Published in final edited form as:

Neurobiol Dis. 2008 March ; 29(3): 422–437. doi:10.1016/j.nbd.2007.10.005.

Cholesterol retention in Alzheimer's brain is responsible for high β - and γ -secretase activities and A β production

Huaqi Xiong^{a,b}, Debbie Callaghan^a, Aimee Jones^a, Douglas G. Walker^c, Lih-Fen Lue^c, Thomas G. Beach^c, Lucia I. Sue^c, John Woulfe^b, Huaxi Xu^d, Danica B. Stanimirovic^{a,b}, and Wandong Zhang^{a,b,*}

^aNeurobiology Program, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6

^bFaculty of Medicine, University of Ottawa, Ottawa, Canada

^cSun Health Research Institute, Sun City, AZ, USA

^dThe Burnham Institute, La Jolla, CA, USA

Abstract

Alzheimer's disease (AD) is characterized by overproduction of A β derived from APP cleavage via β - and γ -secretase pathway. Recent evidence has linked altered cholesterol metabolism to AD pathogenesis. In this study, we show that AD brain had significant cholesterol retention and high β - and γ -secretase activities as compared to age-matched non-demented controls (ND). Over one-half of AD patients had an apoE4 allele but none of the ND. β - and γ -secretase activities were significantly stimulated *in vitro* by 40 and 80 μ M cholesterol in AD and ND brains, respectively. Both secretase activities in AD brain were more sensitive to cholesterol (40 μ M) than those of ND (80 μ M). Filipin-stained cholesterol overlapped with BACE and A β in AD brain sections. Cholesterol (10–80 μ M) added to N2a cultures significantly increased cellular cholesterol, β - and γ -secretase activities and A β secretion. Similarly, addition of cholesterol (20–80 μ M) to cell lysates stimulated both *in vitro* secretase activities. Ergosterol slightly decreased β -secretase activity at 20–80 μ M, but strongly inhibited γ -secretase activity at 40 μ M. Cholesterol depletion reduced cellular cholesterol, β -secretase activity and A β secretion. Transcription factor profiling shows that several key nuclear receptors involving cholesterol metabolism were significantly altered in AD brain, including decreased LXR- β , PPAR and TR, and increased RXR. Treatment of N2a cells with LXR, RXR or PPAR agonists strongly stimulated cellular cholesterol efflux to HDL and reduced cellular cholesterol and β -/ γ -secretase activities. This study provides direct evidence that cholesterol homeostasis is impaired in AD brain and suggests that altered levels or activities of nuclear receptors may contribute to cholesterol retention which likely enhances β - and γ -secretase activities and A β production in human brain.

Keywords

Alzheimer's disease; Cholesterol retention; Nuclear receptors; β - and γ -secretases; A β - production

Introduction

Alzheimer's disease (AD) is characterized by overproduction and deposition of β -amyloid peptides (A β) in the brain. A β peptide is derived from proteolytic cleavage of β -amyloid precursor protein (APP). Three secretases, α , β , and γ , are involved in APP processing (Walter et al., 2001; Selkoe, 2004). Sequential cleavage of APP by β - and γ -secretases yields either A β ₁₋₄₀ or A β ₁₋₄₂ peptide (Walter et al., 2001; Selkoe, 2004); whereas sequential α - and γ -secretase cleavage of APP does not generate A β . It is unknown which factor(s) determines the switch between the two APP processing pathways. Recent studies have linked altered cholesterol metabolism and increased A β production to AD pathogenesis (Miller and Chacko, 2004; Wolozin, 2001, 2004; Puglielli et al., 2005). Epidemiology studies have found that hypercholesterolemia is an early risk factor of AD, and decreased prevalence of AD is associated with use of cholesterol-lowering drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase inhibitors or statins) (Wolozin et al., 2000, 2004; Tan et al., 2003; Puglielli et al., 2005). Treatment with statins reduced serum A β peptides *in vivo* and the release of A β from cultured cells (Refolo et al., 2001; Sjogren et al., 2003; Friedhoff et al., 2001; Kojro et al., 2001). Experimental studies have shown that hypercholesterolemia accelerated AD pathology (Refolo et al., 2000), and cholesterol-lowering drugs reduced A β pathology in transgenic animal models (Refolo et al., 2001). All of these studies suggest a close correlation between cholesterol and AD pathogenesis. Cholesterol may thus be one of the factors that determine APP processing pathways and A β production in AD.

Since α -secretase is located in phospholipid-rich domains and both β - and γ -secretases reside in cholesterol-rich lipid rafts of plasma membrane (Wahrle et al., 2002; Cordy et al., 2003), it is believed that altered levels of cholesterol or/and ratio of cholesterol to phospholipids in cellular membrane could affect secretase activities and determine preferential APP processing pathways (Miller and Chacko, 2004; Wolozin, 2004; Kaether and Haass, 2004). Wolozin hypothesized that high cholesterol inhibits α -secretase and promotes β - and γ -secretase activities (Wolozin, 2001, 2004); while Kaether and Haass (2004) proposed that a moderate low-level of cholesterol increases A β production; whereas extremely poor-cholesterol environment inhibits A β production. Experimental studies yielded contradictory findings about cholesterol and A β generation. Simons et al. (1998) reported that cholesterol depletion inhibited A β production in hippocampal neurons. Cordy et al. (2003) showed that A β secretion was reduced when lipid rafts were disrupted by depleting cellular cholesterol. In another study, cloned β -secretase was purified and reconstituted *in vitro* with liposomes (Kalvodova et al., 2005). The study found that neutral glycosphingolipids, anionic glycerophospholipids, and cholesterol stimulated β -secretase activity. Wahrle et al. (2002) found that γ -secretase activity was located in cholesterol-rich membrane microdomains and was cholesterol-dependent in that cholesterol depletion inhibited γ -secretase activity and cholesterol replacement restored its activity. In contrary, Abad-Rodriguez et al. (2004) reported that cholesterol loss in hippocampal neurons enhanced A β generation. These studies provide the evidence that cholesterol affects β - and γ -secretase activities in cultured cells or reconstituted systems. However, little is known whether cholesterol metabolism and its regulation are altered in AD brain and whether cholesterol can regulate β - and γ -secretase activities in human brain tissue. Several studies suggested that nuclear receptors LXR or RXR affect the expression of a cholesterol transporter ABCA1 and therefore A β production in cultured cells or animals but these studies yielded controversial results (Fukumoto et al., 2002; Koldamova et al., 2003, 2005a,b; Sun et al., 2003). Nevertheless, there is no evidence whether levels or activities of these nuclear receptors are altered in AD brain or not. We hypothesize that cholesterol homeostasis and its regulatory mechanism are impaired in AD brain where the environment enhances β - and γ -secretase activities and APP is processed primarily via β - and γ -secretase pathway. In this report, we provide direct evidence that cholesterol metabolism is altered in AD brain and this alteration is typified by significant cholesterol retention and high levels of

β - and γ -secretase activities. The study demonstrates that cholesterol can regulate β - and γ -secretase activities in human brain and cultured cells. Altered levels or activities of nuclear receptors are likely responsible for impaired cholesterol homeostasis and consequent increases in β - and γ -secretase activities and A β production in AD brain.

Materials and methods

Cell cultures

Mouse neuroblastoma 2a (N2a) and K269 cells stably transfected with a human APP gene were generously provided by Dr. Huaxi Xu at the Burnham Institute and Dr. Dennis Selkoe at the Harvard Medical School, respectively. N2a cells were grown in media containing 50% Dulbecco's Modified Eagle's Medium (DMEM), 50% Opti-MEM, 1 $\times$ penicillin–streptomycin, 200 μ g/ml G418 (GIBCO/Invitrogen, Burlington, ON), and 10% fetal bovine serum (FBS) (HyClone, Logan, UT). K269 cells were grown in DMEM containing 10% FBS and 200 μ g/ml G418. For β - or γ -secretase activity assays, the cells were plated into 60 mm dishes and grown to 90% confluence. The cells were harvested and cell lysates were prepared using β - or γ -secretase lysis buffer from the β - or γ -secretase activity assay kits (R&D Systems, Minneapolis, MN) following manufacturer's instructions. To determine whether cholesterol could affect the activities of β - and γ -secretases in cells, 10–80 μ M of exogenous cholesterol (CAT# C8667, Sigma, Oakville, ON) (dissolved in ethanol) was added to cell cultures, and the cells and media were harvested at 1, 2, 4, and 6 h. Same amounts of solvent (ethanol) were added to control cultures. To deplete cellular cholesterol, the cells were treated with 2.5 mM methyl- β -cyclodextran (CDT) (Sigma) for 2 h. CDT was removed and fresh complete media were added to the cells. The cells and supernatant media were harvested at 4, 8, 12 and 24 h. The cells were also cultured in the presence of nuclear receptors LXR, RXR or PPAR- γ agonists. LXR agonists TO901317 (1 and 5 μ M, Cayman Chemical, Ann Arbor, MI) and 22-R-hydroxylcholesterol (22-RC, 10 μ M), RXR agonist 9-*cis*-retinoic acid (RA, 10 μ M), 22-RC + RA (10 μ M each) (Sigma, Oakville, ON), and PPAR- γ agonist troglitazone (TROG, 10 μ M) were added to cell cultures, respectively, and cells and supernatant media were harvested at 12, 24 and 36 h.

