STUDIES ON APOLIPOPROTEIN E AND HIGH CHOLESTEROL DIET AS RISK FACTORS FOR NEURODEGENERATION

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TO

My father for his uncompromising principles that guided my life

My mother for leading her children into intellectual pursuits

ABSTRACT

Elevated level of plasma cholesterol is a well-established risk factor for cardiovascular disease, and emerging evidence suggest that it plays a role in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD). The relations between dietary habits and neurodegeneration are complex, as the brain is selectively separated from peripheral circulation by the blood brain barrier (BBB). Brain cholesterol is mostly independent from dietary uptake or hepatic synthesis and is almost completely synthesized *in situ*. The enzymatic conversion of cholesterol into 24S-hydroxycholesterol, which fluxes across the BBB into the circulation is the recognized pathway for the elimination and the homeostasis maintenance of brain cholesterol. Apolipoprotein E (apoE) is the major cholesterol transporter in the central nervous system (CNS). From the three human isoforms of apoE (E2, E3 and E4), the presence of apoE4 is the most important known genetic risk factor for sporadic AD and it has also been linked to many other forms of neurodegeneration. The mechanisms by which apoE4 increases the risk for neurodegeneration are largely unknown, but it has been hypothesised that apoE4 may have a deficient function compared with other isoforms. In general, there is a lack of knowledge of how different risk factors affect neurodegenerative disorders, and also of the possible synergistic effect of several risk factors contributing together to these processes.

The work presented in this thesis aims to investigate the mechanisms involving apoE and high cholesterol (HC) diet in neurodegenerative processes. As a model we used wild type (WT) and apoE deficient mice on normal diet or HC diet for 9 months. We have found that HC diet stimulates glial activation and increases expression of IL-6 and caspase-1, and that these effects are potentiated by the apoE deficiency (papers I and II). HC diet also upregulates the expression of antioxidant enzyme NQO1 (paper I). In addition, we found that the combination of HC diet and apoE deficiency induces tau hyperphosphorylation and increases the activity of several tau kinases in the brain (paper III). Finally, we used cDNA microarrays to explore the effects of HC diet on brain gene expression. We found that a high cholesterol intake affects the expression of several genes involved in neurodegeneration (paper IV).

The present study gives insight to the molecular mechanisms by which HC diet and lack of apoE increase the risk for neurodegeneration and suggest that several risk factors may act synergically in these processes.

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Crisby, M*, **Rahman, SMA***, Sylven, C, Winblad, B and Schultzberg, M.
Effects of high cholesterol diet on gliosis in apolipoproteinE knockout mice, implications for Alzheimer´s disease and stroke.
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High cholesterol diet results in increased expression of interleukin-6 and caspase-1 in the brain of apolipoprotein E knockout and wild type mice.
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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
Aβ	β-amyloid
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BBB	Blood brain barrier
CamkII	Calmodulin-dependent kinase II
CE	Cholesterol ester
CNS	Central nervous system
CSF	Cerebrospinal fluid
CIP	cdk5 inhibitory peptide
cdk5	Cyclin-dependent kinase-5
GFAP	Glial fibrillary acidic protein
GSK-3	Glycogen synthase kinase-3
ERK	Extracellular regulatory kinase
HC	High cholesterol
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-methylglutarly-CoA
FC	Free cholesterol
ICE	Interleukin-1β converting enzyme
IL-6	Interleukin-6
JNK	Jun N-terminal kinase
KO	Knock-out
LBD	Lewy body disease
LDL	Low-density lipoprotein
MAPs	Microtubule-associated proteins
LPS	Lipopolysaccharide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
ND	Normal diet
NP	Neuritic plaque
NPC	Niemann-Pick type C disease
NFT	Neurofibrillary tangles
NQO1	NAD(P)H:quinone oxidoreductase
NSAIDs	Non-steroidal antiinflammatory drugs
MS	Multiple sclerosis
OA	Okadaic acid
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PF	Paraformaldehyde
PP	Protein phosphatase
PET	Positron emisson tomography
PSP	Progressive supranuclear palsy
SP	Senile plaque
TBI	Traumatic brain injury
TNF	Tumour necrosis factor
WT	Wild type
VLDL	Very low-density lipoprotein

INTRODUCTION

CHOLESTEROL

Cholesterol is a component of all mammalian cells, and it is vital for the formation and maintenance of cellular membranes, with regard to permeability and fluidity. Cholesterol also plays a role in cellular signalling and as the precursor of regulatory oxysterol biosynthesis (Ikonen and Parton, 2000). It is essential for embryonic development, and deficiencies in cholesterol during embryogenesis and organogenesis cause severe abnormalities (Roux et al., 2000). In eukaryotic cells, most cholesterol is unesterified and is located in the outer or inner leaflet of the plasma membrane and only a small portion of it located in intracellular structures such as the endoplasmic reticulum, Golgi apparatus or nucleus (Dietschy and Turley, 2004). In the plasma membrane, there are microdomains enriched with cholesterol, sphingomyelin, and glycolipids (Maekawa et al., 2003). In the body there are two independent pools of cholesterol, one in peripheral tissues and other in the central nervous system (CNS).

Cholesterol in peripheral tissue

The total cholesterol content of an adult human body (70 kg) is about 140 g, and the daily turnover is about 1% (~1.2 g). New cholesterol is generally derived from *de novo* synthesis and from the diet, but our body has the capacity to synthesize all the cholesterol that is needed. In a typical Western diet, about 1.2 to 1.7 g of cholesterol enter the lumen of the small intestine per day (Lammert and Wang, 2005). About 0.3 to 0.5 g of this sterol is of dietary origin. The remainder mainly comes from the bile, but there is a small portion from sloughed mucosal cells. The average individual absorbs about 50% of all cholesterol, and the liver has to process several hundred milligrams of intestinally derived cholesterol every day (Dawson and Rudel, 1999) Cholesterol also functions as a substrate for the synthesis of bile acids in the liver and as the precursor of steroid hormones and vitamin D (Ohvo-Rekila et al., 2002). Thus, the liver is the principle site for cholesterol synthesis and clearance of low-density lipoprotein (LDL) cholesterol (Dietschy et al., 1993).

