Simvastatin Enhances Learning and Memory Independent of Amyloid Load in Mice

Ling Li, DVM, PhD,[1–4] Dongfeng Cao, PhD,[1] Helen Kim, PhD,[5] Robin Lester, PhD,[4] and Ken-ichiro Fukuchi, MD, PhD[6]

Objective: Normal aging is often associated with a decline in learning and memory functions. This decline is manifested to a much greater extent in Alzheimer's disease. Recent studies have indicated statins, a class of cholesterol-lowering drugs, as a potential therapy for Alzheimer's disease. Our objective was to determine whether administering a statin drug (simvastatin) would protect against the development of behavioral deficits in an established mouse model of Alzheimer's disease.

Methods: Tg2576 mice and their nontransgenic littermates were treated with simvastatin and assessed by behavioral tests and biochemical analyses.

Results: Simvastatin treatment not only reversed learning and memory deficits in the Tg2576 mice, but also enhanced learning and memory in the nontransgenic mice. Moreover, levels of amyloid β protein in the brains of treated mice did not differ from those of untreated mice. Simvastatin treatment was associated with increased expression levels of protein kinase B (Akt) and endothelial nitric oxide synthase in the mouse brain.

Interpretation: Our findings demonstrate that the effects of simvastatin on learning and memory are independent of amyloid β protein levels. The mechanisms by which simvastatin exerts its beneficial effects may be related to modulation of signaling pathways in memory formation.

Ann Neurol 2006;60:729–739

Alzheimer's disease (AD) is a common age-related neurodegenerative disorder characterized clinically by progressive cognitive impairment. Pathological hallmarks of AD brain include neurofibrillary tangles and deposits of aggregated amyloid β protein (Aβ) in neuritic plaques and cerebral vessels.[1] Aβ (39–43 amino acids) is generated from a large transmembrane glycoprotein, amyloid β precursor protein (APP), by proteolytic processing. To date, there is no satisfactory treatment or prevention for AD. In the search for a safe and effective therapy, cholesterol-lowering statin drugs have emerged as a potential medication for AD.

Statins are a class of drugs that inhibit the biosynthesis of cholesterol.[2] Statins have been used successfully to treat hypercholesterolemia and to prevent cardiovascular disease. The connections between AD and vascular disease[3] suggested that statins may confer protection against AD. Retrospective studies have shown a reduced prevalence of AD in people taking statins.[4–9] Prospective studies, however, have produced mixed results. In a 26-week randomized, placebo-controlled, double-blinded trial, treatment with a statin drug (simvastatin) improved cognitive function and reduced $A\beta_{40}$ levels in the cerebrospinal fluid of normocholesterolemic patients.[10] In another study, atorvastatin treatment significantly slowed the decline in cognitive function of AD patients.[11] Other studies, however, showed no protection of statins in preventing AD in a group of patients at risk for cardiovascular disease.[12,13] Moreover, some studies showed a decrease of Aβ after statin treatments,[14,15] whereas others found no effects of statin treatments on Aβ levels.[16–18]

Nevertheless, potential benefits of statins in AD are suggested in in vitro and in vivo studies in which statins modulated APP metabolism. For example, treatment with statins affected activities of APP-processing enzymes in cells and in animal models, resulting in decreased Aβ formation.[19–21] Although the effect of statins on Alzheimer-type histopathology has been investigated in animal models,[19,21,22] the effect of statins on behavior in animal models of AD has not been addressed. In addition, although there are many reports on the potential use of statins for AD therapy, the effects of statins on cognitive functions in normal aging have not been investigated. Here, we studied the effects of a commonly used statin drug, simvastatin, on learn-

From the Departments of [1]Medicine, [2]Genetics, [3]Pathology, [4]Neurobiology, and [5]Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL; and [6]Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine at Peoria, Peoria, IL.

Received May 20, 2006, and in revised form Nov 2. Accepted for publication Nov 6, 2006.

Published online Dec 27, 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21053

Address correspondence to Dr Li, Atherosclerosis Research Unit, Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Alabama at Birmingham, 1530 3rd Avenue South, BDB 658, Birmingham, AL 35294. E-mail: lili@uab.edu

ing and memory in a transgenic mouse model of AD, Tg2576 mice[23] and their nontransgenic (non-Tg) littermates. We found that simvastatin treatment not only reversed learning and memory deficits in Tg2576 mice, but more intriguingly, enhanced learning and memory in normal non-Tg mice.

Materials and Methods

Animals and Diets

Tg2576 mice,[23] a widely used mouse model of AD, were used in this study. These mice overexpress the human APP with a Swedish double mutation (KM670/671NL) under the control of a hamster prion promoter on a SJLxC57BL/6 mixed genetic background.[23] They develop extracellular AD-type amyloid plaques in the cortex and hippocampus concurrent with learning and memory deficits by 11 months of age.[23] In this study, female Tg2576 mice (age, 11.1 $\pm$ 0.8 months) were divided randomly into two groups: the treatment group received simvastatin (added as a diet admixture [0.05%] so that approximately 50mg simvastatin/kg body weight was consumed daily) and the control group received the same diet without simvastatin. In addition to the Tg2576 mice, female nontransgenic littermates treated with or without simvastatin were also included in the study. The mice were treated for 3 months and then subjected to behavioral assessments followed by biochemical and histological analyses. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Assessment of Behavioral Functions

Three AD-related behavioral functions were assessed: spatial learning and memory, exploration of environmental stimuli, and anxiety. The testing schedule included exploration of the T-maze (days 1–10), the open field (days 1–3), the elevated plus-maze (days 4–5), and spatial learning in the Morris water maze (days 6–11). All equipment and software were purchased from SD Instruments (San Diego, CA).

All testing procedures were described previously.[24] In brief, spontaneous alternation was tested in a T-maze containing a central stem and two side arms. On the initial trial, the mice were placed in the stem with the right arm blocked (forced choice). After entering the available arm, the mice were kept in it for 60 seconds by closing the barrier behind them. The mice were then retrieved, and after removing the barrier were immediately placed back in the stem for a free-choice trial. On each of the following 9 days, the same procedure was repeated, except that the blocked arm on the initial trial was changed alternatively from the right to the left. The number of alternations and the latencies before responding were recorded with a cutoff period of 60 seconds per trial.

Motor activity was measured in an open field, made of white acrylic with a 50 $\times$ 50cm surface area and with each wall reaching 38cm in height. The activity was recorded by an overhead video camera and analyzed by video-tracking SMART software (SD Instruments). The mice were placed in the open field for a 5-minute session daily for 3 days.

Anxiety was measured in the elevated plus-maze, consisting of four arms in a cross-shaped form and a central region. Two of the arms were enclosed on three sides by walls, whereas the other two were not. The enclosed or open arms of the maze faced each other. The mice were placed in the central region and their behavior recorded for 5 minutes per session for 2 days. The number of entries and the time spent in either the enclosed or the open arms were measured.