Immunocytochemistry and immunohistochemistry

The collection and use of patients' brain tissues in this study were approved by the Research Ethics Boards of the Sun Health Research Institute (Arizona, USA) and the National Research Council of Canada at Ottawa (Ottawa, Canada). Patients enrolled in an organ donation program at the Sun Health Research Institute were diagnosed by neurologists and neuropathologists before and after death, respectively. The donated brain tissue samples were harvested between 1 and 3.3 h after death, freshly frozen in liquid nitrogen and then stored at -80°C . These samples were collected between year 1999 and 2003. Many of the AD brain samples are characterized by large deposition of A β peptides in brain vascular system, a pathology known as cerebral amyloid angiopathy (CAA) (Preston et al., 2003; Weller and Nicoll, 2003). The brain tissues used in the study were all from occipital cortices, including 12 age-matched non-demented controls (ND) and 13 AD samples with CAA pathology (AD/CAA). Brain tissue samples were frozen-sectioned at 10 μ m thickness and cerebral vessels were visualized by staining with fluorescein-labeled lectin *Ulex Europaeus Agglutinin I* (UEA-I) as described previously (Mojsilovic-Petrovic et al., 2004). Immunohistochemistry was carried out following previously described (Mojsilovic-Petrovic et al., 2004; Zhang et al., 2003). A mouse monoclonal anti-A β antibody (IgG₁) 6E10 (SIGNET Lab, Deham, MA) (dilution 1:50) and a goat anti-mouse secondary antibody conjugated with Alexa 568 (dilution 1:400) (Molecular Probes, Eugene, OR) were used to detect A β deposits in brain parenchyma and vessels. A polyclonal rabbit anti-GFAP (glial fibrillary acidic protein) antibody (dilution 1:50) (CAT# BMDV2023, Accurate Chemical & Scientific Corp., Westbury, NY) and a FITC-conjugated

goat anti-rabbit secondary antibody (dilution 1:400) (Cat# F-2765, Molecular Probes, Eugene, OR) were used to visualize glial cells and gliosis in the brain sections. For BACE staining, the cells or brain tissue sections were incubated with a mouse monoclonal anti-BACE antibody (Cat# sc-33711, Santa Cruz Biotech) at 1:50 dilution and then with a goat anti-mouse secondary antibody conjugated with Alexa 568 (Cat# A-11031, Molecular Probes, Eugene, OR) (1:400 dilution).

Cholesterol content *in situ* was determined using cholesterol-binding fluorescent compound filipin as described (Abulrob et al., 2005). Cells or tissue sections were fixed for 10 min with 4% para-formaldehyde and then stained with 100 µg/ml filipin (Cat# F9765, Sigma) in phosphate-buffered saline for 15 min at 37 °C. Fluorescence of cholesterol-bound filipin was visualized by an Olympus microscope equipped with an UV filter set (360–370 nm excitation, 400 nm dichroic mirror and 425 nm long pass emission filter) as blue color.

Polymerase chain reaction to determine APOE alleles

Genomic DNA samples were isolated from cerebellum tissues of the patients following a standard procedure as described (Hixson and Vernier, 1990). Briefly, the tissues were digested with proteinase K and DNA samples were extracted with phenol-chloroform and precipitated with ethanol. Polymerase chain reaction (PCR) was carried out on DNA samples and HhaI restriction digestion was used to distinguish different apoE genotypes as described (Hixson and Vernier, 1990).

RT-PCR and real-time quantitative RT-PCR

Semi-quantitative RT-PCR and real-time quantitative RT-PCR (qRT-PCR) were used to determine the expression of *ABCA1*, *ABCG1*, and *LXR-β* in the brain tissues or cultured cells as described, respectively (Zhang et al., 1999, 2003). The primers were designed according gene sequences published in the GenBank (Table 1). RNA samples were isolated from brain tissue samples or cultured cells using Trizol (Invitrogen) following manufacturer's instructions. The quality of the samples was confirmed by RNA gel electrophoresis as described (Zhang et al., 1999, 2003). One microgram of RNA from each sample was used in reverse transcription (RT) reaction for first-strand cDNA synthesis and qRT-PCR using an ABI kit (Applied Biosystem Inc., Foster City, CA) was carried out and data analyzed against β-actin as described (Zhang et al., 2003) previously. Semi-quantitative RT-PCR was performed as described using β-actin as an internal control (Zhang et al., 2006). Densitometry analysis of the PCR gels were performed using a Kodak EDAS 290 Gel Documentation System and the Kodak 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT) following manufacturer's instructions.

Western blot

The lysates of human brain tissues or cell membrane fractions were prepared and equal amounts of proteins (50 µg) from each sample were separated on 10% SDS-PAGE gels as described previously (Zhang et al., 2006). The proteins were blotted to nitrocellulose membranes and Western blot analyses were carried out as described (Zhang et al., 2006). For detection of β-secretase (BACE), the membrane was incubated with a mouse monoclonal anti-human BACE antibody at 1:1000 dilutions (#sc-33711, Santa Cruz Biotech) overnight at 4 °C, washed with 1× TBST and then probed with a goat anti-mouse IgG-HRP (#170–6516, BioRad) at 1:3000 for 1 h at room temperature. The blots were washed with 1× TBST, incubated with ECL Plus (#RPN 2132, Amersham Bio-sciences Inc., Quebec, Canada) and exposed to film for 30 min. For detection of ABCA1, a rabbit polyclonal anti-ABCA1 antibody (#sc-20794, Santa Cruz Biotech, Santa Cruz, CA) and a HRP-conjugated goat anti-rabbit secondary antibody (#sc-2004, Santa Cruz Biotech) were used at 1:1000 and 1:5000 dilutions, respectively. ECL

Plus reagents were applied to the membranes which were then exposed to autoradiography films (Kodak) for 30 min.

Cholesterol assays

Cholesterol content in the brain tissue samples of ND and AD and cultured cells was evaluated by the fluorometric enzymatic method using an Amplex Red Cholesterol Assay kit (Molecular Probe/Invitrogen, Eugene, OR) with a minor modification as described (Abulrob et al., 2005). The assay is capable of quantifying both free cholesterol and cholesteryl esters. Briefly, the cell was washed with Hank's balanced salt solution (HBSS) (Sigma) after removing the medium. Cells were collected by centrifugation at 2000 rpm for 2 min at 4 °C and hydrolyzed with 250 µl of 1× cholesterol reaction buffer (0.1 M potassium phosphate, pH 7.4, 0.05 M NaCl, 5 mM cholic acid, 0.1% Triton X-100), and then boiled for 30 min to inactivate endogenous cellular cholesterol esterase. The samples were 1:10 diluted with 1× cholesterol reaction buffer. Fifty microliter of 150 µM Amplex Red reagent, 1 U/ml horseradish peroxidase, 1 U/ml cholesterol oxidase and 1 U/ml cholesterol esterase were added to 50 µL sample. After 30-min incubation in the dark at 37 °C, sample fluorescence was measured using a microplate reader FL600 (Bio-Tek Instruments, Winooski, Vermont) at 530/25 nm excitation and 590/35 nm emission wavelengths. To measure free cholesterol, cholesterol esterase was omitted from the assay. Values obtained from a cholesterol standard curve were normalized for protein content measured by a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Each sample was duplicated in the assays and at least three independent experiments were performed.

The levels of cholesterol in the brain tissue samples of ND and AD were also evaluated by a chemical method as described (Abell et al., 1952; Wiebe and Artiss, 1994). The following reagents were obtained from commercial sources, including absolute alcohol (Commercial Alcohols Inc., Brampton, ON), petroleum ether (Sigma), glacial acetic acid (EM Science), sulfuric acid (EM Science), acetic anhydride (Sigma), and potassium hydroxide (KOH) (Fisher Scientific). For KOH solution, 10 g KOH was dissolved in 20 ml of milli-Q water and kept at room temperature. Alcoholic KOH solution was freshly made before use by adding 6 ml of KOH solution to 94 ml of absolute alcohol. For standard cholesterol solution, 20 mg cholesterol at 0.4 mg/ml concentration (Sigma) was dissolved in 50 ml absolute alcohol and kept in −20 °C. For modified Liebermann–Burchard reagent, 20 volumes of acetic anhydride was chilled to below 10 °C in a glass tube, and one volume of concentrated sulphuric acid was added. The mixture was well shaken and kept cold. Then, 10 volumes of glacial acetic acid was added and the mixture was warmed to room temperature. The reagent was used within 1 h. Briefly, 200 mg human brain tissue was homogenized in 500 µl PBS and the lysate was kept at −20 °C. The homogenized sample (0.5 ml) was transferred to a 25-ml glass tube and 5 ml of alcoholic KOH was added to the tube, well shaken, and the mixture incubated in a water bath at 39 °C for 55 min. After cooling to room temperature, 10 ml of petroleum ether was added to each tube and mixed well with the content of the tube. Five milliliter of Milli-Q water was added to the tube and the tube was shaken vigorously for 1 min. The petroleum ether layer (4 ml) was transferred to a 10 ml glass tube. The petroleum ether was evaporated in a 60 °C water bath until dry. Liebermann–Burchard reagent (1 ml) was added to the tube and the entire inner surface of the tube was washed down with the reagent by vortex. The tube was stood at room temperature for 30 min in the dark. The sample (200 µl) was added to a 96-well plate in triplicate, and the absorbance was measured with a microplate reader at 620 nm (SPECTRAmax 340 Microplate Reader, Molecular Devices Corp., Sunnyvale, CA). For standard curve, 7.5 ml cholesterol standard sample solution (0.4 mg/ml) and 0.3 ml of KOH solution are mixed in a 25-ml glass tube and the mixture incubated for 55 min at 39 °C. Petroleum ether (15 ml) and water (7.5 ml) were added to the tube and then shaken vigorously for 1 min. After centrifugation, 0.5, 1, 2, 3, 4 ml of the petroleum ether layer were measured

out into a 10-ml glass tube separately and evaporated to dryness to provide standards equivalent to 0.1, 0.2, 0.4, 0.6, and 0.8 mg of cholesterol. Liebermann–Burchard reagent (1 ml) was added to each tube, the entire inner surface of the tube was washed down with the reagent by vortex, and the tube was stood at room temperature for 30 min (the samples should not be subjected to intense light during color development). The sample (200 μ l from each) was added to a 96-well plate (in triplicate), and absorbance was measured with the microplate reader at 620 nm. The Liebermann–Burchard reagent was set as 0 μ g cholesterol. The concentration of cholesterol was calculated as described (Abell et al., 1952).

