Interaction of amyloid precursor protein and lipid metabolism with relevance to Alzheimer's disease

Ph.D. Thesis

Annamária Bjelik

Alzheimer's Disease Research Center, Department of Psychiatry,

Albert Szent-Györgyi Center for Medical and Pharmaceutical Sciences,

University of Szeged

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Publications related to the subject of the thesis

I. **Bjelik A.**, Pákáski M., Bereczki E., Gonda Sz., Juhász A., Rimanóczy Á., Zana M., Janka Z., Sántha M., and Kálmán J. *APP mRNA splicing is upregulated in the brain of biglycan transgenic mice.* Neurochemistry International 2007, 50:1-4. **IF: 2.994**

II. **Bjelik A.**, Sántha M., Juhász A., Rimanóczy Á., Zana M., Csont T., Pákáski M., Bereczki E., Boda K., Fedinándy P., Dux L., Janka Z. and Kálmán J. *Human apoB overexpression and a high-cholesterol diet differently modify the brain APP metabolism in the transgenic mouse model of atherosclerosis.* Neurochemistry International 2006, 49:393-400. **IF: 2.994**

III. Juhász A., Rimanóczy Á., Vincze G., Szlávik G., Zana M., **Bjelik A.**, Pákáski M., Janka Z., and Kálmán J. *CYP46 T/C polymorphism is not associated with Alzheimer's dementia in a population from Hungary.* Neurochemical Research 2005, 30:943-948. **IF: 2.187**

Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
ANOVA	one-way analysis of variance
apoB-100	apolipoprotein B-100
apoE	apolipoprotein E
APP	amyloid precursor protein
APP membr	membrane-bound fraction of amyloid precursor protein
APP sol	soluble fraction of amyloid precursor protein
APPs-α	soluble fragment of amyloid precursor protein alfa
APPs-β	soluble fragment of amyloid precursor protein beta
AS	atherosclerosis
Aβ	amyloid beta
BACE	β-secretase
BBB	blood-brain barrier
BChE	butyrylcholinesterase
bg	biglycan
BW 284C51	1,5-bis(N-allyl-N,N-dimethyl-4-ammoniumphenyl)pentan-3-one dibromide
CAT	choline-acetyltransferase
CI	confidence interval
CNS	central nervous system
CNT	sex-matched controls
CSF	cerebrospinal fluid
CVD	cardiovascular disease
CYP46A1	cholesterol 24-hydroxylase
dCTP	deoxycytidine-5-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
HBD	heparin-binding domain
HC	high-cholesterol
HC Tg ($apoB^{+/+}$)	high-cholesterol diet apoB-100 transgenic mice
HC Tg ($apoB^{+/-}$, $bg^{+/-}$)	high-cholesterol diet apoB-100, biglycan transgenic mice
HC Wt	high-cholesterol diet wild-type mice
HDL	high-density lipoprotein
HRP	horseradish peroxidase
HSPG	heparan sulfate proteoglycan
IEL	internal elastic lamina
KPI	Kunitz-type protease
LDL	low-density lipoprotein
MDA	malondialdehyde
MMSE	mini-mental state examination
mRNA	messenger ribonucleic acid
NFTs	neurofibrillary tangles
OD	optical density
OR	odds ratio
oxLDL	oxidized -low-density lipoprotein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PMSF	phenylmethyl sulfonyl fluoride
QRT-PCR	quantitative real-time reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid

RNase	ribonuclease
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDS	sodium dodecylsulfate
SEM	standard error of mean
SMCs	smooth muscle cells
SNP	single nucleotide polymorphism
SPs	senile plaques
TBA	thiobarbituric acid
TBARS	thiobarbituric acid -reactive substances
TBST	Tris-buffered saline containing 0.02% Tween 20
TC	total cholesterol
T/C	thymine/ cytosine
Tg	transgenic
Tg ($apoB^{+/+}$) mice	apoB-100 transgenic mice
Tg ($apoB^{+/-}$, $bg^{+/-}$)] mice	apoB-100, biglycan transgenic mice
Tg ($bg^{+/+}$) mice	biglycan transgenic mice
VD	vascular dementia
Wt	wild-type

CONTENTS

1. Introduction

1.1. Pathomechanism of Alzheimer's disease

Alzheimer's disease (AD), the most common form of dementia in the western - world, currently affects 18 million people worldwide and has been predicted to almost triple by 2050 (Citron, 2004; Hebert et al., 2003). The prevalence of AD increases exponentially with age, with up to 15% of the population older than 65 years and 40% of those over 85 years affected (Zhu et al., 2004). It will be one of the major public health problems during the 21st century because of the increasing age of the elderly population. As populations age, diseases related to aging affect more people and are a source of growing concern.

AD is named after Alois Alzheimer, who in 1907 published an account of rapidly deteriorating mental illness in a 51-year old woman, August D. This disorder, which was later called as an eponyme Alzheimer's Disease, was for a long time not recognized as different from a common dementia. Studies since the 1980's however, have, revealed specific genetic and molecular mechanisms underlying the AD pathogenesis (Selkoe, 2002). AD is characterized by cognitive and emotional impairments associated with a progressive loss of memory, a decrease in linguistic abilities, visuospatial process impairment and personality changes. The history of the disease usually involves a progressive decline, although there may be short plateaus.

The complexity of the disease arises from the different risk factors (both genetic and environmental) that are involved in the development. The early-onset familial AD subtype, which accounts for less than 1-5% of all AD cases, is related to genetic mutations. Mutations have been identified on three genes: the amyloid precursor protein (APP) gene on chromosome 21, the presenilin 1 gene on chromosome 14 and the presenilin 2 gene on chromosome 1 (Suh and Checler, 2002). There are other genes that are considered susceptibility or risk factors for sporadic (late-onset) AD. These include apolipoprotein E (apoE) (Poirier, 1996), the K- variant of butyrylcholinesterase (BChE) (Lehmann et al., 1997) and several mitochondrial genes (Law et al., 2001).

Epidemiological studies have demonstrated the importance of risk factors for AD that include age, female gender, previous head injury and cardiovascular disease (CVD) (Law et al., 2001).

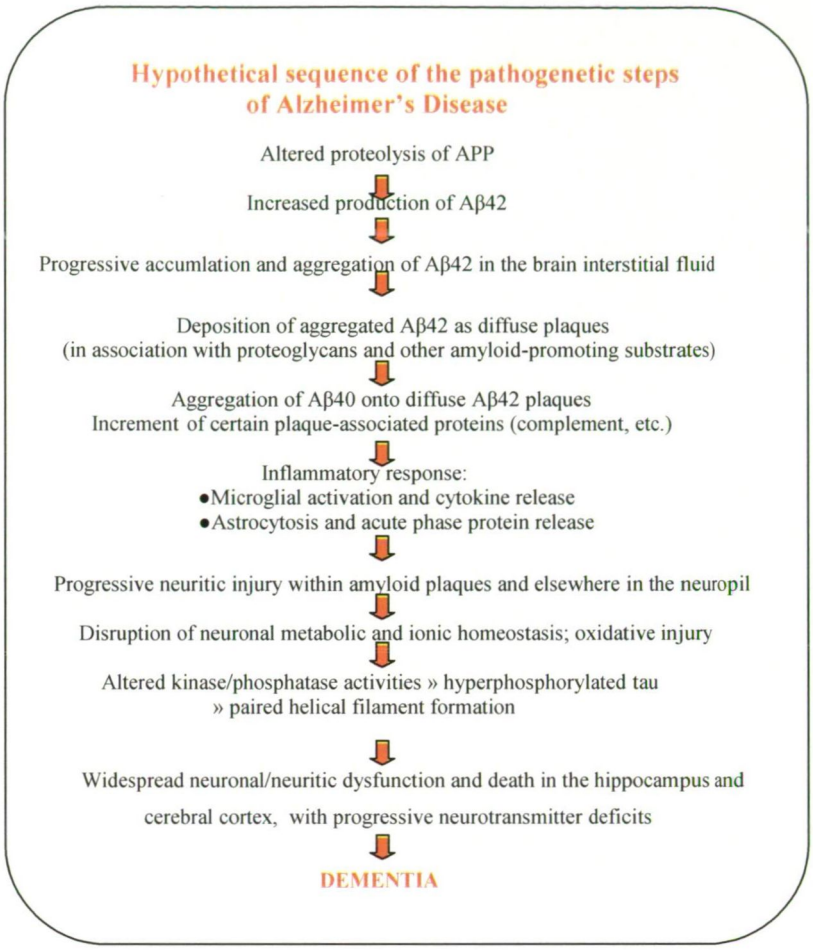

Fig. 1. *The sequence of pathogenic events leading to AD (modified after Hardy and Selkoe, 2002)*

1.2. The amyloid hypothesis

AD is neuropathologically characterized by progressive degeneration of the neuronal cells, particularly cholinergic, especially in the parietal, temporal and frontal cortex, basal forebrain system and hippocampus. The characteristic microscopic features of the disorder are the senile plaques (SPs), and the neurofibrillary tangles (NFTs) and vascular amyoid depositions (Selkoe, 1994). Additional features of AD are deficits in the multiple neurotransmitter signaling system and synaptic loss.

One of the hypotheses concerning the cause of AD, the amyloid cascade hypothesis, which has been widely accepted states that the neurodegenerative process is a series of events triggered by the abnormal processing of the APP (Hardy and Higgins, 1992) (*Fig. 1*). Accordingly, the major event leading to AD is the formation and aggregation in the brain of the amyloid β (Aβ) peptide, a proteolytically derived product of APP (Selkoe, 1994).

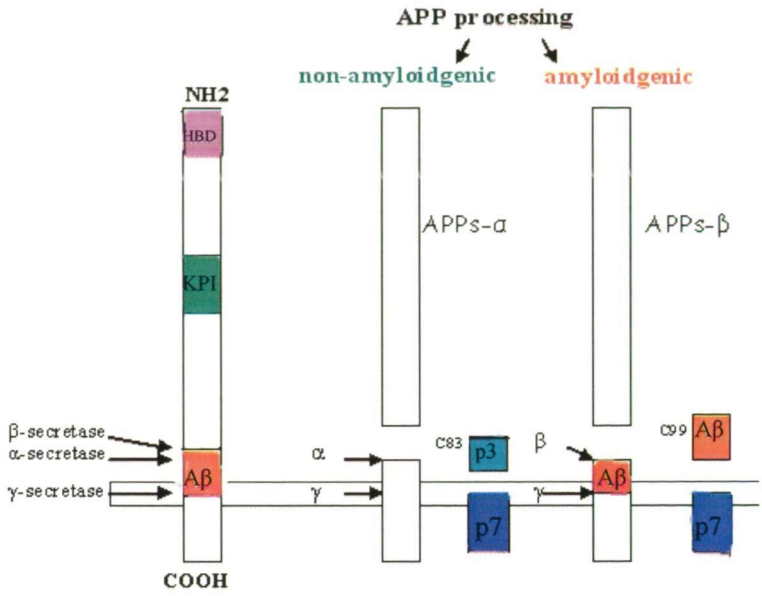

Fig. 2. *Physiological and pathological cleavage of APP*

APP is cleaved sequentially by α- and γ-secretases to produce a soluble version of APP (APPs-α), p3 and an 83-residue COOH-terminal fragment (C83). Alternatively, APP can be cleaved by β- and γ-secretases to generate Aβ, a soluble version of APP (APPs-β) and a 99-residue COOH-terminal fragment (C99).
HBD: heparin-binding domain, KPI: Kunitz-type inhibitor domain (modified after Hardy and Selkoe, 2002)

The Aβ peptide is the predominant constituent of SPs and also deposits in cerebral blood vessels, leading to cerebrovascular dysfunctions (vascular hypothesis of AD; Kalaria, 2002) (Glenner and Wong, 1984).