Spatial orientation was evaluated in the Morris water maze consisting of a round basin (diameter, 112cm) filled with water (22°C) to a height of 31cm. The water was made opaque by mixing in dry milk to camouflage the escape platform (8 $\times$ 8cm). The pool was placed in a room with abundant extramaze visual cues. The acquisition of the spatial task consisted of placing the mouse in the water next to and facing the wall successively in north (N), east (E), south (S), and west (W) positions, with the escape platform hidden 1cm beneath water level in the middle of the NE quadrant. In each trial, the mouse was allowed to swim until it found the hidden platform or until 60 seconds had elapsed, at which point the mouse was guided to the platform. The mouse was then allowed to sit on the platform for 10 seconds before being picked up. The escape latency and swim path length (distance) were recorded by the SMART system for four trials daily for 5 days.

The day after the acquisition phase, a probe trial was conducted by removing the platform and placing the mouse next to and facing the N side. The time spent in the previously correct (target) quadrant was measured in a single 1-minute trial. Two hours later, the visible platform version was evaluated, with the escape platform lifted 1cm above water level and shifted to the SE quadrant. A pole (height, 7cm) was inserted on top of the escape platform as a viewing aid. In an identical manner to the place learning task, escape latencies and swim path length were measured for four trials, except that the visible platform test was conducted in a single day.

Determination of Plasma Cholesterol Concentrations and Transaminase Activities

Blood samples were collected from anesthetized animals by retroorbital bleeding or by cardiac puncture at the end of the experiment. Plasma total cholesterol and high-density lipoprotein cholesterol levels were determined colorimetrically with commercial reagents (Infinity cholesterol reagent; Thermo Electron Corporation, Melbourne, Australia). Plasma glutamic oxalacetic transaminase/aspartate aminotransferase activities were determined with the Transaminase Reagents (Sigma Diagnostics, St. Louis, MO).

Brain Tissue Preparation

Mice were anesthetized with sodium pentobarbital, and blood was collected via cardiac puncture with heparin as an anticoagulant. After transcardial perfusion with ice-cold 0.1M phosphate-buffered saline (pH 7.4), brains were cut sagittally into left and right hemispheres. The right hemisphere was fixed in phosphate-buffered formalin for histological analysis. After removing the olfactory lobe and cerebellum, the left hemisphere was snap frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

Brain Cholesterol, Aβ Enzyme-Linked Immunosorbent Assay, and Immunoblot Analysis

The protocol for brain cholesterol analysis was described previously.[25] In brief, frozen brain samples were Dounce homogenized in 5M guanidine hydrochloride. The homogenate was rocked for 3 to 4 hours at room temperature and then diluted for cholesterol analysis with the Infinity cholesterol reagent (Thermo Electron Corporation, Melbourne, Australia). The levels of $A\beta_{40}$ and $A\beta_{42}$ in the homogenate were determined with $A\beta_{40}$- and $A\beta_{42}$-specific enzyme-linked immunosorbent assay kits (BioSource International, Camarillo, CA) using the manufacturer's protocol. For immunoblot analysis, frozen brain samples were homogenized in sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma, St. Louis, MO), boiled for 5 minutes, then sheared by passage several times through a 26-gauge needle. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of proteins for each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted to polyvinyl difluoride membranes. The membranes were incubated with primary antibodies followed by biotinylated or horseradish peroxidase–conjugated secondary antibodies. Signal was detected by the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA) and quantified by densitometric scanning using the LabWorks image acquisition and analysis software (UVP, Upland, CA). For a loading control, the blots were stripped and reprobed with a mouse anti-actin monoclonal antibody (Sigma, St. Louis, MO). Primary antibodies used for immunoblot analysis included goat polyclonal antibody against mouse apolipoprotein E (ApoE) and PP2B (calcineurin) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibodies against Akt (protein kinase B) (Santa Cruz Biotechnology) and phospho-Akt (Ser473) (Cell Signaling, Danvers, MA), and mouse monoclonal antibody against endothelial nitric oxide synthase (eNOS) (BD Biosciences, San Jose, CA).

Immunohistochemical Analysis and Quantification of Cerebral β-Amyloidosis

Protocols for immunohistochemical analysis were described previously.[24] In brief, formalin-fixed and paraffin-embedded tissue sections were subjected to the avidin-biotin immunoperoxidase method to detect the antigens (eg, Aβ) using Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The primary antibody used for assessing β-amyloidosis was 6E10 (a monoclonal antibody raised against amino acids 1–16 of Aβ; Signet, Dedham, MA). The amyloid load in the cortex and hippocampus of mouse brain were quantified using the histomorphometry system consisting of a Leica DMR research microscope (Leica, Deerfield, IL) equipped for fluorescence, polarizer/analyzer, and bright-field microscopy; a SPOT RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI); and the Image Pro Plus v4 image analysis software (Media Cybernetics, Silver Spring, MD) capable of color segmentation and automation via programmable macros. Multiple images of $1mm^2$ each were captured and analyzed from five coronal brain sections at 500μm intervals from each mouse using a 10× objective lens. A total area of $50mm^2$ giving the highest total Aβ immunoreactivity was chosen to calculate the amyloid load expressed as a percentage of total area covered by Aβ immunoreactivity.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. Comparison of different treatment groups was performed by two-tailed Student's *t* test (for normally distributed data), Mann–Whitney rank-sum test (for nonnormally distributed data), and repeated-measures analysis of variance. In T-maze and probe tests, each group was compared by Mann–Whitney rank-sum test with a theoretical group performing at chance, defined as 50% for the two-choice T-maze test and 25% for the four-choice probe trial. The SigmaStat software (SPSS Science, Chicago, IL) was used for all statistical analyses. $p < 0.05$ was considered statistically significant.

Results

Simvastatin Treatment Decreased Plasma Lipoprotein Cholesterol Levels without Hepatic Toxicity

During the entire study, there were no differences in food intake, body weight, physical appearance, behavior in cage or handling, morbidity, or mortality among groups. Plasma total cholesterol concentrations in mice treated with simvastatin were significantly lower than those of mice on a control diet in both Tg2576 and non-Tg mice (Table 1). Plasma transaminase activity, a

Table 1. Plasma Cholesterol Levels and Transaminase Activities

Plasma Parameters	Treatment Groups			
	Tg2576-C (n = 19)	Tg2576-S (n = 20)	Non-Tg-C (n = 8)	Non-Tg-S (n = 9)
TC, mg/dl	59.6 ± 3.7	50.3 ± 4.2[a]	65.4 ± 4.4	50.6 ± 2.2[b]
HDL-C, mg/dl	57.8 ± 3.6	50.7 ± 3.5	64.7 ± 4.9	48.4 ± 3.7[a]
GOT/AST, IU/L	39.4 ± 3.6	44.6 ± 3.4	41.0 ± 4.5	40.6 ± 3.3

[a]$p < 0.05$ and [b]$p < 0.01$ compared with control mice.