In vitro activity assays for β - or γ -secretases

β -Secretase and γ -secretase activity assay kits were purchased from R&D Systems (Minneapolis, MN). The lysates of human brain tissues and cultured cells were prepared in β -secretase or γ -secretase lysis buffer following manufacturer's protocols. Protein concentrations of the samples were determined by a BioRad protein assay kit (Bio-Rad Lab). The lysate containing 25 μ g of total cellular protein from each sample was used and the assays were carried out following manufacturer's instructions. To test whether cholesterol affects the activities of β - or γ -secretases in the *in vitro* activity assay, exogenous cholesterol (10–80 μ M) (CAT#C8667, Sigma) (dissolved in ethanol) was directly added to the assays and the analyses were performed as above. β -secretase inhibitor III and γ -secretase inhibitor IV (CALBIOCHEM/EMD Biosciences Inc., La Jolla, CA) (dissolved in dimethyl sulfoxide, DMSO) were used in the assays to confirm that β - and γ -secretase activities are detected in human brain and cultured cells. As a control for cholesterol, ergosterol (CAT#45480, Sigma) (dissolved in ethanol) was added to β - and γ -secretase assays at 20, 40, and 80 μ M, respectively. Same amounts of solvent (ethanol or DMSO) were added to control assays. The assay results were normalized by protein concentration in each sample and expressed as activity (fluorescent units)/ μ g protein.

ELISA assays

Beta-amyloid 1–40 ELISA kit (Signet Lab, Deham, MA) was used to determine the levels of A β _{1–40} peptides released into supernatant media from cultured cells. The media collected at different time points were frozen at –80 °C until use. The media were thawed on ice and spun at 10,000 rpm for 10 min at 4 °C to remove cell debris. Supernatant media were diluted 10 times with 1 X dilution buffer and 100 μ l from each sample was used. The ELISA was carried out following manufacturer's instructions. The level of A β _{1–40} peptides in the samples was calculated according to the standard curve prepared on the same ELISA plates according to manufacturer's instructions.

Transcription factor profiling

Nuclear extracts were prepared from brain tissue samples of 10 ND and 10 AD patients using a Panomics Nuclear Extract kit (Panomics Inc., Fremont, CA) following manufacturer's instructions. Equal amounts of the 10 nuclear extracts from ND or AD were mixed and used in the profiling analysis. TranSignal Protein/DNA Array I (CAT# MA1210, Panomics Inc) was used to profile the activities of 54 different transcription factors in the brain tissues. The specific binding element for each transcription factor was spotted four times on the array blot, including two 1 $\times$ spots and two 10 $\times$ dilution spots. The array blots were exposed to X-ray films (Kodak) and the DNA binding activities of the transcription factors (4 replicate spots/transcription factor on each blot) were measured using Kodak EDAS 290 Gel Documentation System and Kodak 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT).

Cholesterol efflux assay

The assays were carried out following a modified protocol as described (Fukumoto et al., 2002; Koldamova et al., 2003; Hirsch-Reinshagen et al., 2004; Wang et al., 2004). Briefly, K269 cells were grown to 80% confluence and labeled overnight with 2 $\mu\text{Ci/ml}$ [1,2- ^3H]-cholesterol (PerkinElmer Life Science, Woodbridge, ON) in complete media. The media containing [1,2- ^3H]-cholesterol were removed, and the cells were washed and incubated (equilibrated) with plain medium DMEM plus 0.2% bovine serum albumin (BSA, Sigma) for 30 min. The media were removed, and fresh plain media containing 30 $\mu\text{g/ml}$ human HDL (CALBIOCHEM/EMD, La Jolla, CA), 1% human lipoprotein-deficient serum (Sigma), and 5 μM LXR agonist TO901317 were added to the cells and incubated for 8 h. The media were collected and centrifuged at 6000 $\times$ g for 10 min at 4 $^{\circ}\text{C}$ to remove cell debris and cholesterol crystals. The cells were lysed in 50–100 μl of 0.1 M sodium hydroxide (NaOH) and 0.1% SDS lysis buffer followed by incubation at room temperature for 20 min. 50 μl of media or cell lysate was added to scintillation vials and the radioactivity was determined by liquid scintillation counting. Protein concentration was determined by a BioRad protein assay kit (Bio-Rad Lab). Efflux was expressed as the percentage of radioactivity in the media relative to the total radioactivity in cells and medium.

Statistical analysis

Measurements in each assay were performed on at least two wells. Each experiment was repeated for 3–6 times. Results are expressed as mean $\pm$ S.D. Multiple sets of data were analyzed by one-way ANOVA, and student t test was used to analyze two sets of data. $p < 0.05$ is considered significant.

Results

Patients' information, immunostaining, and APOE ϵ 4 allele

The average age of ND and AD patients was 88.08 $\pm$ 5.24 and 83.85 $\pm$ 7.02 years, respectively. For 12 ND cases, there were 6 males and 6 females with an average age of 88.83 $\pm$ 13.2 and 87.33 $\pm$ 5.35 years, respectively. For 13 AD patients, there were 10 males and 3 female with an average age of 82.4 $\pm$ 6.8 and 88.67 $\pm$ 6.51 years, respectively.

Anti-A β staining was performed on brain tissue samples to confirm the absence or presence of A β peptide deposition in ND and AD brain samples, respectively. Large amounts of A β deposits were found to be associated with plaques and vessels in AD samples (Fig. 1). GFAP staining shows that the levels and distribution of GFAP in ND and AD brain samples were similar (data not shown). This suggests that there was no significant gliosis in AD brains, which was not a factor affecting the levels of cholesterol in the brain tissues.

Genotyping analysis revealed that, among 12 ND cases, 2 was *apoE*2/3 (16.7%) and 10 was *apoE*3/3 (83.3%); whereas among 13 AD cases, one was *apoE*2/3 (7.7%), five was *apoE* 3/3 (38.5%), six was *apoE* 3/4 (46.2%), and one was *apoE* 4/4 (7.7%). Seven of 13 AD patients (54%) had an *apoE*4 allele; while none of ND cases had an *apoE*4 allele (0%). These results suggest that *apoE*4 allele may be a risk factor of these AD patients.

AD brain exhibited significant cholesterol retention and high levels of β - and γ -secretase activities

Cholesterol has been suggested to be a risk factor of AD and to affect β - and γ -secretase activities in cells. Several experimental studies support the hypothesis (Wahrle et al., 2002; Cordy et al., 2003; Kalvodova et al., 2005; Abad-Rodriguez et al., 2004). However, little is known whether the levels of cholesterol or β - and γ -secretase activities are altered in AD brain as compared to ND brain tissues. Cholesterol measurement by the enzymatic assay showed

that the level of cholesterol content (including both free cholesterol and cholesteryl esters) in AD brain samples (9.43 ± 1.41 $\mu\text{g}/\text{mg}$ protein) was significantly higher than that in ND brain samples (7.9 ± 1.11 $\mu\text{g}/\text{mg}$ protein) (19.37% $\uparrow$, t test, $p = 0.0104$) (Fig. 2A). The total brain cholesterol was also measured by the chemical method, which was found to be significantly higher in AD brain samples (9.34 ± 1.02 $\mu\text{g}/\text{mg}$ protein) than that in ND brain samples (6.61 ± 1.66 $\mu\text{g}/\text{mg}$ protein) (41.3% $\uparrow$, t test, $p < 0.01$) (Fig. 2A). Both methodologies confirm that the level of total cholesterol was significantly higher in AD brain compared to ND samples. The levels of free cholesterol vs. total cholesterol (free cholesterol+cholesteryl esters) were measured by the enzymatic method in the absence or presence of cholesterol esterase. Free cholesterol comprises over 99% of the total brain cholesterol. This finding is consistent with the literature (Bjorkhem and Meaney, 2004). Since GFAP staining showed similar levels of GFAP in ND and AD brain samples, the high level of cholesterol in AD was unlikely a result of gliosis in AD samples. This suggests that altered cholesterol metabolism could be the true cause of increased cholesterol levels in these AD brain samples. Histological analysis shows that filipin-stained cholesterol overlapped with or surrounded beta-amyloid deposits (Fig. 1). *In vitro* activity assays using human brain lysates showed that both β - and γ -secretase activities were 22.2% and 39.8% higher in AD than in ND brain samples ($p < 0.01$) (Fig. 2C), respectively. The activities of both β -secretase and γ -secretase were strongly inhibited (25–30%) by 50 μM β -secretase inhibitor III (CAT#565780, CALBIOCHEM/EMD) or γ -secretase inhibitor IV (CAT# 565761, CALBIOCHEM/EMD), respectively (t test, $p < 0.01$). This confirms that the enzymatic activities detected in human brain samples were from β - or γ -secretases. Western blot analysis revealed that the levels of BACE protein were similar in ND and AD brain samples (data not shown). Therefore, high β -secretase activity in AD samples was not likely a result of increased BACE protein. These results suggest that high cholesterol level may be responsible for increased activities of both β - and γ -secretases in AD brain.

