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Hypercholesterolemia accelerates intraneuronal accumulation of Aβ oligomers resulting in memory impairment in Alzheimer's disease model mice

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ABSTRACT

Aims: Hypercholesterolemia is known to be a risk factor for Alzheimer's disease (AD), and diet-induced hypercholesterolemia has been shown to accelerate amyloid pathology in animals. While growing evidence has shown that synaptic and cognitive dysfunction in AD is associated with intraneuronal accumulation of Aβ, the relationships between hypercholesterolemia, memory impairment, and intraneuronal Aβ remains unclear. The present study aims to clarify this association.

Main methods: Transgenic mice expressing amyloid precursor protein (APP) harboring the Osaka (E693Δ) mutation (APP_{OSK}-Tg mice) were used. These mice exhibit intraneuronal Aβ oligomers and memory impairment from 8 months of age. Five-month-old male APP_{OSK}-Tg mice and non-Tg littermates were fed a high-cholesterol diet for 1 month to induce hypercholesterolemia. At 6 months of age, their cognitive function was evaluated by the Morris water maze. Intraneuronal Aβ, synaptic density, and tau phosphorylation were examined by immunohistochemistry.

Key findings: Serum and brain cholesterol levels were significantly higher in APP_{OSK}-Tg mice and non-Tg littermates that were fed a high-cholesterol diet than in control mice that were fed normal chow, indicating that hypercholesterolemia was successfully induced. Hypercholesterolemic APP_{OSK}-Tg mice, but not control APP_{OSK}-Tg mice or hypercholesterolemic non-Tg littermates, exhibited impaired spatial reference memory, which was accompanied with intraneuronal accumulation of Aβ oligomers, reduced synaptophysin immunoreactivity, and abnormal tau phosphorylation in the hippocampus. Hypercholesterolemia-accelerated accumulation of intraneuronal Aβ oligomers was also observed in another model mouse, Tg2576.

Significance: Our findings suggest that hypercholesterolemia accelerates intraneuronal accumulation of Aβ oligomers and subsequent synapse loss, resulting in memory impairment.

Introduction

Extracellular soluble Aβ oligomers are believed to cause synaptic and cognitive dysfunction in Alzheimer's disease (AD) (Klein et al., 2001; Selkoe, 2002). However, mounting evidence indicates that intraneuronal accumulation of Aβ is an early event in AD (Gouras et al., 2000; Fernández-Vizarra et al., 2004) and Down syndrome (Gyure et al., 2001; Mori et al., 2002) and likely contributes to synaptic and cognitive dysfunction (Wirths et al., 2004; LaFerla et al., 2007; Gouras et al., 2010). For example, morphological alterations of synapses have been shown to occur in association with intraneuronal accumulation of Aβ in brains of AD patients and Tg2576 mice (Takahashi et al., 2002). We also observed that synaptophysin was decreased around the neurons bearing intracellular Aβ in the brains from AD patients (Ishibashi et al., 2006). Furthermore, in the triple transgenic 3xTg-AD mice, synaptic and cognitive dysfunction was shown to be correlated with the accumulation of intraneuronal Aβ before amyloid plaque formation (Oddo et al., 2003; Billings et al., 2005). Such a correlation of synaptic and/or behavioral abnormalities to intraneuronal Aβ accumulation was also demonstrated in other AD model mice (Knobloch et al., 2007; Wegenast-Braun et al., 2009; Tampellini et al., 2010). We also showed that APP_{OSK}-Tg mice, which express amyloid precursor protein (APP) harboring the Osaka (E693Δ) mutation (Tomiyama et al., 2008), exhibited synaptic and cognitive dysfunction and synapse loss at 8 months of age, the time at which the accumulation of intraneuronal Aβ oligomers without forming amyloid plaques began (Tomiyama et al., 2010).

Hypercholesterolemia is known to be a risk factor for AD (Solomon and Kivipelto, 2009; Stefani and Liguri, 2009). Cholesterol

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loading of cells inhibits α-secretase (Bodovitz and Klein, 1996) and causes increased Aβ generation via the activation of both β- and γ-secretases (Frears et al., 1999; Xiong et al., 2008), whereas cholesterol depletion results in reduced Aβ production (Simons et al., 1998; Frears et al., 1999; Grimm et al., 2008). In transgenic mice, diet-induced hypercholesterolemia increased Aβ levels in the brain and thus accelerated extracellular amyloid deposition (Refolo et al., 2000; Shie et al., 2002), whereas treatment with statin attenuated amyloid pathology (Petanceska et al., 2002; Kurata et al., 2011). Despite clear evidence of hypercholesterolemia-induced amyloid pathology, the relationship between hypercholesterolemia and memory impairment in animals is somewhat controversial (Fitz et al., 2010; Ullrich et al., 2010; Schreurs, 2010). Furthermore, little is known about intraneuronal Aβ in hypercholesterolemia.

Therefore, in the present study, we investigated the effects of hypercholesterolemia on the level of intraneuronal Aβ and on cognitive function using APP_{OSK}-Tg mice. We fed 5-month-old APP_{OSK}-Tg mice a high-cholesterol diet for 1 month to induce hypercholesterolemia and examined their phenotypes at 6 months of age when the mice ordinarily show no symptoms or pathology of AD. We found that hypercholesterolemic APP_{OSK}-Tg mice displayed earlier onset of cognitive dysfunction, accelerated accumulation of intraneuronal Aβ oligomers, reduced levels of synaptophysin, and abnormal tau phosphorylation in the hippocampus. Our findings suggest that hypercholesterolemia causes memory impairment by accelerating intraneuronal accumulation of Aβ oligomers.