Tg2576-C = untreated control Tg2576 mice; Tg2576-S = simvastatin-treated Tg2576 mice; Non-Tg-C = untreated control nontransgenic mice; Non-Tg-S = simvastatin-treated nontransgenic mice; TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol; GOT/AST = glutamic oxalacetic transaminase/aspartate aminotransferase.

marker of hepatic toxicity, did not differ among groups (see Table 1), indicating that long-term treatment with daily dose of simvastatin at 50mg/kg body weight was not hepatotoxic in these mice.

Simvastatin Treatment Reversed Spatial Learning and Memory Deficits in Tg2576 Mice and Enhanced Cognitive Functions in Nontransgenic Mice

Because anterograde amnesia is one of the earliest features of AD,[26] the assessment of spatial learning and memory is central to AD diagnosis. Like AD patients, Tg2576 mice display age-related learning and memory deficits.[23] To assess the effect of simvastatin treatment on spatial learning and memory ability of the mice, we conducted the Morris water maze test.[27] The results showed that simvastatin-treated Tg2576 mice acquired the submerged platform significantly faster than untreated control Tg2576 mice (Fig 1A; $F_{(1,152)} = 8.0$; $p = 0.008$). The concurrent shorter path length (distance) traveled by the simvastatin-treated mice indi-

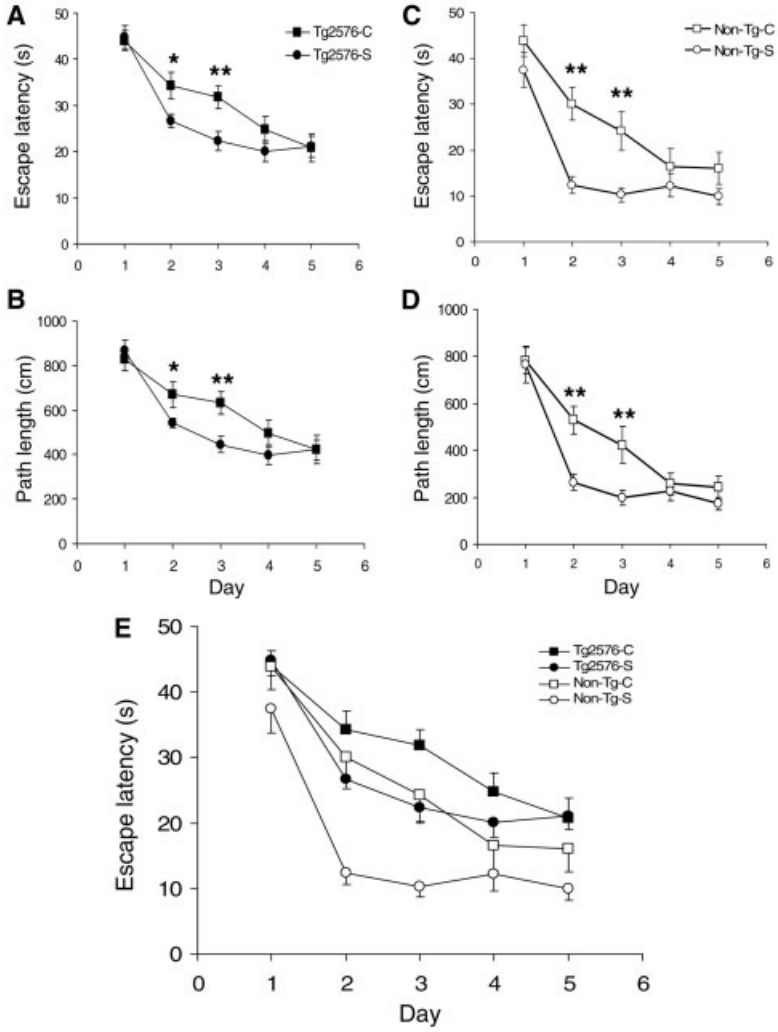

Fig 1. Simvastatin treatment improves performance of Tg2576 and nontransgenic (non-Tg) mice in the acquisition phase of the Morris water maze. (A, B) Escape latencies and path lengths of untreated Tg2576 control (Tg2576-C; solid squares; *n = 20) and treated Tg2576 (Tg2576-S;* solid circles; *n = 20) mice, showing that Tg2576-S mice learned the location of the hidden platform significantly faster than Tg2576-C mice. (C, D) Escape latencies and path lengths of untreated non-Tg control (Non-Tg-C;* open squares; *n = 9) and treated non-Tg (Non-Tg-S;* open circles; *n = 10) mice. Whereas non-Tg-C mice gradually learned the location of the hidden platform, non-Tg-S mice quickly learned the location of the platform with minimal escape latency in the second day of the trial and retained the memory over the next 4 testing days. (E) Superimposition of combined data from A and C. Tg2576-C mice displayed spatial learning deficits in the Morris water maze test compared with normal non-Tg-C mice, whereas Tg2576-S performed similarly as non-Tg-C mice, indicating that simvastatin treatment reversed the learning deficits of Tg2576 mice. In non-Tg mice, non-Tg-S showed superior performance to non-Tg-C mice, indicating that simvastatin treatment further enhanced normal spatial learning and memory. *p < 0.05; **p < 0.01. Error bars indicate standard error of the mean.*

cated a spatial learning improvement as opposed to an increased swimming speed (see Fig 1B; $F_{(1,152)} = 5.0$; $p = 0.031$).

Intriguingly, in non-Tg normal mice, simvastatin treatment resulted in a profound enhancement of spatial learning and memory ability (see Figs 1C, D). In the acquisition phase, although untreated non-Tg mice learned the location of the hidden platform in the course of the 5-day trial, simvastatin-treated non-Tg mice learned the location of the platform significantly faster: The treated mice reached minimal escape latency in the second day of the trial and retained the memory over the next 4 days (see Fig 1C; $F_{(1,68)} = 11.0$; $p = 0.004$). Again, the shorter path length ($F_{(1,68)} = 7.6$; $p = 0.013$) displayed by the simvastatin-treated mice indicated a true spatial learning enhancement as opposed to an increased swimming speed (see Fig 1D). Superimposition of the combined data (see Fig 1E) showed that simvastatin treatment reversed the spatial learning deficits of Tg2576 mice and enhanced learning capability in non-Tg normal mice.

