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Complementary remedy of aged-related learning and memory deficits via exogenous choline acetyltransferase

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Abstract

The present study aimed to examine whether the aged mice with naturally occurring cognitive deficits in learning and memory would benefit from supplementation of choline acetyltransferase (ChAT), the biosynthetic enzyme for neurotransmitter acetylcholine. Delivered by protein transduction domain (PTD), ChAT could pass through the blood–brain barrier, enter the neurons, interact with heat shock protein 70 kDa, and retain enzyme activity. In behavior tests, PTD-ChAT given to the aged and memory-deficient mice almost completely reversed the behavioral changes, such as impairment of memory retention in the step-through test (an index of long-term memory) and prolonged swimming time in water maze test (an index of spatial recognition memory). The results suggest a novel and potential therapeutic use of PTD-ChAT in the age-related cognitive deficits.

Keywords: Choline acetyltransferase; Acetylcholine; Protein transduction domain; Aging; Learning; Memory; Protein therapy

Aging in humans, as well as in experimental animals, is associated with slow deterioration of cognitive performance and, in particular, of learning and memory [1]. Cholinergic neurons have long been proposed to be critically involved in neuropsychic functions. The changes in the central cholinergic system in aging have been suggested to contribute to a variety of adverse behavioral symptoms such as cognitive deficits, depression, and psychosis [2]. Although multiple neurotransmitter pathways are impaired in the course of aging and cholinergic deficiencies do not account for all of the functional deficits observed in aging, one of the most promising therapeutic approaches thus far is aimed at restoring the cholinergic function.

Choline acetyltransferase (acetyl CoA: choline *O*-acetyltransferase, EC 2.3.1.6, ChAT), the biosynthetic enzyme for neurotransmitter acetylcholine (ACh), is presently the most specific indicator for monitoring the functional state of cholinergic neurons in the central and peripheral nervous system [3]. The activity of ChAT is prominently reduced in aged brains, and the degree of reduction of ChAT activity is significantly correlated with the severity of cognitive impairments [4]. The aim of present study was to assess whether it is possible to improve the cognitive performance in aged and memory-deficient mice via supplementation with exogenous ChAT.

However, the delivery of therapeutic protein into brain across the blood–brain barrier is severely limited by the proteins' size and biochemical properties. At present, an approach to deliver therapeutic peptides to brain is the application of fusion proteins linked to so-called protein transduction domains (PTD, an undecapeptide, YGRKKRRQRRR, derived from the human immunodeficiency virus TAT protein), which is able to deliver macromolecules to pass the blood–brain barrier and cell membranes [5,6]. Here, we show that a recombinant PTD-ChAT fusion protein manifests therapeutic benefit for cognitive deficits in aged mice.

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Materials and methods

Preparation of recombinant PTD-ChAT. The plasmid pCRT7/NT-ChAT bearing the human ChAT gene was used as the template. Primer pairs were listed as follows: P1, 5′ GCAGGATCCGCAGGTACCAT GGCAGCAAAAACTCCC 3′; P2, 5′GCTGAATTCTCAAGGTTGG TGTCCCTG 3′ (the restriction sites are underlined). The *pfu Taq* enzyme was used for amplification of the ChAT gene fragments. PCR products were digested with *Bam*HI and *Eco*RI, and cloned into pGEX-4T. Then, the plasmids were cleaved with *Bam*HI and *Kpn*I, and the PTD gene fragments (the sense chain: 5′ GATCCTATGGTCGTAAAAAGCGA CGCCAACGTAGACGTGGTGGTGGTAC 3′; the antisense chain: 5′ CACCACCACGTCTACGTTGGCGTCGCTTTTTACGACCATAG 3′) were incorporated in. The nucleotide sequence of the PCR product was analyzed for correctness by Bioasia (Shanghai, China). The recombinant prokaryotic plasmids were transformed into *Escherichia coli* BL21 and induced with 1 mM isopropyl-D-thiogalactoside (IPTG) at 30 °C for 4 h to express the PTD-ChAT fusion protein. The cells were then harvested and sonicated. The GST-PTD-ChAT fusion protein was purified as previously described [7]. After the GST was removed by thrombin cleavage, the PTD-ChAT protein was analyzed by SDS–PAGE and Western blotting.

