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RESEARCH ARTICLE

Aged wild-type littermates and APPswe+PS1/△E9 mice present similar deficits in associative learning and spatial memory independent of amyloid load

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Abstract

APPswe+PS1/△E9 transgenic (Tg) mice with Aβ plaque formation in neocortex and hippocampus were evaluated in tests measuring exploratory activity, anxiety, and memory ability using open field test (OFT), Y-maze, contextual fear conditioning (CFC), and Morris water maze (MWM). Wild type (WT) and Tg mice over eight months old showed same locomotion activity and anxiety level in novel stimulation, open field, and Y-maze contexts. In other experiments that measured associative memory and spatial memory in Tg mice and their littermates, the subjects also presented similar deficiencies in memory acquisition. These two aged groups showed abnormal freezing level variance especially in CFC test. In comparison to that in non-Tg 8-week-old mice group, the acquisition of spatial memory in MWM task was impaired in aged WT and bigenic Tg mice. Taken together, aged WT littermates and Tg mice present similar deficits in associative learning and spatial memory independent of amyloid plaques.

Keywords APPswe+PS1/△E9 transgenic mice; Alzheimer's disease; Aβ plaque; Learning; Spatial memory

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Introduction

Alzheimer's disease (AD) is the most common form of senile dementia characterized by the presence of amyloid plaques as well as extensive loss of neurons, formation of neurofibrillary tangles, activation of microglial cells, and deficits in the cholinergic system (Whitehouse et al., 1981; Terry and Katzman, 1983; Lee et al., 2003). Impaired learning and memory abilities are the cognitive hallmarks of AD. It is known that in early onset familiar AD in humans, mutations in the genes encoding the amyloid precursor protein (APP) and presenilins 1 and 2 (PS1 and PS2) are linked to development of the disease (Levy-Lahad et al., 1995; Rogaev et al., 1995). Recently, several AD model Tg mice have been developed (Duff et al., 1996; Hsiao et al., 1996). APPswe+PS1/△E9 is a double Tg mouse that can express a chimeric mouse/human APP (Mo/HuAPP695$_{swe}$) and a mutant human PS1 (PS1/△E9), which are both directed to CNS neurons. This double Tg mouse shows a rapid progressive development of plaques within the cortex and hippocampus at as early as 6 months of age (Jankowsky et al., 2004). The availability of Tg mice that mimic human AD has generated interest to perform learning tasks in AD model mouse. However, there are still unanswered questions regarding whether the animal model of AD exhibit learning and memory deficits as a consequence of amyloid β deposits in hippocampus and neocortical structures (Holcomb et al., 1999; Kelly et al., 2003; Jankowsky et al., 2004). Thus, we assessed learning capabilities of Tg mice with CFC and MWM test. We also evaluated their explorative behaviors using OFT and Y-maze.

Material and Method

Animal

Eight week old C57BL/6 male mice were used in MWM task. These animals were purchased from the Orient Bio (Seongnam city, Gyeonggi-do, Korea).

Heterozygous female APPswe+PS1/△E9 (n = 12) and littermate controls (n = 9), male APPswe+PS1/△E9 (n = 12) and littermate controls (n = 14) were used in our behavior experiments. Heterozygous APPswe+PS1/△E9 male mice and WT female littermates which are created on a strain of B6C3 were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). APPswe+PS1/△E9 Tg mice were produced by mating Tg male with WT female littermate, resulting in offspring with APPswe+PS1/△E9 (heterozygous). Their genotype was confirmed by PCR analysis of tail biopsies. The Tg mice and littermate controls were kept inside group cages for over 8 months.

All animals were housed with woodchip bedding under a 12:12 light cycle with food and water provided *ad libitum*. Behavioral experiments were conducted during the light phases. All works were conducted according to the policy and regulation for the care and use of laboratory animals approved by Institutional Animal Care and Use committee in Seoul National University.

Congo-red staining

To confirm the amyloid plaque in brain slices, we used congo-red staining (Sigma-Aldrich, HT60). We performed the assay according to the manufacturer's protocol. To be brief, the brain sections were deparaffinized and hydrated in deionized water. The samples were stained in Mayer's Hematoxylin Solution for 10 minutes. After rinsing in tap water for 5 min, the brain sections were placed in Alkaline Sodium Chloride solution for 20 min. After that, the samples were stained in Alkaline Congo Red solution for 20 min and rinsed in 3 changes of absolute ethanol. After they were cleared in xylene and mount, we observed the brain sections using microscopy.

