Apolipoproteins E and C1 in the brain:
role in Alzheimer's disease

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Apolipoproteins E and C1 in the brain: role in Alzheimer's disease

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The Long Good-Bye

Deep within that hollow stare,
of our presence they're unaware.
A special life that's fading away,
in spite of things we try to convey.

Memories locked up in their mind,
and there it's kept all confined.
The good times spent long ago,
with all their love they did bestow.

For these moments will live forever,
and our pride in them will endeavor.
Seeing them lying there we know why,
Alzheimer's is called the long good-bye.

Bernard Howe
(Poetry from the Heart) 1999—2005

To my parents Adilkhan Abildayev and Goulzhakhan Tumembayeva

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Chapter 1

General Introduction

Neurodegenerative disorders and aging

The life expectancy in the western society has risen significantly in this century, the elderly are an increasing segment of our population, and their health care needs will continue to grow for decades. Neurodegenerative diseases evolve gradually, after a long period of normal brain function, due to progressive degeneration (i.e., nerve cell dysfunction and death) of specific brain regions. Due to the aging population, neurodegenerative diseases have become more common, and finding treatments or cures has become a major focus of researchers worldwide. Although normal aging is characterized by modest reductions in the mass and volume of the human brain, which may be due to the atrophy and/or death of brain cells, these changes are far more profound in the brains of patients who suffer from a neurodegenerative disorder. Several of these neurodegenerative conditions appear in the later stages of life and present with some form of dementia. Most of these diseases are sporadic (i.e., not due to genetic mutations) and of unknown cause, but hundreds of different mutations in many genes have been shown to cause familial (inherited) variants of several neurodegenerative disorders. Many of the dozen or more genes that harbor these mutations were discovered in the quest to determine the genetic basis of neurodegenerative diseases just in the last ten years.

Alzheimer's disease

A well-known neurodegenerative disease is Alzheimer's disease (AD), the most common cause of dementia in the elderly in Western societies, with aging a major contributor to its onset (1). AD is characterized by progressive cognitive deterioration together with declining activities of daily living and neuropsychiatric symptoms or behavioural changes. It is the most common cause of dementia. The most striking early symptom is short-term memory loss (amnesia), which usually manifests as minor forgetfulness that becomes steadily more pronounced with illness progression, with relative preservation of older memories. As the disorder progresses, cognitive (intellectual) impairment extends to the domains of language (aphasia), skilled movements (apraxia), recognition (agnosia), and those functions (such as decision-making and planning) closely related to the frontal and temporal lobes of the brain as they become disconnected from the limbic system, reflecting extension of the underlying pathological process. This consists principally of neuronal loss or atrophy together with an inflammatory response to the deposition of amyloid plaques and neurofibrillary tangles.
United Nation population projections estimate that the number of people older than 80 years will approach 370 million by the year 2050. Currently, it is estimated that 50% of people over age 85 in the USA are afflicted with AD (2). Therefore, if these statistics hold true, in 50 years, more than 100 million people worldwide will suffer from dementia. AD has probably afflicted the elderly for centuries, but it was rare when first reported by Alois Alzheimer in 1907, only because life expectancy was ~50 years then, and few individuals lived to age 60 or longer when the risk of AD increases with

advancing age beyond the 7th decade of life. The symptoms of the disease as a distinct entity were first identified by Emil Kraepelin. The characteristic neuropathology was first observed by Alois Alzheimer (Fig.1) in 1906, a German psychiatrist, whom the disease is named after, in 1910.

Figure 1. Alois Alzheimer

In this sense, the disease was co-discovered by Kraepelin and Alzheimer, who worked in Kraepelin's laboratory. Because of the overwhelming importance Kraepelin attached to finding the neuropathological basis of psychiatric disorders, Kraepelin made the generous decision that the disease would bear Alzheimer's name. For most of the twentieth century, the diagnosis of Alzheimer's disease was reserved for individuals between the ages of 45-65 who developed symptoms of presenile dementia, which was considered to be a more or less normal outcome of the aging process. In the 1970s and early 1980s, because the symptoms and brain pathology were identical, the name "Alzheimer's disease" began to be used, within and outside the medical profession, equally for individuals age 65 and older with senile dementia, and was eventually adopted formally for all individuals with the common symptom pattern and disease course in the psychiatric and neurological nomenclature (http://en.wikipedia.org).

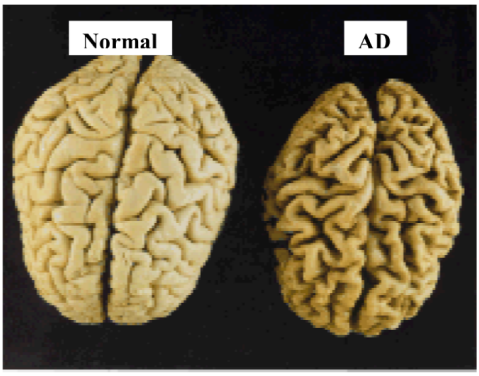
Sporadic AD accounts for ~90% patients with this disorder, and ~10% of patients have familial AD (FAD). About 50% of FAD patients have been shown to have disease caused by mutations in genes on chromosome 14 (Presenilin 1 or PS1), chromosome 1 (Presenilin 2 or PS2), and chromosome 21 (amyloid precursor protein or APP), all serve to transmit AD via autosomal dominant inheritance (2). There are other genes that are considered susceptibility or risk factors for AD. These include: apolipoprotein E (apoE ε4 variant) (3), which is a strongest known risk factor so far; α2-macroglobulin (4), a gene for a component of α-ketoglutarate dehydrogenase (5,6), the K-variant of butyryl-cholinesterase (7,8) and several mitochondrial genes (9,10) and apolipoprotein C1 (apoC1 H2 allele) (11-14). Epidemiological studies that have demonstrated risk factors for AD include: age, sex (females are in greater risk), previous head injury and cardiovascular disease (2).

The cause and progression of both familial and sporadic AD have not been fully elucidated. Neuropathological studies of the end-stage AD brain shows diffuse cerebral atrophy with enlarged ventricles, narrowed cortical gyri and widened sulci (Fig.2).

These changes are attributed to neuronal loss. While the loss of neurons in AD generally exceeds that seen during normal aging, there may be overlap between the AD brain and the brains of age matched normal subjects. However, individual neuronal groups in neurodegenerative disorders and normal aging vary in their susceptibility for degeneration. Specifically, the hippocampal formation is consistently and heavily involved in the pathology of AD, and considerably less affected in normal aging. This

predilection has clinical correlates as well. Since the hippocampus is implicated in the formation of memory, the pathology occurs in a region where it can contribute to the intellectual deficits that are the hallmark of AD.

Figure 2. The brain specimen shown to the right is from a patient with Alzheimer disease. The image illustrates the characteristic gross pathologic changes of cortical atrophy with widening of the cerebral sulci that is usually most obviouse in the frontal, temporal and parietal lobes.



Existing hypotheses

Three major competing hypotheses exist to explain the cause of the disease.

1. The oldest hypothesis is the "cholinergic hypothesis". It states that Alzheimer's begins as a deficiency in the production of acetylcholine. Much early therapeutic research was based on this hypothesis, including restoration of the "cholinergic nuclei" (4,15,16). The possibility of cell-replacement therapy was investigated on the basis of this hypothesis (17-19). All of the first-generation anti-Alzheimer's medications are based on this hypothesis and work to preserve acetylcholine by inhibiting acetylcholinesterases (15,16,20). These medications, though sometimes beneficial, have not led to a cure. In all cases, they have served to only treat symptoms of the disease and have neither halted nor reversed it. These results and other research have led to the conclusion that acetylcholine deficiencies may not be directly causal, but are a result of widespread brain tissue damage, damage so widespread that cell-replacement therapies are likely to be impractical (19,21).

2. The amyloid hypothesis of Alzheimer's disease was proposed in 1984 when the structure of beta-amyloid was discovered by Glenner and Wong (22). The amyloid hypothesis holds that the accumulation of beta-amyloid is the driving force behind amyloid plaques, neurofibrillary tangles, synapse loss, and neuronal cell death. Accumulation is held to occur when the production of beta-amyloid exceeds its clearance from the brain or degradation by enzymes (23). The β- amyloid or senile plaques (SPs) in the extra-cellular space, and tau-rich intraneuronal neurofibrillary tangles (NFTs) are the two hallmark brain lesions of AD (Fig.3). They accumulate most prominently in neocortical and limbic brain regions for reasons that are not well understood, and a diagnosis of definite AD in a demented patient requires detection of abundant SPs and NFTs in the postmortem brain or a brain biopsy. The mechanisms leading to the formation of SPs and NFTs are not fully understood, but it is thought that these lesions form as a result of the conversion of normal Aβ peptides into amyloid fibrils and normal tau into paired helical filaments (PHFs) followed by their aggregation into SPs and NFTs, respectively. The β-amyloid precursor protein (APP) is cleaved sequentally by the proteolytic

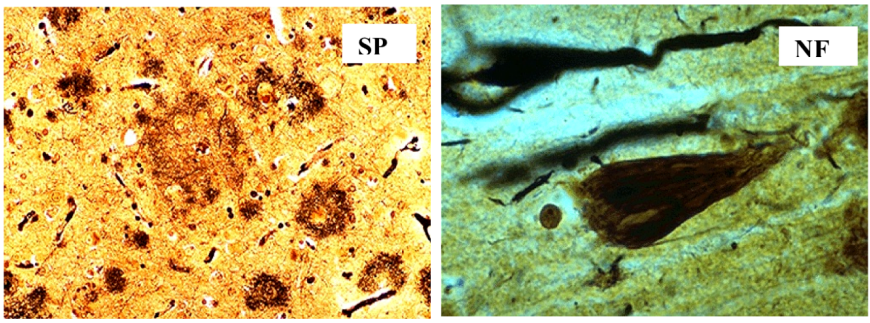


Figure 3. The microscopic features of Alzheimer disease include the presence of senile plaques (SP) with amyloid cores and neurofibrillary tangles (NF) within the cytoplasm of cortical neurons.

enzymes β-secretase and γ-secretase to produce β-amyloid peptides with the $A\beta_{1-42}$ and the $A\beta_{1-40}$ forms being the most prevalent. Secreted Aβ peptides can bind to scavenger receptors and the receptor for advanced glycation end-product (2). Aβ peptides are degraded either via a reuptake mechanism followed by endosomal degradation or by an extracellular insulin-degrading system (2). Overproduction (perhaps only in FAD) or impaired clearance of normal Aβ may exceed a critical "threshold" that "seeds" abnormal amyloid fibril formation followed by deposition in SPs. Significantly, the discovery of FAD mutations in the APP gene in 1991 led to the first transgenic mouse model of AD-like amyloidosis in 1995. The protein BACE (β-site APP cleaving enzyme) has recently been identified as β-secretase (11). The absolute identity of the γ-secretase remains elusive. Accumulated evidence suggests that the γ-secretase activity is mediated by a multi-subunit protein complex containing the PS-1 and PS-2 proteins (12). Mutations in the PS-1, PS-2 or APP genes (linked to FAD) and other mutated genes (linked to sporadic AD) all produce an increase in the $A\beta_{1-42}$ (13-17). Since blocking the activity of β- or γ-secretase can inhibit the production of Aβ peptides, the identification of proteins or compounds that block secretase activity has been and remains a major goal of AD research. Although the amyloid hypothesis is well established in the scientific world, recently some controversial issues have been raised. The amyloid hypothesis predicts that inhibiting presenilins would, in turn, inhibit gamma-secretase, which would, then, decrease the production of beta-amyloid. The amyloid hypothesis holds that decreased presenelin production will lead to decreased accumulation, and thus decreased formation of amyloid plaques. This would result in decreased neurofibrillary tangles and the other characteristics of Alzheimer's that are all supposedly caused by beta-amyloid accumulation. So, drugs that inhibit presenilins should be prime candidates for Alzheimer's. Yet a study by Marjaux et al. (24) proved quite otherwise. Knocking out PS1 in mice specifically in the brain led to a decrease in beta-amyloid, along with mild memory impairment. The absence of both PS1 and PS2 led to "strongly impaired long-term potentiation (LTP), spatial and contextual memory deficits, and, after some time, massive loss of synapses, dendrites, and neurons. Remarkably, this neurodegeneration was accompanied by increased Tau phosphorylation (24).

- While it is true that the accumulation of amyloid plaques are a universal hallmark of Alzheimer's disease, it does not necessarily follow that they are the primary cause of Alzheimer's disease. There are many other signs of pathology, including glutamate mediated phospholipid metabolism (25,26), and proteins involved in synapse formation (27-29). Possibilities outside the conventional amyloid hypothesis include:
- beta-amyloid is an irrelevant byproduct of other damage;
- beta-amyloid accumulation represents an increased burden on the Alzheimer's brain, but is secondary to more primary causes of degeneration;
- beta-amyloid is a byproduct of a neuroprotective mechanism designed to protect against the damage of Alzheimer's;
- beta-amyloid is itself involved in a protective mechanism, or is otherwise necessary, but factors entirely separate from it's production cause it to form fibrillary plaques, which may contribute an additional harmful burden.

3. Another hallmark pathohistological finding in AD is intraneuronal filamentous inclusions, or neurofibrillary tangles (NFT) that are composed of aggregated hyperphosphorilated tau protein (28). Tau proteins belong to the family of microtubule-associated proteins, and six isoforms are differentially expressed in neurons (28). In the normal brain, tau functions to stabilize axonal microtubules, modulate signal transduction, interact with actin cytoskeleton, modulate neurite outgrouth, anchor enzymes such as kinases and phosphatases, and regulate intracellular vesicle transport (29). Abnormal tau modifications found in AD include hyperphosphorelation, glycation, ubiquitination, oxidation and truncation (30). In AD the intraneuronal filamentous inclusions of aggregated tau are called paired helical filaments (PHF) and their constitutive proteins are referred to as PHF-tau proteins (28). Hyperphosphorylation of tau, presumably due to abnormal kinase and/or phosphatase activities, has been implicated in PHF formation followed by aggregation of PHFs into NFTs. NFTs could kill neurons by blocking intracellular transport, or transport may fail because tau in PHFs cannot bind to and stabilize microtubules, which are required for transport. Moreover, the discovery in 1998 of pathogenic tau gene mutations in kindreds with hereditary frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), a rare disorder characterized by prominent intracellular neuronal and glial inclusions formed by PHF-like tau filaments, provided proof that tau abnormalities cause neurodegenerative diseases. Further proof of this has come from recent successful efforts to generate a transgenic mouse model of filamentous tau inclusions similar to the tau-rich tangles found in FTDP-17, AD and related tau diseases (tauopathies), and this should hasten the discovery of new therapies for these diseases.

Potential treatments

Treatment strategies based on molecular knowledge of AD are currently in development. Nevertheless, existing treatments so far are based on the above mentioned hypotheses.

Vaccines for Alzheimer's, unlike typical vaccines, would be used to treat diagnosed patients rather than for disease prevention. Ongoing efforts are based on the idea that, by training the immune system to recognize and attack beta-amyloid, the immune

system might reverse deposition of amyloid and thus stop the disease. Initial results using this approach in animals were promising. However, when the first vaccines were used in humans, a small fraction of participants developed encephalitis and the trials were stopped. Participants in the halted trials continued to be followed, and some showed possible benefit in the form of slower progression of the disease (30-34) (http://www.alz.org/Resources/TopicIndex/AN1792.asp). Work is continuing on less toxic Aβ vaccines, such as a DNA-based therapy that recently showed promise in mice (34).

Vitamin E is an antioxidant that may slow disease progression when taken in large doses. A clear demonstration of the efficacy of vitamin E awaits further study (35)

Proposed alternative treatments for Alzheimer's include a range of herbal compounds and dietary supplements. In general, research on the efficacy of these substances is either non-existent or far too weak to support therapeutic claims of improved memory or slowed disease progression (http://www.alz.org/AboutAD/Treatment/Alternative.asp). Only Ginkgo biloba is currently being tested in a large randomized clinical study (36). Moreover, although they are often advertised as "natural" and available without a prescription, these substances can have serious side effects and interactions with other medications.

Adult neural stem cells One exciting recent development is the discovery that the brain can, in fact, generate new neurons in a process of neurogenesis. In the adult, this process occurs in two areas of the brain, the hippocampus and the subventricular zone, and possibly in others as well. These brain areas contain stem cells, which can divide and migrate to other areas of the brain under certain conditions. These adult neural stem cells can make new neurons and glial cells. This discovery raises the prospect that neural stem cells could be harnessed to replace dying cells or to repair damaged cells and neural circuits in the aging or diseased brain (37-39).

PBT2 compound developed by Prana Biotechnology Limited A team from Australia's Mental Health Research Institute of Victoria have developed a once-a-day pill that they hope could potentially cure Alzheimer's disease. Tests in mice have shown the drug, PBT2, prevents build up of the amyloid protein linked to the disease. Protein levels dropped by 60% within 24 hours of a single dose, and memory performance improved within five days (40,41). It is hoped the drug could be on the market in four years. Human tests are due to start in August 2006 (http://news.bbc.co.uk/2/hi/health/-5210048.stm, http://www.pranabio.com)

Cholesterol metabolism in brain

Recently, a new hypothesis concerning the homeostasis of brain cholesterol and its connection to the pathogenesis of AD has been raised. In general, cholesterol has two major roles: it is an essential constituent of all plasma membranes, and it is the precursor of all steroid hormones. In the brain an additional feature of cholesterol can be discerned: it plays an essential role in synaptic plasticity (42), one of the neuron-specific biological functions. However, cholesterol, unlike other major membrane lipids cannot be synthesized at neuronal terminals (43). It is likely that the synaptic function depends

on whether cholesterol is fully supplied from endogenous sources, i.e. uptake via lipoprotein receptors, and from endogenous sources, i.e. transport via axonal flow.
The brain is the most cholesterol rich human organ, which contains up to 25% of total amount of all free cholesterol in the body, while it makes only 2% of the total body mass (44,45). Most brain cholesterol is unesterified (free) and is found within the specialized membranes of myelin. Since myelin has a very slow turnover rate, myelin-associated cholesterol is virtually immobilized. The remaining brain cholesterol is found in neurons, glial cells and extracellular lipoproteins, and these pools of cholesterol participate in cholesterol homeostasis of the CNS. In 1997 Pfrieger et al (46) observed that neurons formed functioning synapses only once astrocytes were added to the culture. Removing the astrocytes caused the neurons to lose those synapses again. Follow up papers suggested that astrocytes control synapse formation by secreting a mysterious soluble factor (47,48). In 2001, this unknown "glial factor" was identified as cholesterol (43). The researcher's initial hunch that apoE might be the elusive factor proved wrong, but only the next guess—cholesterol—proved right. Further experiments showed that cholesterol increased the frequencies of spontaneous postsynaptic currents and that reducing cholesterol with statin eliminated this effect, as did inhibiting lipoprotein uptake with RAP, a chaperon known to inhibit ligand (i.e. apoE-cholesterol) binding by members of LDLR family.
Cholesterol has also been discovered to play an important role in forming lipid rafts, areas in the plasma membrane of cells that anchor certain proteins important to cell signaling (49). It has been found that several of the proteins anchored in these lipid rafts are responsible for stimulating and guiding the growth of nerve axons. Depriving the membrane of cholesterol selectively inhibited the effect of attractive signaling proteins, which destroyed the axon's ability to grow in the proper direction (50).
Thus, cholesterol is the limiting factor in the ability to form synapses and for nerve growth per se, and is also essential to the regulation of that growth, so that synapses form properly and in the right places.

Cholesterol and Alzheimer's disease

Growing body of evidence suggests that cholesterol plays a major role in the pathogenesis of AD. There is a direct link between Aβ generation and cholesterol metabolism in brain (51-53). Alterations in the cellular cholesterol level modulate the processing of the APP (54,55). Lines of investigation take different directions.
Firstly, elevated plasma cholesterol in humans and animal models is associated with an increased risk of Aβ deposition (56-58), however there are conflicting reports on plasma lipid or lipoprotein levels in AD patients (59-64). A number of epidemiological studies suggest that high levels of cholesterol may contribute to the pathogenesis of AD. Individuals with elevated levels of plasma cholesterol have an increased susceptibility to AD, apparently influenced by the *APOE* 4 genotype (65,66). Moreover, AD patients have increased levels of total serum and low-density lipoprotein (LDL) cholesterol (65,66) along with reduced levels of apoA/high-density lipoprotein (HDL) in their plasma(66,67), as compared to age-matched controls. This is further strengthened by the fact that the activity of lecithin cholesterol acyltransferase (LCAT), an enzyme found in

plasma that catalyzes an acyltransferase reaction on lipoprotein-associated cholesterol and is a key step in reverse cholesterol transport in humans (the process that eliminates cholesterol from peripheral cells), is significantly decreased in AD patients (68). This metabolic profile (high plasma cholesterol with high LDL-cholesterol and low HDL-cholesterol) is commonly found in patients with atherosclerosis. Also, atherosclerosis, intimately related to high blood cholesterol, has been shown to correlate with an increased risk of AD, with higher levels of risk being associated with advanced atherosclerosis (69). Finally, cholesterol abnormally accumulates in the amyloid plaques in the brain of AD patients (70). Similar accumulation of cholesterol has also been found in amyloid plaques of transgenic mice expressing a mutant form of APP695 (Swedish mutation) associated with FAD (70).

Secondly, intracellular cholesterol regulates the generation of Aβ peptides from APP by directly modulating secretase activity or by affecting the intracellular secretase trafficking and/or APP (54,58,71-76) and plays an important role in clearance of Aβ peptides (77-80). Both APP and Aβ are associated, at least in part, with cholesterol-rich domains (CRDs) (81,82), specialized membrane micro-domains characterized by the tight association of cholesterol, sphingomyelin and highly-charged galactosphingolipids. CRDs serve as clustering domains for several membrane-bound proteins, and they have also been proposed to regulate APP processing by favoring the clustering of APP and BACE (73).

Third, cholesterol-lowering drugs reduce the prevalence of AD (83,84) in humans and amyloid burden in animal models of AD (75,85) as well as amyloid forming Aβ peptides in cultured cells (54,74-76,85,86). Two initial reports indicated that statins, which reduce serum levels of cholesterol, also protect against AD (83,87). In one of these initial studies (83), the protective effect of statins was independent of the presence or absence of untreated high lipid levels, suggesting that statins may protect against AD through an unknown mechanism not directly related to cholesterol. In the other (87), lovastatin and pravastatin reduced the risk of AD up to 73%. Despite the preliminary nature of these studies' conclusions, they readily corroborate pre-existing epidemiological data, and were subsequently confirmed by similar studies conducted in independent sets of patients (87-89). Interestingly, recent results from the first 26-week randomized study in 44 AD patients with normal cholesterol showed that simvastatin was able to reduce A40 levels in the cerebrospinal fluid of patients with mild, but not severe, AD (90). A slight improvement in cognitive function reported in this study was not confirmed by a second randomized controlled trial on a much larger scale (91). In conclusion, it should be pointed out that the effects of statins on Aβ deposition in the brain may be independent of the changes in the CNS cholesterol metabolism (92), and may instead involve anti-inflammatory effects of statins (93) or alterations in the cycling of Aβ between the brain and plasma.

Fourth, apoE4 isoform of apoE is a well known cholesterol transporter in the brain, which is a major risk factor for the development of AD.

ApoE, cholesterol and AD

The human APOE gene is located on chromosome 19q13.2, is a polymorphic locus represented by three common alleles, ε2 (APOE2), ε3 (APOE3), ε4 (APOE4). They differ by a single amino acid substitution: APOE2 (Cys112, Cys158), APOE3 (Cys112, Arg158) and APOE4 (Arg112, Arg158).
Lipoproteins are spherical macromolecular particles, in which a hydrophobic core containing triglycerides (fatty acids in brain) and cholesteryl esters is emulsified by a shell composed of phospholipids, unesterified cholesterol, and one of the several specific proteins, termed apolipoproteins (apos) (Fig. 4). Cholesterol and apolipoproteins stabilize the particles, and apolipoproteins have additional roles such as lipid recruitment, modulation of enzyme activity, and modulation of receptor-mediated binding and endocytosis.

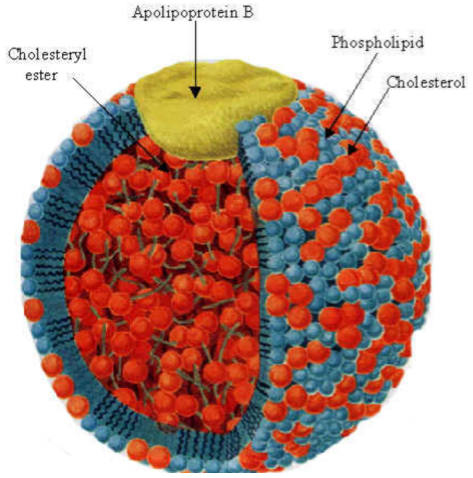


Figure 4. Schematic representation of an apolipoprotein. Apolipoprotein is a protein moiety in it lipid-free form as designated by the prefix, "apo". It combines with lipids to form lipoproteins. Lipoproteins are non-covalent complexes that consist of a lipid core composed of triacylglycerols and cholesterol surrounded by water-soluble phospholipids and apolipoproteins

In human blood four major lipoprotein classes can be distinguished, according to their density determined by ultracentrifugation: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (94,95). These lipoprotein classes differ with respect to size, lipid, and apolipoprotein composition. In the brain there is only one type of lipoprotein, which is similar to HDL in periphery.
Nascent apoE-containing particles secreted by glia are primarily discoidal in shape and contain little core lipid, whereas CSF-associated apoE is associated with spherical particles approximately 15-20 nm in size that also contain peripherally acquired apoA1 and resemble HDL-particles (96-98).
The APOE4 allele is the most significant genetic risk factor associated with AD (3). The ε4 allele of apoE is very common. To date, meta-analysis from the Alzheimer's Research Forum shows that it is present in about 13 percent of the population, but 36 percent of the Alzheimer's population (http://www.alzgene.org. Accessed August 4, 2005).
Although apoE4 occurs nearly three times as frequently in Alzheimer's populations than in the general population, neither one nor two copies of the allele is either necessary or sufficient to cause Alzheimer's disease (23). It is important to understand the significance of apoE in the brain.

Apolipoprotein E is a major lipoprotein in brain. ApoE is a well-characterized 34.2 kDa protein that participates in lipid metabolism in the periphery and the central nervous system. It plays a major role in the transport and redistribution of lipids among tissues and cells. In plasma apoE is a component of triglyceride-rich lipoprotein particles including VLDL and chylomicrons, and cholesterol-rich HDL particles (99), in the brain it is associated with HDL-like particles (100). Transplantation experiments have demonstrated that apoE in synthesized within the brain rather than originating from the periphery (101). In the CNS, apoE is synthesized primarily by astrocytes and by microglia in lesser amount (102,103). ApoE lipoproteins influence membrane lipid raft composition and properties of enzymes, transporter proteins, and receptors mediating Aß production and degradation, tau phosphorylation, glutamate and glucose uptake, and neuronal signal transduction. Thus, lipid rafts may be an important site where Aß, apoE, and tau interact in a way that influences the formation of Aß fibrils and paired helical fragments of phosphorylated tau. ApoE also mediates the transport, fibrilization, and clearance of Aß, and the level and isoform of APOE may influence whether Aß is likely to be metabolized or deposited.

ApoE is involved in the regulation of cholesterol in the outer (exofacial) and inner (cytofacial) leaflets, and in the PUFA content of phospholipid molecular species of the synaptic membrane (104). Changes in synaptic membrane lipid composition result in altered function of membrane-bound enzymes. ApoE lipoproteins may also participate in the efflux of cholesterol from the neuronal cell surface, particularly under circumstances of cellular degeneration. ApoE's primary benefit to the nervous system appears to be its delivery of cholesterol to neurons, which is necessary for synapse formation.

Bohr et al has suggested that apoE4 is less able to efficiently deliver cholesterol to neurons, and that this characteristic contributes to its causal role in Alzheimer's disease (105). Bohr cites Lane and Farlow (106), who observe that apoE4 is less efficient at transporting free fatty acids, and notes that apoE4 is associated with a higher level of cholesterol, which may indicate that cholesterol is not being internalized into cells efficiently. It has been shown that in the presence of beta-migrating very low density lipoproteins (beta-VLDL), apoE3 increased neurite outgrowth, whereas apoE4 decreased outgrowth (107). Yet a study by Ji et al (108) appears, at least at first glance, to contradict this. They compared the effect of apoE3 to that of apoE4 on neuronal cell culture, incubating both forms of apoE in beta- very low-density lipoprotein (beta-VLDL), and found that apoE4 was slightly more efficient at delivering cholesterol to cells, although apoE3, not its associated cholesterol, was retained within the cell at several times the amount that apoE4 was retained. This appeared to occur because apoE4 was quickly released from the cell, not because it was broken down within the cell. Even still, apoE3 enhanced dendrite and axon growth, while apoE4 inhibited dendrite and axon growth.

This appears to suggest that while cholesterol is the limiting factor in the formation of *synapses*, internalized apoE might be the limiting factor in the initial *growth* of the axons and dendrites. It is unknown why there is a difference between the two forms of apoE in this matter, but there are several (speculative) possibilities: apoE or one of its breakdown products is needed by the cell to deliver cholesterol to lipid rafts in the membrane, which are known to be necessary signaling molecules involved in the

growth of dendrites and axons (50), alternatively, apoE is released into the cytosol (the inside of a cell) where it complexes with other proteins necessary for axon and dendrite growth (Suggested by Ji et al (108).) This, in fact, is *critical* to evaluating the observation of whether or not apoE4 is *less* or *more* efficient at delivering cholesterol to cells.

Lane and Farlow note that apoE4 has a preferential affinity to bind to high-triglyceride lipoproteins like chylomicrons and VLDL, as opposed to low-triglyceride lipoproteins such as LDL, IDL, and HDL (106). While they are making a different point, this observation bears an important corollary that has gone unnoticed.

According to Enig, excluding HDL, there is an inverse relationship between cholesterol and triglyceride percentage of a lipoprotein (109). The following data is derived from this table, but excludes irrelevant information:

	Chylomicrons	VLDL	LDL	IDL
Triglyceride %	84-89	50-65	30	7-10
Cholesterol %	4-15	15-25	30	42-50

ApoE4, by binding to high-triglyceride lipoproteins, is also preferentially binding to *low-cholesterol* lipoproteins, because high-triglyceride lipoproteins are also *low-cholesterol* lipoproteins (109). Thus, in a living organism, it appears that apoE4 is less effective than other forms of apoE at delivering cholesterol to cells because it prefers lipoproteins that have less cholesterol. This is consistent with the observation that cholesterol levels are higher in people bearing the apoE4 allele, possibly due to deficient internalization of that cholesterol, and supports Bohr's hypothesis (105).

ApoE4 has other effects as well. ApoE4 has many of the same effects as a high-carbohydrate diet, it has been shown that apoE4 is least common in populations with a long history of agriculture, and most common in present hunter-gatherer populations, ranging from four percent in Israel to 40.7 percent among African Pygmies (106).

It may well be that apoE4 is only a harmful gene *if* it is accompanied by a high-carbohydrate diet that one's ancestors have not partially adapted to by weeding out the apoE4 gene. Is ApoE4 the "cause" of Alzheimer's? As Tanzi and Bertram note (23), even carrying two copies of the allele is not sufficient to cause Alzheimer's, nor is carrying even one copy necessary to cause Alzheimer's. Therefore, there must be dietary and/or environmental (or other genetic) factors that interact with ApoE4's ability to contribute to the cause of Alzheimer's. Apolipoprotein C1 might be such an additional genetic factor.

The role of ApoC1 in the development of the Alzheimer's disease

After apolipoprotein E4 (apoE4) had been identified as a major risk factor for the development of sporadic, late onset Alzheimer's Disease (AD) (3,110), many researchers began looking for additional susceptibility genes for this disease (111). Interestingly, APOE4 is in genetic linkage disequilibrium with the *HpaI* restriction polymorphism in the promoter region of APOC1, which is localized 5 kb downstream of the APOE gene on chromosome 19. Although apoCI is mainly expressed in the liver,

substantial expression has also been detected in other tissues including the brain (112). The *HpaI* polymorphism (so-called H2 allele) leads to a highly significant, 1.5-fold increase in APOC1 gene transcription (113) and was reported to be associated with AD (11-14,114,115). Moreover, the H2 allele of APOC1 was associated with poorer memory and frontal lobe function (116), and with loss of hippocampal volumes (117).
Although apoE4 is a well-established and strong risk factor of AD in Caucasians and in European-Americans, it is not associated with AD in African-Americans. Interestingly, the frequency of APOC1 H2 with apoE4 was 0.85 in European-Americans but only 0.55 in African-Americans, whereas the frequency of APOC1 H2 with apoE3 was 0.02 in European-Americans and 0.08 in African-Americans (113). This led to the hypothesis that the H2 allele of APOC1 may be an independent or an additional risk factor for AD (13,114,118,119), and that not apoE4 but rather apoCI modulates the pathogenesis of AD (120).
The physiological roles of apoCI have not been established in detail. Studies with mice overexpressing human apoCI (121) revealed gene dose-dependent effects on circulating levels of triglycerides (TG), free fatty acids (FFA) and total cholesterol (TC) (122,123). This may be due to interference of apoCI with apoE-dependent clearance of TG-rich lipoproteins by Low Density Lipoprotein-Receptor family in the liver (124-126) and by modulating the activity of enzymes involved in plasma lipid metabolism, (127,128).
The presence of apoCI within the brain at relatively high levels (112) suggests an important role for this protein in brain lipid metabolism. Disturbances in brain lipid metabolism can lead to cognitive impairments in humans and in rodents, indicating that a well-regulated brain lipid metabolism is necessary for normal brain functioning (106,129-132). Therefore, we have investigated learning and memory functions as well as brain lipid metabolism in transgenic mice hemizygous for human apoCI (*hAPOC1*$^{+/0}$) and their wild type littermates. Moreover, we examined the expression of apoCI in post mortem brains of AD and control patients.

Liver X Receptors (LXRs) in the CNS

An aberrant brain cholesterol metabolism may play an important role in the pathogenesis of AD. Hardly anything is known about the regulation of the cholesterol metabolism in brain.
Subsequent studies demonstrated that LXRs are central players in cholesterol homeostasis throughout the periphery (133) and most likely also in brain. The absence of LXRs in mice leads to disturbances in lipid homeostasis in the CNS and age related neuropathological changes such as astrocyte proliferation and disorganized myelin sheaths (134). In the circulation the combined deficiency of LXRalpha and LXRbeta was linked to impaired triglyceride metabolism, increased LDL and reduced HDL cholesterol levels, and cholesterol accumulation in macrophages (foam cells) of the spleen, lung, and arterial wall (135). The characteristic features of the brains of LXR $\alpha^{-/-}\beta^{-/-}$ mice are accumulation of lipid droplets and closure of ventricles. The most severe abnormalities appeared in specific brain areas, where the blood vessels were enlarged and surrounded by lipid deposits. The expression of established LXR target genes were significantly decreased in LXR $\alpha^{-/-}\beta^{-/-}$ mice. These include ABCA1, ABCG1, ABCG8,

SREBP-1c and -2 LDLR, HMG-CoA reductase, farnesyl pyrophosphate synthase, squalene synthase and steaoryl CoA desaturase-1(134). These studies demonstrate that LXRs modulate cholesterol homeostasis in the CNS and are required for normal CNS function.

Moreover, more recent evidence points to additional roles for the LXR nuclear receptors in inflammation (136), glucose metabolism (137-139) and fertility (140). Several genes involved in cholesterol (141) and fatty acid metabolism (142,143) have been found to be regulated by LXR. Some of these genes are: cytochtome P450 7A1, the rate limiting enzyme in bile acid biosynthesis in the liver; the sterol regulatory element-binding protein-1 (SREBP-1); acetyl CoA decarboxylase; fatty acid synthase; and several ATP-binding cassette trasporter proteins (ABCs) (144-149). LXRs also promote the transcription of the cholesterol esther transfer protein (CETP), lipoprotein lipase (LPL), apoE (150) and apoC1 (151). When activated by ligand, LXRs induce CYP7A-1 mediated cholesterol degradation in the liver (rodents) and promote cholesterol efflux from macrophages, hepatocytes and intestinal enterocytes. Enhanced efflux is mediated by LXR-dependent activation of cholesterol/lipid transporters (ABCA1, ABCG1, ABCG5 and ABCG8) and cholesterol acceptor proteins (apoE). These effects are biologically significant as synthetic LXR agonists increase plasma HDL cholesterol, stimulate cholesterol excretion into the bile and the faeces, decrease hepatic cholesterol content and can reduce atherosclerotic lesions by ~50% in various murine models (143,152-155).

Both subtypes are expressed in the CNS at significant levels: LXRα mRNA levels range from 7-29% of that in the liver, while LXRβ levels are 2-5 folds higher in the brain than in liver. The levels of LXRα in cultured neurons and glia are 2% and 17% compared to the liver, and that for LXRβ are 1.1- and 3.8-fold, respectively (156). LXRß, in particular, is broadly expressed in the developing and adult rodent brain (157). Recent studies showed that the LXR agonists 22(R)-hydroxycholesterol and 5-tetradecyloxy-2-furancarboxylic acid induce neuronal differentiation as measured by neurite outgrowth in rat pheochromocytoma cells (158). Astrocytes treated with synthetic LXR ligands exhibit enhanced cholesterol efflux and increased expression of LXR target genes including ABCA1, ABCG1, and apoE (156,159-161).

It has been demonstrated that LXRs can modulate proteolytic-processing of APP, albeit with conflicting conclusions. Fukumoto et al (159) showed that LXR agonists (22(R)-hydroxycholesterol or T0901317) increased both ABCA1 expression and Aβ secretion in Neuro2A cells; exogenous cholesterol carrier was not added. The increase in Aβ was likely mediated via ABCA1 as siRNA targeting this transporter decreased Aβ production. Koldamova et al (160) using two human neuroblastoma cell-lines stably expressing APPswe, observed the expected increase in ABCA1 expression, enhanced cholesterol efflux and subsequent decrease in total cellular cholesterol. However, in sharp contrast to the previous group, LXR agonist (22(R)-hydroxycholesterol) reduced Aβ production. In these experiments, addition of apoA1 or apoE to the culture medium promoted significant cholesterol efflux, which was associated with further reduction in Aβ. It has been found that the LXR agonist (T0901317) enhanced ABCA1 expression and decreased Aβ production in mouse Neuro2A cells expressing human APPswe (162). The reduction in Aβ was related to a decrease in β- and γ-secretase cleavage. Moreover, the decrease in Aβ could be mimicked by overexpression of wild-type

ABCA1 but not by a mutant that was defective in cholesterol transport and apoAI binding. However, ABCA1-mediated reduction in Aβ was observed under conditions where there was little or no cholesterol or phospholipid efflux (i.e. without apoAI or apoE in the media). Thus unlike Fukimoto et al both Koldamova et al and Sun et al found that LXR inhibits Aβ by inducing ABCA1 activity. Some discussion remains as to whether the relevant activity of ABCA1 is intracellular cholesterol redistribution vs. lipid efflux. The reasons for the discrepancies among the 3 groups could be related to differences in cell lines (mouse vs. human; wild-type vs. APPswe+) and/or other methodologies.

Statins, Cholesterol and Alzheimer's disease

A most interesting aspect of the association between cholesterol and AD is that inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, so-called statins, are associated with a reduced risk of developing AD (83,84,88). Epidemiological data show that patients taking statins had a lower risk of developing AD compared with individuals not taking statins, and effects were cholesterol-independent (83,84,88). HMG-CoA reductase is the primary regulatory enzyme in cholesterol biosynthesis

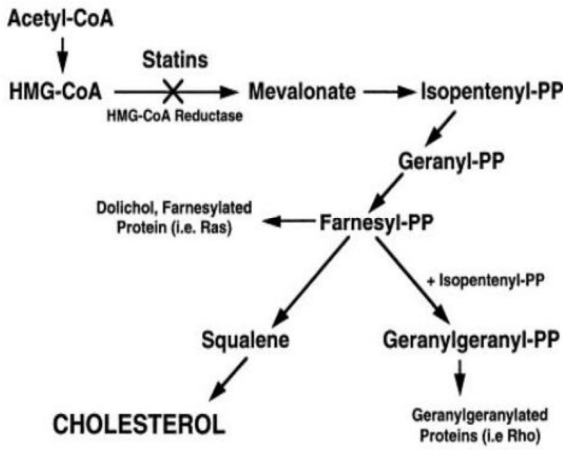


Figure 5 The molecular mechanisms by which statins act include inhibiting the rate of conversion of acetate molecules into cholesterol by the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol biosynthesis. Because a precise amount of cholesterol is required in cells, inhibition of synthesis leads to a homeostatic response in which cells increase the density of LDL receptors on their surface. This increases the clearance rate of LDL particles from the plasma and reduces plasma LDL cholesterol secondarily.

A straightforward explanation for the beneficial effects of statins on reducing the risk of AD is a reduction of brain and serum cholesterol levels. However, it is unclear whether the apparent efficacy of statins is related to simply lowering cholesterol levels. Data on cholesterol levels in brains of AD patients range from less cholesterol, no differences, and more cholesterol when compared with brain tissue of control samples (163,164). Non-statin drugs that lower cholesterol levels do not lower the risk of developing AD (83). Statins appear to reduce the risk of AD; however, the mechanism underlying statin efficacy does not appear to be simply reduction of cholesterol levels. Moreover both clinical and animal studies evaluating the effect of statins on APP are cited as contradictory. Hoglund et al published three studies on the effect of statins on beta-amyloid. In the first study (165), simvastatin was used on AD patients and found to have no effect on beta-amyloid. The second study (166) used simvastatin or atorvastatin on hypercholesterolemics, and found no effect on beta-amyloid. The third study (167) used simvastatin on AD patients and analyzed their cerebro-spinal fluid for a particular AD-related pattern of beta-amyloids, and found no effect. Not only was there not an effect on beta-amyloid, but there was a significant reduction of total plasma and LDL cholesterol, and there was a significant reduction in biomarkers for brain cholesterol levels. Thus, we know the dose of statin was high enough to reduce brain cholesterol levels, yet still did not affect beta-amyloid levels. Yet according to a recent study, high doses of statins inhibit the growth of dendrites and axons and induce neuronal apoptosis (168). On the other hand statins (lovastatin, simvastatin and pravastatin) affect membranous cholesterol homeostasis in the brain, although in different manner (169). It has been hypothesized that statins directly or indirectly induce alterations in the equilibrium of transbilayer cholesterol distribution in the plasma membrane and thereby affect the accessibility of membrane cholesterol to distinct cellular domains (169). Interestingly, lovastatin treatment, for the dose and time investigated, did not alter brain cholesterol in ApoE -/- animals, whereas in C57BL/6J mice, brain cholesterol was significantly reduced by about 30% (170), suggesting the important involvement of apoE in neuronal cholesterol metabolism.

