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Abstract Transgenic mouse has shown great advantages in the study of Alzheimer's disease (AD) and drug screening as AD develops rapidly resent years, while more detail information of these transgenic mice and experience of application are needed. To obtain the basic background information of the B6C3-Tg (APPswe/PSEN1dE9) double-transgenic mouse, which was reported with early onset AD, three- to ten-month-old B6C3-Tg AD mice and normal C57BL/6 mice were selected randomly to test the ability of learning memory by Morris water maze, the brain acetylcholinesterase (AChE) activity by AChE kit, and beta amyloid protein level by immunohistochemistry staining. Compared with the control group, the escape latency time of B6C3-Tg AD mice at 9 and 10 months of age is significantly longer ($P<0.05$) in Morris maze test, and the activity of brain AChE is higher. β-Amyloid plaques were observed at 3 months of age and developed rapidly. Statistical analysis showed a positive correlation between the area of these plaques and the ages of B6C3-Tg AD mouse ($y=0.0355e^{0.5557x}$, $R=0.9557$). The model's behavior is conformed to simulate behaviors of human Alzheimer's disease at the early stage and may provide detail background information a new choice when transgenic mice are needed in the research of AD.

Z. Zhong · X. Tang (✉)
Southern Medical University, Guangzhou, Guangdong Province, China
e-mail: river-t@126.com

Z. Zhong · L. Yang · X. Wu · W. Huang · J. Yan · S. Liu · X. Sun · K. Liu · S. Kuang · X. Tang
Guangdong Medical Laboratory Animal Center, Foshan, Guangdong Province, China

H. Lin
The Chinese University of Hong Kong, Sha Tin, Hong Kong

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Introduction

Alzheimer's disease (AD) is a severe neurodegenerative disorder that mainly characterized by progressive memory loss that leads eventually to dementia. The neuropathology of AD is characterized mainly by extracellular senile plaques and neurofibrillary tangles. In 2006, the worldwide prevalence of AD was 26.6 million, and by 2050, the prevalence will quadruple by which time 1 in 85 persons worldwide will be living with the disease (Brookmeyer et al. 2007). Our health-care systems are facing an increased prevalence of AD and increasing costs, and AD has become a global burden of the world (Selkoe 2001; Chapman et al. 2001; Brookmeyer et al. 2007; Kovács 2009). However, almost all studies have not met expectations, and no new drug has been approved for the treatment of AD in the last decade (Schneider 2013), and all phase 3 drug development programs for disease-modifying agents have failed thus far (Cummings et al. 2013). It is an urgent and important topic to develop drugs for the prevention and treatment of Alzheimer's disease in pharmacology research (Hűll et al. 2006; Pillai and Cummings 2013).

Transgenic mouse has shown great advantages in the study of pathological alterations in AD (Tang and Eckenhoff 2012). However, the deficit of turnout in AD mice and the lack of detail background data limit the application of AD mice in science research. It should be of great help if better and more reliable animal models are constructed with detailed AD data. Several genes have been implicated in Alzheimer's disease in human beings, such as genes that encoding amyloid bA4 precursor protein (APP), presenilin 1(PSEN1), and presenilin 2 (PSEN2) (Hock et al. 2001; Tang and Eckenhoff 2012).

Accordingly, three main transgenic mouse models have been developed in the recent years with single (APP or tau), double (APP and PSEN1), and triple (APP, PSEN1, and tau) mutation. All these three kinds of transgenic mouse have been chosen in scientific research and drug screening. However, the timing of cognitive decline and the appearance of β-amyloid plaques differ from each other (Tang and Eckenhoff 2012).