In the 60-second probe trial for memory retention 24 hours after the acquisition, with the exception of the untreated Tg2576 mice, the other three groups of mice spent significantly greater than chance (25%) of their time in a previously platform-containing (target) quadrant ($p < 0.001$) (Fig 2A). Although the difference between treated and control mice did not reach statistical significance, the general trend was that the simvastatin treatment improved memory retention in both Tg2576 mice and non-Tg mice. A possible reason for nonsignificant effect of simvastatin treatment on probe trial performance is that the probe trials were conducted after all groups of mice had learned the location of the hidden platform in the 5-day acquisition phase of the test. Inside the target quadrant, consistent with the time spent there, simvastatin-treated mice crossed over the previous platform location more often than untreated control mice, indicating that simvastatin-treated mice remembered the platform location more precisely (see Fig 2B). In the visible platform version of the Morris water maze, simvastatin-treated and untreated mice performed similarly, indicating that there were no changes of visual acuities and swimming strategies associated with the simvastatin treatment.

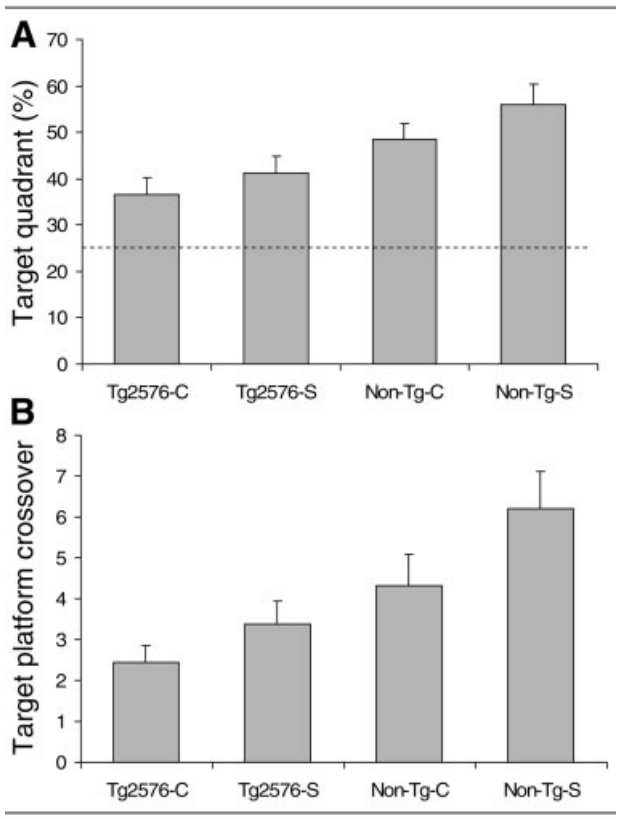

Fig 2. Performance of Tg2576 and nontransgenic (non-Tg) mice in the probe trial (memory retention phase) of the Morris water maze. (A) Percentage time spent in the previously platform-containing (target) quadrant. The dashed line *indicates the chance level (25%) of performance. Except for the untreated control Tg2576 mice (Tg2576-C), the three other groups of mice spent significantly longer than chance of their time in target quadrant* ($p < 0.001$). *(B) Times crossed over the location of previously hidden platform in the target quadrant. Simvastatin-treated mice (Tg2576-S and non-Tg-S) crossed over the previous platform location more often than their untreated control mice (Tg2576-C and non-Tg-C), respectively, indicating that simvastatin treatment improved memory precision in both Tg2576 and non-Tg mice. Error bars indicate standard error of the mean.*

Simvastatin Treatment Restored Habituation of Tg2576 Mice in an Open Field but Had No Significant Effect on Spontaneous Alternations and Anxiety Levels

Before the Morris water maze test, the mice were assessed for motor activity in an open field, willingness to explore the environment in a T-maze, and anxiety in an elevated plus-maze. In the open-field test, there were no differences in activity levels among the four different groups on day 1 (Fig 3). Untreated Tg2576 mice, however, were more active than mice in any other group during the last 2 days ($p < 0.05$). Contrary to the untreated Tg2576 mice, simvastatin-treated Tg2576 mice showed significant day effects for path length ($p < 0.001$), decreasing in the latter 2 days as a result of habituation (see Fig 3). Non-Tg mice, regardless of treatment, also exhibited significant day effects ($p < 0.001$). Thus, statin treatment restored habituation of Tg2576 mice in the open field.

In the T-maze test, no differences were observed in spontaneous alternations among different groups (Table 2). All four groups of mice alternated significantly higher than the 50% chance level ($p < 0.05$). In the

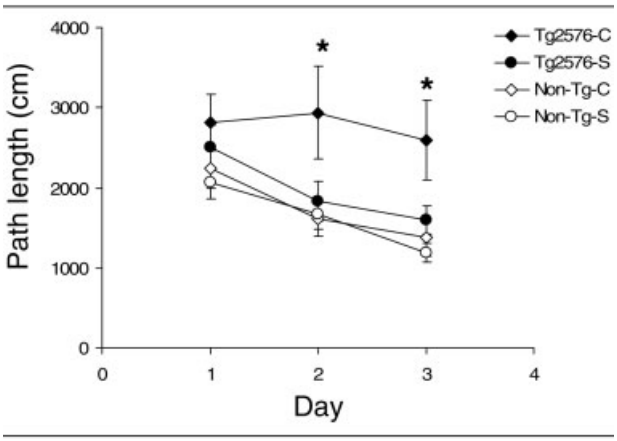

Fig 3. Simvastatin treatment restores intersessional habituation of Tg2576 mice in the open-field test. Untreated Tg2576-C mice (solid diamonds) *were more active than mice in any other groups and did not show intersessional habituation. Simvastatin-treated Tg2576-S mice* (solid circles), *in contrast, performed similarly as non-Tg mice and displayed intersessional habituation shown by decreasing activity in the latter 2 days of testing. Simvastatin treatment had no effect on the activity of non-Tg mice in the open field.* $^*p < 0.05$. *Error bars indicate standard error of the mean.* Open diamonds *represent Non-Tg-C;* open circles *represent Non-Tg-S.*

elevated plus maze tests, whereas Tg2576 mice showed decreased anxiety (more entries to open arms and spent more time there) compared with nontransgenic mice, as observed previously,[28] simvastatin treatment did not affect performance regarding these behavioral functions for either Tg2576 or non-Tg mice (see Table 2). These data thus indicated that enhancement of learning and memory by simvastatin treatment was not due to effects on willingness to explore or anxiety levels.

Simvastatin Treatment Had No Significant Effect on the Steady-State Levels and Depositions of β-Amyloid in the Brain of Tg2576 Mice

To determine whether simvastatin treatment affected the extent of cerebral amyloidosis in the brain of Tg2576 mice, we conducted two independent assays: $A\beta_{40}$- and $A\beta_{42}$-specific enzyme-linked immunosorbent assays were used to quantify the Aβ levels in the cerebral homogenate, and immunohistochemical and morphometrical analyses were used to quantify the Aβ depositions in the brain sections. The results showed that neither Aβ levels nor depositions in the brain differed between simvastatin-treated and untreated control Tg2576 mice (Figs 4A, B). These data indicated that simvastatin improved learning and memory functions independent of amyloidosis status in the brain of Tg2576 mice.