There is a growing recognition that statins have pleiotropic effects beyond lowering cholesterol levels. Pleiotropic effects of statins outside of the central nervous system have been described that include activation of protein kinase Akt, up-regulation of endothelial nitric-oxide synthase expression, anti-inflammatory actions, and antioxidant activity (171). In an experimental model of stroke, brain tissue of rats treated with either simvastatin or atorvastatin displayed increased synaptogenesis, neurogenesis, and angiogenesis that was cholesterol-independent (172). In another study, however, simvastatin, pravastatin, atorvastatin, rosuvastatin, and mevastatin protected mouse neurons from *N*-methyl-D-aspartate-mediated excitotoxicity and cell death and appeared to be cholesterol-dependent because alterations in cholesterol by cyclodextrin modified excitotoxicity (173). The potential neuroprotective effects of statins may include several different pathways involving distinct gene expression patterns that would be difficult to determine by focusing on a few genes or their products in a single study. In addition, gene expression patterns may be specific to a particular statin. Lovastatin has been detected in human cerebrospinal fluid, whereas pravastatin is not thought to cross the blood-brain barrier (174) even though patients taking pravastatin were at a lower risk for developing AD (83,84,88). Statins may accumulate in brain as a result of chronic

administration including pravastatin, and therefore statin levels were determined in the cerebral cortex of mice that were acutely and chronically administered statins (175).

The aim of the thesis

The aim of this thesis was to elucidate possible regulatory mechanisms of the fine tuned brain cholesterol homeostasis. The first part of the study is focused on the role of apoE, the main cholesterol transporter in brain, since apoE4 isoform is the strongest known genetic risk factor for AD. We tested the hypothesis that the apoE transcription, synthesis and secretion in astrocytes are induced by the brain-specific metabolite of cholesterol produced by neurons, i.e. 24(S)-hydroxycholesterol, via LXR mediated pathway.

Since we demonstrated LXRs mediated effect of the natural brain specific 24(S)-hydroxycholesterol in brain cholesterol metabolism using *in vitro* experimental approach, we further addressed our second question to the effect of LXR activation in vivo. We investigated the effects of long-term treatment of C57Bl6/J mice with the synthetic LXR agonist T0901317 on the whole body as well as brain cholesterol turnover.

The third part of the study was to discover the effects of plant sterol accumulation in the brain of mice deficient for ATP-binding cassette transporter G5 (ABCG5) or ABCG8 on brain function.

The physiological role for apoC1 in brain still remains a fascinating and enigmatic question giving rise to the next aspect of the thesis. Since APOE4 is in genetic linkage disequilibrium with the H2 allele of APOC1, which is associated with AD, the hypothesis has been raised that the H2 allele of APOC1, rather than the APOE4 allele, provides a major risk factor for AD.Therefore, in the next two chapters of thesis we attempted to clarify the functions of apoC1 in brain. To investigate a possible role for apoCI in cognitive functions we used *human APOC1*$^{+/0}$ transgenic mice and APOC1$^{-/-}$ knockout mice.

Finally, the last chapter of the thesis gives an overview on vascular aspects in the development of AD. At present, cardiovascular afflictions, hypertension and diabetes constitute accepted risk factors for vascular dementia (VaD) and have gained importance for AD

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Chapter 2

24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux

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Abstract

Both apolipoprotein E (apoE) and 24(S)-hydroxycholesterol are involved in the pathogenesis of Alzheimer disease (AD). It has been hypothesized that apoE affects AD development via isoform-specific effects on lipid trafficking between astrocytes and neurons. However, the regulation of the cholesterol supply of neurons via apoE-containing high density lipoproteins remains to be clarified. We show for the first time that the brain-specific metabolite of cholesterol produced by neurons, i.e. 24(S)-hydroxycholesterol, induces apoE transcription, protein synthesis, and secretion in a dose-and time-dependent manner in cells of astrocytic but not of neuronal origin. Moreover, 24(S)-hydroxycholesterol primes astrocytoma, but not neuroblastoma cells, to mediate cholesterol efflux to apoE. Similar results were obtained using the synthetic liver X receptor (LXR) agonist GW683965A, suggesting involvement of an LXR-controlled signalling pathway. A 10–20-fold higher basal LXRα and -β expression level in astrocytoma compared with neuroblastoma cells may underlie these differential effects. Furthermore, apoE-mediated cholesterol efflux from astrocytoma cells may be controlled by the ATP binding cassette transporters ABCA1 and ABCG1, since their expression was also up-regulated by both compounds. In contrast, ABCG4 seems not to be involved, because its expression was induced only in neuronal cells. The expression of sterol regulatory element-binding protein (SREBP-2), low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl-CoA reductase, and SREBP-1c was transiently up-regulated by GW683965A in astrocytes but down-regulated by 24(S)-hydroxycholesterol, suggesting that cholesterol efflux and synthesis are regulated independently. In conclusion, evidence is provided that 24(S)-hydroxycholesterol induces apoE-mediated efflux of cholesterol in astrocytes via an LXR-controlled pathway, which may be relevant for chronic and acute neurological diseases.

Introduction

Disturbances in brain cholesterol homeostasis are associated with the onset of severe neurological diseases (1) and have recently been suggested to play a key role in the development of Alzheimer disease (AD) (2, 3). The brain, although composing just 2% of the total body mass, contains about a quarter of an individual's whole body unesterified cholesterol. Brain cholesterol originates almost exclusively from *in situ* neosynthesis (1); circulating cholesterol is prevented from entering the brain by the blood-brain-barrier (4). Because cholesterol cannot be degraded and is neurotoxic at high levels, excess cholesterol is secreted from the brain into the circulation (5). Cholesterol is removed from the brain predominantly (about 60%) in the form of the relatively polar brain-specific metabolite, 24(*S*)-hydroxycholesterol, formed by the enzyme cholesterol 24(*S*)-hydroxylase (CYP46) (1). The remaining 40% of cholesterol is secreted from the brain via an unknown pathway that may involve apoE (6). CYP46 is expressed predominantly by neurons (7, 8). Several studies have suggested a role for 24(*S*)-hydroxycholesterol in the pathogenesis of AD (9–11). Polymorphisms of CYP46

have been linked to AD, and the expression of this enzyme appeared to be shifted from neurons to glia in AD patients (12). Finally, increased levels of 24(*S*)-hydroxycholesterol levels have been detected in cerebrospinal fluid of AD patients (13). 24(*S*)-Hydroxycholesterol is a natural ligand of the liver X receptors (LXR), which have recently been identified as central players in the regulation of cholesterol metabolism (14,15). LXR belong to the nuclear hormone receptor superfamily, and two isoforms, *i.e*. LXRα and LXRß, have been identified that are activated by oxysterols. Both isoforms of LXR are expressed in the central nervous system (16) and are thought to be involved in the regulation of brain cholesterol metabolism. LXRα/ß null mice show a variety of central nervous system defects upon aging, including lipid accumulation, astrocyte proliferation, and disorganized myelin sheaths (17). The synthetic LXR ligand T0901317 was found to induce the expression of apoE and of the ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1) in astrocytes (18). However, reported *in vivo* effects of T0901317 on apoE expression in mouse brain are inconsistent (16, 19, 20).

The strongest genetic risk factor known for sporadic AD is apolipoprotein E4 (apoE4), one of the three common apoE variants (apoE2, apoE3, apoE4) in humans (21, 22). ApoE is a key player in the transport of cholesterol in the circulation (23) and is also thought to fulfil such a role within the brain (24). Astrocytes are the predominant source of apoE in the brain. These cells secrete apoE in association with cholesterol and phospholipids in the form of small, high-density lipoprotein-like particles (25). It has been suggested that these particles provide neurons with cholesterol required for the formation of new membranes, *e.g.* during development, regeneration after injury, or during the formation of new synaptic contacts (26). It has been hypothesized that apoE may affect the pathogenesis of AD by isoform-specific effects on lipid trafficking between astrocytes and neurons (27). Indeed, apoE in combination with cholesterol induces the outgrowth of neurites in an isoform-specific manner in neuronal cultures (28). However, factors that regulate the supply of glial-derived apoE-containing lipoproteins are poorly understood.

In this study we tested the hypothesis that 24(*S*)-hydroxycholesterol represents a natural brain-specific LXR ligand that is involved in the regulation of the apoE-mediated lipid supply. For this purpose the effects of 24(*S*)-hydroxycholesterol and the synthetic LXR agonist GW683965A on the expression of apoE and additional LXR target genes involved in cholesterol efflux were compared using human neuroblastoma and astrocytoma cell lines as well as primary astrocytes. We found that 24(*S*)-hydroxycholesterol, like GW683965A, is able to induce the expression of ABCA1, ABCG1, and apoE in astrocytes and to elevate apoE-mediated cholesterol efflux in astrocytoma but not in neuroblastoma cells. Our observations support the hypothesis that 24(*S*)-hydroxycholesterol participates in an LXR-controlled pathway that regulates cholesterol availability in the brain.

Experimental procedures

Chemical Reagents

24(*S*)-Hydroxycholesterol was a kind gift from Dr. D. Lutjohann (Bonn University, Germany), and 22(*R*)-hydroxycholesterol was a kind gift from Dr. J. Plat (Maastricht University, The Netherlands). GW683965A was provided by Glaxo Smith Kline. The following reagents were purchased from Sigma: 9-*cis*-retinoic acid, leupeptin, aprotinin, phenyl methyl sulfonyl fluoride (PMSO), bovine insulin, human transferrin, putrescine, sodium selenite, and progesterone. ApoA-I and apoE were purchased from Calbiochem. Stocks of 24(*S*)-OH cholesterol (10 mM), 22(*R*)-OH cholesterol (10 mM), and cholesterol (10 mM) were dissolved in ethanol. GW683965A (2 mM) and 9-*cis*-retinoic acid (10 mM) were dissolved in dimethyl sulfoxide (DMSO).

Cell Culture Experiments

The human astrocytoma cell line CCF-STTG1 and human neuroblastoma cell line SH-SY-5Y were purchased from European Collection of Cell Cultures (Salisbury, UK). CCF-STTG1 and SH-SY-5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. At 80–90% confluency cells were washed with phosphate-buffered saline (PBS) and treated with different reagents in DMEM/Ham's F-12 medium (1:1) with 10% fetal calf serum for various periods of time as indicated. SH-SY-5Y cells were preincubated for 24 h in the DMEM-Ham's F-12 (1:1) medium without serum containing the N2 supplement (2.5 mg/ml bovine insulin, 10 mg/ml human transferrin (iron-saturated), 0.52 µg/ml sodium selenite, 1.61 mg/ml putrescine, and 0.63 µg/ml progesterone in PBS, pH 7.3). At the end of the treatments conditioned media were collected, and the cells were either lysed in radioimmune precipitation assay buffer (Santa Cruz, CA), or total RNA was isolated as described below for subsequent analyses.

The method of rat primary astrocytes culturing was similar to published procedures (29, 30). Neonatal (postnatal day 1) Lewis rat pups, bred in the animal facilities of Maastricht University, were decapitated, and the neocortex was dissected and cleared of meninges. The tissue was diced in small fragments and incubated in trypsin (0.05% in phosphate-buffered saline) at 37 °C for 15 min. Trypsinization was stopped by adding culture medium, and the tissue was gently centrifuged. The supernatant was discarded, and the pellet was resuspended in 1 ml of culture medium. Single cell dissociation was achieved by 3–5 passes through a 5-ml pipette (Greiner, Germany) and 10–20 passes through a 1-ml pipette (Greiner). Then the tissue was centrifuged very briefly to separate cells from tissue debris. The supernatant containing the cells was then plated into 25 cm^2 cell culture flasks (Corning, NY) at a density of 10^6 cells per flask. Culture medium was refreshed after 4–5 days and every 2 days thereafter. At DIV12 (days *in vitro*) the cultures reached confluence, and contaminating cells were shaken off on a rotary shaker (Rotofix 32, Hettich Zentrifugen). This involves shaking of the flasks for 48 h and refreshment of the medium. At DIV14 the purification is complete and renders ~95% glial fibrillary acidprotein (GFAP)-immunopositive astrocyte cultures.

Primary murine mixed glial cultures were prepared from postnatal day-1–2 C57Bl/6 mice. Brains from individual animals were placed into ice-cold Hanks'-buffered salt

solution (Canadian Invitrogen) containing 6 mg/ml glucose and 10 mM HEPES. Meninges were removed, frontal cortices were dissected, and cells were dissociated by repeated passage through a series of wide to fine bored pipettes. Dissociated cells were plated in DMEM (Invitrogen) with 10% fetal bovine serum, 2 mML-glutamine (Invitrogen) and 100units/ml penicillin-streptomycin (Invitrogen) at one 24-well plate per mouse. Cells were cultured in the presence of 5% CO2 for 12 days when cells were confluent and contained at least 80% astrocytes. Cells were treated with either vehicle-only (ethanol) or increasing concentrations of 24(*S*)-hydroxycholesterol for 24 h. Subsequently, cells were washed once with phosphate-buffered saline, harvested, and lysed using a buffer containing 10% glycerol, 1% Triton X-100, and protease inhibitor (Roche Applied Science) in PBS. Protein concentration was determined by a Dc protein assay (Bio-Rad).

Western Blot Analysis

Cell lysates (25 µg of protein/lane) or conditioned media concentrates (concentrated using Microcon¨ centrifugal filter device, Millipore, Billerica, MA) were subjected to dodecyl sulfate-10 (SDS) or 12% PAGE and then transferred to Protran nitrocellulose transfer membranes (Schleicher & Schuell) or to polyvinylidene membranes (Millipore). After blocking with 5% nonfat dry milk (Protifar-plus, Nutricia Netherland B.V.) in washing buffer (PBS with 0.5% Triton-X100), the membranes were incubated with antibodies against human apoE (1:500, DAKO A/S, Denmark), murine apoE (Santa Cruz), human glutamine synthetase (1:500, BD Transduction Laboratories), glyceraldehyde-3-phosphate dehydrogenase (Chemicon), or a monoclonal anti-ABCA1 antibody raised against the second nucleotide binding domain (NBD2 of ABCA1) (31) overnight at 4 °C. The membranes were then incubated with peroxidase-conjugated secondary antibodies, after which the results were visualized using ECL reagents (Amersham Biosciences) and autoradiography (LAS 3000, Fuji Photo Film Co., Ltd., Japan). Bands were quantitated by densitometry using NIH Image J.

RNA Isolation and Real-time Quantitative PCR (QRT-PCR) Procedures

Total RNA was isolated using the Trizol method (Invitrogen) according to the manufacturer's instructions. Integrity of RNA was checked by agarose gel electrophoresis, and RNA concentration was measured spectrophotometrically (NanoDrop, Witec AG, Littau, Germany). Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector with 1.6.3 software (PerkinElmer Life Sciences) as previously described (32) with modifications (33). Primer sequences are available upon request. Primers were obtained from Invitrogen. Fluorogenic probes labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) were made by Eurogentec (Seraing, Belgium).

Efflux Studies

CCF-STTG1 or SH-SY-5Y cells were cultured in DMEM/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum. AfterwashingwithDMEM/Ham'sF-12medium(1:1),cells were loaded with 30 µg/ml [^{3}H]cholesterol (38 Ci/mmol) in

DMEM/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum for 24 h in the presence or absence of 24(*S*)-hydroxycholesterol or GW683965A. After cells were washed 5 times with PBS-bovine serum albumin 0.2% (w/v), the efflux assay was started by adding 2.5 μg/ml apoE or 5 μg/ml apoA-I in DMEM/Ham's F-12 medium (1:1) to the wells. After 20 h of incubation at 37 °C, the medium was collected and centrifuged. Subsequently, [^{3}H]cholesterol was quantified in the supernatant by liquid scintillation counting. Total cellular [^{3}H]cholesterol was determined after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the radioactive counts in the efflux medium by the sum of the counts in the medium and the cell extract.

Statistical Analysis

Values are presented as the mean ± S.D. Statistical significance was determined by comparing means using an unpaired the Student's t test, the Mann-Whitney U test, and one-way analysis of variance with Newman-Keul's post-test. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

The Natural LXR Ligand 24(S)-Hydroxycholesterol Induces ApoE GeneExpressionand Protein Levels in Astrocytoma Cells and in Primary Astrocytes but Not in Neuroblastoma Cells

To determine the effect of the natural LXR-ligand 24(S)-hydroxycholesterol on apoE expression in astrocytoma- and neuroblastoma cells and in primary astrocytes, cells were incubated with increasing amounts of the compound. We found that the incubation with 24(S)-hydroxycholesterol resulted a dose-dependent increase of APOE mRNA levels in astrocytoma cells (CCF-STTG1), but failed to induce APOE expression in neuroblastoma cells (SH-SY-5Y) even after 72 hours of incubation (Fig. 1A). The synthetic LXR agonist GW683965A (4 μM) also clearly induced APOE expression in astrocytoma cells, but not in neuroblastoma cells (Fig. 1A), strongly suggesting that 24(S)-hydroxycholesterol exerts its effects via the LXR pathway. Importantly, 24(S)-hydroxycholesterol also induced APOE gene expression in primary rat astrocytes in a dose dependent manner (Fig. 1B), supporting the physiological relevance of this process.

The induction of APOE expression (Fig. 2A) by 24(S)-hydroxycholesterol and GW683965A in astrocytoma cells also appeared to be time-dependent, with strong

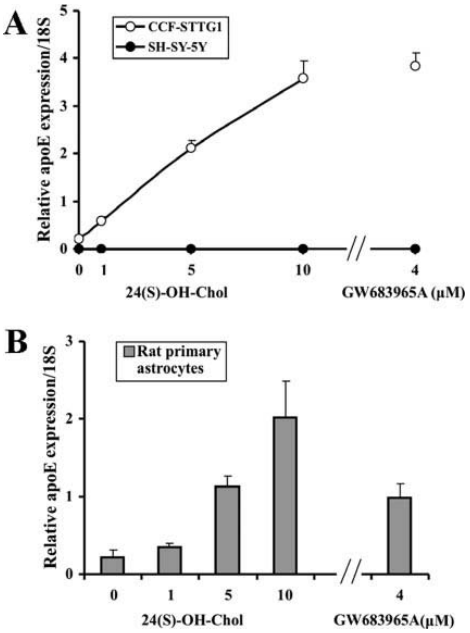


Figure 1. **Concentration dependent effect of 24(S)-hydroxycholesterol on apoE mRNA levels in CCF-STTG1 and SH-SY-5Y cells, and primary rat astrocytes.** Cells were incubated with increasing concentrations of 24(S)-hydroxycholesterol and 4 μM of GW683965A compound for a period of 72 hours. Total RNA was prepared from cells. Expression levels of apoE in astrocytoma and neuroblastoma cells **(A)** and rat primary astrocytes **(B)** were determined by QRT-PCR and expressed as relative gene expression to 18S. Values represent the mean ± S.D., n=4 in all groups.

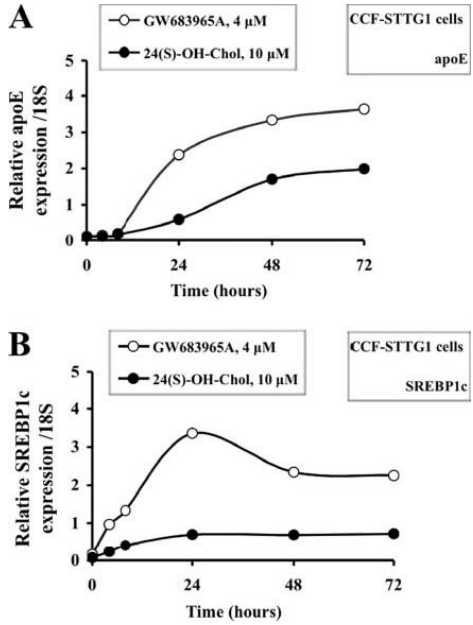


Figure 2. Time-dependent effect of 24(S)-hydroxycholesterol and GW683965A on apoE and SREBP1c expression in CCF-STTG1 cells. Cells were incubated in the presence of 10 μM 24(S)-hydroxycholesterol (●) or 4 μM GW683965A (O) for increasing periods of time. Total RNA was prepared from cells. Expression levels of apoE **(A)** and SREBP1c **(B)** were determined by QRT-PCR and expressed as relative gene expression to 18S. Representative examples of 3 independent experiments are shown.

inducton already occurring after 24 hours and a steady increase up to 72 hours of incubation. The established LXR target gene SREBP-1c was also induced by both compounds, but displayed a different induction profile compared to apoE (Fig. 2B). The expression of APOD, another LXR-target gene which has been suggested to compensate cholesterol transport functions in the absence of APOE (1), was not affected by the natural or synthetic LXR ligand in either cell line (data not shown). Next we determined if induction of APOE mRNA upregulation resulted in increased protein levels. As shown in Fig. 3A, cellular apoE protein levels in astrocytoma cells were also clearly upregulated by 24(S)-hydroxycholesterol in a concentration-dependent manner. Upon incubation of the cells for increasing periods of time in the presence of 10 μM of 24(S)-hydroxycholesterol, apoE protein levels increased up to 48 hours and remained fairly constant thereafter up to 72 hours (Fig. 3B). Similar to 24(S)-hydroxycholesterol, the synthetic LXR agonist GW683965A

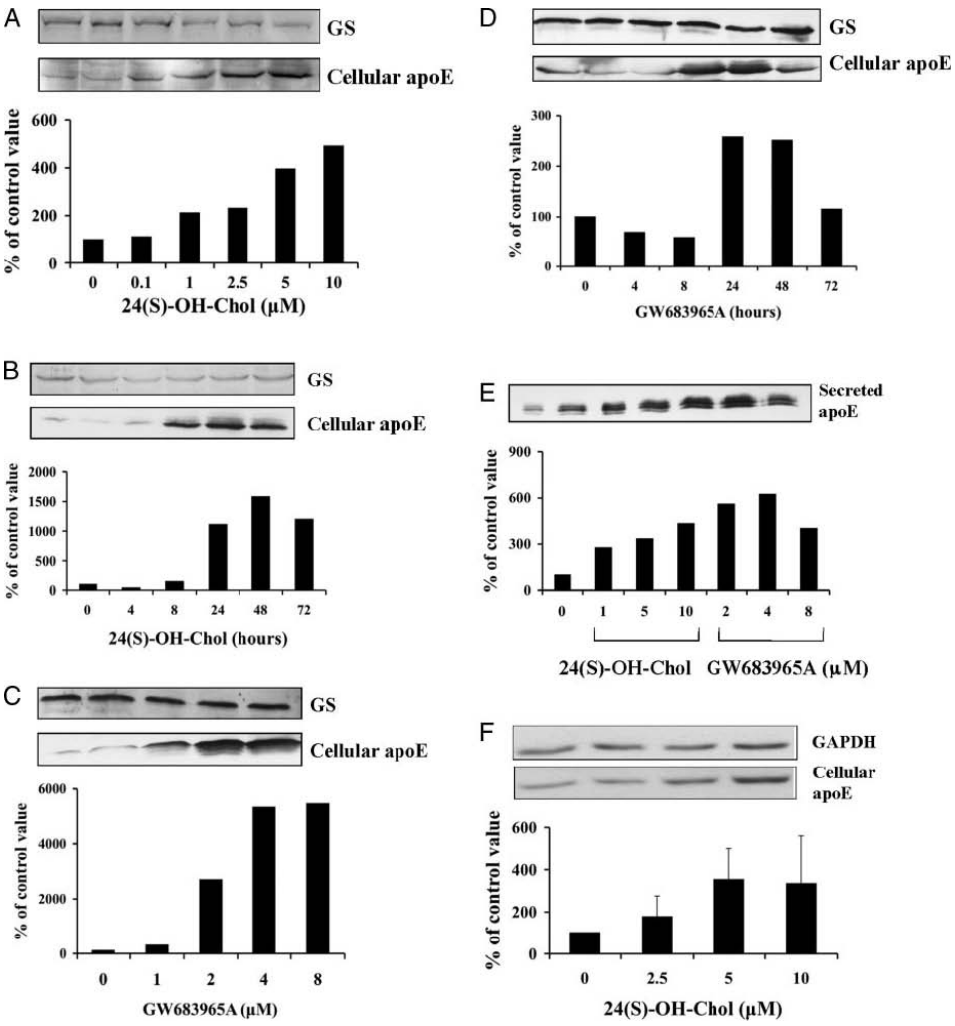


Figure 3. **24(*S*)-Hydroxycholesterol (*Chol*) and GW683965A increase apoE protein levels and apoE secretion in CCF-STTG1 cells**. 24(*S*)-hydroxycholesterol up-regulates apoE protein levels in murine primary glial cultures. CCF-STTG1 cells were treated either with increasing concentrations of 24(*S*)-hydroxycholesterol (*A*) and GW683965A (*C*) for a period of 72 h or with 10 µM 24(*S*)-hydroxycholesterol (*B*) and 4 µM GW683965A (*D*) for increasing periods of time. Conditioned medium was collected after the treatment of CCF-STTG1 cells with different concentrations of 24(*S*)-hydroxycholesterol and GW683965A for a period of 48 h (*E*). Murine primary glial cultures were treated with increasing concentrations of 24(*S*)-hydroxycholesterol for 24 h (*F*). Cell lysates and medium concentrates were subjected to Western blot analysis as described under "Experimental Procedures." ApoE expression was measured using a polyclonal antibody against apoE. Bands were quantified by densitometry, normalized to glutamine synthetase (*GS*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Representative experiments of at least three independent experiments are shown.

increased apoE protein levels in astrocytoma cells in a concentration- and time-dependent manner (Fig. 3C,D). Secretion of apoE by astrocytoma cells into the medium was also strongly induced by 24(S)-hydroxycholesterol and GW683965A in a concentration-dependent manner (Fig. 3E). 24(S)-hydroxycholesterol also upregulated cellular apoE protein levels in murine primary mixed glial cultures containing >80% astrocytes (Fig. 3F), further substantiating our findings. Since differences in expression of LXRs may underlie the differential effects of 24(S)-hydroxycholesterol and GW683965A in the two cell types we next determined their basal expression levels. Significantly higher mRNA levels of LXRα and LXRβ were found in astrocytoma cells compared to neuroblastoma cells (Fig. 4). Also SREBP-1c, an established LXR-target gene involved in cholesterol and fatty acid metabolism (2), was expressed predominantly in astrocytoma cells. In contrast, mRNA levels of SREBP-2, another transcription factor involved in control of cholesterol metabolism, were similar in both cells types (Fig. 4A). Primary rat astrocytes also displayed a similar transcription profile for these genes (Fig. 4B). 24(S)-Hydroxycholesterol is a stronger regulator of intracellular apoE protein levels in astrocytoma cells than retinoic acid (RA) and free cholesterol.

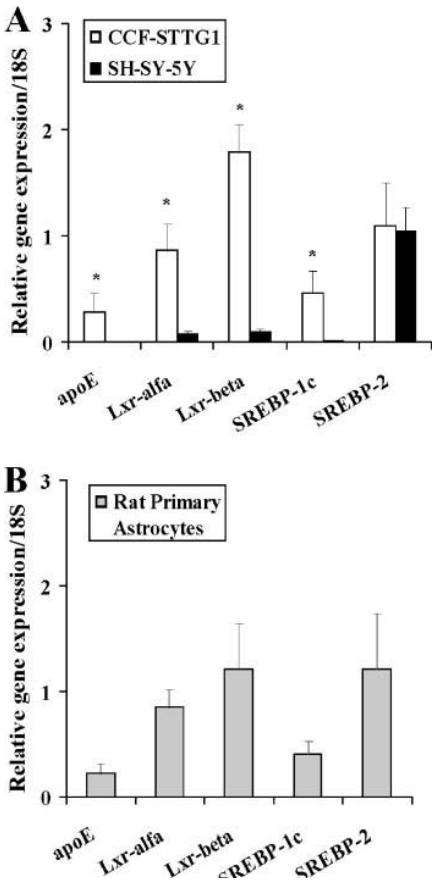
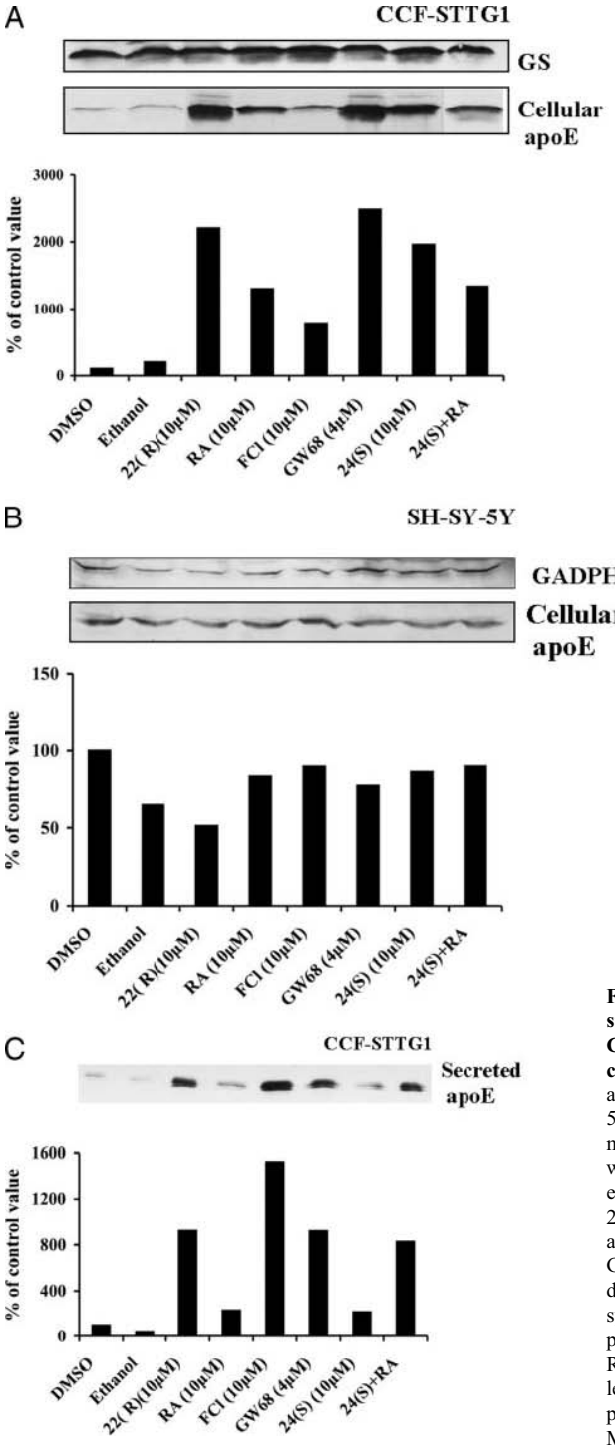


Figure 4. Comparison of the basal expression of apoE, LXRα, LXRβ, SREBP1c and SREBP-2 in CCF-STTG1 and SH-SY-5Y cells, and in rat primary astrocytes. Cells were incubated for 72 hours in DMEM/HAMF12 (1:1) medium containing 10% FCS. Total RNA was prepared from cells. Expression levels of apoE, LXRα, LXRβ, SREBP1c and SREBP2 in CCF-STTG1 and SH-SY-5Y cells (A) and rat primary astrocytes (B) were determined by QRT-PCR and expressed as relative gene expression to 18S. Values represent the mean ± S.D., n=4 in all groups. Mann-Whitney U-test was used to determine significant differences in gene expression. * represents $p < 0.01$.

24(S)-Hydroxycholesterol was compared with other natural LXR/RXR ligands with respect to its effect on intracellular apoE levels. In accordance with the observation that brain apoE is predominantly synthesized by astrocytes (3,4), we observed that the basal expression of the APOE gene in astrocytoma cells was substantially higher than in neuroblastoma cells (Fig. 4A). In astrocytoma cells, 24(S)-hydroxycholesterol and GW683965A are as potent as 22R-hydroxycholesterol in inducing intracellular and secreted apoE, and were considerably more effective than retinoic acid or free cholesterol (Fig. 5A,C). Neither 24(S)-hydroxycholesterol



nor GW683965A, or any of the other LXR/RXR agonists affected apoE synthesis in neuroblastoma cells (Fig. 5B), demonstrating that LXR-mediated upregulation of apoE synthesis and secretion is a pathway specific to astrocytes.

24(S)-Hydroxycholesterol and GW683965A regulate the expression of ABC transporters and other genes related to cholesterol metabolism in a cell type-specific manner.

ABCA1, ABCG1 and ABCG4 are transporters of cholesterol and/or phospholipids from cells to extracellular acceptors that may also be involved in intercellular lipid transport within the brain (5,6). Since all three transporters have been identified as LXR target genes present in the brain (7,8), we examined

Figure 5. 24(*S*)-hydroxycholesterol is a stronger regulator of apoE synthesis in CCF-STTG1 cells than retinoic acid and cholesterol. Shown are Western blot analyses of CCF-STTG1 (*A*) and SH-SY-5Y (*B*) cell lysates and conditioned medium from CCF-STTG1 cells (*C*) that were incubated for 72 h in the presence of either 10 μM 24(*S*)-hydroxycholesterol, 22-(*R*)-hydroxycholesterol, 9-*cis*-retinoic acid, free cholesterol (*FCl*), or 4μM GW683965A. Bands were quantified by densitometry, normalized to glutamine synthetase (*GS*) and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*). Representative experiments are shown; at least three independent experiments were performed for all conditions. *DMSO*, Me2SO.

the effects of 24(S)-hydroxycholesterol and GW683965A on their expression. The results presented in Fig. 6A shows that basal expression of ABCG1 mRNA was comparable in astrocytoma cells and in neuroblastoma cells. The relative expression of ABCA1 mRNA was higher than that of ABCG1 and also 3-fold higher in neuroblastoma than in astrocytoma cells. Likewise, the basal relative expression of ABCG4 mRNA was significantly higher in SH-SY-5Y cells than in astrocytoma cells (Fig. 6A). A pattern of relative ABC transporter expression similar to that in astrocytoma cells was found in primary rat astrocytes (Fig. 6B) Incubation with 24(S)-hydroxycholesterol resulted in a dose-dependent upregulation of the expression of ABCA1 mRNA in astrocytoma, in neuroblastoma cells (Fig. 6C) and in primary astrocytes (Fig. 6D). In contrast, GW683965A induced ABCA1 mRNA only in astrocytoma cells and rat primary astrocytes (Fig. 6C, D). Furthermore, 24(S)-hydroxycholesterol increased ABCA1 protein levels in murine primary glia (Fig. 6E).
ABCG1 mRNA was strongly induced by both 24(S)-hydroxycholesterol and GW683965A in astrocytoma cells (Fig. 6F), and primary astrocytes also demonstrated a significant induction of ABCG1 mRNA (Fig.6G). In contrast, ABCG1 was unresponsive to either compound in neuroblastoma cells (Fig. 6F). Interestingly, ABCG4 was slightly upregulated by 24(S)-hydroxycholesterol but not by GW683965A in neuroblastoma cells, whereas this gene was not affected, or even slightly down-regulated, in astrocytoma cells and in primary astrocytes (Fig. 6H, I). Effects of 24(S)-hydroxycholesterol and GW68395A on the expression of the ABC transporters mentioned were maximal at 24 hours of incubation in astrocytoma cells (data not shown).
Next we determined the effect of 24(S)-hydroxycholesterol or GW683965A on mRNA levels of other genes involved in cholesterol and/or fatty acid metabolism. Incubation of astrocytoma cells with 24(S)-hydroxycholesterol resulted in a transient down-regulation of SREBP-2, LDLR and HMGCoA-R mRNA levels. In contrast, GW683965A induced a transient increase in the expression of these genes with a maximal effect at 24 hours (Fig. 7). SR-BI expression in astrocytoma cells was not affected by either compound (data not shown). Additionally, neither 24(S)-hydroxycholesterol nor GW683965A detectably affected the expression of SREBP-2, HMG-CoA-R and LDLR in neuroblastoma cells (data not shown).

24(S)-Hydroxycholesterol enhances apoE- and apoA-I-mediated cholesterol efflux from astrocytoma cells, while only the apoA-I-mediated cholesterol efflux from neuroblastoma is enhanced.

We observed that basal cholesterol efflux from astrocytoma cells was higher than from neuroblastoma cells (approximately 4% compared to 0.5%, respectively, Fig. 8). The addition of 24(S)-hydroxycholesterol induced apoE- and apoA-I-mediated cholesterol efflux from astrocytoma cells to equivalent levels (Fig. 8A), and similar effects were observed with synthetic LXR agonist GW683965A. Interestingly, neither compound affected apoE-mediated cholesterol efflux from neuroblastoma cells (Fig. 8B), but rather specifically induced apoA-I-mediated cholesterol efflux from neuroblastoma cells (Fig. 8)

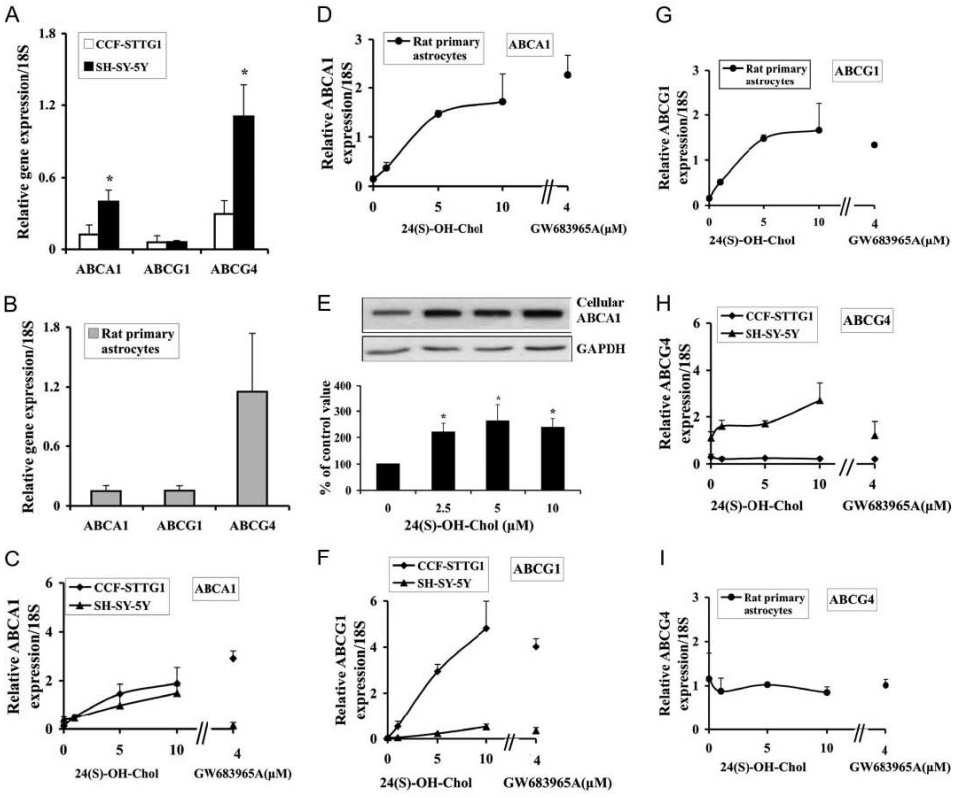


Figure 6. Effect of 24(*S*)-hydroxycholesterol (*Chol*) and GW683965A on mRNA levels ofATP binding cassette transporters in CCF-STTG1 and SH-SY-5Ycellsand rat primary astrocytes. This panel represents a basal gene expression of ATP binding cassette transporters in CCF-STTG1 and SH-SY-5Y cells (*A*) and in rat primary astrocytes (*B*). Total RNA was prepared from the cells as described under "Experimental Procedures." Gene expression relative to 18 S was determined by QRT-PCR. The Mann-Whitney *U* test was used to determine significant differences in gene expression. An *asteris*k indicates that p ≤ 0.01. Astrocytoma and neuroblastoma cells (*C*, *F*, and *H*) and rat primary astrocytes (*D*, *G*, *I*) were incubated for 72 h in the presence of 1, 5, or 10 μM 24(*S*)-hydroxycholesterol or in the presence of 4 μM GW683965A. Total RNA was prepared from the cells as described under "Experimental Procedures." Gene expression of ABCA1 (C and *D*), ABCG1 (F and *G*), and ABCG4 (H and *I*) relative to 18 S was determined by QRT-PCR. Protein levels of ABCA1 in whole lysates of primary mixed glial cultures treated with increasing concentrations of 24(*S*)-hydroxycholesterol for a period of 24 h were determined by Western blot and quantified by densitometry. Intracellular glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal protein loading control (*E*). Data represent the mean ± S.D. of cellular ABCA1 levels. One-way analysis of variance with Newman-Keul's post-test was used to determine significant differences (the *asteris*k represents p ≤ 0.01 compared with vehicle-only).

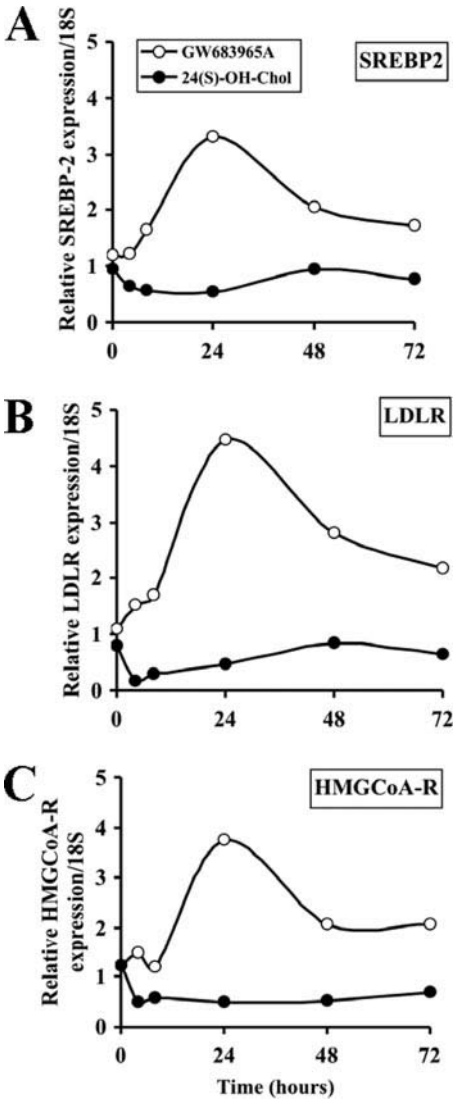


Figure 7. Time-dependent effect of 24(*S*)-hydroxy cholesterol and GW683965A on expression of SREBP2 (*A*), LDLR (*B*), HMG-CoA reductase (*C*) in CCF-STTG1 cells. CCF-STTG1 cells were incubated in the presence of 10 M 24(S)-hydroxycholesterol (●) or 4 μM GW683965A (O) for increasing periods of time. Total RNA was prepared from cells, and gene expression relative to 18S was determined by QRT-PCR.

GW683965A induced a transient increase in the expression of these genes with a maximal effect at 24 h (Fig. 7). SR-BI expression in astrocytoma cells was not affected by either compound (data not shown). Additionally, neither 24(*S*)-hydroxycholesterol nor GW683965A detectably affected the expression of SREBP-2, HMG-CoA reductase, and LDLR in neuroblastoma cells (data not shown).