Dietary cholesterol intake is usually associated with fat consumption. Most of the cholesterol in the food is in the unesterified form, and only 8-15% is in cholesterol esters. Cholesterol is only minimally soluble in aqueous environment and needs to be partitioned into bile salt micelles prior to its transporters to the brush boarder of intestinal mucosal membranes, where it can be absorbed (Hui and Howles, 2005). Cholesterol and fatty acids absorbed by the intestine are assembled with

proteins into large so-called chylomicrons prior to excretion to the lymphatics and the blood circulation. Dietary cholesterol is processed in the liver via chylomicrons and LDL. However, the lipoproteins involved in the transport of free cholesterol (FC) and cholesterol ester (CE) to most tissues is LDL. It is well established that increasing the dietary cholesterol intake causes an inhibitory effect on cholesterol biosynthesis in peripheral tissues and that *de novo* synthesis of cholesterol is stimulated when cellular cholesterol exceeds dietary intake (Burns and Duff, 2003).

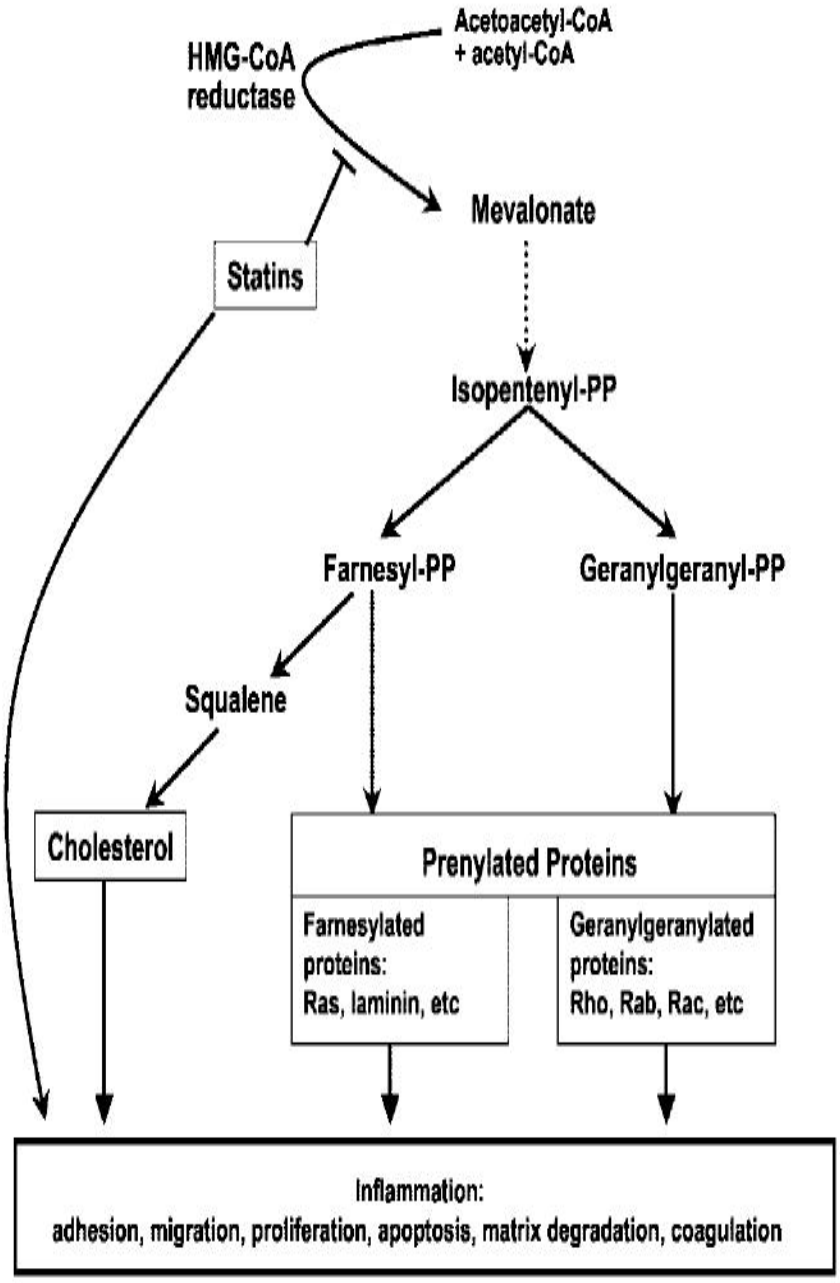

Fig. 1: Cholesterol synthesis pathway

All eukaryotic cells, except for mature red blood cells can synthesize cholesterol from acetate. Cholesterol biosynthesis is a very complicated and multistep process that occurs mainly at the endoplasmic reticulum, and to a less extent at the peroxisomes. Acetyl-CoAs are condensed to 3-hydroxy-methylglutarly-CoA (HMG-CoA) through the successive action of thiolase and HMG-CoA synthase. Conversion of HMG-CoA to mevalonate is a rate-limiting step and HMG-CoA reductase the rate-limiting enzyme for the whole process. Mevalonate is phosphorylated to isopentenyl pyrophosphate (IPP), and other isoprenoid units condense and combine to form squalene. Squalene is converted to lanosterol, which is finally converted to cholesterol (Fig.1) (Burns and Duff, 2003).