To determine whether cholesterol is capable of regulating β - and γ -secretase activities, different concentrations of exogenous cholesterol (20, 40, and 80 μM) was directly added to the lysates of ND and AD brain samples in the *in vitro* assays. Whereas cholesterol of 20 and 40 μM were ineffective, cholesterol at 80 μM concentration significantly stimulated β -secretase activity in ND samples (One-way ANOVA, $p < 0.0001$, Fig. 3A). Both 40 and 80 μM cholesterol significantly enhanced β -secretase activity in AD samples (One-way ANOVA, $p < 0.0001$, Fig. 3A). *In vitro* assays also showed that γ -secretase activity in ND samples was stimulated by 80 μM cholesterol, but its activity in AD lysates was only enhanced by 40 μM cholesterol (One-way ANOVA, $p < 0.0001$, Fig. 3B). The increased secretase activities were inhibited by β - or γ -secretase inhibitors, respectively (data not shown). Interestingly, cholesterol at 20 μM concentration inhibited γ -secretase activity in AD samples (One-way ANOVA, $p < 0.0001$, Fig. 3B). These results demonstrate that cholesterol at a certain range of concentrations could modulate the activities of both β - and γ -secretases in human brain tissues. Both β - and γ -secretases in AD brain were sensitive to a lower cholesterol concentration (40 μM); while a high cholesterol concentration (80 μM) was needed to stimulate the activities of β - and γ -secretases in ND brain. This may be due to higher level of existing cholesterol in AD brain samples. The localization of β -secretase (BACE) and its relationship to beta-amyloid deposits and cholesterol in AD brains were analyzed by immunohistochemistry (Fig. 4). BACE staining is associated with or surrounds beta-amyloid deposits in the AD brains (Figs. 4A–D). Filipin-stained cholesterol is well overlapped with BACE staining (Figs. 4E and F). These findings illustrate a close relationship between BACE, cholesterol, and $\text{A}\beta$ peptides in the AD brain.

Cholesterol increased β - and γ -secretase activities and $\text{A}\beta_{1-40}$ production in N2a cells

In order to confirm the observations on cholesterol and secretase activities obtained from human brain tissues, we studied whether cholesterol added to culture media could affect β - and γ -secretase activities in N2a cells overexpressing hAPP. Cholesterol added to culture media

can be incorporated into cell as shown by cholesterol labeling of K269 cells and efflux assays by us (see below) and others (Fukumoto et al., 2002; Koldamova et al., 2003; Hirsch-Reinshagen et al., 2004; Wang et al., 2004). Approximately 30–40% of [^3H]-labeled cholesterol added to culture media can be incorporated into cells. The incorporated cholesterol may affect cell membrane structure and lipid composition. Different concentrations of exogenous cholesterol (10, 20, 40, and 80 μM) were added to the media of N2a cell cultures, and the cells and media were harvested at 1, 2, 4 and 6 h. Cholesterol assay shows that the cellular cholesterol levels were significantly increased in cholesterol-treated cells as compared to the controls (One-way ANOVA, $p < 0.05$, Fig. 5A). Both β - and γ -secretase activities in cholesterol-treated cells were significantly higher than those in control cells (One-way ANOVA, $p < 0.05$, Fig. 5B and C). As a result of increased levels of cellular cholesterol and elevated activities of secretases, the secretion of $\text{A}\beta_{1-40}$ peptides into media from cholesterol-treated cells was significantly increased compared to controls (One-way ANOVA, $**p < 0.0005$, $***p < 0.0001$, Fig. 5D). It appears that exogenous cholesterol in culture media was more effective in increasing cellular cholesterol and stimulating the secretase activities and $\text{A}\beta$ secretion at earlier time points (1, 2 and 4 h). All of these results show that exogenous cholesterol from the environment can be incorporated into cells and modulate cellular β - and γ -secretase activities, resulting in increased $\text{A}\beta$ production.

The localization of β -secretase (BACE) in N2a and K269 cells was analyzed by immunocytochemistry (Figs. 4G and H). BACE staining is mostly associated with cell membrane (Figs. 4G and H). Some of the BACE staining is also associated with cellular organelle structures (Fig. 4H). It is known that BACE is located in the plasma membrane of endoplasmic reticulum (ER), Golgi apparatus and endosomal compartment (Kinoshita et al., 2003). The finding is consistent with the literature.

Exogenous cholesterol was not only added to culture media but was also tested directly in the *in vitro* assays for its effect on β - and γ -secretase activities. N2a cells were grown under normal conditions and harvested for lysate preparations. Different concentrations of exogenous cholesterol (10, 20, 40 and 80 μM) were mixed with cell lysates in the *in vitro* assays. β -secretase activity was significantly increased by direct addition of cholesterol (20, 40 and 80 μM) in the assay (One-way ANOVA, $p = 0.0198$, Fig. 6A). Cholesterol at 80 μM concentration was stronger than 20 and 40 μM in stimulating β -secretase activity. Cholesterol at 20, 40 and 80 μM concentrations also significantly enhanced γ -secretase activity (One-way ANOVA, $p < 0.0001$, Fig. 6B). The activity of γ -secretase was stimulated to a greater degree in the presence of 20 μM and 40 μM cholesterol than in the presence of 80 μM cholesterol. Both unstimulated and cholesterol-stimulated secretase activities were strongly inhibited by 5 μM β -secretase inhibitor III (~25%) and 10 μM γ -secretase inhibitor IV (~30%) (One-way ANOVA, $p < 0.0001$, Fig. 6C), respectively. This confirms that the activities detected in the assays were from cellular β - and γ -secretases. Neither 10 μM nor 160 μM cholesterol stimulated the activities of the two secretases in the *in vitro* assays (data not shown). As a control for sterol, ergosterol (Sigma CAT#45480, dissolved in ethanol) was added to the assays at 20, 40 and 80 μM , respectively. Ergosterol, also known as provitamin D₂, belongs to phytosterol and is chemically similar to cholesterol. This phytosterol at 20–80 μM concentrations slightly decreased β -secretase activity by 2–5% as compared to controls (not statistically significant) (Fig. 6A). However, ergosterol strongly decreased γ -secretase activity by 25% at 40 μM (One-way ANOVA, $p < 0.0001$) (Fig. 6B). The structures of ergosterol and cholesterol are similar but not identical, which may explain their different effects on secretase activities. The results from cultured N2a cells validate the findings from human brain samples and show that cholesterol at a certain range of concentrations can stimulate activities of both β - and γ -secretases, resulting in increased $\text{A}\beta$ production.

Cholesterol depletion reduced cellular cholesterol, β -secretase activity and $A\beta_{1-40}$ production in N2a cells

To determine whether cholesterol depletion would affect β -secretase activity and $A\beta$ production, N2a cells were treated with 2.5 mM β -methyl cyclodextran (CDT) for 2 h. The media containing CDT were removed, cells washed twice and fresh complete media were added. The cells and media were then harvested at 4, 8, 12, and 24 h thereafter for cholesterol assays, *in vitro* β -secretase activity assay, and $A\beta_{1-40}$ ELISA. Cellular cholesterol was significantly reduced at 4 h post-treatment (One-way ANOVA, $p=0.0442$), but then returned to the control level at 8, 12 and 24 h (Fig. 7A). β -secretase activity and $A\beta$ secretion were significantly decreased at 4 h (One-way ANOVA, $*p=0.0215$, $***p<0.0009$, respectively), but were slightly above the control levels at 8, 12 and 24 h (Fig. 7B and C). These results further confirm that modulation of cellular cholesterol levels affects secretase activities and $A\beta$ production in cells.

Expression levels of cholesterol efflux transporters ABCA1 and ABCG1 in ND and AD

ABCA1 is a large transmembrane protein and mediates cellular cholesterol efflux to apolipoproteins. Several *in vitro* studies suggested that ABCA1-mediated cholesterol efflux affect APP processing and $A\beta$ production in cells (Fukumoto et al., 2002; Koldamova et al., 2003, 2005a,b). In order to explore the link between ABCA1 and AD, we examined the expression of *ABCA1* at both the mRNA and protein levels in ND and AD brain tissues. Realtime qRT-PCR showed that the average levels of *ABCA1* expression were similar in AD and ND brain samples (data not shown). There was a marked variation of *ABCA1* expression among different AD and ND samples. Western blots using human brain lysates failed to detect the signal of ABCA1 in either ND or AD samples (data not shown). The expression of another cholesterol transporter *ABCG1* was also analyzed by qRT-PCR at the mRNA level. AD brains had a slightly increased expression of *ABCG1* as compared to ND controls, but it is not statistically significant (data not shown). All of the analyses on *ABCA1* and *ABCG1* expression were performed at brain tissue level but not at neuronal or glial cell levels.

Transcription factors involved in lipid metabolism were altered in AD brains

Our above experiments showed that cholesterol metabolism and homeostasis was altered in AD brain, resulting in significant cholesterol retention. A number of studies have shown that nuclear receptors play important roles in the regulation of cholesterol and lipid metabolism and in maintaining cholesterol homeostasis in cells and tissues (Schmitz and Langmann, 2005; Edwards et al., 2002; Francis et al., 2003). Nuclear receptors (NR) are ligand-inducible transcription factors composed of a superfamily with six subfamilies and a subfamily O and regulate various cellular activities (Francis et al., 2003). The ligand-activated nuclear receptors can form homodimer or heterodimers with each other and then bind to and activate transcription of the target genes in cells. Nuclear receptors involving cholesterol and lipid metabolism include liver X receptor (LXR), retinoid X receptor (RXR), retinoic acid receptors (RAR), peroxisome proliferation-activated receptors (PPAR), androgen receptors (AR), estrogen receptors (ER), progesterone receptors (PR), glucocorticoid receptors (GR), thyroid receptors (TR), vitamin D receptors (VDR), and sterol regulatory element-binding transcription factor (SREBF, also known as sterol regulatory element-binding protein or SREBP) (Francis et al., 2003). Among these receptors, LXR, RXR, and PPAR have been shown to regulate the expression of the genes encoding lipid/cholesterol transporters and apolipoproteins in cells (Francis et al., 2003; Murthy et al., 2002; Akiyama et al., 2002). The levels of these transcription factors were thus analyzed and compared in ND vs. AD brain samples using Tran-Signal Protein/DNA Array I (Panomics Inc). The array is capable of examining the binding activities of 54 different transcription factors, including almost all of the nuclear receptors described above. Profiling analysis shows that the binding activities of PPAR, TR, and VDR were decreased in AD over two fold as compared to ND brains; while the activities of RXR, AR, ER, and PR were increased

