BACE1 in the retina: a sensitive biomarker for monitoring early pathological changes in Alzheimer’s disease

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Graphical Abstract

Abnormal retinal expression of β-site APP-cleaving enzyme 1 is an early pathological feature in APP/PS-1 transgenic mice

Abstract

Because of a lack of sensitive biomarkers, the diagnosis of Alzheimer’s disease (AD) cannot be made prior to symptom manifestation. Therefore, it is crucial to identify novel biomarkers for the presymptomatic diagnosis of AD. While brain lesions are a major feature of AD, retinal pathological changes also occur in patients. In this study, we investigated the temporal changes in β-site APP-cleaving enzyme 1 (BACE1) expression in the retina and brain to determine whether it could serve as a suitable biomarker for early monitoring of AD. APP/PS-1 transgenic mice, 3, 6 and 8 months of age, were used as an experimental group, and age-matched C57/BL6 wild-type mice served as the control group. In the Morris water maze test, there were no significant differences in escape latency or in the number of crossings in the target area among mice of different ages. Compared with wild-type mice, no changes in learning or memory abilities were detected in transgenic mice at 3 months of age. However, compared with wild-type mice, the escape latency was significantly increased in transgenic mice at 6 months, starting on day 3, and at 8 months, starting on day 2, during Morris water maze training. In addition, the number of crossings of the target area was significantly decreased in transgenic mice. The learning and memory abilities of transgenic mice were further worsened at 8 months of age. Immunohistochemical staining revealed no BACE1 plaques in wild-type mice at 3, 6 or 8 months or in transgenic mice at 3 months, but they were clearly found in the entorhinal cortex, hippocampus and prefrontal cortex of transgenic mice at 6 and 8 months. BACE1 expression was not detected in the retina of wild-type mice at 3 months, but weak BACE1 expression was detected in the ganglion cell layer, inner plexiform layer and outer plexiform layer at 6 and 8 months. In transgenic mice, BACE1 expression in the ganglion cell layer was increased at 3 months, and BACE1 expression in the ganglion cell layer, inner plexiform layer and outer plexiform layer was significantly increased at 6 and 8 months, compared with age-matched wild-type mice. Taken together, these results indicate that changes in BACE1 expression appear earlier in the retina than in the brain and precede behavioral deficits. Our findings suggest that abnormal expression of BACE1 in the retina is an early pathological change in APP/PS-1 transgenic mice, and that BACE1 might have potential as a biomarker for the early diagnosis of AD in humans.

Key Words: nerve regeneration; neurodegenerative disease; Alzheimer’s disease; retina; amyloid-β; β-site amyloid precursor protein cleaving enzyme 1; APP/PS-1 transgenic mouse; neural regeneration
Introduction
Alzheimer’s disease (AD) is the most common cause of dementia in the elderly (Paduraru et al., 2012). Patients afflicted with AD suffer from progressive decline of memory and cognition, as well as changes in behavior and personality (Graff et al., 2012). Neuropathologically, the disease is characterized by the presence of extracellular amyloid plaques composed of amyloid-β (Aβ42) and intracellular neurofibrillary tangles (Paris et al., 2010). Recent developments in central nervous system imaging methods, such as magnetic resonance imaging and positron emission tomography, indicate that it is possible to highlight areas of central nervous system pathology in patients (Handels et al., 2012). However, these methods rely either on the detection of amyloid plaques or on neuronal loss (Handels et al., 2012; Richard et al., 2013). Both amyloid plaques and neuronal loss occur late in disease progression, leaving their role in the early diagnosis of AD questionable (Ni et al., 2013).

The mechanisms underlying the degeneration of neurons in AD remain unclear, but several hypotheses have been proposed, including the amyloid cascade hypothesis (Paris et al., 2010; Graff et al., 2012; Handels et al., 2012). A critical role of β and γ-secretases, the two key enzymes that produce Aβ by sequentially processing APP, is widely accepted. As the main form of the β-secretase, β-site APP-cleaving enzyme 1 (BACE1) is the rate-limiting enzyme in neuronal Aβ generation (Tomiyama, 2010; Nussbaum et al., 2012). In addition, accumulating evidence indicates that abnormalities in BACE1 expression or function may contribute to cerebral amyloidosis by accelerating the generation of neurotoxic Aβ42 in the brain (Bader Lange et al., 2010; Torres et al., 2012). Moreover, Zhao et al. (2007) found that increased BACE1 activity in AD is most likely triggered by the amyloidogenic pathway, driving a neurotoxic positive-feedback loop. Taken together, current research suggests that abnormal BACE1 expression or function is likely one of the earliest pathological changes in AD, preceding Aβ42 aggregation. Accordingly, we reasoned that BACE1 may serve as a sensitive biomarker in the early stage of AD.