APP is an evolutionarily conserved protein which is ubiquitously expressed in human cells including central nervous system (CNS) neurons (Kang et al., 1987). It is a type I integral membrane glycoprotein, with a long luminal N-terminal segment, a single membrane-spanning region and a short intracellular tail. Due to the alternative splicing of three exons (7, 8 and 15), APP exists as several different isoforms, the most common being APP695, APP751 and APP770 (Goldgaber et al., 1987).

In the extracellular part of APP, binding domains for copper, zinc, heparin and collagen have been identified and the isoforms APP751 and APP770 also contain a KPI inhibitor domain (Kitaguchi et al., 1988). APP695, which lacks the KPI - insert, but contains a chondroitin sulfate glycosaminoglycan attachment site, is the splice product most common in neuronal cells (Kang et al., 1987). In the non-amyloidogenic processing pathway, the holo-APP is proteolytically processed by α-secretase, releasing the APPs-α and a membrane-bound C-terminal fragment (*Fig. 2*). α-Secretase cleaves within the Aβ region, precluding the generation of Aβ. The α-secretase activity has both constitutive and inducible components. The inducible α-secretase activity seems to be under the control of

second messenger cascades, such as protein kinase C (PKC) (Skovronsky et al., 2000). In the alternative, amyloidogenic pathway, cleavage in the transmembrane domain of APP by β-secretase (BACE) and an additional cleavage by γ-secretase result in secretion of Aβ. The proteolysis by γ-secretase leads either to a 40 amino acid containing peptide ($A\beta_{40}$) or a 42 amino acid containing isoform ($A\beta_{42}$). The longer and more hydrophobic $A\beta_{42}$ is much more prone to fibril formation than is $A\beta_{40}$ and is the major Aβ species in the SPs (Jarrett et al., 1993).

1.3. The cholinergic hypothesis

The cognitive deficits observed in AD are closely associated with losses in the cholinergic function of the brain, including a dramatic loss of choline-acetyltransferase activity (CAT, EC 2.3.1.6), acetylcholine (ACh) levels, the high-affinity choline uptake in the neocortex and hippocampus and a reduced number of cholinergic neurons in the basal forebrain and basal nucleus of Meynert (Cummings and Kaufer, 1996). As a neurotransmitter, ACh plays a critical role in learning and memory functions. It is synthesized from acetyl-CoA and choline by CAT and released into the synaptic cleft. Finally, it is degraded there by acetylcholinesterase (AChE, EC 3.1.1.7) into choline and acetic acid. Choline is taken up again into the presynaptic neurons for use in ACh synthesis (Fishman et al., 1986). BChE (EC 3.1.1.8), also known as pseudo- or non-neuronal cholinesterase, is very similar in sequence, tertiary structure and function to AChE (Saxena et al., 1999). BChE is expressed in most human tissues and can be also found in the human amygdala, thalamus and hippocampal formation (Darvesh et al., 2003; Kálmán et al., 2004), yet its function is unknown.

The AChE activity is reduced in AD overall (Fishman et al., 1986), though, it is increased in the SPs and NFTs in the early stages of the disease (Martzen et al., 1993). On the other hand, BChE activity in the brain increases with age over 60 years and it is further elevated in AD (Perry, 1980). It has been suggested that AChE may promote the aggregation of Aβ into a more toxic amyloid form (Munoz and Inestrosa, 1999). BChE is also associated with the SPs and NFTs and with amyloid angiopathy in AD (Mesulam and Geula, 1994).

It has additionally been suggested that BChE may be involved in the lipid metabolism (Kutty and Payne, 1994) since elevated serum BChE activity has been found in type IIa, IIb and IV hyperlipoproteinemias (Chu et al., 1978; Cucuianu et al., 1975; Jain et al., 1983). The results of animal experiments involving diabetic rats and mice, indicate

that BChE may have a role in the regulation of the lipoprotein metabolism (Kutty and Payne, 1994; Patel et al., 1990). It has further been reported that there is an association between the inheritance of apoE ε4 and the extent of the cholinergic dysfunction in AD (Cedazo-Minguez and Cowburn, 2001). ApoE ε4 allele carriers are reported to exhibit poorer responses to AChE inhibitors, as compared with apoE ε2-3-positive subjects (Soininen et al., 1995). Consequently, impaired lipid delivery, especially of cholesterol, has been proposed as one mechanism for the apoE association to the cholinergic system (Poirier et al., 1995).

1.4. The role of cholesterol in Aβ peptide generation

Cholesterol is an integral component of all eukaryotic cell membranes and is essential for normal cellular functions, including raft and caveola formation (Smart et al., 1994). In these capacities, cholesterol is essential for life. However, an elevated concentration of plasma cholesterol is a well-established risk factor for CVD, and the emerging evidence suggests that the cholesterol metabolism plays a direct role in the pathogenesis of AD (Sparks, 1997).

Cholesterol also affects physical properties of cell membranes, such as increasing order and rigidity and decreasing permeability and lateral diffusion (Yeagle, 1991). The compartmentation of the APP molecule within the cell membrane is regulated by the cholesterol content of the bilayers (Kálmán and Janka, 2005). It has been hypothesized that APP is present in two cellular pools, one associated with lipid rafts, in which Aβ is generated, and another cholesterol-poor membrane domain outside the rafts, where α-cleavage takes place (Simons et al., 1998). Hereby, the elevated cholesterol content of the cell membrane facilitates the β- and γ-cleavage of APP (Simons et al., 1998). Moreover, diet-induced hypercholesterolemia and atherosclerosis (AS) are both associated with β-amyloidosis in the transgenic (Tg) mouse model of AD (Li et al., 2003). Furthermore, there is evidence that cholesterol-lowering drugs can reduce the prevalence of human AD (Wolozin et al., 2000; Zandi et al., 2005) and beneficially interfere with the abnormal amyloid metabolism in Tg animal models of this type of dementia (Refolo et al., 2000).

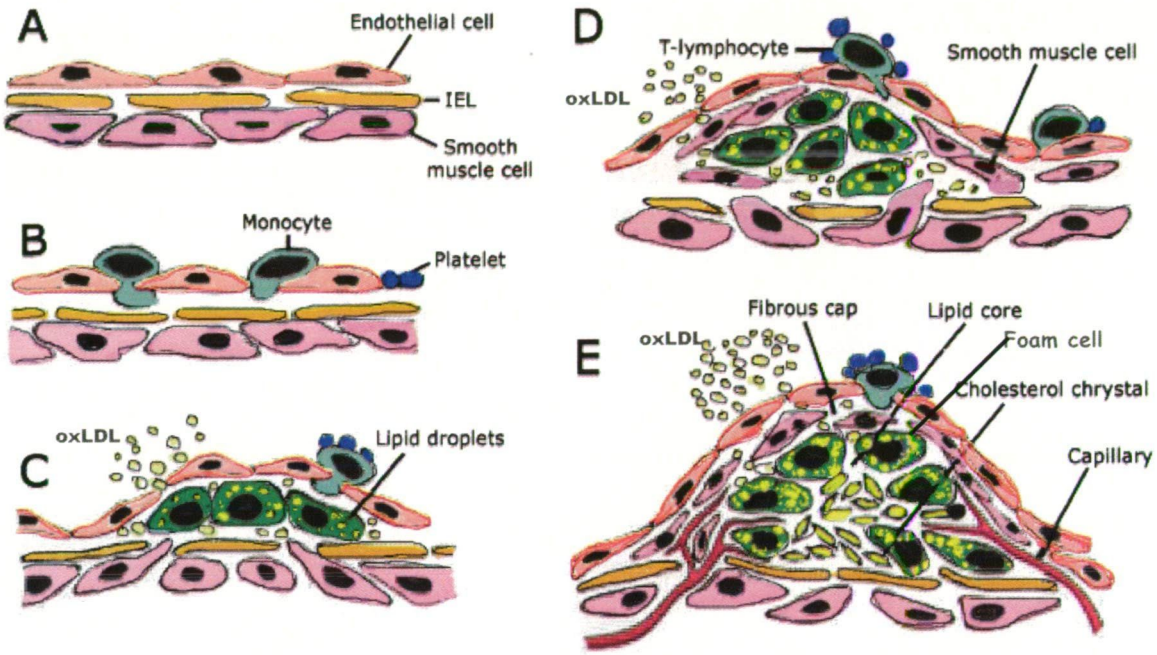

Fig.3. *Schematic diagram showing the formation of atherogenic plaque*
IEL: internal elastic lamina, oxLDL: oxidized -low-density lipoprotein (modified after Xu, 2006)

1.5 Common pathogenic factors in the formation of atherogenic plaque and Alzheimer's disease

1.5.1. The roles of cholesterol and apoB-100 in the formation of atherogenic plaque

In the periphery, apolipoprotein B-100 (apoB-100) is the major vehicle for cholesterol transport. It is synthesized in the liver and small intestine (Pitas et al., 1987). ApoB-100 is the key component in all classes of lipoproteins, including low density lipoprotein (LDL), intermediate-density lipoprotein and very low-density lipoprotein remnants considered to be atherogenic (Kim and Young, 1998).

The key event in the development of atherogenic plaque is the formation of fatty streaks, i.e. the subendothelial accumulation of lipid-laden foam cells (*Fig. 3*). This process begins as the subendothelial retention of cholesterol-rich, atherogenic lipoproteins, LDL and its oxidation into oxLDL. Unsaturated fatty acids are rapidly oxidized to lipid hydroperoxides, which are converted during the decomposition phase to a variety of other products, including reactive aldehydes (e.g. malondialdehyde [MDA]) (Esterbauer et al., 1987). Oxidatively modified LDL is rapidly taken up by macrophages. These aldehydes may react with lysine residues in the LDL apoB-100 moiety, resulting in a decreased affinity of apoB-100 for the LDL receptor and an increased affinity for scavenger receptors. This shift in receptor recognition leads to cellular uptake of the LDL by

receptors that are not regulated by the cholesterol content of the cell. The result is a massive accumulation of cholesterol in the macrophages and smooth muscle cells (SMCs). Such cholesterol-loaded cells have a foamy cytoplasm and develop the foam cells (Brown and Goldstein, 1990).

1.5.2. The role of platelet APP in the formation of atherogenic plaque

The Aβ and Aβ-like peptides are also present in human atherosclerotic plaques (De Meyer et al., 2002). The platelet phagocytosis that occurs within human atherosclerotic plaques can activate macrophages, and it has been suggested that the platelet constituent APP is involved (Jans et al., 2006). *In vitro* studies indicate that platelet phagocytosis also leads to macrophage activation and suggest that platelet-derived APP is proteolytically processed to Aβ, resulting in inducible nitric oxide synthase induction. Accordingly, the hypothesis has been suggested that platelet phagocytosis evokes macrophage activation via the proteolytic processing of platelet-derived APP, similar to APP processing in microglia in brain tissue. Moreover, it has been shown that the messenger ribonucleic acid (mRNA) of BACE is also expressed in human atherosclerotic plaques and macrophages (De Meyer et al., 2002). This hypothesis represents a novel mechanism for macrophage activation and provides a biochemical link between AS and AD, as proposed in the vascular hypothesis of AD by Kalaria (1996) and Farkas (2001).