Cholesterol in the brain

The CNS accounts for 2% of the whole body mass, but contains up to 25% of unspecified body cholesterol (Dietschy and Turley, 2001). In the brain, the cholesterol is mainly located in plasma membranes of glial cells and neurons, and in the myelin. Most of the cholesterol in the brain is acquired in the process of myelination, which starts in the early stages of development. It has been estimated that up to 70% of the brain cholesterol is associated with myelin (Björkhem and Meaney, 2004). Brain cholesterol is mostly independent of dietary uptake or hepatic synthesis and it is almost completely synthesized *in situ* (Dietschy and Turley, 2001). There is no evidence for a net transfer of sterol from blood into the brain and spinal cord (Dietschy and Turley, 2004). Cholesterol turnover in the brain is low and the half-life is estimated to be 2-4 months (Björkhem et al., 1997). The enzymatic conversion of cholesterol to 24S-hydroxycholesterol and the flux of this oxysterol across the blood brain barrier (BBB) into the circulation is the recognized pathway for the elimination and the homeostasis maintenance of brain cholesterol (Björkhem et al., 1998). Cholesterol 24S-hydroxylase, the enzyme responsible for the conversion of 24S-hydroxycholesterol, is almost exclusively expressed in the brain. Like other oxysterols, 24S-hydroxycholesterol is efficiently converted into normal bile acids or excreted in bile in its sulfated and glucuronidated form (Björkhem et al., 2001). Plasma 24S-hydroxycholesterol levels are only 20 to 30 times higher than in the CSF. It was recently shown that 27-hydroxycholesterol can pass the BBB efficiently (Heverin et al., 2005), and that there is a significant correlation between CSF and plasma levels of 27-hydroxycholesterol in humans (Leoni et al., 2004).

HIGH CHOLESTEROL DIET AS RISK FACTOR FOR NEURODEGENERATION

Epidemiological studies have indicated several factors that can increase the risk of neurodegenerative disorders. The majority of these studies have focussed on Alzheimer's disease (AD). Established risk factors for AD include: higher age, family history of AD and APOE4 allele. Other proposed AD risk factors, for example include fewer years of formal education, female sex, low serum levels of folate and vitamin B_{12}, elevated plasma homocysteine levels, smoking and vascular factors (Clarke et al., 1998; Evans et al., 1997; Fratiglioni et al., 1993; Guo et al., 2000). Increased risk of AD, in countries where dietary fat intake is high suggested the view of dietary component in AD (Chandra and Pandav, 1998). A population-based, longitudinal study showed that raised midlife blood pressure, high serum cholesterol levels and obesity at midlife increased the risk of AD in later life (Kivipelto et al., 2001; Kivipelto et al., 2005). Dietary fat, and to a lesser extent, total energy (caloric intake), has been found to be risk factors for the development of AD, while fish consumption has been found to be a protective factor (Grant et al., 2002; Morris et al., 2005). However, the literature is often controversial in this field. For example, data from the Rotterdam study suggested that high saturated fat and cholesterol intake increases the risk of dementia, whereas fish consumption may decrease this risk (Kalmijn et al., 2004). In contrast, earlier reports from the same study did not show any correlation between different diets and increased risk for dementia during a longer follow-up period (Engelhart et al., 2002). Another study showed that high intake of unsaturated, unhydrogenated fats may be protective against AD, whereas intake of saturated or trans-unsaturated fats may increase the risk (Morris et al., 2003).

Studies in animal models have shown that dietary restriction extends the lifespan and increases the resistance of neurons to degeneration (Mattson, 2000). Furthermore, it has been demonstrated that diet-induced hypercholesterolemia accelerates, while cholesterol-lowering drugs reduce β-amyloid pathology in a transgenic mouse model for AD (Refolo et al., 2000; Refolo et al., 2001). Only few studies have examined the relationship between high cholesterol (HC) diet and memory in animal models of neurodegeneration. A recent study showed that memory performance is severely affected in rabbits subjected to a HC diet in the presence of copper (Sparks and Schreurs, 2003). A strong linear association was found between levels of late-life high-density lipoprotein (HDL) cholesterol and the number of neocortical neuritic plaques (Launer et al., 2001). Oxidative

stress, which can be modified by diet, increases with ageing and may participate in the AD pathological mechanisms.

Statins are inhibitors of HMG-CoA reductase, and are widely used as cholesterol-lowering drugs. Cross-sectional analyses have documented a decreased prevalence of AD associated with use of statins e.g. (Wolozin et al., 2000). Results from randomised controlled studies on the positive effect of lipid–lowering treatment for prevention of AD are still lacking. The Heart Protection Study of the Medical Research Council/British Heart Foundation (MRC/BHF) revealed no effect on cognition by long-term treatment with the lipid-lowering drug simvastatin. However, follow-up time in this study was short and incidence of AD very low among the participants. A recent prospective observational study suggested that statins may not decrease the risk of AD in the overall population, but only in those younger than 80 years (Li et al., 2004). Careful analysis of the pleiotropic effects of statins on the brain will be necessary in order to evaluate their potential use in AD therapy. All of these observations suggest a potentially important role for diet in the development of neurodegenerative disorders such as AD.

Atherosclerosis is associated with high lipid content in the blood, particularly LDL. This extremely widespread disease predisposes for myocardial infarction, cerebral thrombosis, and other serious illnesses. Individuals with elevated levels of LDL have higher incidence of atherosclerosis and its complications, whereas individuals with elevated levels of HDL have a lower incidence (Ong and Halliwell, 2004). In addition to coronary and peripheral atherosclerosis, individuals with high levels of LDL cholesterol are particularly disposed to cerebral atherosclerosis (Nubiola et al., 1981). Atherosclerotic (multi-infarct) dementia or vascular dementia refers to an impairment of intellectual functions due to multiple infarcts (Iadecola and Gorelick, 2003). Vascular dementia may be preventable by good control of vascular factors in middle age, including dietary intervention.