The eye, as an extension of the brain, has been a target of AD research, having the potential to reflect pathologic changes in the brain (Guo et al., 2010; Huang et al., 2012). In particular, it is more easily accessible for observation and evaluation than the brain (Koronyo-Hamaoui et al., 2011). Increasing evidence shows that the eye, particularly the retina, is also affected in AD. Aβ deposits, a hallmark feature of AD, have been also found in postmortem retinas of AD patients (Perez et al., 2009; Koronyo et al., 2012). Accumulation of β-amyloid in neurons in the retinal ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) has been detected in the retinas of APP/PS-1 transgenic mice and TG2576 transgenic mice (Lu et al., 2010; Williams et al., 2013). However, the temporal profile of BACE1 expression in the retina and brain has not been investigated in transgenic mouse models of AD, and the association between BACE1 and behavioral deficits are unclear (Lu et al., 2010; Koronyo et al., 2012; Gallagher et al., 2013; Williams et al., 2013). In the present study, we evaluated the hypothesis that early changes in BACE1 levels occur in the retina, preceding changes in the brain. Our findings suggest that BACE1 may serve as a sensitive biomarker of early neuropathological changes in AD.

Materials and Methods

Animals
APP/PS1 transgenic mice were generated as previously described (Gallagher et al., 2013). These mice (obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Peking Union Medical College, China) overexpress the Swedish (K594M/N595L) mutation of APP, together with a mutant presenilin 1 (PS1) containing an exon 9 deletion, in a C57BL/6J genetic background. In this study, C57/BL6 wild-type mice (obtained from the Laboratory Animal Center of Central South University, China) served as controls. APP/PS1 transgenic mice and C57BL/6 wild-type mice (equal number of males and females) were divided into three groups for analysis at 3, 6 and 8 months of age, with each group containing 12 animals (24 eyes and 12 brains per group). The APP/PS1 transgenic mice were tail-snipped and genotyped using PCR with primers specific for the APP and PS1 sequences (Table 1).

All mice were fed a 4% fat diet and housed under a 12-hour light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee of Central South University, China.

Morris water maze
A mouse version of the Morris water maze (Panlab, Holliston, MA, USA) was used to assess spatial learning and memory. The test was always performed in the same room and at the same time to ensure environmental consistency. All mice were habituated to the water and apparatus before water maze testing.

Table 1 Primers specific for the APP and PS1 sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
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| APP   | Forward: 5′-GAC TGA CCA CTC GAC CAG GGT CTG-3′  
Reverse: 5′-CTT GTA AGT TGG ATT CTC ATA TCG G-3′ | 344               |
| PS1   | Forward: 5′-AAT AGA GAA CGG CAG GAG CA-3′  
Reverse: 5′-GCC ATG AGG GCA CTA ATC AT-3′  
Reverse: 5′-GCA CCA TTC GCT CAA ACA-3′  
Reverse: 5′-ACC ACA GTC CAT GCC ATC AC-3′  
Reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′ | 608               |
| GAPDH | Forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′  
Reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′ | 452               |
Figure 1 Memory ability and β-site APP-cleaving enzyme 1 (BACE1) immunoreactivity in the brain of APP/PS-1 transgenic (TG) and wild-type (WT) mice.