Biotinylation assay

Biotinylation assay for examining the surface AMPAR was performed essentially as described previously (Kim et al., 2007). Briefly, 400 μm hippocampal slices were stabilized into oxygenated ACSF (124 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM glucose) for 1 hr at room temperature. And then, slicers were than incubated with ACSF containing 1 mg/ml Sulfo-NHS-SS-biotin (Pierce) for 45 min at 4℃. After a brief wash with cold ACSF, slices were washed again with cold ACSF containing 100 mM glycin two times for 20 min. Before lysis, slices were rinsed briefly with cold TBS twice. To purify the crude synaptosomal P2 fraction, hippocampal slices were homogenated using TEVP buffer (320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM Tris pH 7.4). After centrifuge at 1,000 g for 10 min, S1 fraction was centrifuged for purification of P2 at 10,000 g for 15 min at 4℃; and then P2 fraction was solubilized into RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.5 % sodium dioxycholate, 0.5 % Triton X-100, 0.1 % SDS) containing protease inhibitor cocktail. Bradford assay was performed for protein quantification. Equal amounts of P2 fraction were diluted into detergent free buffer (50 mM Tris pH 7.6, 150 mM NaCl) and then incubated with 50% Neutravidin agarose (Pierce) at 4℃ overnight. After washing, bound proteins were eluted using a SDS sample buffer. Isolated biotinylated proteins were analyzed by an immunoblot using anti-GluR1 (1:2,000, Santacruz), anti-GluR2 (1:1,000, Abcam), actin (1:5,000, Sigma). The density of immunoblot band was measured using the Image J program.

Behavioral procedure

Female APPswe+PS1/△E9 mice: After adaptation to handling for 5 days, the behavioral tests were conducted over a 24-day period. OFT was performed first, followed by Y-maze (day 2), handling (day 3−4), CFC (day 5−7), rest (day 8−10), handling (day 11−13), and MWM (day 14−24).

Male APPswe+PS1/△E9 mice: After adaptation to handling for 4 days, only CFC tests were performed on the male WT and Tg mice. For a control experiment, they were all injected with phosphate buffered saline with glycerol as vehicle, 24 hours before conditioning.

Open field test: Locomotion activity of the mice was tested using an open-field made of white acrylic with 40 × 40 cm surface area and 40 cm high walls. Their activity in the central (with in 10 cm^2 or 20 cm^2) and peripheral zones was recorded by an overhead video camera and analyzed by video-track software (Ethovision Pro 3.1, Noldus Co.). The mice were placed at the center of the open field context for a single 10 min trial.

Y-maze: Each mouse was placed at the center and allowed to move freely through the Y-maze during an 80 min session. The maze was made of transparent acrylic with black paper attachment. Each arm was 30 cm long, 15 cm high, 6 cm wide and converged at an equal angle. The series of arm entries was recorded visually. An alternation was defined as entries

into all three arms on consecutive occasions. The maximum number of alternations was therefore the total number of arm entries minus two and the percentage of alternation was calculated as (actual number of alternations/maximum number of alternations) × 100. For example, if the arms were called A, B, C and the mouse performed ABCACBACCAB, the number of arm entries would be 11, and the successive alternations would be: ABC, BCA, ACB, CBA, BAC, CAB. Therefore, the percent alternation would be [6/(11−2)] × 100 = 66.7.

Contextual fear conditioning: Prior to CFC, mice were handled in the holding room for two consecutive days for adaptation. On the next day, the mice were placed in the CFC chamber (Coulbourn, H10-24T) for 5 min in order to habituate them to the procedures used during training and testing. One day after the completion of handling and habituation, mice were trained. During training mice were placed in the conditioning chamber for 5 min. After 2 min they were presented with 3 unsigned foot shocks (2-sec duration, 0.5 mA, 1 min apart). The mice were then returned to the conditioning chamber for fear memory testing 24 hr after conditioning (retrieval). The freezing behavior was recorded using video-based FreezeFrame fear-conditioning system and scored by Actimetrics Software.