Ischemic stroke ranks among the most important causes of death and disability in developed countries. Stroke is associated with AD among elderly individuals. Ischemic stroke results when blood flow is reduced in some part of the brain, leading to depletion of metabolic stores, intracellular calcium accumulation, oxidative stress, and profound inflammatory responses (Zheng et al., 2003). Epidemiological studies have not demonstrated a clear relationship between stroke and serum cholesterol levels (Lancet, 1995), and serum cholesterol is a poor predictor of total stroke risk (Ansell, 2000). However, there is some evidence indicating that elevated serum

cholesterol levels increase the relative risk for thromboembolic stroke, probably by contributing to both cerebrovascular and coronary atherosclerosis (Benfante et al., 1994). The reduction in stroke events in clinical trials of statins is much greater than would be expected from the week associations between serum cholesterol levels and risk of stroke reported in observational studies (Crouse et al., 1998).

In summary, high levels of cholesterol in serum have been associated with AD and vascular dementia. The molecular mechanisms underlying these associations are currently unknown. It is also suggested that high levels of serum cholesterol could contribute to other neurodegenerative disorders, but this hypothesis needs to be explored.

APOLIPOPROTEIN E (APOE)

Intercellular transport of lipids requires packaging of hydrophobic molecules with water-soluble carriers called lipoproteins. Lipoproteins consist of distinct proteins called apolipoproteins and are present in plasma, CSF,the interstitial space of the brain and in other body fluids (Fig. 2). ApoE is a component of several plasma lipoproteins, including chylomicrons, VLDL and HDL. ApoE plays a major role in transport and metabolism of cholesterol, triacylglycerols and phospholipids (Mahley, 1988). Plasma apoE is primarily derived from liver parenchymal cells and to a much lesser extent from macrophages throughout the body (Lin et al., 1986). In humans, apoE has three major isoforms E2 (cys^{112}, cys^{158}), E3 (cys^{112}, arg^{158}) and E4 (arg^{112}, arg^{158}),

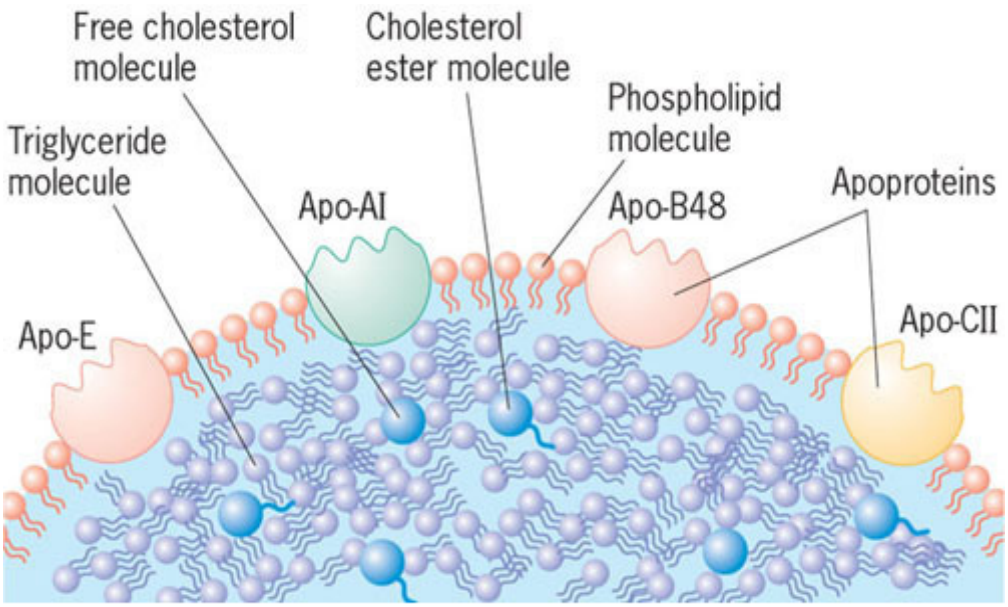

Fig. 2: Structure of a lipoprotein with apolipoproteins and cholesterol

all of which are encoded by a single gene on chromosome 19 (Mahley, 1988). Mice have only one isoform of apoE (Strittmatter and Bova Hill, 2002).

ApoE mediates the binding of lipoproteins to LDL receptors, LDL receptor-related proteins and apoER2 receptors. ApoE binds to lipoproteins in an isoform-specific manner (Dong and Weisgraber, 1996) and each apoE isoform has different affinity for each of these receptors (Davignon et al., 1988). Structurally, apoE have two independently folded domains separated by a hinge region. The NH_2-terminal domain contains the receptor-binding region and the COOH-terminal domain has a lipid-binding region. Compared to apoE3, the apoE2 and apoE4 isoforms are more potent in altering lipid metabolism (Siest et al., 1995). Subjects carrying the apoE2 isoform have lower levels, and apoE4 carriers have higher levels, of total plasma cholesterol and LDL cholesterol as compared to apoE3 carriers (Mahley and Rall, 2000).