over two fold in AD brain samples as compared to ND samples (Figs. 8A and B). The levels of sterol regulatory binding-element transcription factor (SREBF/SREBP), GR and RAR were similar in ND and AD samples (Figs. 8A and B). Since 5 female and 5 male ND brain samples were used in the control group, and 7 male and 3 female AD brain samples were used in the test group, increased AR binding activity in AD group may not be a valid observation because more male samples were used. The role of two other sex hormone receptors (ER and PR) and vitamin D receptor (VDR) in brain cholesterol metabolism is not clear, but decreased PPAR and TR and increased RXR (Fig. 8C) may be more important and relevant in the regulation of brain lipid and cholesterol metabolism. Since LXR was not included in the TranSignal DNA/Protein Array I, we analyzed LXR- β expression by semi-quantitative RT-PCR. There are two LXR isoforms, LXR- α and LXR- β (Francis et al., 2003). RT-PCR shows that the expression of LXR- β was generally decreased in AD compared to ND samples (Fig. 9). Quantitative densitometry analysis shows that the average levels of LXR- β expression in ND and AD samples are $61.15 \pm 13.12\%$ and $39.48 \pm 9.62\%$, respectively, normalized against internal control β -actin (100%). The level of LXR- β is significantly lower in AD (100%) than in ND (156%) (*t* test, $p < 0.01$). These results suggest that altered levels or activities of the nuclear receptors may contribute to the disordered regulation of cholesterol metabolism, leading to cholesterol retention in AD brain.

LXR, RXR and PPAR agonists reduced cellular cholesterol and β - and γ -secretase activities in cells

LXR, RXR and PPAR are principal nuclear receptors that regulate the metabolism of lipids and cholesterol. A number of studies have shown that LXR or/and RXR agonists can up-regulate the expression of lipid/cholesterol metabolism-related genes and ABCA1 and therefore reduce cellular cholesterol (Fukumoto et al., 2002; Koldamova et al., 2003; Sun et al., 2003; Wang et al., 2004; Schmitz and Langmann, 2005). One study showed that LXR agonists increased ABCA1 expression and A β production in neural cells (Fukumoto et al., 2002). Two others reported that LXR agonists induced ABCA1 expression, but reduced A β production in cells (Koldamova et al., 2003; Sun et al., 2003). To clarify these contradictory results and evaluate the significance of our findings on altered nuclear receptors in AD brain, we used cultured cells to test whether LXR or/and RXR could affect the levels of cellular cholesterol and β - and γ -secretase activities. K269 or N2a cells were cultured in the presence of LXR agonists TO901317 (1 μ M and 5 μ M) or 22-*R*-hydroxylcholesterol (22-RC) (10 μ M), RXR agonist 9-*cis*-retinoic acid (RA) (10 μ M) or 22-RC (10 μ M) + RA (10 μ M) for 12, 24, and 36 h. Expression of LXR, RXR, ABCA1 and ABCG1 was strongly up-regulated in both cell types as measured by semi-quantitative RT-PCR (data not shown). Cholesterol efflux assay using K269 shows that 42.06% more [3 H]-labeled-cholesterol in cells treated with 5 μ M TO901317 for 8h was transported to HDL compared to controls (One-way ANOVA, $p < 0.0001$) (Fig. 10A). Treatments of N2a cells with the agonists strongly reduced cellular cholesterol (One-way ANOVA, $*p < 0.05$, Fig. 10B). As a result of reduced cellular cholesterol, both β - and γ -secretase activities were significantly decreased in the cells (One-way ANOVA, $*p < 0.05$, $**p < 0.01$, Fig. 10C and D). It appears that RXR agonist 9-*cis*-retinoic acid was less potent than LXR agonists (TO and 22-RC) in affecting β - and γ -secretase activities in cells (Fig. 10C and D). Treatment of N2a cells with 10 μ M PPAR- γ agonist troglitazone also significantly reduced the activities of both β - and γ -secretases (Oneway ANOVA, $*p = 0.0108$; $***p < 0.0001$) as compared to the controls (Fig. 11A and B). These results demonstrate that the activation of nuclear receptors LXR, RXR or PPAR can reduce cellular cholesterol and therefore inhibit the activities of both β - and γ -secretases and support the notion that the altered nuclear receptors play a role in disordered regulation of cholesterol metabolism, leading to cholesterol retention and high levels of β - and γ -secretase activities and A β production in AD brain.

Discussion

Altered cholesterol metabolism has been linked to AD patho-genesis. However, little is known whether cholesterol metabolism is altered in AD brain or not. This study provides direct evidence that altered cholesterol metabolism does exist in AD brain. There are two APP processing pathways, α - and γ -secretase and β - and γ -secretase pathways. Cholesterol may serve as a determinant for switching the two pathways. This is supported by the findings that AD brains had significant cholesterol retention and high levels of β - and γ -secretase activities and that cholesterol can regulate β - and γ -secretase activities in human brain and cultured cells. Compared to other tissues, brain is unique in terms of cholesterol metabolism and requirements. It contains 25% of a total body amount of unesterified cholesterol although it accounts for only 2% of the body mass, and over 99% of the total cholesterol is unesterified in the brain (Dietschy and Turley, 2001; Bjorkhem and Meaney, 2004). Brain tissue is separated from blood circulation and other tissues by the blood-brain barrier (Zhang and Stanimirovic, 2005), and its cholesterol is almost completely synthesized *de novo* (Dietschy and Turley, 2001; Pfrieger, 2003; Bjorkhem and Meaney, 2004). Glial cells are considered to be the main source of cholesterol synthesis in the brain (Pfrieger, 2003). Brain lipids are primarily present in cell membranes and are constantly replaced. Brain cholesterol is actively turned over among neuronal and glial cells and is essential for many important brain functions (Koudinov and Koudinova, 2001). Impaired cholesterol homeostasis in AD brain suggests that this fine-tuned regulation is disturbed. This could occur at several levels, including inefficient cholesterol redistribution, altered expression of cholesterol transporters and/or nuclear receptors in the brain.

Nuclear receptors, such as LXR, RXR and PPAR, play a central role in the regulation of cholesterol/lipid metabolism (Schitz and Langmann, 2005; Francis et al., 2003). However, little is known about the roles of these receptors in cholesterol metabolism of Alzheimer's brain. This study provides the first evidence that the levels or activities of LXR- β , RXR, PPAR and TR are altered in AD brain. PPAR has three isoforms, α , β , and γ . Activation of PPAR γ was shown to reduce cholesterol synthesis in hepatic and intestinal cells (Klopotek et al., 2006). PPAR γ agonists reduced A β levels in cultured cells and AD mice (Landreth, 2006). Several recent studies have found that TR activation has beneficial effects on serum cholesterol and weight loss (Johansson et al., 2005; Webb, 2004; Kraiem, 2005). There are two TR isoforms, TR α 1 and TR β 1, of which TR β 1 is more involved in lipid/cholesterol metabolism. Selective agonists have been designed to reduce cholesterol and weight (Webb, 2004; Kraiem, 2005). There are three RXR isoforms, α , β , and γ . Since RXR form heterodimers with LXR, PPAR and TR, the levels and ratios of RXR monomers may be important in the regulation of cholesterol/lipid metabolism. Increased activity of RXR in AD brain observed in this study may affect heterodimer formation and disturb the regulation of cholesterol metabolism. Thus, altered levels or activities of LXR- β , PPAR, RXR, and TR in AD brain may contribute to impaired cholesterol homeostasis, which leads to cholesterol retention and increased β -/ γ -secretase activities and A β genesis.

Another factor that may contribute to altered cholesterol metabolism in AD brain is ApoE. ApoE is a multifunctional protein with three isoforms (ApoE2, ApoE3, and ApoE4) and plays an important part in lipid metabolism/homeostasis (Huang, 2006). ApoE is involved in lipid redistribution in the brain and is a component of HDL found in cerebral spinal fluid (CSF) (Bjorkhem and Meaney, 2004; Huang, 2006). ApoE4 is known to be associated with a high risk of AD (Huang, 2006). The structure of ApoE4 is bulky and may not be efficient in mediating cholesterol transport and redistribution as compared to other ApoE isoforms (Huang Y, personal communication). A recent study showed that ApoE4 enhances A β production in cultured neuronal cells (Ye et al., 2005). Genotyping result from this study revealed that over

one-half of the AD patients examined had an *apoE4* allele but none of the ND. This suggests a possible role of ApoE in altered cholesterol metabolism in AD brain.

Inefficient cholesterol efflux from cells to apolipoproteins may also contribute to cholesterol retention in AD brain. One of the major cholesterol efflux transporters is ABCA1. Fukumoto et al. (2002) reported that induction of *ABCA1* expression in N2a cells significantly increased $A\beta_{1-40}$ and that RNAi knockdown of *ABCA1* reduced $A\beta_{1-40}$ secretion. In contrast, two other studies showed that induction of *ABCA1* expression reduced $A\beta$ peptide in cells (Koldamova et al., 2003; Sun et al., 2003). More recently, a study has shown that T0901317 stimulated *ABCA1* expression and reduced the levels of soluble $A\beta$ peptides in APP23 mice (Koldamova et al., 2005a,b). ABCA1 is involved in the regulation of ApoE expression and *ABCA1* deficiency decreased soluble ApoE levels but did not affect $A\beta$ deposition in Tg-SwDI/B and APP/PS1 mice (Hirsch-Reinshagen et al., 2004, 2005). Another study showed that disruption of *ABCA1* decreased ApoE level and increased $A\beta$ deposition in APP23 mice (Koldamova et al., 2005a,b). Genetic studies suggested that single nucleotide polymorphisms of *ABCA1* modulate CSF cholesterol levels and affect the risk and onset of AD (Wollmer et al., 2003; Katzov et al., 2004; Sundar et al., 2006). Collectively, these studies suggested that ABCA1 plays a role in AD pathogenesis. However, this study failed to detect the difference of ABCA1 expression between ND and AD brains. This suggests that ABCA1 may not be a major factor responsible for cholesterol retention in AD brains examined in this study.