Our study is to observe the dynamic change of β-amyloid plaques in B6C3-Tg AD mice brain with the growth of age and to analyze the correlation between the activity of acetylcholinesterase (AChE) and β-amyloid plaques so that researchers could choose the right age of AD mice and delivery time appropriately for medical research and drug screening when they know the material. The B6C3-Tg (APPswe/PSEN1dE9) AD mouse line are bred over four generation in Guangdong Medical Laboratory Animal Center (GDMLAC) which was provided by the Model Animal Research Center of Nanjing. The AD mouse is easy to identify and reproduce by cross-hybridizing male B6C3-Tg AD mice with female wild-type mice. So, our center could supply reliable and a large number of animal models for the AD researcher with detailed AD data.

Materials and Methods

Animals

Twenty-four male APPswe/PSEN1dE9 mice and 24 male C57BL/6 counterparts were provided and used at GDMLAC (certification number: 44007200000555). Both two kinds of animals were randomly divided into eight groups with three animals per group. All animals were provided with a standard diet and housed in an approved facility with climate control and a 10-h light/14-h dark cycle. The protocols of animal test were approved by the Institutional Animal Care and Use Committee of GDMLAC.

PCR Identification

Tail of each mouse was cut (about 5 mm) and digested using a lysis solution (20 mM Tris–HCl, 5 mM EDTA, 400 mM NaCl, 1%SDS, and protein K 5 mg/mL) at 55 °C for 12 h. DNA of each mouse was extracted by phenol-chloroform method. Polymerase chain reaction (PCR) was performed to detect APPswe gene of each mouse for 30 cycles. Primers for APPswe were 5′-GACTGACCACTCGACCAGGTTCTG-3′ and 5′-CTTGTAAGTTGGATTCTC ATATCCG-3′ (Zhang et al. 2009a, b).

Morris Water Maze Test

Morris water maze test was utilized to examine the behavior of mice. Each pair of groups (B6C3-Tg AD mice and normal C57BL/6) was tested one time at 3, 4, 5, 6, 7, 8, 9, 10 months age, respectively. The movement of each mouse was recorded by a video camera suspended above the center of the water maze. This camera was connected to a computer, and the movement of each mouse was analyzed using a video tracking system (Academia Sinaca, China).

Their performance was evaluated by testing the spatial and temporal navigation abilities of each mouse. Briefly, each mouse swam to find a hidden platform. This test was carried out in a circular pool that filled with warm water (powdered milk) with four quadrants virtually. Before the first training session in the water maze, all mice were allowed to swim freely in the maze to assess swimming abilities for 2 min, twice per day, and total 2 days. After pre-training, all mice underwent a spatial learning test with a hidden platform placed in the water maze for 5 days with 1 test per day. The platform was kept in the same place of the maze during this phase. In the test, each mouse was gently lowered into the water from each quadrant facing the wall of the maze and was allowed to swim for 90 s to find the hidden platform. Mouse that reached the platform within 90 s was allowed to rest on the platform for 10 s, while the mouse that failed to find the platform within 90 s was guided to the platform and also allowed to rest on it for 10 s. Latency time that took a mouse to reach and climb the platform was chosen and recorded to evaluate the performance of each mouse. Twenty-four hours after the last test, the platform was removed from the maze, and each mouse received a 90-s swim probe trail. Each mouse was lowered into the water from the quadrant that farthest away from the platform site facing the wall of the maze. The movement of each mouse was recorded by video camera. The spatial memory for the platform location during probe trails was evaluated by the dwelling time that each mouse crossed over the platform site the first time and the number of crossed over the platform site within 90 s. All these tests were performed in the barrier environment with stable temperature and humidity the same as housing condition so that we could obtain accurate information of these mice.

Examination of Brain AChE

Following completion of Morris water maze test, all animals were sacrificed by decapitation. The brain of each mouse was removed carefully and homogenized in sodium chloride (brain: sodium chloride = 3:7). The mixtures were centrifuged at 6,000 rpm for 20 min at 4 °C. The supernatants were collected, and the brain AChE activity was examined using an AChE kit (Jiancheng Co., Nanjing, China).