1.5.3. Interaction between Aβ peptide and biglycan

Several lines of evidence suggest roles for biglycan in AS and it is also probably involved in the pathogenesis of AD. Biglycan, member of the small leucine-rich protein family (Olin et al., 2001) is believed to be a critical molecule in the initiation of AS. The biglycan binds and retentions via ionic interactions of apoB in the LDL and may be especially important for extracellular lipid accumulation in the endothelium. In the brain, biglycan is present in the cytoplasm of the neurons, in the pia mater, in the ependyma and also in the tela choroidea vascular endothelial cells (Stichel et al., 1995). It has been suggested that extracellular matrix signaling molecules, such as heparan sulfate proteoglycans (HSPGs), regulate APP expression (Scholefield et al., 2003). Furthermore, APP has at least four heparin-binding domains (Clarris et al., 1997) and highly sulfated HSPGs may promote the amyloidogenic cleavage of APP (Leveugle et al., 1997). It was demonstrated earlier that biglycan is derived from endothelial cells and binds Aβ with low affinity (Snow et al., 1995). The selective affinity of vascular cell-derived biglycan and

other proteoglycans for Aβ may account for the accumulation of Aβ in conjunction with biglycan in cerebrovascular amyloid deposits in AD brain.

1.6. Cholesterol homeostasis in the brain

1.6.1. Brain cholesterol metabolism

Cholesterol plays an essential role in the brain, eg. in synaptic plasticity (Koudinov and Koudinova, 2001), and optimal neurotransmitter release (Mauch et al., 2001; Yanagisawa, 2002). The CNS is unique as compared with other peripheral organs in regard of the cholesterol metabolism and cholesterol requirements (Dietschy and Turley, 2001) for the following reasons: First, it accounts for only 2% of the entire body mass, but it is very rich in cholesterol: about 25% of the total amount of unesterified cholesterol in the entire body is contained in the CNS. Second, the brain is considered to be isolated from the blood and other organs by the blood-brain barrier (BBB). Cholesterol is synthesized *de novo* by a endoplasmic reticulum rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (Brown et al., 1973), present in both the neuronal and glial cells.

1.6.2. Association of apoE polymorphism and Alzheimer's disease

In the normal brain, apoE is the principal cholesterol carrier protein; it is predominantly synthesized and secreted by astrocytes and microglia (Beffert et al., 1998). In humans, there are three common alleles of the apoE gene: ε2, ε3 and ε4. The protein isoforms produced by these alleles differ in the amino acids at positions 112 and/or 158: ε2 (Cys 112, Cys 158), ε3 (Cys 112, Arg 158), which is the most common, and ε4 (Arg 112, Arg 158), which is present in at least one copy in ~25% of the population (Tanzi and Bertram, 2001).

ApoE-mediated effluxes of cholesterol from both neurons and astrocytes have been shown to be isoform-dependent. Furtheremore, apoE ε2 is the most, and ε4 isoform is the least efficient at inducing cholesterol secretion (Michikawa et al., 2000). The binding of Aβ is also isoform-specific: ε2 > ε3 > ε4, thus permitting its aggregation and toxicity. In addition, both apoE ε3 and ε2 bind Aβ with higher affinities to facilitate clearance of the peptide following its release from the cells (Jordan et al., 1998). Accordingly, the polymorphism of the apoE protein, which plays roles in the redistribution of lipids and the control of cellular cholesterol homeostasis, is one of the major risk factors of AD.

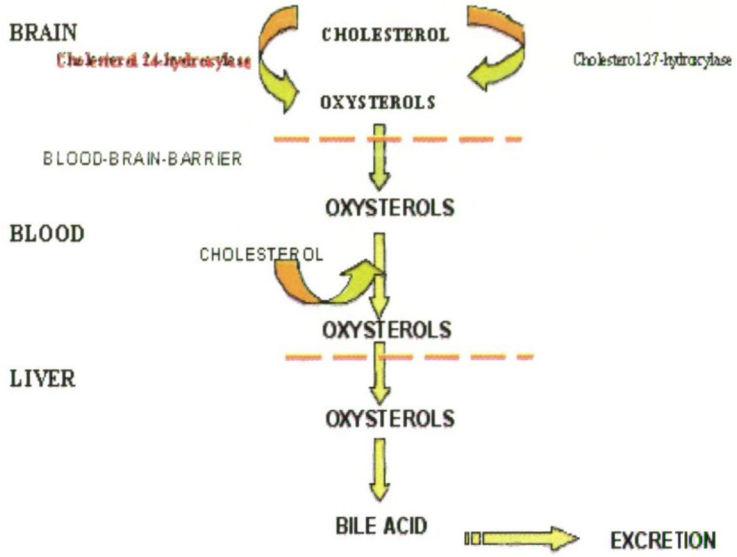

Fig.4. *Schematic diagram showing the origin and distribution of the oxysterols in the human body, (modified after Reiss et al., 2004)*

1.6.3. Removal of cholesterol from the brain and its relationship to Alzheimer's disease

Brain cholesterol is not removed via lipoproteins, but by conversion to 24-hydroxycholesterol, a soluble oxysterol that can diffuse across the BBB (*Fig. 4*). The enzyme suggested to perform this conversion is cholesterol 24-hydroxylase (CYP46A1), a new subfamily member of the cytochrome P450 enzymes. CYP46A1 is highly expressed in the brain (Lund et al., 1999), mainly in neurons in the cerebral cortex, hippocampus, and dentate gyrus (Papassotiropoulos et al., 2002), the same neurons that are preferentially targeted in AD. In the circulation, 24(S)-hydroxysterol is cleared in the liver, by conversion into 7α-hydroxylated intermediates. In addition to its role in cholesterol efflux, 24-hydroxycholesterol has a second potential role in the brain as it is a ligand for the nuclear hormone receptors liver X receptor, which are potent activators of several genes involved in the lipid metabolism (Bretillon et al., 2000).

Furthermore, a single nucleotide polymorphism (SNP), thymine/ cytosine (T/C), in the intron of CYP46A1 gene (located on chromosome 14q32.1), has been reported to be associated with an increased amyloid load in the brain of AD patients, together with elevated tau and amyloid levels in the cerebrospinal fluid (CSF) (Papassotiropoulos et al., 2003). In addition, Kolsch et al. (2002) found a C/T transition in intron 3, 43 base pairs upstream of exon 3 of CYP46A1. Carriers of the C allele of this polymorphism were more frequent in AD patients as compared with healthy controls, and carriers of the CC genotype had significantly higher 24-hydroxycholesterol/cholesterol ratios in the CSF than

carriers of the CT and TT genotypes. One possible explanation for the effect of CYP46A1 T/C polymorphism on the development of AD is the decreased activity of the enzyme expressed from the C variant of the gene, a subsequent increase in the cholesterol content of neuronal membranes leading to a shift to the amyloidogenic β-cleavage of the APP (Kálmán et al., 2004; Simons et al., 2001). The interaction of the CYP T/C SNP with the ε4 allele of the apoE gene has been proposed as a combined risk factor for AD (Wang et al., 2004).

1.7. Hypercholesterolemia and atherosclerosis. The Alzheimer's disease connection

Vascular risk factors, such as an elevated serum cholesterol level and hypertension, are shared risk factors for both AD, vascular dementia (VD) and AS (Engelberg, 2004). Moreover, VD frequently overlaps with primary neurodegenerative diseases, such as AD. The common cause of cognitive impairment and dementia is a chronically reduced blood supply to the brain, generally resulting from cerebral AS. In the circulation, the blood platelets are the major sources of APP and Aβ peptide released into the blood, which may trigger the key pathological events in AD pathogenesis (Chen et al., 1995). The Aβ peptides often form deposits in the walls of the cerebral capillaries, arterioles and arteries of AD patients, leading to cerebral amyloid angiopathy and, in some, hemorrhagic stroke (Ghiso and Frangione, 2001).

The main risk factors for AS are the high levels of both apoB-100 and LDL cholesterol in the plasma (Sniderman et al., 1980). The LDL cholesterol and apoB-100 levels are also increased in AD and correlate with the amount of Aβ deposited in the brain (Caramelli et al., 1999). In the normal brain, apoE and apolipoprotein A-I are the most abundant lipoproteins, with less apolipoprotein J (Raffai and Weisgraber, 2003) and a very limited amount of apoB (Pitas et al., 1987). In contrast, abnormal levels of apoB-100 and cholesterol accumulate in the brain of AD patients and have been found as core components of the mature plaques (Houlden et al., 1995; Mori et al., 2001; Puglielli et al., 2003). This discrepancy may be due to the disruption of the BBB which occurs in a number of pathological conditions, including stroke (Hulthe et al., 1997) and possibly AD (Skoog et al., 1998). Accordingly, these results suggested that AS involving hypercholesterolemia influences the risk of development of AD.

1.8. High-cholesterol diet and transgenic mouse model of atherosclerosis

Numerous animal species have been used to study the pathogenesis and potential treatment of the lesions of AS (Jawien et al., 2004). The most useful models have been restricted to relatively large animals, such as nonhuman primates, swine and rabbits (Drobnik et al., 2000). Rats and dogs are not good models for AS because they do not develop spontaneous lesions and require heavy modifications of the diet to produce vascular lesion (Drobnik et al., 2000). In recent years, in the number of *in vivo* studies used Tg mouse models to study atherogenic mechanisms because the mouse as a model supremely suffices for as criteria (Breslow, 1996). Furthermore, uniquely in mice, it is possible to knock out or replace endogenous genes; this is one of the main advantages of working with the mouse model.

Accordingly, in our experimental design, Tg mouse lines overexpressing the human apoB-100 and biglycan genes were generated. Among the inbred mouse strains, the strain C57B6 has been found to be the most susceptible to the development of AS lesions upon administration of a high-cholesterol (HC) diet (Jawien et al., 2004). Therefore, the apoB-100 Tg mice backcrossed twice with the C57/B6 x CBA strain was used in our studies.

On the other hand, it is important to acknowledge that many noteworthy differences exist between mice and humans. The serum lipid profile in mice is very different from that in humans. These rodents carry most of the cholesterol on high-density lipoprotein (HDL). Furtheremore, since they have low LDL cholesterol levels, they are protected against hypercholesterolemia and do not develop AS without dietary or genetic manipulations (Breslow, 1996). The human apoB-100 Tg mice has mildly increased LDL and total cholesterol (TC) levels, as confirmed by FPLC analysis, their lipoprotein profile showing a distinct LDL peak, in contrast with wild-type (Wt) mice, which have only a distinct HDL (Voyiaziakis et al., 1998). These mice also furnish a very diet-responsive AS model. AS is not observed when the mice are on a regular diet, but it develops in response to feeding with either the HC-diet or a western-type diet (Purcell-Huynh et al., 1995). This mouse model permits study of the APP metabolism due to the presence of the human apoB-100, which results in a "human-like" lipoprotein profile (Grass et al., 1995).

1.9. Serum and brain cholesterol. Possible connections

Several studies with various animal models, such as rabbits, guinea-pigs and Aβ-depositing Tg mice, have provided further evidence that changes in peripheral cholesterol

metabolism modulate the APP processing in brain, possibly as a result of alterations in the brain cholesterol content (Fassbender et al., 2001; Howland et al., 1998; Petanceska et al., 2002; Refolo et al., 2000; Refolo et al., 2001; Shie et al., 2002; Sparks, 1996). Accordingly, it has been demonstrated that hypercholesterolemia results in a significant increase in the accumulation of Aβ in double PSAPP Tg mice (Refolo et al., 2000). Treatment of Tg mice exhibiting an AD Aβ phenotype with the cholesterol-lowering drug BM15.766 results in hypocholesterolemia associated with reduced Aβ accumulation (Refolo et al., 2001). Furthermore, clinical and epidemiological studies have indicated that patients with elevated cholesterols level have an increased susceptibility to AD (Jarvik et al., 1994) and that the incidence of AD is higher in countries with HC and high-calorie diets (Kalmijn et al., 1997). Accordingly, in our studies we set out to prove that the alterations in the peripheral cholesterol influence the brain APP metabolism.

2. Aims

The aims of our studies were:

1. *1.1.* To examine whether a HC diet affects the serum TC, LDL cholesterol and MDA levels in Wt and Tg ($apoB^{+/+}$) mice.