In the CNS, apoE is synthesized locally, primarily by glial cells (Boyles et al., 1985). Neurons can express apoE under certain conditions, such as excitotoxic stress (Boschert et al., 1999). Glial cells deliver lipids to neurons for membrane synthesis during synaptogenesis and repair by secreting lipoproteins (Pfrieger, 2003). ApoE is involved in the regulation of cholesterol content in the outer and inner leaflets of the plasma membrane and also in the transport of phospholipids and polyunsaturated fatty acids to the synapses (Igbavboa et al., 2002). The transport of cholesterol by apoE is important for the regulation of development, remodeling and regeneration of the nervous system. Using *in vitro* neuronal models of the CNS and peripheral nervous system (PNS), it has been demonstrated that apoE can promote neurite outgrowth (Buttini et al., 1999; Holtzman et al., 1995; Ignatius et al., 1987; Nathan et al., 1994). ApoE levels were demonstrated to increase many folds in response to peripheral nerve injury and to a lesser extent to CNS injury (Boyles et al., 1989; LeBlanc and Poduslo, 1990). Several other roles of apoE in the brain have been described. For example, apoE has isoforms-specific effects on signaling, in the regulation of neuronal calcium (Hartmann et al., 1994) and of several signalling pathways, including protein kinase C (PKC) and glycogen synthase kinase-3β (GSK-3β) (Cedazo-Minguez and Cowburn, 2001; Cedazo-Minguez et al., 2003). ApoE isoforms have also been reported to have anti-oxidant and anti-inflammatory functions (Laskowitz et al., 1998b). Biochemical studies showed that apoE deficiency is associated with enhanced lipid peroxidation and with chronic oxidative stress in the brain (Lomnitski et al., 1997; Shea et al., 2002). In a cell culture system, physiologically relevant concentrations of human recombinant apoE have been shown to regulate glial activation and the release of NO and TNFα in a dose dependent manner (Laskowitz et al., 2001). Moreover, studies

on transgenic mice showed that apoE regulates gliosis in an isoform-dependent manner, by apoE3, but not by apoE4 (Ophir et al., 2003).

APOE AND NEURODEGENERATION

ApoE gene polymorphism represents the strongest known genetic risk factor for sporadic AD (Poirier et al., 1993; Saunders et al., 1993). ApoE3 is the most common isoform in the general population (70-80%) while E4 and E2 account for 10-15% and 5-10%, respectively (Roses, 1996). Numerous studies have demonstrated that the frequency of APOE4 carriers is increased in patients with late-onset AD, as compared to other apoE isoform carriers. Individuals with two copies of APOE4 have an approximately eight-fold risk for having AD (Roses, 1996). In addition, AD patients carrying apoE4 have a lower age of onset (Roses, 1996).

Recent evidence identified the presence of the E4 allele also as a major risk factor for several other neurodegenerative disorders, including stroke, poor out-come due to head injury and spontaneous intracerebral hemorrhage (Laskowitz et al., 1998a). ApoE4 has also been shown to influence the age of onset and the severity of Parkinson's disease (PD) (Zareparsi et al., 1997). An increased frequency of APOE4 has been observed in patients with vascular dementia (Frisoni et al., 1994; Isoe et al., 1996). Other studies did, however, not find such an association with apoE4 and vascular dementia (Higuchi et al., 1996; Kawamata et al., 1994). There are controversial reports, regarding the influence of apoE4 on other neurodegenerative disorders, such as progressive supranuclear palsy (PSP), multiple system atrophy, frontotemporal dementia and Lewy body disease (LBD) (Nielsen et al., 2003).

The neurobiological mechanisms linking the APOE4 allele to AD and other diseases are not well understood. Most of the effects of apoE4 have been seen in relation to the AD pathogenesis, and several hypotheses have been formulated. These hypotheses support a putative role for apoE4 in the formation of both neuropathological hallmarks of AD, i.e. senile plaques (SP) and neurofibrillary tangles (NFTs). ApoE binds to Aβ, the major constituent of the SP, in an isoforms-specific manner, and it has been suggested that apoE4 enhances amyloid fibrillar stability and facilitates the plaque formation, and/or that apoE3 is more efficient in the clearance of Aβ (LaDu et al., 1994; Strittmatter et al., 1993a; Strittmatter et al., 1993b). ApoE3 was shown to bind the tau protein better than apoE4 does, and thereby apoE3 could provide better protection against tau hyperphosphorylation and NFT generation (Strittmatter et al., 1994). ApoE could be

also implicated in AD pathogenesis through its involvement in atherosclerosis and vascular disease (Hofman et al., 1997). Several studies have also suggested that apoE4 may have deleterious functions or a lack of trophic or antioxidant activities as compared to the other apoE isoforms (reviewed in Cedazo-Minguez et al. 2001). In addition, evidence from *in vivo* and *in vitro* studies suggest that the apoE modulation of CNS inflammatory responses may another mechanism (Laskowitz et al., 2001).