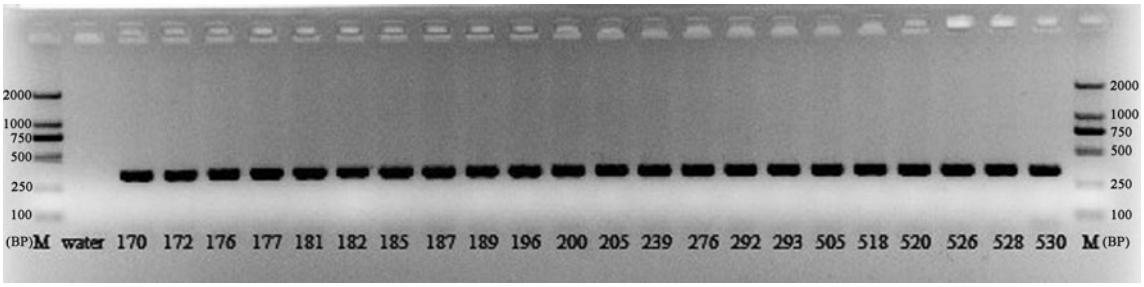

Fig. 1 PCR identification of newly born AD mice that was used in this study (N=22, two more mice were also detected and the same result was observed but not shown in Fig. 1)

Immunohistochemistry Staining of β-Amyloid Plaques

For quantitative image analysis of β-amyloid plaques, the left part of the brain of each mouse mentioned before was collected and cut into slices along the sagittal plane. Immunohistochemistry was performed on a serial of 4 μm paraffin sections ranging from the frontal to the occipital poles. Antibody (β-amyloid, no. 9888, cell signaling) against human APP was used to access plaque load, followed by washing with PBS containing 0.5 % Tween-20 and incubation with biotinylated secondary goat anti-rabbit antibody. After the incubation of antibodies and washing, the sections were treated with diaminobenzidine and hematoxylin. These sections were recorded using OLYMPUS BX41 microscope equipped with a CCD camera. HMIAS-2000 analysis system was used to examine the plaques of these sections.

Results

PCR Identification

As B6C3-Tg Alzheimer mice were constructed by cross-hybridizing the heterozygote with the wild-type mice, we need to take an examination to identify each AD mouse. Newly born mice were examined using PCR and selected according to the results of PCR in this study. As shown in Fig. 1, the AD mice was expressing the gene of human APP, showing that the heterozygotes inherited the AD-related genes from B6C3-Tg AD mice.

Spatial Learning Test

As shown in Table 1, the result of Morris water maze test shows that there is no significant difference of latency time

Table 1 Latency time of spatial learning test and the probe test of C57BL/6 and B6C3-Tg AD mice of different ages by using Morris water maze