1.2. To investigate how a HC diet and human apoB-100 gene overexpression, either individually or in combination, affect the brain APP metabolism in Tg mice.

1.3. To examine whether human biglycan gene overexpression can affect the brain APP metabolism in Tg mice.

1.4. To explore whether the individual and combined effects of a HC diet and overexpression of the human apoB-100 and biglycan genes modulate the brain APP metabolism in the double Tg mouse model.

1.5. To investigate whether the individual and combined effects of a HC diet or human apoB-100 and biglycan interact with the brain BACE and PKC levels in Tg mice.

2. To study whether a HC diet and overexpression of the human apoB-100 and biglycan genes influence the activity of two major ACh-degrading ezymes of the cholinergic system, AChE and BChE, in the serum, liver and brain of Tg animals.

3. *3.1.* To examine whether inheritance of the CYP46 C allele is associated with AD in a Hungarian cohort.

3.2. To determine whether an interaction exists between the apoE ε4 and CYP46 C alleles in this Hungarian population.

3. Materials and methods

3.1. Materials

The substances used in this study were (listed in alphabetical order): 1,5-bis(N-allyl-N,N-dimethyl-4-ammoniumphenyl)- pentan-3-one dibromide (BW 284C51) from Wellcome Research Labs (Beckenham, UK); anti-Alzheimer precursor protein A4 (Mab 22C11), anti-protein kinase C (anti-PKC) and -β-secretase (anti-BACE) polyclonal antibody from Chemicon International Inc. (Temecula, CA, USA); BioMax light autoradiographic film from Sigma–Aldrich (St Louis, MO, USA); *CfoI* from Sigma-Aldrich Ltd. (Poole, UK); cholesterol colorimetric assays from Diagnosticum Ltd. (Budapest, Hungary); ethopropazine from Wellcome Research Labs (Beckenham, UK); ethylenediaminetetraacetic acid (EDTA) from Sigma-Aldrich (St Louis, MO, USA); GeneStormR Expression-Ready clone expressing the human biglycan gene from Invitrogen (Carlsbad, CA); HMW-SDS marker kit from Amersham Pharmacia Biotech (Uppsala, Sweden); horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA); Hybond ECL nitrocellulose membrane from Amersham Pharmacia Biotech (Uppsala, Sweden); leupeptin from Sigma–Aldrich (St Louis, MO, USA); *Msp*I Gibco BRL (Paisley, UK); pepstatin A, phenylmethylsulfonyl fluoride (PMSF) from Sigma-Aldrich (St Louis, MO, USA); protease K solution from Boehringer Mannheim (Indianapolis, IN, USA); Ready-to-Go kit was from Pharmacia (Uppsala, Sweden); Rotor-Gene 2000 from Corbett Research (Sydney, Australia); ribonuclease (Rnase) from Sigma–Aldrich (St Louis, MO, USA); sodium dodecylsulfate (SDS) from Merck (Darmstadt, Germany); Standard Lipid Controls from Sentinel (Milano, Italy); Supersignal® West Pico Chemiluminescent Substrate from Pierce (Rockford, IL, USA); *Taq* DNA polymerase from Fermentas (St Leon-Rot, Germany); [α-32P]deoxycytidine-5-triphosphate (dCTP) was from Izotóp Ltd. (Budapest, Hungary); RevertAid™ First Strand cDNA Synthesis Kit from Fermentas (St Leon-Rot, Germany); Trizol reagent from Sigma-Aldrich (St Louis, MO, USA); Tween 20 from ICN Biomedicals (Eschwege, Germany); all other reagents were commercial products of analytical grade with the highest purity available.

3.2. Subjects

125 AD patients (46 males and 79 females; mean age 74.4 years; standard deviation (SD): 6.38 years) who met the National Institute of Neurological and

Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association (McKhann et al., 1984) and Diagnostic and Statistical Mannual of Mental Disorders 4th edition criteria for probable AD and 102 age- and sex-matched controls (CNT) (45 males and 57 females; mean age 73.5 years; SD 5.28 years) were enrolled in the study. The AD probands had late-onset (over the age of 65 years) sporadic type of AD. Their Mini-Mental State Examination (MMSE) (Folstein et al., 1975) scores were 17.3 ± 5.43 points (mean ± SD). The CNT group had significantly (p=0.0001) higher MMSE scores, (28.3 ± 1.01 points, mean ± SD). All subjects were Hungarian and Caucasian. All venous blood samples for genotyping were obtained in accordance with the written informed consent procedures approved by the local ethics committee (Institutional Review Board of the University).

3.3. Transgenic animals and diet

Fertilized oocytes from Wt female mice (C57/B6 x CBA F1) were collected and injected with purified P1-phagemid deoxyribonucleic acid (DNA) containing the entire 43 kb human apoB-100 gene in a concentration of 1 ng/ml and a GeneStorm^R Expression-Ready clone expressing the human biglycan gene in a concentration of 2 ng/μl, using the standard pronucleus microinjection technique (Hogan et al., 1996). This GeneStorm^R Expression-Ready clone construct involves human biglycan cDNA fused to a cytomegalovirus promoter, a V5 epitope and a 6X His Tag sequence at the 3' end of the cDNA. A 3440 bp fragment including the transcription unit was separated from the vector backbone.

Microinjected eggs were implanted into the oviduct of pseudopregnant Swiss female mice. Tg founders were identified by using polymerase chain reaction (PCR) analysis and dot blot hybridization on tail DNA samples. The apoB-100 Tg mice and the biglycan Tg mice were homozygous for the human apoB-100 and biglycan transgene. The primers for PCR analysis are shown in *Table 1*. For dot blot hybridization, a 824 bp, Nru I-Hind III fragment was labeled with [α-^{32}P] dCTP and hybridized to 5 μg of genomic DNA. All animal experiments were performed in accordance with institutional guidelines.

Transgene expression from different tissues (liver, brain, heart and muscle) of Tg mice was tested by using quantitative reverse transcriptase PCR (QRT-PCR) and in the case of Tg apoB-100 the clone 485 and in the case of Tg biglycan the clone 1052, which expressed the human apoB-100 or the biglycan mRNA at the highest level, was selected for further study.

Table 1. Primers for RT-PCR and QRT-PCR

Targets for RT-PCR	Forward primer	Reverse primer	Expected size (bp)	Reference
APP695	5'-GCACTAACTTGCACG-ACTATGGCATGCTGCTG-CCCTG-3', 500-537 bp	5'-GCTGGCTGCCGTCGTGG-GAACTCGGACTACCTCCT-CCACA-3', 861-901 bp	242	(Shivers et al., 1988; Kang et al., 1987, Sola et al., 1993)
APP770	5'-CTACCACTGAGTCT-GTGGAG-3', 848-868 bp	5'-CTTGAGTAAACTTTGGG-ATGACACGCTGCCACAC-ACCGCC-3', 1028-1068 bp	401	
β-actin	5'-GTGGGCCGCTCTA-GGCACAA-3', 25-45 bp	5'-CTCTTTGATGTCACGCA-CGATTTC-3', 564-540 bp	537	
apoE	5'-TCCAAGGAGCTGCA-GGCGGCGCA-3'	5'-ACAGAATTCGCCCCGG-CCTGGTACACTGCCA-3'	217	(Crook et al., 1994)
CYP46A1	5'-TGA AAA CGA GTTTCC- CGT CC-3'	5'-GTG TGA CCA GGT AA-CAGT CA-3'	285	(Borroni et al., 2004)
Targets for QRT-PCR				
apoB-100	5'-CCCAAGAGGTATTTAA-AGCCATT-3'	5'-CGTAGCACCTCTGTGGT-CTTG-3'	Designed by our group.	
biglycan	5'-GGA CTC TGT CAC ACC- CAC CT-3'	5'-AGC TCG GAG ATGTCG- TTGTT- 3'		
β-actin	5'-CCAGCAGATGTGGATC-AGCA-3'	5'-CTTGCGGTGCACGAT-GG-3'		

Homozygous apoB-100 ($apoB^{+/+}$) and biglycan ($bg^{+/+}$) Tg mice were crossed in order to obtain hemizygous double Tg ($apoB^{+/-}$ x $bg^{+/-}$) littermates. Both the Wt F1 and the Tg F1 generation mouse lines were backcrossed twice with the C57B6 strain in order to approach closer to the susceptible genetic background. The 6-week-old Wt, Tg ($apoB^{+/+}$) and double Tg ($apoB^{+/-}$, $bg^{+/-}$) mice were fed with a standard laboratory diet supplemented or not with 2% cholesterol (5 mice/group) for 17 weeks under standard laboratory conditions (Csont et al., 2002). Accordingly, we used seven animal groups: Wt, apoB-100 Tg [Tg ($apoB^{+/+}$)], biglycan Tg [Tg ($bg^{+/+}$)], apoB-100, biglycan Tg [Tg ($apoB^{+/+}$, $bg^{+/+}$)] mice and their matches with a HC diet: HC Wt, HC Tg ($apoB^{+/+}$) and HC Tg ($apoB^{+/+}$, $bg^{+/+}$). The animals were anesthetized after the treatment period and blood samples were obtained intracardially. Brains were removed without perfusion and the whole cerebral cortex was isolated and stored at -70 °C until used. The experimental protocol was approved by the local animal care ethical committee.

3.4. Selection of the transgenic apoB-100 and biglycan mouse lines

The apoB-100 gene was detected by means of QRT-PCR, and the mouse β-actin gene was applied as an internal control. One Tg line (No. 485) exhibited a very high expression of human apoB-100 transgene (arbitrary rate: 0.78-fold) in the liver. In the case of biglycan, the clone 1052 expressed a very high amount of the human biglycan gene. These Tg mouse lines (No. 485 and No. 1052) were therefore used to generate the double Tg ($apoB^{+/-}$ x $bg^{+/-}$) mice.

3.5. DNA extraction from white blood cells

DNA was isolated from the white blood cells by using the anticoagulated, EDTA-treated whole blood samples. The blood samples were stored at -70 °C. Frozen samples were thawed at 4 °C, incubated with a 10-fold volume of lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na_2EDTA, pH 8.2) at 4 °C for 5 min and centrifuged at 15 000 x *g* for 20 min. After removal of the supernatant, the pellet was washed twice with phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 5 000 x *g* for 10 min at 4 °C. The cell lysates were digested overnight at 55 °C with 0.2 ml of 10% SDS and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na_2EDTA). After digestion was complete, the resuspensed pellet was extracted with equal volumes of phenol by vigorous shaking for 5 min, and centrifugation at 6 000 x *g* for 10 min at 4 °C. The precipitated protein pellet was discardedand the supernatant was extracted twice with equal volumes of chloroform–isoamyl alcohol (24:1) by vigorously shaking for 5 min and centrifuged at 6 000 x *g* for 10 min at 4 °C. To the aqueous supernatant, was added one-tenth volume of 3.0 M sodium acetate, and the DNA was precipitated with ethanol at -20 °C with inversion of several times. The precipitated DNA strands were removed with a glass pipet and were washed once with 70% alcohol. The DNA pellet was resuspended in Trishydrochloride buffer (containing 10 mM Tris-HCl, 0.2 mM Na_2EDTA, pH 7.5). The DNA was allowed to dissolve for 2 h at 37 °C, followed by digestion with a 100-fold volume of RNase (1000 μg/ml). The concentration was determined by ultraviolet spectrophotometry.