APOE KNOCK-OUT MICE AS A MODEL

ApoE knock-out (apoE KO) mice were originally developed as a model for atherosclerosis (Zhang et al., 1992), but it has also facilitated the understanding of apoE functions in the brain. ApoE KO mice have normal growth and development, but an advanced atherosclerosis and xanthomatosis that may indirectly reduce their life span (Moghadasian et al., 2001). ApoE KO mice on normal chow diet have at least five-fold more total plasma cholesterol levels, than their wild-type counterparts. In contrast, triglyceride levels are only slightly decreased, or normal. When given a high fat diet with mildly elevated cholesterol, these mice have even a further three-fold rise in total plasma cholesterol (Wouters et al., 2005). As discussed previously, it is believed that human apoE4 has a lack of function as compared with other isoforms of apoE, and this could contribute to the pathology of neurodegenerative processes. Therefore, apoE-deficient mice may serve as a limited model for understanding the molecular mechanisms by which a deficit in apoE function could lead to neurodegeneration. Indeed, apoE KO have a defective spatial learning and memory compared to wild-type in the Morris water maze test (Oitzl et al., 1997). However, there are conflicting results from different laboratories regarding the presence or absence of neurodegeneration in these mice. The brains of apoE KO mice have synaptic alterations, but do not have SP or NFTs (Masliah et al., 1995). Increased tau hyperphosphorylation in apoE KO mice has been reported by some investigators, but not by others (Genis et al., 1995; Mercken and Brion, 1995). There are also controversial reports regarding choline acetyltransferase activity in the brain of these mice. Gordon et al. reported a decrease in choline acetyltransferase activity in the hippocampus and frontal cortex of apoE KO mice (Gordon et al., 1995) as compared to WT animals, while others reported no significant differences (Anderson and Higgins, 1997). Interestingly, a significant reduction in cerebral cortical phospholipids and their constituent fatty acids, as well as an elevation of lipid peroxidation products, have been observed in apoE KO mice (Montine et al., 1999). In agreement with these observations, Ramassamy et al., (2001) reported that the levels of apoE influence the balance between oxidants and antioxidants in the

hippocampus. These observations suggest that a deficiency in apoE induces increased oxidative stress in the brain. Importantly, an increase in oxidative stress markers have demonstrated in the brain of apoE4 carriers (Ramassamy et al., 2000) which further supporting the lack of function of this isoform.

ApoE KO mice have also been shown to have increase susceptibility to closed head injury, ischemic lesion and brain lesions. Recently, loss of BBB integrity has been described in these mice (Fullerton et al., 2001). These observations suggest that lack of apoE is not the sole cause of these abnormal phenotypes. Several environmental factors like dietary intake or physical activity could modify the phenotypes of apoE deficient mice. Understanding these modifications would provide important insights to the mechanisms by which apoE influence the CNS.

CHOLESTEROL HOMEOSTASIS IN NEURODEGENERATIVE CONDITIONS

Growing evidence support the link between alterations in cholesterol metabolism and neurodegeneration. AD and Niemann-Pick disease type C (NPC) are the best-studied clinical conditions and represent cholesterol homeostasis alterations in neurodegeneration.

AD is the most common cause of dementia, affecting up to 15 million individuals worldwide (Puglielli et al., 2003). Only about 5% to 10% AD case of are due to hereditary factors and occur at an early age; the remaining cases are considered to be idiopathic and appear in later age (Selkoe, 2001). Clinically, AD is diagnosed by different sets of criteria, but a confirmed diagnosis still depends on post-mortem analysis of the presence of SP and NFTs in the brain. These lesions are accompanied by neuronal and synaptic loss. A reduction in brain weight of 20% or more is characteristic for the AD brains. The typical AD pathology is found most commonly in the entorhinal cortex, hippocampus, parahippocampal gyrus, amygdala, and the frontal, temporal and occipital association cortices (Braak and Braak, 1992). SP are microscopic foci mainly formed by extracellular deposition of Aβ and associated with axonal and dendritic injury. SP are generally found in large numbers in limbic and association cortices (Selkoe, 2001). An increased production of Aβ is thought to be a central event in the pathophysiology of AD, according to the amyloid hypothesis (Hardy and Higgins, 1992). Aβ derives from a large type I transmembrane protein, the amyloid precursor protein (APP). APP is cleaved by α–, β– and γ–secretases. The cleaved products of α– and γ-secretases do not result in abnormal brain pathology. In contrast, the products generated by the β- and γ-secretases are the Aβ-40 and Aβ-42 fragments, that are

amylodogenic and result in formation of SP. Cholesterol metabolism has been shown to be altered in the brain of AD patients. In normal brain, 24S-hydroxylase is expressed in neurons (Lund et al., 1999), but in the AD brain, a very strong expression of this enzyme is observed in some reactive astrocytes (Bogdanovic et al., 2001). The concentration of 24S-hydroxycholesterol is elevated in the CSF in AD and early stages of dementia (Papassotiropoulos et al., 2002). In contrast, the levels are reduced in advanced AD and in chronic stages of multiple sclerosis (MS). Cholesterol accumulates in mature SP in the AD brain as well as in the brains of transgenic mice with mutant APP (Mori et al., 2001). In rabbits and APP transgenic mice, a HC diet induces Aβ-deposition in the brain (Refolo et al., 2000; Sparks et al., 1994). Several studies using immortalized and primary cell lines have demonstrated that increased cholesterol levels can enhance the activity of the β-secretase pathway, leading to an accumulation of extracellular amyloid deposits (Bodovitz and Klein, 1996; Frears et al., 1999; Howland et al., 1998). The observation that reduced levels of cellular cholesterol inhibits the formation of Aβ in hippocampal neurons, by enhancing α-secretase activity, provides further support for the involvement of cholesterol in AD (Simons et al., 1998). On the other hand, cholesterol is also suggested to stimulate the generation of Aβ, since β– and γ–secretase complexes are enriched in cholesterol-rich microdomains (lipid rafts). Thus, access of α– and β-secretase to APP, and therefore Aβ generation, may be determined by dynamic interactions of APP with lipid rafts (Ehehalt et al., 2003).