Animals. Mice, Kunming species (Grade II, Certificate NO. 01-3023), female, aged 15–16 months, weighing 64 ± 5 g, were provided by the Animal Breeding Center Affiliated to AMMS, China. Animal studies were approved by the Animal Care and Use Committee, Ministry of Science and Technology, China. Animals were housed in plastic cages ($420 \times 240 \times 170$ mm), with free access to standard laboratory food and water, and kept in a regulated environment (23 ± 1 °C). The aged mice were pretested in the behavior assessment to ascertain the aged-induced cognitive impairment at least 3 weeks prior to the trials. The mice with memory deficiency were used throughout the experiments.

Immunohistochemistry analysis. Mice were anesthetized and fixed by intracardical perfusion of 4% paraformaldehyde in PBS (pH 7.4). The cerebral cortices were removed and kept at −20 °C ready for use. The cortex tissues were cryoprotected in 30% sucrose. The tissue section slices (20 μm) were incubated stepwise in 10% normal rabbit serum diluted in PBS for 1 h at room temperature, in the goat anti-human ChAT monoclonal antibody diluted in PBS (1:500) at 4 °C overnight, and then in the horseradish peroxidase (HRP)-labeled rabbit anti-goat IgG diluted in PBS (1:1000) for 5 h. The PBS (pH 7.2) was used to wash the slices before each addition. Finally, the slices developed the color in the DAB solution. Then, the cerebral cortex slices were dehydrated in a series of graded ethanol solutions and coverslipped with Permount. Images were obtained with Zeiss microscope.

Co-immunoprecipitation. Mice were killed by decapitation, the cerebral cortices were removed, homogenized in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mg/ml PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1% NP-40), left for 1 h in ice, and centrifuged at 14,000*g* for 10 min at 4 °C. The supernatant was immunoprecipitated using the goat or mouse normal IgG, goat anti-human ChAT, and the mouse anti-human heat shock protein 70 (HSP70), respectively, for 4 h at 4 °C. Subsequently, protein A/G–agarose beads (Santa Crutz) were added, and the incubation was continued for another 4 h. Immunocomplexes were washed four times with lysis buffer and subjected to Western blotting.

ChAT activity measurement. The ChAT activity was determined spectrophotometrically according to Chao [8,9]. Briefly, ten percent (1:10, w/v) cerebral cortex homogenates in cold saline were prepared (10,000 rpm, 10 s for twice with 30 s interval) in ice. The reaction mixture contained 20 μl of each of 0.5 M sodium phosphate buffer (pH 7.0), 6.2 mM acetyl coenzyme A, 1.0 M chloride choline, 0.76 mM physostigmine, 3 M NaCl, 1.1 mM EDTA, 0.5 M hydrochloric creatinine, 6.5 mM DTT, and distilled water to a total volume of 400 μl. The mixture was preincubated at 37 °C for 5 min, then mixed with 200 μl of the cortex homogenates, and incubated at 37 °C for 20 min, and finally the reaction was stopped in boiling water. Sodium arsenate (2.5 mM, 800 μl) was added to each tube for precipitation. One milliliter of each supernatant was mixed with 20 μl of 3 nM 4-PDS and incubated at 25 °C for 15 min. Absorbances were read at 324 nm, and calibrated with the blank and the standard (0.11 mM CoA, instead of the cerebral cortex homogenates). Protein concentration was determined according to Lowry.

Assay of ACh. ACh was determined by using the method of Hestrin [10]. In brief, the aliquots (0.8 ml) of cerebral cortex homogenates were mixed with 1.4 ml distilled water, 0.2 ml of 1.5 mM physostigmine, and 0.8 ml of 1.84 M trichloroacetic acid blending adequately. After centrifugation, 1 ml of each supernatant was added to 1 ml of basic hydroxylamine. The mixture was incubated for 15 min at 25 °C and then 4 M HCl 0.5 ml and 0.37 M $FeCl_3$ 0.5 ml were added. Absorbance were read at 540 nm, and calibrated with the blank and the standard (0.2 μmol/ml ACh, instead of the cerebral cortex homogenates).