Cholesterol is a major component of lipid bilayer membrane. Since both β - and γ -secretases are located in cholesterol-rich lipid rafts of cell membrane, their activities are subjected to cholesterol changes. Lipid rafts are small, specialized areas in the membranes where glycosphingolipids and cholesterol form lipid bilayer. Since lipid bilayer is somewhat thicker in the rafts due to stiffer side chains of glycosphingolipids and cholesterol, certain membrane proteins with long membrane-spinning segments preferentially partition into lipid rafts. An important observation from this study is that certain concentration range of cholesterol stimulated β - and γ -secretase activities in human brain lysates and cultured cells, but cholesterol also inhibited γ -secretase activity in human brain lysates at 20 μ M concentration. This may explain the contradictory results on the relation of cholesterol and $A\beta$ production from reported studies (Wahrle et al., 2002; Cordy et al., 2003; Kaether and Haass, 2004; Simons et al., 1998; Fukumoto et al., 2002; Koldamova et al., 2003; Sun et al., 2003). Cholesterol may affect lipid composition and structure of membrane lipid bilayer and therefore the activities of β - and γ -secretases. Cholesterol at an optimal range of concentrations may enhance intrinsic activities of the secretases or increase the numbers of lipid rafts for optimal secretase activities. Other possible explanation is that cholesterol and lipid rafts may stabilize β - or γ -secretases and reduce their degradation. Ehehalt and colleagues' work (2003) suggested that there are two APP pools, e.g., APP inside raft clusters is cleaved by β -secretase, while APP outside rafts is cleaved by α -secretase. Optimal cholesterol level could make APP more accessible to β - and γ -secretase cleavage. Lipid rafts may also be involved in clearance of $A\beta$ by amyloid-degrading enzymes, such as plasmin or neprilysin (Cordy et al., 2006). All of these indicate that cholesterol may have multiple effects on secretase activities and APP processing. However, the fact that β - or γ -secretase could cleave more substrates in the presence of exogenous cholesterol suggests that cholesterol most likely makes the enzymes more active in the reactions.

An interesting observation is that β - and γ -secretases in AD brain are more sensitive to cholesterol than those in ND brain. This may be due to higher cholesterol levels already existing in AD brain and less cholesterol being therefore required to stimulate their activities to maximal level. Another possible explanation is that β - and γ -secretases in AD brain may have intrinsic sensitivity to cholesterol and therefore low cholesterol levels are needed to stimulate their activities. Single nucleotide polymorphisms (SNP) in genes encoding γ -secretase components

modify AD risk (Yasuda et al., 1999; Dermaut et al., 2002; Howell and Brookes, 2002). Whether any SNP affects secretase sensitivity to cholesterol requires further investigation.

In summary, this study supports our initial hypothesis and provides direct evidence that cholesterol homeostasis is impaired in AD brain with significant cholesterol retention, which leads to high levels of β - and γ -secretase activities and increased A β production. The results suggest that altered levels or activities of the nuclear receptors may contribute to cholesterol retention in the AD brain where β - and γ -secretase activities are stimulated to maximum for A β overproduction, and that these nuclear receptors could be therapeutic targets for restoring cholesterol homeostasis in the AD brain.

Acknowledgments

The authors thank Dr. Dennis Selkoe at the Harvard Medical School for providing K269 cells for the study and Ms. Ewa Baumann for doing some of the brain tissue sections and staining. The study was supported by funding from a Canadian Research Program "Vascular Health & Dementia" sponsored by Heart & Stroke Foundation of Canada, Canadian Institutes of Health Research, Alzheimer Society of Canada, and Pfizer.

References

- Abad-Rodriguez J, Ledesma MD, Craessaerts K, Perga S, Medina M, Delacourte A, Dingwall C, De Strooper B, Dotti CG. Neuronal membrane cholesterol loss enhances amyloid peptide generation. *J. Cell Biol* 2004;167(5):953–960. [PubMed: 15583033]
- Abulrob A, Tauskela JS, Mealing G, Brunette E, Faïd K, Stanimirovic D. Protection by cholesterol-extracting cyclodextrins: a role for N-methyl-D-aspartate receptor redistribution. *J. Neurochem* 2005;92(6):1477–1486. [PubMed: 15748165]
- Akiyama TE, Sakai S, Lambert G, Nicol CJ, Matsusue K, Pimprale S, Lee YH, Ricote M, Glass CK, Brewer HB Jr, Gonzalez FJ. Conditional disruption of the peroxisome proliferator-activated receptor gamma gene in mice results in lowered expression of ABCA1, ABCG1, and apoE in macrophages and reduced cholesterol efflux. *Mol. Cell. Biol* 2002;22(8):2607–2619. [PubMed: 11909955]
- Bjorkhem I, Meaney S. Brain cholesterol: long secret life behind a barrier. *Arterioscler. Thromb. Vasc. Biol* 2004;24:806–815. [PubMed: 14764421]
- Cordy JM, Hooper NM, Turner AJ. Exclusively targeting beta-secretase to lipid rafts by GPI-anchor addition up-regulates beta-site processing of the amyloid precursor protein. *Proc. Natl. Acad. Sci. U. S. A* 2003;100(20):11735–11740. [PubMed: 14504402]
- Cordy JM, Hooper NM, Turner AJ. The involvement of lipid rafts in Alzheimer's disease. *Mol. Membr. Biol* 2006;23(1):111–122. [PubMed: 16611586]
- Dermaut B, Theuns J, Sleegers K, Hasegawa H, Van den Broeck M, Vennekens K, Corsmit E, StGeorge-Hyslop P, Cruts M, Van Duijn CM, Van Broeckhoven C. The gene encoding nicastrin, a major gamma-secretase component, modifies risk for familial early-onset Alzheimer disease in a Dutch population-based sample. *Am. J. Hum. Genet* 2002;70(6):1568–1574. [PubMed: 11992262]
- Dietschy JM, Turley SD. Cholesterol metabolism in the brain. *Curr. Opin. Lipidol* 2001;12:105–112. [PubMed: 11264981]
- Edwards PA, Kast HR, Anisfeld AM. BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. *J. Lipid Res* 2002;43:2–12. [PubMed: 11792716]
- Ehehalt R, Keller P, Haass C, Thiele C, Simons K. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. *J. Cell Biol* 2003;160:113–123. [PubMed: 12515826]
- Francis GA, Fayard E, Picard F, Auwerx J. Nuclear receptors and the control of metabolism. *Annu. Rev. Physiol* 2003;65:261–311. [PubMed: 12518001]
- Friedhoff LT, Cullen EI, Geoghagen NS, Buxbaum JD. Treatment with controlled-release lovastatin decreases serum concentrations of human beta-amyloid (A β) peptide. *Int. J. Neuropsychopharmacol* 2001;4(2):127–130.