In addition, a relation between cholesterol and NFTs has been shown in NPC. NPC is an autosomal recessive disorder, typically associated with abnormal intracellular cholesterol and glycosphingolipids in a number of tissues. NPC is characterized by compromised liver and spleen function as well as widespread neurological deficits, including ataxia, dystonia, seizure, dementia and reduced cognitive functions, eventually leads to premature death (Ikonen and Holtta-Vuori, 2004). In affected brains, the brain pathology is characterized by many ballooned neurons and a considerable nerve cell loss, leading to neurological deterioration and dementia. Filipin fluorometry analysis showed that neurons accumulate high levels of cholesterol in the NPC brain (Distl et al., 2001). In addition, NFTs are found in almost all juvenile/adult NPC cases but there is no Aβ deposition (Ohm et al., 2003). Mutations in the NPC1 gene occur in about 95% of NPC cases and NPC2 mutations are found in the remaining 5%. These genes encode for proteins that interact with cholesterol and mutations result in the NPC genes result in considerable intraneuronal cholesterol accumulation. In NPC mouse models, tau is hyperphosphorylated in a

site-specific manner and accompanied by activation of mitogen-activated kinase (MAPK) (Sawamura et al., 2001). In the same models, this MAPK activation has been shown to decrease cholesterol levels in the plasma membrane and lipid rafts (Sawamura et al., 2003).

TAU AND NEUROFIBRILLARY TANGLES

Hyperphosphorylation of tau leading to the formation of NFTs is a hallmark of not only of AD and NPC, but also of several other neurodegenerative disorders.

Structure and biological function of tau

Tau is primarily, although not exclusively, a neuronal protein, abundant in the central and peripheral nervous system (Avila et al., 2004). A single gene located on chromosome 17q21 encodes the human tau proteins (Neve et al., 1986). By alternative mRNA splicing of exons 2, 3 and 10, six tau isoforms are produced in the CNS with apparent molecular weights between 50 and 70 kDa (Fig. 3). These isoforms differ by the absence or presence of one or two short inserts in the NH_2-terminal half (0N, 1N, and 2N, respectively), and have either three or four microtubule-binding repeat motifs in the COOH-terminal half (3R- and 4R-tau) (Gong et al., 2005). In fetal brain, only the shortest tau isoforms (3R-tau) is expressed, whereas all isoforms are present in adult brain. Fetal tau is more extensively phosphorylated than adult tau (Lee et al., 2001). A striking feature of the primary structure of tau is the presence of three or four imperfectly repeated stretches of 31 and 32 residues in the COOH-teriminal half, which constitutes the core of the microtubule-interacting unit (Lee et al., 1989). The acidic NH_2-terminal region projects from the microtubule surface (when tau is bound to microtubules), and may interact with other cytoskeletal elements and plasma membrane components.

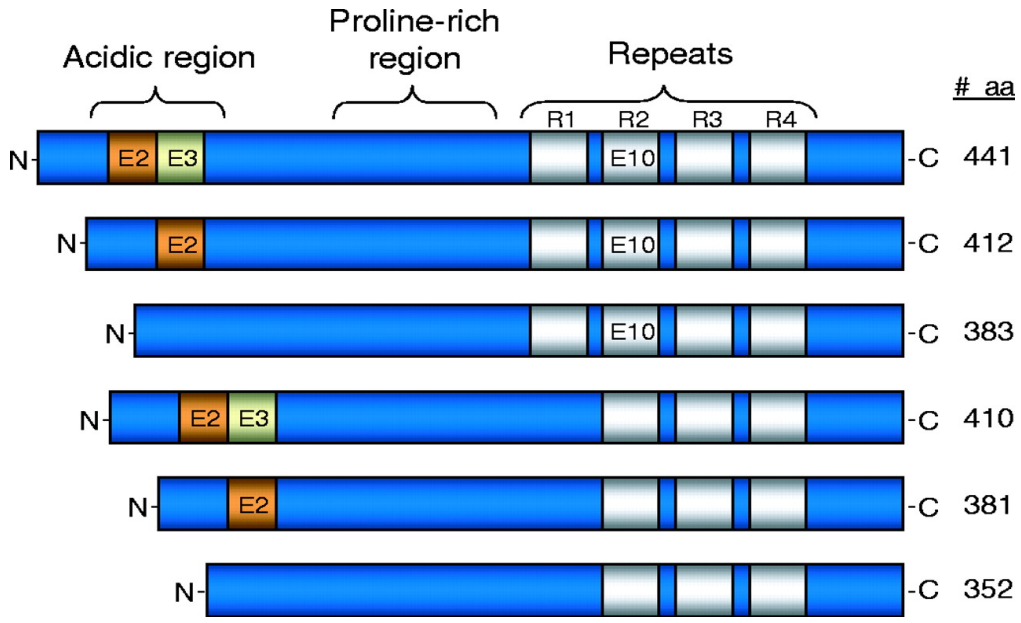

Fig. 3: Schematic diagram showing the organization of the six predominant isoforms of tau in adult human brain

Tau protein binds to microtubules and promote microtubule stability and assembly (Billingsley and Kincaid, 1997). Findings from cell culture studies indicate a fundamental role of tau in neurite outgrowth and stabilization (Baas et al., 1991; Drubin et al., 1985; Knops et al., 1991). There are conflicting results regarding the role of tau expression and axon development and elongation from studies on tau KO mice. The nervous system of tau KO mice appeared normal histologically, and axon growth was not affected in cultured hippocampal neurons according to some reports (Harada et al., 1994). However, other studies have shown a significant delay in axonal and neuritic extensions in embryonic hippocampal cultures from these mice (Dawson et al., 2001). The expression of human tau proteins was shown to rescue axonal sprouting and extension, in tau KO mice, confirming the role of tau in neurite extension (Dawson et al., 2001). There is significant plasticity and redundancy among the microtubule-associated proteins (MAPs), and this may be the reason behind the normal phenotype of the tau KO mice. Growing evidence suggest the role of tau, in association with actin, in the regulation of cellular cell shape, motility and microtubule plasma membrane interactions. As a linker protein, tau interacts with components of neural plasma membrane through its NH_2-terminal non-microtubule binding domain, which protrudes from the microtubule surface when tau interacts with microtubules (Brandt et al., 1995). *In vitro*, tau was shown to interact with spectrin, which may provide another link to the membrane skeleton (Carlier et al., 1984). Tau also appears to interact with src-family non-receptor tyrosine

kinases, such as fyn and phospholipase C-γ via its proline-rich region (Lee et al., 1998). However, the physiological significance of these interactions remains to be investigated.