3.6. ApoE and CYP46 genotyping

3.6.1. ApoE genotyping

The apoE genotyping were determined by PCR from genomic DNA with the specific primers (*Table 1)*. The final volume of PCR solution was 25 µl, containing 20 µM of two primers, 1.25 µl of each, 50-300 ng of genomic DNA, 1.25 µl of deoxynucleoside triphosphates (dNTPs) (20 mM), consisting of a mix of 5 mM of each, 1.5 µl (25 mM) of $MgCl_2$, 2.5 µl (5%) of dimethyl sulfoxide, and 0.5 U of *Taq* DNA polymerase, in 67 mM Tris-HCl buffer (pH 8.8). The initial denaturation was 5 min at 95 °C, followed by 30 cycles of 30 s at 94 °C denaturation, 22 s at 63 °C, and annealing and extension for 30 s at 72 °C. A final extension for 3 min at 72 °C completed the amplification procedure. The amplified DNA was digested with 5 U of *Cfo*I overnight at 37 °C, and the DNA fragments (91, 81, 72 and 48 base pairs) were separated on 8% non-denaturing acrylamide gel. The gel was stained with 0.5 µg/ml of the ethidium bromide, and the apoE genotype was determined by the pattern of DNA fragments presented.

3.6.2. CYP46 genotyping

The 285 bp PCR product containing CYP46A1 was determined by PCR from 100 ng of genomic DNA with the specific primers (*Table 1)*.

The final volume of PCR solution was 25 µl, containing 0.2 µM of the two primers, 1 µl of each, 0.25 µl of dNTPs (200 µM), consisting of a mix of 5 mM of each, 1.5 µl of (25 mM) $MgCl_2$, and 0.2 µl of *Taq* DNA polymerase (1 U), in 67 mM Tris-HCl buffer (pH 8.8). After the initial denaturation at 95 °C for 5 min, the reaction mixture was subjected to 32 cycles of 30 s denaturation at 95 °C, 30 s annealing at 53 °C, and 30 s extension at 72 °C, followed by a 10 min extension step at 72 °C. Amplified products were digested by *Msp*I restriction enzyme. A 209 bp and a 76 bp fragments were recognized as C, while the 285 bp undigested product corresponded to the T allele, respectively. The results of amplification and the digestion fragments were revealed by 2% and 3% agarose gels with 0.5 µg/ml of ethidium bromide, respectively.

3.7. Determination of serum lipid and cholesterol levels in Wt and Tg ($apoB^{+/+}$) mice

Blood samples of Tg mice were obtained intracardially and the serum lipids, TC and LDL cholesterol levels were measured in triplicate, using commercially available colorimetric assays adapted to a 96-well plate kit. LDL cholesterol was determined

following the selective protection of LDL. The accuracy of the assays was monitored by using standard lipid controls. Results are expressed as mM/l of serum.

3.8. Measurement of serum malondialdehyde levels in Wt and Tg (apoB$^{+/+}$) mice

Thiobarbituric acid (TBA)-reactive substances (TBARS) were assayed in order to measure the level of lipid peroxidation (Buege and Aust, 1978). Serum samples were mixed with 2 volumes of a stock solution of 15% w/v trichloroacetic acid, 0.375% w/v TBA and 0.25 M HCl, heated for 30 min at 95 °C. After cooling and centrifugation at 1000 × *g* for 10 min, the supernatants containing TBARS were measured spectrophotometrically at 535 nm. Freshly diluted tetramethoxypropane, which yields MDA, was used as external standard. Results are expressed as nmol MDA/ml serum.

3.9. Measurement of AChE and BChE activities in the serum, liver and brain of Wt and Tg (apoB$^{+/+}$) mice

AChE and BChE activities were determined by spectrophotometric assay (Ellman et al., 1961). BW 284C51 (10^{-5} M) and ethopropazine (10^{-4} M) were used as specific AChE and BChE inhibitors, respectively. Each sample was measured in triplicate, with an intra-assay variation of less than 10%. Protein was measured by the dye-binding assay of Bradford (1976). The enzyme activities are expressed as nmol substrate hydrolyzed/min/mg protein.

3.10. Quantitation of brain APP mRNAs by the reverse transcriptase polymerase chain reaction

Total cellular ribonucleic acid (RNA) was extracted from the brain cortex of Wt and Tg mice with the guanidium thiocyanate phenol/chloroform method (Chomczynski and Sacchi, 1987). Five μg of total mRNA was transcribed into cDNA with oligo $(dt)_{18}$ primers. The cDNA was amplified by PCR with oligonucleotides for β-actin, APP770 and APP695 (539, 401 and 242 bp of PCR products, respectively). Primer sequences are shown in *Table 1*. Reactions were performed with a My Cycler™ Thermal Cycler. PCR was carried out in a final volume of 25 μl, containing 2.5 μl of 10x PCR buffer, 2 μl of 25 mM $MgCl_2$, 4 μl of 5 mM dNTP, 3 pmol of primers specific for β-actin and APP695, and 10 pmol of primers for APP770, 2 μl of the template cDNAs described above and 0.25 μl of *Taq* DNA polymerase (5 units/μl). Thirty cycles consisted of denaturation (30 s at 95

°C), annealing (30 s at 55 °C) and extension (1 min at 72 °C). PCR products were separated by 1.3% agarose gel electrophoresis. The ratios APP695/β-actin and APP770/β-actin mRNA were calculated.

3.11. Quantitation of human apoB-100 and biglycan mRNAs by the real-time reverse transcriptase polymerase chain reaction

The expression of the human apoB-100 and the human biglycan transgenes were detected by using QRT-PCR. The total RNA from the liver of Tg mice was purified with Trizol reagent and reverse transcribed (RevertAid™ First Strand cDNA Synthesis Kit). Primer sequences for QRT-PCR are shown in *Table 1*. PCR reactions were run on a Rotor-Gene 2000, using the green dye SYBR to detect double-stranded products. Curves were analyzed with Rotor-Gene software, using dynamic tube and slope correction methods, data from cycles close to the baseline being ignored. Relative expression ratios were normalized to β-actin. The analysis of the results was performed by the Pfaffl method (Pfaffl, 2001).

3.12. Sample preparation for Western blotting

For APP and PKC detection, five cortices of Wt and Tg mice from each group were homogenized in 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 2 mM PMSF, 2 mM EDTA, 2 μg/ml of leupeptin and 1 μg/ml of pepstatin by using a glass-teflon potter (1500 rpm, 1 min). The cortical homogenates were centrifuged at 100 000 x *g* for 30 min at 4 °C. The supernatant represented the soluble fraction, while the pellet was resuspended in the same volume of buffer containing 1% SDS and centrifuged as before. The new supernatant represented the membrane fraction.

For BACE level measurement, the soluble and membrane-bound fractions were not separated. Cortices were homogenized in 50 mM Tris buffer pH 7.5 containing 0.15 M NaCl, 2 mM PMSF, 2 mM EDTA, 2 μg/ml of leupeptin, 1 μg/ml of pepstatin and 1% SDS. The homogenates were centrifuged at 10 000 x *g* for 30 min at 4 °C. The supernatants were used for protein assay (Hess et al., 1978)

3.13. Western blotting

The cortical protein samples were loaded onto 9% SDS-polyacrylamide gels. After the electrophoresis, proteins were electrotransferred to nitrocellulose membranes by using

the BioRad Mini Protean II System. Blocking was carried out with Tris-buffered saline containing 0.02% Tween 20 (TBST) supplemented with 5% non-fat dry milk solution. For the labeling of APP, PKC and BACE, monoclonal 22C11 antibody (5 μg/ml; against residues 68-81 of APP), monoclonal PKC antibody (1:2000) and BACE polyclonal antibody (1:1000) were applied. After three washes with TBST, sheep anti-mouse IgG-HRP (1:1000), or goat anti-rabbit IgG-HRP (1:5000) was added for 1 h. The nitrocellulose membrane was washed again three times with TBST. In the cases of APP, PKC and BACE, the bands were detected with the Supersignal® West Pico Chemiluminescent Substrate and then exposed to Kodak autography film. The optical densities (ODs) of immunoreactive bands were quantified by means of the National Institutes of Health Image Analyzer System.

3.14. Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Results were considered to be significantly different at a probability level of $p<0.05$.

The Western blotting, RT-PCR, serum lipid, MDA levels, and AChE and BChE activity values of the examined groups were compared by one-way analysis of variance (ANOVA), with the Sidak probe for pairwise comparisons.

The differences between the mean age and MMSE values of the AD probands were tested by the Mann-Whitney U test. Hardy-Weinberg equilibrium of the examined genes was calculated by the chi square (χ^2) test. Gender differences and allele frequencies were compared with the Pearson χ^2 test. In the case of tables with small-expected frequencies, *p*-values were also computed by using exact methods (i.e. Fisher's test for 2 x 2 tables). The χ^2 test was used to determine whether gender is associated with the CYP46A1 T/C SNP. Odds ratios (ORs) as estimates of the relative risk of disease were calculated according to the Wilson formula (Wilson, 1927) for the 95% confidence intervals (CIs) of the percentages of CYP46 C/T genotypes and alleles. A logistic regression model was used to test for the interaction between the CYP46 C and apoE ε4 alleles.

4. Results

4.1. Serum lipid and TC levels in the Wt and Tg (apoB$^{+/+}$) mice

The HC Tg (apoB$^{+/+}$) animals had a significantly higher serum TC level (4.8 ± 0.17 mM/l) as compared with the Wt, the Tg (apoB$^{+/+}$) and the HC Wt groups (3.1 ± 0.17 mM/l, p=0.047, 3.2 ± 0.44 mM/l, p=0.048, and 3.1 ± 0.32 mM/l, p=0.049, respectively). The serum TC and LDL levels were not altered in the HC Wt mice as compared with the normal diet-fed Wt animals. The HC diet significantly increased the serum concentration of atherogenic LDL cholesterol in the HC Tg (apoB$^{+/+}$) mice (2.3 ± 0.29 mM/l) as compared with the Wt, the Tg (apoB$^{+/+}$) and the HC Wt groups (1.1 ± 0.14 mM/l, p=0.004, 1.2 SEM ± 0.15 mM/l, p=0.019 and 1.5 ± 0.05 mM/l, p=0.05, respectively).

4.2. Serum malondialdehyde levels in Wt and Tg (apoB$^{+/+}$) mice

The MDA levels were significantly higher in the HC Wt (45.6 ± 1.5; p=0.006) and in the HC Tg (apoB$^{+/+}$) (48.2 ± 2.2; p=0.006) mouse groups than in the Wt group (34.2 ± 3.02, p=0.008). The MDA level was not altered in the Tg (apoB$^{+/+}$) animals (39.8 ± 3.2, p= 0.34).

Table 2. *Effect of HC diet on serum, liver and brain AChE and BChE activities in the Wt, Tg (apoB$^{+/+}$), Tg (bg$^{+/+}$) and Tg (apoB$^{+/-}$, bg$^{+/-}$) Tg mice*

	Wt (n=5)	**Tg (apoB$^{+/+}$)** (n=5)	**Tg (bg$^{+/+}$)** (n=5)	**Tg (apoB$^{+/-}$, bg$^{+/-}$)** (n =5)	**HC diet-fed Wt** (n=5)	**HC diet-fed Tg (apoB$^{+/+}$)** (n=5)	**HC diet-fed Tg (apoB$^{+/-}$, bg$^{+/-}$)** (n=5)
Serum							
AChE	5.11±0.9	2.75±0.1	2.84±0.2	5.09±0.9	6.24±0.8	2.63±0.2	5.31±0.8
BChE	55.4±1.5	49.3±1.8	52.0±2.2	46.8±3.7	58.4±1.1	48.8±2.4	52.6±5.2
Liver							
AChE	0.70±0.1	0.94±0.6	0.93±0.6	0.62±0.6	0.90±0.1	0.88±0,1	0.55±0.9
BChE	15.0±0.6	19.1±0.8*	17.6±1.6	14.1±0.8	15.6±0.4	17.0±1.2	15.5±0.4
Brain							
AChE	165.0±1.4	153.7±2.6	151.5±2.9	166.9±2.7	157.9±1.3	165.4±5,7	168.9±5.2
BChE	1.04±0.1	0.97±.03	1.03±0.9	0.90±0.2	0.97±0.1	0.85±0.8	0.80±0.7

AChE and BChE activities were expressed in nmol/min/mg protein. The values are expressed as means ± SEM. Asterisks (*) indicate $p<0.05$.