Step-through test. The step-through test was performed in the JZZ94 multifunction passive avoidance apparatus (PA M1 O'Hara & Co. Ltd.). The apparatus consisted of two compartments separated by a black wall with a hole in the lower middle part. One of the two chambers was illuminated and the other, dark. The test was conducted for 2 consecutive days including one training trial (d1), each mouse was placed in the illuminated compartment, facing away from the dark compartment and left for 5 min to habituate to the apparatus. One hour after the adaptation trial, the mouse received the training trial. It was similar to the adaptation trial, except that when the mouse entered the dark compartment it would suffer an electric foot shock (DC, 0.3 mA, 30 V, 1 s) through the stainless steel grid floor. In the testing trial (d2), the same test procedure was performed, the time intervals between the placement in getting out to the illuminated compartment and the entry into the dark compartment were recorded as memory retention, and the entering events per testing trial were recorded as the entries into dark compartment. Mice were allowed to stay in the illuminated compartment for 30 s in the training trial and 120 s in the testing trial.

Water maze test. The water maze apparatus (Chinese Academy of Medical Sciences, China) was a black plexiglass rectangular box ($130 \text{ cm} \times 85 \text{ cm} \times 50 \text{ cm}$), including a starting point, a terminal platform, and six non-exits. Near the platform was the safe region and an invisible ladder was located for rest. The swimming time of mice was recorded from the starting point (the farthest non-exit to the end) to the terminal platform. The maze was filled with water to depth 25 cm and the temperature was kept at (20 ± 1 °C). At training trial on d1, each mouse stayed on the terminal platform 30 s for recognizing the location, and then placed at the starting point of the box facing the wall, and the swimming time to find the ladder was recorded up to 3 min. If the mouse did not find the ladder within 3 min, the swimming time was assigned as 3 min, and the mouse was manually led to the ladder, left for 30 s, and returned to its home cage. The testing trials were carried out 2 h after the training trial (d1), and on d2 and d3 as well. The time spent for each mouse to reach the ladder from the starting point (swimming time) was recorded.

Statistical. Data were expressed as means ± SEM and were evaluated for statistical significance with two-way ANOVA followed by Duncan's multiple range tests.

Results

Preparation of PTD-ChAT

To produce the genetic in-frame PTD-ChAT fusion protein, we constructed a bacterial expression vector, pGEX-PTD-ChAT. The vector consists of genes of glutathione *S*-transferase (GST), undecapeptide PTD, and linked up via three glycine residues (for free bond rotation of the domain) to ChAT. A specific thrombin recognition site in the GST-PTD-ChAT fusion protein was located just before the PTD, so that the GST could be easily cleaved by thrombin, thereof no additional residues would attach to the purified PTD-ChAT protein (Fig. 1).

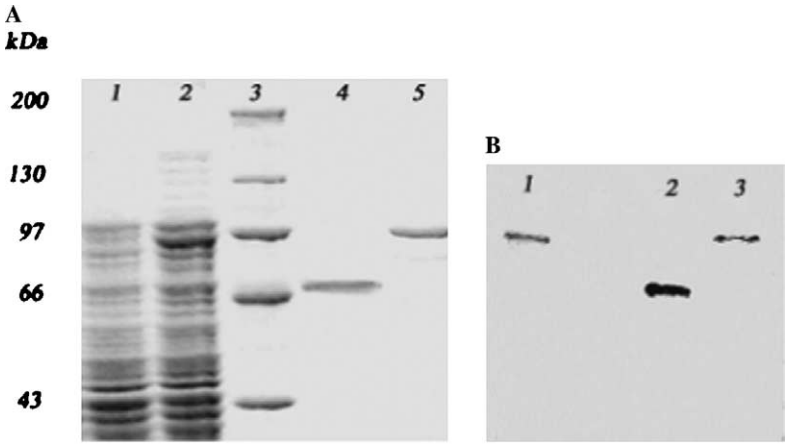

Fig. 1. Expression and purification of PTD-ChAT. (A) Lane 1, uninduced *E. coli*; lane 2, induced *E. coli*; lane 3, marker proteins; lane 4, purified PTD-ChAT; lane 5, purified GST-PTD-ChAT. The molecular weight standard is listed on the left. (B) Western blotting of GST-PTD-ChAT and PTD-ChAT. The goat anti-human ChAT McAb was used. Lanes 1 and 3, GST-PTD-ChAT; lane 2, purified PTD-ChAT.

