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Age-Related Increase of Insulin-Degrading Enzyme Is Inversely Correlated with Cognitive Function in APPswe/PS1dE9 Mice

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Manuscript Preparation E
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Background: Insulin-degrading enzyme (IDE) is an important regulator for A β clearance and diabetes. Although it is indispensable in removing plaques related to onset Alzheimer's disease (AD) and in degrading insulin related to diabetes, there have been few studies on the dynamic level of IDE in different stages of AD.


Material/Methods: The present study explored the level IDE protein in different stages of APPswe/PS1dE9 mice and their correlations with cognitive decline. The 4-month-old, 10-month-old, and 18-month-old mice were used as the different age stages of mice. Cognitive function was evaluated using the Morris water maze test. We also observed the level of A β plaques in brain regions of different stages.

Results: The data revealed that the expression of IDE was dramatically higher than in age-matched wild mice at the age of 10 months and 18 months. In terms of distribution, A β plaques were deposited mostly in the cortex and hippocampus, especially in 10-month-old and 18-month-old APPswe/PS1dE9 mice. The cognitive function of 4-month-old APPswe/PS1dE9 mice was not significantly differ in spatial learning. However, the cognitive function, both spatial learning and spatial memory, was dramatically lower in 10-month-old and 18-month-old groups.

Conclusions: There was a positive correlation between the expression of IDE and spatial memory in 10-month-old and 18-month-old APPswe/PS1dE9 mice. The study of this protein may provide reference values for the further study of IDE in Alzheimer's disease.

MeSH Keywords: **Alzheimer Disease • Amyloid beta-Peptides • Insulysin • Mild Cognitive Impairment**

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Background

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is already a major focus of research attention. AD causes functional impairment, like loss of cognitive functions and behavioral changes, which has severe effects on AD patients [1,2]. It was reported that over 46 million elderly people have cognitive impairment and memory loss caused by AD [3,4]. Furthermore, this trend may be increased to more than 131 million by 2050, and expenditure on AD patients with dementia is more than \$800 billion every year [5,6]. Cognitive decline has become the most important problem with AD patients [7].

Cognitive functions are attributed to different types of cerebral activities, which include learning memory, spatial perception, reasoning, judgement, and evaluation [8]. The brain areas involved in cognitive functions include the amygdala, hypothalamus, hippocampus, cingulate gyrus, and thalamus [9,10]. The decline in these functions, especially in the hippocampus, is considered to be an aggravating factor leading to progression of dementia from the mild cognitive impairment (the MCI phase) phase to Alzheimer's disease (the DAT phase) [11,12].

Although factors promoting cognitive decline have been identified, such as age and genetic predisposition, the key pathogenic mechanism of AD memory loss is the accumulation of amyloid- β ($A\beta$) in the brain [13,14]. This compound is a cleavage product of the amyloid precursor protein (APP) by β - and γ -secretase. $A\beta$ -induced neuron apoptosis and autophagy are considered to be the core pathological mechanism underlying neuronal damage in AD [15–17]. Recent research presents the consensus that $A\beta_{42}$, the abundant cleavage product in the parenchyma plaques, is the primary amyloidogenic form in AD [18]. The lost dynamic balance between $A\beta$ removal and production causes the abnormal accumulation of $A\beta$ in the brain, triggering neuronal dysfunction and cell death [19]. The production of $A\beta$ is believed to be important in the AD pathological process, but its clearance is also useful in maintaining $A\beta$ steady-state levels [20,21].

Insulin-degrading enzyme (IDE) is a thiol-sensitive zinc metalloproteinase that is a major endogenous $A\beta$ -degrading enzyme [22]. The level and enzymatic activity of IDE are negatively correlated with the size of the amyloid plaques and AD pathology [23]. There is evidence showing IDE-KO that mice had a more than 95% reduced rate of degradation of physiological $A\beta$ levels and it also increased the levels of endogenous cerebral $A\beta$ in an IDE-KO animal model [24]. Owing to the dual function of IDE in reducing insulin secretion and the role of plaque removal, it might show the different alteration of expression level compared to ordinary $A\beta$ -degrading enzymes such as the metalloproteinases neprilysin (NEP) and endothelin-converting enzyme (ECE) in different stages of the

AD pathological process (mature adult, middle age, and old age) [25]. The level of this protein could be affected not only by the gradual accumulation of $A\beta$ plaques, but also by insulin-related intervention. Thus, its expression level might have its own specificity. However, there have been few studies on the actual expression of IDE in different stages (juvenile, adult, old age) in APPswe/PS1dE9 double-transgenic mice and their correlation with cognitive decline.

In the present study, 4-month-old, 10-month-old, and 18-month-old APP/PS1 AD mice were used to explore the dynamic alteration of IDE in the AD pathophysiological process. In the AD animal model, the age span phases in C57BL/6J mice is divided into 3 stages, which include 3–6 months of age (mature adult), 10–15 months of age (middle age), and 18–24 months of age (old age) [26]. Meanwhile, APP/PS1 mice show amyloid plaques formation at around 4 months of age [27]. Thus, 4-, 10-, and 18-month-old mice can stand for the AD model mice stage as mature adult, middle age, and old phases, respectively. Furthermore, we also detected the cognitive function of AD model mice and $A\beta$ plaque accumulated in the brain areas in charge of the cognitive functions (hippocampus and cortex). Meanwhile, the correlation between cognition and IDE was also analyzed.