24(S)-Hydroxycholesterol Enhances ApoE- and ApoA-I-mediated Cholesterol Efflux from Astrocytoma Cells, whereas Only the ApoA-Imediated Cholesterol Efflux from Neuroblastoma Is Enhanced

We observed that basal cholesterol efflux from astrocytoma cells was higher than from neuroblastoma cells (4% compared with 0.5%, respectively; Fig. 8). The addition of 24(*S*)-hydroxycholesterol induced apoE-and apoA-I-mediated cholesterol efflux from astrocytoma cells to equivalent levels (Fig. 8*A*), and similar effects were observed with synthetic LXR agonist GW683965A. Interestingly, neither compound affected apoE-mediated cholesterol efflux from neuroblastoma cells (Fig. 8*B*) but, rather, specifically induced apoA-I-mediated cholesterol efflux from neuroblastoma cells (Fig. 8).

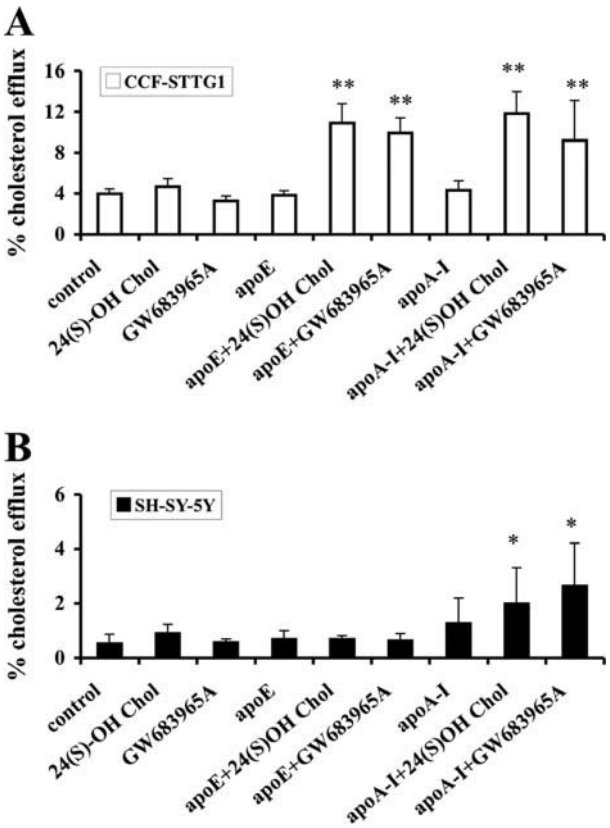


Figure 8. **24(*S*)-Hydroxycholesterol (*Chol*) and GW683965A induce apoE-and apoAI-mediated cholesterol efflux from CCF-STTG1 cells but not from SH-SY-5Y cells**. After loading the cells with [^{3}H]cholesterol for 24 h, the apoE-or apoA-I-mediated cholesterol efflux over a period of 20 h from CCF-STTG1 cells (*A*) and SH-SY-5Y cells (*B*) was determined. [^{3}H]Cholesterol was quantified in medium and in cell extracts. Values are the mean ±S.D. of three independent experiments, each measured in duplicate. Student's t test was used to determine significant differences of cholesterol efflux levels. The single asterisk represents p 0.05, and the double asterisk represents p≤ 0.001.

Discussion

In this paper we addressed the regulation of genes involved in apoEmediated cholesterol trafficking between astrocytes and neurons, an important process during regeneration and synaptic plasticity (25). We observe that 24(*S*)-hydroxycholesterol is capable of inducing apoE and ABC transporter expression in astrocytic cells as well as in primary rat and murine astrocytes. In contrast, 24(*S*)-hydroxycholesterol did not induce expression of these genes in SHSY5Y neuronal cells. Notably, 24(*S*)-hydroxycholesterolis a

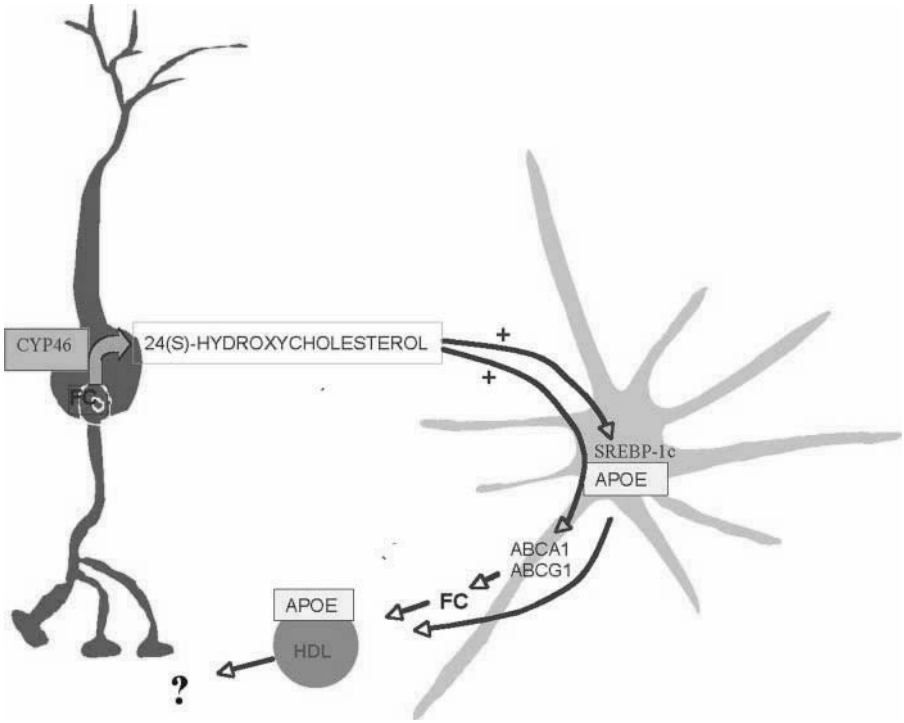


Figure 9. Schematic presentation of the differential effects in astrocytes and neurons of 24(*S*)-hydroxycholesterol on the expression of apoE and ABC transporters expression as well as apoE-mediated cholesterol efflux. 24(*S*)-Hydroxycholesterol up-regulates apoE synthesis as well as apoE-mediated cholesterol efflux in astrocytes but not in neurons. Up-regulation of ABCA1 and ABCG1 in astrocytes by 24(*S*)-hydroxycholesterol suggests involvement of these transporters in the efflux of cholesterol. *HDL*, high density lipoprotein

natural LXR ligand found in the brain, and elevated levels of 24(*S*)-hydroxycholesterol levels are often associated with neuronal injury. Our results are consistent with a model in which release of 24(*S*)-hydroxycholesterol from neurons can induce the secretion of apoE-associated cholesterol from astrocytes. This glial-derived cholesterol would then be available for neuronal uptake during the process of dendritic and axonal extension and regeneration of synapses (26).

Because the potent synthetic LXR agonist GW683965A also induced apoE, ABCA1, and ABCG1 mRNA expression in astrocyte-derived cells, our results suggest that 24(*S*)-hydroxycholesterol mediates its action through LXR activation. Furthermore, differential expression of the nuclear hormone receptor LXR isoforms, *i.e.* LXRα and -ß, between astrocytoma and neuroblastoma cells may also account for the difference in sensitivity to these agonists between astrocytic and neuronal cells. Our results suggest that 24(*S*)-hydroxycholesterol-mediated induction of ABCG1 and ABCA1 primes the astrocytes to deliver cholesterol to apoE or apoA-I, since their expression was robustly induced

by both compounds. In neuroblastoma cells, neither of the two compounds stimulated apoE-mediated cholesterol efflux, but both compounds induced apoA-I-mediated cholesterol efflux, probably involving ABCA1 actions.

It has been proposed that, after differentiation of astrocytes, neurons reduce their endogenous cholesterol synthesis and rely predominantly on cholesterol delivery by astrocytes via lipoprotein-like particles that contain astroglia-derived apoE (47). Cholesterol delivery may at least in part participate in regulating the number of synapses formed (42). A continuous turnover of cholesterol in neurons facilitates cell ability for efficient and quick adaptation of cholesterol homeostasis required for dynamic structural changes of neurons, their extensions, and their synapses during synaptic plasticity (43). Conversion of cholesterol into 24(*S*)-hydroxycholesterol, which is also a brain-specific LXR ligand, by CYP46 represents a major route for cholesterol turnover in neurons (44).

A common feature that astrocytes share with macrophages and adipocytes is their large content of free cholesterol (45, 46). In agreement with what has been reported for macrophages (47), we found that activation of LXR by 24(*S*)-hydroxycholesterol or GW683965A stimulates cellular cholesterol efflux through the coordinated regulation of ABCA1, ABCG1, and apoE. Some aspects of these pathways may be specific to astrocytes, however, because apoD was unresponsive to LXR agonists in astrocytes, whereas it is induced in adipocytes (48).

GW683965A up-regulated the expression of HMG-CoA reductase, LDLR, and SREBP2 in astrocytoma cells, supposedly to maintain cellular cholesterol homeostasis during excessive loss by efflux. However, 24(*S*)-hydroxycholesterol down-regulated the expression of these genes. It is long since known that oxysterol s reduce the activity of HMG-CoA reductase and are more potent inhibitors of cholesterol synthesis than cholesterol (49). However, the mechanisms by which cholesterol and oxysterols reduce cholesterol synthesis differ (50). Cholesterol directly interferes with SREBP cleavage-activating protein, inducing a conformational change that prevents the processing of SREBP to its active form. Oxysterols have similar effects without directly interacting with SREBP cleavage-activating protein. Thus, 24(*S*)-hydroxycholesterol may not exert its effect exclusively via the LXR pathway. The observation that 24(*S*)-hydroxycholesterol, but not GW683965A, enhanced the expression of ABCG4 in neurons suggests that LXR-independent pathways may be involved. Apparently, cholesterol efflux from astrocytes is not directly driven solely by the rate of cholesterol biosynthesis, because afflux was induced to a similar level by 24(*S*)-hydroxycholesterol and GW683965A. A concomitantly enhanced cholesterol efflux, up-regulation of ABCA1 and ABCG1, and impaired synthesis of cholesterol has recently also been reported as a consequence of statin treatment in macrophages (47).

Numerous studies have demonstrated that ABCA1 is necessary for the efflux of cellular cholesterol to lipid-poor apoA-I (51). Recently, ABCA1 was found to facilitate the efflux of central nervous system cholesterol to apoE as the absence of ABCA1 compromised apoE secretion from both astrocytes and microglia. In addition, apoE that is present in the cerebrospinal fluid of ABCA1-deficient animals is poorly lipidated (38, 52). In contrast to ABCA1, ABCG1 and ABCG4 are thought to facilitate the efflux of cholesterol to high density lipoprotein rather than to lipid-poor apolipoproteins (53, 54). A relationship between ABCG1 and the secretion of apoE was suggested by the

observation that treatment of macrophages with antisense oligonucleotides to ABCG1 decreased the efflux of cholesterol and phospholipids to high density lipoprotein and, surprisingly, also the secretion of apoE (53, 55). Although ABCG1 and ABCG4 may function both as homodimers and heterodimers (56, 57), expression of ABCG1 and ABCG4 overlaps in some but not all tissues assayed (54), which may indicate different functions in different tissues. The expression of ABCG4appearsto belargely restricted to nervous tissue (56). Our results strongly suggest that apoA-I-mediated cholesterol efflux from astrocytes involves ABCA1 and that apoE mediates cholesterol efflux via ABCG1 and possibly also ABCA1, but not via ABCG4. Although 24(*S*)-hydroxycholesterol also stimulated apoE synthesis and secretion from astrocytic cells, it remains to be established why 24(*S*)-hydroxycholesterol treated resulted in observable cholesterol efflux only in the presence of exogenous apoE or apoA-1. A possible explanation may be that the levels of endogenous apoE secreted from 24(*S*)-hydroxycholesterol-treated cells is ~100-fold lower than the concentrations of exogenous apoE added as a lipid acceptor under our experimental conditions. Alternatively, endogenously secreted apoE may already be lipidated and thereby act a less efficient cholesterol acceptor.

Neurons are thought to dispose their cholesterol by conversion into 24(*S*)-hydroxycholesterol, which is more polar than cholesterol and, as a result, may easily traverse the blood-brain barrier and perhaps the neuronal plasma membrane itself (4). However, how oxysterols are transported across membranes and through the intracellular water phase is not yet known. The selective up-regulation of ABCG4 in neuroblastoma cells by 24(*S*)-hydroxycholesterol suggests a possible role for this transporter in oxysterol transport. Both 24(*S*)-hydroxycholesterol and also GW683865A enhanced apoA-I-mediated cholesterol efflux from neuronal cells, suggesting this is another neuronal pathway to dispose of cholesterol. However, GW683965A had only a limited effect on ABCA1 expression in these cells. Rebeck *et al.* (57) recently reported up-regulation of neuronal ABCA1 expression by the synthetic LXR ligand T0901317. A role for apoA-I in the disposal of cholesterol from neurons is in line with its well-known role in so called "reverse cholesterol transport." ApoA-I is present in brain and in cerebrospinal fluid and has been detected in senile plaques in AD patients (58, 59). So far apoA-I synthesis within the brain has only been ascribed to endothelial cells of the blood brain barrier (60). It remains to be established why 24(*S*)-hydroxycholesterol does not alone, but only in concert with apoE or apoAI increase cholesterol efflux from astrocytes. A possible explanation may be the relatively small amounts (~100-fold) of apoE that are secreted by the cells in comparison with the amount of apoE that is added as cholesterol acceptor. Alternatively, secreted apoE may be lapidated and thereby become a less efficient cholesterol acceptor.

LXR isoforms differ in their pattern of expression (61). In the brain LXR ß levels are 2–5-fold higher than in the liver, whereas LXRα levels are 3.5–14-fold lower than in the liver (62–64). However, 24(*S*)-hydroxycholesterol and GW683965A up-regulated LXRα but not LXR ß expression in astrocytoma cells (data not shown), similar to what has been reported for macrophages and adipocytes, but not liver and muscle (65, 66). These results suggest the possibility that the autoregulation of LXRα that has been suggested to occur in adipocytes to coordinate expression of target genes such as APOE (66) may also occur in brain.

In conclusion, our results provide evidence indicating that 24(*S*)-hydroxycholesterol acts as a signalling molecule that induces the apoE mediated cholesterol efflux from astrocytes but not from neurons (Fig. 9). Our findings also suggest a role for ABCA1 and ABCG1 in mediating cholesterol efflux from astrocytes. Thus, in the intact brain, 24(*S*)-hydroxycholesterol derived from neurons may signal astrocytes to increase production of lipidated apoE particles in order to supply neurons with additional cholesterol during synaptogenesis or neuritic remodeling. Moreover alterations in the transcriptional regulation role of 24(*S*)-hydroxycholesterol on apoE-mediated cholesterol efflux may affect the progression of neurodegenerative diseases including AD.

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Chapter 3

Long-term treatment with the Liver X Receptor ligand T0901317 in C57BL/6 mice upregulates whole-body cholesterol turnover and modulates brain cholesterol metabolism

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Submitted

Abstract

The liver X receptor (LXR) plays a central role in the control of cholesterol metabolism. Here we show that long-term treatment of C57Bl6/J mice with the LXR agonist T0901317 persistently enhances whole body cholesterol turnover and also affects brain cholesterol metabolism.
Feeding mice T0901317 (0.015% w/w) for a period of 4 weeks increased faecal sterol excretion (+250%) and plasma cholesterol and precursors, indicative for enhanced cholesterol biosynthesis. Hepatic cholesterol precursor levels were significantly increased (lanosterol: +487%; lathosterol: +209%; desmosterol: +56%) while total hepatic cholesterol levels were decreased (-48%). Interestingly, T0901317 treatment also significantly increased brain cholesterol precursor levels (lathosterol: +24%; desmosterol +29%), while brain cholesterol levels were not affected. Upon treatment no significant changes were observed in brain levels of 24(S)-hydroxycholesterol (-39%; p=0.132), the form in which cholesterol is predominantly secreted from the brain. This suggests additional secretion of cholesterol via alternative pathways. T0901317 upregulated expression of Abca1 (2.5-fold), Abcg1 (1.6-fold) and, surprisingly, of Apoc1 (2.7-fold). These genes may be involved in the increased sterol flux within the brain or across the blood-brain barrier. Our results strongly suggest that prolonged administration (4 weeks) of the LXR-agonist T0901317 to wild-type C57BL/6 mice enhances whole body cholesterol turnover including that in the brain. Thus, pharmacological interference with the LXR system may be applicable to assess the role of cholesterol metabolism in the brain in health and in neurodegenerative disease, including Alzheimer's disease.

Introduction

The liver X receptor α (LXRα) and LXRβ are members of the nuclear receptor superfamily involved in the control of cholesterol and fatty acid metabolism. LXRα is expressed predominantly in liver, fat tissue and macrophages, whereas LXRβ is ubiquitously expressed, and is the predominant form expressed in the developing and adult rodent brain (9,10). Use of natural as well as synthetic ligands has provided more insight into metabolic consequences of LXR activation. Studies in enterocytes and macrophages have shown that activation of LXR results in increased expression of genes involved in cholesterol metabolism, predominantly in cellular efflux, including the ATP-binding cassette (ABC) cholesterol transporters Abca1, Abcg1, Abcg5, Abcg8, and Apolipoprotein e (Apoe). Furthermore, LXR activation in the liver results in expression of genes that play key roles in cholesterol disposal, i.e., Abcg5 and Abcg8 (biliary excretion) and Cyp7a1 (conversion to bile salts) (11). As a consequence, activation of LXR with synthetic ligands for a period of 3-7 days has been shown to accelerate cholesterol disposal from the body. The long-term consequences hereof have remained elusive so far.

It present very little is known with respect to the functions of LXR in the brain. The brains of aged Lxrα/β-deficient mice showed closed lateral ventricles that were lined with lipid-laden cells as well as enlarged blood vessels, especially in the pars reticularis of the substantia nigra and in the globus pallidus, upon aging (9). In addition, brains of Lxrα/β-deficient mice showed excessive lipid deposits, proliferation of astrocytes, loss of neurons, disorganized myelin sheaths and, in aged mice, accumulation of lipid vacuoles in astrocytes surrounding blood vessels (9). This unmistakably illustrates the important role of LXR in cholesterol and lipid homeostasis in the brain. The synthetic LXR-agonist T0901317 penetrates the blood-brain barrier with a brain:plasma ratio of ~0.5 (personal communications with Dr. D. Hill, Organon, UK). Short-term treatment of mice with T0901317 was found to induce expression of LXR target genes Abca1, Abcg1 and Srebp-1 in the brain (12). In vitro, it was found that LXR activation leads to an increase of cholesterol efflux from astrocytes, but not from neurons (12,13).
Disturbances in cerebral cholesterol metabolism can lead to severe neurological diseases. Accumulating evidence indicates an important role for an aberrant brain cholesterol metabolism in the development of Alzheimer's disease (AD). A major neuropathological hallmark of AD are so-called plaques, which are extracellular protein deposits, of which amyloid beta (Aβ) is regarded as the key protein (14). Interestingly, the synthetic LXR-agonist T0901317 was found to decrease Aβ levels *in vitro* (15). Also *in vivo* LXR-agonists decreased brain Aβ levels as well as its deposition (15). It was suggested that these effects are mediated via the LXR activation-induced upregulation of the expression of Abca1 (15,16).
In the present paper we investigated the effects of prolonged oral administration (4 weeks) of the LXR-agonist T0901317 in wild-type C57BL/6 mice on the sterol profile in brain as compared to liver and plasma, using gas-chromatography/mass spectrometry. In addition the expression of LXR-target genes involved in cholesterol metabolism within the brain was examined.

Experimental procedures

Animals and diets

Male, three-month-old C57BL/6J mice were purchased from Harlan (Horst, The Netherlands). Animals were housed in temperature-controlled rooms (21°C) with 12 hours light cycling and received a semi-synthetic diet and water *ad libitum* for 2 weeks (run-in period). Mice were assigned to two treatment groups (6 animals per group) and were fed the specific diets (control or T0901317 diet) for 4 weeks. The composition of T0901317 diet was similar to the control diet but contained 0.015 g/kg T0901317 (Cayman Chemicals (Ann Arbor, MI, USA)). Animal procedures were performed with approval of the institutional ethical committee for the use of experimental animals of the University Medical Center Groningen according to governmental guidelines.

Sample preparation

After 4 weeks, feces were collected for 24 hours. Feces were weighed and homogenized to a powder. Aliquots of fecal powder were used for analysis of total neutral sterol and bile salt content according to Arca et al. (17) and Setchell et al. (18), respectively. The mice were subsequently anaesthetized by intraperitoneal injection with Hypnorm® (fentanyl/fluanisone, 1 ml/kg) and diazepam (10 mg/kg). Blood was collected by cardiac puncture in EDTA-containing tubes. The brain hemispheres and livers of all mice were immediately shock-frozen in liquid nitrogen and stored at -80°C.

Sterol profile determination

Prior to sterol analysis, brains were spun in a speed vacuum dryer (12 mbar) (Savant AES 1000) for 48 hours to relate individual sterol concentrations to dry-weight. The sterols were extracted from the dried tissue by placing them in a 5 ml mixture of chloroform-methanol (2:1) for 48 hours. One ml of the sterol extract of the brains, upon addition of internal standards, was evaporated to dryness under a stream of nitrogen at 63ºC, and hydrolyzed for 1 hour with 1 ml of 1 M NaOH in 90% ethanol at 50ºC. One ml of distilled water was added to the samples. To extract the neutral sterols, 3 ml of cyclohexane was added twice. The combined cyclohexane phases were again evaporated to dryness under a stream of nitrogen at 63ºC, and the sterols were dissolved in 100 µl n-decane. After transfer to gas-chromatography (GC)-vials, the sterols were converted to trimethylsilylethers by adding 40 µl of TMSi reagent (pyridine-hexamethyldisilazan-trimethylchlorosilane, 9:3:1 v/v/v) and incubated at 60ºC for one hour (19). The sterols were extracted from 100 µl plasma in the same manner. Levels of cholesterol were determined in a gas-chromatograph-flame ionization detector (GC-FID) with 5α-cholestane as internal standard. Levels of cholesterol precursors (lanosterol, lathosterol and desmosterol), cholesterol metabolites (24(S)-hydroxycholesterol and cholestanol) and dietary plant sterols (campesterol, sitosterol) were determined using gas chromatography-mass spectrometry (GC-MS) as described previously (19) using epicoprostanol as internal standard.

Real time (RT)-PCR RNA-measurements

Total RNA was extracted from frozen brains of C57BL/6J mice with Tri-Reagent (Sigma, St. Louis, Mo, USA). cDNA synthesis was using recombinant M-MLV reverse transcriptase (10 U/µl), the appropriate buffer, dNTPs (500 µM), random nonamers (1 µM), RNAse inhibitor (2 U/µl; all from Sigma) and total RNA (50 mg/µl). Real-time quantitative PCR was performed using Applied Biosystems 7700 sequence detector as previously described (20) with modifications (21). Primers were obtained from Invitrogen (Breda, The Netherlands). Fluorogenic probes labelled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA) were made by Eurogentec (Seraing, Belgium). Primers and probes used in these studies have been described elsewhere (*Abca1*, *Abcg5*, *Abcg8*, *Hmgcr*, *Lxrα*, *Srebp1c*, *Srebp2*, *Acat1*, *Acat2*, *Srb1*, *Lpl, 18S rRNA*, (21); *Abcg1*, *Niemann-Pick-C1-like protein 1 (Npc1l1)*: (22); *Apoe*: (23); *Apoc1*: (24). Sequences of primers and probes for *Lxrβ*, *Abcg4*, *Cyp46, Hmg-CoA-synthase (Hmgcs), mevalonate kinase (Mvk), phosphomevalonate*

kinase (Pmvk), squalene epoxidase (Sqle) are available on request. The mRNAs of six brains of both the control and T0901317 mice were pooled to determine gene expression of *Insulin-induced gene (Insig) 1, Insig 2, Apod, Apoa1 and Stearoyl-CoA desaturase (Scd)* and therefore no standard deviation or p-value are given for the expression levels of these genes. All expression data were standardized for the housekeeping gene 18s, which was analyzed in separate runs

Fatty acid profile determination

The fatty acid composition of phospholipids in the brain were determined as described previously (25). In short, lipids were extracted from vacuum-dried brain by using a chloroform/methanol solution (2:1). The internal standard (dinonadecanoyl phosphatidylcholine) was added to 100 µl of the brain lipid extract, and phospholipids were isolated by using a modified version (26) of Folch *et al.* (27), which involved a solid-phase extraction on aminopropyl-silica columns (28). The isolated phospholipids fractions were hydrolyzed and the fatty acids were methylated with boron trifluoride in methanol (29). The fatty acids were quantified by capillary gas chromatography using a polar capillary column (50 m x 0.25 mm CPSIL 88; Chrompack, Middelburg, The Netherlands), with helium as carrier gas.

Statistical analysis

The statistical test used in this study is a Mann-Whitney U test using SPSS 11.0.2 for Macintosh OS X. A p-value smaller that 0.05 was considered statistically significant. All data is presented as mean value ± standard deviation (sd).

Results

T0901317 persistently increases faecal sterol loss

Long-term treatment of mice with T0901317 resulted in a very strong induction of daily faecal neutral sterol (cholesterol and its intestinal metabolites) (5.1±1.3 vs. 23.2±5.3 µmol/day) and bile salt loss (6.0±1.1 vs. 16.2±4.9 µmol/day), indicating a state of permanently accelerated cholesterol turnover in the treated mice.

T0901317 induces increased circulating levels of cholesterol and cholesterol precursors

As expected, circulating levels of cholesterol increased significantly upon treatment with T0901317, similar to what has been reported in earlier studies with shorter treatment periods (152,154,187) (Fig. 1A). Levels of cholesterol precursors were also increased upon T0901317 administration (lathosterol: 0.06±0.01 vs. 0.14±0.02 mg/dl plasma; p=0.002; lanosterol: 0.15±0.03 vs. 0.37±0.13 mg/dl plasma; p=0.002 and

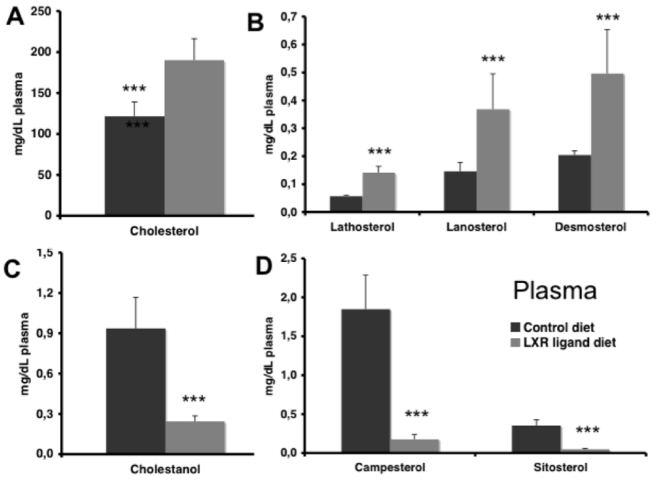

Figure 1. Plasma levels of cholesterol, cholesterol precursors (lathosterol, lanosterol and desmosterol), cholestanol and plant sterols campesterol and sitosterol in C57Bl6/J mice fed a normal chow diet or a diet containg LXR ligand T0901317. Values represent means ± standard deviations and are presented as mg/dL plasma (*P≤0.01; ***P≤ 0.001).

desmosterol: 0.20±0.01vs. 0.50±0.16 mg/dl plasma; p=0.002) (Figure 2A). Plasma levels of the cholesterol metabolite cholestanol were decreased (0.94±0.23 vs. 0.24±0.04 mg/dl plasma; p=0.002) (Figure 3A). Also the plant sterols campesterol (1.85±0.44 vs. 0.18±0.06 mg/dl plasma; p=0.002) and sitosterol (0.35±0.08 vs. 0.05±0.01 mg/dl plasma; p=0.002) were decreased (Figure 4A).

T0901317 increases cholesterol precursor levels, but decreases cholesterol levels in liver

Similar to observations in plasma, administration of T0901317 increased levels of cholesterol precursors, lanosterol in particular, in the liver (Figure 2B). However, levels of total cholesterol were reduced in the liver by 48% as a result of T0901317 administration (Figure 1B). Cholestanol levels (Figure 3B) and levels of sitosterol were significantly decreased in the liver of the LXR-treated mice, while campesterol levels remained unchanged (Figure 4B).

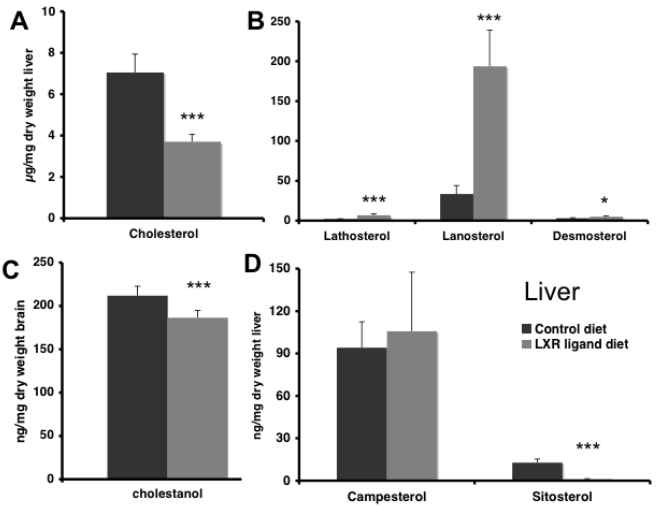

Figure 2. Liver levels of cholesterol, cholesterol precursors (lathosterol, lanosterol and desmosterol), cholestanol and plant sterols campesterol and sitosterol in C57Bl6/J mice fed a normal chow diet or a diet containing LXR ligand T0901317. Values represent means ± standard deviations and are presented as ng/mg dry weight tissue (*P≤0.01; ***P≤ 0.001).

Administration of T0901317 results in elevated brain cholesterol precursor levels

Treatment of mice for a period of 4 weeks with T0901317 significantly increased brain levels of the cholesterol precursors lathosterol (84±7 vs. 104±7 ng/mg brain; p=0.002) and desmosterol (301±30 vs. 389±72 ng/mg brain; p=0.026), while lanosterol levels were not significantly altered (96±8 vs. 108±12 ng/mg brain; p=0.180) as compared to mice that were fed a control diet (Figure 2C). Brain cholesterol levels remained similar in both groups (66±3 vs. 64±2 µg/mg brain, in untreated vs. treated mice, respectively) (Figure 1C). This resulted in significantly higher ratios of precursors to cholesterol in T0901317-fed mice, (e.g. lathosterol/cholesterol: 1.3±0.1 vs. 1.6±0.1; p=0.015; lanosterol/cholesterol: 1.5±0.1 vs. 1.7±0.2; p=0.002; desmosterol/cholesterol: 4.6±0.4 vs. 6.0±1.1; p=0.002) indicating an increased cholesterol synthesis in the brains of these mice. T0901317 treatment did not significantly alter brain levels of 24(S)-hydroxycholesterol (31±10 vs. 19±9 ng/mg brain; p=0.132). On the contrary, levels of the cholesterol metabolite cholestanol decreased significantly in brains of these animals (chow: 212±11; T0901317: 186±8 ng/mg brain; p=0.002) (Figure 3C). Levels of the dietary plant sterol campesterol were significantly decreased in the brains of T0901317-treated mice (49±6 vs. 30±4 ng/mg brain; p=0.009) (Figure 4D), whereas levels of sitosterol remained unaffected (9±4 vs. 11±7 ng/mg brain; p=0.132) (Figure 4D).

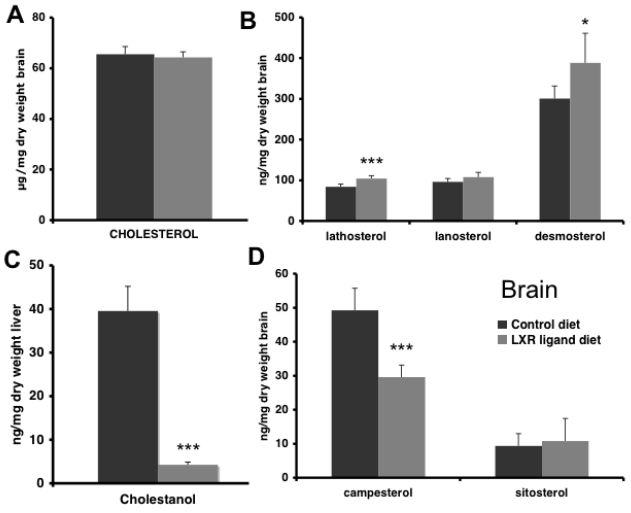

Figure 3. Brain levels of cholesterol, cholesterol precursors (lathosterol, lanosterol and desmosterol), cholestanol and plant sterols campesterol and sitosterol in C57Bl6/J mice fed a normal chow diet or a diet containing LXR ligand T0901317. Values represent means ± standard deviations and are presented as ng/mg dry weight tissue (*P≤0.01; ***P≤ 0.001).

T0901317 modulates the expression of genes involved in cholesterol metabolism in the brain

Since cholesterol synthesis appeared to be increased upon T0901317 treatment, as indicated by higher levels of cholesterol precursors in the brain, we next examined the effect on relative expression levels of genes involved in cholesterol synthesis (Table 1). Surprisingly, no alterations in gene expression levels of the enzyme Hmg-Coa-reductase

TABLE 1. Gene expression levels in brains of control versus T0901317 diets in C57BL/6 mice normalized to the 18S housekeeping gene. Values represent means ± standard deviations.

Gene	C57BL/6 mice + 4 weeks control diet	C57BL/6 mice + 4 weeks T0901317 diet	p-value
Abca1	1.00 ± 0.36	2.54 ± 0.40	0.006 *
Abcg1	1.00 ± 0.29	1.57 ± 0.16	0.011 *
Abcg4	1.00 ± 0.30	0.90 ± 0.10	1.000
Apoe	1.00 ± 0.21	1.29 ± 0.06	0.068
Apoc1	1.00 ± 0.39	2.68 ± 0.88	0.006 *
Hmgcr	1.00 ± 0.30	0.91 ± 0.10	0.855
Lxrα (Nr1h3)	1.00 ± 0.27	1.06 ± 0.08	0.465
Lxrβ (Nr1h4)	1.00 ± 0.17	1.21 ± 0.22	0.127
Srebp1c	1.00 ± 0.20	2.35 ± 0.28	0.006 *
Srebp2	1.00 ± 0.24	0.99 ± 0.14	0.855
Npc1l1	1.00 ± 0.26	0.93 ± 0.06	0.715
Acat1	1.00 ± 0.38	0.88 ± 0.07	0.855
Sr-b1	1.00 ± 0.23	1.06 ± 0.11	0.584
Lpl	1.00 ± 0.32	1.08 ± 0.07	0.584
Cyp46a1	1.00 ± 0.22	1.06 ± 0.14	0.596
Insig1[#]	1.00	1.16	nd
Insig2[#]	1.00	0.95	nd
Apod[#]	1.00	1.77	nd
Apoa1[#]	1.00	1.13	nd
Scd1[#]	1.00	1.86	nd

[#]measured in pooled samples. *considered significant ($p \leq 0.05$)

(Hmgcr) or of other enzymes involved in the cholesterol synthesis pathway (Hmgcs, mevalonate kinase, phosphomevalonate kinase and squalene epoxidase) were detected. Administration of the LXR-agonist T0901317 also did not alter relative gene expression levels of Insig1 and 2, whose products post-translationally control cholesterol biosynthesis (194), in the brain. Neither were there any effects of T0901317 on expression levels of other genes involved in cholesterol metabolism, including Apoa1, Lxrα, Lxrβ, Abcg4, Srebp2, Sr-b1, Npc1l1, Acat1, Lpl and Cyp46 in the brain. Brain expression levels of Apoe were not significantly altered (1.3-fold; p=0.068). Gene expression levels of Abcg5, Abcg8 and Acat2 were too low in the brain for reliable measurements. However, T0901317 did induce relative gene expression levels of Abca1 (2.5-fold; p=0.006), Abcg1 (1.6-fold; p=0.011), Srebp-1c (2.4-fold; p=0.006) and, interestingly, of Apoc1 (2.7-fold; p=0.006) (Table 2). Furthermore, relative gene expression levels of Apod and Scd1 appeared to be increased (1.8-fold and 1.9-fold, respectively) (Table 1).

Table 2. Fatty acid composition of total brain phospholipids of wild type C57BL/6 mice.

Fatty acids	Control diet	LXR-agonist T0901317 diet	
	% total fatty acids		
C16:0	22.0 ± 0.7	23.0 ± 0.8	
C17:0	6.1 ± 0.5	5.6 ± 0.2	
C18:0	18.3 ± 0.7	18.0 ± 0.7	
C20:0	2.1 ± 0.2	2.2 ± 0.3	
Other SFA	3.5 ± 0.7	3.1 ± 0.7	
∑SFA	52.0 ± 0.4	51.9 ± 0.3	
C18:1(n-9)	14.8 ± 0.5	14.3 ± 0.4**	
C18:1(n-7)	3.5 ± 0.1	3.9 ± 0.1**	
C24:1(n-9)	2.5 ± 0.3	2.4 ± 0.3	
Other MUFA	2.2 ± 0.3	2.1 ± 0.3	
∑MUFA	23.5 ± 0.7	23.0 ± 0.9	
C20:4(n-6)	7.9 ± 0.2	8.5 ± 0.5*	
C22:5(n-6)	2.6 ± 0.1	2.1 ± 1.2	
∑ (n-6)	10.8 ± 0.3	11.3 ± 0.5	
C22:6(n-3)	13.7 ± 0.3	13.9 ± 0.5	

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; * $p \leq 0.05$; ** $p \leq 0.01$

T0901317 alters fatty acid composition of phospholipids in the brain of mice

LXR also controls cellular fatty acid metabolism, and, as described above, gene expression levels of Srebp1c and Scd1, known for their role in fatty acid synthesis, were increased in the brain of C57BL/6 mice after T0901317 administration. Therefore, we determined the effect of LXR activation on the fatty acid composition of phospholipids in brain. The results presented in Table 2 indicate a small but significant increase in levels of C18:1(n-7) ($p \leq 0.01$) and C20:4(n-6) (arachidonic acid) ($p \leq 0.05$) and slightly, but significantly decreased levels of C18:1(n-9) (oleic acid) ($p \leq 0.01$) upon T0901317 administration.

DISCUSSION

Our results demonstrate that long-term administration of the synthetic LXR-agonist T0901317 via the diet to wild-type C57BL/6J mice leads to a state of permanently accelerated whole body cholesterol turnover, as evidenced by the massively increased faecal sterol loss. Interestingly, also brain cholesterol metabolism is affected since long-

term administration of T0901317 induces a small but significant increase of cholesterol precursor levels in the brain.

The induction of faecal sterol excretion at 4 weeks was similar to what has been observed after short-term (3-7 days) LXR-agonist treatment (8,21). The strongly elevated levels of neutral sterols and bile salts in the faeces reflect the metabolic consequences of increased biliary cholesterol excretion in conjunction with reduced intestinal cholesterol absorption, both mainly due to induction of hepatic and intestinal Abcg5/Abcg8 expression (11), and of stimulated hepatic bile salt synthesis (21). To compensate for this massive loss of cholesterol, cholesterol synthesis must be upregulated. Indeed, we detected strongly increased levels of cholesterol precursors in plasma and liver, indicative for a compensatory upregulation of cholesterol synthesis (30,31). Cholesterol levels in the liver of LXR-agonist treated mice were decreased, suggesting that the accelerated loss from hepatocytes in the form of bile salts exceeded maximal hepatic cholesterol synthesis capacity. We did not examine the liver with respect to pathology.

Plant sterol levels were decreased in plasma of T0901317-treated mice, supporting a lower sterol uptake at the intestinal level as a result of upregulation of Abcg5 and Abcg8 expression (32).

Here we show for the first time that, in the brain, long-term administration of T0901317 induces significantly upregulated levels of cholesterol precursors, without affecting cholesterol levels. In previous studies, in which there was short-term treatment with T0901317, the effects on brain cholesterol metabolism have not been examined. The ratios of cholesterol precursors to cholesterol were increased in the brains of T0901317 treated mice, which is indicative of increased cholesterol synthesis in the brain (31,33, Miettinen, 2003 #290,34). Yet, we did not detect any difference in gene expression of Hmgcr, nor of other genes encoding enzymes that are involved in the cholesterol synthesis pathway between treated and control mice. Intriguingly, although hepatic and plasma levels of cholesterol precursors were several fold more increased in liver and plasma than in brain upon long-term T0901317 treatment, we also did not find elevated mRNA levels of Hmgcr in the liver and intestine (*data not shown*) of these mice. In earlier studies, with short-term treatments, we found only a moderate upregulation of Hmgcr expression in liver (~1.5-fold) (21) and no induction in the intestine (22).

This may be explained by a post-transcriptional modulation of cholesterol synthesis upon LXR activation, distinct from the well-established Srebp2-controlled response to fluctuations in cellular cholesterol content. Recently, it was found that Hmgcr protein is ubiquinated and degraded in a process modulated by ER proteins called Insigs (35). However, levels of Insig1 and Insig2 mRNA were similar in the brains of control and T0901317-fed mice. This obviously does not exclude differential effects on Insig actions. Yet, further studies are clearly required to decipher the molecular mechanisms underlying upregulation of cellular cholesterol synthesis upon LXR activation.

The possibility remains that the cholesterol precursors that are detected within the brain are derived from plasma. Björkhem *et al.* (36) have suggested that several sterols can move from plasma into the CNS. Recently, we have reported that plant sterols, which can only be derived from the diet, do accumulate within the brain (37). We found that campesterol in particular accumulates in the brain. Also after treatment with T0901317 the brain levels of campesterol decrease and brain levels of sitosterol remain unchanged,

while the plasma levels of both types of plant sterol decrease in plasma. This indicates, as also suggested by Björkhem *et al.* (38), that the different sterols are not transported to an equal extend into or out of the brain.

Although levels of cholesterol precursors were increased in the brain, this was not associated with an increase in brain cholesterol levels. The major pathway (60%) by which cholesterol is eliminated from the brain is via the secretion of the cholesterol metabolite 24(S)-hydroxycholesterol (39). The remaining 40% is excreted via an unknown pathway. Brain levels of 24(S)-hydroxycholesterol were not significantly decreased as a result of T0901317 treatment. In contrast with extracerebral tissues brain cholesterol homeostasis is strictly regulated. This is supported by the observation that in a number of transgenic mouse models, that display dramatic alterations in plasma sterol profiles, brain sterol levels remain absolutely unchanged. Previously, we found that, in apoE-deficient mice, dramatically increased plasma levels of cholesterol precursors do not lead to increased levels in the brain (unpublished data). This underlines the importance of our finding that 4 weeks treatment with T0901317 does affect brain cholesterol metabolism.