Simvastatin Treatment Did Not Affect the Steady-State Levels of Cholesterol and Apolipoprotein E in the Brain

To determine whether simvastatin treatment affected cholesterol homeostasis in the brain, we measured total cholesterol content of the cerebral homogenate. The results showed no significant differences in cholesterol content between the brains of treated and untreated mice (18.7 ± 0.6 vs 17.7 ± 0.5μg/mg cerebral wet weight). Western blot analysis of ApoE content in the brain also did not show any differences because of simvastatin treatment (Fig 5).

Effect of Simvastatin Treatment on the Steady-State Levels of Memory-Related Signaling Molecules in the Brain

The observation that simvastatin treatment resulted in significant enhancement of learning and memory in

Table 2. Exploratory Activities and Anxiety Levels

Tests	Treatment Groups			
	Tg2576-C (n = 20)	Tg2576-S (n = 20)	Non-Tg-C (n = 9)	Non-Tg-S (n = 10)
Spontaneous alternation (T-maze)				
Rate, %	61.0 ± 3.2	60.0 ± 2.7	65.6 ± 5.0	73.0 ± 5.0
Latency, sec	4.3 ± 1.0	6.8 ± 1.9	10.1 ± 4.3	3.7 ± 1.0
Anxiety (elevated plus-maze)				
Entries to open arms				
Day 1	11.6 ± 1.6	10.9 ± 1.2	6.9 ± 1.6	8.4 ± 0.5
Day 2	9.4 ± 2.0	6.6 ± 0.8	5.3 ± 2.1	5.4 ± 0.8
% Time in open arms				
Day 1	53.1 ± 6.2	51.3 ± 4.8	25.3 ± 6.2	26.1 ± 3.6
Day 2	25.7 ± 5.7	21.0 ± 3.6	13.1 ± 5.5	11.3 ± 2.1

Tg2576-C = untreated control Tg2576 mice; Tg2576-S = simvastatin-treated Tg2576 mice; Non-Tg-C = untreated control nontransgenic mice; Non-Tg-S = simvastatin-treated nontransgenic mice.

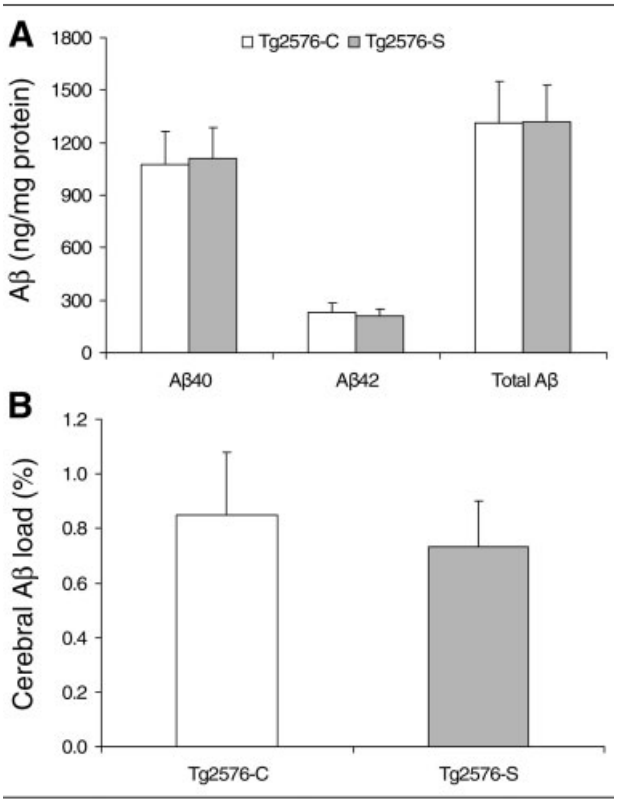

Fig 4. Simvastatin treatment does not change the status of cerebral amyloidosis in Tg2576 mice. (A) Cerebral amyloid β protein (Aβ) levels measured by enzyme-linked immunosorbent assay. The steady-state levels of $A\beta_{40}$, $A\beta_{42}$, and total Aβ did not differ between control (Tg2576-C; open bars*) and simvastatin-treated (Tg2576-S;* gray bars*) mice. (B) Cerebral Aβ loads determined by immunohistochemical and morphometric analyses, showing no significant differences between Tg2576-C and Tg2576-S mice. Error bars indicate standard error of the mean.*

both Tg2576 and non-Tg mice without causing significant changes on brain Aβ and cholesterol/ApoE levels led us to hypothesize that simvastatin might have directly modulated signal transduction pathways pertinent to the learning and memory processes through its cholesterol-independent (pleiotropic) effects. One of the known pleiotropic effects of statins is increased protein kinase B (Akt) activity resulting from inhibition of mevalonate production and activation of phosphoinositol 3-kinase.[29] Akt, particularly the phosphorylated Akt (P-Akt), is involved in regulating a wide range of biological functions[30]; more recently, it has been implicated in synaptic plasticity and spatial memory formation.[31,32] Thus, we assessed the levels of total Akt and P-Akt by immunoblot analysis of cerebral homogenates. We found that chronic simvastatin treatment increased both total Akt and P-Akt levels in the brains of treated mice (see Fig 5).

Another major pleiotropic effect of statins is upregulation of eNOS resulting from decreased production of isoprenoids such as geranylgeranylphosphate and subsequent inhibition of intracellular trafficking and activity of the small guanosine triphosphate–binding protein Rho.[29,33] Some of the neuroprotective effects of statins have been attributed to an increase in nitric oxide (NO) bioavailability and improved endothelial function resulting from the upregulation of eNOS.[34,35] NO has also been suggested to act as a potential retrograde messenger that modulates synaptic function during memory formation.[36] We observed an increase of steady-state levels of eNOS in the brains of simvastatin-treated mice (see Fig 5).