Material and Methods

Animal Model

APPswe/PS1dE9 (Jackson Laboratory, stock No. 004462, n=30, male) were purchased from Nanjing Biomedical Research Institute of Nanjing University. All animal experiments conformed to the National Institutes of Health guidelines. All animal procedures were approved by the Ethics Committee of Xuanwu Hospital of Capital Medical University. The model mice were kept with accessible water and fed under a 12-h light-dark cycle. In the AD animal model, the age span phases in C57BL/6J mice is divided into 3 stages: 3–6 months of age (mature adult), 10–15 months of age (middle age), and 18–24 months of age (old age) [26]. Thus, 4-, 10-, 18-month-old mice can represent the AD model mouse stage of mature adult, middle age, and old phases respectively [26,28]. We separated these mice into 3 groups: a 4-month-old group (AD, n=10), a 10-month-old group (AD, n=10), and an 18-month-old (AD, n=10). We used C57BL/6J mice of corresponding ages as wild-type groups (WT, n=10 per group).

Western blot

The hippocampus was taken out together with the inferior temporal cortex and prefrontal cortex (abbreviated hereafter as cortex). Then, we homogenized the tissue in neuronal

Protein Extraction Reagent (Thermo Scientific, 87792) containing a cocktail of protease and phosphatase inhibitors (Thermo Scientific, 87786). Protein concentrations were determined using BCA protein assay (Thermo Scientific, 23227). Equal amounts of total protein were analyzed by 12% SDS-PAGE gels. Then, we transferred it to nitrocellulose (NC) membranes (Solarbio, Beijing, China). The primary antibodies rabbit anti-IDE (Abcam, 1: 1000) were used. Image Lab (Bio-Rad) was utilized for protein band densitometry.

Immunohistochemistry analysis

Immunohistochemical examinations were checked to determine the level of IDE in hippocampus and cortex of model mice. We separated 4 mice per group and primed them with saline solution. Then, perfusion was conducted with 4% paraformaldehyde. Brain tissues were removed and cut through the mid-sagittal plane. Hemispheres of the brain were fixed with 4% paraformaldehyde overnight, then we embedded them with paraffin. The paraffin sections were dried for 1 h at 60°C and dewaxed with xylene. After a graded series of ethanol solutions, antigen repair, incubation with 3% H₂O₂, and blocking (5% BSA), slides were incubated with primary antibody (anti-IDE, 1: 50) overnight at 4°C, then rinsed with PBS and incubated with secondary antibody for 20 min at room temperature [29]. We visualized them with diaminobenzidine. The mean integral optical density (IOD) was calculated in 3 fields of the hippocampus and cortex for each slide. Each field was imaged at 400× using a microscope (Leica, DM3000) equipped with Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Thioflavin T assay

Brain tissue sections were washed 3 times with TBS for 5 min each time. Then, we incubated them for 10 min at room temperature with 10 µg/ml thioflavin T (ThT, Sigma, No. T3516). The sections were washed with 50% C₂H₅OH 3 times for 5 min each time. After that, we washed them with TBS 1 time for 5 min and kept them in the dark during the entire procedure. This reagent was used to stain amyloid Aβ deposits and must be freshly prepared and filtered. In the presence of amyloid fibrils, there is a bright fluorescence with this dye at the excitation and emission maxima of 450 and 482 nm, respectively [30].

Morris water maze

Hippocampal-dependent spatial learning and memory were evaluated using the Morris water maze, as described previously [28]. All mice in groups were individually coded. The measurements were performed by investigators blinded to group designations. Each group of mice (4-month-old, n=10; 10-month-old, n=10; 18-month-old, n=10 WT and APPswe/PS1dE9 mice) received 5 consecutive days of training to find the submerged

platform (in the second quadrant). Before the training, we kept the mice in the water for adaptation and carefully observed the movement and trajectory of each mouse. We excluded mice that were visually impaired or weak according to the observation of swim speed, swim path to a visible platform, and behavioral phenotypes that related to visual and motor functions. The experimental environment remained constant during behavioral testing. Swimming trajectories were videotaped. Escape latency and time spent in the target quadrant during probe trials were analyzed. After that, we put all the mice back for a 7-day rest. Then, the brains were removed for subsequent experiments.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.01; CA, USA). The data presented for each experimental condition were obtained from at least 3 independent experiments. All data are presented as the mean ±S.D. Statistical significance was analyzed by the *t* test. The group differences in the escape latency were analyzed using two-way ANOVA in the Morris water maze test. A 2-tailed *t* test was used to evaluate and compare the 2 groups for time spent in the target quadrant during the last day. Linear regression analysis was carried out to evaluate correlations between the levels of IDE and spatial memory. Statistical significance was set at *p*<0.05.