As expected, the expression of the cholesterol transporters Abca1 and, to a lesser extend, also of Abcg1 was clearly upregulated in the brain as a result of T0901317 administration. It has been demonstrated that these transporters are expressed in astrocytes and neurons (40, Fukumoto, 2002 #49), but also in the blood-brain barrier (BBB) (41) and thus may be instrumental in the transport of sterols. For example the secretion of campesterol from the brain across the BBB may be mediated by one of these transporters. We found that ApoE mRNA levels were not significantly increased in the brain as a result of 4 weeks of T0901317 treatment. In line with this, Whitney *et al.* (12) reported upregulation of expression of Abca1, Abcg1, but not ApoE in the cerebellum and hippocampus of wild-type C57BL/6 mice that were fed 50 mg/kg LXR-agonist T0901317 in the diet for 3 or 7 days. Liang *et al.* (42), on the other hand, found a moderate but significant increase (about 1.5-fold) in brain Apoe mRNA levels after administration of 10 mg/kg T0901317 for a period of 7 days, while we found a trend of 1.3-fold increase of ApoE mRNA. T0901317 and also another LXR-agonist GW683965A have been shown to induce Apoe expression and cholesterol efflux from astrocytes *in vitro* (12, Liang, 2004 #27,42). The timing of measuring the effects of T0901317 on ApoE mRNA expression may be critical. The consequences of T0901317-induced changes in brain ApoE mRNA levels and the relation with cholesterol secretion from the brain remain to be established in a concentration- and time-dependent manner.

Surprisingly, upon T0901317 treatment, the expression of Apoc1, which belongs to the same gene cluster as Apoe, increased almost 3-fold. The functions of Apoc1 in the brain remain to be clarified, but may be related to cholesterol and/or fatty acid metabolism (43). Apoc1 has been reported to affect the activity of several enzymes involved in cholesterol and fatty acid metabolism including cholesteryl ester transfer protein (Cetp), lecithin-cholesterol acyltransferase (Lcat) and lipoprotein lipase (Lpl) (43). Moreover, we found a 1.8-fold increased expression of Apod. Apod has been suggested to be involved in cholesterol transport during regeneration or reinnervation of the CNS (44), and thus may be involved in the secretion of cholesterol from the brain. In the brain of AD patients it is highly induced and it has been detected in compact Aβ deposits (45).

An aberrant cholesterol metabolism has been implicated in the development of AD (46). Alterations in cellular cholesterol metabolism have been shown to affect Aβ generation (42). T0901317 induces the expression of Abcg1, Abca1 and Apoc1. This may take place in neurons, astrocytes and at the levels of the BBB (Figure 5). In line with this, it was reported that T0901317 reduces the levels and deposition of Aβ40 and Aβ42 in brain in an animal model of AD, most likely via an effect on Abca1 expression and brain cholesterol metabolism (15). Puglielli *et al.* (47) proposed that ACAT1, the protein that converts free cholesterol to its ester, is implicated in the processing of APP to Aβ. Yet, we did not detect any differences in expression levels of Acat1 mRNA as a result of T0901317 treatment. We propose that the reduced levels of Aβ40 and Aβ42 in brain after T0901317 treatment may also be the result of effects on the LXRα and/or LXRβ pathway at the level of the BBB or brain-CSF. Therefore, the decrease in the deposition of Aβ in the brain as a result of T0901317 treatment, may result from an increased cholesterol- and possibly Aβ efflux, in combination with decreased production and secretion of Aβ by neurons.

Activation of LXR by T0901317 induced the expression of Srebp1c and Scd1, which may alter the fatty acid composition of specific brain cells (48). Scd1 catalyzes the rate-limiting step in the overall de novo synthesis of monounsaturated FA, mainly oleic acid and palmitoleic acid from stearoyl- and palmitoyl-CoA, respectively (49). Yet, our measurements showed that T0901317 administration resulted in a slightly decreased brain levels of C18:1(n-9) (oleic acid) and increased levels of C18:1(n-7). These findings, and the small increase in C20:4(n-6) (arachidonic acid) can not be explained by increased activity of Srebp1c or Scd1. Since Srebp1c and Scd1 are likely expressed in specific cell types only, it may be that biologically relevant changes in fatty acid composition were induced in these cells.

In conclusion, the here presented results, obtained after long-term administration of the LXR agonist T0901317 to C57Bl/6 mice, together with published observations (21,22), strongly suggest that LXR-activation leads to an overall increased flux of cholesterol in the body. In the brain, LXR-activation increased the expression of Abca1 and Abcg1 mRNA, which suggests a role for these gene products in cholesterol transport and, for instance, may be involved in the elimination of cholesterol from the brain by efflux to HDL and/or ApoA1. Besides this, ApoC1 or ApoD may be involved in the lipid transport at the level of the BBB, since these are increased as a result of T0901317 treatment. Yet, we found that LXR-activation does not alter total cholesterol levels in the brain, although a small increase in cholesterol precursor levels was found. We suggest that, as a result of increased Abca1 and Abcg1 expression in the brain, an increased flux of cholesterol from the brain may take place, which may contribute to the reduction in the deposition of Aβ in the brain of AD patients and animal models of AD. LXR-activation may therefore prove to be an interesting tool in the research on neurodegenerative disorders like AD.

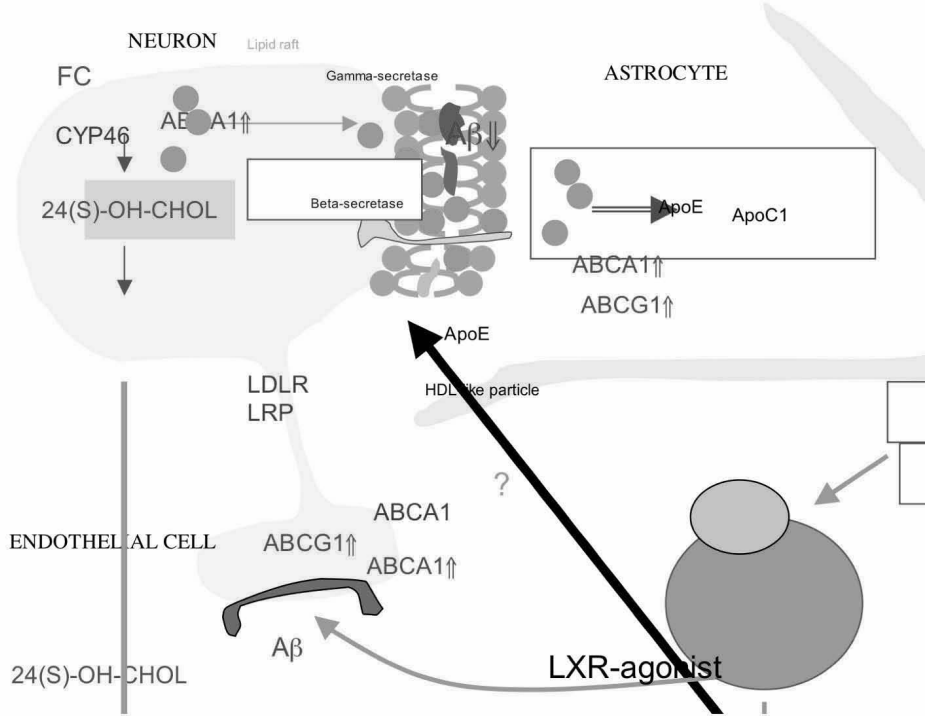

Figure 4. Schematic representation of effects of administration of LXR-agonist T0901317 on cholesterol metabolism and amyloid β in the brain. T0901317 upregulates expression of Abca1, Abcg1, Apoc1 and possibly ApoE, and increase transport of cholesterol. Also the efflux of main brain cholesterol metabolite 24(S)-OH-Cholesterol is increased. The LXR-agonist was found to reduce the formation and deposition of amyloid β. This may result from reduced production by neurons, or from increased efflux at the level of the BBB. In both cases there may be a prominent role for Abca1 or Abcg1. *Adapted from Shobab et al. Lancet Neurology 2005.*

Acknowledgements

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Chapter 4

Dietary plant sterols accumulate in the brain

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Abstract

Dietary plant sterols and cholesterol have a comparable chemical structure. It is generally assumed that cholesterol and plant sterols do not cross the blood–brain barrier, but quantitative data are lacking. Here, we report that mice deficient for ATP-binding cassette transporter G5 (Abcg5) or Abcg8, with strongly elevated serum plant sterol levels, display dramatically increased (7-to 16-fold) plant sterol levels in the brain. Apolipoprotein E (ApoE)-deficient mice also displayed elevated serum plant sterol levels, which was however not associated with significant changes in brain plant sterol levels. Abcg5-and Abcg8-deficient mice were found to carry circulating plant sterols predominantly in high-density lipoprotein (HDL)-particles, whereas ApoE-deficient mice accommodated most of their serum plant sterols in very low-density lipoprotein (VLDL)-particles. This suggests an important role for HDL and/or ApoE in the transfer of plant sterols into the brain. Moreover, sitosterol upregulated apoE mRNA and protein levels in astrocytoma, but not in neuroblastoma cells, to a higher extend than cholesterol. In conclusion, dietary plant sterols pass the blood–brain barrier and accumulate in the brain, where they may exert brain cell type-specific effects.

Introduction

Plant sterols comprise a group of sterols that enter the mammalian body only via the diet. Relatively large amounts of plant sterols are present in plant oils, nuts, and avocados. The structure of plant sterols is very similar to that of cholesterol. They differ from cholesterol only by an additional methyl-group (campesterol) or ethyl-group (sitosterol) at the C24-position, or by an additional double bond at the C22-position (brassicasterol, stigmasterol, respectively). Yet, this small structural difference leads to very divergent metabolic fates of plant sterols and cholesterol in mammals [1]. Both cholesterol and plant sterols are internalized by intestinal mucosa cells via the Niemann–Pick C1-Like1 (NPC1L1)-transporter [2]. Cholesterol is transported to the endoplasmic reticulum, where it is esterified by the action of acyl-CoA:cholesterol O-acyltransferase 2 (Acat2) for incorporation into chylomicrons [3]. However, plant sterols are poor Acat2 substrates and hence are transported back to the luminal membrane to be re-secreted into the lumen of the intestine by the heterodimeric ATP-binding cassette (Abc) half transporters g5 and g8 [4]. Abcg5 and Abcg8 are present at the apical membrane of enterocytes and are also expressed in liver [5]. In the liver, the Abcg5/Abcg8 transporter mediates efflux of plant sterols and cholesterol into bile [6,7]. The formation of this heterodimer becomes impossible in the absence of either Abcg5 or Abcg8 and thus leads to a dysfunction of the transporter system. Mutations in the genes encoding either of these half transporters lead to sitosterolemia, an inborn error of metabolism characterized by high levels of sitosterol, campesterol and stigmasterol [5,7,8]. It has been reported previously that Abcg5-deficient mice display massively elevated serum levels of campesterol and sitosterol and 50% lower serum cholesterol levels compared to wild-type mice [7]. Mice deficient for both Abcg5 and Abcg8 also

display dramatically elevated circulating levels of campesterol and sitosterol as well as reduced serum cholesterol levels as compared to wild-type mice [8].
It is widely assumed that, in contrast with other tissues, the brain does not retrieve cholesterol from the circulation, because this is normally prevented by the blood–brain barrier (BBB), and instead synthesizes all its cholesterol in situ [9]. Yet, indications of close communications between extracerebral and brain cholesterol have been reported previously [10]. In line with the fact that the structure of plant sterols is very similar to that of cholesterol, and the subsequent assumption that they do not seem to enter the brain, only very low levels of plant sterols in brain have been reported [8,11]. However, consequences of high dietary plant sterol intake on brain plant sterol levels have not been reported so far.
In the present study, we have determined levels of cholesterol and plant sterols (campesterol and sitosterol) in serum and brain of wild-type mice that were fed either a normal chow diet or a diet rich in plant sterols for 4 weeks. Interestingly, we observed a small but significant increase in brain campesterol levels in mice fed the plant sterol-enriched diet. Subsequently, we examined three mouse models with strongly elevated serum plant sterol levels, i.e., Abcg5-deficient mice, Abcg8-deficient mice and apolipoprotein E (ApoE)-deficient mice [4,5,7]. Surprisingly, we found that high plant sterol levels in serum were associated with significantly elevated plant sterol levels in brain of Abcg5and Abcg8-deficient mice, but not in brain of ApoE-deficient mice. Plant sterols have been reported to affect HMG-CoA reductase activity, although the results are inconsistent [12,13]. Therefore, we investigated the effect of plant sterols on total brain cholesterol metabolism in vivo and their effects on ApoE protein and mRNA levels in vitro, using astrocytoma and neuroblastoma cells. Our results suggest that an accumulation of plant sterols in the brain may exert brain cell type-specific effects and as a consequence may affect brain functioning.

Materials and methods

Animals

Experiment 1

Twelve male, three month old C57BL/6J mice were purchased from Harlan (Horst, The Netherlands). Animals were housed in temperature-controlled rooms (21 ¡C) with 12-h light cycles and received a semisynthetic diet and water ad libitum. All animals were fed the semisynthetic diet for 2 weeks (run-in period). Afterwards, mice were assigned to two treatment groups (6 animals per group) and were fed the specific diets (control or plant sterol-rich diet) for 4 weeks. Analysis of sterol composition revealed that the control diet contained 0.03% (w/w) sterols, mainly beta-sitosterol (53.8% w/w of all sterols). The plant sterol enriched diet contained 0.12% cholesterol (w/w) and 0.83% plant sterol esters (w/w); plant sterol esters were mainly beta-sitosterol, campesterol and stigmasterol (36.5%, 20.4% and 14.9%, respectively).

Experiment 2

Male Abcg5-deficient mice (n = 10), Abcg8-deficient mice (n= 5) and apoEdeficient mice (n= 7) and their respective littermate controls were maintained on a standard laboratory chow diet and were allowed to eat and drink ad libitum. The Abcg5-deficient mice and littermate control mice were generated by Deltagen (Redwood City, CA, USA) and were fed a normal chow diet (Arie Blok, Woerden, The Netherlands) as described by Plšsch et al. [7]. The Abcg8deficient mice and littermate control mice were generated as described by Klett et al. [14]. The ApoE-deficient mice (Breslow mouse line) were obtained from Charles River (Someren, The Netherlands). The three different mouse models were from three different laboratories and sterol profiles were therefore examined in littermate controls from the respective institute. All animal procedures were performed with approval of the respective institutional ethical committees for the use of experimental animals of the Universities of Groningen, Amsterdam and Maastricht, according to governmental guidelines.

Tissue sample preparation

The mice were anaesthetised with Nembutal (CEVA, Maassluis, The Netherlands) in a dose of 180 µg/g body weight. In the Abcg5-and ApoEdeficient mice the whole body was perfused with phosphate buffered saline (137 mM NaCl; 2.7 mM KCl; 6.5 mM Na2HPO4; 1.5 mM KH2PO4; pH = 7.3) (PBS) [15]. Blood was collected before perfusion and spun at 1400 rpm to obtain serum or plasma, which was snap frozen in liquid nitrogen. The Abcg8-deficient mice were not perfused. However, brains were carefully rinsed in PBS and peripheral blood was collected by puncturing of the heart. The brain hemispheres of all mice were immediately shock-frozen in liquid nitrogen, while submerged in isopentane, and stored at −80 ¡C. Prior to sterol analysis, brains were spun in a speed vacuum dryer (Savant AES 1000) at 12 mbar for 48 h to relate individual sterol concentrations to dry weight. The sterols were extracted from the dried tissues by placing them in a 5-ml mixture of chloroform–methanol (2:1) for 48 h.

Sterol profile determination

One ml of the brain sterol extracts was evaporated to dryness under a stream of nitrogen at 63 ¡C, and hydrolyzed for 1 h with 1 ml of 1 M NaOH in 90% ethanol at 50 ¡C. One ml of distilled water was added to the samples. To extract the neutral sterols 3 ml of cyclohexane was added twice. The combined cyclohexane phases were again evaporated to dryness under a stream of nitrogen at 63 ¡C, and the sterols were dissolved in 100 µl n-decane. After transfer to gas-chromatography (GC)-vials, the sterols were converted to trimethylsilylethers (TMSis) by adding 40 µl of TMSi reagent (pyridine–hexamethyldisilazan– trimethylchlorosilane, 9:3:1 v/v/v) and incubated at 60 ¡C for 1 h [11]. The sterols were extracted from 100 µl serum by the same protocol. Levels of cholesterol were determined in a gas-chromatograph-flame ionization detector (GC-FID) with fifty µl5α-cholestane-solution (1 mg/ml 5α-cholestane in cyclohexane) as internal standard. Levels of plant sterols (campesterol, sitosterol), cholesterol precursors (lanosterol, lathosterol and desmosterol) and cholesterol metabolites (24S-OH-cholesterol and cholestanol) were determined using gas chromatography-mass

spectrometry (GC-MS) as described previously [11] using epicoprostanol as internal standard.

FPLC

We determined the lipoprotein fractions in serum of ApoE-deficient mice (n = 4), and plasma of Abcg5-deficient mice (n = 3) and Abcg5-wild type controls (n = 5) using fast protein liquid chromatography (FPLC). Equal volumes of serum or plasma (50 µl) were injected on a Superose 6 HR 10/30 column (Amersham Biosciences), which was connected to an AKTA Basic FPLC system (Amersham Biosciences). Lipoproteins were eluted at a constant flow rate of 0.05 ml/min with Dulbecco's phosphate buffered saline (Sigma-Aldrich, St. Louis, U.S.A.) containing 1 mM EDTA. Fractions 8–14 corresponded to VLDL-, 15–22 to LDL-and 23–31 to HDL-sized particles. These fractions were pooled for the different genotypes and analysed with GC-MS for cholesterol and plant sterol levels.

Cell culture

The human astrocytoma cell line CCF-STTG1 and human neuroblastoma cell line SH-SY-5Y were purchased from ECACC (Sallisbury, UK). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/ HamF12 (1:1) with 10% fetal calf serum (FCS). At 80–90% confluency cells were washed with PBS (pH 7.3) and treated with plant sterols in a concentration-or in a time-dependent manner in fresh DMEM/HamF12 (1:1) with 10% FCS. SH-SY-5Y cells were pre-incubated for 24 h in the DMEM-HamF12 (1:1) medium containing N2 supplement (2.5 mg/ml bovine insulin, 10 mg/ml human transferrin (iron-saturated), 0.52 µg/ml sodium selenite, 1.61 mg/ml putrescine, 0.63 µg/ml progesterone in PBS, pH 7.3). The plant sterols were a mix of two plant sterols: 92% sitosterol and 8% campesterol, and will be referred to from here on as sitosterol. GW683965A, a synthetic LXR-agonist, was obtained from GlaxoSmithKline (Stevenage, UK). Tissue culture flasks were purchased from Corning (Corning, NY). Sitosterol was dissolved in ethanol and GW683965A was dissolved in DMSO. The concentrations used for sitosterol were 0, 1, 5 and 10 µM. A concentration of 10 µM was incubated with the cell lines for 8, 24 and 48 h. Finally, CCF-STTG1 cells were incubated with 10 µM campesterol (Sigma), 10 µM sitosterol or 10 µM cholesterol (Sigma) in the absence or presence of 100 mM mevalonate, in order to compare the effects of cholesterol versus plant sterols. At the end of the treatment, the cells were either lysed in RIPA buffer (Santa Cruz, Ca) or total RNAwas isolated using Trizol (Invitrogen, Breda, The Netherlands) for subsequent analyses.

Western blot analysis

Cell lysates from CCF-STTG1 and SH-SY-5Y cell lines were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 25 µg protein/lane) and then transferred to Protran nitrocellulose transfer membranes (Schleicher and Schuell BioScience GmbH, Dassel, Germany). After blocking with 5% non-fat dry milk (Protifar-Plus, Nutricia, The Netherlands) in washing buffer (150 mM NaCl, 10 mM TRIS, 0.05% Tween 20), the membranes were incubated with polyclonal rabbit-anti-human ApoE antibody (1:500) (A0077, DAKO A/S, Glostrup, Denmark). The

membranes were then washed and incubated with peroxidase-conjugated secondary antibody swine-anti-rabbit (1:100) (DAKO A/S, Glostrup, Denmark), after which the results were visualized using Enhanced Chemiluminescence (ECL) reagents (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, UK) and autoradiography (LAS 3000, Fuji Photo Film Co., LTD, Japan).

Real time quantitative PCR

Total RNA was extracted from frozen brains of C57BL/6J mice, Abcg5deficient mice and wild type littermates and from cell lysates using Trizol (Invitrogen, Carlsbad) and quantified using Ribogreen (Molecular Probes, Eugene). cDNA synthesis was performed according to Bloks et al. [16]. Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector with 1.6.3 software (Perkin-Elmer Corp., Foster City, Calif., USA) as previously described [17] with modifications [18]. Primers were obtained from Invitrogen (Carlsbad, Germany). Fluorogenic probes labelled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA) were made by Eurogentec (Seraing, Belgium). Primer sequences used in these studies are available upon request (Abcg5, Abcg8, ApoE, Ldlr, Cyp46, ApoC1, AbcA1, Hmg-CoA-reductase and Srebp1c). All expression data were subsequently standardized to 18S ribosomal RNA, which was analyzed in separate runs.

Statistics

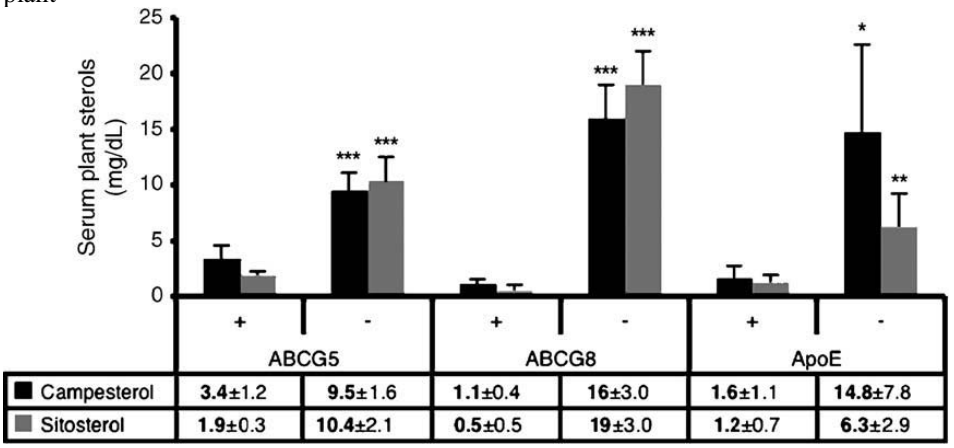
The statistical test used to evaluate differences in sterol profiles was ANOVA with Bonferroni-correction using SPSS 11.0.2 for Macintosh OS X. Differences in mRNA levels were evaluated using the Mann–Whitney U-test. A P-value lower than 0.05 was considered significant. Data are presented as the mean value ± standard deviation (SD).

Results

Increased brain campesterol levels as a result of a plant sterol enriched diet in wild type mice

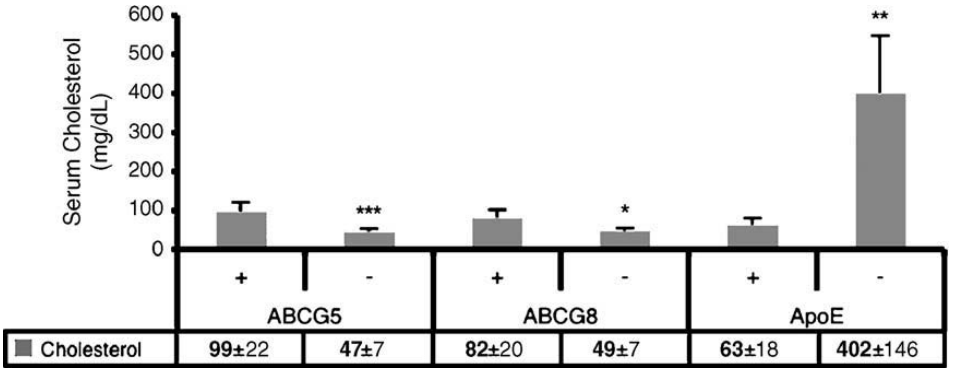
Feeding C57BL/6J wild type mice a plant sterol enriched diet for a period of 4 weeks resulted in an increase of serum campesterol levels from 1.85 ± 0.44 mg/dl to 4.28 ± 1.19 mg/dl ($P \leq 0.001$), while plasma sitosterol levels showed a slight, nonsignificant increase (0.35 ± 0.07 mg/dl to 0.44 ± 0.11 mg/dl). Serum cholesterol levels were comparable in chow-fed (122 ± 17 mg/dl) and plant sterol diet fed mice (142 ± 20 mg/dl). Interestingly, as a result of consumption of the plant sterol enriched diet, brain campesterol levels increased significantly from 49 ± 6 ng/mg to 64 ± 9 ng/mg dry weight brain ($P \leq 0.004$). Brain sitosterol levels did not differ significantly between control (9.4 ± 3.6 ng/mg dry weight brain) and plant sterol fed mice (9.6 ± 2.0 ng/mg dry weight brain) ($P \leq 0.271$) and also no differences were observed in brain cholesterol levels (66 ± 3 µg/ mg and 66±5 µg/mg in chow and plant sterol-fed mice, respectively).
Elevated serum plant sterol levels in Abcg5-, Abcg8-and ApoE-deficient mice

Since the above mentioned results suggest that plant sterols can enter the brain, we assessed the effects of more pronouncedly elevated serum plant sterol levels on brain plant



	ABCG5		ABCG8		ApoE	
Campesterol	3.4±1.2	9.5±1.6	1.1±0.4	16±3.0	1.6±1.1	14.8±7.8
Sitosterol	1.9±0.3	10.4±2.1	0.5±0.5	19±3.0	1.2±0.7	6.3±2.9

Fig. 1. Elevated plant sterol levels in serum of Abcg5-, Abcg8-and ApoE-deficient mice as compared to their respective littermate controls. Values represent means± standard deviations and are presented as mg/dl serum. *P-value ≤0.05; **P-value ≤0.01; ***P-value ≤0.001.

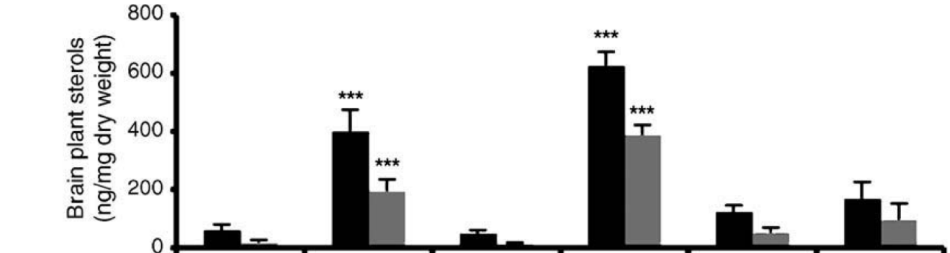


	ABCG5		ABCG8		ApoE	
Cholesterol	99±22	47±7	82±20	49±7	63±18	402±146

Fig. 2. Serum cholesterol levels are reduced in Abcg5-and Abcg8-deficient mice, and upregulated in ApoE-deficient mice compared to their respective littermate controls. Values represent means±standard deviations and are presented as mg/dl serum. *P-value ≤0.05; **P-value ≤0.01; ***P-value ≤0.001.

sterol concentrations. For this purpose, Abcg5-deficient mice, Abcg8-deficient mice, ApoE-deficient mice and their respective littermate controls were used. All three groups of transgenic mice displayed strongly increased serum plant sterol levels (Fig. 1) in comparison to their wild type littermates. Abcg5-deficient mice displayed serum levels of campesterol and sitosterol that were 3-and 5-fold higher, respectively, than levels of their littermate controls (Fig. 1). The absence of Abcg8 resulted in a 15-and 35-fold

increase of campesterol and sitosterol levels, respectively (Fig. 1). Both Abcg5-and Abcg8-deficient mice displayed approximately 40–50% lower serum levels of cholesterol (Fig. 2) than their controls, in accordance with previously described results [8]. ApoE-deficient mice displayed 9-fold higher serum levels of campesterol and 5-fold higher levels of sitosterol as compared to their wild-type littermates (Fig. 1). Yet, in the ApoE-deficient mice serum sitosterol levels are lower and campesterol levels tended to be more variable than in Abcg5-and Abcg8-deficient mice. In contrast to the Abcg5-and Abcg8-deficient mice, they also displayed 6-fold higher serum levels of cholesterol (Fig. 2), as reported previously [19].



	ABCG5 +	ABCG5 -	ABCG8 +	ABCG8 -	ApoE +	ApoE -
Campesterol	61±18	399±75	49±12	624±49	125±21	170±57
Sitosterol	18±9	196±39	14±3	389±33	52±17	96±58

Fig. 3. Brain plant sterol levels are dramatically increased in Abcg5-and Abcg8-deficient mice, but not in ApoE-deficient mice as compared to their respective littermate controls. Values represent means ± standard deviations and are presented as ng/mg brain (dry weight). *P-value ≤0.05; **P-value ≤0.01; ***P-value ≤0.001.

Dramatic increases in brain plant sterol levels in Abcg5and Abcg8-deficient mice but not in ApoE-deficient mice

A dramatic increase was observed in brain levels of campesterol and sitosterol in both the Abcg5-and Abcg8-deficient mice (Fig. 3). The level of campesterol was 7-fold higher in the brains of Abcg5-deficient mice and 13-fold higher in the brains of Abcg8-deficient mice when compared to their respective controls. The level of sitosterol was 11-fold higher in the brains of Abcg5deficient mice and 16-fold higher in the brains of Abcg8-deficient mice. Surprisingly, in contrast with these findings, the ApoEdeficient mice did not show a significant increase in brain levels of campesterol and sitosterol (Fig. 3), although their serum campesterol levels were as high as those of Abcg5-and Abcg8deficient mice (Fig. 1).

Unchanged brain cholesterol levels in Abcg5-, Abcg8-and ApoE-deficient mice

No significant differences were observed in brain cholesterol levels of Abcg5-, Abcg8-, or ApoE-deficient mice in comparison to their respective controls (Fig. 4). Consequently, the ratio of campesterol to cholesterol and the ratio of sitosterol to

cholesterol were elevated in the brains of Abcg5-deficient mice (3-fold and 5-fold, respectively) and Abcg8-deficient mice (4-fold and 5fold, respectively), but not in the ApoE-deficient mice.

Abcg5-deficient mice retain plant sterols in HDL particles, but ApoE-deficient mice retain plant sterols in VLDL particles

The divergence between plasma and brain concentrations of plant sterols in Abcg5/g8-deficient and ApoE-deficient mice may be due to differences in the classes of lipoproteins that retain the plant sterols in the circulation. Therefore, the lipoprotein

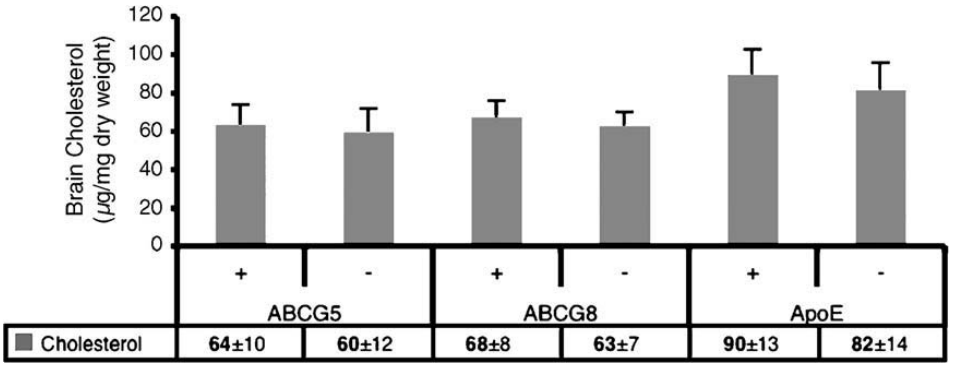


Fig. 4. Brain cholesterol levels are unaltered in Abcg5-, Abcg8-, and ApoE-deficient mice as compared to their respective littermate controls. Values represent means± standard deviations and are presented as µg/mg brain (dry weight). *P-value ≤0.05; **P-value ≤0.01; ***P-value ≤0.001.

fractions in serum and plasma of the different groups of mice, as well as the relative cholesterol and plant sterol content in each of these fractions was analyzed. Our results confirm that in ApoEdeficient mice VLDL constitutes the largest cholesterol-containing fraction, while in Abcg5-deficient mice, as well as in wild type mice, HDL particles are the predominant cholesterol carriers (Fig. 5). Cholesterol and plant sterols were distributed equally over the different lipoprotein fractions. Whereas approximately 50% of total campesterol and sitosterol was present in HDL particles in Abcg5-deficient mice, this was only 3.5% and 5.6%, respectively, in ApoE-deficient mice. These mice carried approximately 70% of their plant sterols in VLDL particles, which contained relatively more cholesterol (cholesterol/plant sterol ratio: 28.1) than the HDL particles of Abcg5deficient mice (cholesterol/plant sterol ratio: 1.4).

Increased brain plant sterol levels do not affect total brain cholesterol metabolism

To establish potential effects of elevated plant sterol levels on brain cholesterol synthesis and catabolism, we measured the levels of the cholesterol precursors lanosterol, lathosterol and desmosterol as indicators of cholesterol synthesis rate in the brain. No significant differences were observed in the levels of the various precursors in

the Abcg5-deficient, the Abcg8deficient or the ApoE-deficient mice as compared to their wild type littermates (Table 1).
Absence of Abcg5 or ApoE did also not significantly affect the levels of the major brain cholesterol metabolites 24Shydroxycholesterol and cholestanol (Table 1). However, a trend was observed indicating a reduction of 24S-hydroxycholesterol levels in brains of Abcg8-deficient mice (Abcg8+/+: 215 ± 58 vs. Abcg8−/−: 144 ± 7; P ≤0.053) (Table 1). In agreement, the ratio of 24S-hydroxycholesterol to cholesterol was significantly decreased in the brains of the Abcg8-deficient mice compared to controls (Abcg8+/+: 3.1 ± 0.7 vs. Abcg8−/−: 2.3 ± 0.1; P ≤0.039).

Abcg5 or Abcg8 mRNA is not expressed in C57BL/6J mouse brain

We examined whether the increase in brain plant sterol levels in Abcg5-and Abcg8-deficient mice might be attributable to the presence of the Abcg5/8 heterodimer in cells of the blood–brain barrier of wild type mice as a potential means to re-secrete plant sterols from the brain into the circulation. However, in line with published results [20], we could not detect Abcg5/8 mRNA in wild type C57BL/6J mouse brains using real time PCR (data not shown). Since Abcg5/8 may be expressed selectively in endothelial cells of the blood–brain barrier, we determined Abcg5/8 mRNA levels in endothelial cells isolated from rat brain. Abcg5 and Abcg8 mRNA levels were more than 350 and 370 times lower, respectively, in endothelial cells from rat brain as compared to rat liver, whereas Abca1 mRNA levels were 2

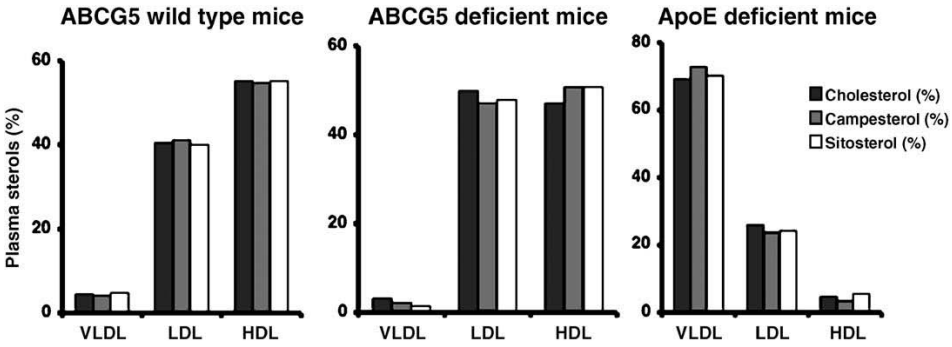


Fig. 5. Cholesterol and plant sterol composition (%) of lipoprotein fractions (VLDL, LDL, HDL) in plasma of Abcg5 wild type mice, Abcg5-deficient mice and serum of ApoE-deficient mice.

Table 1 No detectable effect of high plant sterol levels on brain cholesterol metabolism of Abcg5-, Abcg8-and ApoE-deficient mice compared to their respective littermate controls

Brain	ABCG5		ABCG8		APOE	
	+/+	−/−	+/+	−/−	+/+	−/−
Lanosterol	90±23	111±41	109±62	66±15	17±3	15±3
Lathosterol	73±8	85±22	70±17	71±8	139±31	116±24
Desmosterol	527±77	496±51	482±67	430±43	352±61	350±68
Cholestanol	158±44	175±46	156±26	172±11	621±248	686±318
24(S)OH-Chol	181±41	167±29	215±58	144±7	140±29	122±12

Values represent means±standard deviations and are presented as ng/mg brain (dry weight). times higher in brain endothelial cells than in liver (data not shown). This indicates that expression levels of Abcg5 and Abcg8 are extremely low at the level of the blood–brain barrier.

Deficiency for Abcg5 does not affect cholesterol-related gene expression

To further examine potential effects of increased brain plant sterol levels on brain cholesterol metabolism, we determined the effect of deficiency for Abcg5 on the relative expression levels of a number of genes involved in cholesterol metabolism in whole brain. No differences were observed in the expression levels of genes encoding ApoE or ApoC1, the low density lipoprotein-receptor (Lldr), 3-hydroxy-3-methyl-glutaryl-coenzyme A-reductase (Hmg-CoAr), cytochrome P450 46A1 (Cyp46A1), ATP-binding cassette transporter A1 (Abca1), or in the sterol regulatory binding protein 1c (Srebp1c) (Table 2).

Table 2 No differences in mRNA expression in whole brain samples of Abcg5-deficient mice as compared to wild type littermates

mRNA	**ABCG5+/+**	**ABCG5−/−**	
ApoE	1.00 ± 0.20	1.03 ± 0.08	
LDLr	1.00 ± 0.16	1.03 ± 0.14	
CYP46	1.00 ± 0.12	0.97 ± 0.15	
ApoC1	1.00 ± 0.18	0.79 ± 0.11	
ABCA1	1.00 ± 0.17	0.92 ± 0.03	
HMGCoAr	1.00 ± 0.15	1.03 ± 0.11	
SREBP1c	1.00 ± 0.15	0.93 ± 0.21	

Values represent the relative expression of the respective gene to housekeeping gene 18S in relation to wild type mice and are represented as mean value ± standard deviation.

Sitosterol upregulates apoE expression in astrocytoma cell line

Although we did not observe differences in gene expression patterns in whole brain samples, the possibility remains that plant sterols exert local effects. We examined effects of plant sterols on specific brain cell types, using astrocytoma (CCFSTTG1) and

neuroblastoma (SH-SY-5Y) cell lines. Incubation with various concentrations of sitosterol induced mRNA and protein expression of ApoE in astrocytoma cells in a concentration-and time-dependent manner as shown by real time PCR (Fig. 6A) and Western blotting (Fig. 7A, B). The synthetic liver-X receptor (Lxr) ligand GW683965A, which was used as a control, also induced ApoE-expression in these cells (Fig. 7A). Neither sitosterol nor GW683965A affected ApoE expression in neuroblastoma cells (data not shown). Furthermore, sitosterol strongly down-regulated the levels of Ldl-receptor (Lldr) mRNA in astrocytoma cells in a

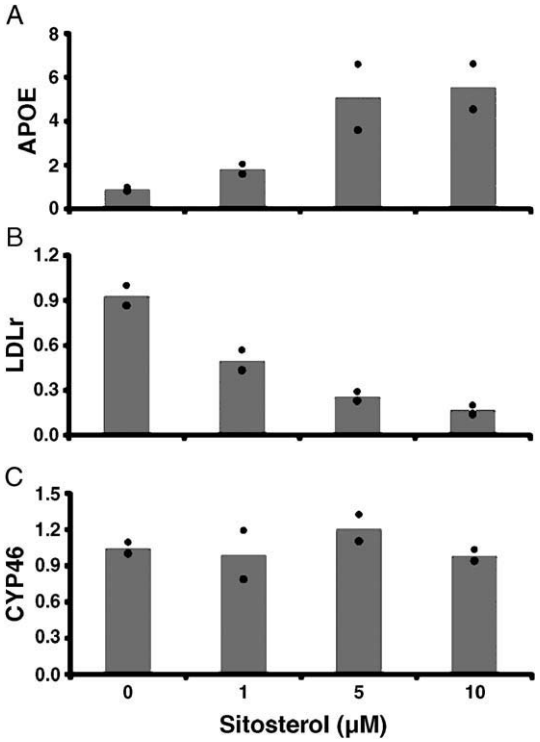


Fig. 6. Real time PCR displaying concentration-dependent effects of sitosterol on expression of APOE (A), LDLr (B) and CYP46 (C) in astrocytoma cell line (CCF-STTG1). Data from two independent experiments (dots) as well as average outcome (bars) are shown for the respective genes, normalized to 18S mRNA

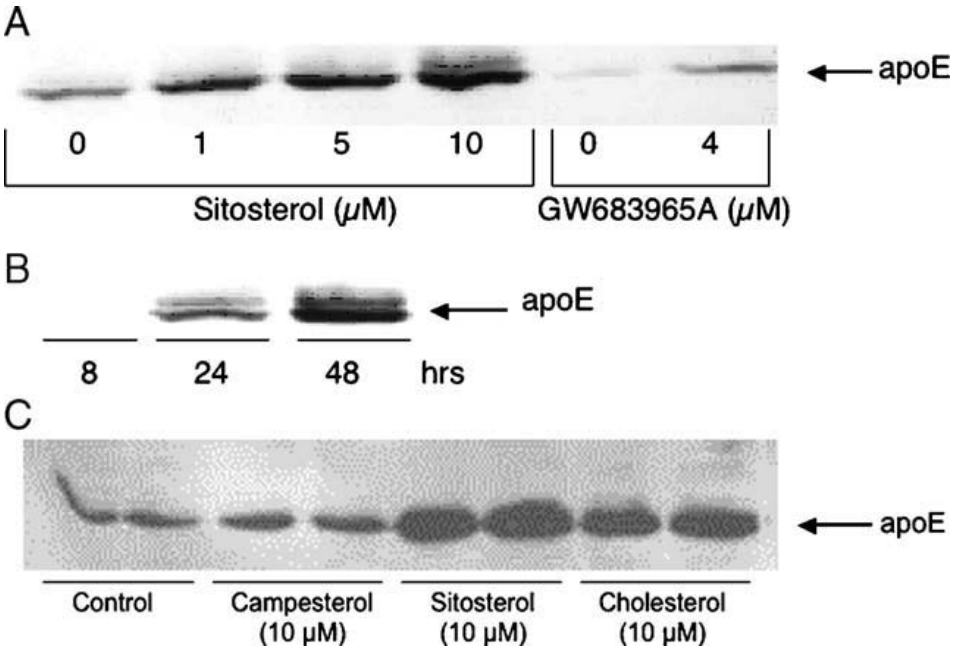


Fig. 7. Western blots showing ApoE protein expression in astrocytoma cell line (CCF-STTG1). (A) Concentration-dependent increase of ApoE-expression as a result of sitosterol and GW683965A. (B) Time-dependent increase of ApoEexpression as a result of sitosterol (10 μM). (C) ApoE-expression after incubation with 10 μM campesterol, 10 μM sitosterol and 10 μM cholesterol. concentration-dependent manner (Fig. 6B), whereas expression of CYP46 was not affected (Fig. 6C). Comparison of equal concentrations of campesterol, sitosterol and cholesterol indicated that sitosterol upregulated ApoE-expression in astrocytoma cells more effectively than cholesterol did, while essentially no effect of campesterol could be detected (Fig. 7C).