Morris water maze: A circular water maze tank was used for assessing spatial memory. The pool, which was 140 cm in diameter, was filled to a depth of 30 cm with water made opaque by the addition of white non-toxic paint. The water temperature was maintained at 24℃. A circular escape platform (10 cm in diameter) was submerged below the water surface and located in one of the quadrants. The pool was surrounded by dark red curtains that had distinct cues hung on them. Each animal underwent 10 days of acquisition blocks. On each of these days, the mice underwent four trials, during which they were required to locate a hidden platform randomly placed in one quadrant of the pool. The animals began each trial in a different cardinal position of the maze and used the constant spatial cues to locate the platform. A maximum latency of 60 sec was allowed to locate the platform in each trial, and there was a 20 min rest period between trials. Mice unable to locate the platform in the allotted time were manually guided to it and placed on the platform. All mice were allowed to remain on the platform for 20 sec at the conclusion of each trial. During the acquisition trials, the daily escape latency for each mouse was calculated as the average of the four trials. On day 11, the hidden platform was removed, and each mouse was subjected to a single (60 sec) probe trial. Occupancy was assessed using a video analysis system (Ethovision 3.1 Pro, Noldus Co.).

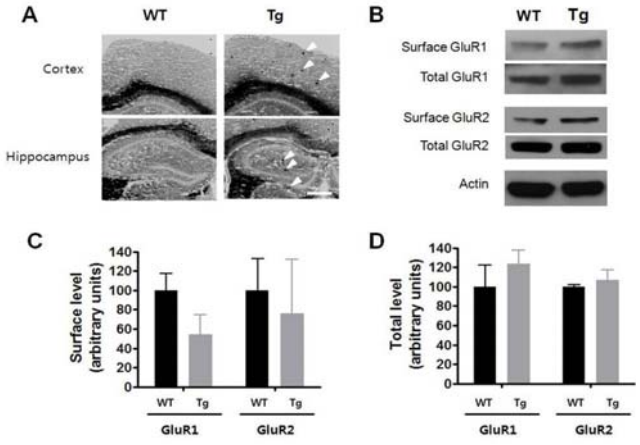

Figure 1. Biochemical characterization of Tg mouse. (A) Amyloid burden was measured using congo-red staining method. Brain section from Tg mouse expressed a lot of amyloid plaques (Arrow-head) in the cortex and hippocampus. Unlike the hetero, there was no stained amyloid plaque in WT brain section. Scale bar, 500 μm. (B) Surface GluR1 and GluR2 levels were similar in WT and Tg mice. No difference was observed in total levels of GluR1 and GluR2 as well. (C) Quantification of surface level of GluR1 (p = 0.19; unpaired t-test; n = 3) and GluR2 (p = 0.73; unpaired t-test; n = 3). (D) Total level of GluR1 and GluR2 (p > 0.05; unpaired t-test; n = 3).

Results

Biochemical characterization of APPswe+PS1/△E9 mouse

Congo-red staining of brain sections verified that the cortex and hippocampus of only Tg mice contained amyloid plaques (Fig. 1A), as shown in other studies using amyloid antibody (Jankowsky et al., 2005) or thioflavin-S (Roberson et al., 2007). In addition, we examined the surface α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) level in AD mice since it was reported that decreased level of AMPAR is presumably one cause of cognitive problem in AD mice (Chang et al., 2006). As shown in Figure 1B-D, the groups did not exhibit a significant difference. The hippocampus of AD mice showed a tendency to decrease in the surface GluR1 level compared to age-matched wildtype (p = 0.19; unpaired t-test). However, the levels of total GluR1 were not different between groups (p = 0.43; unpaired t-test). Moreover, we observed the same pattern in GluR2 expression in the hippocampus of AD mice (Surface GluR2 p = 0.73, Total GluR2 p = 0.60; unpaired t-test).