Results

Age-dependent changes of IDE in different stages of APPswe/PS1dE9 mice

Increasing evidence shows that Aβ degrading enzymes contribute to the AD pathological process [31]. The levels of IDE in the hippocampus and cortex, which are the regions vulnerable to amyloid β protein accumulation, of all mice were first detected by Western blot analysis (Figure 1). The level of IDE in APPswe/PS1dE9 double-transgenic mice dramatically increased compared with age-matched control mice at the age of 10 months (*p*<0.01) and 18 months (*p*<0.05), but not at 4 months (Figure 1A, 1D). There were significant age-dependent group differences in the levels of IDE between 4-month-old and 10-month-old groups, showing the phases of the AD pathological development process in AD model mice (Figure 1B, *p*<0.01). However, no obvious changes were found among the 3 age groups of control mice (Figure 1C). All these data indicated that the expression level of IDE was associated with AD, especially in the aged phases. IDE protein expression was up-regulated with age in APP/PS1 mice (Figure 1B, *p*<0.01).

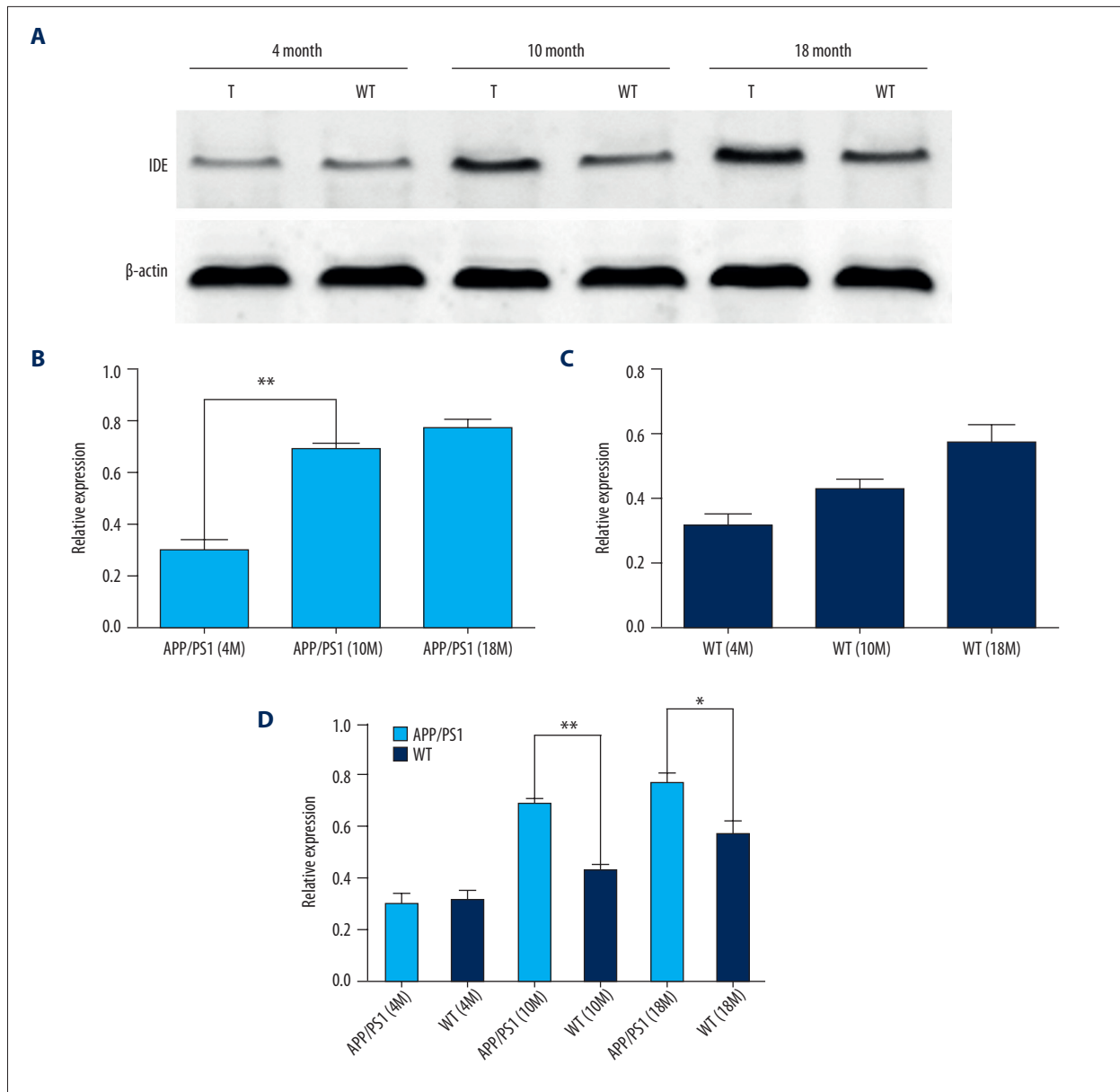
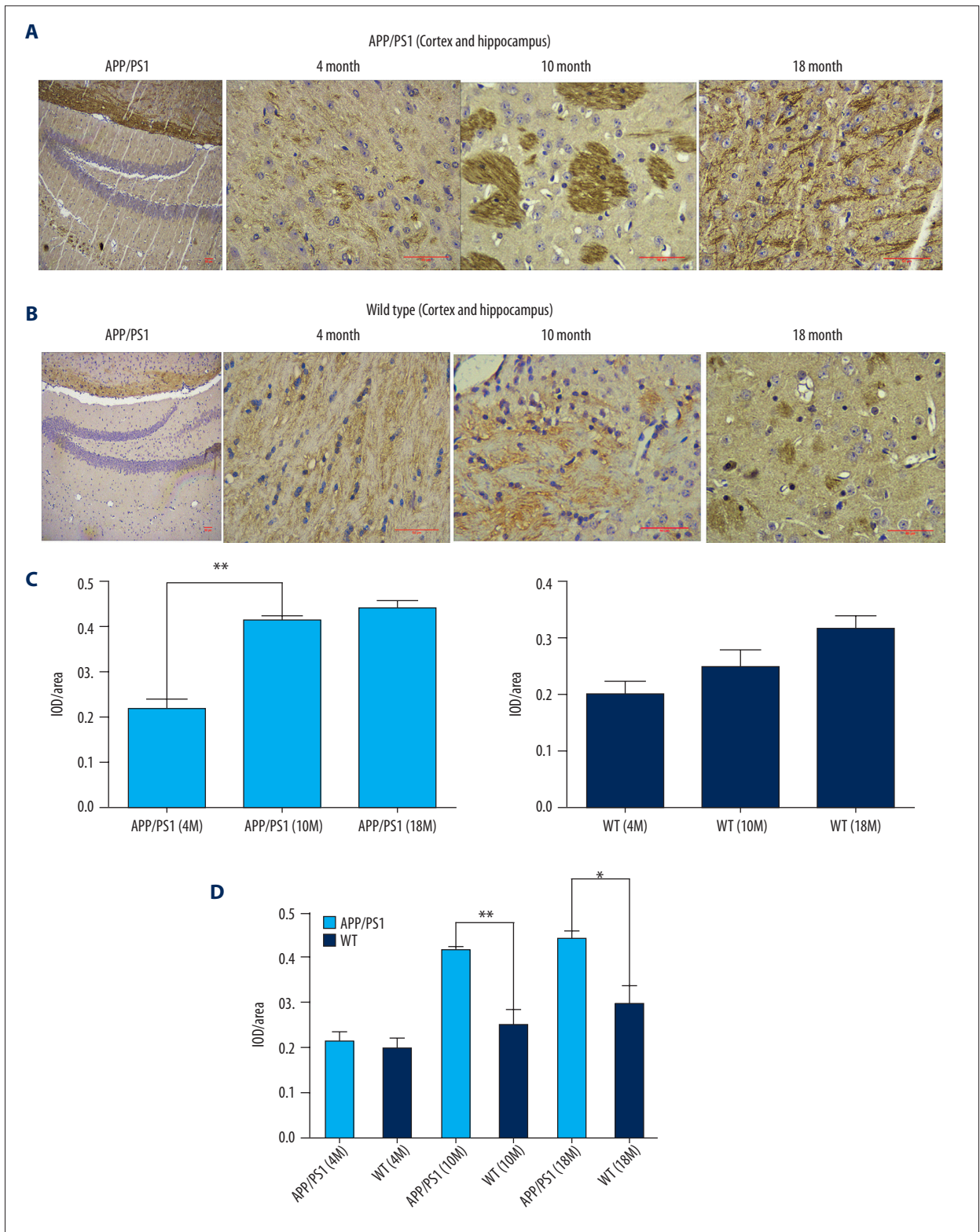