Materials and methods

Antibodies

Rabbit polyclonal antibody to the N-terminus of Aβ (β001; Lippa et al., 1999) was prepared in our laboratory and was confirmed to bind to both human and mouse Aβ in western blots. Mouse monoclonal antibody NU-1 (Lambert et al., 2007) was used to detect Aβ oligomers. Rabbit polyclonal antibody to the repeat domains of tau (pool 2; Endoh et al., 1993) was prepared in our laboratory and was confirmed to bind to both human and mouse tau in western blots. Mouse monoclonal antibody to phosphorylation at Ser396/Ser404 of tau (PHF-1; Greenberg et al., 1992) was kindly gifted by Dr Peter Davies (Department of Pathology, Albert Einstein College of Medicine, Bronx, NY). Mouse monoclonal antibody to synaptophysin (SVP-38; Sigma, St. Louis, MO) was purchased.

Animals

Five-month-old male APP_{OSK}-Tg mice (n = 16) and non-Tg littermates (n = 16) were divided into 2 groups (n = 8 each), such that the mean body weight was not significantly different between the 2 groups. One group was fed a high-cholesterol diet (2% cholesterol and 4% fat; CLEA Japan, Inc., Tokyo, Japan) for 1 month to induce hypercholesterolemia, while the control group was fed normal chow (0.1% cholesterol and 4% fat; CLEA Japan). At the end of the month, blood samples were collected from their tail veins and serum cholesterol levels were measured using the Cholesterol Assay Kit (BioVison, Inc., Mountain View, CA). Three mice of high-cholesterol-fed APP_{OSK}-Tg group died during the diet period; hence, this group contained only 5 mice. After behavioral tests, the mice were killed, and their brains were removed for the measurement of brain cholesterol and Aβ and for immunohistochemical analyses of intraneuronal Aβ and synaptophysin. Hippocampal tissues were dissected for measurement of cholesterol levels using the Cholesterol Assay Kit, as described previously (Umeda et al., 2010). In another experiment, 7-month-old male and female Tg2576 mice (n = 6; Taconic, Hudson, NY) were divided into 2 groups (n = 3 each) and were fed high cholesterol diet or normal chow for 1 month. After euthanasia, their brains were removed for immunohistochemical analysis of intraneuronal Aβ. All animal experiments were approved by the committee of Osaka City University and were performed in accordance with the Guide for Animal Experimentation, Osaka City University. Every effort was made to minimize the number of animals used and their suffering.

Behavioral tests

Spatial reference memory was assessed at 6 months of age using the Morris water maze, as described previously (Tomiyama et al., 2010). Male mice were trained to swim to the platform in a pool with a diameter of 96 cm for 5 consecutive days. Training consisted of 5 trials per day with intertrial intervals of 30 s. At day 6, retention of spatial memory was assessed by a probe trial consisting of a 30 s free swim in the pool without the platform. Locomotor activities of the mice were examined by an open-field test, as described previously (Tomiyama et al., 2010). During the period of behavioral tests, mice were maintained on their specified high cholesterol diet or normal chow.

Immunohistochemistry

Mouse brains were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and deparaffinized with xylene and ethanol. For Aβ staining only, sections were pretreated by boiling in 0.01 N HCl (pH 2) for 10 min to expose epitopes. After washing with 100 mM Tris–HCl, pH 7.6, 150 mM NaCl (TBS), the sections to be stained with horseradish peroxidase (HRP) were treated with 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidases. The sections were then blocked with 20% calf serum in TBS for 1 h. Aβ and tau were stained with corresponding antibodies (β001, NU-1, or PHF-1) followed by biotin-labeled secondary antibodies (Vector Laboratories, Inc., Burlingame, CA), HRP-labeled avidin–biotin complex (Vector Laboratories), and the substrate DAB (Dojindo, Kumamoto, Japan). Synaptophysin was stained with SVP-38 antibody followed by FITC-labeled secondary antibody (Jackson ImmunoResearch Labs Inc., West Grove, PA). The specimens were observed under a BZ-8000 fluorescence microscope (Keyence, Osaka, Japan). Synaptic density in the hippocampal CA3 region was estimated by quantifying synaptophysin fluorescence intensity in an area of 30 μm × 60 μm using NIH imageJ software obtained from a public website (National Institutes of Health; http://rsb.info.nih.gov/nih-image/).

Aβ ELISA and tau western blot

Hemispheres of the cerebral cortex including hippocampus were homogenized by sonication in 4 volumes of TBS containing protease inhibitor cocktail (P8340; Sigma) and phosphatase inhibitor cocktail (06863-01; Nacalai tesque, Kyoto, Japan). 400 μl of the homogenates was centrifuged at 100,000 × *g* at 4 °C for 1 h, and the supernatants were harvested as TBS soluble fractions. The precipitates were dissolved by sonication in 200 μl of 70% formic acid and centrifuged at 100,000 × *g* at room temperature for 1 h. The supernatants were harvested as TBS insoluble fractions and diluted 20-fold in 1 M Tris solution. Aβ concentrations in the TBS soluble and insoluble fractions were determined using human β amyloid (1–40) and (1–42) ELISA kits (298–64601 and 296–64401; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Since APP_{OSK}-Tg mice express the mutant Aβ, synthetic Aβ E22Δ peptides were used as standards. The levels of tau were examined by western blot. Aliquots of each fraction were mixed with an equal volume of SDS sample buffer containing β-mercaptoethanol and boiled for 5 min. The samples were subjected to SDS-PAGE (1 μl/lane for TBS soluble fractions and 10 μl/lane for insoluble fractions) with 7% NuPage Tris-Acetate gels (Invitrogen, Carlsbad, CA) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Total and phosphorylated taus were probed with pool 2 and PHF-1 antibodies, respectively, followed by HRP-labeled second antibodies and the chemiluminescent substrate Immobilon Western (Millipore). Signals were visualized using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

Statistical analysis

Comparisons of means between 2 groups were performed using the unpaired Student's *t*-test (for Aβ), while those among the 4 groups were performed with ANOVA (for cholesterol, synaptophysin, and probe trial) or two-factor repeated measures ANOVA (for memory acquisition) followed by Fisher's PLSD test. Differences with a p value of less than 0.05 were considered significant.