Immunohistochemical detection of ChAT in cerebral cortices of mice after systematically administering the PTD-ChAT

To determine whether the purified PTD-ChAT intravenously (i.v.) injected could pass through the blood–brain barrier, the ChAT activity in the cerebral cortices of mice was detected using the immunohistochemistry assay 1 h after injection of PTD-ChAT or ChAT (4 mg/kg). The results showed that intensive staining was observed in the slices of cerebral cortex after injection of PTD-ChAT (Fig. 2B), sharply contrasting with that of the control mice injected ChAT without bearing PTD (Fig. 2A).

Involvement of heat shock protein 70 in the renaturation of PTD-ChAT in vivo

Monoclonal antibodies of goat anti-human ChAT and mouse anti-heat shock protein 70 (HSP70) were alternately used in the co-immunoprecipitation assay as described under Materials and methods. The results showed that no matter what antibody was first used, the ChAT–HSP70 complex was always precipitated from the cerebral cortex lysate (Fig. 3), whereas in the normal IgG controls and ChAT (without bearing PTD) controls, no immunoprecipitation was shown in the Western blots. It implies that HSP70 involves somehow in the renaturation of the enzyme activity.

Increase of brain ChAT and ACh levels after injection of PTD-ChAT

The cerebral cortices were dissected at 0, 0.5, 1, 2, 5, 8, and 12 h, respectively, after i.v. injection of PTD-ChAT or ChAT (4 mg/kg). The time-effect curve showed that the ChAT activity increased along with time, peaked at 0.5 h, and then declined to the intrinsic level in 12 h (Fig. 4A), whereas the control did not show any increase on the basis of the endogenous level 55.4 ± 4.1 nmol/h/mg protein. The time-effect curves of the ACh level behaved similarly to the curves of ChAT activity, nevertheless with a peak at 1 h

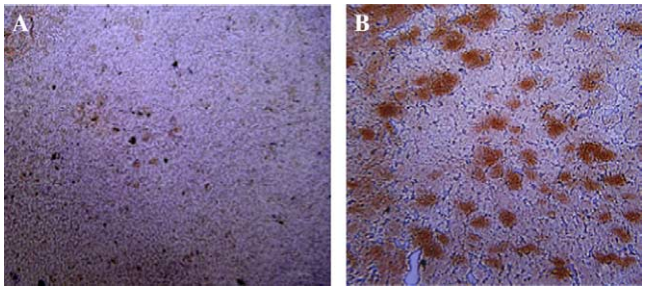

Fig. 2. Immunohistochemistry analysis of ChAT in the cerebral cortex of mice 1 h after i.v. injection of recombinant human PTD-ChAT (4 mg/kg, saline was used as vehicle). Immunohistochemical assay was performed using goat-anti-human ChAT monoclonal antibody and HRP labeled rabbit anti-goat IgG, and visualized with DAB. (A) Control, injection of ChAT without PTD; (B) injection of PTD-ChAT.

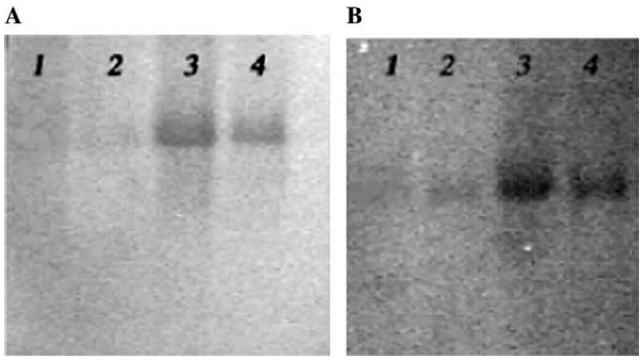

Fig. 3. Interaction between ChAT and HSP70 in vivo. ChAT–HSP70 complexes were immunoprecipitated from the tissue lysate of mice cortex with (A) anti-ChAT antibody and detected by immunoblotting with anti-HSP70 antibody; (B) anti-HSP70 antibody and detected by immunoblotting with anti-ChAT antibody. Lane 1, IgG control; lane 2, ChAT (without PTD) control (1 h after injection); lane 3, PTD-ChAT (1 h after injection); lane 4, PTD-ChAT (5 h after injection). ChAT (without PTD) or PTD-ChAT was i.v. injected 4 mg/kg, saline was used as vehicle.