Figure 1. The expression level of IDE in the cortex and hippocampus of APPswe/PS1dE9 mice at different ages. **(A)** Representative bands of IDE in the cortex and hippocampus of APPswe/PS1dE9 AD model mice included 4-month-old (4 M), 10-month-old (10 M), and 18-month-old (18 M) mice. T stand for APP/PS1 mouse groups. WT stand for wild-type groups. **(B)** The relative expression of IDE in the cortex and hippocampus of APPswe/PS1dE9 AD model mice. **(C)** The relative expression of IDE in the cortex and hippocampus of wild-type mice. **(D)** The relative expression of IDE in the cortex and hippocampus at different ages (* $p < 0.05$, ** $p < 0.01$).

The expression level of IDE has obvious discrepancy in the different areas of the brain in APPswe/PS1dE9 mice

We continued to confirm the detailed and specific relationship of IDE with AD pathogenesis by immunohistochemistry (IHC) using paraffin sections of the APP/PS-1 mouse brain tissues (Figure 2). Expression of this protein in 10-month-old ($p < 0.01$) and 18-month-old ($p < 0.05$) mice were also higher than that in

the age-matched mice in the control group, especially in the hippocampus and cortex (Figure 2A, 2B, 2D). Meanwhile, there were obviously differences in expression distribution of IDE in different brain regions. The level of IDE was increased significantly in the hippocampus and cortex of APPswe/PS1dE9 mice in the 10-month-old group compared to 4-month-old AD mice (Figure 2C, $p < 0.01$). This evidence shows that IDE expression changes with age, especially in the hippocampus and cortex.