Wild type and APPswe+PS1/△E9 mice showed same tendencies in open field test

Anxiety levels and exploratory behavior can be measured us-

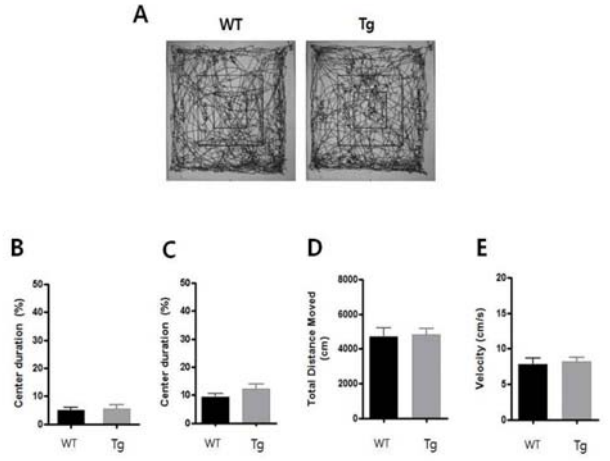

Figure 2. No difference observed in OFT between WT (n = 9) and Tg (n = 12) mice. (A) Representative path tracings (small square: 10 cm boundary, large square: 20 cm boundary). (B-C) Percent of time in center area. (B) in 10 cm^2 (C) in 20 cm^2. (D) Total movement (cm). (E) Mean velocity (cm/s).

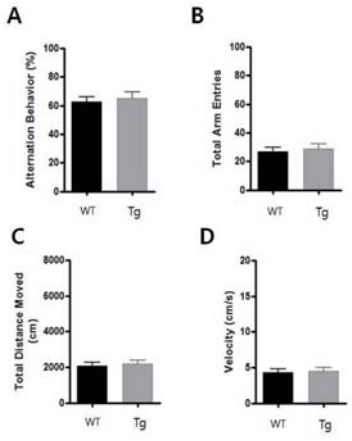

Figure 3. Similar locomotion activity in Y-maze context. (A) Spontaneous alternation values of WT (n = 9) and Tg (n = 12). Both groups exhibited approximately 60% of successful alternation. Other locomotion activities, total arm entries (B), total distance movement (C), and velocity (D) were same in Y-maze context as well.

ing OFT apparatus (Belzung and Griebel, 2001; Prut and Belzung, 2003). Tg mice had normal activity tendencies in the OFT (Fig. 2). The center/total time percentages were approximately equal in both 10 cm^2 (Fig. 2B) and 20 cm^2 sections (Fig. 2C) ($p>0.05$; unpaired *t*-test), although AD patients exhibited abnormal degree of anxiety (Hollingworth et al., 2006). In addition the overall movements and mean velocity of Tg mice did not differ from those of controls (Fig. 2D and 2E) ($p>0.05$; unpaired *t*-test). These results suggest that both WT and Tg mice show similar anxiety levels and locomotion activity in OFT.

No difference between APPswe+PS1/△E9 mice and wild type littermates in Y-maze test

Spatial working memory and locomotion activities of Tg mice were assessed using Y-maze test (Bertholet and Crusio, 1991; King and Arendash, 2002; Savonenko et al., 2003). Spontaneous alternation, the tendency of mice to switch arm choices on successive trials, was evaluated. The Tg mice performed the entire task as well as WT control in Y-maze (Fig. 3). The mean spontaneous alternation rate of the control group in Y-maze was significantly above the chance level. The alternation behavior of the Tg mice group was at the same level as that of WTs (Fig. 3A) ($p>0.05$; unpaired *t*-test). No difference in locomotion was observed between the groups in terms of the number of arm entries, total distance moved, and velocity during the 8 minute sessions (Fig. 3B-D) ($p>0.05$; unpaired *t*-test). These data suggest that both WT and Tg mice show same explorative behaviors in Y-maze context.

Wild type and APPswe+PS1/△E9 mice showed same associative learning capacity in contextual fear conditioning test

In the third series of experiments, we compared the learning capabilities of old WT and Tg mice using CFC paradigm. As illustrated in Figure 4A, the mean values of freezing levels were similar between the two groups ($p>0.05$; unpaired *t*-test). Interestingly, the variation in freezing level within each group was much greater than other studies using Tg mice have shown (Saura et al., 2005; Gruart et al., 2008). We also observed same freezing variation in male Tg mice (Fig. 4B). Nevertheless, the mean values of freezing levels were not different in WT and Tg groups ($p>0.05$; unpaired *t*-test).