- Fukumoto H, Deng A, Irizarry MC, Fitzgerald ML, Rebeck GW. Induction of the cholesterol transporter ABCA1 in central nervous system cells by liver X receptor agonists increases secreted Abeta levels. *J. Biol. Chem* 2002;277(50):48508–48513. [PubMed: 12384498]
- Hirsch-Reinshagen V, Zhou S, Burgess BL, Bernier L, McIsaac SA, Chan JY, Tansley GH, Cohn JS, Hayden MR, Wellington CL. Deficiency of ABCA1 impairs apolipoprotein E metabolism in brain. *J. Biol. Chem* 2004;279:41197–41207. [PubMed: 15269218]
- Hirsch-Reinshagen V, Maia LF, Burgess BL, Blain JF, Naus KE, McIsaac SA, Parkinson PF, Chan JY, Tansley GH, Hayden MR, Poirier J, Van Nostrand W, Wellington CL. The absence of ABCA1 decreases soluble apoE levels but does not diminish amyloid deposition in two murine models of Alzheimer's disease. *J. Biol. Chem* 2005;280(52):43243–43256. [PubMed: 16207707]
- Hixson JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *J. Lipid Res* 1990;31(3):545–548. [PubMed: 2341813]
- Howell WM, Brookes AJ. Evaluation of multiple presenilin 2 SNPs for association with early-onset sporadic Alzheimer disease. *Am. J. Med. Genet* 2002;111(2):157–163. [PubMed: 12210343]
- Huang Y. Molecular and cellular mechanisms of apolipoprotein E4 neurotoxicity and potential therapeutic strategies. *Curr. Opin. Drug Discov. Dev* 2006;9(5):627–641.
- Johansson L, Rudling M, Scanlan TS, Lundasen T, Webb P, Baxter J, Angelin B, Parini P. Selective thyroid receptor modulation by GC-1 reduces serum lipids and stimulates steps of reverse cholesterol transport in euthyroid mice. *Proc. Natl. Acad. Sci. U. S. A* 2005;102(29):10297–10302. [PubMed: 16006512]
- Kaether C, Haass C. A lipid boundary separates APP and secretases and limits amyloid beta-peptide generation. *J. Cell Biol* 2004;167(5):809–812. [PubMed: 15583026]
- Kalvodova L, Kahya N, Schwillle P, Eehalt R, Verkade P, Drechsel D, Simons K. Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro. *J. Biol. Chem* 2005;280(44):36815–36823. [PubMed: 16115865]
- Katzov H, Chalmers K, Palmgren J, Andreassen N, Johansson B, Cairns NJ, Gatz M, Wilcock GK, Love S, Pedersen NL, Brookes AJ, Blennow K, Kehoe PG, Prince JA. Genetic variants of ABCA1 modify Alzheimer disease risk and quantitative traits related to beta-amyloid metabolism. *Hum. Mutat* 2004;23(4):358–367. [PubMed: 15024730]
- Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT. Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *J. Cell Sci* 2003;116(Pt 16):3339–3346. [PubMed: 12829747]
- Klopotek A, Hirche F, Eder K. PPAR gamma ligand troglitazone lowers cholesterol synthesis in HepG2 and Caco-2 cells via a reduced concentration of nuclear SREBP-2. *Exp. Biol. Med.* (Maywood) 2006;231(8):1365–1372. [PubMed: 16946405]
- Kojro E, Gimpl G, Lammich S, Marz W, Fahrenholz F. Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha-secretase ADAM 10. *Proc. Natl. Acad. Sci. U. S. A* 2001;98(10):5815–5820. [PubMed: 11309494]
- Koldamova RP, Lefterov IM, Ikonovic MD, Skoko J, Lefterov PI, Isanski BA, DeKosky ST, Lazo JS. 22R-hydroxycholesterol and 9-*cis*-retinoic acid induce ATP-binding cassette transporter A1 expression and cholesterol efflux in brain cells and decrease amyloid beta secretion. *J. Biol. Chem* 2003;278(15):13244–13256. [PubMed: 12547833]
- Koldamova R, Staufenbiel M, Lefterov I. Lack of ABCA1 considerably decreases brain ApoE level and increases amyloid deposition in APP23 mice. *J. Biol. Chem* 2005a;280(52):43224–43235. [PubMed: 16207713]
- Koldamova RP, Lefterov IM, Staufenbiel M, Wolfe D, Huang S, Glorioso JC, Walter M, Roth MG, Lazo JS. The liver X receptor ligand T0901317 decreases amyloid beta production in vitro and in a mouse model of Alzheimer's disease. *J. Biol. Chem* 2005b;280(6):4079–4088. [PubMed: 15557325]
- Koudinov AR, Koudinova NV. Essential role for cholesterol in synaptic plasticity and neural degeneration. *FASEB J* 2001;15:1858–1860. [PubMed: 11481254]
- Kraiem Z. Selective agonists and antagonists to thyroid hormone action. *Thyroid* 2005;15(4):336–339. [PubMed: 15876155]
- Landreth G. PPAR γ agonists as new therapeutic agents for the treatment of Alzheimer's disease. *Exp. Neurol* 2006;199(2):245–248. [PubMed: 16733054]

- Abell LL, Levy BB, Brodie BB, Kendall FE. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem* 1952;195:357–366. [PubMed: 14938387]
- Miller LJ, Chacko R. The role of cholesterol and statins in Alzheimer's disease. *Ann. Pharmacother* 2004;38(1):91–98. [PubMed: 14742802]
- Mojsilovic-Petrovic J, Nesic M, Pan A, Zhang W, Stanimirovic DB. Development of rapid staining protocols for laser capture microdissection of brain vessels from human and rat coupled to gene expression analyses. *J. Neurosci. Methods* 2004;133:39–48. [PubMed: 14757343]
- Murthy S, Born E, Mathur SN, Field FJ. LXR/RXR activation enhances basolateral efflux of cholesterol in CaCo-2 cells. *J. Lipid Res* 2002;43(7):1054–1064. [PubMed: 12091489]
- Pfriege FW. Outsourcing. in the brain: do neurons depend on cholesterol delivery by astrocytes? *BioEssays* 2003;25(1):72–78.
- Preston SD, Steart PV, Wilkinson A, Nicoll JA, Weller RO. Capillary and arterial cerebral amyloid angiopathy in Alzheimer's disease: defining the perivascular route for the elimination of amyloid beta from the human brain. *Neuropathol. Appl. Neurobiol* 2003;29:106–117. [PubMed: 12662319]
- Puglielli L, Tanzi RE, Kovacs DM. Alzheimer's disease: the cholesterol connection. *Nat. Neurosci* 2005;6(4):345–351. [PubMed: 12658281]
- Refolo LM, Malester B, LaFrancois J, Bryant-Thomas T, Wang R, Tint GS, Sambamurti K, Duff K, Pappolla MA. Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. *Neurobiol. Dis* 2000;7(4):321–331. [PubMed: 10964604]
- Refolo LM, Pappolla MA, LaFrancois J, Malester B, Schmidt SD, Thomas-Bryant T, Tint GS, Wang R, Mercken M, Petanceska SS, Duff KE. A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of Alzheimer's disease. *Neurobiol. Dis* 2001;8(5):890–899. [PubMed: 11592856]
- Schmitz G, Langmann T. Transcriptional regulatory networks in lipid metabolism control ABCA1 expression. *Biochim. Biophys. Acta* 2005;1735(1):1–19. [PubMed: 15922656]
- Selkoe DJ. Alzheimer disease: mechanistic understanding predicts novel therapies. *Ann. Intern. Med* 2004;140(8):627–638. [PubMed: 15096334]
- Simons M, Keller P, De Strooper B, Beyreuther K, Dotti CG, Simons K. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A* 1998;95(11):6460–6464. [PubMed: 9600988]
- Sjogren M, Gustafsson K, Syversen S, Olsson A, Edman A, Davidsson P, Wallin A, Blennow K. Treatment with simvastatin in patients with Alzheimer's disease lowers both alpha- and beta-cleaved amyloid precursor protein. *Dement. Geriatr. Cogn. Disord* 2003;16(1):25–30. [PubMed: 12714796]
- Sun Y, Yao J, Kim TW, Tall AR. Expression of liver X receptor target genes decreases cellular amyloid beta peptide secretion. *J. Biol. Chem* 2003;278(30):27688–27694. [PubMed: 12754201]
- Sundar PD, Feingold E, Minster RL, Dekosky ST, Kamboh MI. Gender-specific association of ATP-binding cassette transporter 1 (ABCA1) polymorphisms with the risk of late-onset Alzheimer's disease. *Neurobiol. Dis* 2006;22(1):1–10. [PubMed: 16444444] (May 22 Electronic publication ahead of print)
- Tan ZS, Seshadri S, Beiser A, Wilson PW, Kiel DP, Tocco M, D'Agostino RB, Wolf PA. Plasma total cholesterol level as a risk factor for Alzheimer disease: the Framingham Study. *Arch. Intern. Med* 2003;163(9):1053–1057. [PubMed: 12742802]
- Wahrle SE, Das P, Nyborg AC, McLendon C, Shoji M, Kawarabayashi T, Younkin LH, Younkin SG, Golde TE. Cholesterol-dependent gamma-secretase activity in buoyant cholesterol-rich membrane microdomains. *Neurobiol. Dis* 2002;9(1):11–23. [PubMed: 11848681]
- Walter J, Kaether C, Steiner H, Haass C. The cell biology of Alzheimer's disease: uncovering the secrets of secretases. *Curr. Opin. Neurobiol* 2001;11(5):585–590. [PubMed: 11595492]
- Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A* 2004;101(26):9774–9779. [PubMed: 15210959]
- Webb P. Selective activators of thyroid hormone receptors. *Expert Opin. Investig. Drugs* 2004;13(5):489–500.
- Weller RO, Nicoll JA. Cerebral amyloid angiopathy: pathogenesis and effects on aging and Alzheimer brain. *Neurol. Res* 2003;25:611–616. [PubMed: 14503015]

- Wiebe, DA.; Artiss, JD. Lipids, lipoproteins, and apolipoproteins. In: McClatchey, KD., editor. Clinical Laboratory Medicine. Baltimore: Williams and Wilkins; 1994. p. 287-302.
- Wollmer MA, Streffer JR, Lutjohann D, Tsolaki M, Iakovidou V, Hegi T, Pasch T, Jung HH, Bergmann K, Nitsch RM, Hock C, Papassotiropoulos A. ABCA1 modulates CSF cholesterol levels and influences the age at onset of Alzheimer's disease. *Neurobiol. Aging* 2003;24(3):421–426. [PubMed: 12600718]
- Wolozin B. A fluid connection: cholesterol and A β . *Proc. Natl. Acad. Sci. U. S. A* 2001;98(10):5371–5373. [PubMed: 11344276]
- Wolozin B. Cholesterol and the biology of Alzheimer's disease. *Neuron* 2004;41:7–10. [PubMed: 14715130]
- Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Arch. Neurol* 2000;57(10):1439–1443. [PubMed: 11030795]
- Yasuda M, Hirano N, Maeda K, Imamura T, Mori E, Tanaka C. Case-control study of presenilin-1 intronic polymorphism in sporadic early and late onset Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatry* 1999;66(6):722–726. [PubMed: 10329743]
- Ye S, Huang Y, Mullendorff K, Dong L, Giedt G, Meng EC, Cohen FE, Kuntz ID, Weisgraber KH, Mahley RW. Apolipoprotein (apo) E4 enhances amyloid beta peptide production in cultured neuronal cells: apoE structure as a potential therapeutic target. *Proc. Natl. Acad. Sci. U. S. A* 2005;102(51):18700–18705. [PubMed: 16344478]
- Zhang, W.; Stanimirovic, DB. The transport systems of the blood–brain barrier. In: De Vries, E.; Prat, A., editors. *The Blood-Brain Barrier and its Microenvironment: Basic Physiology to Neurological Disease*. New York: Taylor & Francis Publisher Group; 2005. p. 103-142.
- Zhang W, Smith C, Shiparo A, Monette R, Hutchison J, Stanimirovic D. Increased expression of bioactive chemokines in human cerebrovascular endothelial cells and astrocytes subjected to simulated ischemia in vitro. *J. Neuroimmunol* 1999;101:148–160. [PubMed: 10580798]
- Zhang W, Mojsilovic-Petrovic J, Andrade M, Zhang H, Ball M, Stanimirovic D. Expression and functional characterization of ABCG2 in brain endothelial cells and vessels. *FASEB J* 2003;17(14):2085–2087. [PubMed: 12958161]
- Zhang W, Mojsilovic-Petrovic J, Callaghan D, Jones A, Cui H, Howlett C, Stanimirovic D. Evidence that hypoxia-inducible factor-1 (HIF-1) mediates transcriptional activation of interleukin-1 β (IL-1 β) in astrocyte Cultures. *J. Neuroimmunol* 2006;174:63–73. [PubMed: 16504308]

Fig. 1. Detection of A β peptides and cholesterol in human brain samples. Brain sections were reacted with a primary 6E10 antibody and then with a secondary antibody conjugated with Alexa 568. Beta-amyloid is visualized as red color. Filipin is used to stain cholesterol in brain sections. Filipin-stained cholesterol is visualized as blue color. Overlapping staining of cholesterol and beta-amyloid shows blue-reddish haze color. Panels A and B are ND samples. Panels C–F are AD brain samples. All of the images were taken at 200 $\times$ magnifications.