Distribution of increased A β plaques in brain regions of different stages in APPswe/PS1dE9 mice

The deposition of A β plaques plays a very important role in the occurrence and development of AD [32]. The previous results have demonstrated that the level of IDE increased with age. IDE is one of the most important A β protein-degrading enzymes in AD; therefore, we sought to confirm the plaque formation at different ages in APPswe/PS1dE9 mice. The A β plaques in the brains of APPswe/PS1dE9 mice at 4, 10, and 18 months of age and the age-matched wild-type mice were analyzed with thioflavin T staining. The number of A β plaques were observed after staining, which revealed dense core amyloid plaques [33] (Figure 3). As illustrated in Figure 3A and 3B, the APPswe/PS1dE9 mice displayed greater numbers and area of A β plaques with age. There were significant age-dependent group differences in the numbers and area of A β plaques among the 3 groups of APPswe/PS1dE9 mice (Figure 3C, $p < 0.01$). Furthermore, there were more plaques deposited in the cortex and hippocampus, especially in 10-month-old and 18-month-old groups of APPswe/PS1dE9 mice (Figure 3A, 3C, 3D, $p < 0.01$).

APPswe/PS1dE9 double-transgenic mice dramatically decreased cognitive ability with age

Previous studies have found that A β plaques can induce memory loss [34,35]. To evaluate spatial memory, we performed behavioral testing with the 3 age groups of mice using the Morris water maze, which can assess spatial learning and memory associated with the hippocampus [28]. The analysis of mean escape latencies during the 5 days of training are exhibited in Figure 4A–4C. The data showed longer escape latencies in the 10-month-old (Figure 4B, group factor effect: $F_{(1, 18)} = 5.64$, $p < 0.05$) and 18-month-old groups (Figure 4C, group factor effect: $F_{(1, 18)} = 14.94$, $p < 0.01$) groups of APPswe/PS1dE9 mice compared with age-matched WT mice. However, there were no obvious differences between the 4-month-old APPswe/PS1dE9 mice and WT mice (Figure 4A, group factor effect: $F_{(1, 18)} = 3.76$, $p > 0.05$). Meanwhile, there was no significant difference in swimming speed of APPswe/PS1dE9 double-transgenic mice compared with corresponding control mice (4 M: 11.11 ± 1.77 vs. 10.19 ± 2.16 , $p > 0.05$; 10 M: 8.28 ± 1.62 vs. 9.58 ± 1.76 , $p > 0.05$; 18 M: 7.35 ± 1.14 vs. 7.50 ± 1.78 , $p > 0.05$). This implies the escape latency differences in the water maze test were not due to motor deficits. Moreover, the visible platform trials in the water maze also suggested there were no significant differences in visual acuity and swimming speed between APP/PS1 mice and control mice at the 3 age groups.

Additionally, spatial memory was evaluated using the probe trial at the sixth day to check the memory retention. As expected, all 3 age groups of APPswe/PS1dE9 mice spent less time in the target quadrant compared with age-matched

controls (Figure 4D, $p < 0.01$). Collectively, these data indicate that 10-month-old and 18-month-old APPswe/PS1dE9 mice had obvious cognitive deficits when compared with the age-matched WT mice.

Expression of IDE is inversely correlated with cognitive function

The potential role of IDE in the cognitive deficits was analyzed by correlation analysis. The time spent in the target quadrant in the Morris water maze was used to evaluate spatial memory ability. The correlations between the expression of IDE and spatial memory are shown in Figure 5.

There was a significant positive correlation between the expression of IDE and spatial memory in 10-month-old (Figure 5B, $r^2 = 0.6264$, $p < 0.01$) and 18-month-old (Figure 5C, $r^2 = 0.5484$, $p < 0.05$) APPswe/PS1dE9 mice. However, the level of IDE in 4-month-old APPswe/PS1dE9 mice did not significantly correlate with spatial memory performance (Figure 5A, $p > 0.05$). These data indicate that the expression of IDE is inversely correlated with cognitive function in APPswe/PS1dE9 mice.

Discussion

A β plaques are one of the most important risk factors of advanced cognitive impairment in the pathogenesis of AD. They can be produced by neuronal cells and peripheral cells [36]. Almost all patients with Alzheimer's disease, which includes familial AD and sporadic AD, have A β plaques deposited in brain tissue. Greater A β plaques accumulation also causes enhanced neuronal loss and leads to cognitive impairment [1]. Therefore, reducing the production of A β and promoting A β degradation have become one of ways to control the progression of AD.

A β oligomers could be degraded by various enzymes, which include neutral endopeptidase (NEP), endothelin-converting enzyme (ECE), and insulin-degrading enzyme (IDE). It was reported that NEP, which is located on the cell surface, plays a more important role in resisting A β accumulation in infant and juvenile mice than does endothelin-converting enzyme (ECE) [37]. Compared with other A β -degrading enzymes, IDE not only affects the pathogenesis of AD, but also is associated with type 2 diabetes [38], and in-depth study may help understand the actual pathological changes and the prognosis of related senile diseases. The affinity of IDE for insulin is much higher than that for A β , but its rate of hydrolysis of insulin is very slow. Because IDE has a strong affinity for insulin, the scientific community has been paying more attention to its role in diabetes. It has begun to find that IDE actually has its own unique effect on AD in recent years. Meanwhile, the scientific community has found that patients with type 2 diabetes

