juvenile mice.

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