Results

Diet-induced hypercholesterolemia in APP_{OSK}-Tg mice

Five-month-old male APP_{OSK}-Tg mice and non-Tg littermates were fed a high-cholesterol diet for 1 month to induce hypercholesterolemia. Control APP_{OSK}-Tg mice and non-Tg littermates were fed normal chow. At the end of the month, serum cholesterol levels of the APP_{OSK}-Tg mice and non-Tg littermates that were fed the high-cholesterol diet were significantly higher than those of their counterparts fed a normal diet (Fig. 1A), indicating that hypercholesterolemia was successfully induced. Brain cholesterol levels in these mice were also measured after behavioral tests. Both hypercholesterolemic APP_{OSK}-Tg mice and hypercholesterolemic non-Tg littermates showed higher cholesterol levels in the hippocampus than control mice (Fig. 1B), although this increase was significant in the APP_{OSK}-Tg mice only.

Cognitive dysfunction in hypercholesterolemic APP_{OSK}-Tg mice

To determine whether hypercholesterolemia affects cognitive function in mice, we studied the spatial reference memory of mice using the Morris water maze when mice were 6 months of age, a time when APP_{OSK}-Tg mice ordinarily show no symptoms or pathology of AD. Mice were trained for 5 days to remember the location of a hidden platform in a swimming pool (memory acquisition), and were tested for memory retention at day 6 in a probe trial with the platform removed. Hypercholesterolemic non-Tg littermates showed performance similar to or slightly better than control non-Tg littermates in both memory acquisition (Fig. 2A) and retention tests (Fig. 2B). Control APP_{OSK}-Tg mice also showed performance similar to or slightly worse than control non-Tg littermates, whereas hypercholesterolemic APP_{OSK}-Tg mice displayed significant deficits in performance with longer escape latencies in the memory acquisition test and lower time occupancy in the target quadrant in the probe trial than control non-Tg littermates. These results indicate that hypercholesterolemia does not significantly affect cognitive function in wild-type mice at 6 months of age, but causes earlier onset of memory impairment in APP_{OSK}-Tg mice. After the water maze task, locomotor activities of mice were examined by an open field test. No significant difference in locomotion among the 4 groups was recorded (data not shown), indicating that the memory impairment observed in hypercholesterolemic APP_{OSK}-Tg mice was not due to motor dysfunction.

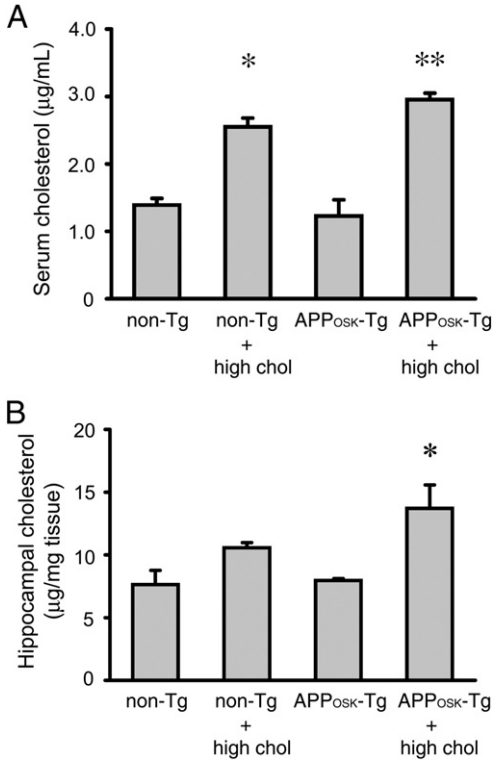

Fig. 1. Serum and brain cholesterol levels in mice. Five-month-old APP_{OSK}-Tg mice and non-Tg littermates were fed a high-cholesterol diet or normal chow for 1 month. (A) Serum cholesterol levels were determined before behavioral tests. The results are means ± S.E.M. (n = 5 for hypercholesterolemic APP_{OSK}-Tg mice; n = 8 for other groups). *p < 0.0001 vs non-Tg littermates fed normal chow; p < 0.0001 vs APP_{OSK}-Tg mice fed normal chow; not significant vs APP_{OSK}-Tg mice fed high-cholesterol diet, **p < 0.0001 vs non-Tg littermates fed normal chow; p < 0.0001 vs APP_{OSK}-Tg mice fed normal chow. (B) Brain cholesterol levels were determined from the hippocampal tissues after behavioral tests. The results are means ± S.E.M. (n = 3 per group). *p = 0.0031 vs non-Tg littermates fed normal chow; p = 0.0043 vs APP_{OSK}-Tg mice fed normal chow; not significant vs non-Tg littermates fed high-cholesterol diet.