Age	Group	N	Latency time (s)					Probe test	
			First day	Second day	Third day	Fourth day	Fifth day	First cross time (s)	Number
3 months	C57BL/c	3	59.8±36.2	69.0±19.6	54.0±18.1	41.4±41.9	43.7±17.2	19.4±21.3	2.0±0
	AD	3	78.7±15.8	70.5±19.1	53.2±32.0	74.7±5.6	61.9±6.8	78.6±19.8*	0.33±0.58*
4 months	C57BL/c	3	64.8±26.0	62.8±15.8	72.9±7.3	75.9±12.1	81.9±14.0	49.9±42.8	1.0±1.0
	AD	3	58.6±27.5	76.2±17.6	63.9±32.2	55.1±30.8	60.6±20.0	53.1±33.0	1.67±1.53
5 months	C57BL/c	3	90.0±0	78.8±9.6	79.8±6.3	71.1±5.7	79.2±18.2	47.7±38.8	0.67±0.58
	AD	3	86.2±6.6	69.1±22.5	76.4±15.2	79.0±10.1	87.6±1.9	90.0±0	0
6 months	C57BL/c	3	56.8±21.0	55.1±31.4	54.9±43.2	49.4±39.7	55.2±42.9	41.1±44.7	3.67±5.51
	AD	3	63.8±25.5	69.1±25.7	71.2±32.6	74.7±26.4	65.4±42.5	90.0±0	0
7 months	C57BL/c	3	80.7±11.7	76.0±23.6	69.0±36.3	63.5±45.7	66.4±40.8	61.8±48.9	1.0±1.7
	AD	3	64.3±33.6	89.8±10.7	80.9±8.6	87.2±4.8	78.5±19.8	63.6±45.7	0.33±0.58
8 months	C57BL/c	3	65.5±25.0	65.3±24.6	55.5±21.0	53.6±27.7	57.2±39.4	67.6±38.8	0.67±1.15
	AD	3	71.8±22.6	83.7±10.9	81.7±6.8	89.9±0.1	75.7±21.1	87.5±4.4	0.33±0.58
9 months	C57BL/c	3	76.7±13.7	53.6±21.5	54.9±24.6	41.0±17.9	47.2±17.6	38.6±45.4	2.0±1.7
	AD	3	52.2±14.0	56.9±12.9	74.4±15.3	82.0±12.1*	82.8±12.3*	90.0±0	0
10 months	C57BL/c	3	77.0±12.2	50.6±19.2	67.7±22.4	44.5±21.6	40.7±17.0	19.6±16.7	1.33±0.58
	AD	3	54.1±38.4	46.7±40.0	51.3±33.5	82.1±8.1*	75.4±12.2*	58.6±27.4	0.33±0.58

*P<0.05 compared with C57BL/c group

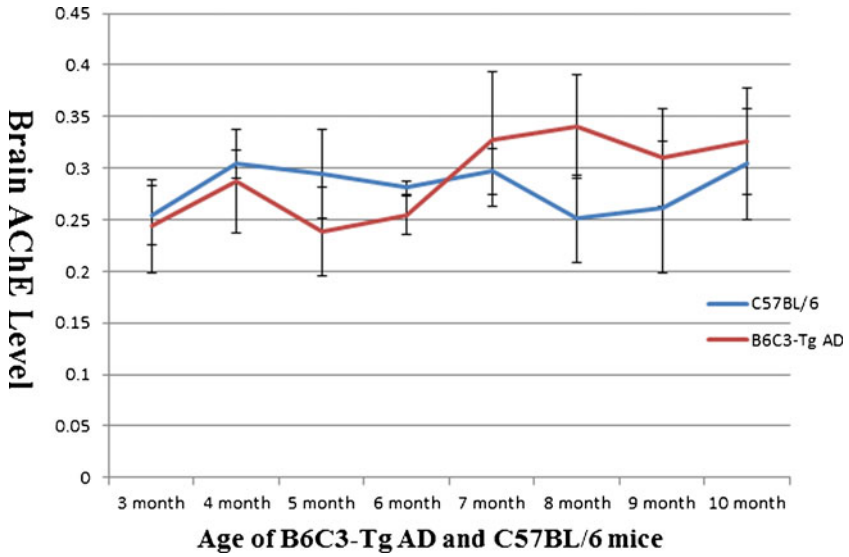

Fig. 2 Brain AChE activities of B6C3-Tg AD and the C57BL/6 mice at different ages