Discussion

In contrast to the general assumption that plant sterols do not enter the brain, this study demonstrates that increased circulating levels of plant sterols, as a result of intake of a plant sterol-enriched diet in wild type mice or as a consequence of Abcg5-or Abcg8-deficiency, are associated with elevated levels of plant sterols in the brain. Evidence is provided that plant sterols are only transferred into the brain when they are carried by small HDL particles, as is the case in Abcg5-and Abcg8-deficient mice and not when plant sterols are present in the larger VLDL particles, as is the case in ApoE-deficient mice. The increased plant sterol concentrations in the brain do not detectably affect overall brain cholesterol metabolism. Yet, it is important to note that they may exert cell-specific effects, which is supported by the finding that sitosterol specifically upregulates the expression of apoE in cultured human astrocytoma cells, but not in neuroblastoma cells.

The question arises how plant sterols are transported into the brain in the Abcg5-and Abcg8-deficient mice, while they are not in the ApoE-deficient mice. First, Abcg5-and Abcg8-deficient mice displayed approximately 50% lower serum cholesterol

concentrations, while ApoE-deficient mice display about 6-fold higher serum cholesterol levels than their respective littermate controls [7,8]. Therefore, theoretically, cholesterol may compete with plant sterols for transport across the blood–brain barrier. Also, the possibility remains that ApoE itself is involved in the transport of plant sterols from the blood into the brain.

Secondly, the mice strains studied differ in the lipoprotein fraction that retains the plant sterols. The majority of plant sterols are carried by HDL particles in Abcg5-and Abcg8-deficient mice, while the plant sterols shifted to the larger lipoproteins, i.e. VLDL and LDL, in ApoE-deficient mice. VLDL particles of ApoE-deficient mice were shown to have a higher ratio of cholesterol to plant sterols than HDL of Abcg5deficient mice. This suggests that the relative amount of plant sterols and possibly also the dissociation of plant sterols from HDL particles are much greater than from the LDL particles. This may explain the higher levels of plant sterols in the brain of Abcg5-and Abcg8-deficient mice. Although in vitro evidence has been obtained indicating that transport of HDL across the blood–brain barrier into the brain takes place [21], hardly anything is known with respect to the transfer of HDL particles into the brain in the in vivo situation. Remarkably, we found differences in the ratio of campesterol to sitosterol in serum and brain, which is in favour of initial transfer of plant sterols from HDL to the endothelial cells of the blood–brain barrier. Endothelial cells may subsequently re-secrete sterols from the basolateral plasma membrane, resulting in accumulation in the brain. Since campesterol is more similar in structure to cholesterol than sitosterol, the preference of campesterol transfer into the brain as compared to sitosterol is remarkable. In line with this, it was shown that the esterification rate of campesterol in macrophages was 20% of that of cholesterol, while β sitosterol was not detectably esterified [22]. These differences in esterification may play a role in the preferential transport of campesterol into the circulation and also into the brain.

Thirdly, the Abcg5/8 heterodimeric transporter may be present in cells of the blood–brain barrier, where it may re-secrete plant sterols from the brain into the circulation. However, in line with previous publications [20,23], there was no detectable expression of Abcg5 or Abcg8 mRNA in total brains of C57BL/6J mice, and very low expression levels in endothelial cells isolated from rat brain. Thus, at the level of the blood–brain barrier, expression levels of Abcg5 and Abcg8 are extremely low and are very unlikely to play a role in plant sterol transport.

Abca1 is a likely candidate to be involved in the transport of plant sterols into the brain, since it has been detected in brain endothelial cells at the basolateral side where it mediates cholesterol efflux [24]. Abca1 mediates the cellular efflux of free cholesterol to ApoA-I or to ApoE as extracellular sterol acceptors [25–27], and does not discriminate between cholesterol and sitosterol [28]. Considering this, the lack of increase in brain plant sterol levels of ApoE-deficient mice could be explained by assuming that ApoE derived from astrocytes acts as an acceptor for Abca1-mediated sterol efflux from blood– brain barrier endothelial cells. Yang et al. [1] found 10-fold increased Abca1 expression levels in the adrenal glands of Abcg5/g8-deficient mice. The fact that we did not detect any effect of the absence of Abcg5 or Abcg8 on the expression of Abca1 in whole brain may be due to the relatively low levels of plant sterols in the brain.

The levels of brain plant sterols were relatively low in Abcg5-and Abcg8-deficient mice as compared to cholesterol, but they still were about 2-fold higher than the levels of 24Shydroxycholesterol. Elevated 24S-hydroxycholesterol levels in cerebrospinal fluid and plasma as well as polymorphisms in CYP46, the enzyme that transforms cholesterol into 24Shydroxycholesterol, have been associated with neurodegenerative diseases, including Alzheimer's disease [29,30]. 24Shydroxycholesterol is a natural brain-specific activator of the Lxr and thereby able to affect cerebral cholesterol and fatty acid metabolism [31]. Similarly, plant sterols have been reported to activate Lxr [1,32]. Therefore, we hypothesized that plant sterol levels, as high as reported in the Abcg5-and Abcg8-deficient mice of this study, might have an effect on mRNA and/or protein expression.

High levels of plant sterols increase HMG-CoA reductase and disrupt cholesterol metabolism in extracerebral tissues [12]. In the brains of Abcg5-and Abcg8-deficient mice, however, the elevated levels of plant sterols did not have any detectable effects on overall brain cholesterol metabolism. Neither did we detect an effect on the mRNA levels of ApoE, ApoC1, Abca1, Ldlr, Hmg-CoAr, Cyp46 or Srebp1c in the whole brain of the Abcg5-deficient mice as compared to the wild type mice. This confirms the strict regulation of the maintenance of cholesterol balance in the brain. Yet, it is important to note that local sitosterol accumulation may exert cell-specific effects, since sitosterol enhanced ApoE mRNA expression and down-regulated Ldlr mRNA expression specifically in astrocytoma cells, but not in a neuroblastoma cell line. Sitosterol was a stronger regulator of ApoE protein expression than cholesterol, while campesterol did not detectably affect ApoE protein levels. In agreement with this, sitosterol has been reported to affect membrane characteristics due to a less favourable interaction with phospholipids than cholesterol [33].

The implications of our findings for the human situation remain to be established. In humans, plant sterols are predominantly transported by LDL particles [34]. Salen et al. [35] found that campesterol levels represent 0.6% and sitosterol levels 0.9% of the total sterol content in the brain of a sitosterolemic patient, whereas no plant sterols were detected in the brain of control patients. Actually, this indicates the presence of a relatively high level of plant sterols in the sitosterolemic brain. The single sitosterolemic subject that has been examined did not show any gross neurological dysfunction [35]. More extensive studies, particularly employing appropriate and more refined neurological tests are required to properly investigate the effect of plant sterols on brain functions in humans. Patients with familial hypercholesterolemia due to defective Ldlr that consume a plant sterol rich diet in combination with the use of statins, also display dramatically elevated serum plant sterol levels [36]. Whether or not this affects their brain plant sterol levels remains to be determined.

In conclusion, we demonstrate that increased levels of plant sterols in the circulation can result in increased levels of plant sterols in the brain. The results strongly suggest a role for HDL in the transport of plant sterols into the brain. This may either be by crossing the blood–brain barrier or by delivering plant sterols to endothelial cells of the blood–brain barrier and subsequent resecretion. ApoE may play a decisive role in these processes, either by influencing the lipoprotein particle size and composition in serum or by transport of plant sterols at the level of the blood–brain barrier. Furthermore, our results suggest that plant sterols exert effects within the brain by affecting expression of

genes, including ApoE and Ldlr, in a cell-type specific manner. Further investigations are needed to elucidate the mechanisms by which plant sterols are transported across the blood–brain barrier and whether or not they have a significant influence on brain functions.

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Chapter 5

Human apolipoprotein CI expression in mice impairs learning and memory functions

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Submitted

Abstract

APOE4 is associated with the development of sporadic, late onset Alzheimer's disease (AD). The H2 allele of APOC1, giving rise to increased gene expression of apolipoprotein CI (apoCI), is in genetic disequilibrium with the APOE4 allele. This has led to the hypothesis that the H2 allele of APOC1, rather than the APOE4 allele, provides a major risk factor for AD. We found that the apoCI protein is present mainly in astrocytes and endothelial cells within hippocampal regions of both control and AD brains. Surprisingly, it also co-localized with β-amyloid in plaques in brain specimens of AD patients. In vitro apoC1 peptide was found to inhibit Aβ peptide aggregation. We next investigated the effect of human apoCI expression in mice on their cognitive functions. In brains of human APOC1 expressing (*hAPOC1*$^{+/0}$) mice, the human apoCI protein was also detected predominantly in astrocytes and endothelial cells. As compared to their wild-type littermates, *hAPOC1*$^{+/0}$ mice showed impaired hippocampal-dependent learning and memory functions as judged from their performance in the object recognition task (P=0.012) and in the Morris water maze task (P=0.01). These effects of apoCI on memory may depend on its well-known inhibitory properties towards apoE-dependent lipid metabolism. However, no effects of hAPOC1 on brain mRNA or protein levels of endogenous apoE were detected. Neither were there any detectable differences in the overall sterol or phospholipids profile in the brain. In addition, analysis of gene expression profiles did not indicate gross changes in brain lipid metabolism or inflammation. In conclusion, apoCI expression impairs cognitive functions in mice independent of apoE expression, which strongly suggests that apoCI has a modulatory effect during the development of Alzheimer's disease.

Introduction

After apolipoprotein E4 (apoE4) had been identified as a major risk factor for the development of sporadic, late onset Alzheimer's Disease (AD) (50,51), many researchers began looking for additional susceptibility genes for this disease (52). Interestingly, APOE4 is in genetic linkage disequilibrium with the *HpaI* restriction polymorphism in the promoter region of APOC1, which is localized 5 kb downstream of the APOE gene on chromosome 19. Although apoCI is mainly expressed in the liver, substantial expression has also been detected in other tissues including the brain (53). The *HpaI* polymorphism (so-called H2 allele) leads to a highly significant, 1.5-fold increase in APOC1 gene transcription (54) and was reported to be associated with AD (55-60). Moreover, the H2 allele of APOC1 was associated with poorer memory and frontal lobe function (61), and with loss of hippocampal volumes (62).

Although apoE4 is a well-established and strong risk factor of AD in Caucasians and in European-Americans, it is not associated with AD in African-Americans. Interestingly, the frequency of APOC1 H2 with apoE4 was 0.85 in European-Americans but only 0.55 in African-Americans, whereas the frequency of APOC1 H2 with apoE3 was 0.02 in European-Americans and 0.08 in African-Americans (54). This led to the hypothesis

that the H2 allele of APOC1 may be an independent or an additional risk factor for AD (57,58,63,64), and that not apoE4 but rather apoCI modulates the pathogenesis of AD (65).
The physiological roles of apoCI have not been established in detail. Studies with mice overexpressing human apoCI (66) revealed gene dose-dependent effects on circulating levels of triglycerides (TG), free fatty acids (FFA) and total cholesterol (TC) (67,68). This may be due to interference of apoCI with apoE-dependent clearance of TG-rich lipoproteins by Low Density Lipoprotein-Receptor family in the liver (43,69,70) and by modulating the activity of enzymes involved in plasma lipid metabolism, (71,72).
The presence of apoCI within the brain at relatively high levels (53) suggests an important role for this protein in brain lipid metabolism. Disturbances in brain lipid metabolism can lead to cognitive impairments in humans and in rodents, indicating that a well-regulated brain lipid metabolism is necessary for normal brain functioning (73-77). Therefore, we have investigated learning and memory functions as well as brain lipid metabolism in transgenic mice hemizygous for human apoCI (*hAPOC1*$^{+/0}$) and their wild type littermates. Moreover, we examined the expression of apoCI in post mortem brains of AD and control patients.

Materials and Methods

Immunohistochemistry.

Post mortem human brain samples were obtained from the Netherlands Brain Bank (Amsterdam, The Netherlands, coordinator Dr. R.Ravid). Brains were characterized with respect to their neuropathological status following criteria established by Khachaturian (78). Samples from nondemented subjects displaying no neuropathological changes related to AD or any other neurodegenerative disorder was designated as control cases. All AD cases showed similar severity of dementia. The cryostat sections (35 μm) of hippocampal region were stored at -80°C until further use. Immunohistochemical procedures were similar to that used in mice brain. Mice were anesthetized (Nembutal, Ceva Sante Animale BV, Maassluis, The Netherlands, 180 μg/g body weight, i.p.) and perfused transcardially with PBS followed by Somogyi fixative as described (79). After removal, the brain was transected midsagittally and the lateral block was postfixed in the same fixative (+4°C, overnight) and cryoprotected in sucrose (10% for 24 hours followed by 30% for 1-2 weeks) in 0.1M phosphate buffer. The half brain was cut in 35 μm coronal sections on a cryostat. The sections were sampled systematically throughout the entire hippocampus with a random start. The sections were kept at -80°C. Free floating sections were incubated overnight at 4°C with following primary antibodies: goat anti human apoC1 (Academy Bio-Medical Co, Houston, TX), rabbit anti-glial fibrillary acidic protein (GFAP) (DAKO, A/S, Denmark), rabbit anti-amyloid β (Aβ) (Calbiochem Immunochemicals, San Diego, Ca), rabbit anti-von Willebrand factor (Abcam, Cambridge, UK), Rabbit anti-human apoE (DAKO), goat anti-mouse apoE (Santa Cruz Biotechnology, Inc, Santa Cruz, Ca) or for 1 hour at room temperature with biotinylated-*Lycopersicon Esculentum* (tomato) lectin (Vector Laboratories, Inc, Burlingame, Ca). For control purposes, sections were

simultaneously processed in the absence of the primary antibody. Subsequently, sections were incubated for 1 hour at room temperature with secondary antibodies: FITC-conjugated donkey anti-goat (Santa Cruz Biotechnology) or Texas Red conjugated donkey anti-rabbit (Abcam). Sections were also incubated with donkey anti-rabbit biotin (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) followed by avidin-Texas Red (Vector Laboratories). All antibodies were diluted in PBS containing 1% of BSA (Sigma, Zwijndrecht, Netherlands). Auto fluorescence was blocked by incubation for 75 seconds in 0.3% Sudan black (Sigma) solution in ethanol. Finally sections were mounted in 90% glycerol, 0.02 M Tris–HCl pH 8.0, 0.8% NaN_3 and 2%1,4-di-azobicyclo-(2,2,2)-octane (DABCO; Merck, Darmstadt, Germany) containing DAPI (Sigma). Slides were covered with thin glass coverslips and stored for further analysis. Fluorescent samples were imaged using a Bio-Rad MRC600 confocal microscope (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) equipped with an air-cooled Argon–Krypton mixed gas laser and mounted onto an Axiophote microscope (Zeiss), using oil-immersion objectives (40x). The laser-scanning microscope was used in the dual parameter set-up, according to the manufacturer's specifications, using dual wavelength excitation at 488 and 568 nm. Emission spectra were separated by the standard sets of dichroic mirrors and barrier filters.

In situ hybridization.

In situ hybridization with human APOC1-specific end-labeled oligonucleotide probes was performed as described (80). Twenty-micrometer brain sections containing dorsal hippocampus were cut on a cryostat, thaw-mounted on poly-L-lysine coated microscope slides, and kept at -80^{o}C until further use. One hundred microliters of the riboprobe mix (murine probe 5' CTTCAGTTTATCCGGTATGCTCTCCAATGTTCCGGACAAATCC; mismatch murine probe: 5'CgTCAGTgTATCCtGTATGaTCTCC cATGTTaCGGACcAATCC; human probe 1: 5' TGTGTTTCCAAACTCCTTC AGCTTATCCAAGGCACTGGAGACG; mismatch humane probe-1: 5'TtTGTTTaCAAACgCCTTCcGCTTAgCCAAGtCACTGtAGACG) were applied to each slide, which was then covered with a standard microscopic coverslip and put in a moist chamber for overnight hybridization at 53°C. The next day, the slides were washed, treated with RNAse A and dehydrated in a graded ethanol series, put in a cassette and a X-OMAT AR film (Kodak, Rochester, NY) was exposed to the sections for 14 days.

Animals.

Transgenic mice with high expression of human APOC1 in the liver and a moderate expression in the brain were generated as described previously (122,214). Female human apoC1 (*hAPOC1*$^{+/0}$) transgenic mice (n=12) and their wild-type littermates (n=14) were bred at TNO-PG in Leiden, The Netherlands. Mice were housed under standard conditions in conventional cages and given food (standard chow diet) and water *ad libitum*; lights on from 07.00 to 19.00 h in a temperature and humidity controlled room. The ethical board of the Maastricht University approved of all experimental procedures.

Behavioral testing.

Mice were subjected to behavioral tests when they were 12 months old. Testing took place between 9.00 and 15.00 h. The experimenters were unaware of the genetic background of the mice. There were no differences in the results of the mice tested early or late on the days of the experiment.

Object recognition task.

The object recognition task measures whether a mouse remembers an object it has explored in a previous learning trial. The task was performed in a circular arena with a diameter of 49 cm, as described previously (81,82). A testing session comprised two trials of 3 min each. During the first trial (T1), the testing site contained two identical objects (samples). A mouse was always placed in the arena facing the wall in the centre of the transparent front segment. Subsequently, after a delay interval of 1 hour, the mouse was put back in the arena for the second trial (T2), but now with two dissimilar objects, a familiar one (the sample) and a new one. The times spent exploring each object during T1 and T2 were recorded manually with a personal computer. The discrimination index (d2) represents the relative measure of discrimination between the new object (b) and the familiar object (a), which corrects for the absolute discrimination (b-a) for the total exploration activity. The animals recognize the familiar object if the discrimination index is higher than zero. Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behaviour. In addition, all combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects.

Morris water escape task.

Eight days before the beginning of the behavioral testing, the mice were housed individually and were allowed to adapt to the room were the testing took place. The spatial discrimination performance of mice was assessed in a circular gray water tank (diameter 81 cm) filled with clear tap water (22-24°C). A video camera was centrally positioned above the water tank. It automatically recorded movements of the mice at a rate of five samples per second (via a tracking system EthoVision™, Noldus, Wageningen, The Netherlands). First, the mice were given one trial in which a white platform (diameter 8 cm) was visible. Subsequently, the mice were given a total of 16 trials (four trials a day), using four different starting positions (assigned in a random order between mice), in which a gray platform was submerged. If a mouse did not find the platform within 60 s, it was guided to the platform and the mouse had to climb onto the platform. After 3 s it was removed from the water tank. If a mouse found the platform, it was left there for 3 s and removed from the water tank. To reveal memory effects and strategies followed the following day, the mice were subjected to a probe trial. For this purpose, the platform was removed from the water tank and the behavior of the mice, i.e. time spent in the vicinity of the place of the platform, was registered. The procedures and analysis of the data are described elsewhere (83,84).

Phospholipid profile determination.

One ml of the chloroform-methanol (2:1) extract of the brains was dried under a nitrogen stream and was stored at -80°C until shipment in dry ice and phospholipid analysis. The extracted lipids were dissolved in methanol:chloroform (2:1) for separation and quantification by high performance thin layer chromatography (HPTLC). The measurements were performed without knowledge of the genetic background of the mice. Thin layer chromatography (TLC) was performed as described previously by Weerheim et al (85) with minor modifications. The density of the spots obtained after charring of the phospholipids was analyzed by photodensitometric scanning (GS-800 Calibrated Densitometer, Bio-Rad, Hercules, CA), and quantified using Quantity One software version 4.2.2 (Bio-Rad). Results were expressed as nmol phospholipids/mg brain dry-weight. Phospholipid standards were obtained from Sigma (St. Louis, MO). Chloroform, ethyl acetate, acetone, methanol, ethanol, dichloromethane, isopropanol, acetic acid (all HPLC grade) were obtained from Merck. All other chemicals were of analytical quality.

Sterol profile determination.

Blood was collected before perfusion of the mice. Serum was snap frozen in liquid nitrogen. The brains of all mice were frozen in liquid nitrogen and stored at –80°C until subsequent analysis. Prior to sterol analysis, brains were spun in a speed vacuum dryer (12 mbar) (Savant AES 1000) for 48 hours to relate individual sterol concentrations to dry-weight. The sterols were extracted from 100µl serum in the same manner. Levels of cholesterol were determined in a gas-chromatograph-flame ionization detector (GC-FID) with 5α-cholestane as internal standard. Levels of plant sterols (campesterol, sitosterol), cholesterol precursors (lanosterol, lathosterol and desmosterol) and cholesterol metabolites (24S-OH-cholesterol and cholestanol) were determined using gas chromatography-mass spectrometry (GC-MS) as described previously (39) using epicoprostanol as internal standard.

RNA isolation and Real-time quantitative PCR (QRT-PCR) procedures:

Total RNA was isolated using the Trizol method (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Integrity of RNA was checked by agarose gel electrophoresis, and RNA concentration was measured spectrophotometrically (NanoDrop, Witec AG, Littau, Germany). QRT-PCR was performed using an Applied Biosystems 7700 sequence detector with 1.6.3 software (Perk-Elmer Corp., Foster City, CA, USA) as previously described (40) with modifications (41). Primer sequences are available upon request. Primers were obtained from Invitrogen. Fluorogenic probes, labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), were made by Eurogentec (Seraing, Belgium).

Western blot analysis:

Tissue lysates (25 µg protein/lane) were subjected to dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Protran nitrocellulose transfer membranes (Schleicher & Schuell BioScience GmbH, Dassel,

Germany). After blocking with 5% non-fat dry milk (Protifar-plus, Nutricia Netherlands B.V.) in washing buffer (PBS with 0.5% Triton-X100), the membranes were incubated with antibodies against murine apoE (Santa Cruz Biotechnology), GAPDH (Chemicon, California, USA) overnight at 4°C. The membranes were then incubated with peroxidase-conjugated secondary antibodies, after which the results were visualized using ECL reagents (Amersham Biosciences, Little Chalfont, UK) and autoradiography (LAS 3000, Fuji Photo Film Co., Ltd, Japan).

Thioflavin-T (Th-T) fluorimetric assay

The fibril formation of Aβ(1-40) peptide was measured by a Th-T binding assay as previously described (42, 43). In brief, a 100 μM Aβ(1-40) solution (Bachem, Weil am Rhein, Germany) was incubated at 37°C for 16 hours in phosphate-buffered saline (pH 7.4) in the absence or presence of apoC1 or apoA-I peptides at a molar ratio of 1:100. After incubation, a 10-μl aliquot of solution was added to 3.0 μM Th-T (Sigma) in a final volume of 1 ml of 50 mM phosphate buffer (pH 6.0). Fluorescence was monitored immediately at excitation and emission wavelengths of 450 and 482 nm, respectively, using a Shimadzu RS 1501 spectrofluorimeter.

Statistics.

Group differences in behavioral tests were analyzed using a one or two factorial ANOVA (Group or Group and Day, respectively). To determine the spatial bias in the probe trial of the Morris task, the time the mice spent in the different quadrants was evaluated. Group differences in sterol/ phospholipids analysis, body weight and temperature changes were evaluated using a *t* test. Statistical significance in QRT-PCR experiments was determined by comparing means using an unpaired Student's *t*-test and Mann-Whitney U-test.

Results

Expression and cellular localization of human apoCI in brain of hAPOC1$^{+/0}$ transgenic mice.

Human *APOC1*$^{+/0}$ transgenic mice were used as an experimental model to study the effect of apoCI on cognitive functioning. *In situ* hybridization experiments demonstrated that human APOC1 mRNA is present throughout the brain of *hAPOC1*$^{+/0}$ transgenic mice. The specificity of the riboprobe for human apoCI was evidenced by the absence of staining of brains of wild-type mice (Fig. 1). Human apoCI protein could be demonstrated immunohistochemically in the hippocampus of *hAPOC1*$^{+/0}$ transgenic mice, whereas no immunopositive cells in hippocampus of wild-type mice were observed (data not shown). To verify whether the distribution of human apoCI over the specific brain cell types reflects that in humans, we first performed a double staining with both anti-human apoCI and anti-GFAP antibodies. Indeed, apoCI was expressed in astrocytes, as evidenced by co-localization of human apoCI and GFAP, e.g. in the

hippocampal region (Fig. 2A). Moreover, it was detected in endothelial cells, as revealed by double staining with anti-human apoCI and anti-von Willebrand factor antibodies (Fig.2B). More specifically, human apoCI was detected at the basolateral side of endothelial cells stained with anti-tomatolectin, a marker for microglia that also stains endothelial cells of blood vessels (44, 45) (Fig.2C).

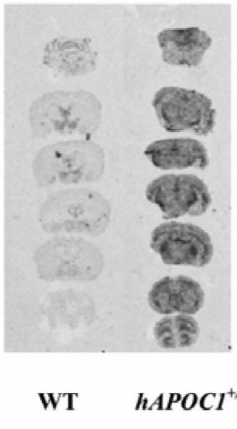

Figure 1. *In situ* detection of human APOC1 mRNA in mice brains. *In situ* hybridization with human APOC1-specific end-labeled oligonucleotide probes. Detection of human APOC1 mRNA in the brains of *hAPOC1*$^{+/0}$ transgenic mice and wild-type mice by *in situ* hybridization showing the presence of hAPOC1 mRNA throughout the brain of *hAPOC1*$^{+/0}$transgenic mice.

Human APOC1 expression does not affect overall behavioral appearance.

The *hAPOC1*$^{+/0}$ transgenic and wild-type mice that participated in this study were littermates with a highly homogeneous genetic background, obtained by backcrossing at least 10 times to the C57Bl/6 background. The *hAPOC1*$^{+/0}$ mice appeared healthy and there were no gross differences between the *hAPOC1*$^{+/0}$ transgenic mice and their wild-type littermates with respect to overall appearance, behavior in cage, handling response, or body weights. The weight of the mice was 26.0±1.7 g for the *hAPOC1*$^{+/0}$ transgenic mice and 25.0±1.8 g for their wild-type littermates, prior to the beginning of the behavioral experiments at the age of 12 months.

Human APOC1 expression impairs cognitive performance in the object recognition task.

Human APOC1$^{+/0}$ transgenic mice and wild-type littermates were subjected to the object recognition task (ORT). One hour after a first trial using two identical objects, the wild-type mice were able to discriminate between the familiar and the new object (Fig. 3). In contrast, the *hAPOC1*$^{+/0}$ mice failed to discriminate between both objects, as their discrimination index, which is a measure for selective exploration of the new object, did not differ from zero (*hAPOC1*$^{+/0}$ versus wild-type, p=0.012). This indicates that human APOC1 expression in mice significantly impairs object memory.

Human APOC1 expression impairs cognitive performance in the Morris water escape task.

Mice were subjected to a swimming trial, in which the platform was visible in a circular water tank. Subsequently the platform was submerged and mice were subjected to the spatial discrimination training trial consisting of four sessions per day on four successive days. During the acquisition of the Morris water escape task, the *hAPOC1*$^{+/0}$ transgenic mice swam on average a longer distance ($p<0.01$) (Fig. 4A) and needed more time ($p<0.01$) (Fig. 4B) to find the platform than the wild-type mice. The *hAPOC1*$^{+/0}$ mice

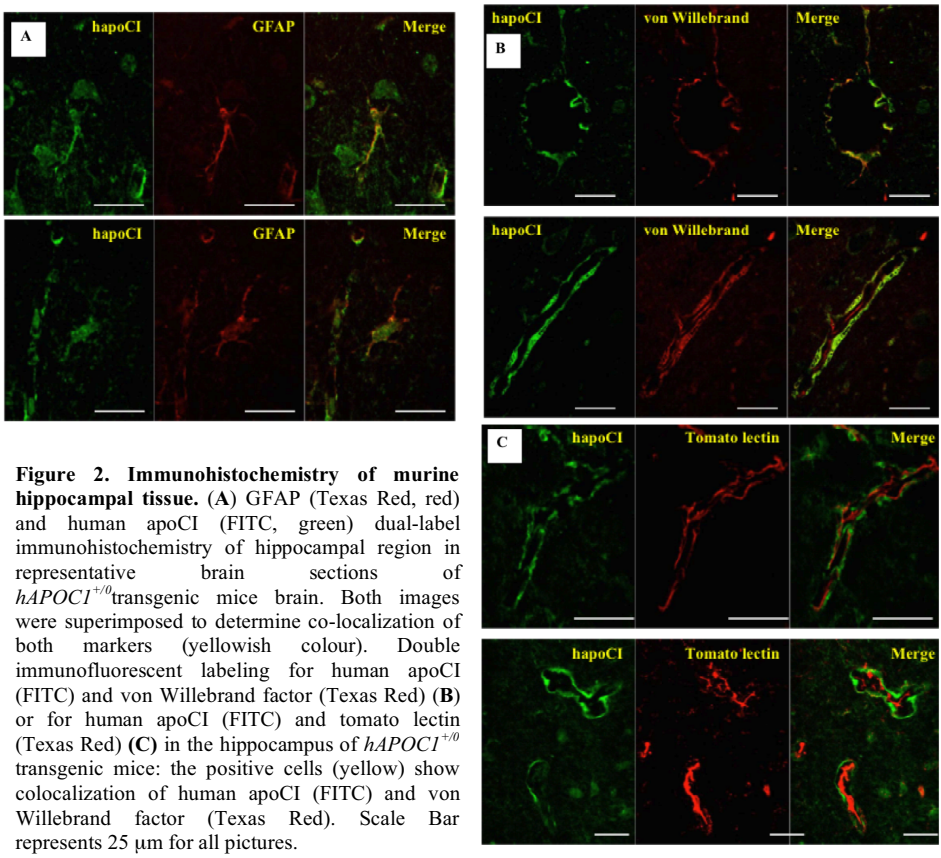

Figure 2. Immunohistochemistry of murine hippocampal tissue. (**A**) GFAP (Texas Red, red) and human apoCI (FITC, green) dual-label immunohistochemistry of hippocampal region in representative brain sections of *hAPOC1*$^{+/0}$transgenic mice brain. Both images were superimposed to determine co-localization of both markers (yellowish colour). Double immunofluorescent labeling for human apoCI (FITC) and von Willebrand factor (Texas Red) (**B**) or for human apoCI (FITC) and tomato lectin (Texas Red) **(C)** in the hippocampus of *hAPOC1*$^{+/0}$ transgenic mice: the positive cells (yellow) show colocalization of human apoCI (FITC) and von Willebrand factor (Texas Red). Scale Bar represents 25 μm for all pictures.

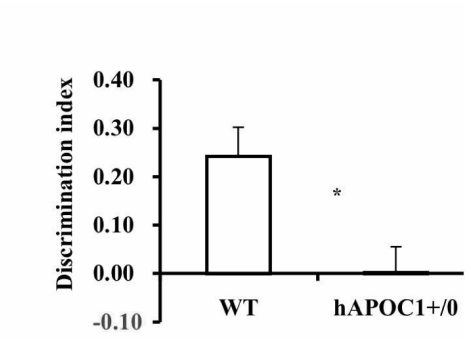

Figure 3. *hAPOC1* expression impairs cognitive performance in the object recognition task. One hour after a first trial using two identical objects, mice were subjected to a second trial with dissimilar objects. In this second trial, the discrimination index was determined from the time spent exploring the new object relative to the familiar object. Data represent mean ± SEM. *p=0.012

thus acquired this task less well than the wild-type mice. There was no significant difference in average swimming speed between the groups (Fig. 4C), although the *hAPOC1*$^{+/0}$ mice swam slower than wild-type mice on the second day ($p<0.05$) (Fig. 4C). In the probe trial, the *hAPOC1*$^{+/0}$ mice spent less time and swam a shorter distance in the target quadrant, albeit that the difference reached significance only for the distance moved in the target quadrant ($p<0.01$) (Fig. 4D, E).

Human APOC1 expression affects sterol levels, but not phospholipid profiles, in the brain.

Since apoCI is known to affect plasma TG, FFA and TC levels, we determined brain phospholipid and sterol profiles to assess whether the presence of human apoCI affects brain lipid metabolism and, thereby, may have altered brain functioning. In the brain, fatty acids are mostly retained by phospholipids (46-48). Therefore, we measured brain phospholipid levels in *hAPOC1*$^{+/0}$ transgenic and wild-type mice. No difference in phospholipid profiles could be detected between the two groups of mice (Table I). As expected, plasma sterol levels were elevated in the *hAPOC1*$^{+/0}$ transgenic mice in comparison with wild-type littermates (Table II). Yet, this was not associated with similar changes in brain sterol levels. As shown in Table III, the sterol distribution in brains of *hAPOC1*$^{+/0}$ and wild-type mice was comparable, except for a small but significant increase in the amount of the plant sterols campesterol ($p<0.05$) and sitosterol ($p<0.05$).

Human APOC1 expression does not dramatically affect expression of genes involved in cholesterol and fatty acid metabolism, cellular stress and inflammation.

Since APOC1 is encoded by the same gene cluster as APOE, and apoE levels in the brain may affect learning and memory processes (49-52), we determined the APOE mRNA levels in the brains of *hAPOC1*$^{+/0}$ and wild-type mice by quantitative real-time PCR (QRT-PCR). No significant difference could be detected between the two groups (Table IV). In addition, no differences were detectable in the protein levels of apoE in the brains of the two groups of mice, as determined by Western immunoblot (Fig. 5). In addition, gene expression profiles of key regulatory, metabolic and transporter encoding genes involved in cholesterol metabolism (hydroxy-methylglutaryl coenzyme A reductase (HMGCoA-R), low-density lipoprotein receptor (LDL-R), scavenger receptor type BI (SR-BI), ABC-transporters ABCA1, ABCG1, ABCG4, Lecithin:Cholesterol Acyltransferase (LCAT), phospholipid transfer protein (PLTP), cholesterol 24-

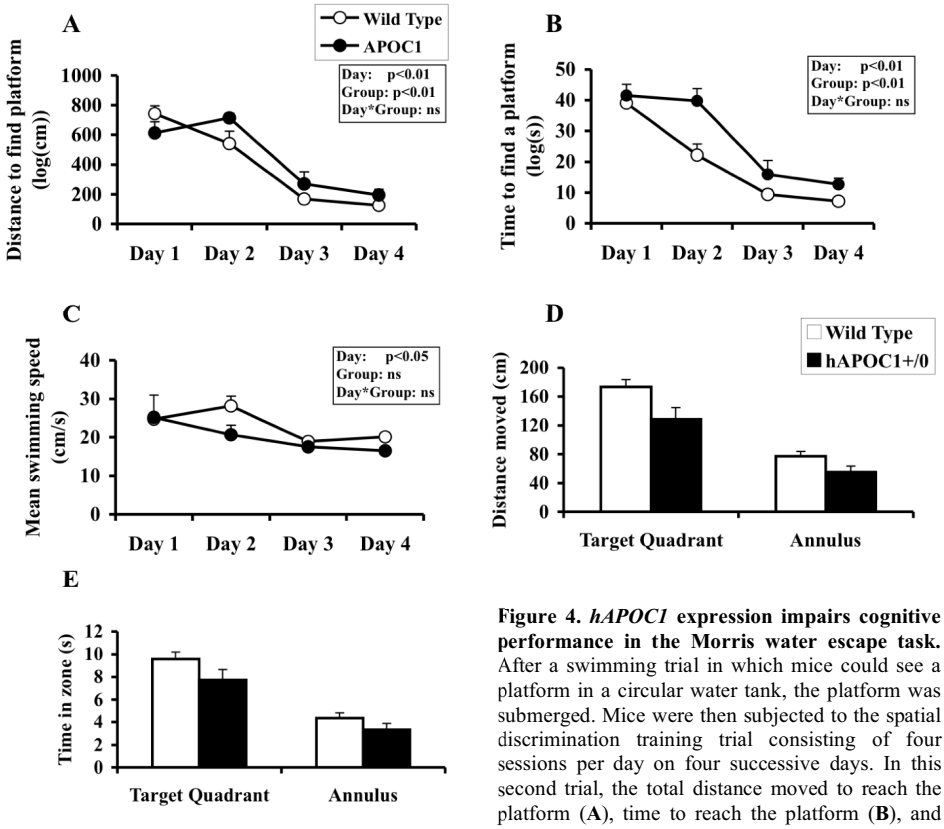

Figure 4. *hAPOC1* expression impairs cognitive performance in the Morris water escape task. After a swimming trial in which mice could see a platform in a circular water tank, the platform was submerged. Mice were then subjected to the spatial discrimination training trial consisting of four sessions per day on four successive days. In this second trial, the total distance moved to reach the platform (**A**), time to reach the platform (**B**), and the mean swimming speed (**C**) were evaluated on each day. Twenty-four hours after this trial, the mice were given a probe trial, in which the distance swam (**D**) and time spent **(E)** in the quadrants (target quadrant and annulus) were measured. Data represent means ± SEM. *p<0.01.

hydroxylase (CYP 46)) revealed no significant difference between two groups of animals except for very limited changes in the levels of HMGCoA-R and ABCG4 mRNA (Table IV). Expression of genes involved in fatty acid metabolism - fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC1), lipoprotein lipase (LPL), stearoyl-CoA desaturase 1 (SCD1) did not differ between two groups of mice and neither were there any significant differences between transgenic mice and wild-type mice in expression of genes that act as regulators of lipid metabolism: liver X receptor alfa (LXRα), peroxisome proliferator-activated receptors (PPARα, PPARγ), sterol regulatory element-binding proteins (SREBP-1a, SREBP1c, SREBP-2), carbohydrate response element binding protein (ChREBP) except for a very modest difference in the expression of 9-cis-retinoic acid receptor alfa (RXRα). We also tested genes involved in cellular stress (heme oxygenase-1 (HO-1)) and inflammation (IL6, TNFα, iNOS). No differences were detected between the groups (Table IV). Cellular localization of apoCI in human control- and AD brains

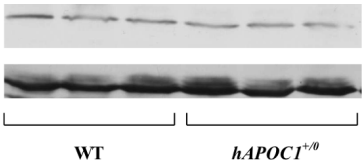

Figure 5. Human APOC1 expression does not affect protein levels in brains of *hAPOC1*$^{+/0}$ transgenic mice. Brain lysates were subjected to Western blot analysis as described in Methods and apoE expression was measured using a polyclonal antibody against apoE. GADPH was used as an internal standard.

Subsequently we performed an immunohistochemical analysis of apoCI on human hippocampal sections (CA1 region) of control subjects and AD cases. ApoCI protein was detectable primarily in astrocytes in control brain and also in AD brain (Fig.6A), as evident from co-localization with GFAP. In addition, in the AD- as well as in the control brain, apoCI was detected in endothelial cells lining the blood vessels, as demonstrated by co-localization with von Willebrand factor (Fig.6B). Interestingly, we observed that human apoCI-staining overlapped with the staining for amyloid β (Aβ) protein, the key protein of senile plaques, one of the hallmarks of AD (Fig. 6C), albeit that not all Aβ-positive plaques were positive for apoCI. Moreover, human apoCI co-localized with apoE in the same plaques in the brain of AD patients (Fig.6C).

Table I Brain phospholipid levels of *hAPOC1*$^{+/0}$ transgenic and Wild type mice

	Wild-type (n=14)	*hAPOC1*$^{+/0}$ (n=12)
Lysophosphatidylcholine	6.7±2.3	6.9±1.7
Sphingomyelin	6.5±1.3	7.3±1.2
Phosphatidylcholine	96.8±10.0	99.0±10.2
Lysophosphatidylethanolamine	37.9±16.1	38.2±16.1
Phosphatidylserine	60.3±8.2	61.7±10.3
Phosphatidylinositol	8.1±1.5	7.9±1.4
Phosphatidylethanolamine	140.2±15.6	142.5±16.6
Total	356.5±29.2	363±30.8

No differences were detected in brain phospholipid profiles of *hAPOC1*$^{+/0}$ and wild-type mice. The extracted brain lipids were dissolved in methanol:chloroform (2:1) for separation and quantification by high performance thin layer chromatography (HPTLC). Values represent as means±SD, expressed in nmol/mg brain.

Table II Serum sterol levels of *hAPOC1*$^{+/0}$ transgenic and wild type mice

	Wild type (n=14)	*hAPOC1*$^{+/0}$ (n=11)
Cholesterol (mg/dl)	57.1±8.0	152.2±14.9*
Lathosterol (μg/dl)	15.1±10.5	133.2±29.9*
Lanosterol (μg/dl)	53.2±14.1	168.7±42.4*
Desmosterol (μg/dl)	76.5±7.9	165.3±24.5*
24(S)-OH-Chol (μg/dl)	268.1±0.9	271.7±1.8*
Cholestanol (μg/dl)	76.1±35.9	421.6±91.8*
Campesterol (mg/dl)	1.4±0.3	4.9±9.2*
Sitosterol (mg/dl)	0.2±0.05	0.8±0.1*

Levels of cholesterol, plant sterols, and other sterols are strongly elevated in serum of *hAPOC1*$^{+/0}$ mice in comparison with their wild-type littermates. Values are presented as means ± SD, expressed in μg/dl and mg/dl for cholesterol. Significant differences ($p<0.001$) between the two groups of mice are indicated by "*" asterisks.

Table III Brain sterol levels of *hAPOC1*$^{+/0}$ transgenic and wild type mice

	Wild type (n=14)	*hAPOC1*$^{+/0}$ (n=12)
Cholesterol (μg/mg)	61.6±2.3	63.2±1.5
Lathosterol (ng/mg)	1624.1±148.5	1726.7±241.6
Lanosterol (ng/mg)	78.1±16.5	75.2±13.6
Desmosterol (ng/mg)	485.9±57.5	537.1±79.2
24(S)-OH-chol (ng/mg)	164.0±18.3	155.1±11.0
Cholestanol (ng/mg)	169.5±29.0	185.8±32.8
Campesterol (ng/mg)	74.5±12.9	87.8±16.8*
Sitosterol (ng/mg)	11.2±1.2	12.4±1.5*

Expression of human apoC1 has a small but significant effect on brain levels of cholesterol and plant sterols (campesterol and sitosterol). Values are presented as means ± SD. Cholesterol is expressed in μg/mg and other sterols are expressed in ng/mg brain (dry weight). Significant differences ($p<0.05$) between the two groups of mice are indicated by asterisks.