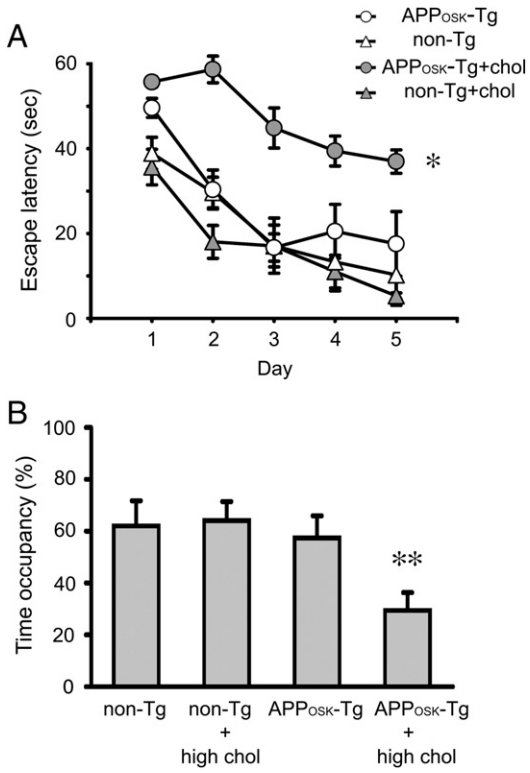

Fig. 2. Memory impairment in hypercholesterolemic APP_{OSK}-Tg mice. Six-month-old APP_{OSK}-Tg mice and non-Tg littermates with or without hypercholesterolemia were tested for spatial reference memory. Memory acquisition (A) and retention (B) were assessed using the Morris water maze. In probe trials (B), time spent in the target quadrant was measured. The results are means ± S.E.M. (n = 5 for hypercholesterolemic APP_{OSK}-Tg mice; n = 8 for other groups). *p < 0.0001 vs normal non-Tg littermates; p < 0.0001 vs hypercholesterolemic non-Tg littermates; p = 0.0003 vs normal APP_{OSK}-Tg mice, **p = 0.0209 vs normal non-Tg littermates; p = 0.0148 vs hypercholesterolemic non-Tg littermates; p = 0.0443 vs normal APP_{OSK}-Tg mice.

Intraneuronal accumulation of Aβ oligomers in hypercholesterolemic APP$_{OSK}$-Tg mice

We previously showed that cognitive dysfunction in APP$_{OSK}$-Tg mice was associated with intraneuronal accumulation of Aβ oligomers and subsequent synapse loss (Tomiyama et al., 2010). To investigate the effect of hypercholesterolemia on intraneuronal Aβ, we examined the brain sections of mice by immunohistochemistry using β001 antibody that recognizes the N-terminus of Aβ and NU-1 antibody that selectively recognizes Aβ oligomers. Sections from control non-Tg littermates, control APP$_{OSK}$-Tg mice and hypercholesterolemic non-Tg littermates were negative for staining with β001 (Fig. 3A) and NU-1 (Fig. 3B) in the hippocampus and cerebral cortex. In contrast, hypercholesterolemic APP$_{OSK}$-Tg mice exhibited marked staining with β001 and NU-1 within neurons in the hippocampal CA3 region and some staining in the cerebral cortex at 6 months of age. In our previous observation, these brain regions showed Aβ oligomer accumulation only from 8 months of age in APP$_{OSK}$-Tg mice (Tomiyama et al., 2010). Thus, hypercholesterolemia accelerated accumulation of intraneuronal Aβ oligomers in APP$_{OSK}$-Tg mice. Hypercholesterolemia-induced accumulation of brain Aβ was confirmed by ELISA. The levels of Aβ1-40 and Aβ1-42 in TBS soluble fractions were significantly increased in hypercholesterolemic APP$_{OSK}$-Tg mice, compared with control APP$_{OSK}$-Tg mice (Fig. 3C). TBS insoluble Aβ1-40 and Aβ1-42 were also increased, although the changes were not significant.

Synapse loss in hypercholesterolemic APP$_{OSK}$-Tg mice

We next examined whether synapse loss also occurred in hypercholesterolemic APP$_{OSK}$-Tg mice at 6 months of age. Brain sections were stained with an antibody to the presynaptic marker synaptophysin and the intensities were quantified. Neither control APP$_{OSK}$-Tg mice nor hypercholesterolemic non-Tg littermates showed significant changes in synaptophysin levels in the hippocampus compared with control non-Tg littermates (Fig. 4A and B). In contrast, hypercholesterolemic APP$_{OSK}$-Tg mice displayed a marked decrease in synaptophysin