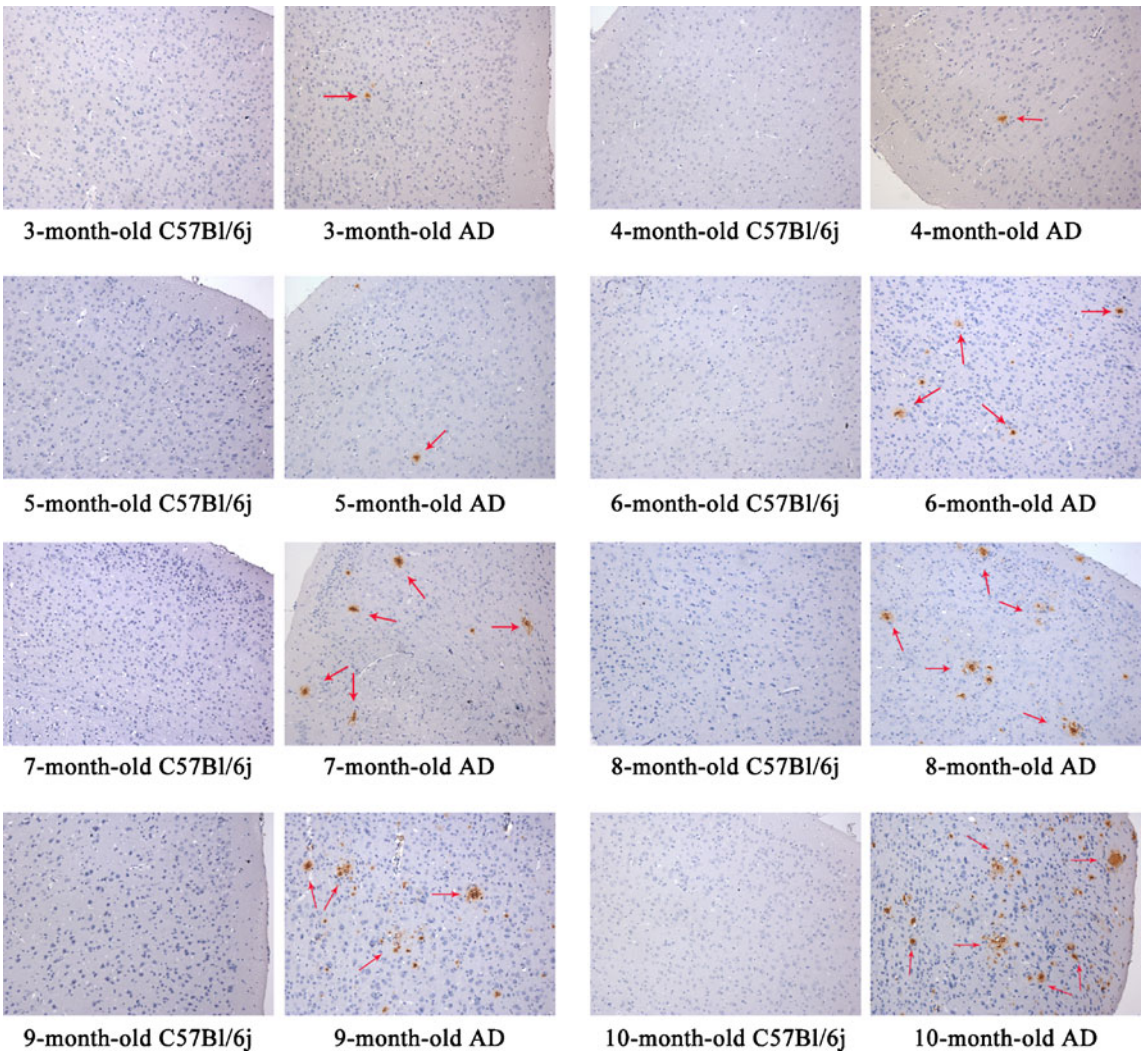

Fig. 3 The β-amyloid plaque in the cerebral cortex of C57BL/6 and AD mice at different ages. None of the β-amyloid plaque was observed in the brain of all C57BL/6 mice; in contrast, various β-amyloid plaques were observed in the brain of AD mice at all ages (*arrow*). The number and the area of the β-amyloid plaques increased with aging. At any age, the β-amyloid plaque was higher in the cerebral cortex than any region of the brain

between B6C3-Tg AD mouse and normal C57BL/6 mouse during the whole test from the third to eighth month ($P>0.05$) because the statistical number is small. However, B6C3-Tg AD mice (9- and 10-month-old) take more time to reach the platform in the fourth and fifth day of Morris water maze test ($P<0.05$), suggesting a loss of memory.