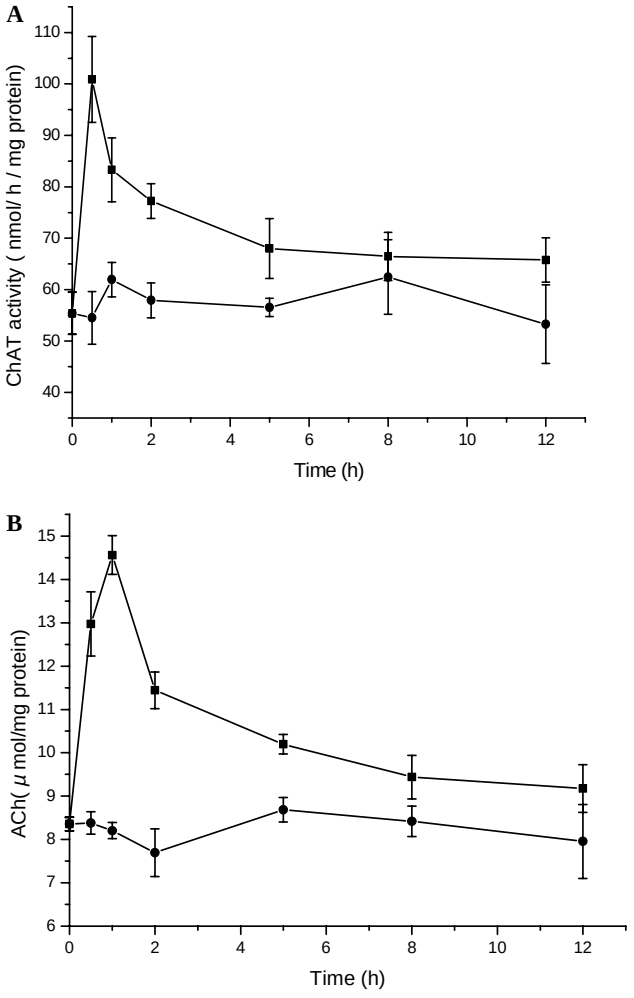

Fig. 4. ChAT activity and ACh level in cerebral cortices of mice after i.v. injection of PTD-ChAT or ChAT (4 mg/kg, saline as vehicle). (A) ChAT activity; (B) ACh contents. (■), PTD-ChAT; (●), ChAT. $\bar{x} \pm$ SEM, $n = 3$.

after injection (Fig. 4B). These results indicated that denatured and dispersed recombination human ChAT mediated by PTD well passed the blood–brain barrier, entered the neurons in the cerebral cortices of mice, and renatured the enzyme activity.

Effects of PTD-ChAT on aged mice with memory-deficiency

To determine whether supplementation with ChAT could improve learning and memory in the aged mice, we examined the potential reversal of amnesia with denatured and dispersed recombination human PTD-ChAT and ChAT. Cholinesterase inhibitor Huperzine A (Hup) was used as positive-control medicine [11]. The aged mice were assessed in the step-through and water maze tests.

PTD-ChAT and ChAT were i.v. injected (4 mg/kg), respectively, to the aged and memory-deficient mice 0.5 h before each testing trial. Hup was intraperitoneally (i.p.) injected (0.1 mg/kg) 40 min before testing. The results showed that in the step-through test, the entries into the dark compartment of the mice in the saline and ChAT (without bearing PTD) controls significantly elevated to 2.1 ± 0.7 and 2.0 ± 0.6, as compared with the normal young mice 0.6 ± 0.7 ($P < 0.05$), whereas in the PTD-ChAT-treated mice it significantly decreased to 0.6 ± 0.5 as compared with the saline control ($P < 0.05$), and slightly better than that of the Hup-treated mice (0.8 ± 0.4) (Fig. 5A). The memory retention of the mice in saline and ChAT (without harboring PTD) controls significantly decreased to 26.8 ± 25.6 and 29.0 ± 16.8 s as compared with the normal young mice 116.0 ± 7.2 s ($P < 0.01$), whereas in the PTD-ChAT treated mice it significantly increased to 119.3 ± 2.1 s as compared with the saline control ($P < 0.01$), and is also slightly better than that of the Hup-treated mice 89.0 ± 18.1 (Fig. 5B).