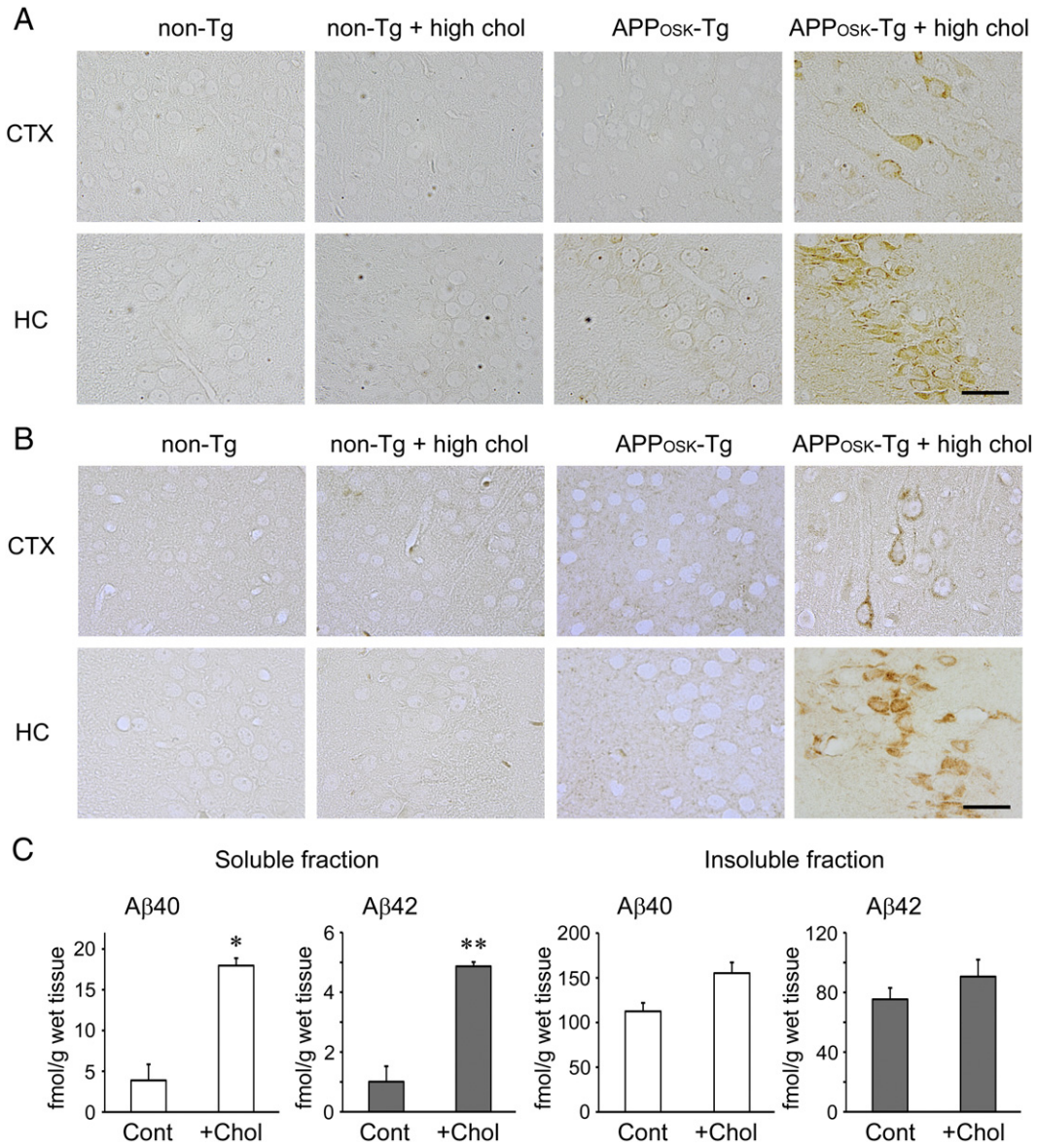

Fig. 3. Intraneuronal accumulation of Aβ oligomers in hypercholesterolemic APP$_{OSK}$-Tg mice. Brain sections from 6-month-old APP$_{OSK}$-Tg mice and non-Tg littermates with or without hypercholesterolemia were stained with antibodies to Aβ (β001, A) and Aβ oligomers (NU-1, B). The photographs show the cerebral cortex (CTX) and hippocampal CA3 regions (HC). Scale bar = 30 μm. (C) Aβ concentrations in brain TBS soluble and insoluble fractions from APP$_{OSK}$-Tg mice with or without hypercholesterolemia were measured by ELISA. The results are means ± S.E.M. (n = 3 per group). *p = 0.0019 and **p = 0.0020 vs control APP$_{OSK}$-Tg mice.

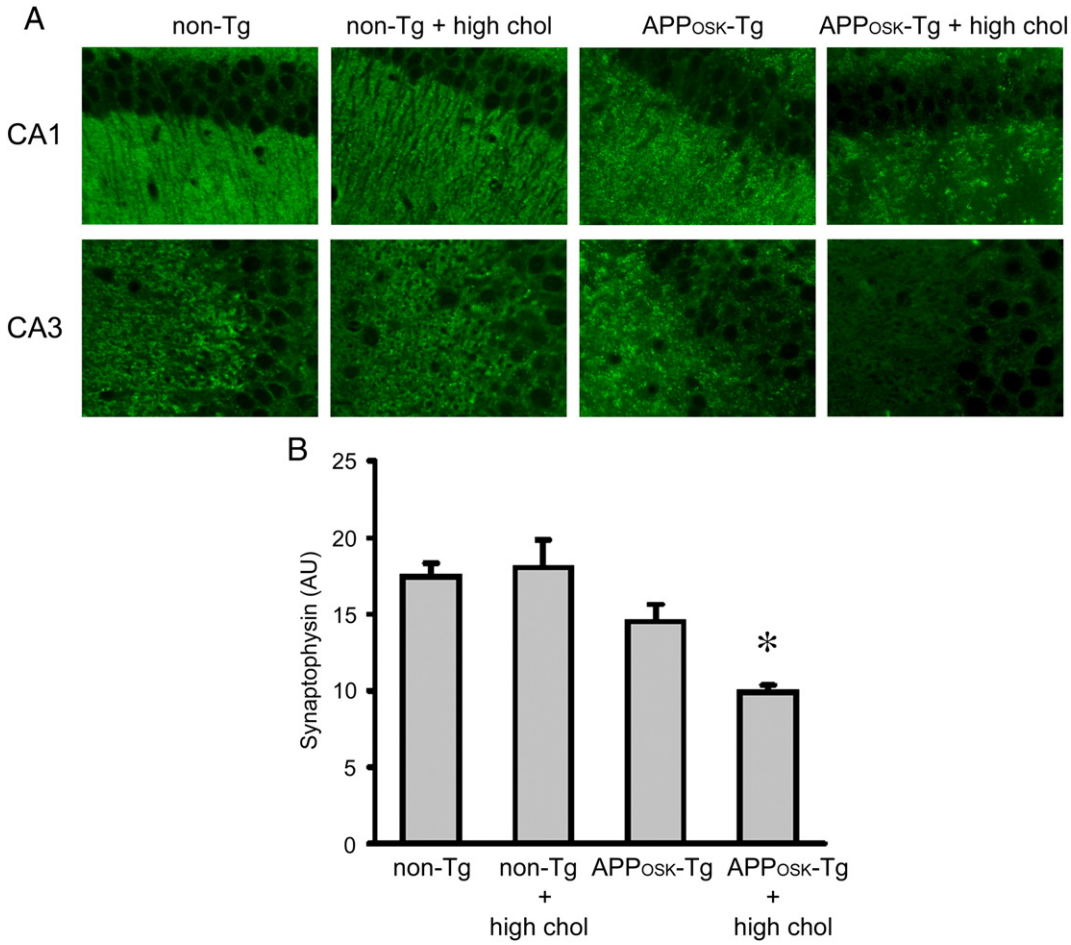

Fig. 4. Decrease in synaptophysin levels in hypercholesterolemic APP_{OSK}-Tg mice. (A) Brain sections from 6-month-old APP_{OSK}-Tg mice and non-Tg littermates with or without hypercholesterolemia were stained with antibody to synaptophysin. The photographs show the hippocampal CA1 and CA3 regions. (B) The synaptophysin fluorescence intensities in the hippocampal CA3 regions were quantified and shown in arbitrary units (AU). The results are means ± S.E.M. (n = 3 per group). *p = 0.0019 vs normal non-Tg littermates; p = 0.0012 vs hypercholesterolemic non-Tg littermates; p = 0.0239 vs normal APP_{OSK}-Tg mice.

levels in the hippocampus. Again, since our previous study showed that hippocampal synapse loss in APP_{OSK}-Tg mice occurred from 8 months of age (Tomiyama et al., 2010), this collectively indicates that hypercholesterolemia also accelerated synaptic alteration.