Calcineurin (PP2B) is a phosphatase and has been shown to be a negative regulator in memory formation.[37] Use of calcineurin inhibitors is associated with

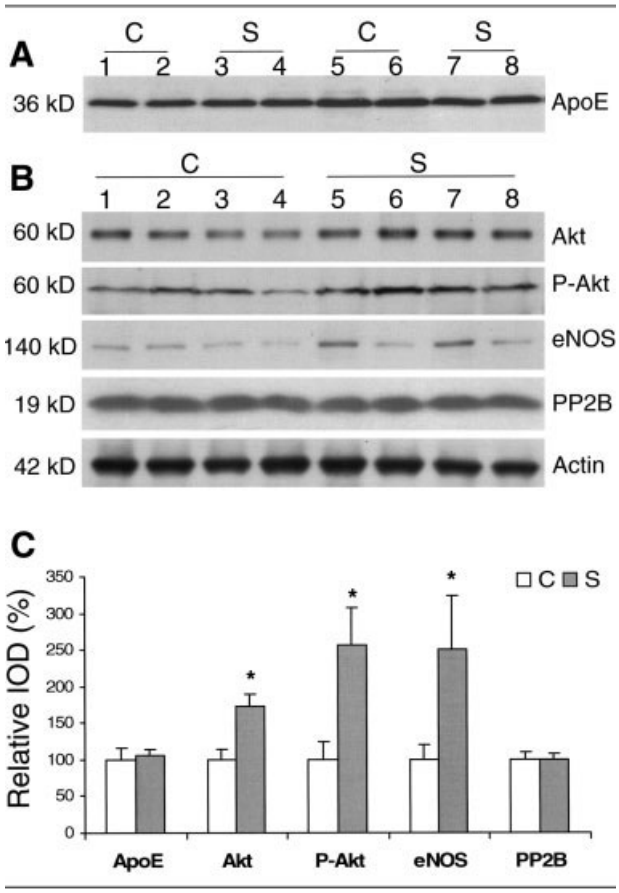

Fig 5. Steady-state levels of cerebral apolipoprotein E (ApoE), Akt, phosphorylated Akt (P-Akt), endothelial nitric oxide synthase (eNOS), and PP2B in nontransgenic mice. (A) Immunoblot analysis of ApoE. (B) Images of immunoblots with antibodies against Akt, P-Akt, eNOS, and PP2B, respectively. (C) Densitometric analysis of immunoblots with the levels in control (C; open bars) *mice set as 100%. Levels of ApoE were not affected by simvastatin* (S; gray bars) *treatment. Levels of Akt, P-Akt, and eNOS were increased significantly in simvastatin-treated mice. Levels of PP2B did not differ between control and simvastatin-treated mice.* $^*p < 0.05$. *Error bars indicate standard error of the mean.*

cardiovascular complications,[38] and simvastatin treatment attenuates calcineurin inhibitor–induced vascular dysfunction.[39] To determine whether simvastatin treatment had any effect on cerebral PP2B, we measured the level of PP2B in the brains of our experimental mice. Our results showed no significant differences in the steady-state level of cerebral PP2B between simvastatin-treated and untreated mice (see Fig 5), excluding the involvement of PP2B in modulating learning and memory in these mice.

Discussion

In this study, we showed that long-term treatment of simvastatin reversed spatial learning and memory deficits in Tg2576 mice and, more intriguingly, enhanced learning and memory abilities in normal non-Tg mice. These beneficial effects of simvastatin on cognitive function were not associated with any significant changes in noncognitive function such as willingness to explore and anxiety levels in treated mice.

To our knowledge, there have been no other studies on the effects of statins on behavioral function in transgenic mouse models of AD. A few reports, however, showed that statins (simvastatin and atorvastatin) promoted the restoration of spatial learning and memory after hypoxic-ischemic or traumatic brain injuries in rats.[40–42] Recently, lovastatin was shown to reverse the learning and attention deficits in a mouse model of neurofibromatosis type 1.[43]

To address the potential mechanisms by which simvastatin exerted cognition protective effects, we analyzed several physiological parameters in this study. Cholesterol analyses showed that the simvastatin treatment reduced the plasma total cholesterol levels by 16 and 23% in Tg2576 and non-Tg mice, respectively. The brain cholesterol content, however, was not affected by the simvastatin treatment. The steady-state levels of ApoE in the brain also did not change. In previous animal studies with statin treatments, results on brain cholesterol contents were conflicting. One study showed a slight reduction of cholesterol levels in the brains of young C57BL/6 mice treated with simvastatin and pravastatin, but not with lovastatin.[44] In the PS1 (presenilin 1)/APP double-transgenic mice, atorvastatin treatment had no effect on brain cholesterol levels even though the plasma cholesterol levels were decreased by 59%.[21] In guinea pigs, no changes in the total brain cholesterol levels were observed even with a high dose of statins (simvastatin and pravastatin).[19,45] In the latter studies, however, the levels of cholesterol precursor lathosterol and its ratio to cholesterol were significantly decreased in the brain of animals treated with statins,[19,45] indicating that statins can reduce brain cholesterol synthesis. Notably, however, changes in lathosterol levels were in the nanomolar range, whereas the total brain cholesterol levels were several orders of magnitude higher. Levels of lathosterol in the brains of our mice were not determined. It is possible that statins decrease brain cholesterol synthesis without significantly affecting brain total cholesterol contents because the turnover rate of cholesterol in the brain is very slow.[46] In addition, studies on synaptosomal plasma membranes showed that statins affected cholesterol distribution in certain microdomains in the membrane rather than the total cholesterol content.[47,48] Therefore, effects of statins on brain functions may not be reflected by the total cholesterol content in the brain.

One of the most intriguing findings in our study is that simvastatin improved spatial learning and memory without significantly affecting the Aβ levels in the brain of Tg2576 mice. Several in vitro studies have shown that statins modulated the processing of APP and decreased the production of Aβ in cells.[19,49] In animals, whereas two studies reported that statins (simvastatin and atorvastatin) decreased the production of Aβ in the brain of guinea pigs and in the PS1/APP double-transgenic mice,[19,21] one study showed that lovastatin increased Aβ production in female Tg2576 mice but had no effect in male Tg2576 mice.[22] In the study with guinea pigs,[19] the dose (0.5% in diet) of simvastatin was 10 times greater than what we used (0.05%) in this study. In a preliminary study, we found that a dose of simvastatin at 0.3% was toxic and lethal to Tg2576 mice (data not shown). In the study with PS1/APP double-transgenic mice, atorvastatin treatment was initiated at an age before the manifestation of amyloidosis in the brain.[21] In a preliminary study with a group of younger Tg2576 mice, we found that when simvastatin treatment was started at the age of 9 months (when no amyloid deposition could be detected), the Aβ levels in the brain were significantly decreased after 3 months of the treatment (data not shown). Therefore, whether statins reduce Aβ levels in the brain may depend on the age of the animals and the status of amyloidosis at the initiation of the statin treatment.

Reversal of learning and memory deficits by simvastatin treatment without affecting the Aβ levels suggests that the beneficial effect of simvastatin may be independent of molecular events downstream of Aβ. The dramatic enhancement of spatial learning and memory by simvastatin treatment in non-Tg mice is consistent with this concept. Whether statins can modulate learning and memory in normal aging awaits further investigation.