Aged wild–type littermates and APPswe+PS1/△E9 mice present similar deficits in spatial memory task in Morris water maze

Spatial learning capacity was analyzed in the MWM with fixed platform position across 10 days of training. As shown in Figure 5A, the amount of time that aged Tg mice spent to find the platform did not differ from that spent by the WT littermates ($p>0.05$; unpaired *t*-test). However, young non-Tg mice group (B6) swam for a shorter amount of time to reach the hidden platform than their aged counterparts on day3 trial block ($*p<0.05$; unpaired *t*-test).

In a probe trial on day 11 in which the platform was removed, the mice were given 1 minute to explore the pool (Fig. 5B). Aged WT and Tg mice had no apparent spatial memory of the platform location. The amount of time spent in non-target quadrants and the target quadrant did not vary in these groups. In comparison to aged WT and Tg mice, eight-week-

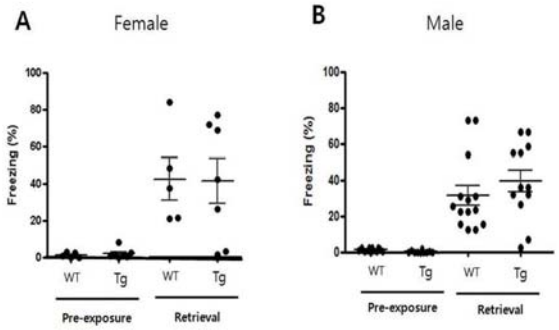

Figure 4. Same degree of defect in CFC task in WT and Tg. (A) WT (n = 5) and Tg (n = 7) female mice of more than eight months of age were tested in three-shock CFC task. Average freezing levels were not different in the two groups. Both groups also showed great variation in freezing levels. (B) Six to ten month-old male mice showed abnormal variation in freezing behavior as well as same mean value of freezing levels in the WT (n = 14) and Tg (n = 12) groups.

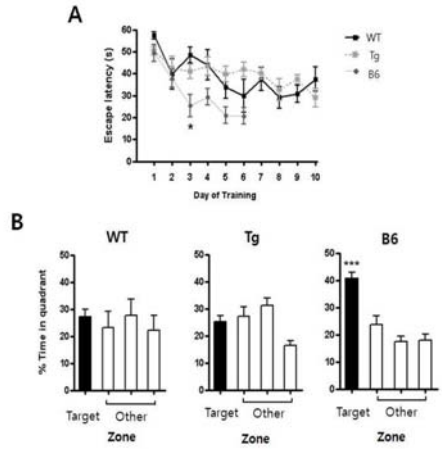

Figure 5. Deficits in spatial learning and memory in MWM task. (A) Time taken to find the platform is displayed as the averages of daily block of 4 platform trials. Note that old WT and Tg mice were less efficient in finding the platform than young, non-Tg control (B6) (*$p < 0.05$; unpaired *t*-test; n = 5−8 for each group). (B) In the probe test on day 11, only young mice group (B6) spent more time searching the target zone (black) compared to the time they spent in other (white) zones [***$p < 0.0001$; one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison test] whereas aged WT and Tg mice searched non-selectively in the pool region (p>0.05; one-way ANOVA and Newman-keuls multiple comparison test).

old B6 group spent more time in the trained quadrant when the platform was removed [***$p < 0.0001$; one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison test]. These data indicate that aged WT and Tg mice present similar deficits in spatial memory.

Discussion

Bigenic Tg mice were compared to the control in terms of exploratory activity using OFT and Y-maze. Curiosity, which conflicts with their fear of the unknown on one hand, motivates mice to explore novel stimuli such as the OFT context or Y-maze context on the other hand. In OFT, mice generally move along the walls as a mechanism for protection against possible predators and for easy escape. Using this instinct of mice, we can measure their anxiety level and locomotion activity (Belzung and Griebel, 2001; Prut and Belzung, 2003). In some studies, WT and Tg AD mouse models showed equal ratio of periphery to total time in OFT, though Tg mice spent more time in the open arms and had higher open/total duration ratio than controls in the elevated plus maze test (Lalonde et al., 2003; Lalonde et al., 2004). In accordance with this, we could not detect any difference between WT and Tg mice when they were put in the OFT context.