Abnormal tau phosphorylation in hypercholesterolemic APP_{OSK}-Tg mice

Our previous study revealed that APP_{OSK}-Tg mice developed abnormal tau phosphorylation soon after the beginning of intraneuronal accumulation of Aβ oligomers (Tomiyama et al., 2010). It is of interest to clarify whether hypercholesterolemia also accelerates tau pathology. Brain sections were stained with PHF-1, an antibody to phosphorylated tau. Control and hypercholesterolemic non-Tg littermates and control APP_{OSK}-Tg mice displayed no staining in the hippocampus and cerebral cortex (Fig. 5A). In contrast, hypercholesterolemic APP_{OSK}-Tg mice exhibited PHF-1-positive staining in hippocampal Mossy fibers at 6 months of age. These results indicate that hypercholesterolemia accelerated not only Aβ but also tau pathology, and that the latter pathology was probably mediated by intraneuronal Aβ oligomers, not directly by hypercholesterolemia itself. Hypercholesterolemia-accelerated abnormal tau phosphorylation was confirmed by western blot. While total tau levels did not differ between hypercholesterolemic and control APP_{OSK}-Tg mice, the levels of PHF-1-positive phosphorylated tau were apparently increased in hypercholesterolemic APP_{OSK}-Tg mice in both TBS soluble and insoluble fractions (Fig. 5B).

Hypercholesterolemia-accelerated intraneuronal Aβ oligomers in Tg2576 mice

Aβ with the Osaka (E22Δ) mutation has a particular tendency to accumulate within cells, forming oligomers in contrast to wild-type Aβ (Nishitsuji et al., 2009; Ito et al., 2009; Tomiyama et al., 2010; Umeda et al., 2011). This raises the question of whether or not other mouse models expressing wild-type Aβ also show hypercholesterolemia-accelerated accumulation of intraneuronal Aβ oligomers as we observed in APP_{OSK}-Tg mice. To address this question, we examined the effect of hypercholesterolemia on intraneuronal Aβ oligomers in a well-known AD model mouse, Tg2576. This mouse has been reported to start amyloid deposition from 9 to 10 months of age (Hsiao et al., 1996). We fed 7-month-old Tg2576 mice the high-cholesterol diet for 1 month and examined their amyloid pathology at 8 months of age by immunohistochemistry. Control Tg2576 mice that were fed normal chow showed intracellular β001- and NU-1-positive staining in the cerebral cortex and rarely in the hippocampus but no extracellular amyloid deposition (Fig. 6A and B). On the other hand, Tg2576 mice that were fed the high-cholesterol diet exhibited intensely intracellular β001- and NU-1-positive staining in both the cerebral cortex and hippocampus, and furthermore, a few extracellular amyloid deposits in the cerebral cortex and entorhinal cortex (Fig. 6C). Notably, some of these deposits were tiny, atypical in morphology, and closely associated with neuronal cell bodies as if they had overflowed the cells. These results indicate that hypercholesterolemia-accelerated accumulation of intraneuronal Aβ

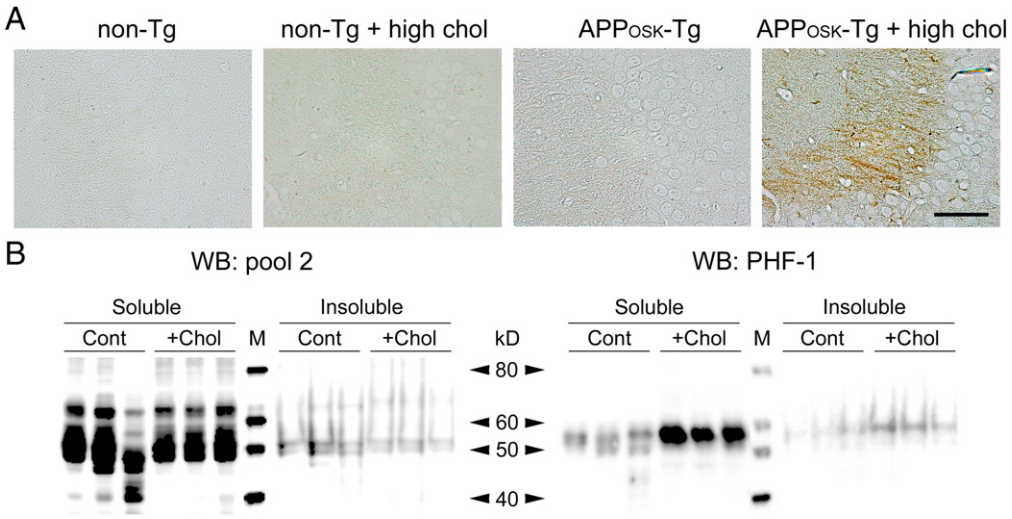

Fig. 5. Abnormal tau phosphorylation in hypercholesterolemic APP_{OSK}-Tg mice. (A) Brain sections from 6-month-old APP_{OSK}-Tg mice and non-Tg littermates with or without hypercholesterolemia were stained with antibody to phosphorylated tau (PHF-1). The photographs show the hippocampal CA3 regions. Scale bar = 50 μm. (B) The levels of total and phosphorylated tau in brain TBS soluble and insoluble fractions from APP_{OSK}-Tg mice with or without hypercholesterolemia were examined by western blot using pool 2 and PHF-1 antibodies, respectively. M, MagicMark XP western standard (Invitrogen).

oligomers is not restricted to APP_{OSK}-Tg mice but also occurs in other model mice.