(A) Comparison of Morris water maze test results for TG and WT mice at 3, 6 and 8 months of age: (a) average escape latency in the visible platform test; (b) escape latency in the hidden platform test on days 1–4; and (c) the number of crossings of the target quadrant in 1 minute. (B) BACE1 immunohistochemical staining in the brain of TG and WT mice at 3, 6 and 8 months of age. BACE1 immunoreactivity (brown granules) was higher in the entorhinal cortex and prefrontal cortex. No significant change was observed in the average gray value of BACE1 staining in the hippocampal pyramidal layer (a, b1). BACE1 plaques (a, b2) were clearly visible in the entorhinal cortex and prefrontal cortex at 6 months, and were more intense at 8 months, in the brain of TG mice. The boxed regions are shown magnified 4× in the images on the right. The results are presented as the mean ± SEM. *P < 0.05, vs. WT mice; #P < 0.05, vs. TG mice at 3 months of age. One-way analysis of variance (ANOVA) was used to determine the average gray value (per 0.5 mm²) of BACE1 staining in the hippocampal pyramidal cell layer and the relative area (% of 0.5 mm²) of BACE1 plaques in the brain, followed by Tukey post hoc analysis (12 brains per group).

The visible platform test was performed first. A circular tank with a diameter of 2 m was filled with water (22 ± 2°C) made opaque with milk powder, and extra-maze cues were used. The tank was divided into four quadrants and contained a circular platform with a diameter of 20 cm placed 1.5 cm above the water surface in the center of one quadrant. Mice were placed in the pool from one of four start positions located at the interaction of the quadrants and were allowed 60 seconds to locate the hidden platform and remain on the platform 20 seconds prior to subsequent trials. If the mouse did not locate the platform within 60 seconds, it was manually guided to the platform. The time taken to find the platform, i.e. escape latency, was recorded. Mice with reduced vision or an inability to swim were removed from the experiment.

In the hidden platform task, the platform was placed in
the opposite quadrant and hidden 1.5 cm below the water surface. The procedure was identical to that for the visible platform task. After 4 consecutive days of training, the platform was removed from the pool, and a 60-second probe trial was conducted to examine how well the mice had learned the location of the platform. The number of entrees into the target quadrant was recorded during the probe trial. During all water maze trials, the movement of the mouse in the water maze was captured by an overhead video camera (Canon, Beijing, China). Movements were digitally tracked using EthoVision software.

Tissue preparation
Following the Morris water maze test, all mice were intraperitoneally anesthetized with 10% chloral hydrate (0.4 mL/kg). Following euthanasia, thoracotomy was performed, and a blunt needle was introduced into the ascending aorta from the left ventricle. The animals were perfused transcardially with saline and 4% paraformaldehyde. Eyeballs were removed to produce optic cups, and postfixed with 4% paraformaldehyde for 24 hours after dehydration with 15% and 30% sucrose solutions. Brains were removed and treated similarly. The optic cups were embedded in O.C.T. embedding medium and sliced into 20-μm-thick slices on a freezing microtome (Thermo Scientific, Waltham, MA, USA). Brains were sliced into 25-μm-thick slices.

BACE1 immunohistochemistry
For BACE1 immunohistochemistry, cross sections of the retina and brain were incubated with 50% formamidine and 50% sodium citrate buffer for 1 hour in a 65°C water bath for antigen retrieval. Retinal sections were soaked in 1% H2O2 for 30 minutes at room temperature to abolish endogenous peroxidase activity. After rinsing with PBS, sections were treated with a blocking solution containing 5% bovine serum albumin and 0.1% Triton X-100 for 1 hour at room temperature. Then, sections were stained overnight at 4°C with a rabbit polyclonal BACE1 antibody (1:100; Abcam, Hong Kong, China) diluted in blocking solution. Thereafter, sections were rinsed in PBS/0.1% Triton X-100 for 5 minutes, and then incubated with goat anti-rabbit secondary antibody (1:200; Vector, Burlingame, CA, USA) for 2 hours at room temperature. Sections were washed with PBS/0.1% Triton X-100 for 5 minutes, and then incubated with avidin-biotin-peroxidase complex (1:200; ABC Standard Elite, Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Staining was evaluated using conventional optical microscopy (Olympus, Tokyo, Japan) after the DAB color reaction. In control sections, the primary antibody was omitted.

Nissl staining of mouse retina
Retinal sections were treated with toluidine blue (Biyuntian, Shanghai, China) at room temperature for 5 minutes. After rinsing with distilled water, retinal sections were dehydrated and mounted with Permount. Nissl-positive cells in the GCL were quantitatively analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA).