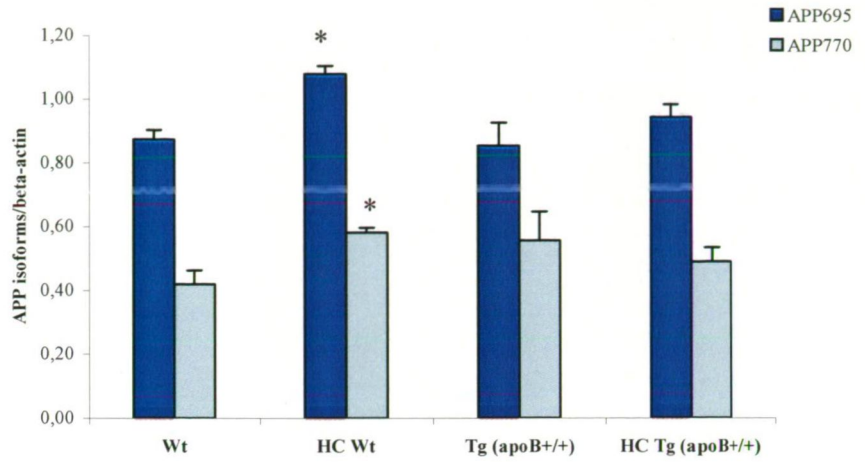

Fig. 5. *APP/β-actin ratios in the normal diet-fed Wt, HC Wt, the Tg (apoB$^{+/+}$) and the HC Tg (apoB$^{+/+}$) mice. The ratios of OD values are expressed as means ± SEM (n=5). Asterisks (*) indicate p<0.05.*

4.3. Effects of HC diet on AChE and BChE activities in the serum, liver and brain of Wt and Tg (apoB$^{+/+}$) mice

The BChE activity was significantly increased in the liver the Tg (apoB$^{+/+}$) group (19.1 ± 0.98 nmol/min/mg protein) as compared with the Wt group (16.0 ± 0.46 nmol/min/mg protein, p<0025) (*Table 2*). We did not find any other statistically significant differences between the different groups as concerns other examined tissues.

4.4. Effect of HC diet on the cortical APP metabolism

The HC diet significantly increased the APP695 (123%, p=0.027) and APP770 (138%, p=0.042) mRNA levels in the cortex as compared with the Wt animals (*Fig. 5*). The HC diet decreased the APP levels in the soluble fraction (87%, p=0.006) in the Wt animals, while the membrane-bound APP levels increased significantly (114%, p=0.036) (*Fig. 7*).

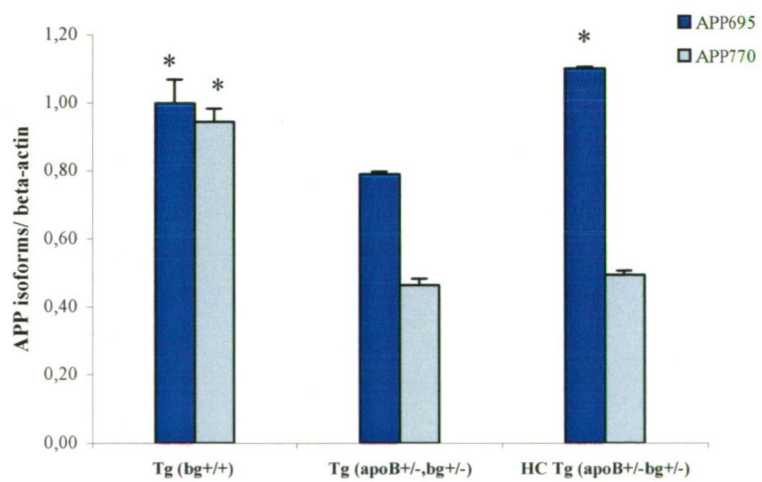

Fig. 6. *APP/β-actin ratios in the Tg (bg$^{+/+}$), the Tg (apoB$^{+/+}$bg$^{+/+}$) and the HC Tg (apoB$^{+/-}$bg$^{+/-}$) mice. The ratios of OD values are expressed as means ± SEM (n=5). Asterisks (*) indicate p<0.05.*

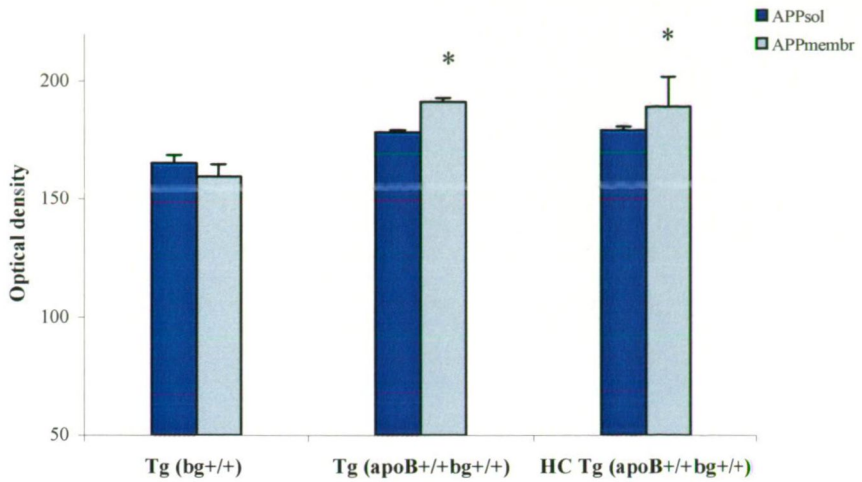

Fig. 8. *OD values of the APP isoforms in the APPsol and the APPmembr fractions. Semiquantitative evaluation of the Western blots of the Tg ($bg^{+/+}$), the Tg ($apoB^{+/+}$,$bg^{+/+}$) and the HC Tg ($apoB^{+/-}$,$bg^{+/-}$) mice. Values are expressed as means ± SEM (n=5). Asterisks (*) indicate $p<0.05$ as compared with the Wt group.*

4.7. Combined effect of the human apoB-100 and biglycan transgenes on the cortical APP metabolism

The APP695 mRNA levels were not altered in the cortex of the double Tg ($apoB^{+/-}$, $bg^{+/-}$) mice relative to the Wt animals (*Fig. 6*). A slight, statistically not significant increase in the level of APP770 mRNA could be observed in the cortex of the Tg ($apoB^{+/-}$, $bg^{+/-}$) mice as compared with the Wt animals *(Fig. 6).* Quantitative analysis of immunoblots demonstrated that the membrane-bound APP isoforms in the membrane-bound fraction were significantly increased (122%, p=0.035) in the Tg ($apoB^{+/-}$, $bg^{+/-}$) mice as compared with the Wt mice (*Fig. 8*). No differences were found in the soluble fraction of the cortex of the Tg ($apoB^{+/-}$, $bg^{+/-}$) mice (*Fig. 8*).

4.8. Combined effect of HC diet with the human apoB-100 transgene on the cortical APP metabolism

The common effect of the HC diet and the overexpression of apoB-100 did not significantly alter the cortical APP695 and APP770 mRNA levels in any of the studied groups (*Fig. 5*). Quantitative analysis of the Western blots demonstrated that the HC diet significantly increased (171%, p=0.0001) the levels of the membrane-bound APP isoforms in the HC Tg ($apoB^{+/+}$) animals relative to the Tg ($apoB^{+/+}$) group. In the HC Tg ($apoB^{+/+}$) mice, the membrane-bound APP isoforms were also increase (134%, p=0.004) relative to the Wt controls (*Fig. 7*).

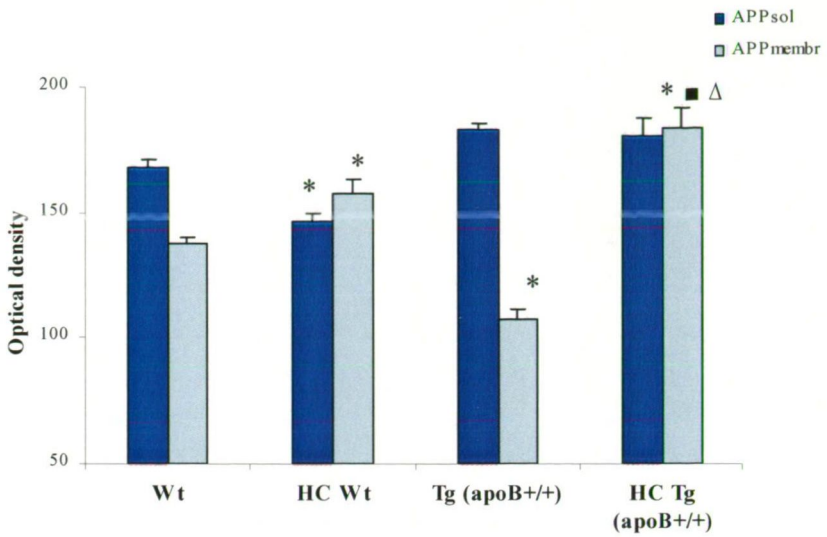

Fig.7. *OD values of the APP isoforms in the soluble (APPsol) and the membrane-bound (APPmembr) fractions. Semiquantitative evaluation of the Western blots of the Wt, the HC Wt, the Tg (apoB$^{+/+}$) and the HC Tg (apoB$^{+/+}$) mice. Values are expressed as means ± SEM (n=5). Asterisks (*) indicate p<0.05 as compared with the Wt group; the square (■) indicates p<0.05 as compared with the Tg (apoB$^{+/+}$) group; and the triangle (Δ) indicates p<0.05 as compared with the HC Wt group.*

4.5. Effect of the human apoB-100 transgene on the cortical APP metabolism

We did not observe significant differences in the levels of the mRNA isoforms in the cortex of the Tg (apoB$^{+/+}$) mice as compared with the Wt animals (*Fig. 5*). The levels of the membrane-bound APP isoforms were significantly decreased in the Tg(apoB$^{+/+}$) animals (78%, p=0.005) relative to the Wt group (*Fig. 7*). The APP levels in the soluble fraction of the cortex were not altered in the Tg (apoB$^{+/+}$) mice (*Fig. 7*).

4.6. Effect of the human biglycan transgene on the cortical APP metabolism

Significantly increased APP695 mRNA isoform levels (122%, p=0.012) were observed in the cortex of the Tg (bg$^{+/+}$) mice as compared with the Wt animals (*Fig. 6*). The extent of the increase in the APP770 mRNA level was much higher, since the biglycan transgene caused a 1.5-fold elevation (p=0.0001) relative to the control value (*Fig. 6*). No significant changes were found in the APP isoforms in either the soluble or the membrane-bound fraction in the cortices of the Tg (bg$^{+/+}$) mice (*Fig. 8*).

However, the HC diet did not have a significant effect on the levels of the APP isoforms in the soluble fraction of the cortices of the HC Tg (apoB$^{+/+}$) group as compared with those in the Tg (apoB$^{+/+}$) mouse and the Wt mouse group (*Fig. 7*).

4.9. Combined effect of HC diet, and the human apoB-100 and biglycan transgenes on the cortical APP metabolism

The APP695 isoforms were significantly (125%, p=0,002) increased while the APP770 mRNA level remained constant in the HC Tg (apoB$^{+/-}$, bg$^{+/-}$) mouse group relative to the Wt animals (*Fig. 6*).