Discussion

In the present study, we investigated the relationships between hypercholesterolemia, memory impairment, and intraneuronal Aβ using our AD model mouse, APP_{OSK}-Tg. A high-cholesterol diet successfully induced hypercholesterolemia in normal and AD model mice. Brain cholesterol levels were also increased, suggesting that they reflect the levels of serum cholesterol. It is generally believed that cholesterol itself does not easily cross the blood brain barrier and that brain and peripheral cholesterol levels are independently regulated. However, brain cholesterol can be excreted into the circulation via its conversion into 24S-hydroxycholesterol, and inversely, peripheral cholesterol can be taken up by the brain via its conversion into 27-hydroxycholesterol (Björkhem, 2006). These transports are presumed to occur by diffusion due to the concentration gradient of each oxysterol between the brain and the circulation (Björkhem, 2006). Thus, high levels of serum cholesterol may cause an increased flux of cholesterol from the circulation into the brain through the conversion between cholesterol and oxysterol, leading to increased levels of brain cholesterol. We found that hypercholesterolemia accelerates intraneuronal accumulation of Aβ oligomers and subsequent synapse loss resulting in memory impairment. Control APP_{OSK}-Tg mice that were fed normal chow showed neither intraneuronal Aβ oligomers nor memory impairment at the same age (6 months old). Our findings suggest that intraneuronal Aβ, particularly its oligomeric forms, play an important role in the onset of cognitive dysfunction in AD.

It has been shown that high levels of cellular cholesterol inhibit α-secretase (Bodovitz and Klein, 1996) and increase Aβ generation via activation of both β- and γ-secretases (Frears et al., 1999; Xiong et al., 2008) and that diet-induced hypercholesterolemia increases Aβ levels in the brain and thus accelerates extracellular Aβ deposition in transgenic mice (Refolo et al., 2000; Shie et al., 2002). The present study shows that diet-induced hypercholesterolemia also enhances the intraneuronal accumulation of Aβ in both APP_{OSK}-Tg and Tg2576 mice. Aβ is generated in various intracellular compartments such as the ER, Golgi apparatus, endosomes, and autophagosomes, in addition to the plasma membrane (Nishitsuji et al., 2009). Enhanced Aβ generation in these organelles stimulated by high cholesterol intake would result in intracellular accumulation of Aβ. We previously proposed that Aβ upregulation by high cholesterol stimulation occurs to maintain cellular cholesterol levels (Umeda et al., 2010). That is, Aβ mediates cholesterol efflux from cells by forming high-density lipoprotein (HDL)-like particles with excess cellular cholesterol during its secretion. This apolipoprotein-like function of Aβ requires interaction with ATP-binding cassette transporter A1 (ABCA1), a transmembrane protein as a cholesterol donor. In general, cholesterol is taken up by cells via receptor-mediated endocytosis and is transported from endosomes to the ER and the plasma membranes. Thus, increased levels of cellular cholesterol may promote interaction between Aβ and ABCA1 within the plasma membrane or intracellular compartments such as endosomes and ER, which may lead to intracellular accumulation of Aβ. It has also been proposed that Aβ regulates cellular cholesterol levels by inhibiting the cholesterol biosynthesis enzyme, hydroxymethylglutaryl-CoA (HMG-CoA) reductase (Grimm et al., 2005). Since this enzyme primarily localizes to the ER, the Aβ produced in response to high levels of cellular cholesterol may accumulate in the ER to inhibit further cholesterol synthesis.

The mechanism underlying intraneuronal Aβ-induced memory impairment remains unclear. We have investigated the mechanisms by which intraneuronal accumulation of Aβ oligomers cause cell death in APP_{OSK}-Tg mice (Umeda et al., 2011). Aβ oligomers accumulated within the ER, endosomes/lysosomes, and mitochondria in hippocampal neurons of APP_{OSK}-Tg mice and caused ER stress, endosomal/lysosomal leakage, and mitochondrial dysfunction at 18 months of age. These insults presumably lead to eventual neuronal death, i.e. at 24 months of age in APP_{OSK}-Tg mice. At younger ages, the damage caused by intracellular Aβ oligomers would be less, but even mild aberration in these organelles might profoundly affect synaptic function, because these organelles play crucial roles in cellular Ca^{2+} control and endocytosis/exocytosis, which are both involved in synaptic function. In addition, APP_{OSK}-Tg mice were demonstrated to develop abnormal tau phosphorylation soon after the beginning of intraneuronal accumulation of Aβ oligomers (Tomiyama et al., 2010), suggesting that intraneuronal Aβ oligomers trigger the pathological alteration of tau. Abnormal phosphorylation of tau would affect its function in axonal transport and in dendrites, leading to synaptic dysfunction. Hippocampal neurons in culture respond to toxic Aβ oligomers with increased tau phosphorylation (De Felice et al., 2008) and impaired axonal transport (Decker et al., 2010). Hypercholesterolemia is presumed to accelerate these synaptotoxic processes by enhancing Aβ generation.