PTD-ChAT, ChAT and Hup were administered to mice in the same way as above mentioned, The results showed

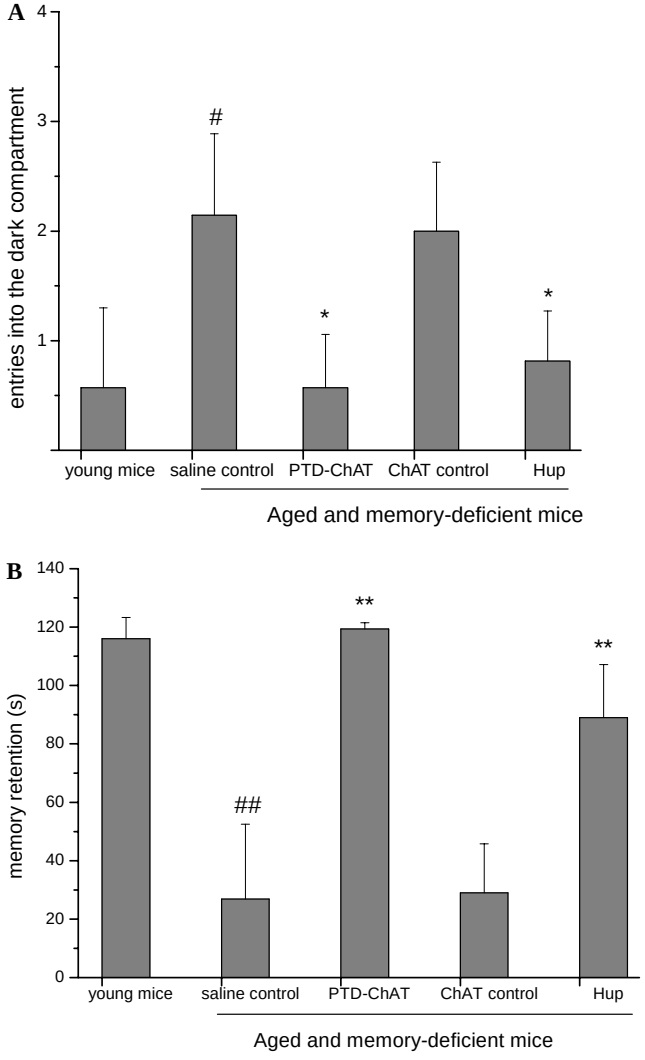

Fig. 5. Reversal effects of PTD-ChAT on aged and memory-deficient mice (step-through test). (A) Entries into the dark compartment of mice; (B) memory retention of mice. PTD-ChAT and ChAT were i.v. injected, respectively. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with young mice; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the saline and ChAT controls. $\bar{x} \pm$ SEM, $n = 10$.

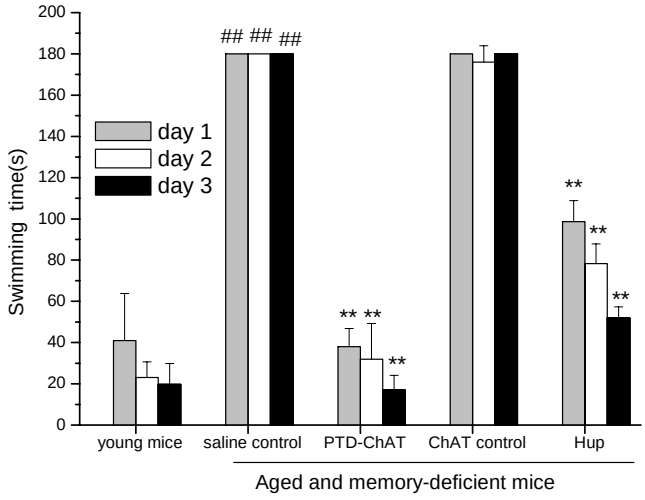

Fig. 6. Reversal effects of PTD-ChAT for aged and memory-deficient mice in water maze test. PTD-ChAT and ChAT were i.v. injected, respectively. $^{\#\#}P < 0.01$ compared with young mice; $^{**}P < 0.01$ compared with control. $\bar{x} \pm \mathrm{SEM}$, $n = 10$.