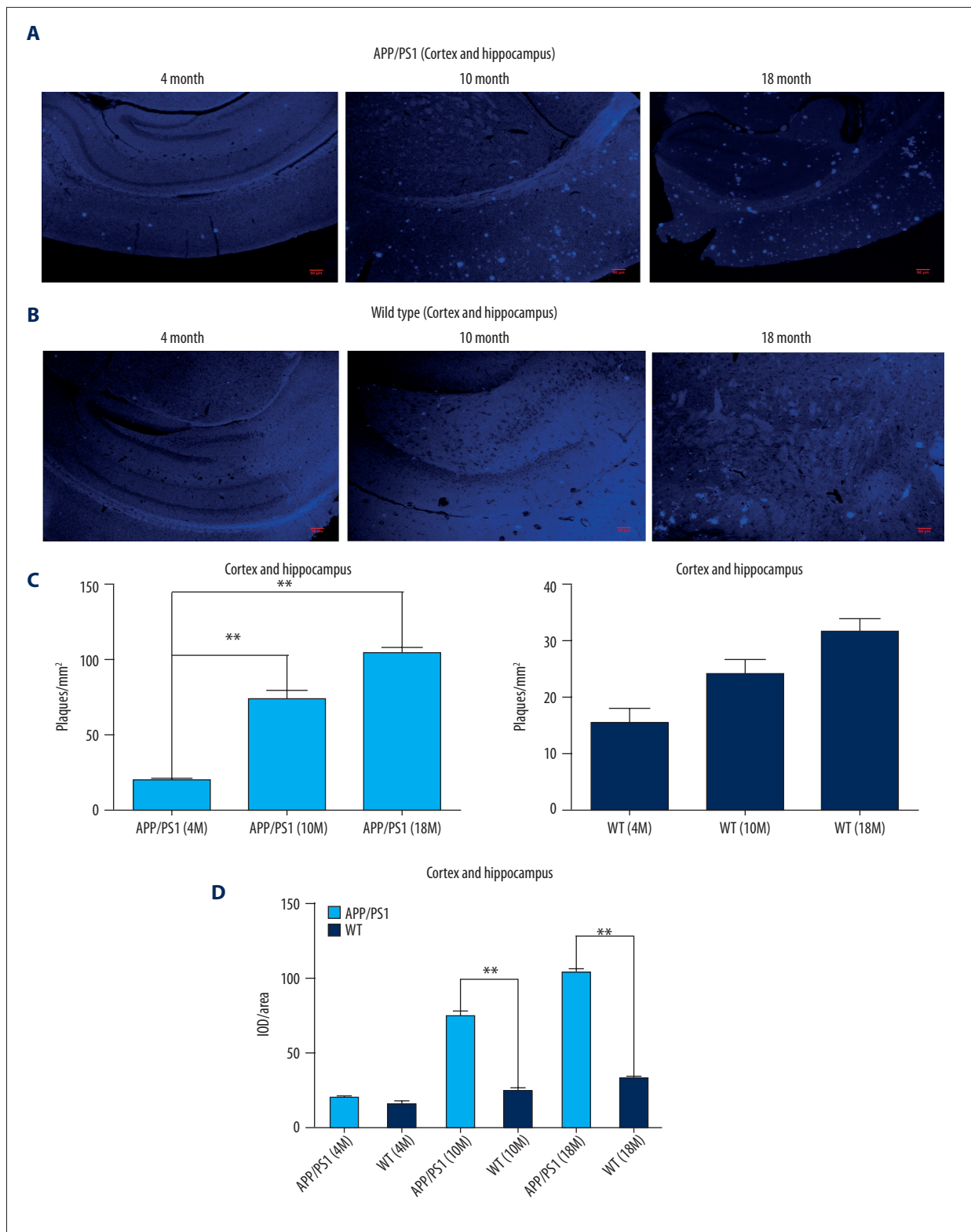


Figure 3. Distribution of increased Aβ plaques in brain regions (cortex and hippocampus) at different stages. **(A, B)** Representative epifluorescence microphotographs showing the β amyloid plaques density between APPswe/PS1dE9 and wild-type brains stained with thioflavin T, scale bars 50 μm (n=4 per group). **(C, D)** Quantification of the number of thioflavin-positive plaques in the cortex and hippocampus at different ages (* p<0.05, ** p<0.01).