A large body of evidence has indicated that the beneficial effects of statins in preventing coronary heart disease extend beyond their plasma cholesterol-lowering ability.[50,51] This also appears to be the case in this study of the effects of simvastatin on learning and memory. Simvastatin has been shown to increase

phosphorylation of Akt at Ser473,[52] and this particular form of P-Akt is implicated in synaptic plasticity and memory consolidation in rats.[53] We found that chronic simvastatin treatment increased both total Akt and P-Ser473-Akt levels in the brain of mice. In other studies,[52,54] simvastatin treatment increased only the level of P-Akt, but not total Akt. Those studies, however, investigated only the acute (minutes to hours) effects of simvastatin treatment. We treated our mice for 3 months, and this long-term treatment appears to have changed the expression level of Akt, as well as the level of its posttranslational modifications such as phosphorylation. The importance of Akt in synaptic plasticity and memory has been demonstrated recently in a number of studies. Akt has been shown to mediate the effect of phosphoinositol 3-kinase on the expression of long-term potentiation in the hippocampal CA1 region of rats.[31] The level of P-Akt in the hippocampus of rats increases in parallel with spatial memory formation.[32] More recently, P-Ser473-Akt has been shown to be necessary for phosphoinositol 3-kinase–mediated synaptic plasticity and memory consolidation by promoting cell survival and protein synthesis in rats.[53] Therefore, an increase of Akt may be part of the mechanism by which simvastatin enhanced learning and memory in this study.

Another major cholesterol-independent effect of statins is in the upregulation of eNOS and a subsequent increase in NO bioavailability and improvement in endothelial function.[55] NO may also act as a potential retrograde messenger that modulates synaptic function during memory formation.[36] In addition, eNOS has been shown to generate NO within the postsynaptic cell during long-term potentiation.[56] Our results showed an increase of steady-state levels of eNOS in the brains of simvastatin-treated mice, which might also have contributed to the enhanced learning and memory in these mice.

Whether the changes of Akt and eNOS result from linked effects is uncertain. Although it is clear that both of these changes are related to the cholesterol-independent inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by simvastatin, changes in Akt are most likely caused by the reduced level of mevalonate, whereas changes in eNOS may be more related to the reduced level of geranylgeranylphosphate.[29] Because eNOS is a potential downstream substrate of Akt,[57,58] it is possible that an increased level of P-Akt (activated form) may further increase the activity of eNOS. Certainly, additional studies are needed to enable drawing of clear conclusions.

Finally, it is possible that other previously documented pleiotropic effects of statins such as antineuroexcitotoxic effects,[59,60] antiinflammatory effects,[61] antioxidant effects,[61] antiapoptotic effects,[44] and prosynaptogenesis effects[41,62] also facilitated learning and memory in the simvastatin-treated animals. Further biochemical and electrophysiological studies are under way to investigate the molecular and cellular mechanisms by which statins enhance learning and memory.

In conclusion, this study showed that simvastatin treatment reversed learning and memory deficits in Tg2576 mice independent of cerebral Aβ levels and enhanced cognitive function in normal-aged non-Tg mice. Our findings suggest that simvastatin may exert its beneficial effects directly through modulation of signaling pathways in memory formation.

This study was supported by the NIH (National Institute of Aging, AG025949, L.L.), the American Federation for Aging Research, 637417, L.L.; the Alzheimer's Disease Research Center of the University of Alabama at Birmingham, AG016582; the American Heart Association (0455304B, L.L.), and the Alzheimer's Association (IIRG-05-13139, L.L.).

We thank Dr K. Hsiao Ashe for providing Tg2576 breeders, Merck & Co. for providing simvastatin, Dr R. Lalonde for helpful discussion on the assessment of behavioral functions, and Dr P. Sanders for his careful reading of our manuscript.

References

1. Selkoe DJ. The genetics and molecular pathology of Alzheimer's disease: roles of amyloid and the presenilins. Neurol Clin 2000;18:903–922.
2. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986;232:34–47.
3. Casserly I, Topol E. Convergence of atherosclerosis and Alzheimer's disease: inflammation, cholesterol, and misfolded proteins. Lancet 2004;363:1139–1146.
4. Wolozin B, Kellman W, Ruosseau P, et al. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. Arch Neurol 2000;57:1439–1443.
5. Jick H, Zornberg GL, Jick SS, et al. Statins and the risk of dementia. Lancet 2000;356:1627–1631.
6. Hajjar I, Schumpert J, Hirth V, et al. The impact of the use of statins on the prevalence of dementia and the progression of cognitive impairment. J Gerontol A Biol Sci Med Sci 2002;57: M414–M418.
7. Rodriguez EG, Dodge HH, Birzescu MA, et al. Use of lipid-lowering drugs in older adults with and without dementia: a community-based epidemiological study. J Am Geriatr Soc 2002;50:1852–1856.
8. Rockwood K, Kirkland S, Hogan DB, et al. Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. Arch Neurol 2002;59: 223–227.
9. Zamrini E, McGwin G, Roseman JM. Association between statin use and Alzheimer's disease. Neuroepidemiology 2004;23: 94–98.
10. Simons M, Schwarzler F, Lutjohann D, et al. Treatment with simvastatin in normocholesterolemic patients with Alzheimer's disease: a 26-week randomized, placebo-controlled, double-blind trial. Ann Neurol 2002;52:346–350.
11. Sparks DL, Sabbagh MN, Connor DJ, et al. Atorvastatin for the treatment of mild to moderate Alzheimer disease: preliminary results. Arch Neurol 2005;62:753–757.