that in the water maze test, the mice in the saline and ChAT (without bearing PTD) controls failed to find out the way to reach the platform in the water maze within the observed 3 min, whereas the PTD-ChAT-treated mice and the young mice only needed 38.0 ± 8.8 and 41.0 ± 22.8 s, respectively, to reach the platform. It did sharply contrast with the swimming times of the mice in the control groups ($P < 0.01$) and is much better than that of the Hup-treated mice 98.7 ± 10.1 s (Fig. 6).

Discussion

Delivery of protein therapeutics using PTD offers a new and exciting strategy to treat diseases [12,13]. This technology allows efficient delivery into a variety of different cells and tissues in a rapid, concentration-dependent manner. When fused to PTD, the peptide or protein of interest can be readily delivered into many tissues in animal models [14]. In our study, ChAT mediated by TAT PTD well passed the blood–brain barrier and entered into the neurons.

Although PTD had been used to deliver biologically active cargo as shown in various animal studies, the mechanism of transduction remained unknown. Because of the PTD's strong cell-surface binding, early assumptions regarding cellular uptake suggested a direct penetration mechanism across the lipid bilayer by a temperature- and energy-independent process [15]. However, Wadia et al. reported recently that PTD-fusion proteins are rapidly internalized by lipid raft-dependent macropinocytosis after an initial ionic cell–surface interaction, followed by a pH drop and destabilization of the integrity of the macropinosome vesicle lipid bilayer. Although the exact nature of TAT-mediated release from macropinosomes into the cytosol remains unclear, macropinosomes are thought to be inherently leaky vesicles compared with other types of endosomes. Notably, although the pH of macropinisomes decreases, macropinosomes do not fuse into lysosomes to degrade their contents [16].

Here we showed that PTD-ChAT fusion protein interacted with HSP70 after entering the cells. Extensive researches had indicated that a major function of HSP70 was that of a molecular chaperone [17,18]. The HSP70 chaperone in cells plays roles in solubilization of denatured protein aggregates, facilitation of the restoration of the function of the renatured proteins, and transportation of proteins to different compartments. In our study, the denatured recombination fusion protein PTD-ChAT was genetically expressed in and purified from the inclusion bodies of *E. coli*, refolded efficiently in mice after injection, and retained the enzyme activity.

As normal human aging, rodents also show an age-dependent cognitive decline associated with neurodegenerative changes in the central cholinergic neurons. The aged and memory-deficient mice are suitable models for the assessment of the function of the cholinergic system [19]. When increasing the ChAT and ACh levels in brain by injection of PTD-ChAT, the aged and amnesia mice showed less entry into the dark compartment and longer memory retention in the step-through test (an index of long-term memory), and a shorter swimming time in water maze test (an index of spatial recognition memory) as compared with control mice ($P < 0.01$). These results indicate that age-related cognitive deficits in learning and memory were remedied by reinforcement of ChAT and ACh in the brain.

We speculate that synapse may have the ability to package ACh in the vesicles after ACh level increment. Since the the aged and memory-deficient mice do not have sufficient ACh, the vesicles will be brimming, and can release more transmitters after ACh supplementation. ACh systems in the prefrontal cortex or in the hippocampus are activated during the performance of memory tasks, while enough ACh release can modulate aged cortical and hippocampal networks adequately to reverse cognitive deficits in learning and memory [20–22]. Furthermore, it is well known that cholinergic systems take part in many tasks, such as retrieving the age-related losses in agility and reduction in the incidence and severity of tardive dyskinesia. Supplementation of ChAT and ACh in brain can probably increase the physical activities (motility) in aging animals.

Exogenous ChAT allows us to incorporate in various kinds of cells, supplementation of ChAT has demonstrated that ACh release from cholinergic neuron can modulate aged cortical networks adequately to reverse the cognitive deficiency in learning and memory. These findings conclude that ChAT supplementation in the brain is sufficient to ameliorate aged-related cognitive deficits in mice.

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