Table IV

		Wild-type (n=8)	*hAPOC1*$^{+/0}$ (n=8)
Cholesterol metabolism			
Synthesis	HMG-CoA	1.0 ± 0.16	1.26 ± 0.27*
	LDLR	1.0 ± 0.18	1.32 ± 0.34
	SR-BI	1.0 ± 0.22	1.29 ± 0.33
Efflux	ABCA1	1.0 ± 0.16	1.24 ± 0.35
	ABCG1	1.0 ± 0.13	1.20 ± 0.24
	ABCG4	1.0 ± 0.13	1.23 ± 0.24*
	LCAT	1.0 ± 0.18	1.27 ± 0.37
	PLTP	1.0 ± 0.17	1.17 ± 0.22
	ApoE	1.0 ± 0.09	1.17 ± 0.15
Catabolism	CYP46A1	1.0 ± 0.17	1.08 ± 0.21
Fatty acid metabolism			
Synthesis	FAS	1.0 ± 0.24	1.23 ± 0.31
	ACC1	1.0 ± 0.19	1.14 ± 0.29
	SCD1	1.0 ± 0.26	1.23 ± 0.45
Uptake	LPL	1.0 ± 0.28	1.20 ± 0.34
Regulators of lipid metabolism			
	RXR α	1.0 ± 0.12	1.23 ± 0.24*
	LXR α	1.0 ± 0.19	1.21 ± 0.38
	PPAR α	1.0 ± 0.19	1.25 ± 0.45
	PPAR γ	1.0 ± 0.18	1.16 ± 0.26
	SREBP-1a	1.0 ± 0.21	1.11 ± 0.36
	SREBP-1c	1.0 ± 0.31	1.20 ± 0.31
	SREBP-2	1.0 ± 0.21	1.13 ± 0.28
	ChREBP	1.0 ± 0.23	1.22 ± 0.45
Cellular stress			
	HO-1	1.0 ± 0.42	1.33 ± 0.29
Inflammation			
	IL6	1.0 ± 0.29	1.12 ± 0.45
	TNFalfa	1.0 ± 0.30	0.91 ± 0.32
	iNOS	1.0 ± 0.27	1.16 ± 0.45

Quantitative real-time PCR was performed as described in Methods; n=8 per group. All data were standardized for 18S ribosomal RNA. Expression in control mice was set to 1.00. Values represent means ± SD. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$)

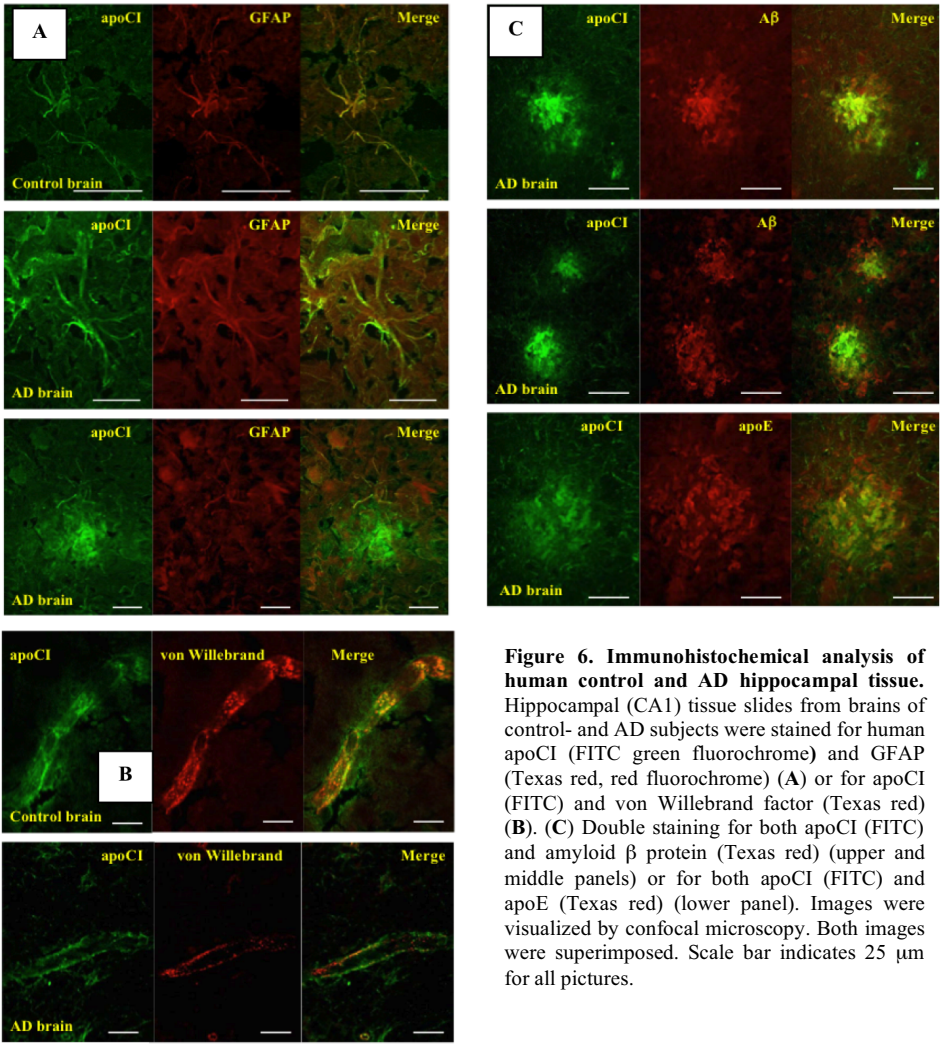

Figure 6. Immunohistochemical analysis of human control and AD hippocampal tissue. Hippocampal (CA1) tissue slides from brains of control- and AD subjects were stained for human apoCI (FITC green fluorochrome) and GFAP (Texas red, red fluorochrome) (**A**) or for apoCI (FITC) and von Willebrand factor (Texas red) (**B**). (**C**) Double staining for both apoCI (FITC) and amyloid β protein (Texas red) (upper and middle panels) or for both apoCI (FITC) and apoE (Texas red) (lower panel). Images were visualized by confocal microscopy. Both images were superimposed. Scale bar indicates 25 μm for all pictures.

ApoC1 peptides inhibit Aβ aggregation in vitro

In order to examine whether apoCI might directly interfere with the aggregation Aβ, we incubated Aβ in the absence or presence of apoCI or apoA-I peptides and measured the aggregate formation by Th-T fluorescence. Figure 7A shows the time course and the extent of aggregate formation, illustrating the different kinetics of aggregate formation for Aβ peptide alone and with the association with apoC1 or apoA-I peptides. Aggregate formation for Aβ peptide alone as well as with apoA-I begins after 110 min whereas the mixture of Aβ and apoC1 peptide delays the aggregation up to 840 min. Figure 7B clearly shows reduced Aβ (1-40) fibril formation in samples containing apoC1 peptides, whereas the presence of apoA-I peptide doesn't prevent fibril formation.

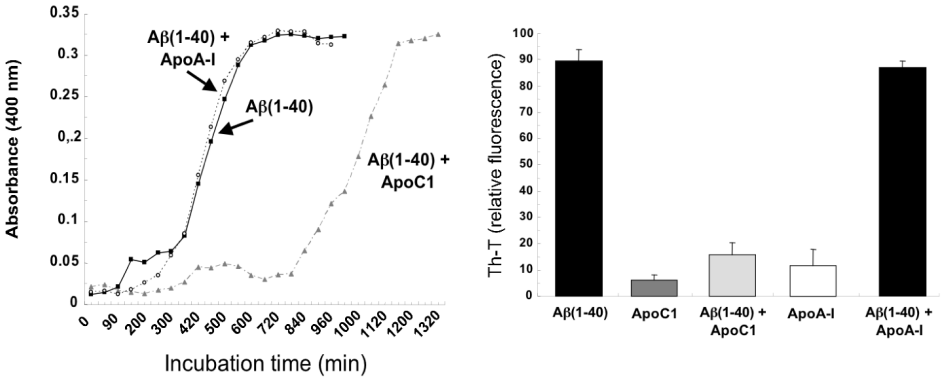

Figure 7. ApoC1 peptides inhibit Aβ aggregation in vitro. Aβ (1-40) peptide at 100 μM was incubated at 37°C in PBS buffer (pH 7.4) for the indicated time in the absence or presence of apoC1 or apoA-I at a molar ratio of 1:100. The aggregation was monitored as a function of time by measuring the turbidity of the solution at 400 nm against a blank containing only buffer and apoC1 or apoA-I **(A).** Aβ (1-40) peptide at 100 μM was incubated at 37°C in PBS buffer (pH 7.4) for 16h in the absence or presence of apoC1 or apoA-I at a molar ratio of 1:100. The level of Aβ

Discussion

In order to investigate a possible role for apoCI in cognitive functions we used *human APOC1*$^{+/0}$ transgenic mice. These mice display APOC1 mRNA throughout their brains and moderate levels of human apoCI protein predominantly in astrocytes and endothelial cells lining the cerebral microvessels, which reflects the distribution of apoE observed in the hippocampal region of human brains. This work shows, for the first time, that expression of human apoCI impairs learning and memory functions in mice. These findings underscore the hypothesis that the H2 allele of APOC1, giving rise to increased expression of the gene, may be an independent or additional risk factor for the development of AD. In this regard, it is of interest that in H2-allelic AD patients, who

are usually also apoE4 carriers, apoCI protein levels in the CSF were significantly higher than in H1-allele carriers (53).
In comparison with their wild-type littermates, the *hAPOC1*$^{+/0}$ mice displayed impaired hippocampal-dependent memory functions as indicated by the results obtained from the object recognition task. The learning tested in the spatial Morris water maze task, as also applied in the present study, is generally accepted to be highly dependent on the neuronal plasticity of the hippocampus. In the Morris task, the *hAPOC1*$^{+/0}$ mice displayed an impaired learning as indicated by the statistically significant differences between the two groups in the acquisition of the task. Also in the probe trial, the *hAPOC1*$^{+/0}$ mice moved a shorter distance in the target quadrant and thus performed less well than the wild-type mice, indicating an impaired memory. Previously, it has been reported that homozygous *hAPOC1*$^{+/+}$, but not the hemizygous *hAPOC1*$^{+/0}$ mice used in the present study, transgenic mice have a thinner hair-coat than their non-transgenic littermates (19). Since the test results in the water maze may have been confounded by the mice becoming hypothermic, especially since transgenic female mice were used (54). We performed thermoregulation studies on both groups of mice. However, there were no differences in thermoregulation between the hemizygous *hAPOC1*$^{+/0}$ transgenic mice and their wild-type littermates. The body temperature of the two groups of mice remained comparable when the mice were kept up to 6 hours at 4°C.
It is interesting to speculate about the mechanism(s) that underlie the observed effects of human apoCI expression on cognitive function. ApoCI and apoE are both produced by the same gene cluster (i.e. APOE/APOC1/APOC2/APOC4) on chromosome 19. In the circulation, apoE facilitates the clearance of lipoproteins by the LDLR and LRP by hepatocytes within the liver (22, 55). Similarly, apoE is thought to deliver cholesterol to neurons for the outgrowth of synapses (56-58). In the circulation, human apoCI inhibits the hepatic clearance of lipoproteins by interfering with their apoE-mediated binding to the LDLR or the LRP on hepatic cells (22, 55). Similarly, it can be speculated that, in the brain, human apoCI retards the uptake of the apoE-containing HDL-like lipoproteins by neurons.
Since it has previously been reported that apoE-knockout mice show impaired learning and memory functions (49-52), the effect of human apoCI expression on these cognitive functions could be explained by either reduced expression of mouse apoE in the brain or by counteracting the effects of apoE on lipid distribution within the brain. However, no differences were observed in brain *apoE* mRNA and protein levels between *hAPOC1*$^{+/0}$ transgenic mice and their wild-type littermates, and neither was there any detectable difference in the distribution of apoE throughout the brain determined by immunohistochemistry (data not shown) The possibility remains that apoCI has an effect on the apoE function independent of apoE expression, thereby modulating synaptic plasticity and learning and memory processes.
To obtain further insight into a potential role of apoCI in modulating the apoE-dependent transport and distribution of lipids, we determined sterol concentrations in the brain. However, whereas the levels of all sterols were increased in serum, only levels of campesterol and sitosterol were slightly, but significantly, increased in the brains of *hAPOC1*$^{+/0}$ mice. Recently, we have shown that increased serum plant sterol levels can result in increased brain plant sterol levels (59). Therefore, the increased brain levels of campesterol and sitosterol in *hAPOC1*$^{+/0}$ mice most likely are due to the

elevated levels of plant sterols in the circulation. Since hardly anything is known with respect to effects of plant sterols on brain functions, it remains to be established whether this increase contributes to the changes observed in learning and memory.
Besides affecting the apoE-dependent cellular lipid uptake, apoCI has profound effects on triglycerides (TG) and free fatty acid (FFA) levels in the circulation, due to its inhibitory effect on the action of lipoprotein lipase (LPL) (24). LPL is also found within the brain, where it is associated with neuronal and vascular endothelial cells (60) it may play a role in regeneration after brain injury and in neurite extension (61-66). In the brain, fatty acids are not retained in TG, but predominantly in phospholipids. However, we could not detect any effect of expression of human apoCI on total brain phospholipid profiles. The possibility remains that the effects of apoCI on brain phospholipids are restricted to specific brain regions, such as the hippocampus or cell types, and therefore, have been missed in our analysis using whole brain homogenates.
Previous research has raised the hypothesis that the H2 allele of APOC1 might be an independent or an additional risk factor next to apoE4 for the development of AD (5-9, 15, 53, 67). In line with previous observations (53), we demonstrate that apoCI is present predominantly in astrocytes but also in endothelial cells lining the blood vessels in human control as well as in AD brains. In fact, we observed co-localization of apoCI with apoE and Aβ in plaques in the brains of AD patients, moreover, we show that the incubation of apoC1 with Aβ peptides inhibits Aβ (1-40) fibril formation in vitro, whereas the incubation with apoA-I peptide didn't prevent fibril formation, despite the presence of apoA-I in senile plaques (68). It has been demonstrated that the in vitro interaction between apoE and Aβ is apoE isoform specific (69, 70). The apoE2 and E3 isoforms formed stable complexes in vitro with Aβ, whereas apoE4 isoform did not interact with Aβ. Since APOE4 is in genetic disequilibrium with H2 allele of APOC1 and H2 allele is associated with increased apoCI protein levels in brains of AD patients (53), it is tempting to speculate that apoCI forms stable complexes with Aβ instead of apoE4. The role of apoE in the etiology of AD might be related to a protective role of apoE2 and E3 isoforms by complexing Aβ and inducing the clearance of Aβ from the extracellular space, limiting in that way Aβ peptide aggregation and neurotoxicity. The association of apoC1 and apoE with Aβ peptide indicate that both apolipoproteins may somehow be involved in generation and processing of plaques. Recent studies demonstrated that apolipoproteins (apoA1, apoB, apoD, apoJ) are involved in the pathogenesis of senile plaques (71-76). However, the exact role of apolipoproteins in the complex interaction of microglial and astroglial cells with Aβ plaques remains to be determined. Apolipoproteins may function to facilitate aggregation of Aβ into the fibrillar β-pleated sheet conformation, or to sequester already aggregated fibrillar Aβ for removal from the brain parenchyma. ApoD could also function to transport molecules to the site of neuronal injury in AD brain, to facilitate remyelination and neuronal regeneration (76).
APOC1 is a Liver X receptor (LXR) target gene. Interestingly, the expression of apoCI is highly induced by LXR activators in macrophages (77, 78). We recently found that administration of the synthetic LXR agonist T0901317 to C57BL6 wild type mice via the diet induced murine APOC1 expression in brain 2.7-fold (unpublished data), further supporting an important role for apoC1 in brain. LXRs belong to the nuclear hormone receptor superfamily and play a key role in the regulation of the cellular cholesterol and

FA levels by modulating target genes involved in lipid homeostasis (79) and also inflammatory processes (80). Two LXR isoforms, i.e. LXRα and LXRβ, are expressed in the central nervous system (81) and are thought to be involved in the regulation of brain cholesterol metabolism (81-83), but may also regulate inflammation processes (80, 84). Thus naturally occurring oxysterols in the brain, such as 24(S)-hydroxycholesterol may not only modulate the expression of apoE (85, 86), but also of apoCI.

Besides a potential effect on the lipid homeostasis in the brain, apoCI may affect cognitive function by its proinflammatory properties. On the one hand, activation of LXRs leads to anti-inflammatory activities and promotes macrophage survival in bacterial infection (87). On the other hand, we recently found that apoCI increases proinflammatory responses in mice and protects mice against mortality from bacterial infection by enhancing the early antibacterial attack (88). Given the fact that AD is an inflammatory disease, it may be possible that chronic elevation of apoCI levels in the brain (e.g. in subjects expressing the H2 allele) may lead to a chronic inflammatory state that ultimately leads to the development of AD. The characteristics of plaques positive for apoCI remain to be clarified. In this respect, it would be interesting to investigate the effects of chronic LXR activation of cognitive function. Yet, no effects of hapoCI on brain expression of IL6 and TNFα we found under non-stressed conditions.

In conclusion, expression of human apoCI in the mouse brain results in impaired learning and memory processes, supporting a modulatory effect of apoCI on the development of AD. In the brain, apoCI may be involved in regulating lipid metabolism and/or inflammatory processes, but the underlying molecular mechanisms remain to be clarified.

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Chapter 6

Apolipoprotein CI knock-out mice exhibit impaired learning and memory functions

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Abstract

The APOE4 allele, which is a well established genetic risk factor for the development of Alzheimer's Disease (AD), is in genetic disequilibria with the H2 allele of APOC1, giving rise to increased gene expression of apolipoprotein CI (apoCI). This has led to the hypothesis that the H2 allele of APOC1, rather than the APOE4 allele, provides a major risk factor for AD. In line with this we previously found that mice overexpressing human apoCI display impaired learning and memory functions.
Here we tested the hypothesis that the absence of apoCI expression in mice may improve learning and memory functions. In contrast with our expectations, *apoc1*$^{-/-}$ mice also showed impaired hippocampal-dependent learning and memory functions as judged from their performance in the object recognition task (P<0.001) as compared to their wild-type littermates. We didn't detect any differences in overall brain morphology or in brain levels of cholesterol, its precursors and metabolites. In addition, the absence of APOC1 affected the expression of APOE (0.75 ± 0.12, p<0.05), which could underly the impaired behavior. In line with a role for apoCI in inflammatory processes, we observed significantly increased expression of a proinflammatory marker TNFα and the oxidative stress related heme oxygenase 1 (HO-1) protein.
In conclusion, the absence of apoCI results in impaired memory functions in mice, which is supportive of an important role for apoCI in brain and in the development of AD.

Introduction

Apolipoprotein E4 (apoE4) is the strongest known risk factor for AD (1, 2). Interestingly, since APOE4 is in genetic linkage disequilibrium with the *HpaI* restriction polymorphism in the promoter region of APOC1 it was hypothesized that not apoE4, but the H2 allele of APOC1 is the risk factor for AD. The *HpaI* polymorphism (so-called H2 allele) leads to a highly significant, 1.5-fold increase in APOC1 gene transcription and was reported to be associated with AD (3-8). Moreover, the H2 allele of APOC1 was associated with poorer memory and frontal lobe function (9) and with loss of hippocampal volumes (10). In line with these observations we recently found that overexpression of human apoCI in mice results in impaired learning and memory functions (Abildayeva et al, submitted).
Pathogenesis of senile plaques in AD may involve interactions between amyloid-beta (Aβ) peptide and apolipoproteins. The level of a number of apolipoproteins are elevated in AD brains, including apoE, apoCI, apoD, apoJ (11-16) and apoE, apoD, and apoA-1 have been detected in plaques. (17-20). Recently we have found that also apoCI co-localizes with Aβ and with apoE in plaques in the CA1 region of hippocampus of AD brain. Moreover, we obtained *in vitro* evidence indicating a direct interaction of apoCI with Aβ peptide (Abildayeva et al, submitted). The physiological roles of apoCI in brain have not been established in detail.

A gene dose-dependent effect on circulating levels of triglycerides (TG), free fatty acids (FFA) and total cholesterol (TC) has been reported (21-23). ApoCI may modulate plasma lipid levels by interference with apoE-dependent clearance of TG-rich lipoproteins by the liver (24-26) and by modulating the activity of enzymes involved in plasma lipid metabolism, including lecithin:cholesterol acyl transferase (LCAT) (27), lipoprotein lipase (LPL) (28, 29), hepatic lipase (HL) (30, 31) and cholesteryl ester transfer protein (CETP) (32, 33).
The presence of apoCI within the brain at relatively high levels (34) suggests an important role for this protein in brain lipid metabolism and/or inflammatory responses. In the present study we have investigated the effect of apoCI deficiency on learning and memory functions, brain sterol profiles and gene expression. Our data indicate, in contrast with our expectations, that apoCI-knockout mice (*apoc1*$^{-/-}$) display impaired memory functions in comparison with wild-type littermates**,** establishing an important role for this apolipoprotein in normal brain functioning.

Results

Absence of apoCI expression does not affect overall behavioral appearance.

We used *apoc1*$^{-/-}$ mice as an experimental model to investigate if the absence of apoCI would affect learning and memory functions. The *apoc1*$^{-/-}$ mice appeared healthy and there were no gross differences between the *apoc1*$^{-/-}$ mice and their wild-type littermates with respect to overall appearance, behavior in cage, handling response, or body weights. The weight of the mice was 25.7±1.4 g for the *apoc1*$^{-/-}$ mice and 24.0±1.9 g for their wild-type littermates, prior to the beginning of the behavioral experiments at the age of 12 months.

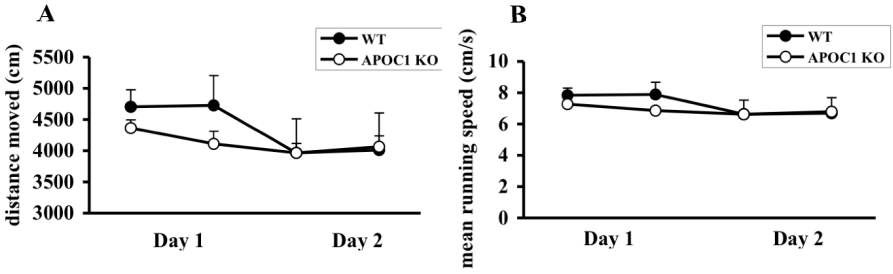

Figure 1. Open field test. Animals were allowed to explore an open square for 20 minutes. Time spent in the different zones (TIZ) and the distance moved were recorded. **A.** Distance moved in 20 minutes. **B.** Mean running speed. Distance moved nor running speed does not differ significantly between the apoc1$^{-/-}$ mice and the wild types.

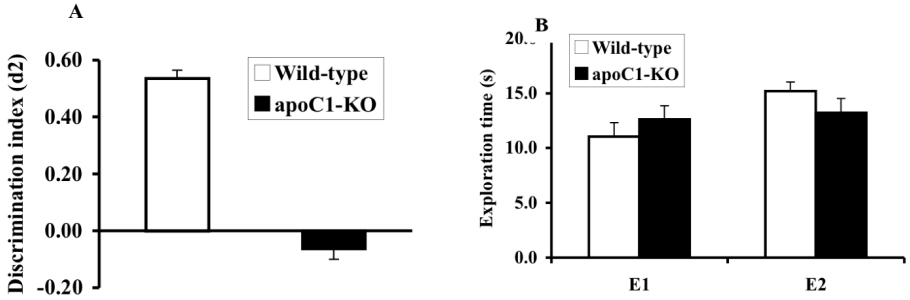

Figure 2. The absence of APOC1 expression impairs cognitive performance in the object recognition task. One hour after a first trial using two identical objects, mice were subjected to a second trial with dissimilar objects. Total exploration time **(A)** was similar between apoc1$^{-/-}$ and wild type mice, where E1- total exploration time to both objects in the first trial, E2- total exploration time to both objects in the second trial. **(B)** In the second trial, the discrimination index was determined from the time spent exploring the new object relative to the familiar object. Data represent mean ± SEM. *p=0.012

Table I

	Wild type (n=14)	***apoc1*$^{-/-}$ (n=12)**
Cholesterol (µg/mg)	72.5±2.3	75.2±4.2
Lathosterol (ng/mg)	90.9±7.0	93.6±11.4
Lanosterol (ng/mg)	5.4±0.4	5.7±0.7
Desmosterol (ng/mg)	169.6±15.1	172.9±8.4
24(S)-OH-chol (ng/mg)	198.2±13.5	191.0±8.9
27(OH)-chol (ng/mg)	2.0±0.7	2.2±0.7
Cholestanol (ng/mg)	230.2±25.7	257.3±35.1
Campesterol (ng/mg)	105.2±16.7	105.9±14.5
Sitosterol (ng/mg)	22.5±2.2	22.4±2.2

The absence of apoC1 has no effect on brain levels of cholesterol and plant sterols (campesterol and sitosterol). Values are presented as means ± SD. Cholesterol is expressed in µg/mg and its precursors and metabilites are expressed in ng/mg brain (dry weight). Significant differences ($p<0.05$) between the two groups of mice are indicated by asterisks.

No difference in locomotor activity and exploratory behaviour between apoc1$^{-/-}$ knockout and wild type mice in the open field test.

There were no differences between *apoc1*$^{-/-}$ and wild type mice in distance moved (1A) nor in running speed (1B) (Fig.1), indicating that the absence of apoCI does not significantly affect locomotor activity and exploratory behaviour.

Apoc1$^{-/-}$*knockout mice display impaired memory functions in the object recognition task.*

Apoc1$^{-/-}$ mice and wild-type littermates were subjected to the object recognition task (ORT). One hour after a first trial using two identical objects, the wild-type mice were

able to discriminate between the familiar and the new object (Fig. 2A). In contrast, the *apoc1*$^{-/-}$ mice failed to discriminate between both objects (*apoc1*$^{-/-}$ versus wild-type, $p<0.001$). The exploration activity was similar in both groups (Fig. 2B).
This indicates that the absence of apoCI expression in mice significantly impairs object memory.

Table II

	Ct	Wild-type (n=8)	*apoc1*$^{-/-}$(n=8)
ABCA1	26.5	1.00 ± 0.16	1.15 ± 0.16
ABCG1	23.7	1.00 ± 0.15	1.10 ± 0.17
ABCG4	24.2	1.00 ± 0.17	1.10 ± 0.24
ApoD	21.4	1.00 ± 0.20	1.06 ± 0.09
ApoE	19.9	1.00 ± 0.16	0.75 ± 0.12 *
LPL	27.2	1.00 ± 0.13	1.18 ± 0.20
HO1	29.2	1.00 ± 0.16	1.24 ± 0.21 *
IL6	33.3	1.00 ± 0.42	1.31 ± 0.55
TNFα	32.5	1.00 ± 0.24	1.74 ± 0.62 *

Quantitative real-time PCR was performed as described in Methods; n=8 per group. All data were standardized for 18S ribosomal RNA. Expression in control mice was set to 1.00. Values represent means ±SD. * indicates significant difference (Mann-Whitney-U-test, $p < 0.05$)

The absence of apoCI does not affect brain levels of cholesterol, its precursors and metabolites

Alterations in brain lipid metabolism may affect cognitive functions (35-38). In order to investigate the mechanism(s) that may underlie the observed effects of the absence of mouse apoC1 on cognitive function, we determined brain sterol profiles, since apoCI is known to regulate several enzymes involved in lipid metabolism, which are also present in brain (29, 39, 40). As shown in Table I, no differences were detected in levels of cholesterol, its precursors (desmosterol, lathosterol and lanosterol) and its metabolites (24(S)-hydroxycholesterol, 27-hydroxycholesterol, cholestanol) in brains of *apoc1*$^{-/-}$ and wild type mice. Neither was any difference in the cholesterol:cholesterylester ratio indicating that there is no difference in the rate of cholesterolesterification.

The absence of APOCI affects expression of genes involved in cholesterol metabolism, cellular stress and inflammation.

Since APOC1 is encoded by the same gene cluster as APOE, and apoE and apoCI levels are tightly balanced (34, 41), we determined the APOE mRNA levels in the brains of *APOC1*$^{-/-}$ and wild-type mice by quantitative real-time PCR (QRT-PCR). APOE mRNA levels in the brains of *APOC1*$^{-/-}$ mice were significantly decreased in comparison with wild-type mice (Table II).
Further, because apoCI afects the activity of several enzymes involved in lipid metabolism, we analyzed gene expression profiles of genes involved in fatty acids

metabolism: LPL, APOD; transporter-encoding genes: ABCA1, ABCG1 and ABCG4. No differences were detected between two groups. Earlier it has been hypothesized that apoCI is a physiological protector against infection by enhancing the early inflammatory response (42), moreover, the treatment with LPS elicited an increase in HO-1 mRNA in cultured glial cells (43), therefore we analyzed gene expression profiles of genes involved in inflammation (tumour necrosis factor-alpha (TNFα), interleukin 6 (IL6) and in cellular stress (heme oxygenase-1 (HO-1). Indeed, we found significant differences in expression of HO-1 and TNFα, IL6 (Table II).

Discussion

We recently found that the moderate overexpression of human apoCI leads to impaired learning and memory functions in mice. Therefore, we hypothesized that apoCI-deficiency in mice may have the opposite effect and may enhance these functions. However, surprisingly, this work shows that the absence of apoCI also impairs memory functions in mice. These results could not be explained by differences in brain sterol profiles, but may be partly explained by reduced expression of APOE.

The *apoc1*$^{-/-}$ mice displayed impaired hippocampal-dependent memory functions as indicated by their performance in object recognition task. These results were unexpected since transgenic mice that overexpress human apoCI showed a comparable behavioural pattern: impaired performance in the object recognition task. Nevertheless, other studies have shown similar behaviour in apoE-knockout and transgenic mice expressing human apoE. Spatial memory of apoE-knockout mice was severely impaired (44) as well as the spatial memory of mice carrying human apoE4 isoform (45, 46). Moreover, mice that are either deficient for PPARα or that overexpress it in muscle are protected against high-fat diet induced insulin resistance (47, 48), showing that overexpression and deficiency of a specific gene can have comparable effects.

Growing evidence suggests that cellular cholesterol homeostasis is causally involved in different steps leading to pathological events in the brain of AD patients indicating that a well-regulated brain lipid metabolism is necessary for normal brain functioning (49-53). Although the absence of apoC1 results in reduced circulating cholesterol levels and in slightly reduced TG levels in the circulation of 12 weeks old mice (Rensen et al, unpublished data) in the brain of *apoc1*$^{-/-}$ knockout mice, the apoC1 deficiency did not affect overall levels of cholesterol, its precursors or metabolites. Neither was there any difference in the percentages of free and esterified cholesterol. This suggests that apoC1 does not affect memory functions via a modulatory effect on brain cholesterol metabolism. In brain apoC1 is present predominantly in astrocytes and endothelial cells of blood brain barrier (Abildayeva et al, unpublished data), while in the circulation it is in dynamic equilibrium with HDL, VLDL and chylomicron particles (54, 55). ApoC1 activates the plasma enzyme LCAT (56), which converts discoidal HDL particles to spherical particles (27, 57). Paradoxically, both over-expression (22) and absence (58, 59) of apoCI in mice reduces VLDL clearance. (25, 26). These studies highlight the need for a more detailed understanding of the functional properties of apoC1in brain.

Since it has previously been reported that apoE-knockout mice show impaired learning and memory functions (44, 60-62), we examined if knocking out the APOC1 gene

affects apoE expression. Indeed, we demonstrated significant decrease of APOE mRNA levels in the brains of *apoc1*$^{-/-}$ knockout mice; therefore the effect of the absence of apoC1 expression on memory functions may be, to a certain extend, explained by reduced expression of mouse apoE in the brain.
Previous studies demonstrated that the levels of apoD mRNA in the brains of apoE-deficient mice are 50-fold those of wild type control mice (14). In the absence of apoE, apoD mRNA and protein levels are upregulated suggesting compensatory role for apoD (Jansen et al, unpublished data). However, we didn't detect differences in APOD expression between two groups of mice.
ApoCI is involved in proinflammatory responses in mice and protects mice against mortality from bacterial infection by enhancing the early antibacterial attack (42). Our study showed that the expression of TNFα was significantly elevated ($p<0.05$) in the brains of *apoc1*$^{-/-}$ knockout mice. Moreover, we found that HO-1 mRNA levels were also significantly increased in the brains of knockout animals. It has been shown that in primarily cultured rat glial cells the treatment with LPS elicited an increase in HO-1 mRNA (43). Thus, our data suggests that apoC1 is actively involved in the inflammatory processes.
In conclusion, our findings underscore the hypothesis that APOC1 plays an important role in brain, however the molecular and cellular mechanisms underlying the learning and memory deficits observed in *apoc1*$^{-/-}$ mice remain to be fully elucidated.

Methods

Animals.

apoc1-/- knockout mice were generated as described previously (22, 58). Female apoc1-/- knockout mice (n=12) and their wild-type littermates (n=14) were bred at TNO-PG in Leiden, The Netherlands. (59). The apoc1-/- knockout mice and wild-type mice that participated in this study were littermates with a highly homogeneous genetic background, obtained by backcrossing at least 10 times to the C57Bl/6 background. Mice were housed under standard conditions in conventional cages and given food (standard chow diet) and water ad libitum; lights on from 07.00 to 19.00 h in a temperature and humidity controlled room. The ethical board of the Maastricht University approved of all experimental procedures.

Behavioral testing.

Mice were subjected to behavioral tests when they were 12 months old. Testing took place between 9.00 and 15.00 h. The experimenters were unaware of the genetic background of the mice. There were no differences in the results of the mice tested early or late on the days of the experiment.

Open field test.

Open-field behavioral assays are commonly used to test both locomotor and exploratory activities in rodents.

We used a 100 x 100-cm arena with 45-cm-high walls as described previously (63). A video camera was placed straight above the arena to record trials, and the tracking software noted the location of the mouse every 0.055 s for 20 min. We defined a minimum displacement of 2.5 cm to constitute a change in location, a minimum angle of 30° to constitute a turn, and a 3-cm-wide wall zone. Trials were 3 min in duration and were performed in a dimly lit room (5 lux on the floor of the testing arena) to optimize digitization. At the start of each trial, a mouse was grasped by the tail and placed in the center of the arena. The arena floor was wiped with a moist sponge (water only) between trials and allowed to dry before the next trial, as is standard protocol in open-field testing. Each mouse was weighed after its first trial. In addition to the computer-recorded variables, we recorded the number of fecal boli and urine pools deposited on the arena floor.

Object recognition task.

The object recognition task measures whether a mouse remembers an object it has explored in a previous learning trial. The task was performed in a circular arena with a diameter of 49 cm, as described previously (64, 65). Half of the 40 cm high wall was made of grey polyvinyl chloride, the other half of transparent polyvinyl chloride. The light intensity (20 lux) was equal in the different parts of the arena. Two objects were placed in a symmetrical position about 15 cm away from the grey wall. We used four different sets of objects. The different objects were: (1) a standard transparent glass bottle (diameter 2.5 cm, height 10 cm) filled with sand; (2) a truncated cone (diameter 6 cm, total height 3.5 cm); (3) a massive metal parallelepiped (7.5×5.0×2.5 cm) with two holes (diameter 1.5 cm), and (4) a massive aluminium cube with a tapering top (4.5×4.5×8.5 cm). Mice could not displace the objects. In the first week, the animals were handled daily and were adapted to the procedure in 2 days, i.e., they were allowed to explore the testing site (without any objects) twice for 3 min each day. A testing session comprised two trials of 3 min each. During the first trial (T1), the testing site contained two identical objects (samples). A mouse was always placed in the arena facing the wall in the centre of the transparent front segment. After the first exploration period, the mouse was put back in its home cage. Subsequently, after a delay interval of 1 hour, the mouse was put back in the arena for the second trial (T2), but now with two dissimilar objects, a familiar one (the sample) and a new one. The times spent exploring each object during T1 and T2 were recorded manually with a personal computer. The discrimination index (d2) represents the relative measure of discrimination between the new object (b) and the familiar object (a), which corrects for the absolute discrimination (b-a) for the total exploration activity. The animals recognize the familiar object if the discrimination index is higher than zero. Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behaviour. In order to avoid the presence of olfactory trails, the objects were always thoroughly cleaned. Moreover, each object was available in triplicate so none of the two objects from the first trial had to be used as the familiar object in the second trial. In addition, all combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects.

Sterol profile determination.

The mice were anaesthetised with Nembutal (Ceva Sante Animale BV, Maassluis, The Netherlands) (180 μg/g body weight) and were perfused with phosphate buffered saline (PBS) (66). Blood was collected before perfusion and spun at 1400 rpm to obtain serum, which was snap frozen in liquid nitrogen. The brains of all mice were placed in 2-methylbutane solution (Sigma-Aldrich, Stainheim, Germany) and frozen in liquid nitrogen and stored at –80 °C for subsequent analysis. Prior to sterol analysis, brains were spun in a speed vacuum dryer (12 mbar) (Savant AES 1000) for 48 hours to relate individual sterol concentrations to dry-weight. The sterols were extracted from the dried tissue by placing them in a 5 ml mixture of chloroform-methanol (2:1) for 48 hours. One ml of the sterol extract of the brains, in combination with internal standards, was evaporated to dryness under a stream of nitrogen at 63ºC, and hydrolyzed for 1 hour with 1 ml of 1 M NaOH in 90% ethanol at 50ºC. One ml of distilled water was added to the samples. To extract the neutral sterols 3 ml of cyclohexane was added twice. The combined cyclohexane phases were again evaporated to dryness under a stream of nitrogen at 63ºC, and the sterols were dissolved in 100μl n-decane. After transfer to gas-chromatography (GC)-vials, the sterols were converted to trimethylsilylethers (TMSis) by adding 40 μl of TMSi reagent (pyridine-hexamethyldisilazan-trimethylchlorosilane, 9:3:1 v/v/v) and incubated at 60ºC for one hour (67). The sterols were extracted from 100μl serum in the same manner. Levels of cholesterol were determined in a gas-chromatograph-flame ionization detector (GC-FID) with 5α-cholestane as internal standard. Levels of plant sterols (campesterol, sitosterol), cholesterol precursors (lanosterol, lathosterol and desmosterol) and cholesterol metabolites (24S-OH-cholesterol and cholestanol) were determined using gas chromatography-mass spectrometry (GC-MS) as described previously (67) using epicoprostanol as internal standard.

RNA isolation and Real-time quantitative PCR (QRT-PCR) procedures:

Total RNA was isolated using the Trizol method (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Integrity of RNA was checked by agarose gel electrophoresis, and RNA concentration was measured spectrophotometrically (NanoDrop, Witec AG, Littau, Germany). Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector with 1.6.3 software (Perk-Elmer Corp., Foster City, CA, USA) as previously described (68) with modifications (69). Primer sequences are available upon request. Primers were obtained from Invitrogen (Breda, The Netherlands). Fluorogenic probes, labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), were made by Eurogentec (Seraing, Belgium).

Statistics.

Group differences in behavioral tests were analyzed using a one or two factorial ANOVA (Group or Group and Day, respectively). Group differences in sterol/ phospholipids analysis and body weight were evaluated using a *t* test. Statistical

significance in QRT-PCR experiments was determined by comparing means using an unpaired Student's *t*-test and Mann-Whitney U-test.

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Chapter 7

ApolipoproteinE, the blood-brain barrier and Alzheimer's disease

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Abstract

Vascular aspects in the development of Alzheimer's disease (AD) have recently received increased attention. At present, cardiovascular afflictions, hypertension and diabetes constitute accepted risk factors for vascular dementia (VaD) but these have gained importance for AD. Another well established susceptibility factor with regard to AD in particular is the e4 allele of the apolipoprotein E (APOE) gene, which encodes one of three common isoforms of apolipoprotein E (apoE) in humans. The presence of APOE e4 is also associated with an augmented probability rate for cardiovascular disorders, hypertension and diabetes. Combined with vascular disease, APOE e4 further increases the risk of AD (Table 3). The APOE genotype may, therefore, be a potential element that modifies the vascular function in the periphery as well as in the brain during ageing, possibly amplifying the responsiveness to pathogenic processes in AD. This infers a strong role for high-density lipoproteins (HDL) in the pathogenic processes of AD. In this context other apolipoproteins including A-I and C-I, and the HDL-binding proteins that are also expressed by cells, which form the blood-brain barrier (BBB) may be instigated in the pathogenesis.

I. Cerebrovascular aspects in AD

Ia. Vascular risk and AD

AD is the most common cause of dementia. It is characterized by a progressive loss of higher cognitive functions. The strongest risk factor for sporadic AD is old age, which also coincides with the increased occurrence of cerebrovascular lesions (Table 1). Additional risk factors associated with AD vascular alterations include some forms of heart disease, atherosclerosis, high plasma cholesterol, increased fat intake, history of hypertension, diabetes mellitus, head injury, stroke, systemic inflammation and the APOE e4 (1,2). The APOE genotype is the prime susceptibility factor for sporadic AD (3), that has been linked to an increased risk and reduced age of onset of AD. So too, it has been associated with VaD, mild cognitive impairment (MCI) (4), impaired recovery after brain trauma, amyotrophic lateral sclerosis and Pick's disease and, more recently, also with the risk and age of onset of Parkinson's disease (5). ApoE4 was found to interfere with cognitive functions in non-diseased adult men carrying apoE4 in comparison with non-carriers (6,7). Memory deteriorates in apoE4 carriers before the symptomatic occurrence of MCI, prior to age 60 (8). It remains to be clarified how apoE4 affects cognitive functioning and neurodegenerative processes. Nevertheless, the APOE genotype may be a leading aspect in the modification process of cerebrovascular functions during ageing and the increase of the predisposition to the pathogenesis of AD (9).

Table 1

Emerging Common Risks for Alzheimer's disease and Vascular dementia
Age
Family history of dementia
Transient ischaemic attacks (TIA)
Strokes
Atherosclerosis and coronary heart disease
Increased markers if peripheral vascular disease- homocysteine, cholesterol
Deregulation of blood pressure- hypertension or hypotension
Diabetes type II
Smoking
Presence of Apolipoprotein E- ε4 allele

The odds ratios for the various factors have been determined to be 1.7- 17.0 (summarized from several references, see (9,1,43).

Ib. Cerebrovascular changes in AD

Neuropathological hallmarks comprise extracellular deposits of amyloid-b in form of amyloid plaques and cerebral amyloid angiopathy (CAA) and intracellular accumulation of hyperphosphorylated tau, which characterises neurofibrillary tangles. Other features include substantial loss of synapses, a decline in cholinergic transmission, increased markers of oxidative stress and low grade inflammatory responses (10). However, the patients with Alzheimer's lesions may also bear evidence of cerebrovascular pathology. While these cases may be diagnosed as mixed dementia or VaD if they predominantly exhibit CAA it is increasingly evident that similarities in pathological, symptomatic and neurochemical features as well as cholinergic deficits between AD and VaD exist (11). Indeed, more than 30% of AD cases may exhibit cerebrovascular pathology, which involves the cellular entities that represent the BBB. Profound morphological and biochemical changes of the microvasculature have been observed in brains of late-onset AD subjects. These entail degenerative changes in endothelial and vascular smooth muscle cells, degrees of macro and micro-infarction and white matter rarefaction related to small vessel disease (Table 2). CAA involves the degeneration of the larger perforating arterial vessels as well as the cerebral capillaries as seat of the BBB. CAA is also associated with an increased susceptibility to intracerebral haemorrhages. Moreover, changes were observed in the glucose transporters, Na+/K+ ATPase, adhesion molecules such as the intercellular adhesion molecule-1, collagen components, perlecans, carnitine acetyltransferase, a-actin and amyloid-b of the cortical microvasculature in late-onset AD patients. Moderate changes in other components associated with BBB function including alkaline phosphatase, g-glutamyl transpeptidase, acetylcholinesterase and butrylcholinesterase have also been identified. While the BBB abnormalities may be induced by pathological changes within the brain parenchyma the BBB appears particularly vulnerable in AD patients who exhibit peripheral vascular abnormalities attributed to cardiovascular disease, hypertension and diabetes (1). Thus cerebrovascular incongruities underscore the role of the BBB in the

pathogenesis of AD. These vascular anomalies may relate to the long-term peripheral influences associated with cardiovascular disease or peripheral vascular disease.