In this study, we observed the very beginning of amyloid plaque formation in 8-month-old hypercholesterolemic Tg2576 mice, at

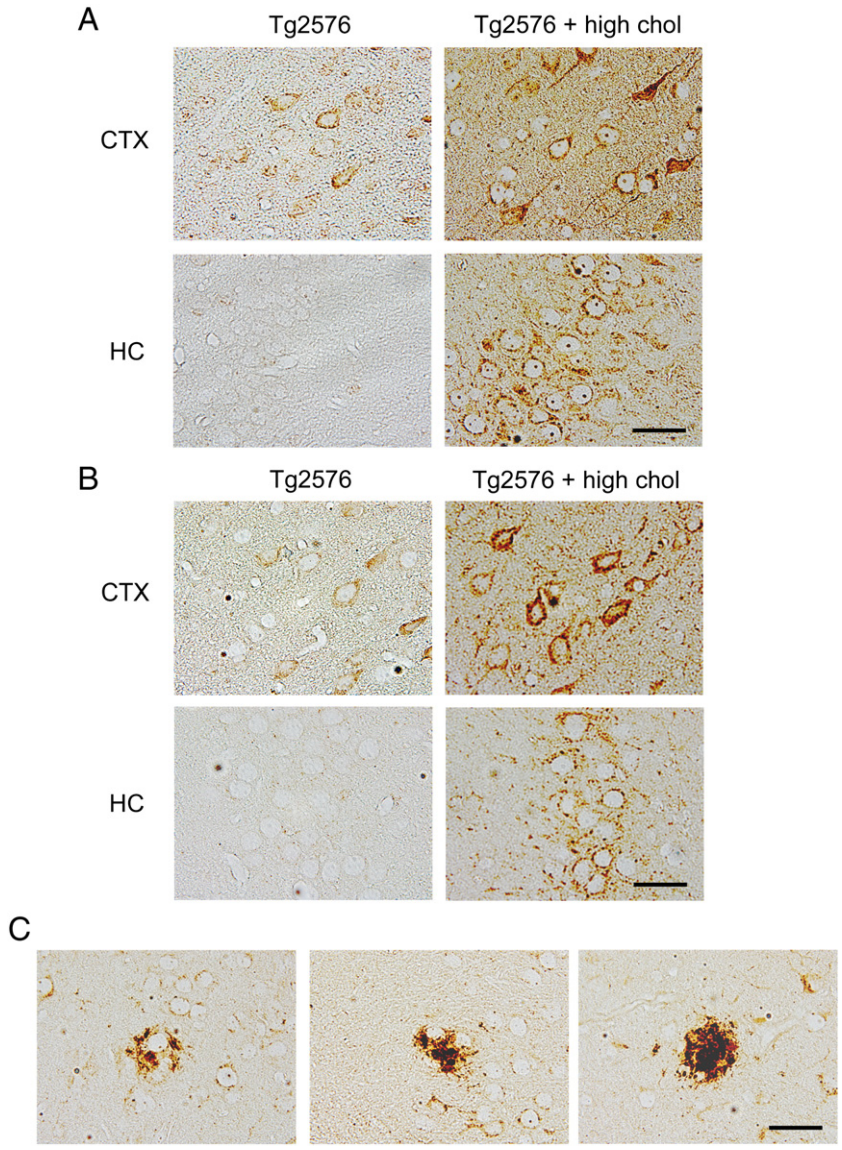

Fig. 6. Intraneuronal accumulation of Aβ oligomers in hypercholesterolemic Tg2576 mice. Seven-month-old Tg2576 mice were fed a high-cholesterol diet or normal chow for 1 month. Their brain sections were stained with antibodies to Aβ (β001, A and C) and Aβ oligomers (NU-1, B). (A, B) The photographs show the cerebral cortex (CTX) and hippocampal CA3 regions (HC). (C) Extracellular amyloid deposits were detected in the cerebral cortex and entorhinal cortex of hypercholesterolemic mice. Scale bar = 30 μm.

which age intraneuronal accumulation of Aβ was already detected, even in normal Tg2576 mice. It is noteworthy that some of these extracellular Aβ deposits were closely associated with neuronal cell bodies, implying the possibility that they originated from intracellular Aβ pools. Similar observations have been reported in AD patients (D'Andrea et al., 2001) and other mouse models (Oakley et al., 2006). In addition, previous studies have suggested that intraneuronal Aβ serves as a source for extracellular amyloid deposits (Oddo et al., 2006) and that amyloid seeds are formed by intracellular concentration and aggregation of Aβ within endosomal/lysosomal compartments (Hu et al., 2009; Friedrich et al., 2010). Taken together, it is likely that enhanced accumulation of intraneuronal Aβ and its subsequent leakage from cells into the extracellular space during neurodegenerative processes led to the accelerated extracellular amyloid deposition in hypercholesterolemic mice (Refolo et al., 2000; Shie et al., 2002; and the present study).

Our findings imply that intracellular Aβ oligomers play important roles in synaptic and cognitive dysfunction. However, we cannot exclude the possibility that both extracellular and intracellular Aβ oligomers contribute to the synaptic pathology of AD. For example, Tg2576 mice have been reported to exhibit memory impairment at 6 months of age accompanied with the appearance of extracellular soluble Aβ oligomers termed Aβ56 (Lesné et al., 2006). On the other hand, we detected intraneuronal accumulation of Aβ in Tg2576 mice at the same age (6-month-old) using human Aβ-specific monoclonal antibody 82E1, although the immunoreactivities were very faint (unpublished observation). In APP_{OSK}-Tg mice and 3xTg-AD mice, memory impairment appeared to be closely correlated with intraneuronal Aβ accumulation. Nevertheless, further studies are required to elucidate the possible existence of extracellular synaptotoxic Aβ species associated with memory impairment in these mice.

Conclusion

Diet-induced hypercholesterolemia in APP_{OSK}-Tg mice caused earlier onset of cognitive dysfunction, which was accompanied with accelerated accumulation of intraneuronal Aβ oligomers and subsequent synapse loss in the hippocampus. Our findings suggest that hypercholesterolemia causes memory impairment by accelerating intraneuronal accumulation of Aβ, particularly its oligomeric form.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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