The overexpression of the human apoB-100 and biglycan significantly (121%, p=0.001) increased the levels of the membrane-bound APP isoforms in the Tg (apoB$^{+/-}$, bg$^{+/-}$) mice (*Fig. 8*). The combined effect of the HC diet and the overexpression of the human apoB-100 and biglycan also significantly (119%, p=0.003) increased the levels of the membrane-bound APP isoforms relative to the Wt animals (*Fig. 8*). In the HC Tg (apoB$^{+/-}$, bg$^{+/-}$) group, the HC diet did not have a significant effect as compared with that in the soluble fraction of the cortices in the Wt controls (*Fig. 8*).

Table 3. *Effects of HC diet on the PKC and the BACE levels in cerebral cortex in the Wt, apoB Tg (apoB$^{+/+}$), [Tg (bg$^{+/+}$)], and Tg (apoB$^{+/-}$, bg$^{+/-}$) mouse groups*

	Wt (n=5)	Tg (apoB$^{+/+}$) (n=5)	Tg (bg$^{+/+}$) (n=5)	Tg (apoB$^{+/-}$, bg$^{+/-}$) (n =5)	HC diet-fed Wt (n=5)	HC diet-fed Tg (apoB$^{+/+}$) (n=5)	HC diet-fed Tg(apoB$^{+/-}$, bg$^{+/-}$) (n=5)
PKC levels (OD)							
Soluble fraction	159.8±5.3	151.5±4.0	158.3±5.6	162.7±4.8	162.9±12.1	170.0±14.7	143.5±8.9
Membrane-bound fraction	170.1±7.1	171.9±6.5	162.2±4.9	149.7±9.7	177.7±3.0	150.8±9.7	168.9±6.5
BACE levels (OD)	154.1±4.3	133.3±2.7	149.5±11.4	159.6±8.6	141.2±5.5	147.9±9.0	139.3±12.3

OD: Optical density. Values are expressed as means ± SEM.

4.10. PKC and BACE levels after dietary and genetic manipulations

In order to investigate the possible regulation of the APP metabolism, the PKC and BACE protein levels were also monitored in the cortical samples. The HC diet did not change either the PKC (in the soluble or the membrane-bound fractions) or the BACE levels as compared with the Wt group (*Table 3*). In the case of overexpression of the human apoB-100 and biglycan genes, either individually or combined, no significant changes were found in either the soluble or the membrane-bound PKC levels. Similarly to

the PKC results, the BACE levels remained constant in each of the examined groups (*Table 3*). No differences were found either in the soluble or in the membrane-bound PKC and the BACE levels when the HC diet-fed Tg ($apoB^{+/+}$) and the HC Tg ($apoB^{+/-}$, $bg^{+/-}$) animals were examined (*Table 3*).

4.11. ApoE polymorphism in a Hungarian cohort of Alzheimer's disease patients

ApoE genotype and allele frequencies are presented in *Table 4*. The apoE allele frequencies were in Hardy-Weinberg equilibrium both in the CNT (χ^2=9.04, df=5, p=0.107) and AD groups (χ^2=4.837, df=5, p=0.436). The ε4/4 genotype and ε4 allele were over-represented in the AD group as compared with the CNT group (8% vs. 3.9% and 23.2% vs. 11.3%, respectively).

Table 4. *ApoE genotypes and allele frequencies in CNT subjects and AD patients*
Figures in parentheses indicate frequencies (percentages {95% CI by Wilson's formula})

apoE genotypes*	CNT (n = 102) (n % {CI})	AD (n = 125) (n % {CI})
2/2	-	1 (0.8% {0.004-5.0})
2/3	14 (13.7% {7.9-22.3})	10 (8% {4.1-14.6})
2/4	-	1 (0.8% {0.004-5.0})
3/3	69 (67.6% {57.5-76.4})	66 (52.8 {43.7-61.7})
3/4	15 (14.7% {8.73-23.4})	37 (29.6% {21.9-38.5})
4/4	4 (3.9% {1.26-10.3})	10 (8% {4.1-14.6})
Allele**		
ε2	14 (6.9% {3.9-11.5})	13 (5.2% {2.9-8.9})
ε3	167 (81.9% {75.7-86.6})	179 (71.6% {65.5-77.0})
ε4	23 (11.3% {7.4-16.6})	58 (23.2% {18.2-29.0})

**CNT vs. AD Pearson χ^2=12.409, df=5, p=0.03, exact p=0.015*
***CNT vs. AD Pearson χ^2=11.029, df=2, p=0.004.*

4.12. CYP46 T/C polymorphism in a Hungarian cohort of Alzheimer's disease patients

There was evidence of deviation from Hardy-Weinberg equilibrium in the case of CYP46 T/C polymorphism, when the AD population alone was examined (χ^2=7.671, df=2, p=0.022). However, no significant difference was found in the case of the CNT group (χ^2=4.92, df=2, p=0.085). CYP46 T/C genotype and allele frequencies are presented in *Table 5*. No significant differences were observed between the distributions of CYP46 T/C genotypes and alleles, when the CNT and AD groups were compared (*Table* 5).

***Table* 5.** *CYP46 genotypes and allele frequencies in CNT subjects and AD*
Figures in parentheses indicate frequencies (percentages, {95% CI by Wilson's formula})

CYP46 T/C genotypes*	CNT (n = 102) (n % {CI})	AD (n = 125) (n % {CI})
T/T	50 (49% {39.5-58.6})	54 (43.2% {34.8-51.9})
T/C	49 (48% {38.5-57.6})	66 (52.8% {44.1-61.3})
C/C	3 (3% {1.0-8.2})	5 (4% {1.7-9.0})
Allele**		
T	149 (73% {65.5-78.6})	174 (70% {63.6-74.9})
C	55 (27% {21.3-33.4})	76 (30% {25.0-36.3})

**CNT vs. AD Pearson χ^2=0.845, df=2, p = 0.655, exact p=0.712*
***CNT vs. AD Pearson χ^2=0.647, df=1, p = 0.421, exact p=0.466, OR=0.845, 95% CI (0.561, 1.274)*

4.13. The interaction between the apoE ε4 and CYP46 C alleles

Table 6 lists thes frequencies and ORs for the interaction between the CYP46 C and apoE ε4 alleles in the CNT and AD groups, divided into subgroups according to the apoE status (one or two ε4+ or ε4- carriers). The OR for the presence of one or two CYP46 C without the

ε4 allele was 1.628 (95% CI: 0.864-3.066) as compared with subjects with neither ε4 nor CYP46 C. Since the OR 3.714 (95% CI: 1.549-8.908) for the presence of ε4 without CYP46 C was similar to the OR 3.492 (95% CI: 1.401-8.707) for those with both ε4 and CYP C, we can exclude the synergistic effect of the two SNPs on the risk of AD in the examined population.

Table 6. *ORs for interaction between CYP46 C and apoE ε4 alleles in AD and CNT subjects*

apoE ε4 allele	CYP46 C allele	CNT n (%)	AD n (%)	OR (95% CI)	*p*
-	-	40 (39.2)	28 (22.4)	reference	
-	+	43 (42.2)	49 (39.2)	1.628 (0.864-3.066)	0.131
+	-	10 (9.8)	26 (20.8)	3.714 (1.549-8.908)	0.003
+	+	9 (8.8)	22 (17.6)	3.492 (1.401-8.707)	0.007

Model χ^2=13.176, df=3, p=0.004.

There were no significant differences in mean age by groups of CYP46 T/C alleles ($F(2,220)=0.657$, $p=0.519$), nor by groups of disease ($F(1,220)=2.015$, $p=0.157$). The interaction was not significant between the age and the CYP46 T/C alleles ($F(2,220)=0.818$, $p=0.442$). Gender was not significantly associated with CYP46T/C SNP either in the CNT group ($\chi^2=1.121$, df=2, $p=0.571$, exact $p=0.564$), or in the AD group ($\chi^2=0.231$, df=2, $p=0.891$, exact $p=0.897$).

5. Discussion

5.1. Effects of HC diet on the serum TC and LDL cholesterol and MDA levels in Wt and apoB-100 Tg mice

The Wt mouse lines are resistant to the effect of the HC diet. These animals are therefore protected against diet-induced hypercholesterolemia and do not develop AS (Breslow, 1996). However, the mouse Wt (C57BL/6) line used in our study is the most susceptible to the development of AS lesions upon administration of a HC diet (Jawien et al., 2004). In our experimental design, Tg mouse lines overexpressing the human apoB-100 were generated by using this HC diet-susceptible Wt (C57BL/6) mouse line.

In accordance with the expected results, we found a relative resistance in the serum TC and LDL cholesterol levels of the Wt animals to the HC diet, whereas in response to the HC diet, the serum TC and LDL cholesterol levels were increased in the apoB-100 Tg mice, due to the fact that cholesterol was carried by their human apoB-100-containing lipoproteins. These and previous observations indicate that the reason for the serum TC and LDL cholesterol differences between the Wt and the Tg mice may be their different lipoprotein profiles and cholesterol metabolisms (Kim and Young, 1998; Purcell-Huynh et al., 1995).

In addition, a previous study revealed that the MDA levels were increased in both the plasma and the aorta after feeding with the HC diet (1% w/w) for 2 months (Balkan et al., 2002). In our study, the HC diet resulted in concomitant increases in serum markers of oxidative stress in the Wt and the apoB-100 Tg animals. Furthermore, several studies have confirmed that the HC diet is accompanied by an increased oxLDL level (Berliner et al., 1995; Schwenke and Carew, 1989; Young and Parthasarathy, 1994). Our finding is consistent with an earlier observations of significant increases in the levels of MDA in mice fed with cholesterol (1.5%) for a period of 6 weeks and after a cholesterol-treatment period of 8 weeks in Watanabe heritable hyperlipidemic rabbits (Lauridsen and Mortensen, 1999). Moreover, others found that the brain MDA was increased in New Zealand white rabbits that received 1% cholesterol plus regular chow (Aydemir et al., 2000). Our findings indicate that cholesterol feeding influenced the prooxidant-antioxidant status in the organism. Unfortunately, no data are available regarding the effect of a HC diet on the MDA level in the brain in the experimental model of AD.

5.2. Isolated and combined effects of HC diet and human apoB-100 gene overexpression on cortical APP metabolism in Tg mice

We found that, despite the reported relative resistance in the serum cholesterol levels of the Wt (C57BL/6) animals, the HC diet increased the levels of both APP695 and APP770 mRNAs in their cortex, which remained on the same level even in the apoB-100 Tg group. In the only published study on primary glial and neuronal cell lines, cholesterol treatment significantly decreased the total APP mRNA levels (Galbete et al., 2000). However, no synergistic effect was found in the case of the APP mRNA levels when the two atherogenic factors, the HC diet and the overexpression of the human apoB-100 gene, were applied together in our study. No other data are available regarding the effect of the overexpression of the human apoB-100 gene on cerebral APP mRNA levels. It is not known why the HC treatment did not further increase the APP mRNA levels in the apoB-100 Tg animals. Further investigations are necessary to study the different regulatory mechanisms of gene transcription.

Additionally, we investigated the extent of translation of the total APP products by immunoblotting in the soluble and membrane-bound fractions of the cerebral cortex. The membrane-bound APP levels were significantly elevated by the HC diet in both the Wt and the apoB-100 Tg animals. It is noteworthy, however, that in response to the HC diet the membrane-bound APP levels were even more elevated in the apoB-100 Tg and in the double Tg mice than in the Wt animals.