Table 2

Vascular lesions and small vessel disease in Alzheimer type of dementia
Presence of Aβ-CAA and CAA-related cerebral haemorrhages
Degeneration and intracellular changes, e.g. mitochondria, tight junctions, in the cerebral endothelium
Basement lamina thickening and collagen accumulation
Small vessel disease including hyalinosis, fibroid necrosis and perivascular changes
Localisation of inflammatory mediators and cell adhesion molecules
Ischaemic white matter lesions
Microinfarcts and lacunes
Presence of lobar and intracerebral haemorrhages

This summary is supported by data from several studies (9,11,133,216,1).

Ic. The Blood-Brain Barrier in health and AD

The cerebral capillary endothelium is the anatomical equivalent of the BBB, isolating the brain neuropil from the systemic circulation. The cerebral endothelium lining the blood vessel lumen consists of a single layer of cells joined together by tight intercellular junctions. This layer of cells is supported by a tough basement membrane, which is the laminar structure formed by the fusion of the endothelial and glial vascular basement membrane (VBM). The end feet of astrocytes make up a discontinuous sheath at the abluminal surface of the VBM. Pericytes, likely of macrophage lineage, wrap around endothelial cells and are embedded in the VBM. They play an essential role in the structural stability of the vessel wall (12). The brain capillary endothelium embodies a crucial element in the supply of oxygen, glucose and other vital nutrients that are instrumental in maintaining a stable internal milieu, and it is important for the catabolic outflow of CNS waste products (13,14). Disturbances in these processes may result in chronic restrictions in capillary blood flow or disrupt the BBB to contribute to the progression of AD (15). This is supported by the facts that microvascular changes are located in the densely vascularized layers of the brain that correspond to areas with a high metabolic rate and increased density of Alzheimer lesions such as the hippocampus and temporoparietal areas (16-18).

II. Apolipoprotein E (ApoE) and AD

IIa. ApoE: gene and protein

The APOE gene is a member of the apolipoprotein gene family, which comprises several genes that regulate functions related to lipoprotein metabolism. The gene is located at chromosome 19q13.2 and is closely linked to the APO C-I/C-II gene

complex. The DNA sequence consists of four exons and three introns spanning 3597 nucleotides. The transcripts of the three common alleles (1156 bp) exist as three isoforms, E2, E3 and E4 each with 299 residues. ApoE2 differs from E3 by a single cysteine substituted for an arginine at position 158. E4 differs from E3 by an arginine substitution for a cysteine residue at position 112 (19). An overview of the putative transcription factor sites in the APOE promoter was recently provided by Lahiri et al. (20). The e4 allele of the APOE gene is considered to be the most important genetic factor in non-familial AD. The allele has moderate specificity for AD with estimates ranging from 0.75 to 0.81. However, APOE status is a strong predictor of outcome once the patients have been diagnosed with memory impairment but the correlation is not perfect. The mechanisms underlying the effect of this allele in AD and CAA pathogenesis (22) are being intensively investigated but is far from clear. However, both in vivo and in vitro evidence suggest the interaction between ApoE and Ab causes peptide conformation conversion and increased cellular toxicity that also pertains to the cerebral vasculature (21-23).

ApoE is a key player in the distribution of cholesterol throughout the body. The plasma concentration ranges 40-60 mg/L and the protein is associated with several classes of lipoproteins; chylomicrons, very low-, intermediate- and high-density lipoproteins (VLDL, IDL and HDL). ApoE mediates their interaction with cellular receptors, including the low density lipoprotein (LDL) and the VLDL receptors, and the LDL receptor-related protein (LRP) (24-29). Its absence induces increased cholesterol in the circulation and atherosclerosis in humans and in mice (30). In addition to its role in lipid transport, apoE appears involved in a number of processes, including the deposition and clearance of amyloid-b, the aggregation of tau, inflammatory processes, removal of cellular debris, neuroendocrine- and oxidative functions, signal transduction and even apoptosis (31).

IIb. ApoE functions, cholesterol and the brain

ApoE is produced by nearly all cell types in the body (32). Apart from the liver, the brain is the largest source of apoE. ApoE is predominantly synthesized by astrocytes but there is evidence to suggest that microglia, endothelial cells and pericytes in all regions of the brain may also produce it (27). It is thought that under normal conditions neurons do not synthesize apoE. However, recent reports suggest cortical and hippocampal neurons express apoE, which may be taken up (see below) under diverse physiological and pathological conditions (33,34). In normal adult subjects the cerebrospinal fluid (CSF) concentration is about 10 mg/L with CSF-serum ratio of 1:5.

ApoE within the brain is separated from the extracerebral pool by the BBB. This is corroborated by the observation that subsequent to liver transplantation with a different APOE genotype, patients exhibit a change in apoE isoform in the circulation but not in the CSF (35). This suggests that all apoE in the brain is synthesized locally and is not necessarily derived from the circulation. Conversely, there is no empirical evidence to suggest that apoE originating in the brain ends up in the circulation. Within the brain, apoE plays a major part in the re-distribution of cholesterol, and possibly phospholipids, during regenerative processes after brain injury and in synaptic plasticity (24). An

altered cholesterol metabolism may be central in the pathogenesis of AD (36). Alterations in cholesterol metabolism affect the production of amyloid-b, which is thought to have a significant influence in the pathophysiology of AD. The activity of the enzymes responsible for the cleavage of amyloid, b- and g-secretases, which reside in cholesterol-rich lipid domains within the cell membrane, is sensitive to membrane cholesterol content (37).

The brain contains almost 23% of all free unesterified cholesterol retained within the body, while it only represents 2% of all body mass. Cholesterol is synthesized locally with minimal if any derivation from the circulation (38). Brain lipids are primarily present in cell membranes where they are, contrary to initial thoughts constantly being replaced. In man the daily turnover of brain cholesterol is estimated to be in the order of 6 mg. This corresponds to nearly 1% of the turnover in the rest of the body. Since cholesterol cannot be degraded compared to other lipids the excess is secreted (27). About 40% of the cholesterol is secreted in the form of 24S-hydroxycholesterol (39,40) and the rest 60% through another yet undefined mechanism (41,42). ApoE may also modulate this process (43). The regulation of the cholesterol flux is important as excess cholesterol can form solid crystals, which are toxic as is the formation of oxysterols.

To date there is meagre information on cholesterol metabolism in the brain, especially during ageing and progression of AD. However, alterations or defects in cholesterol metabolism or trafficking underlie several severe neurological disorders including Niemann-Pick C1 (44), Smith-Lemli-Opitz syndrome (45) and Cerebrotendinous Xantomatosis (46). Surprisingly, a viable transgenic cholesterol-free mouse was generated only recently indicating lack of cholesterol is not necessarily fatal (47). Increased levels of 24S-hydroxycholesterol as one of the main metabolites were reported in plasma and in CSF of patients with AD and VaD (48-50). However, the brain concentrations of 24S-hydroxycholesterol were reduced in AD compared to normal controls. Interestingly, patients with a defective BBB display markedly increased up to 10-fold absolute levels of 24S-hydroxycholesterol (51). Furthermore, a polymorphism in the cholesterol 24S-hydroxylase (CYP46) gene was reported to be associated with AD. CYP46 is the enzyme that catalyzes the conversion of cholesterol into 24S-hydroxycholesterol, and is largely expressed in neurons (50,52,53). Clinical evidence suggests that high plasma cholesterol levels and/or a high fat intake are associated with an increased risk of AD. However, contradictory results have been reported in studies relating to cholesterol, HDL-cholesterol and LDL-cholesterol levels in AD (54,55) that may be explained by changes during disease progression. It is unclear if changes in plasma cholesterol and other lipids have any direct link in the modulation of brain cholesterol metabolism (56).

Astrocytes provide both structural and metabolic support to neurons. They supply neurons with cholesterol to form new membranes. Astrocytes secrete small, primarily discoid HDL-like particles that contain a little core lipid, with both apoE and apolipoprotein J (apoJ) on their surface (57). The lipoprotein particles differ from those detected in the CSF by their larger size and their content of apolipoprotein A (apoA-I) (58). These HDL-like lipoproteins secreted by astrocytes interact with apoE-binding receptors, such as the LDL receptor, LRP (59) or the heparan sulphate proteoglycans (HSPG) or perlecans (60,61). Similar to that in macrophages, the secretion of HDL-like particles by astrocytes are affected by the APOE genotype (62). Moreover, apoE

expression and secretion by astrocytes was found to be facilitated by the liver X receptor and retinoid X receptor heterodimer (63). Experiments in apoE4 and apoE3 knock-in mice have revealed that apoE3 generates similarly sized lipid particles with fewer numbers of molecules than in apoE4 mice (62). If the secretion of cholesterol and apoE from glial cells is prevented, the growth stimulating effect of a glial cell-conditioned medium on axon (neuritic) extension is also prevented (64). The introduction of glia-derived lipoproteins containing apoE to distal axons, but not to cell bodies, enhances the axonal outgrowth. Interestingly, ApoE enhances the outgrowth of neurites in an isoform-specific manner in the presence of a source of lipids (60,61,65,66). ApoE3 strengthens neurite growth where as it is inhibited by apoE4. Since the LDL receptor may be involved in the uptake of the apoE-containing lipoproteins, the divergent interaction of apoE4 and apoE3 with the LDL receptor may explain these disparities. Alternatively, a variation in the interaction with HSPGs may be involved (67).

It has been demonstrated that apoE can be re-secreted from cells after being endocytosed (68) and the recycling of apoE is accompanied by a cholesterol efflux. In hepatocytes remarkably a significant amount of apoE4 was found to be retained within hepatocytes after endocytosis while apoE3 is re-secreted (Heeren J, personal communication). This may also apply to neurons. The increased levels of apoE in neurons after CNS injury or pathological lesions may therefore be due to increased uptake of apoE, amplified retention or augmented synthesis.

ApoE levels in the brain are upregulated as a consequence of almost any type of CNS injury. It is also increased in a number of degenerative diseases including amyotrophic lateral sclerosis, schizophrenia, Niemann-Pick (69-72). Recently, Harris et al (73) provided evidence suggesting that certain factors secreted by astrocytes regulate apoE expression in neuronal cells. It is known that specific alterations in the periphery can also modulate apoE levels in the brain. High fat diet can lead to upregulation of apoE levels (30) whereas chronic changes in total plasma cholesterol concentrations as a result of dietary or pharmacological intervention may alter apoE mRNA levels in the brain (74). Secretion of apoE by astrocytes is regulated in a downward fashion by statins, which inhibit hydroxymethylglutaryl-conenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the cholesterol-synthesis pathway (75), but the cholesterol-lowering drug probucol boosts apoE production in the hippocampus (76). Epidemiological studies indicate that statins reduce the risk of AD (77) and the amyloid burden in the brains of transgenic mice over expressing the amyloid precursor gene to model AD (78). This may be achieved by possibly reducing the levels of membrane cholesterol (78). Statins not only decrease plasma cholesterol levels but they also influence cholesterol and apoE levels in the brain albeit in guinea pigs. On the other hand, statins did not affect cholesterol levels in the brain of apoE-knockout mice, suggesting that the therapeutic effect of simvastatin depends on the presence of a functional apoE (79).

IIc. ApoE, HSPGs and Alzheimer pathology

ApoE is variably localised in amyloid deposits characteristic of both cerebral and systemic amyloidosis (80). In AD, apoE immunoreactivity has also been detected in

cerebrovascular amyloid-b deposits and neurofibrillary pathology (81-83). ApoE is a common constituent of amyloid fibrils and may facilitate different types of amyloidosis by reducing the solubility of amyloid and stabilizing the b-pleated structure (84-86). It may therefore also affect the clearance of amyloid.
In the absence of other specific protein interactions with amyloid-b, ApoE and dimeric soluble amyloid-b complexes have been observed in supernatants of brain tissue from AD patients (87). Binding studies suggest apoE binds avidly to amyloid-b peptides and enhances the formation of amyloid-b fibrils (84-86,88-90). ApoE3 binds amyloid-b with greater affinity than apoE4, which may have consequences for both its deposition and clearance. Whereas there is a strong association between the APOE e4 allele and CAA, it is not clear if its product affects the extent of CAA, atherosclerosis or myelin loss. However, its effect on the distribution within the occipital cortex was more severely affected by all of these pathological changes (91). It is plausible that apoE may indirectly influence amyloid-b fibril formation or deposition through a modulatory effect on cerebrovascular functions or via brain cholesterol metabolism.
The HSPG or perlecans have been suggested to play a central role in the deposition of amyloid-b by promoting fibrillogenesis (92). They are a major component of the VBM of the cerebral vessels. HSPGs, similar to apoE, are usually detected in amyloid deposits of any type of amyloidosis. They can bind both apoE and amyloid and are known to be involved in the clearance of lipoproteins by the liver. They may also play a role in the apoE-mediated effect on neurite outgrowth. Thus like the other lipid receptors do HSPGs influence the mobilisation of apoE and amyloid?

IId. APOE e4 as a vascular risk factor for AD?

The hypothesis that the modifying effect of APOE e4 on the vascular system is related to the increased risk of AD is supported by the observation that other vascular factors linked to atherosclerosis increase the risk of AD. These include hypertension, diabetes and stroke (93,94,1). All of these are potential modifiers of the vasculature. It is thought that atherosclerosis in extracranial vessels causes sustained hypoperfusion in the brain and may thereby indirectly contribute to the progression of AD (1). Patients with diabetes and hypertension may exhibit reduced blood flow to the brain (95). In support of this, an increased prevalence of cerebral senile plaques in the brains of cognitively intact individuals with hypertension or critical coronary artery disease in comparison with age-matched controls without heart disease was observed (96). Epidemiological evidence indicates that atherosclerosis may also represent a risk for CAA (97).
APOE e4 is currently viewed as a compounding factor in the development of cardiovascular disease because of its association with increased plasma cholesterol levels and with atherosclerosis (30), which may also attract amyloid-b itself within the atherosclerotic plaques. Moreover, hypertension results more often in white matter lesions in APOE e4 carriers than in non-carriers (98). This implicates an interaction between APOE e4 and atherosclerosis in the aetiology of AD. Thus, APOE e4 may affect AD through a modulatory effect on the vascular wall. ApoE4 combined with hypertension, atherosclerosis, peripheral vascular changes or diabetes presumably further increases the risk for cognitive dysfunction. Alternatively, similar mechanisms may underlie the increased susceptibility of individuals with the APOE4 genotype to

vascular diseases and to AD, or APOE e4 allele or apoE4 may modulate the pathogenesis of AD through more than one pathway.
The role of APOE in cerebrovascular disease, which may exhibit CAA but not other AD lesions, however, is not clear (99). A meta analysis revealed significantly higher APOE e4 allele frequencies with more than six-fold greater risk in patients diagnosed with ischaemic cerebrovascular disease compared to age and gender-matched controls. These findings suggest a role for APOE genotype in the pathogenesis of cerebrovascular disease (100). Frisoni et al (101) had previously implicated comparably high APOE- e4 allele frequencies in cerebrovascular disease associated with dementia but subsequent clinical reports have not confirmed this finding. Indeed, pathologically confirmed studies showed that e4 allele frequencies did not differ between Binswanger's disease and other forms of vascular dementia (102). However, the APOE e 4-allele frequency may increase the risk of dementia in stroke-survivors and that e 4 homozygotes exhibit extensive hypoperfusion related to lesions in the deep white matter than those with other genotypes (103). The latter is, however, not a consistent finding. An interaction between arterial disease and APOE e4 was similarly indicated by the finding of a nine-fold increase in cardiac ischaemia in e4 homozygotes (104) compared to those with e3. These observations appear in accord with the notion that the e4 allele or its product may exert its effects in tandem with hypoperfusion. Above all evidence for a direct role through pathological alterations in the vascular wall rather than by secondary mechanisms via cardioembolic or thrombotic changes is strong but a recent epidemiological study implicated that the effect of APOE gene in dementia is not through atherosclerosis or other vascular disease but yet unknown actions ((22), (105).

III. Cerebrovascular changes in mouse models of AD

IIIa. ApoE-knockout mice

ApoE-knockout mice develop severe atherosclerosis (106) but controversy abounds concerning the AD like behavioural and neuropathological anomalies (107,108) suggesting the involvement of other genetic and/or environmental factors. ApoE-knockout mice also display long term potentiation which may be linked to the impaired learning and memory functions (109-111). In accord with this, in-vitro studies demonstrate that astrocytes from apoE-knockout mice also do not secrete HDL-like particles (112). In addition, aging knockout mice show progressively reduced number of presynaptic boutons compared to wild mice. However, no alterations were found in the number of presynaptic boutons in transgenic mice that express apoE4 or apoE3 in astrocytes. Only upon environmental enrichment do apoE4 transgenic mice fail to produce increases in synapse number whereas apoE3 transgenic mice do not. Similarly, in double transgenic mice expressing apoE4 and an APP mutant, reduced number of synapses was observed in comparison with mice expressing the apoE3 and the same APP mutant (113). Whereas overexpression of apoE4 in astrocytes does not cause severe neurodegeneration, overexpression of apoE4 in neurons results in axonal degeneration with gliosis (114). ApoE has been implicated in the maintenance of BBB integrity during ageing (115,116). In accord with this apoE protects against cerebral

lesions induced by a high fat diet. Moreover, when apoE-knockout mice were fed a western type diet for a period of 10 months, dramatic immunopositive staining for IgG was observed in the brains indicating breach of the BBB. Additionally, the brain volumes were appreciably reduced compared with wild type mice kept on the same diet. However, high plasma cholesterol levels and atherosclerosis were not considered to be the causative factors. It is not yet known whether these effects are due to lack of apoE within the brain or systemic organs, or if there is an indirect effect of chronic inflammatory process in apoE-knockout mice.

The lack of apoE also appears to induce extracerebral endothelial dysfunction. Compared to young in aged apoE-knockout mice higher nitric oxide synthase (NOS) activity levels were reported (117). This suggests increased nitric oxide (NO) production with consequent vasodilatation. ApoE-knockout mice fed on a western diet exhibit impaired endothelium-dependent relaxation responses to acetylcholine action in aortic vessels (118). D'Uscio et al. (119) reported that this impairment of aortic endothelial function was due to increased O2- levels resulting in significantly reduced endothelial NOS activity and cGMP in the apoE-knockout mice. Interestingly, NO production in macrophages and microglial cells is also influenced by the APOE genotype (120). In monocyte-derived macrophages from AD patients carrying the APOE e4 allele significant increases in NO production were observed as opposed to AD patients with APOE e3 allele or age-matched controls.

Recent evidence also suggests that apoE may modulate angiogenesis. Pola et al (87) have identified compromised cerebral angiogenesis subsequent to ischaemia in apoE-knockout mice. This was linked to lower post-ischaemic induction of vascular endothelial growth factor. Consistent with this larger infarct volumes were evident in apoE-knockout mice subjected to focal stroke (121). Not surprisingly, overexpression of human APP751 in the apoE-knockout mice further augments the infarct volume. This could be attenuated by the coexpression of a human apoE isoform, possibly due to inhibition of microgliosis (122). However, treatment with a peroxisome proliferator-activated receptor-a activator (123) reduced the propensity of stroke in apoE-knockout mice.

III B. Amyloid precursor protein-transgenic (APP) mice

Several transgenic mice overexpressing single or double APP mutant genes have been generated. As in AD, these mice develop cerebral amyloid-b deposits, which are apoE positive as well as associated with activated astrocytes (124). Changes in learning and memory processes have been documented in some models (125-128). Some APP transgenic mice have also been shown to exhibit compromised BBB. Aliev et al (129) also reported mitochondrial DNA deletions in endothelial and perivascular cells juxtaposed to regions with high amyloid deposition in transgenic mice and in AD brains. Changes in the integrity of the BBB were also indicated by age-related increases in the levels of plant sterols (campesterol and sitosterol) in transgenic mice carrying the Swedish APP mutation (APP23) (130). Poduslo et al (131) described structural alterations at the BBB, but no differences in the permeability to human amyloid-b1-40 in APP transgenic mice. The increase in BBB permeability was found to precede amyloid plaque formation in the Tg2576 transgenic mice, however (132).

In AD patients (133), the APOE e4 allele compared to the APOE e3 is significantly associated with a greater degree of CAA and amyloid-b deposition. In accord with this the murine apoE also promotes the formation of CAA and associated vessel damage. In other studies (134) APP transgenic mice exhibiting brain amyloid deposits treated with antibodies against amyloid were more prone to bleeds compared to untreated mice. Interestingly, age-dependent CAA associated with microhaemorrhaging in APP transgenic mice could be substantially reduced if mice were exposed to an apoE-deficient environment (135). The ratio of amyloid-b1-40 to amyloid-b1-42 and total amyloid-b was significantly reduced in the absence of apoE in these mice that also exhibited impediments in learning abilities and memory functions. This suggests that the interaction of apoE with amyloid-b induces a loss of functional apoE. Thus, these studies strongly support a role for apoE, both in the deposition of amyloid-b and in its secretion from the brain into the circulation (43). It has been hypothesised that amyloid-b is removed, possibly in association with HDL, from the brain parenchyma to the microvascualture where it binds to e.g. HSPG in the vascular basement membrane subsequently to be internalized by pericytes via the LRP. The intracellular accumulation of amyloid-b in pericytes may result in a loss of vessel stability, making them more vulnerable to haemorrhages.

IV. A plausible central role for HDL

Collective evidence suggests HDL plays a substantial role in the pathogenesis of AD (43) than realised. HDL is a key element in diabetes and inflammation (136), and cardiovascular disorders, which are also considered risk factors for AD. Brain HDL may originate from at least three different sources. While small HDL may be derived from the circulation across the BBB (137,138), astrocytes and cerebral endothelial cells may be the other two sources. In addition to apoE, astrocytes synthesize several enzymes involved in the metabolism of HDL. One of these is cholesteryl ester transfer protein (CETP), a glycoprotein that facilitates the transfer of cholesteryl esters, phospholipids and triglycerides between lipoproteins and regulates plasma HDL levels. CETP has previously been identified to astrocytes localized in the white matter. However, in AD subjects CETP-positive reactive astrocytes were detected in the grey as well as white matter (139). Lecithin: cholesterol acyltransferase (LCAT), an enzyme that is involved in the esterification of cholesterol in HDL or remodeling of HDL, has also been reported to be present in the CSF. Moreover, astrocytes synthesize several other proteins (Table 3) involved in HDL metabolism including apoA-I (140), apoCI and LPL and HDL-binding receptors, including the low density lipoprotein receptor (LDLR), the LRP, scavenger receptor class B, type 1 (SR-B1) and CD36.

IVa. Apolipoprotein A-I and AD?

ApoA-I is the major protein component of HDL, predominantly synthesized in the liver and intestine (141). Together with apoE, it is a major apolipoprotein of HDL in CSF (27). While the origin of apoA-I in the CSF remains to be determined ApoA-I may also be produced by the cerebral endothelium (142). There is evidence to validate the notion that lipid-free apoA-I or HDL lacking apoE but associated with apoA-I can be

transcytosed across the cerebral endothelium (143,138). ApoA-I activates LCAT, thereby facilitating the transport of excess cholesterol from tissues. High LCAT activities have been found in rat brains (93) and in the cerebellum and cerebral cortex of baboons (144). LCAT activities were found to be reduced by as much as 50% in CSF of AD patients compared with aging controls (58).
ApoA-I along with small HDL typically facilitate the efflux of cholesterol from tissues and induce the translocation of cholesterol from intracellular membranes to the cell surface (145). Perhaps not surprisingly ApoA-I also promotes the secretion of apoE by macrophages (146). The ATP binding cassette transporter (ABCA1) plays a major role in the elimination of tissue cholesterol, because it enables the apolipoprotein-dependent transfer of intracellular cholesterol and phospholipids to lipid-free apoA-I (147). ABCA1 and apoA-I appear to bear a distinct role in the basolateral efflux of cholesterol. ABCA1 expression as well as the secretion of apoA-I, generally associated with HDL, from the basolateral compartment of cultured endothelial cells can be induced by 24S-hydroxycholesterol (148), a ligand for the nuclear liver X receptor. This receptor dimerizes with the retinoic acid receptor and boosts transcription of proteins important for cholesterol and fatty acid metabolism (149). Alternatively, the basolateral compartment may be involved in efflux of phospholipids alone. It has been suggested that endothelial cells reassemble and secrete either intracellular or ABCA1-dependent HDL-like lipoproteins. Interestingly, variants of ABCA1 may increase the risk of AD (150).
The ABCG2 too has been detected in cells associated with the BBB (151). Other ATP-binding cassette transporters involved in lipid metabolism that have been detected in the brain include ABCG1/ABCG4, which mediate the efflux of cholesterol to HDL, ABCA2, which was found to regulate LDLR and HMGCoA synthase expression, and ABCA7, which mediates the efflux of phospholipids, but not cholesterol (152,153). Since both apoA-I and apoE fulfil important roles in the transfer of cholesterol from cells to HDL, it is plausible that this is also occurs in the CNS (154,155,27).
Several other functions for apoA-I have been implicated. These include the binding of lipopolysaccharide (LPS), engendering of antiviral activity, nerve regeneration (156) and regulation of the complement system (157). Furthermore, apoA-I may directly interact with endothelial NOS (158) to increase its activity through multisite phosphorylation changes in endothelial cells. An apoA-I mimetic peptide was also found to suppress the infection-induced trafficking of macrophages into arteries (159).
Like amyloid-b the amyloid protein product of apoA-I causes a distinct amyloidosis originating from the specific cleavage of the precursor (160,161). Point mutations or deletions in the apoA-I gene cause a very rare disease known as hereditary non-neuropathic systemic amyloidosis, which involves deposition of polymers of apoA-I (162,141). Although there is no clear evidence for APOA-I gene to be associated with risk of AD (163), a previous study (164) reported apoA-I immunoreactivity in endothelial cells, hippocampal pyramidal neurons, astrocytes and also in cortical amyloid-b plaques in AD subjects. Whether these cellular localizations bear any influence on the decreased serum HDL cholesterol and apoA-I concentrations found to be correlates with severity of AD (165) is unclear. However, this appears consistent with the finding that individuals who reach a very old age in relatively good health

exhibit significantly higher levels of HDL cholesterol and apoA-I compared to age-matched diseased subjects (166).

IVb. Apolipoprotein CI in AD

The APOE e4 allele is considered to be in disequilibrium with the H2 allele of APOCI, which is localized immediately downstream from the APOE gene, and results in 50% increased expression of apoCI (167). The H2 allele of APOCI may be an independent or an additional risk factor for AD (168,169) (Table 3). ApoCI is primarily expressed in the liver, while low expression was reported in lung, skin, testes and spleen (170), and it has been detected in astrocytes. Perhaps not surprisingly apoCI blocks the interaction of apoE with all of its known receptors and, consequently delays the clearance of apoE-containing lipoproteins (171). ApoCI may also activate cholesterol esterification via LCAT (172) and it is a known inhibitor of CETP (173).
ApoCI may play a role in the pathogenesis of AD but the underlying mechanisms have yet to be defined. ApoCI could retard uptake of the apoE-containing HDL-like lipoproteins by neurons and affect delivery of cholesterol required for the outgrowth of synapses. However, it was recently reported that apoCI protein levels are elevated, while those of apoE4 are reduced in the brains of AD patients carrying an APOE e4 allele (174). Additionally, the effect of APOCI polymorphism on hippocampal volumes, memory and frontal lobe function in subjects with age-associated memory impairment (175,176) has been described.

V. HDL receptors and the cerebral endothelium

Va. Low Density Lipoprotein Receptor

In the brain the LDLR has been predominantly detected in astrocytes but other cell types including neurons and endothelial cells also express the receptor (59,177,178,27,179) In contrast to the extracerebral endothelium, its expression in brain endothelial cells remains robust during ageing. The LDLR enables the cellular interaction and internalization of apoE- and/or apoB100-containing lipoproteins (180). Malfunctioning of this receptor in humans gives rise to familial hypercholesterolemia and causes cardiovascular disease (181). Patients with familial hypercholesterolemia that carry the APOE e4 allele are further disadvantaged (182). Several case reports suggest patients with familial hypercholesterolemia develop xanthoma in the brain (183-185).
Although the extracerebral functions of the LDLR have been previously investigated, at present very little is known with respect to the extent of its influence on the CNS. The primary ligand for the LDLR present in brain is apoE as apoB100 does not cross the BBB or does not appear to be synthesized within the brain (186). Another ligand for the LDLR in the brain is likely lipoprotein lipase (LPL) (187). Lipases are involved in the formation of endogenous canabinoids that modulate learning and memory abilities (188). The hippocampus and to a lesser degree cerebral microvessels are the main sites

for expression of LPL (189,190). LPL was also found to be involved in synaptic remodeling (191). Its expression in the hippocampus relative to other brain regions is regulated by severe diet restriction (192). Furthermore, LPL has been shown to affect the uptake and transcytosis of LDL albeit in an in-vitro model of the BBB (193). An association between LPL polymorphism and AD was reported (194) but was not confirmed by Fidani et al. (195) and others (Kalaria et al, unpublished observations).
The LDLR is an important receptor for apoE in the brain and actively facilitates the distribution of lipids (59). Consistent with the occurrence of small HDL-like lipoproteins the interaction between apoE and LDLR increases with small particle size (196). A clear indication of the potential role for the LDLR in the apoE-mediated distribution of cholesterol is the observation that both LDLR-deficient and apoE-deficient mice display an altered distribution of cholesterol in brain synaptic plasma membranes with a higher percentage in the exofacial leaflet (197). Furtheromore, LDLR-knockout mice were shown to display impaired memory and a reduced number of presynaptic boutons in the hippocampus CA1 (198). Retz et al (199) have reported a trend toward the association between an LDLR polymorphism and AD (Table 3).

Table 3

Currently reported associations between late-onset AD and polymorphisms in APOE and other genes involved in lipid metabolism and transport

Gene	Allele(s)	Association	Product(s)	Expression in brain
APOE	ε4, ε2	positive	apoE4, apoE2	astrocytes, EC, neurons
APOAI	various	none	apoA	nc
APOB	Various	unclear	apoB	glial cells
APOCI	H2 insertion	mostly positive	apoC1	astrocytes
APOCIII	Exon 4	none	apoC	nc
CYP46	2 sites	not confirmed	C24SH	neurons, glia
LDLR	H2	not confirmed	LDLR	astrocytes, EC
LRP	Exon 3	unvlear	LRP	neurons, EC, perycites
LPL	H1, H2	unclear	LPL	hippocampus, EC

For original data see references (3,51,30,133,162,163,167,96,173,174,192,199,202,203) Polymorphisms in *HMG-CoA* have also been reported to be weakly associated with AD. Abbreviations: C24SH, cholesterol 24S-hydroxylase; EC, endothelial cells; nc, not clear; other abbreviations see main text.

Vb. Low density lipoprotein receptor-related protein

In addition to the LDLR, the LRP is a multifunctional endocytic receptor expressed predominantly in neurons. However, it has also been detected in capillaries (200) and pericytes. It has been reported that the tissue-type plasminogen activator induces opening of the BBB via interaction with the LRP (201). Several studies have tested associations between the LPR gene and late-onset AD exist but there is no clear indication for a correlation between LRP exon 3 polymorphism and AD or brain LRP levels (202,203).

Vc. Scavenger receptor class B, type I

The SR-BI is detectable almost exclusively at the apical membrane of cerebral endothelial cells. It is also expressed by astrocytes (204,205), however . SR-BI, a member of the CD36 superfamily, is a high affinity receptor for HDL stimulating selective uptake of cholesteryl esters and the efflux of unesterified cholesterol from cells (196). HDL cholesteryl ester uptake has been demonstrated in all tissues with the exception of the brain (206). Therefore it is likely that SR-BI has an important role in the efflux of cholesterol from the brain. Such effect on free cholesterol efflux presumably occurs independently from binding to HDL. SR-BI may in effect modulate membrane free cholesterol domains to provoke cholesterol flux between cells and HDL (207). The SR-BI was also found to stimulate NO production in cerebral endothelium indicating a positive effect on the vasculature (208). Interestingly, the efflux of 24S-hydroxycholesterol from the apical surface of endothelial cells in the presence of HDL is worsened by overexpression of SR-BI (148). However, so far no abnormalities in the CNS have been detected in SR-BI-deficient mice (209).

Vd. CD36: a scavenger receptor

Similar to SR-BI, CD36 is another scavenger receptor. Both SR-BI and CD36 have common ligands. However, the specific function of CD36 is to facilitate the uptake of long chain fatty acids (208). CD36 is strongly expressed in normal brain capillary endothelium (210). CD36 also binds HDL, most likely via apoAII (211). Notably, small dense apoE-free HDL3 seems to be involved in brain entry of polyunsaturated fatty acids, which can be used for membrane synthesis (212). This is compatible with the HDL3 enhanced conversion of phosphatidylethanolamine into phosphatidylcholine by stimulating phospholipase A2. However, CD36 was originally identified as a receptor for oxidized LDL. Although normally the larger lipoproteins do not pass the BBB, oxidized LDL has been detected in astrocytes surrounding cerebral infarcts (213). Oxidized LDL also stimulates the secretion of interleukin-6 from astrocytes. CD36 is apparently also localized in macrophages and microglia. The interaction of amyloid-b with CD36 bearing macrophages was found to trigger H2O2 production (214). Not surprisingly, a high CD36 count was established in AD and cognitively normal subjects with diffuse amyloid plaques that correlate with amyloid-b deposition, but not with AD (215).

VI. Conclusions

Recent advances indicate the pathology of AD includes cerebrovascular abnormalities, which may modify the mobilization, metabolism and storage of brain lipids and lipid carriers. The specialized localization within reactive glia and endothelial cells indicates the brain retains its own repertoire of proteins and receptors linked to lipid transport for handling lipids and proteolipids during brain injury and repair. Since blood to brain traffic of lipids is restricted, impairment of the BBB has implications on mobilization

and sequestration of lipids during injury and impact upon neuronal repair processes. While variable associations have been reported in polymorphisms of genes for lipid metabolic enzymes and receptors the e4 allele of the APOE gene appears the most robust in increasing the burden of disease. Gene knockout and transgenic mice models suggest a variety of ligands and receptors associated with lipid transport or metabolism are vital for neuronal growth and maintenance, and have implications in the pathogenesis of AD.

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Chapter 8

General discussion

The importance of cholesterol in the nervous system was recognized as early as 1834, when Couerbe's observations lead him to regard cholesterol as *un element principal* of the nervous system (1). Only during the last decade cholesterol metabolism in brain attracted attention, but the regulation of the fine tuned cholesterol homeostasis still remains one of the most enigmatic question in the field of brain research.
This thesis provides new insights into brain cholesterol homeostasis regulation via apoE and apoC1 in the brain.http://atvb.ahajournals.org/cgi/content/full/24/5/806—R1-0330
The central nervous system is unique when compared to other peripheral organs in regard to cholesterol metabolism and cholesterol requirements (2) for the following reasons:

- The CNS comprising only 2% of the entire body mass is very rich in cholesterol. About 25% of the total amount of unesterified cholesterol in the entire body is contained in CNS.
- The CNS is considered to be isolated from blood and other organs by a blood brain barrier (BBB) and can obtain cholesterol only via de novo synthesis. Although cholesterol synthesis in the developing CNS is relatively high, it declines to a very low level in the adult state, which can be explained by an efficient recycling of brain cholesterol via apolipoproteins and their receptors http://atvb.ahajournals.org/cgi/ijlink?linkType=ABST&journalCode=jlr&resid=39/8/1594. As a consequence, most of the brain cholesterol has an extremely long half-life. In the adult human brain, the half-life of the bulk of cholesterol has been estimated to be at least 5 years (3), whereas a small fraction (0.02% in humans and approximately 0.04% in mice) turns over every day (2).
- Cholesterol plays an important role in synaptic plasticity (4,5). However, unlike other major membrane lipids, cholesterol cannot be synthesized at neuronal terminals (6). It has been proposed that, after differentiation of astrocytes, neurons reduce their endogenous cholesterol synthesis and rely predominantly on cholesterol delivery by astrocytes via lipoprotein-like particles that contain astroglia-derived apoE (7). Cholesterol delivery may, at least in part, participate in regulating the number of synapses being formed (8). A continuous turnover of the small fraction of cholesterol in neurons facilitates the cells ability for efficient and rapid adaptation of cholesterol homeostasis, required for dynamic structural changes of neurons, their extensions, and their synapses during synaptic plasticity (9).
- Despite the efficiency of cholesterol re-utilization, enzymatic conversion of cholesterol into 24(S)-hydroxycholesterol, which is able to traverse the blood-brain barrier, by CYP46 represents a major route for cholesterol turnover in neurons (10). Depletion of CYP46 results in a slower rate of cholesterol excretion, which is compensated by the suppressing mevalonate pathway leading to the reduction of de novo cholesterol synthesis (11). Moreover, CYP46 knockout mice exhibit severe defects in learning indicating the importance of cholesterol turnover in brain for normal functioning (12). Noticeably, 24(S)-hydroxycholesterol is a natural LXR ligand found in the brain and elevated levels of 24(S)-hydroxycholesterol are often associated with neuronal injury. Neurons are thought to dispose their cholesterol by conversion into 24(S)-hydroxycholesterol, which is more polar than cholesterol and as a result may more easily traverse the BBB and the neuronal plasma membrane

(13). However, the mechanisms that allow oxysterols to be transported across membranes and through the intracellular water phase are yet to be discovered.

Growing evidence from recent studies suggests that altered cholesterol homeostasis in the brain may underlie neuropathological processes. Mutations in the ubiquitously expressed Niemann-Pick C (NPC-1 or NPC-2) genes involved in intracellular trafficking of cholesterol lead to Niemann-Pick disease, a fatal neurovisceral cholesterol storage disease. In the past, it was generally accepted that this accumulation did not occur in the CNS. Recent studies indicate, however, that cholesterol accumulation does indeed occur in NPC neurons (14). Studies using a mouse model of the disease, have suggested that the turnover of cholesterol is also increased under these pathological conditions (15). The net effect is that neurons are unable to adequately respond to increased cholesterol levels via storage or excretion, with the consequence of vastly accelerated development of neurofibrillary tangles. These are essentially identical to those observed in Alzheimer's disease (AD).

Disturbances in cholesterol biosynthesis lead to the Smith–Lemli–Opitz syndrome (SLOS), an autosomal recessive multiple congenital anomaly and mental retardation disorder caused by an inborn error of post-squalene cholesterol biosynthesis. Deficient cholesterol synthesis in SLOS is caused by inherited mutations of the 3β-hydroxysterol-Δ7 reductase gene (DHCR7). DHCR7 deficiency impairs both cholesterol and desmosterol production, resulting in elevated cholesterol precursors 7-dehydrocholesterol and its spontaneous isomer 8-dehydrocholesterol (7DHC/8DHC) levels, typically decreased cholesterol levels and, importantly, developmental dysmorphology. Since cholesterol is known to be an important component of membrane lipid rafts, any subtle defect in raft formation could conceivably alter signaling pathways (16,17). An appropriate composition of cholesterol in the cell membranes is crucial for optimal enzymatic activity, ion and metabolite transport or channelling, protein–protein and protein–lipid interactions, and signal transduction. The pathogenetic mechanisms of cholesterol deficiency caused by abnormal cholesterol biosynthesis may extend beyond disruption of morphogenic pathways. For example, the role of cholesterol in normal synaptogenesis is independent of the cholesterol biosynthesis pathway (18).

Despite the prominence of apoE as the most well established genetic risk factor of AD, the precise mechanisms underlying the relationship of APOE alleles to risk of AD remain a subject of thorough investigation. Recently, several hypotheses have been proposed, the most prominent being that apoE is the key mediator of amyloid β (Aβ) metabolism (19-25).

Understanding the molecular mechanisms of apoE in relation to the onset or progression of AD pathology is one of the challenging topics in neuropathological research.

This thesis proposes that apoE is the key player in cholesterol trafficking between astrocytes and neurons undergoing structural changes in their synapses during synaptic plasticity. Since cholesterol delivery participates in regulating the number of synapses being formed, a continuous turnover of cholesterol in neurons seems to be important for cholesterol homeostasis. The total number of synapses on a neuron is not an intrinsic property, but can be profoundly regulated by extrinsic signals. In the absence of glia, neurons in culture have only a limited ability to form synapses. Astrocytes greatly increase the number of structurally mature, functional synapses and are necessary to

maintain synaptic stability. During normal embryonic development, initially formed synapses may be immature and highly plastic, with the postnatal appearance of astrocytes serving to increase synapse number and to lock synaptic circuitry in place (26). Recent studies indicate that the ability of CNS neurons to form synapses is limited by the availability of cholesterol (5). Massive synaptogenesis requires large amounts of cholesterol and thus depends on cholesterol production by glial cells and its delivery via apoE-containing lipoproteins.

In **Chapter 2** we provide evidence supporting the model in which release of 24(S)-hydroxycholesterol from neurons can induce the secretion of apoE-associated cholesterol from astrocytes. This glial-derived cholesterol would then be available for neuronal uptake during the process of dendritic and axonal extension and regeneration of synapses (27). We observed that 24(S)-hydroxycholesterol induces apoE and ABC transporter expression in astrocytic cells, as well as in primary rat and murine astrocytes via an Liver X Receptor (LXR) mediated mechanism. The potent synthetic LXR agonist GW683965A also induced apoE, ABCA1, and ABCG1 mRNA expression in astrocyte-derived cells. In contrast, 24(S)-hydroxycholesterol did not regulate expression of these genes in cultured neuronal cells. LXR isoforms differ in their pattern of expression in brain. Two LXR isoforms, i.e. LXRα and LXRβ, expressed in the central nervous system (28), are involved in the regulation of brain cholesterol metabolism (28-30), but may also regulate inflammation processes (31,32). In the brain LXRβ levels are 2-5 fold higher than in the liver, while LXRα levels are 3.5-14 fold lower than in the liver. (33-35). However, 24(S)-hydroxycholesterol and GW683965A upregulated LXRα but not LXRβ expression in astrocytoma cells, similar to what has been reported for macrophages and adipocytes, but not liver and muscle (36,37). These results suggest the possibility that the autoregulation of LXRα, suggested to occur in adipocytes to coordinate expression of target genes such as APOE (37), may also occur in the brain. Results presented in Chapter 2 provide evidence indicating that 24(S)-hydroxycholesterol acts as a signalling molecule that induces the apoE-mediated cholesterol efflux from astrocytes, but not from neurons. Our findings also suggest a role for ABC transporters ABCA1 and ABCG1 in mediating cholesterol efflux from astrocytes. 24(S)-hydroxycholesterol-mediated induction of ABCG1 and ABCA1 primes the astrocytes to deliver cholesterol to apoE or apoA-I, since their expression was robustly induced by both compounds. Our data is in agreement with the previous observation (38) that indicated that in glia ABCG1 is the primary determinant of cholesterol release to apoE for lipoprotein formation. ApoE is the major extracellular acceptor of glia-derived cholesterol that is secreted from the glia in a partially lipidated state. Moreover, ABCA1 mediates the initial lipidation of extracellular apoA1/apoE, and ABCG1 participates in the further lipidation of pre-formed apoE-containing lipoproteins secreted by the glia and of pre-formed apoA1-containing lipoproteins formed by the action of ABCA1.