In spontaneous alternation test, mice generally prefer the novel one to the familiar maze arm to enhance their chances of finding food, water, and mates (Sarter et al., 1988). Conversely, choosing the same arm repeatedly might increase their chance of being captured by predators. In our study, Tg mice showed normal rate of alternation behavior in Y-maze. This result is in accordance with what was found in APP$_{K670N,M6711}$+PS1 Tg mice (Arendash et al., 2001) or APPswe mice (Savonenko et al., 2003). However, other studies have reported altered alternation rate in mutant APP or APP+PS1 Tg mice tested between 3 and 9 months of age (Hsiao et al., 1996; Holcomb et al., 1998; Holcomb et al., 1999). Total numbers of arm entries of WT and AD Tg mice were different in many other studies as well (Arendash et al., 2001; Roberson et al., 2007). This inconsistency could be a reflection of methodological differences or the inherent sensitivity of this task.

In the third experiment, we performed CFC in which mice form an association between a distinctive place (context) and an aversive event (typically the delivery of shock). When mice return to the conditioned context, presence of contextual memory is inferred from an increase in freezing behavior (Kim and Fanselow, 1992; Wang et al., 2009). In our CFC experiment, subjects showed abnormal variation of 1% to 85% in freezing behavior. We also could not observe any difference in mean values of freezing behavior in the two groups. This might be a result of the difference in methods that were used. Other studies using two 1.5 mA-shocks (Jacobsen et al., 2006) or one 1 mA-shock (Saura et al., 2005) showed normal freezing variance as well as difference between WT and Tg groups. However, we applied three 0.5 mA-shocks to mice. These rela-

tively mild shocks might account for the variation in freezing behavior with no difference between groups. In addition to shock intensity, old age of over eight months might be another reason for the great variation in freezing levels (Rapp and Amaral, 1992). Moreover, our data confirm the phenomenon in which individual differences are observed in the cognitive decline with dementia of Alzheimer type (Haxby et al., 1992).

MWM was designed as a device to investigate spatial learning and memory (Morris, 1984). MWM performance can be influenced by many factors such as the apparatus or training procedure (Morris, 1984; Brandeis et al., 1989; Wenk, 2004) as well as by the characteristics of the experimental animals–sex, species/strain, age (Brandeis et al., 1989), exposure to stress or infection (Grauer and Kapon, 1993; Holscher, 1999), etc. In our study, we could distinguish the spatial memory ability of aged mice from that of young mice. This result was in accordance with the display of age-related decline in spatial learning in control and APP Tg mice (Saura et al., 2005). However, we could not observe any significant difference between WT and Tg mice, though there have been many studies confirming spatial memory impairment of AD model mice through water maze experiment (Jankowsky et al., 2005; Roberson et al., 2007; Liu et al., 2008). These incompatible results might be due to several factors. As mentioned above, MWM performance can be influenced by many elements. Therefore, different experimental environment and methodological changes could lead to inconsistent results.

Taken together, we could not observe any apparent memory impairment related to the presence of amyloid β deposits. However, the amounts of amyloid plaques were not different with other study (Nilsson et al., 2001). These indicate that amyloid plaques alone might be not sufficient to cause severe associative and spatial memory impairment in Tg mice. It appears that factors other than plaque deposits are involved in the memory deficits observed in AD model mice. It has been proposed that the reduced mRNA expression of several genes associated with memory consolidation is directly related to memory deficits in double Tg (APP+PS1) mice even before the degeneration of synapse and neurons (Dickey et al., 2003). In addition, choline acetyltransferase fibers are more closely related to cognitive deficits than amyloid plaque load (Buttini et al., 2002). Changes in cholinergic terminals in the cerebral cortex and hippocampus were observed in AD model mouse (Wong et al., 1999).

In addition, it is possible that reorganization of brain pathways and recruitment of atypical brain pathways can overcome the amyloid plaque deposition (Buckner, 2004; Gilpin et al., 2008). Although there were amyloid plaques in hippocampus and cortex of Tg mice, other brain regions and pathways could compensate the damaged region caused by amyloid plaque. In conclusion, our study suggests that there can be a lot of complicated processes other than amyloid plaque deposition that affect AD patients and Tg AD mouse model.

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