The results presented here indicate that the levels of the APP isoforms in the soluble fraction decrease in response to a HC diet. This perturbation may be a result of the stiffening of the cell membrane by the cholesterol load, possibly inhibiting the lateral movement of the APP and its cleaving enzymes and decreasing the required contact between the secretases and their APP substrate (Bodovitz and Klein, 1996). In addition, APPs-α may serve as a neuroprotective agent against hypoglycemic damage and oxidative toxicity (Araki et al., 1991; Mattson et al., 1993), and its reduction could exacerbate cell death in AD (Bodovitz and Klein, 1996). In conclusion, the overexpression of the human apoB-100 gene did not change the APP metabolism, whereas the HC diet individually and in combination with the overexpression of the human apoB-100 gene could alter the mode of APP processing into the route of Aβ production.

5.3. Human biglycan gene overexpression interferes with the cortical APP metabolism in Tg mice

It is tempting to speculate that biglycan may promote the extracellular lipid accumulation and formation of the oxidatively modified LDL in the endothel. As a consequence, it is proposed that this interaction may contribute to the processes of AS and subsequent VD and AD. Our results indicated that biglycan increased the APP770 mRNA level relatively more than the APP695 mRNA level in the cortex. This is an important finding, since a similar tendency was observed in the AD brain. Earlier reports suggested that isoforms containing KPI are preferentially expressed in the AD brain, whereas APP695 is reduced (Rockenstein et al., 1995). Other authors have also demonstrated that the APP-770/APP-695 mRNA ratio is generally increased during AD (Johnson et al., 1990). However, according to our findings overexpression of the human biglycan did not have a significant effect on the levels of the APP isoforms in either the soluble or the membrane-bound fractions in the cortices of the biglycan Tg mice. It is not known why the overexpression of the human biglycan did not alter the levels of the APP isoforms. Biglycan may also be important in modifying APP mRNA alternative splicing in the brain. Further experiments are required to study which regulatory mechanisms of gene transcription are followed.

5.4. Atherogenic risk factors modulate the cortical APP metabolism in the double Tg mouse model of atherosclerosis

The overexpression of the human apoB-100 and biglycan genes and the HC diet increase the membrane-bound APP levels in the double Tg mice. To best of our knowledge, this is the first study in which the interaction between the HC diet and the apoB-100 and biglycan in the transgenic mice was examined. The human apoB-100 and biglycan extra genes or the HC diet together might therefore have a direct or indirect synergistic influence on the subcellular localization of the APP isoforms. One possible explanation for the increased amount of APP isoforms in the membrane-bound fractions is that the BACE activity might be changed in both the HC diet-fed Wt, the apoB-100 Tg and the double Tg mice. Taken together, the HC diet individually and in combination with the overexpression of the human apoB-100 and biglycan genes may promote the abnormal processing of APP and probably leads to the formation of Aβ.

5.5. Atherogenic risk factors do not interact with the brain PKC and BACE levels

It was previously found that BACE is up-regulated by increased intracellular cholesterol levels, and down-regulated by the inhibition of cholesterol biosynthesis *in vitro* (Sidera et al., 2005). Surprisingly, the BACE levels were not altered in the cortices of the animals treated with the HC diet and the peripheral overexpression of the human apoB-100 or biglycan genes. One possible explanation for this discrepancy is that the previous results were obtained from different model systems. Another possible explanation is that not the BACE levels *per se,* but rather the activity of this membrane-integrated protein was modified by the treatment applied in the present study.

PKC is known to be a regulator of the α-secretory proteolytic processing of the APP molecule (Skovronsky et al., 2000). In our experiment, the levels of the APP isoforms in the soluble fraction were found to be decreased by the HC diet in the Wt animals. For this reason decreased cortical PKC proteins would also be expected. In contrast, there were no changes in either the soluble or the membrane-bound fractions in the studied groups. These findings indicate that neither the HC diet nor the increased expression of the human apoB-100 and biglycan genes is likely to interfere with the APP metabolism through the PKC signal transduction pathway.

5.6. Atherogenic risk factors do not influence the serum, liver and brain AChE and BChE activities of Tg animals

AChE may also be involved in the lipid metabolism and the BChE activity is positively correlated with the serum triglyceride, cholesterol and apoB levels (Alcantara et al., 2002). It has also been reported that there is an association between the inheritance of the apoE ε4 allele and the extent of cholinergic dysfunction in AD (Cedazo-Minguez and Cowburn, 2001). These studies suggest that mice deficient in apoE may exhibit impaired central cholinergic function (Anderson and Higgins, 1997). We, therefore investigated the effects of the HC diet on the AChE and BChE levels in the brain, liver and serum. However, our results did not confirm the earlier observations. No other *in vivo* data are available regarding the effects of the overexpression of the human apoB-100 or the biglycan genes on the AChE and BChE levels. It is unlikely, therefore, the examined dietary and genetic atherogenic risk factors interfere with the activities of the enzymes responsible for the degradation of ACh.

5.7. The polymorphism of CYP46 A1 is not associated with Alzheimer's disease in the Hungarian population

Although the CYP46 gene is a good candidate for potential involvement in the proposed pathomechanism of AD, we provide here evidence that the intron 2 and T/C SNP do not confer susceptibility to this type of dementia in the Hungarian ethnic group. Other studies have reported similar findings, a negative association between CYP46 T/C polymorphism and AD (Chalmers et al., 2004; Desai et al., 2002; Kabbara et al., 2004; Kolsch et al., 2002) in different African-American and Caucasian populations from North America and Europe. The opposite finding, a positive association of the CYP46 polymorphism, was found to be associated with AD in independent populations and different ethnic groups from Asia and Europe (Borroni et al., 2004; Combarros et al., 2004; Johansson et al., 2004; Kolsch et al., 2002; Wang et al., 2004). The discrepancy between the different studies may be a result of ethnic or methodological differences or both.

5.8. No interaction exists between the apoE ε4 and CYP46 C alleles in the Hungarian Alzheimer's disease population

There is a synergistic increase in the risk of AD in persons who carry CYP46C and apoE ε4 alleles. Those carrying CYP46C and apoEε4 variants are almost 10 times more likely to develop AD than those with neither variation (Papassotiropoulos et al., 2003). Our case-control study was therefore performed on subjects from Hungary, to test the interaction of these alleles in AD.

It is pertinent to mention here that the normal Hungarian population is considered to differ from other European ethnic groups in connection with another risk gene for AD, the apoE ε4 allele distribution (Hallman et al., 1991). Perhaps this is the reason why the frequency of the allele apoE ε4 was lower (23%) even in the Hungarian AD population in the present study than is other AD groups reported from Europe. However, the proportion of the ε4 allele carriers (11%) was still significantly higher in the Hungarian AD population than in the CNT group. The percentage distribution of the apoE genotypes and frequencies in our present investigation confirmed the findings of our previous studies on different control and AD groups from the same country (Kálmán et al., 1998; McConathy et al., 1997). Our findings are not in accord with a previous report where the CYP46 C and ε4 alleles together are risk factors for AD (Borroni B. 2004). In summary, our data

indicate that it is unlikely that the intron 2 CYP46 T/C polymorphism, either alone or together with the ε4 allele, is a risk factor for AD in the Hungarian population.

6. Limitations

6.1. One possible limitation of the present study is that we did not determine the brain cholesterol levels in the Wt and the Tg animals. Recent evidence has been reported, however, that marked changes can be induced by a 2% HC diet in the fatty acid composition of the phospholipids of the brain of C57/B6 x CBA mice (Puskás et al., 2004).

6.2. Another weakness of our work was that the HC diet was not applied to the biglycan Tg mice. Further investigations are therefore necessary with the overexpression of biglycan Tg mice after feeding with a HC diet.

6.3. An additional limitation of our SNP studies may be the relatively small number of the examined Hungarian population.

7. Summary of the major findings and conclusions

7.1.1. The Wt (C57BL/6) mice exhibited a relative resistance to the effect of the HC diet. The

significantly elevated serum MDA levels indicate more oxidized lipids which might play role in the formation of AS plaque.

Due to the overexpression of the human apoB-100, the HC diet increased the TC and the LDL cholesterol levels in the Tg ($apoB^{+/+}$) mice. The increased MDA level in the Tg ($apoB^{+/+}$) mice denotes oxidative stress and lipid peroxidation caused by the HC diet.

7.1.2. The HC diet increased the brain APP695 and APP770 mRNA levels in the Wt mice,

whereas the HC diet also increased the membrane-bound fraction of APP in the Wt and

Tg ($apoB^{+/+}$) animals. These results indicate that the changes in peripheral cholesterol metabolism individually and in combination with the overexpression of the human apoB-100 gene may result in an elevated cholesterol content of the cell membrane and therefore alter the gene expression of APP and/or facilitate the β- and γ-cleavage of APP. However, the overexpression of the human apoB-100 gene individually did not interfere with the APP metabolism.

7.1.3. The human biglycan gene overexpression influences the gene expression of APP. Overexpression of this transgene increased the APP770 mRNA level relatively more than the APP695 mRNA level in the cortex of the Tg ($bg^{+/+}$) mice.

7.1.4. Individually, overexpression of the human apoB-100 and biglycan genes increased the membrane-bound fraction of APP in the double Tg mouse model of AC. The combined effect of the human apoB-100 and biglycan or the HC diet also increased the APP695 mRNA and the membrane-bound fraction of APP. These results indicate that the HC diet and these atherogenic factors possibly enhance the gene expression of APP and/or facilitate its β- and γ-cleavage.

7.1.5. The BACE levels were not altered in the cortices of the animals treated with the HC diet or by the overexpression of the human apoB-100 or biglycan genes. The HC diet and the increased expression of human apoB-100 and biglycan genes are not likely to interfere with the APP metabolism through the BACE levels or the PKC signal transduction pathway.

7.2. Overexpression of the human apoB-100 and biglycan genes or the HC diet did not influence the AChE and BChE activities in the serum, liver and brain of the Tg animals. It is unlikely, therefore, that under these conditions either the overexpression of the human apoB-100 and biglycan genes or the HC diet modulates the activities of these enzymes.

7.3.1. Inheritance of the CYP46A1 C allele was not associated with AD in the examined Hungarian population. Accordingly the polymorphism of CYP46 A1 is unlikely to confer a higher risk of sporadic AD in this ethnic group.

7.3.2. We did not observe an interaction between the apoE ε4 and CYP46 C alleles in the examined Hungarian population. However, the proportion of the ε4 allele carriers was still significantly higher among the AD probands. Hence, it is unlikely that intron 2 CYP46 T/C polymorphism, either alone or together with the ε4 allele, is a risk factor for AD in the Hungarian population.

In our study, a new transgenic mouse (apoB-100 and biglycan) model of AS was developed. This mice should furnish a possibility for study of the the role of the HC diet, AS and the APP metabolism in the brain. Furthermore, these transgenic animal models could facilitate future investigations of the interaction between the APP metabolism and

the cholesterol metabolism in the CNS, and might provide further information relating to pathomechanism of AD. Focusing on the relationship between cholesterol and APP metabolism would be important not only for etiological, but also for therapeutic purposes. Our study has confirmed the concept that anti-atherogenic therapy, including dietary regimens, may be effective in the prevention and treatment of such type of dementia.

Further, in the examined Hungarian group we demostrated that inheritance of the apoE E4 allele is an independent risk factor for AD, but we could not confirm the same effect in the case of another gene (CYP46) associated with lipid metabolism.

The improved knowledge resulting from such studies may promote an understanding of the function of the HC diet in the progress of AD, and may facilitate the development of drugs associated with the lipid metabolism as potential candidates for the treatment of AD.

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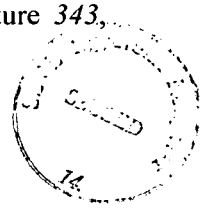

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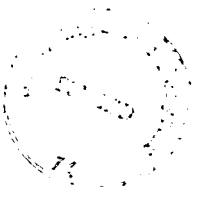

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