Probe Test

As shown in Table 1, B6C3-Tg AD mice spent more time to cross over the platform site at the first time, and the result of annulus-crossing index showed that B6C3-Tg AD mice crossed the platform site less than C57BL/6 group from 4- to 10-month-old.

Brain AChE Level

There was no significant difference of brain AChE enzyme activity between C57BL/6 mice at different ages and no trend was observed. In contrast, brain AChE enzyme activity of AD mice increased at 7 to 10 months. Meanwhile, the level of *AChE* in AD mice was lower than normal C57BL/6 mice from the third to seventh month and became higher from the eighth to tenth month though in a small range (Fig. 2).

Immunohistochemistry Staining of β-Amyloid

To evaluate the level of β-amyloid in the brain of B6C3-Tg AD mice and normal C57BL/6 mice, immunohistochemistry and quantitative analysis were performed. Judging from the result of immunohistochemistry, no β-amyloid plaque was found in the brain sections of C57BL/6 mice. In contrast, β-amyloid plaques were found in the brain of each B6C3-Tg AD mice from 3- to 10-month-old, and the quantitative of these β-amyloid plaques showed an increase as these B6C3-Tg AD mice grow older (Fig. 3). Areas of these plaques were calculated (Fig. 4), and regression analysis was performed to detect the linear dependency of β-amyloid and age of B6C3-Tg AD mouse ($y=0.0.0355e^{0.5557}$, $R^2=0.9557$).

Discussion

Transgenic AD mouse has been developed in recent years and can be used as a replacement of traditional AD animal models for medical research and drug screening. Compared with traditional AD animal models, transgenic AD mouse is not only much more accurate, reliable, and economic but also can shorten the screening time. However, the deficiency of productivity and lack of dynamic of AD mouse limit the researcher to use transgenic AD mouse in study. In our reproduction system, we reproduce AD mouse by cross-hybridizing male B6C3-Tg AD mice with female wild-type mice and identify the gene of human APP gene by PCR. Thus, we can provide a large number of reliable AD mice for scientific research and drug screening.

Until now, definitive diagnosis of AD mouse required postmortem examination of brain tissue, which makes the diagnosis more difficult. Thus, the background data of the learning ability and memory of AD mouse, along with the