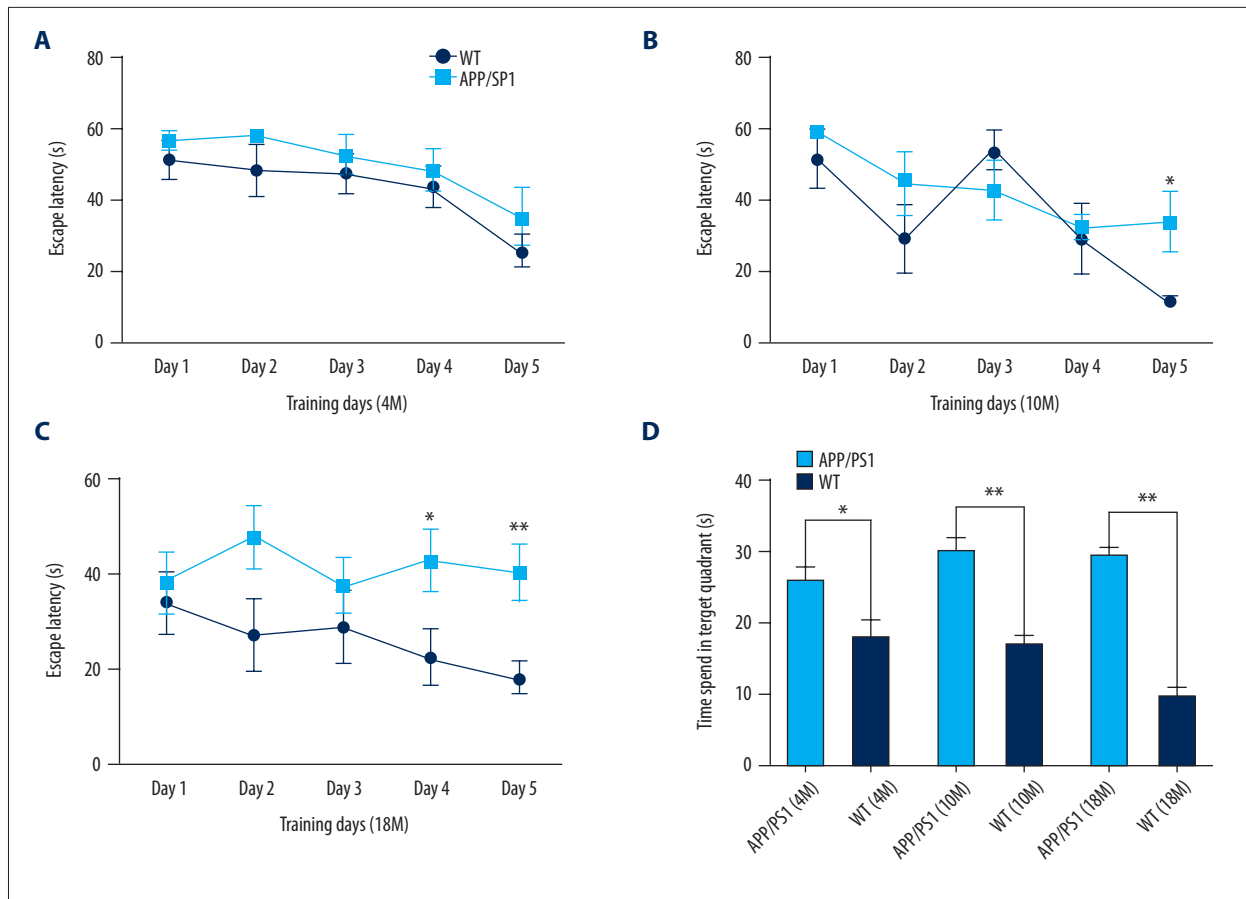


Figure 4. Spatial learning and memory declined in APPsw/PS1dE9 mice. (A) The escape latency showed spatial learning in 4-month-old mice. (B) Spatial learning ability of 10-month-old mice. (C) Spatial learning ability of 18-month-old mice. (D) Time spent in the target quadrant (the second quadrant) tested by probe trial (n=10 per group, * p<0.05, ** p<0.01).

have an accelerated decline in cognitive function, and the risk of dementia is increased by 1.3 to 1.4 times [39]. Because of this close relationship between diabetes and AD, IDE is also a substance that might play a role in both diseases. This protein could affect the pathogenic core factors (insulin and A β) in both diseases. So, the actual changes in the expression level of IDE might be more complex than the changes in other A β -degrading enzymes, but few studies have reported on this.

IDE, composed of 4 similar domains, is a zinc metallopeptidase that can degrade many physiological peptides. Many studies have reported that IDE is secreted in exosomes from astrocytes in the brain [38]. In this study, we demonstrated that the expression of IDE was dramatically higher with increased age in APPsw/PS1dE9 AD model mice (Figure 1). The same trend was also found in paraffin sections of the mouse brain tissue (Figure 2A, 2B). The AD susceptibility genes APP, PS-1, and PS-2 have all been found to increase A β production [40]. Our team used APPsw/PS1dE9 double-transgenic mice, which showed amyloid plaques formation at around 4 months of age [27]. Results obtained using the Western blot-extracted protein from

the hippocampus and cortex of model mice and those obtained through the immunohistochemical method that used paraffin sections of the mouse brain tissue showed that IDE is up-regulated at different stages of APPsw/PS1dE9 mice, depending on age (Figure 1A, 1B). A possible mechanism for this trend might be that neurotoxicity damage caused by A β causes cellular dysfunction or cell apoptosis in which a small percentage of astrocytes become lysed. This mechanism might increase the release of IDE induced by altering membrane integrity.

The expression of APPsw/PS1dE9 promotes the rapid accumulation of A β plaques in the brain of AD model mice. A β can form senile plaques outside the cell and produce strong cytotoxicity, leading to neuronal apoptosis in the cells. Owing to this cytotoxicity, the neurons in important brain regions associated with cognitive functions are gradually lost [41]. The data showed the APPsw/PS1dE9 mice displayed strongly increased numbers and area of A β plaques with age. Furthermore, there were more plaques deposited in the cortex and hippocampus, especially in 10-month-old and 18-month-old groups of APPsw/PS1dE9 mice (Figure 3A, 3C, 3D).