Fig. 2.

Levels of cholesterol and the activities of β - and γ -secretases in ND and AD brain samples. Panel A: Enzymatic assay shows that the level of cholesterol in AD is significantly higher (19.37% $\uparrow$) than that in ND brain samples (t test, * $p=0.0104$). Panel B shows the results of chemical method analysis of total brain cholesterol. AD brains have significantly higher cholesterol (41.3% $\uparrow$) than ND controls (t test, $p<0.01$). Panel C: Brain tissue lysates were prepared for β - and γ -secretase assays following manufacturer's instructions as described in Materials and methods. The activities of both β -secretase and γ -secretases are significantly higher in AD than in ND brain samples (t test, ** $p<0.01$).

Fig. 3.

Effects of exogenous cholesterol on β - and γ -secretase activities in human brain tissue lysates. Panel A shows that 80 μ M cholesterol (Cho) strongly stimulated β -secretase activity in ND brain lysates (One-way ANOVA, *** p <0.0001); while 40 μ M and 80 μ M cholesterol significantly enhanced β -secretase activity in AD brain lysates (One-way ANOVA, *** p <0.0001), respectively. Panel B shows that 80 μ M cholesterol strongly stimulated γ -secretase activity in ND samples (One-way ANOVA, *** p <0.0001); while 40 μ M cholesterol significantly stimulated γ -secretase activity in AD samples (One-way ANOVA, *** p <0.0001). Cholesterol at 20 μ M concentration did not affect β -secretase activity, but inhibited γ -secretase activity in AD brain samples (*** p <0.0001).

Fig. 4. Immunostaining for BACE, beta-amyloid, and cholesterol. AD brain sections were immunoreacted with a primary anti-human BACE antibody and then with a secondary antibody conjugated with Alexa 568. BACE is visualized as red color. Panels A and C are BACE staining for AD brain sections. Panels B and D are sister slides of panels A and C sections (respectively) and were reacted with a primary beta-amyloid antibody 6E10 and then with a secondary antibody conjugated with Alexa 568. The beta-amyloid in Panels B and D is also visualized as red color. Panels E and F show double-staining of AD brains with filipin for cholesterol (blue color) and BACE (red color). Panels G and H show BACE staining for N2a and K269 cells. All of the images were taken at 200× magnifications.

Fig. 5.

Effects of exogenous cholesterol in culture media on β - and γ -secretase activities in N2a cells. Different concentrations of exogenous cholesterol (Cho) were added to the culture media of N2a cells and incubated for 1, 2, 4, and 6 h. Cells were harvested for lysate preparations. Panel A: Cellular cholesterol was significantly higher in cholesterol-treated cells than in controls (One-way ANOVA, $*p < 0.05$). Panels B and C: The in vitro assays show that cholesterol added to culture media significantly stimulated β - and γ -secretase activities in N2a cells (One-way ANOVA, $*p < 0.05$). Panel D: A β_{1-40} peptides released into the media from cholesterol-treated cells were significantly increased at all the time points compared to controls (One-way ANOVA, $***p < 0.0005$, $***p < 0.0001$).

Fig. 6.

In vitro β - and γ -secretase activity assays in the presence of exogenous cholesterol. Cell lysates were prepared from N2a cells following manufacturer's instructions as described in Materials and methods. Different concentrations of exogenous cholesterol or ergosterol were added to the *in vitro* assays for β - or γ -secretase activities, respectively. Panel A: The activity of β -secretase was significantly stimulated in the presence of 20–80 μ M cholesterol as compared to controls (One-way ANOVA, $*p=0.0198$). Ergosterol at 20, 40 and 80 μ M slightly decreased β -secretase activity. Panel B: Cholesterol at 20, 40 and 80 μ M strongly stimulated γ -secretase activity (One-way ANOVA, $***p<0.0001$). It appears that 20–40 μ M cholesterol was stronger in stimulating γ -secretase activity than 80 μ M cholesterol. Ergosterol at 40 μ M strongly

decreased γ -secretase activity (One-way ANOVA, *** $p < 0.0001$). Panel C: The activities of β - and γ -secretases were significantly inhibited by β -secretase inhibitor III and γ -secretase inhibitor IV, respectively (One-way ANOVA, *** $p < 0.0001$). The inhibitors inhibited 25–30% of β - and γ -secretase activities no matter in the presence or absence of cholesterol.

Fig. 7.

Effects of cholesterol depletion on levels of cellular cholesterol, β -secretase activity and A β production in N2a cells. Panel A shows that cellular cholesterol was significantly decreased at 4 h post-treatment with 2.5 mM cyclodextran (CDT) (One-way ANOVA, $*p=0.0442$), returned to control level at 8, 12, and 24 h. Panel B shows that β -secretase activity in CDT-treated cells was reduced at 4 h (One-way ANOVA, $*p=0.0215$), but returned to control levels at 8, 12 and 24 h. A β_{1-40} peptides released from CDT-treated cells were significantly lower at 4 h (One-way ANOVA, $**p<0.0009$), but slightly higher at 8, 12 and 24 h than controls (Panel C).

Fig. 8.

Activation of transcription factors in ND vs. AD brain samples. Panels A and B show transcription factor (TF) profiling using TranSignal protein/ DNA array blot I analysis (Panomics Inc.). The boxed transcription factors are involved in the regulation of lipid and cholesterol metabolism in cells. The binding activities of PPAR, TR, and VDR were decreased over 2-fold in AD; while the activities of RXR, ARE, ERE, and PRE were increased over 2-fold in AD brain samples as compared to ND brain samples (t test, $p < 0.05$). The levels of sterol regulatory binding element transcription factor (SREBF or SREBP), GR and RAR were similar in ND and AD samples. Panel C shows the fold changes for PPAR, RXR, and TR in AD brain samples. The activity of the relevant transcription factor in ND brain samples was set as 1.

Fig. 9.

Level of LXR- β in ND vs. AD brain samples. Semi-quantitative RT-PCR was carried out to analyze the expression of LXR- β in ND and AD brain samples. The levels of LXR- β were generally lower in AD than in ND brain samples. The graph shows quantitative densitometry analysis of LXR- β expression in ND vs. AD brain samples normalized against an internal control β -actin. The average level of LXR- β in AD is 1.56-fold higher than that in ND (t test, ** $p < 0.01$).

Fig. 10.

Effects of LXR and RXR agonists on cholesterol efflux and β - and γ -secretase activities in cells. Cholesterol assays using K269 cells (Panel A) show that more cholesterol (41%) was transported to HDL after 8 h incubation of the cells with 5 μ M LXR agonist TO901317 (TO) (One-way ANOVA, *** p < 0.0001). In the absence of TO, only a small fraction of cholesterol (22%) was shed to culture media compared to controls (One-way ANOVA, *** p < 0.0001). Panel B shows that cholesterol levels were decreased in N2a cells treated with LXR or/and RXR compared to controls (One-way ANOVA, * p < 0.05). Since cellular cholesterol was decreased in LXR- and RXR-treated N2a cells, β - and γ -secretase activities were significantly reduced in the cells (One-way ANOVA, * p < 0.05, ** p < 0.01) (Panels C and D).

Fig. 11.

Effects of PPAR- γ agonist troglitazone on β - and γ -secretase activities in N2a cells. Both β -secretase (Panel A) and γ -secretase (Panel B) activities were significantly decreased in N2a cells treated with 10 μ M troglitazone (TROG) as compared to controls (One-way ANOVA, Panel A, * $p=0.0108$; Panel B, *** $p<0.0001$) at 24 and 36h post-treatment

Table 1

Primers for RT-PCR and real-time qRT-PCR

Gene	Sense primers	Anti-sense primers
LXR- β (RT-PCR)	5'-GGC GGC AGC TTC TTG TCC-3'	5'-GGA CGA CTT CCA CCG TGC-3'
ABCA1 (RT-PCR)	5'-TCA TCT TCA TCT GCT TCC AGC-3'	5'-GTG CTG GGG ATC TTG AAC AC-3'
ABCA1 (qPCR)	5'-TCA TCT TCA TCT GCT TCC AGC-3'	5'-GTG CTG GGG ATC TTG AAC AC-3'
ABCG1 (RT-PCR)	5'-TTC TTC TCC ATG CTG TTC CTC -3'	5'-GCC AGG TAG TAG GCC TTC AG-3'
β -actin (qPCR)	5'-TGT CCA CCT TCC AGC AGA TGT-3'	5'-AGT CCG CCT AGA AGC ATT TGC-3'
β -actin (RT-PCR)	5'-GTC ACC CAC ACT GTG CCC ATC T-3'	5'-ACA GAG TAC TTG CGC TCA GGAG-3'