In contrast, GW683965A upregulated and 24(S)-hydroxycholesterol down-regulated the expression of HMGCoA-R, LDLR and SREBP2 in astrocytoma cells, supposedly to maintain cellular cholesterol homeostasis during excessive loss by efflux. Rebeck et al. (39) recently reported upregulation of neuronal ABCA1 expression by the synthetic LXR ligand T0901317. A role for apoA-I in the disposal of cholesterol from neurons is in line with its well-known role in the so called “reverse cholesterol transport”. ApoA-I

is present in brain and cerebrospinal fluid, and has been detected in senile plaques in AD patients (40,41). So far apoA-I synthesis within the brain has only been ascribed to endothelial cells of the BBB (42).
Thus, in the intact brain, 24(S)-hydroxycholesterol derived from neurons may signal astrocytes to increase production of lipidated apoE particles in order to supply neurons with additional cholesterol during synaptogenesis or neuritic remodelling. We elucidated another aspect of 24(S)-hydroxycholesterol, which is crucial for cholesterol turnover in brain and therefore essential for learning and memory processes (12). Moreover alterations in apoE-mediated cholesterol efflux may affect the progression of neurodegenerative diseases including AD.
Since in **Chapter 2** we elucidated LXRs mediated effect of the natural brain specific 24(S)-hydroxycholesterol in brain cholesterol metabolism using *in vitro* experimental approach, in **Chapter 3** we addressed the effect of LXR activation in vivo.
We demonstrated that long-term administration of the synthetic LXR-agonist T0901317 via the diet to wild-type C57BL/6J mice leads to a state of permanently accelerated whole body cholesterol turnover, as evidenced by the massively increased faecal sterol loss. We showed for the first time that, in the brain, long-term administration of T0901317 induces significantly upregulated levels of cholesterol precursors, without affecting cholesterol levels. Brain levels of 24(S)-hydroxycholesterol were not significantly decreased as a result of T0901317 treatment indicating that the secretion of cholesterol via this pathway is not upregulated.
As expected, the expression of the cholesterol transporters ABCA1 and, to a lesser extend, also of ABCG1 was clearly upregulated in the brain as a result of T0901317 administration. It has been demonstrated that these transporters are expressed in astrocytes and neurons (43,44), but also in the BBB (45) and may thus be instrumental in the transport of sterols from the periphery into the CNS. We found that APOE mRNA levels were not significantly increased in the brain as a result of 4 weeks of T0901317 treatment. In line with this, Whitney *et al.* (28) reported upregulation of expression of ABCA1, ABCG1, but not APOE in the cerebellum and hippocampus of wild-type C57BL/6 mice that were fed 50 mg/kg LXR-agonist T0901317 in the diet for 3 or 7 days. Liang *et al.* (30), on the other hand, found a moderate but significant increase (about 1.5-fold) in brain APOE mRNA levels after administration of 10 mg/kg T0901317 for a period of 7 days, while we found a trend of 1.3-fold increase of APOE mRNA. T0901317 and also another LXR-agonist GW683965A have been shown to induce APOE expression and cholesterol efflux from astrocytes *in vitro* (28,30). We found the strongest effect on apoCI mRNA. Interesting in this respect is the recent finding that apoCI can mediate cholesterol efflux with a similar efficiency as the established cholesterol acceptors apoAI and apoE (Westerterp et al, personal communication).
Cholesterol is transported to the endoplasmic reticulum, where it is esterified by acyl-coenzyme-A cholesterol acyl transferase 2 (ACAT2) for incorporation in to chylomicrons. Since, plant sterols are poor substrates for ACAT2 they will immediately be re-secreted by transporters like the ATP Binding Cassette G5/G8 (ABCG5/8) heterodimer in the intestinal lumen and bile (46). Therefore, plant sterols are normally hardly found in the human serum. Sitosterol and campesterol are two of various sterols, exclusively derived from plants (47). Yet, although partly functionally related to

cholesterol, small differences lead to very divergent metabolic fates of plant sterols and cholesterol in mammals (48). In contrast to cholesterol, plant sterols are not synthesized by the human body, but are entirely derived from the diet. Both humans and mice with a genetic disturbance in ABCG5 or ABCG8 have increased serum plant sterol levels, called sitosterolemia (49). Up to now, it is considered that these plant sterols cannot reach the brain, similar to cholesterol. Nevertheless, we found that campesterol in particular accumulates in the brain. After treatment with T0901317 the brain levels of campesterol decrease and brain levels of sitosterol remain unchanged, while the plasma levels of both types of plant sterol decrease in plasma. This indicates, as also suggested by Björkhem *et al* (13), that the different sterols are not transported to an equal extend into or out of the brain.

In contrast to the general assumption that plant sterols do not enter the brain, this study demonstrates that increased circulating levels of plant sterols, as a result of intake of a plant sterol-enriched diet in wild type mice or as a consequence of ABCG5- or ABCG8-deficiency, are associated with elevated levels of plant sterols in the brain.

Since plant sterols have been reported to affect HMG-CoA reductase activity (49,50), we investigated the effect of plant sterols on total brain cholesterol metabolism in vivo and their effects on ApoE protein and mRNA levels in vitro, using astrocytoma and neuroblastoma cells. Our results described in **Chapter 4** suggest that an accumulation of plant sterols in the brain may exert specific effects and as a result may affect brain functioning.

Evidence is provided that plant sterols are only transferred into the brain when they are carried by small HDL particles, as is the case in ABCG5- and ABCG8-deficient mice and not when plant sterols are present in the larger VLDL particles, as is the case in apoE-deficient mice. The increased plant sterol concentrations in the brain do not detectably affect overall brain cholesterol metabolism. Yet, it is important to note that they may exert cell-specific effects, which is supported by the finding that sitosterol specifically upregulates the expression of apoE in cultured human astrocytoma cells, but not in neuroblastoma cells. Since campesterol is more similar in structure to cholesterol than sitosterol, the preference of campesterol transfer into the brain as compared to sitosterol is remarkable. In line with this, it was shown that the esterification rate of campesterol in macrophages was 20% of that of cholesterol, while β-sitosterol was not detectably esterified (51). These differences in esterification may play a role in the preferential transport of campesterol into the circulation and also into the brain.

The member of the ABC (ATP binding cassette) transporter family ABCA1 is a likely candidate to be involved in the transport of plant sterols into the brain, since it has been detected in brain endothelial cells at the basolateral side where it mediates cholesterol efflux (45). ABCA1 mediates the cellular efflux of free cholesterol to apoA-I or to apoE as extracellular sterol acceptors (52-54), and does not discriminate between cholesterol and sitosterol (55). Considering this, the lack of increase in brain plant sterol levels of apoE-deficient mice could be explained by assuming that apoE derived from astrocytes acts as an acceptor for ABCA1-mediated sterol efflux from blood–brain barrier endothelial cells. Yang et al. (48) found 10-fold increased ABCA1 expression levels in the adrenal glands of ABCG5/G8-deficient mice. The fact that we did not detect any effect of the absence of ABCG5 or ABCG8 on the expression of ABCA1 in whole brain may be due to the relatively low levels of plant sterols in the brain.

The levels of brain plant sterols were relatively low in ABCG5- and ABCG8-deficient mice as compared to cholesterol, but they still were about 2-fold higher than the levels of 24S-hydroxycholesterol. Similarly, plant sterols have been reported to activate LXRs (48,56). Therefore, we hypothesized that plant sterol levels, as high as reported in the ABCG5- and ABCG8-deficient mice of this study, might have an effect on mRNA and/or protein expression, although we did not detect an effect on the mRNA levels of APOE, APOC1, ABCA1, LDLR, HMG-CoAR, Cyp46 or SREBP1c in the whole brain of the ABCG5-deficient mice as compared to the wild type mice. This confirms the strict regulation of the maintenance of cholesterol balance in the brain. Yet, it is important to note that local sitosterol accumulation may exert cell-specific effects, since sitosterol enhanced APOE mRNA expression and down-regulated LDLR mRNA expression specifically in astrocytoma cells, but not in a neuroblastoma cell line. Sitosterol was a stronger regulator of apoE protein expression than cholesterol, while campesterol did not detectably affect apoE protein levels. In agreement with this, sitosterol has been reported to affect membrane characteristics due to a less favorable interaction with phospholipids than cholesterol (57).

In conclusion, we demonstrate that increased levels of plant sterols in the circulation can result in increased levels of plant sterols in the brain. The results strongly suggest a role for HDL in the transport of plant sterols into the brain. This may either be by crossing the BBB or by delivering plant sterols to endothelial cells of the BBB and subsequent resecretion. ApoE may play a decisive role in these processes, either by influencing the lipoprotein particle size and composition in serum or by transport of plant sterols at the level of the BBB. Furthermore, our results suggest that plant sterols exert effects within the brain by affecting expression of genes, including APOE and LDLR, in a cell-type specific manner. In *in vitro* experiments sitosterol was a stronger regulator of apoE protein expression than cholesterol, while campesterol did not detectably affect ApoE protein levels. In agreement with this, sitosterol has been reported to affect membrane characteristics due to a less favorable interaction with phospholipids than cholesterol (57) indicating its possible role in synaptic plasticity. Further investigations are needed to elucidate the mechanisms by which plant sterols are transported across the BBB and whether or not they have a significant influence on brain functions.

In **Chapter 3** we demonstrated that upon T0901317 treatment, the expression of apoC1, which belongs to the same gene cluster as apoE, increased almost 3-fold, further sustaining an important role for apoC1 in brain. Until now the role of apoCI has been extensively studied in the respect to atherosclerosis. The physiological role for apoC1 in brain still remains a fascinating and enigmatic question, especially due to the fact that there were mostly epidemiological studies performed in the area. Interestingly, APOE4 is in genetic linkage disequilibrium with the *HpaI* restriction polymorphism in the promoter region of APOC1, which is localized 5 kb downstream of the APOE gene on chromosome 19. Although apoCI is mainly expressed in the liver, substantial expression has also been detected in other tissues including the brain (58). The *HpaI* polymorphism (so-called H2 allele) leads to a highly significant, 1.5-fold increase in APOC1 gene transcription (59) and was reported to be associated with AD (60-65). Moreover, the H2 allele of APOC1 was associated with poorer memory and frontal lobe function (66), and with loss of hippocampal volumes (67).

Therefore, in **Chapters 5** and **6** we attempted to clarify the functions of apoC1 in brain. To investigate a possible role for apoCI in cognitive functions we used *human APOC1*$^{+/0}$ transgenic mice (Chapter 5) and APOC1$^{-/-}$ knockout mice **(Chapter 6).** *APOC1*$^{+/0}$ transgenic mice display APOC1 mRNA throughout their brains and moderate levels of human apoCI protein predominantly in astrocytes and endothelial cells lining the cerebral microvessels, which reflects the distribution of apoE observed in the hippocampal region of human brains.

In **Chapter 5** we demonstrated for the first time that expression of human apoCI impairs learning and memory functions in mice. In comparison with their wild-type littermates, the *hAPOC1*$^{+/0}$ mice displayed severely impaired hippocampal-dependent memory functions as indicated by the results obtained from the object recognition task.

The learning tested in the spatial Morris water maze task, as also applied in the present study, is generally accepted to be highly dependent on the neuronal plasticity of the hippocampus. In the Morris task, the *hAPOC1*$^{+/0}$ mice displayed an impaired learning as indicated by the statistically significant differences between the two groups in the acquisition of the task as well as in the probe trial, indicating an impaired memory.

These findings underscore the hypothesis that the H2 allele of APOC1, giving rise to increased expression of the gene, may be an independent or additional risk factor for the development of AD. In this regard, it is of interest that in H2-allelic AD patients, who are usually also apoE4 carriers, apoCI protein levels in the CSF were significantly higher than in H1-allele carriers (68). Our next step was to hypothesize that apoC1-deficiency in mice might enhance these functions (Chapter 6). However, surprisingly, this work shows that the absence of apoCI also impairs memory functions in mice. In comparison with their wild-type littermates, the APOC1$^{-/-}$ mice displayed impaired hippocampal-dependent memory functions as indicated their performance in an object recognition task. Therefore it can be concluded that the APOC1$^{-/-}$ mice are impaired in their learning and (recognition) memory processes. These results were unexpected since the transgenic mice expressing human apoC1 showed a comparable to APOC1$^{-/-}$ mice behavioral pattern in the object recognition task.

It is interesting to speculate about the mechanism(s) that underlie the observed effects of human apoCI expression or the absence of APOC1 on cognitive function. ApoCI and apoE are both produced by the same gene cluster (i.e. APOE/APOC1/APOC2/APOC4) on chromosome 19. In the circulation, apoE facilitates the clearance of lipoproteins by the LDLR and LRP on hepatocytes (69,70). Similarly, apoE is thought to deliver cholesterol to neurons for the outgrowth of synapses (9,27,71). In the circulation, human apoCI inhibits the hepatic clearance of lipoproteins by interfering with their apoE-mediated binding to the LDLR or the LRP (69,70). Similarly, it can be speculated that, in the brain, human apoCI retards the uptake of the apoE-containing HDL-like lipoproteins by neurons. ApoCI may have similar functions in the brain as in the circulation, where it is in dynamic equilibrium with HDL, VLDL and chylomicron particles (72,73). This ability to interact at lipid surfaces underlies a number of functional properties ascribed to apoCI. ApoCI activates the plasma enzyme LCAT (74), which converts discoidal HDL particles to spherical particles, although this role appears to be secondary to the role played by apoAI (75,76). Paradoxically, both over-expression (77) and under-expression (78,79) of human apoCI in transgenic mice reduces VLDL clearance, an effect that has been interpreted as apoCI-mediated

disruption of apoE-dependent β-VLDL–receptor interactions (70,80). Recent studies establish a more direct and unique function for apoCI with submicromolar levels inhibiting the activity of cholesterol ester transfer protein (81). This property has special significance as small molecule inhibitors of cholesterol ester transfer protein inhibit the progression of atherosclerosis in rabbits (82). These studies highlight the need for a more detailed understanding of the functional properties of apoCI in brain.

In line with previous observations (68), in **Chapter 5** we demonstrate that apoCI is present predominantly in astrocytes, but also in endothelial cells lining the blood vessels in human control as well as in AD brains. In fact, we observed co-localization of apoCI with apoE and Aβ in plaques in the brains of AD patients. Moreover, we show that the incubation of apoC1 with Aβ peptides inhibits, whereas apoAI does not prevent Aβ (1-40) fibril formation *in vitro*, despite the presence of apoA-I in senile plaques (83). It has been demonstrated that *in vitro* interaction between apoE and Aβ is apoE isoform specific (84,85). The apoE2 and E3 isoforms formed stable complexes *in vitro* with Aβ, whereas apoE4 isoform did not interact with Aβ. The role of apoE in the etiology of AD might be related to a protective role of apoE2 and E3 isoforms by complexing Aβ and inducing the clearance of Aβ from the extracellular space, limiting in that way Aβ peptide aggregation and neurotoxicity. The association of apoC1 and apoE with Aβ peptide indicate that both apolipoproteins may somehow be involved in generating and processing of plaques.

Since it has previously been reported that apoE-knockout mice show impaired learning and memory functions (86-89), the effect of human apoCI expression on these cognitive functions could be explained by either reduced expression of mouse apoE in the brain or by counteracting the effects of apoE on lipid distribution within the brain. However, no differences were observed in brain apoE mRNA and protein levels between $hAPOC1^{+/0}$ transgenic mice and their wild-type littermates, and neither was there any detectable difference in the distribution of apoE throughout the brain determined by immunohistochemistry. The possibility remains that apoCI has an effect on the apoE function independent of apoE expression, thereby modulating synaptic plasticity and learning and memory processes. We did not find any alterations in expression of apoE in $APOC1^{-/-}$ mice in comparison with their wild-type littermates, but the apoD expression around blood vessels was elevated in the brain of $APOC1^{-/-}$ knockout mice in comparison to wild-type mice. Previous studies demonstrated that the levels of apoD mRNA levels in the brains of apoE-deficient mice are 50-fold increased as compared to those of wild type control mice (90). In addition, it has been shown that apoD in AD cases, compared to controls, showed elevated vascular and intracellular apoD immunostaining which localized primarily to cells clustered within plaques and around large blood vessels (91). Since apoC1 and apoD are found in the plaques and both can influence plaque deposition, it was interesting to detect increased apoD expression in brain of $APOC^{-/-}$ mice.

To obtain further insight into a potential role of apoCI in modulating the apoE-dependent transport and distribution of lipids, we determined sterol concentrations in the brains of transgenic and knockout animals. However, whereas the levels of all sterols were increased in serum, only levels of campesterol and sitosterol were significantly increased in the brains of $hAPOC1^{+/0}$ mice. In **Chapter 4** we have shown that increased serum plant sterol levels can result in increased brain plant sterol levels.

Therefore, the increased brain levels of campesterol and sitosterol in hAPOC1$^{+/0}$ mice are most likely due to the elevated levels of plant sterols in the circulation. Since hardly anything is known with respect to effects of plant sterols on brain functions, it remains to be established whether this increase contributes to the changes observed in learning and memory.
The absence of apoC1 results in reduced circulating cholesterol levels and in slightly reduced triglyceride levels in the circulation of 12 weeks old mice (Rensen et al, personal communication). Though, in the brain of APOC1$^{-/-}$knockout mice, the apoC1 deficiency did not affect overall levels of different sterols. Neither was there any difference in the percentages of free and esterified cholesterol.
APOC1 is a Liver X receptor (LXR) target gene. Interestingly, the expression of apoCI is highly induced by LXR activators in macrophages (92,93). Besides a potential effect on the lipid homeostasis in the brain, apoCI may affect cognitive function by its proinflammatory properties. On the one hand, activation of LXRs leads to anti-inflammatory activities and promotes macrophage survival in bacterial infection (94). On the other hand, we recently found that apoCI increases proinflammatory responses in mice and protects mice against mortality from bacterial infection by enhancing the early antibacterial attack (95). Given the fact that AD is an inflammatory disease, it may be possible that chronic elevation of apoCI levels in the brain (e.g. in subjects expressing the H2 allele) may lead to a chronic inflammatory state that ultimately leads to the development of AD. The characteristics of plaques positive for apoCI remain to be clarified. In this respect, it would be interesting to investigate the effects of chronic LXR activation on cognitive function. Yet, no effects of hapoCI on brain expression of IL6 and TNFα were found under non-stressed conditions.
In conclusion, expression of human apoCI in the mouse brain as well as the absence of apoCI results in impaired learning and memory processes, supporting an important role for apoCI in the brain. In the brain, apoCI may be involved in regulating lipid metabolism and/or inflammatory processes, but the underlying molecular mechanisms remain to be clarified.
Finally, **Chapter 7** gives an overview on vascular aspects in the development of AD. At present, cardiovascular afflictions, hypertension and diabetes constitute accepted risk factors for vascular dementia (VaD) and have gained importance for AD. Another well established susceptibility factor with regard to AD in particular is the ε4 allele of the APOE gene. The presence of APOE ε4 is also associated with an augmented probability rate for cardiovascular disorders, hypertension and diabetes. Combined with vascular disease, APOE ε4 further increases the risk of AD. The APOE genotype may, therefore, be a potential element that modifies the vascular function in the periphery as well as in the brain during ageing, possibly amplifying the responsiveness to pathogenic processes in AD. This infers a strong role for high-density lipoproteins (HDL) in the pathogenic processes of AD, which, similar to APOE, is thought to play a central role in cardiovascular disorder, hypertension and diabetes as well as in inflammatory processes. In this context other apolipoproteins including A-I and C-I, and the HDL-binding proteins that are also expressed by cells, which form the BBB, may be instigated in the pathogenesis of AD.

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Summary

Alzheimer's disease (AD) is a devastating neurodegenerative condition affecting increasing amount of elderly worldwide. Accumulating body of evidence demonstrates an important role for cholesterol in the pathogenesis of AD. The role of cholesterol in brain and its connection to AD is thoroughly explained in the General Introduction chapter as well as the detailed background of existing hypothesis of AD. The chapter also gives an overview on the role of apolipoproteins E and C1 in brain and on the regulation of LXR-mediated genes involved in cholesterol metabolism. Furthermore, potential treatments of AD as well as critical aspects of the action of statins have been described in the Introduction part of the thesis.

Since apoE4 is a well known risk factor for the development of AD and apoE is a key lipid transporter in brain, it has been hypothesized that apoE affects AD development via isoform-specific effects on lipid trafficking between astrocytes and neurons. In this thesis we show **(Chapter 2)**, for the first time, that the brain-specific metabolite of cholesterol produced by neurons, i.e. 24(S)-hydroxycholesterol induces apoE transcription, protein synthesis and secretion via LXR mediated pathway. Moreover, 24(S)-hydroxycholesterol primes astrocytoma, but not neuroblastoma cells, to mediate cholesterol efflux to apoE. Furthermore, apoE-mediated cholesterol efflux from astrocytoma cells may be controlled by the ATP-binding cassette transporters ABCA1 and ABCG1, since their expression was also upregulated by 24(S)-hydroxycholesterol.

Since we demonstrated LXRs mediated the effect of the natural brain specific 24(S)-hydroxycholesterol in brain cholesterol metabolism using *in vitro* experimental approach, we further addressed the effect of LXR activation in vivo. **In Chapter 3** we show that long-term treatment of C57Bl6/J mice with the synthetic LXR agonist T0901317 persistently enhances whole body cholesterol turnover and also affects brain cholesterol metabolism. T0901317 treatment significantly increased brain cholesterol precursor levels (lathosterol and desmosterol), while brain cholesterol levels were not affected. Upon treatment no significant changes were observed in brain levels of 24(S)-hydroxycholesterol suggesting additional pathway of cholesterol secretion. T0901317 upregulated the expression of ABCA1, ABCG1 and apoC1. These genes may be involved in the increased sterol flux within the brain or across the blood-brain barrier.

Up to now, it is considered that plant sterols cannot reach the brain; similar to cholesterol. Nevertheless, we found that campesterol in particular accumulates in the brain. After the treatment with T0901317 the brain levels of campesterol decrease and brain levels of sitosterol remain unchanged, while the plasma levels of both types of plant sterol decrease in plasma. Our results strongly indicate that prolonged administration of the LXR-agonist T0901317 to wild-type C57BL/6 mice enhances whole body cholesterol turnover including that in the brain. Thus, pharmacological interference with the LXR system may be applicable to assess the role of cholesterol metabolism in the brain in health and in neurodegenerative disease, including Alzheimer's disease.

Our results described in **Chapter 4** suggest that an accumulation of plant sterols in the brain may exert specific effects and as a result may affect brain function. Dietary plant sterols and cholesterol have a comparable chemical structure. It is generally assumed that cholesterol and plant sterols do not cross the blood–brain barrier, but quantitative data are lacking. We demonstrate that mice deficient for ATP-binding cassette transporter G5 (ABCG5) or ABCG8, with strongly elevated serum plant sterol levels, display dramatically increased (7- to 16-fold) plant sterol levels in the brain. Although apoE-deficient mice also displayed elevated serum plant sterol levels, the brain plant sterol levels were not significantly changed. ABCG5- and ABCG8-deficient mice were found to carry circulating plant sterols predominantly in HDL-particles, whereas apoE-deficient mice accommodated most of their serum plant sterols in VLDL-particles. This suggests an important role for HDL and/or ApoE in the transfer of plant sterols into the brain. Moreover, sitosterol upregulated apoE mRNA and protein levels in astrocytoma, but not in neuroblastoma cells, to a higher extend than cholesterol. In conclusion, dietary plant sterols pass the blood–brain barrier and accumulate in the brain, where they may exert brain cell type-specific effects.

In **Chapter 3** we demonstrated that upon T0901317 treatment, the expression of apoC1, which belongs to the same gene cluster as apoE, increased almost 3-fold, further sustaining an important role for apoC1 in brain. The physiological role for apoC1 in brain still remains a fascinating and enigmatic question, especially due to the fact that there were mostly epidemiological studies performed in the area. Interestingly, APOE4 is in genetic linkage disequilibrium with the *HpaI* restriction polymorphism in the promoter region of APOC1, which is localized 5 kb downstream of the APOE gene on chromosome 19. The *HpaI* polymorphism (so-called H2 allele) leads to a highly significant, 1.5-fold increase in APOC1 gene transcription and was reported to be associated with AD. Moreover, the H2 allele of APOC1 was associated with poorer memory and frontal lobe function, and with loss of hippocampal volumes. This has led to the hypothesis that the H2 allele of APOC1, rather than the APOE4 allele, provides a major risk factor for AD. Therefore, in **Chapters 5** and 6 we attempted to clarify the functions of apoC1 in brain. To investigate a possible role for apoCI in cognitive functions we used *human APOC1*$^{+/0}$ transgenic mice (Chapter 5) and APOC1$^{-/-}$ knockout mice **(Chapter 6).**

In Chapter 5 we demonstrated for the first time that expression of human apoCI impairs learning and memory functions in mice as indicated by the results obtained from the object recognition and Morris water maze tasks. These effects of apoCI on memory may depend on its well known inhibitory properties towards apoE-dependent lipid metabolism. However, no effects of hAPOC1 on brain mRNA or protein levels of endogenous apoE were detected. Neither were there any detectable differences in the overall sterol or phospholipids profile in the brain. In addition, analysis of gene expression profiles did not indicate gross changes in brain lipid metabolism or inflammation. We found that the apoCI protein is present mainly in astrocytes and endothelial cells within hippocampal regions of both control and AD brains. Surprisingly, it also co-localized with β-amyloid in plaques in brain specimens of AD patients. In vitro apoC1 peptide was found to inhibit Aβ peptide aggregation. In conclusion, apoCI expression impairs cognitive functions in mice independent of apoE

expression, which strongly suggests that apoCI has a modulatory effect during the development of Alzheimer's disease.
Our next step was to hypothesize that apoC1-deficiency in mice might enhance cognitive functions (Chapter 6). However, surprisingly, this work shows that the absence of apoCI also impairs memory functions in mice. In comparison with their wild-type littermates, the $APOC1^{-/-}$ mice displayed impaired hippocampal-dependent memory functions as indicated their performance in an object recognition task. Therefore it can be concluded that the $APOC1^{-/-}$ mice are impaired in their learning and (recognition) memory processes. These results were unexpected since the transgenic mice expressing human apoC1 showed a comparable to $APOC1^{-/-}$ mice behavioral pattern in the object recognition task.
Finally, **Chapter 7** gives an overview on vascular aspects in the development of AD. At present, cardiovascular afflictions, hypertension and diabetes constitute accepted risk factors for vascular dementia (VaD) and have gained importance for AD.
The results described in this thesis elucidate partly the mechanism(s) underlying the regulation of apoE-mediated neuronal cholesterol supply by astrocytes, providing insight into a role of the natural brain specific 24(S)-hydroxycholesterol as a key regulator. Moreover, we provide evidence that not only APOE4, but also the H2-allele of APOC1 is a risk factor for AD. We showed that apoCI is present in plaques in AD brains and that apoCi directly interacts with amyloid. Overexpression of apoCI in mice impairs learning and memory processes supporting an important role for apoCI in the brain. Together, these findings provide novel insights into the role of apoE and apoC1 in the brain and into the development of AD.
In conclusion, in **Chapter 8**, the main findings are summarized, discussed and related to current literature.

Samenvatting

Alzheimerdementie (AD) is een zeer invaliderende neurodegeneratieve aandoening die almaar meer ouderen over de hele wereld treft. Wetenschappelijk onderzoek levert steeds meer aanwijzingen dat cholesterol een belangrijke rol speelt in de pathogenese van AD. De rol van cholesterol in de hersenen en het verband met AD worden uitgebreid uitgelegd in het hoofdstuk **Algemene inleiding**, waar ook de gedetailleerde achtergrond wordt gegeven van bestaande hypotheses voor AD. Het hoofdstuk geeft ook een overzicht van de rol bij het cholesterol metabolisme betrokken eiwitten en genen zoals apolipoproteïnen E (apoE) en C1 (apoC1), eiwitten die een rol spelen in het cholesterol metabolisme, in de hersenen en de regulatie van LXR-gemedieerde genen. Verder worden mogelijke behandelingen voor AD en de specifieke aspecten van de werking van statines beschreven in de **Inleiding** van de proefschrift.

Aangezien apoE4 is een bekende risicofactor is voor de ontwikkeling van AD en apoE een belangrijke lipidetransporter is in de hersenen, werd de hypothese gesteld dat apoE de ontwikkeling van AD beïnvloedt doordat de isovormen een verschillend effect zouden hebben op het verkeer van lipiden tussen astrocyten en neuronen. In deze thesis (**Hoofdstuk 2**) tonen we voor het eerst aan dat de metaboliet van cholesterol die specifiek in de hersenen wordt geproduceerd door neuronen (namelijk 24(S)-hydroxycholesterol) de transcriptie, eiwitsynthese en de uitscheiding van apoE induceert via de reacties gemedieerd door LXR route. Bovendien activeert 24(S)-hydroxycholesterol in astrocyten, maar niet neuronen, de uitscheiding van cholesterol en apoE. De apoE-gemedieerde efflux van cholesterol uit astrocyten gereguleerd te worden door de ABC-transporteiwitten (ATP-bindende cassette) ABCA1 en ABCG1, aangezien hun expressie ook opgereguleerd werd door 24(S)-hydroxycholesterol.

Sinds we *in vitro* hebben aangetoond dat LXR het effect van de natuurlijke hersenspecifieke 24(S)-hydroxycholesterol op het cholesterolmetabolisme in de hersenen medieert, hebben we het effect van de activering door LXR *in vivo* verder bestudeerd. In **hoofdstuk 3** tonen we aan dat langdurige behandeling van C57Bl6/J-muizen met de synthetische LXR-agonist T0901317 de turnover van cholesterol niet alleen in de periferie maar ook in de hersenen versnelt. Behandeling met T0901317 verhoogt significant de concentraties van cholesterolprecursors in de hersenen (lathosterol en desmosterol), waarbij de hersencholesterolspiegel onveranderd blijft. Na de behandeling werden er geen significante veranderingen waargenomen in de hersenconcentraties van 24(S)-hydroxycholesterol, wat erop wijst dat cholesterol via een andere weg wordt uitgescheiden. T0901317 versterkte de expressie van ABCA1, ABCG1 en apoC1. Deze genen zouden betrokken kunnen zijn bij de sterolfluxen in de hersenen of over de bloed-hersenbarrière.

Tot nu toe werd verondersteld dat plantensterolen evenmin als cholesterol doordringen in de hersenen. Wij hebben echter vastgesteld dat van de plantsterolen vooral campesterol in de hersenen accumuleert. Na de behandeling met T0901317 daalde de hersenspiegel van campesterol. De hersenspiegel van sitosterol verandert niet, terwijl de

plasmaspiegel van beide types plantensterolen wel daalt in het plasma. Onze resultaten wijzen er sterk op dat langdurige toediening van de LXR-agonist T0901317 aan wild-type C57BL/6 muizen de turnover van cholesterol in het hele lichaam, en dus ook in de hersenen, versnelt. Farmacologische interferentie met het LXR-systeem kan worden toegepast om de rol te evalueren van het cholesterolmetabolisme in gezonde hersenen en bij neurodegeneratieve aandoeningen, waaronder ook de ziekte van Alzheimer.
In de resultaten beschreven in **Hoofdstuk 4** vinden we aanwijzingen dat een stapeling van plantensterolen in de hersenen specifieke effecten kan hebben op hersenfuncties. Plantensterolen in de voeding en cholesterol hebben een vergelijkbare chemische structuur. Zoals gezegd wordt algemeen aangenomen dat cholesterol en plantensterolen niet door de bloed–hersenenbarrière dringen, maar kwantitatieve gegevens ontbreken nog. We tonen aan dat bij muizen met een deficiëntie voor de ABC-transporteiwitten (ATP-bindende cassette) G5 (ABCG5) of ABCG8 bij zeer hoge serumplantensterolspiegels de concentratie van deze sterolen in de hersenen zeer sterk stijgt (7 tot 16 keer). Hoewel apoE-deficiënte muizen ook een verhoogde serumplantensterolspiegel vertonen, veranderde de plantensterolconcentratie in de hersenen niet. ABCG5- en ABCG8-deficiënte muizen bleken de circulerende plantensterolen overwegend te transporteren in high density lipoprotein (HDL)-partikels, terwijl apo-E-deficiënte muizen de meeste van hun serumplantensterolen meedroegen in very low density lipoprotein (VLDL)-partikels. Dit wijst op een belangrijke rol voor HDL en/of apoE in het transport van plantensterolen in de hersenen. Bovendien werden de spiegels van apoE mRNA en -eiwit door sitosterol opgereguleerd in astrocyten-, maar niet in neuronen, en dit in hogere mate dan door cholesterol. De conclusie is dus dat plantensterolen door de bloed–hersenbarrière dringen en zich opstapelen in de hersenen, waar ze celtypespecifieke effecten kunnen uitoefenen.
In **hoofdstuk 3** hebben we aangetoond dat na behandeling met T0901317 de uitdrukking van apoC1, dat tot dezelfde gencluster behoort als apoE, bijna verdrievoudigde, wat een verdere aanwijzing is voor de belangrijke rol van apoC1 in hersenen. De fysiologische rol van apoC1 in de hersenen blijft nog steeds een intrigerend mysterie, want er zijn alleen nog maar epidemiologische studies uitgevoerd op dit gebied. Interessant is dat het genetische evenwicht tussen apo-E4 en het polymorfisme in de *HpaI*-restrictie in het promotorgebied van apoC1, 5 kb stroomafwaarts van het apo-E-gen op chromosoom 19, verstoord is. Het *HpaI*-polymorfisme (in het H2-allel) leidt tot een zeer significante stijging met een factor 1,5 in de transcriptie van het apoC1 gen. Dit werd ook geranden bij AD. Bovendien ging het H2-allel van apo-C1 gepaard met een slechter geheugen en functioneren van de frontale kwab, en met een volumeverlies van de hippocampus. Dit heeft geleid tot de hypothese dat het H2 allel van apoC1, en niet het apoE4 allel, een belangrijke risicofactor voor AD zou zijn. Daarom hebben we in **hoofdstukken 5 en 6** geprobeerd om de functies van apoC1 in de hersenen op te helderen. Om de mogelijke rol voor apoC1 te onderzoeken voor de cognitieve functies, hebben we *humaan apoC1*$^{+/0}$ transgene muizen **(hoofdstuk 5)** en apo-C1$^{-/-}$ knockout-muizen **(hoofdstuk 6)** gebruikt.
In **hoofdstuk 5** hebben we voor het eerst aangetoond dat de expressie van humaan apoCI bij muizen de leer- en geheugenfuncties verstoort, wat bleek uit de resultaten voor objectherkenning staak “Morris water maze”. Deze effecten van apoCI op het

geheugen kunnen afhangen van de bekende inhiberende eigenschappen op het apoE-afhankelijke lipidemetabolisme. Er werden echter geen effecten van hapoC1 op de mRNA- of eiwitconcentraties van endogeen apoE in de hersenen gedetecteerd. Ook viel er geen verschil te onderscheiden in het algemene sterol- of fosfolipidenprofiel in de hersenen. Bovendien wees de analyse van genexpressieprofielen niet op globale veranderingen in het lipidemetabolisme of op ontstekingsprocessen in de hersenen. We hebben ontdekt dat het apoC1 eiwit hoofdzakelijk voorkomt in astrocyten en endotheelcellen in de hippocampus van hersenen met en zonder AD. Vreemd genoeg viel dit ook samen met de β-amyloïdplaques in hersenen van AD patienten. *In vitro* bleek dat het apoC1 eiwit de aggregatie van het Aβ-peptide remde. We concludeerden dat de overexpressie van humaan apoC1 de cognitieve functies bij muizen verstoorte en dat dit onafhankelijk is van effecten van apoE, wat er sterk op wijst dat apo-C1 een modulerende invloed heeft tijdens de ontwikkeling van AD.
Onze volgende stap was het testen van de hypotheses dat apo-C1-deficiëntie bij muizen de cognitieve functies zou verbeteren **(hoofdstuk 6)**. Tot onze verbazing bleek dat de afwezigheid van apo-C1 evenzeer de geheugenfuncties verstoort bij muizen. In vergelijking met hun wild-type broertjes vertoonden de apo-C1$^{-/-}$ muizen verstoorde hippocampusafhankelijke geheugenfuncties zoals bleek uit hun prestaties in objectherkenningstaken. Dit duidt wederom op een belangrijke rol voor apoC1 vij leer- en geheugen functies.
Hoofdstuk 7 geeft een overzicht van de vasculaire aspecten in de ontwikkeling van AD. Sinds enige tijd zijn cardiovasculaire aandoeningen, hypertensie en suikerziekte algemeen aanvaarde risicofactoren voor vasculaire dementie (VaD) en de kennis over hun rol in AD neemt ook toe.
De resultaten beschreven in dit proefschrift bieden nieuwe inzichten in de mechanismen die ten-grondslag liggen aan de regulatie van de apoE-gemedieerde cholesterol voorziening van neuronen door astrocyten. De data duiden erop dat 24(S)-hydroxycholesterol, de hersenspecifieke metaboliet van cholesterol, hierbij een sleutelrol speelt. Verder zijn er aanwijzingen dat niet alleen APOE4, maar ook het H2-allel van APOC1 een risicofactor is voor AD. We laten zien dat apoCI aanwezig is in plaques in AD hersenen en dat apoCI direct een interaktie kan aangaan met amyloid. Overexpressie van apoCI in muizen leidt tot verstoorde leer- en geheugenfuncties. Samengevat, leiden deze bevindingen tot meer inzicht in de rol van zowel apoE als apoCI in gezonde hersenen en bij de ontwikkeling van AD.
Tot slot worden in **hoofdstuk 8** de voornaamste bevindingen samengevat, besproken en in verband gebracht met de bestaande literatuur.

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Finally it's done! The Acknowledgements' chapter is not easy to write, but it is the most pleasant and popular chapter of the thesis. This part is usually read first and sometimes the only chapter one reads. No thesis is ever the product of a single person's efforts, and certainly this one was no different. I owe a great deal to colleagues, friends and members of my family who, through their own research, life experience, comments and questions have encouraged, supported and enlightened me. There are plenty of them who should be mentioned and as usual there is not enough room (or this Chapter would look more like a list from a telephone directory) to mention them all. Therefore, to begin with, I would like to thank all the people who are not named below but who have been very helpful in creating this thesis. However I would like to acknowledge some people personally.

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Curriculum Vitae

Karlygash Abildayeva was born on May 25th, 1971, in Almaty, Kazakhstan. She attended physico-mathematical high school in Almaty. After obtaining her high school diploma (with distinction) in 1988, she enrolled to study biology at Kazakh National State University in Almaty, Kazakhstan. Karlygash performed her Master's thesis on the G proteins expression in murine kidneys with nephrogenic diabetes insipidus at the Department of Physiology (Institute of Cytology and Genetics, Russian Academy of Sciences, Novosibirsk, Russia) under supervision of Prof. Dr. L. Ivanova and Dr. E. Solenov in 1993.

In 1993 she started a project on investigation of bacterial flora in contaminated soils from Kazakhstan oil sources under supervision of Prof. Dr. R. Bersimbayev at the Department of Molecular Biology and Genetics (Kazakh National State University, Almaty, Kazakhstan). The project has been continued further at the Department of Clinical Microbiology, Hadassah Medical School, Hebrew University, Jerusalem, Israel, supported by a grant #TA-MOU-CA 13-006 (US Agency for International Development, US-Israel CDR Program, Central Asian Initiative). The study topic was to develop phospholipids-liposomes for bioremediation of contaminated soils and to investigate molecular mechanisms of microbial degradation of aromatic hydrocarbons in collaboration with Prof. Dr. Y. Barenholz.

In 1995 Karlygash started to work as a research fellow at the Department of Endocrinology and Metabolism, Hadassah medical School, Jerusalem, Israel. The area of research interest was to characterize of functional domains of PDX-1-transcriptional activator of insulin gene.

In 2000 she got a position of a research assistant at the Department of Psychiatry and Neuropsychology, Maastricht University, The Netherlands.

The author started with her PhD research on the role of apoE in the brain in 2001. This work was supervised by Prof. Dr. F.Ramaekers, Dr. M. Mulder and Dr. J. de Vente.

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Apolipoprotein E production by astrocytes is regulated by 24(S)-hydroxycholesterol
3rd Dutch Endo-Neuro-Psycho Meeting 1-4 June 2004, Doorwert, The Netherlands (poster presentation)

ABBREVIATIONS

Aβ Amyloid beta
ABC A1, G1, G4 ABC-transporters
ACAT Acyl-CoA cholesterol acyltransferase
ACC1 Acetyl CoA carboxylase 1
AD Alzheimer's Disease
APP Amyloid Precursor Protein
ApoE Apolipoprotein E
ApoC1 Apolipoprotein C1
ApoD Apolipoprotein D
ATP Adenosine-Tri-Phosphate
ABC ATP-Binding-Cassette transporter

BBB Blood-Brain Barrier

CNS Central Nervous System
ChREBP Carbohydrate response element binding protein
CSF Cerebrospinal fluid
CYP46 Cholesterol 24(S)-hydroxylase

DG Dentate Gyrus
DIV Days in vitro

ECL Enhanced Chemmiluminescence

FAS Fatty acid synthase
FCS Fetal calf serum
FPLC

GC-MS Gass-Chromatography Mass-Spectrometry
GFAP Glial Fibrillary Acidic Protein

HO-1 Heme oxygenase 1
HMG-CoA 3-Hydroxy-3-Methylglutaryl-CoA
HMGCoAS HMG-CoA synthase
HDL High-Density Lipoprotein
HSPG Heparan Sulfate Proteoglycane

IDL Intermediate Density Lipoprotein
IL6 Interleukin 6

kD kiloDalton

LCAT Lecithin-cholesterol acyltransferase
LDL Low-Density Lipoprotein
LDLr LDL receptor
LPL Lipoprotein Lipase
LRP LDL-receptor related protein
LXR Liver X receptor

MVK Mevalonate kinase

iNOS Inducible nitric oxide synthase

NPC1L1 Niemann-Pick type C1-Like 1

PBS Phosphate Buffered Saline
PMVK Phosphomevalonate kinase
PPARα, β Peroxisome proliferators-activated receptors
PS1/2 Presenilin 1/2
PVDF Polyvinylidene

RT Room Temperature
RT-PCR Real-time-polymerase chain reaction
RXR Retinoid X Receptor

SCD Steary;-CoA desaturase
SDS-PAGE Sodium-Dodecyl-Sulfate polyacrylamide gel
SREBP 1a, 1c, 2 Sterol Regulatory Element Binding Proteins
SR-B1 Scavenger Receptor B Type I

TBS Tris Buffered Saline

VaD Vascular Dementia
VLDL Very Low-Density Lipoprotein