Data analysis
All data are presented as the mean ± SEM and were analyzed using GraphPad Prism 5.0 software for Windows (GraphPad Software, San Diego, CA, USA). Three retinal slices centered on the optic nerve were used for each animal and image-captured from six symmetrical portions. For each brain, 3 equally-spaced coronal sections containing the dorsal hippocampus (400 mm apart) were used for sampling. One-way analysis of variance was used to determine the average gray value (per 0.5 mm2) of BACE1 staining in the retina and pyramidal cell layer in the hippocampal regions, followed by Tukey post hoc analysis. The relative area (% of 0.5 mm2) of BACE1 plaques in the cortex and hippocampus were determined by one-way analysis of variance followed by Tukey post hoc analysis. Repeated measures one-way analysis of variances with the least significant difference test for post hoc comparisons was used to determine the escape latency of all groups in the Morris water maze test. The number of entrees into the platform-containing quadrant was analyzed with one-way analysis of variance followed by Tukey post hoc analysis. Significance was set at P < 0.05.

Results
Learning and memory dysfunction correlates with the formation of BACE1 plaques in the brain of AD transgenic mice
To exclude the possibility that non-learning and memory deficits effect the performance of AD transgenic mice in the Morris water maze, the visible platform test was performed. No significant differences were found between wild-type and transgenic mice of the same age (P > 0.05). Transgenic mice displayed normal perception, motivation and motor ability compared with age-matched wild-type mice.

Compared with wild-type mice, no changes in learning and memory were detected in transgenic mice at 3 months of age (P > 0.05). However, compared with wild-type mice, the escape latency was significantly increased in transgenic mice at 6 months from day 3, and at 8 months from day 2, during Morris water maze training (P < 0.05). Morris water maze probe testing showed that the number of entrees into the target area was decreased in transgenic mice older than 6 months, compared with wild-type mice (P < 0.05). These results suggest that transgenic mice have significantly worse learning and memory abilities at 6 and 8 months, compared with controls (P < 0.05) (Figure 1A).

Immunohistochemical staining showed that BACE1 was expressed widely in the hippocampus of wild-type mice. No BACE1 plaques were detected in these animals. In contrast, BACE1 plaques were detected in the cortex and hippocampus of AD transgenic mice at 6 months of age. The average gray value of BACE1 staining in the hippocampal pyramidal cell layer was not significantly different between wild-type and transgenic mice (Figure 1B). BACE1 plaques were clearly visible in the entorhinal cortex and prefrontal cortex at 6 months and were more intense at 8 months in transgenic mice.

Abnormal retinal BACE1 expression appears earlier than BACE1 cortical plaques in AD transgenic mice

The average gray values of BACE1 in the retinal GCL, inner plexiform layer (IPL) and outer plexiform layer (OPL) were calculated. Weak BACE1 staining, but no plaques, was detected in the retina of wild-type mice. Compared with wild-type mice, BACE1 expression was significantly increased in the retinal GCL of transgenic mice at 3, 6 and 8 months of age, and in the IPL and OPL of transgenic mice at 6 and 8 months of age ($P < 0.05$) (Figure 2).

Nissl staining revealed similar structures in the GCL, OPL and ONL in both wild-type and transgenic mice. Nissl-positive cells in the GCL were not significantly decreased in AD transgenic mice at 3, 6 and 8 months, compared with age-matched wild-type mice ($P > 0.05$) (Figure 3). This suggests that aberrant retinal expression of BACE1 appears earlier than cytoarchitectural changes or retinal ganglion cell loss in AD transgenic mice.

Discussion

In the present study, we investigated the time course of pathological changes in the retina and brain as well as cognitive dysfunction in APP/PS-1 transgenic mice. Our results showed that (1) abnormal expression of BACE1 in the retina can be detected in the AD transgenic mice from 3 months of age, and (2) BACE1 plaques in the cortex and cognitive impairment appear in AD transgenic mice from 6 months of age. These results suggest that abnormal expression of BACE1 in the retina is an early pathological change in AD transgenic mice.