12. Shepherd J, Blauw GJ, Murphy MB, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002;360:1623–1630.
13. Collins R, Armitage J, Parish S, et al. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 2002;360:7–22.
14. Buxbaum JD, Cullen EI, Friedhoff LT. Pharmacological concentrations of the HMG-CoA reductase inhibitor lovastatin decrease the formation of the Alzheimer beta-amyloid peptide in vitro and in patients. Front Biosci 2002;7:a50–a59.
15. Sjogren M, Gustafsson K, Syversen S, et al. Treatment with simvastatin in patients with Alzheimer's disease lowers both alpha- and beta-cleaved amyloid precursor protein. Dement Geriatr Cogn Disord 2003;16:25–30.
16. Fassbender K, Stroick M, Bertsch T, et al. Effects of statins on human cerebral cholesterol metabolism and secretion of Alzheimer amyloid peptide. Neurology 2002;59:1257–1258.
17. Hoglund K, Wiklund O, Vanderstichele H, et al. Plasma levels of beta-amyloid(1-40), beta-amyloid(1-42), and total beta-amyloid remain unaffected in adult patients with hypercholesterolemia after treatment with statins. Arch Neurol 2004;61: 333–337.
18. Hoglund K, Syversen S, Lewczuk P, et al. Statin treatment and a disease-specific pattern of beta-amyloid peptides in Alzheimer's disease. Exp Brain Res 2005;164:205–214.
19. Fassbender K, Simons M, Bergmann C, et al. Simvastatin strongly reduces levels of Alzheimer's disease beta-amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. Proc Natl Acad Sci U S A 2001;98:5856–5861.
20. Kojro E, Gimpl G, Lammich S, et al. Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha-secretase ADAM 10. Proc Natl Acad Sci U S A 2001;98: 5815–5820.
21. Petanceska SS, DeRosa S, Olm V, et al. Statin therapy for Alzheimer's disease: will it work? J Mol Neurosci 2002;19: 155–161.
22. Park IH, Hwang EM, Hong HS, et al. Lovastatin enhances Abeta production and senile plaque deposition in female Tg2576 mice. Neurobiol Aging 2003;24:637–643.
23. Hsiao K, Chapman P, Nilsen S, et al. Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 1996;274:99–102.
24. Li L, Cao D, Garber DW, et al. Association of aortic atherosclerosis with cerebral beta-amyloidosis and learning deficits in a mouse model of Alzheimer's disease. Am J Pathol 2003;163: 2155–2164.
25. Cao D, Fukuchi KI, Wan H, et al. Lack of LDL receptor aggravates learning deficits and amyloid deposits in Alzheimer transgenic mice. Neurobiol Aging 2006;27:1632–1643.
26. Price DL. Aging of the brain and dementia of the Alzheimer type. In: Kandel ER, Schwartz JH, Jessell TM, eds. Principles of neural science. 4th ed. New York: McGraw-Hill, 2000.
27. Morris RG, Garrud P, Rawlins JN, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. Nature 1982;297: 681–683.
28. Lalonde R, Lewis TL, Strazielle C, et al. Transgenic mice expressing the betaAPP695SWE mutation: effects on exploratory activity, anxiety, and motor coordination. Brain Res 2003;977: 38–45.
29. Vaughan CJ. Prevention of stroke and dementia with statins: effects beyond lipid lowering. Am J Cardiol 2003;91:23B–29B.
30. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905–2927.
31. Sanna PP, Cammalleri M, Berton F, et al. Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. J Neurosci 2002;22:3359–3365.
32. Mizuno M, Yamada K, Takei N, et al. Phosphatidylinositol 3-kinase: a molecule mediating BDNF-dependent spatial memory formation. Mol Psychiatry 2003;8:217–224.
33. Laufs U, Liao JK. Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. J Biol Chem 1998;273:24266–24271.
34. Endres M, Laufs U, Huang Z, et al. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. Proc Natl Acad Sci U S A 1998;95:8880–8885.
35. Laufs U, Gertz K, Huang P, et al. Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice. Stroke 2000;31:2442–2449.
36. Malenka RC, Nicoll RA. Long-term potentiation—a decade of progress? Science 1999;285:1870–1874.
37. Kandel ER. The molecular biology of memory storage: a dialogue between genes and synapses. Science 2001;294: 1030–1038.
38. Moore R, Hernandez D, Valantine H. Calcineurin inhibitors and post-transplant hyperlipidaemias. Drug Saf 2001;24: 755–766.
39. Inman SR, Davis NA, Olson KM, Lukaszek VA. Simvastatin attenuates renal ischemia/reperfusion injury in rats administered cyclosporine A. Am J Med Sci 2003;326:117–121.
40. Balduini W, Mazzoni E, Carloni S, et al. Prophylactic but not delayed administration of simvastatin protects against long-lasting cognitive and morphological consequences of neonatal hypoxic-ischemic brain injury, reduces interleukin-1beta and tumor necrosis factor-alpha mRNA induction, and does not affect endothelial nitric oxide synthase expression. Stroke 2003; 34:2007–2012.
41. Lu D, Goussev A, Chen J, et al. Atorvastatin reduces neurological deficit and increases synaptogenesis, angiogenesis, and neuronal survival in rats subjected to traumatic brain injury. J Neurotrauma 2004;21:21–32.
42. Qu C, Lu D, Goussev A, et al. Effect of atorvastatin on spatial memory, neuronal survival, and vascular density in female rats after traumatic brain injury. J Neurosurg 2005;103:695–701.
43. Li W, Cui Y, Kushner SA, et al. The HMG-CoA reductase inhibitor lovastatin reverses the learning and attention deficits in a mouse model of neurofibromatosis type 1. Curr Biol 2005; 15:1961–1967.
44. Johnson-Anuna LN, Eckert GP, Keller JH, et al. Chronic administration of statins alters multiple gene expression patterns in mouse cerebral cortex. J Pharmacol Exp Ther 2005;312:786–793.
45. Lutjohann D, Stroick M, Bertsch T, et al. High doses of simvastatin, pravastatin, and cholesterol reduce brain cholesterol synthesis in guinea pigs. Steroids 2004;69:431–438.
46. Dietschy JM, Turley SD. Cholesterol metabolism in the brain. Curr Opin Lipidol 2001;12:105–112.
47. Eckert GP, Kirsch C, Mueller WE. Differential effects of lovastatin treatment on brain cholesterol levels in normal and apoE-deficient mice. Neuroreport 2001;12:883–887.
48. Kirsch C, Eckert GP, Mueller WE. Statin effects on cholesterol micro-domains in brain plasma membranes. Biochem Pharmacol 2003;65:843–856.
49. Simons M, Keller P, De Strooper B, et al. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc Natl Acad Sci U S A 1998;95:6460–6464.

50. Bellosta S, Ferri N, Bernini F, et al. Non-lipid-related effects of statins. Ann Med 2000;32:164–176.
51. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. Arterioscler Thromb Vasc Biol 2001;21:1712–1719.
52. Kureishi Y, Luo Z, Shiojima I, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 2000;6:1004–1010.
53. Horwood JM, Dufour F, Laroche S, Davis S. Signalling mechanisms mediated by the phosphoinositide 3-kinase/Akt cascade in synaptic plasticity and memory in the rat. Eur J Neurosci 2006;23:3375–3384.
54. Wolfrum S, Dendorfer A, Schutt M, et al. Simvastatin acutely reduces myocardial reperfusion injury in vivo by activating the phosphatidylinositide 3-kinase/Akt pathway. J Cardiovasc Pharmacol 2004;44:348–355.
55. Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 1998;97:1129–1135.
56. O'Dell TJ, Huang PL, Dawson TM, et al. Endothelial NOS and the blockade of LTP by NOS inhibitors in mice lacking neuronal NOS. Science 1994;265:542–546.
57. Fulton D, Gratton JP, McCabe TJ, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 1999;399:597–601.
58. Dimmeler S, Fleming I, Fisslthaler B, et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601–605.
59. Zacco A, Togo J, Spence K, et al. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors protect cortical neurons from excitotoxicity. J Neurosci 2003;23:11104–11111.
60. Bosel J, Gandor F, Harms C, et al. Neuroprotective effects of atorvastatin against glutamate-induced excitotoxicity in primary cortical neurones. J Neurochem 2005;92:1386–1398.
61. Cucchiara B, Kasner SE. Use of statins in CNS disorders. J Neurol Sci 2001;187:81–89.
62. Chen J, Zhang ZG, Li Y, et al. Statins induce angiogenesis, neurogenesis, and synaptogenesis after stroke. Ann Neurol 2003;53:743–751.