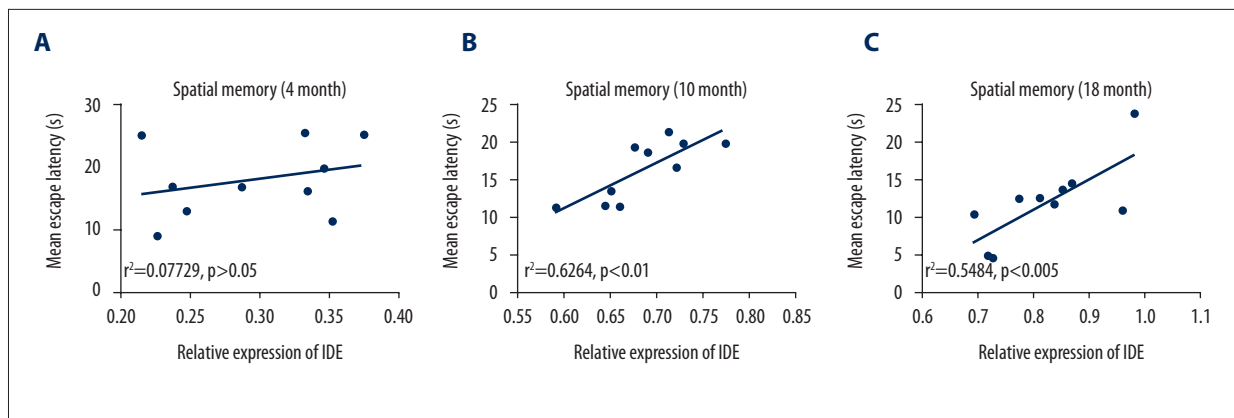


Figure 5. Relationship between the level of IDE and cognitive impairment in APPsw/PS1dE9 mice. **(A)** Correlation between the expression of IDE and spatial memory function was observed in the cortex and hippocampus of 4-month-old APPsw/PS1dE9 mice. ($r^2=0.07729$, $p>0.05$). **(B)** Correlation between the expression of IDE and spatial memory function of 10-month-old APPsw/PS1dE9 mice ($r^2=0.6264$, $p<0.01$). **(C)** Correlation between the expression of IDE and spatial memory function of 18-month-old APPsw/PS1dE9 mice ($r^2=0.5484$, $p<0.005$).

The cortex and hippocampus are considered to be important regions for AD pathological variation [42]. We found region- and age-related changes in the level of IDE in the cerebral cortex and hippocampus. The protein of IDE in the cerebral cortex and hippocampus was significantly increased in the APPsw/PS1dE9 mice older than 10 months of age (Figure 1A, 1B, 2A, 2B), which strongly matched the increase in A β plaques indicated by thioflavin T staining (Figure 3A, 3B).

Cognitive impairment is one of the main clinical manifestations of Alzheimer's disease and seriously decreases the quality of life of people with AD [42]. According to the Morris water maze test results, 10-month-old and 18-month-old APPsw/PS1dE9 mice showed obvious cognition deficits, revealing significantly reduced time spent in the target quadrant compared with control mice (Figure 4D). These results also matched the trend of A β plaques accumulation detailed above.

Results of the present study demonstrate that the level of IDE is associated with AD pathogenesis, especially with A β plaques, which were known to be one of the causes of cognitive decline [43]. Our evaluation of the relationships between cognitive decline and expression of IDE showed the expression of IDE is inversely correlated with cognitive function in 10-month-old (Figure 5B) and 18-month-old (Figure 5C) APPsw/PS1dE9 mice. A possible mechanism for this might be associated with increased IDE in the specific brain regions in which A β accumulated. On the one hand, the increased expression of IDE makes more A β be decomposed in part of cortex and hippocampus. This trend reduced the neurotoxicity effect induced by A β in these areas. More neurons survived owing to the elevated level of IDE. On the other hand, IDE, as the insulin-degrading

enzyme, reduces the regional brain insulin level. Insulin is the most important hormone that lowers the level of blood glucose. Because of the decreased level of insulin, the level of brain blood glucose increased. The elevated blood glucose could help more neurons survive A β -induced neurotoxicity.

Although the association of IDE and cognitive decline was shown in our study, our results do not prove a causal relationship. It should be noted that this study examined only APPsw/PS1dE9 AD model mice, and our results do not suggest that the same applies to clinical AD patients in the corresponding age stages. Further research is needed to elucidate the relationship between IDE and cognitive function in the AD pathological process. Our findings suggest that this protein might provide a reference value for earlier diagnosis of cognitive impairment and timely therapy to correct A β plaques accumulation.

Conclusions

These observations, together with those of alterations, suggest that IDE affected AD pathogenesis in an age-dependent manner. In summary, the expression of IDE is negatively associated with cognitive decline. The study of this protein may provide reference values for the further study of IDE in Alzheimer's disease.

Conflicts of interest

None.

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