The Morris water maze test is commonly used to evaluate spatial learning and memory abilities (Minkeviiciene et al., 2008; Hooijmans et al., 2009; Gallagher et al., 2013). We used this test to assess changes in learning and memory abilities in AD transgenic mice over time (Stover and Brown, 2012). Compared with wild-type mice, the escape latency was significantly increased at 8 months of age, from day 2, and at 6 months of age, from day 3, in AD transgenic mice during training. During the probe test, the number of entries into the target area was decreased at 6 and 8 months in the transgenic mice. The normal performance of the transgenic mice in the visible platform task indicates that the observed impairment in spatial learning reflects a cognitive dysfunction and not a deficit in normal perception, motivation, motor ability or visual acuity (Billings et al., 2005). Our results are similar to previous reports. For example, Minkeviiciene et al. (2008) found that transgenic mice carrying mutant copies of the human APP and presenilin genes (APPdE9) exhibit spatial memory impairments at 6 and 15 months of age. The triple-transgenic mouse model of AD (3xTg-AD) exhibits cognitive impairments at 4 months (Minkeviiciene et al., 2008; Graff et al., 2012; Su et al., 2012; Williams et al., 2013).

Interestingly, the time course of cognitive dysfunction in the APP/PS-1 transgenic mice corresponds to that of the appearance of BACE1 plaques in the brain. We detected BACE1 plaques in the brain of transgenic mice at 6 months of age. Meyer-Luehmann et al. (2008) found that Aβ plaques begin to appear in APPswe/PS1d9xYFP (B6C3-YFP) transgenic mice at 5–6 months, and Waters et al. (2009) found BACE1 plaques in 3xTG-AD mice starting at 9 months (Waters et al., 2009; Yan et al., 2012). Increased Aβ plaques were also observed in AD patients in a postmortem histological study (Bourgeat et al., 2015). Taken together, these observations suggest that abnormal BACE1 expression plays a very important role in AD disease progression.

In the present study, we found that compared with wild-type mice, BACE1 expression was significantly increased in the retinal GCL of transgenic mice at 3 months, and expression widened to include the IPL and OPL at 6 and 8 months of age. Abnormal BACE1 expression appeared earlier in the retina than in the brain in transgenic mice. This suggests that BACE1 upregulation in the retina may be an early pathological change in AD.

Considering the pivotal role of the enzyme in Aβ generation, retinal BACE1 may have potential as a biomarker for the early detection of AD. Many studies have shown that PiB-PET and MRI imaging are valuable for AD diagnosis, but these methods have practical limitations for population screening (Fletcher et al., 2013; Martins et al., 2013). Our findings demonstrate that the abnormal expression of BACE1 in the retina occurs prior to behavioral changes or expression changes in the brain. Future research should focus on the development of BACE1 probes for in vivo labeling. Combining these probes with modern imaging techniques such as MRI and PET (Martins et al., 2013) may permit the early presymptomatic diagnosis of AD in humans.

Author contributions: XGL, IMS, HW and JFH conceived and designed this study. LL, YQC, JL, DC, JBT, LPZ and JX performed experiments and analyzed the data. LL wrote the paper. DC, JBT, LL, HW and JFH were responsible for fundraising. All authors approved the final version of the paper.

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Figure 2 BACE1 immunoreactivity in the retina of transgenic (TG) and wide-type (WT) mice.
(A) BACE1 immunohistochemical staining in the retina of TG and WT mice at 3, 6 and 8 months of age. BACE1 immunoreactivity (brown granules) was obvious in the ganglion cell layer (GCL), inner plexiform layer (IPL) and outer plexiform layer (OPL) in TG mice but not in WT mice. (B) BACE1 expression was significantly increased in the GCL (a) in the retina of TG mice at 3, 6 and 8 months compared with age-matched WT mice (*P < 0.05). One-way analysis of variance (ANOVA) was used to determine the average gray value (per 0.5 mm²) of BACE1 staining in the retina, followed by Tukey post hoc analysis (24 eyes per group). The distribution of BACE1 was significantly increased, and expanded to include the IPL (b) and OPL (c) in the retina of TG mice at 6 and 8 months compared with 3 months (#P < 0.05).

Figure 3 Nissl staining in the retina of transgenic (TG) and wide-type (WT) mice.
(A) Nissl bodies were visible as blue granules in the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) of the retina. (B) There was no significant change in the number of Nissl-positive cells in the GCL of TG mice (24 eyes per group).