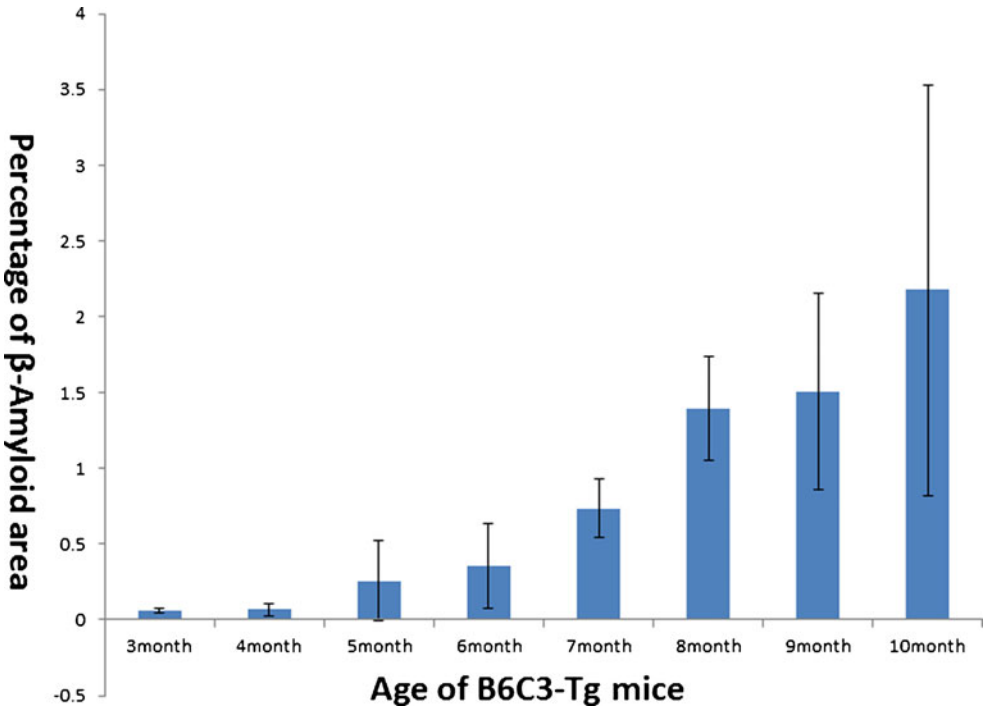

Fig. 4 Percentage (%) of β-amyloid area in the brain of AD mice at different ages. The area of β-amyloid plaques increased with aging

level of brain AChE and the dynamic of β-amyloid, is important for user in scientific research. In this study, three mice of each group were selected randomly considering the 3R principles of animal experiments and small individual variation of transgenic AD and C57BL/6 mice. Morris water maze test was used to examine the behavioral changes of AD mouse. The result of Morris water maze test shows that the latency time of B6C3-Tg AD mice is longer than normal C57BL/6 mice during the whole test from the sixth to eighth month ($P>0.05$), especially the B6C3-Tg AD mice (9- and 10-month-old) in the fourth and fifth day of Morris water maze test ($P<0.05$), and the result of annulus-crossing index showed that B6C3-Tg AD mice crossed the platform site less than C57BL/6 group from 4- to 10-month-old. These results suggest a loss of memory.

One reason that might cause AD is the damage of cholinergic transmission of the cerebral cortex. This damage often leads to the deficiency of acetylcholine (Ach) and then cause decline of memory. Thus, to inhibit AChE activity, delay the rate of hydrolysis, or raise the activity of Ach should help in treating and preventing AD (Lane et al. 2006; Anand and Singh 2013). Examination was performed to detect the brain AChE level of both AD mice and C57BL/6 mice at different ages. Judging from the results, we can figure out that the brain AChE activities of AD mice at the age of 3 to 6 months were lower than the normal C56BL/6 mice, while higher at 7 to 10 months of age. The results basically coincide with the results of water maze test.

AD is an age-related disease which usually happens in the old ages of human beings. Another factor that might cause AD is the formation of β-amyloid plaques. It is reported that a transgenic mouse line with mutation of APPswe and PSEN1 develops plaque around 6–7 months and cognitive deficits at 7 months (Jankowsky et al. 2001). Another transgenic mice line with single mutation of APP produces amyloid plaque at 6 months of age (Sturchler-Pierrat et al. 1997). In our study, β-amyloid plaques were found in the brain of AD mice at 3 months of age. The number and the area of these plaques increased rapidly as AD mice growing up by judging from the immunohistochemistry staining and the regression analysis. All these results suggest that B6C3-Tg AD mouse model is age dependent. It is reported that the activity of AChE in AD patients are totally lower than normal people but higher in and around β-amyloid plaques (Ulrich et al. 1990) and high enzyme activity of AChE promotes β-amyloid plaques in turn (Rees et al. 2003). In this study, we observed that enzyme activity of AD mice brain AChE increased from 7 to 10 months of age when β-amyloid plaques developed rapidly. This consistency suggests that the enzyme activity of AChE and the development of β-amyloid plaques are mutually improved.

As AD, an early start of treatment enhances the chance of success (Salomone et al. 2012). Thus, an early onset transgenic AD mouse model seems to be more effective and reliable for the research of AD and drug screening, especially for early onset form of Alzheimer's disease, and the detailed information provided in this study should help in the research of AD mouse, especially when AD mouse is ready for drug screening.

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