Tau hyperphosphorylation and neurodegeneration

NTFs are mainly composed of hyperphosphorylated tau. The distribution and the numbers of NFTs closely correlated with the degree of dementia (Braak and Braak, 1992; Braak et al., 1993). NFTs, in the absence of SP, are also seen in other neurodegenerative diseases including NPC, progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease and frontotemporal dementia with parkinsonism linked to chromosome-17 (Lee et al., 2001). In these tauopathies, abnormally hyperphosphorylated tau is relocalized from axons to somatodendritic compartments, where it accumulates as PHFs and eventually assemble into NFTs (Buee et al., 2000; Goedert et al., 1995). In some of the tauopathies including progressive supranuclear palsy, corticobasal degeneration, multiple system atrophy, and some familial forms of frontotemporal dementia, fibrillary inclusions of tau are also seen in glial cells (Komori, 1999). Tau is a phosphoprotein that normally contains 2-3 moles of phosphate per mole (Gong et al., 2005). The longest form in the adult human brain has 80 Ser or Thr residues and 5 Tyr residues; therefore, almost 20% of the molecule has the potential to be phosphorylated (Goedert et al., 1989). A balanced phosphorylation state of tau maintains its activity to bind to microtubules and stimulate their assembly. Dynamic site-specific phosphorylation of tau is essential for its optimal functioning, whereas the hyperphophorylated tau looses its biological acitivity (Johnson and Stoothoff, 2004). Tau isolated from AD brains has 3-4 times higher levels of phosphorylation, than in control brains (Köpke et al., 1993). In the AD brain, all six isoforms of tau are aggregated into PHFs in abnormally hyperphosphorylated forms (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b). Thus, hyperphophorylation of tau represents a critical event leading to mislocation and abnormal aggregation of tau proteins in affected neurons. Tau hyperphosphorylation is also observed in physiologically reversible responses of the brain to stressful conditions, such as cold-water stress, heat shock, starvation or hybernation (Arendt et al., 2003; Korneyev et al., 1995; Papasozomenos, 1996; Yanagisawa et al., 1999).

Tau Kinases and phosphatases

Several protein kinases and protein phosphatases (PPs) have been shown to be involved in the regulation of tau phosphorylation/dephosphorylation state. Among these kinases, glycogen

synthase kinase-3 (GSK-3), cyclin-dependent kinase-5 (cdk5), extracellular regulatory kinase1/2 (ERK1/2), calmodulin-dependent kinase II (CamkII), and the stress-activated kinases c-Jun N-terminal kinase (JNK) and MAP-kinase p38 have been shown to phosphorylate tau (See Table 1). Among the protein phosphatases, PP2A, PP2B and PP1 are known to dephosphorylate it (Liu et al., 2005).

GSK-3 plays an important role in regulating tau phosphorylation in physiological and pathological conditions. Mammalian GSK-3 exists as two isoforms, α and β, sharing 98% homology in their catalytic domain. High levels of GSK-3β expression in brain and neurons, make it suitable to access tau (Leroy and Brion, 1999). Increased levels (50%) of GSK-3β have been found in AD postmortem brain tissue as compared with the non-diseased human brain (Pei et al., 1997). GSK-3 overexpressing transgenic mice exhibit persistent tau hyperphosphorylation, pretangle-like somatodendritic localization of tau, neuronal cell death in the hippocampus and cognitive deficits (Lucas et al., 2001; Spittaels et al., 2000). Treatment of cells with lithium, a selective inhibitor of GSK-3 reduces tau hyperphosphorylation (Lovestone et al., 1999).

In vitro, tau has been shown to act as a substrate for cdk5 and immunohistochemical experiments showed cdk5 localized to tangle-bearing neurons in the AD brain (Pei et al., 1998). Increased immunoreactivity in neurons with pretangles and early tangle stage has been shown to occur in early phases of AD pathogenesis. Overexpression of p25, a regulatory protein of cdk5, in transgenic mice has been found to increase both cdk5 activity and tau phosphorylation (Ahlijanian et al., 2000). Double transgenic mice generated by crossing p25 transgenic mice with the P301L mouse (a model for frontotemporal dementia with enhanced tau phosphorylation) were found to express increased levels of GSK-3β (Noble et al., 2003). A recent study has demonstrated that neuronal infections with cdk5 inhibitory peptide (CIP) selectively inhibited p25/cdk5 activity and suppressed aberrant tau phosphorylation in cortical neurons (Zheng et al., 2005).

There is evidence that tau can be phosphorylated by cAMP-dependent protein kinase A (PKA) *in vivo*. Activation of PKA in the rat brain by intracranial injection of isoproterenol and forskolin resulted in tau hyperphosphorylation (Sun et al., 2005). The levels of activated JNK are significantly increased in the AD brain (Zhu et al., 2001). Phospho-JNK and phospho-p38 are colocalized with hyperphosphorylated tau (Atzori et al., 2001), suggesting that these kinases may play a role in the development of degenerative diseases with tau pathology. Tau can also be phosphorylated by ERK in various neuronal cultures and in a brain slice model (Ekinci and Shea, 1999; Lu and Wood, 1993).

Table I: Different kinase directed tau phosphorylation sites

Kinases	Sites on tau they phosphorylate
GSK-3β	S46, T50, T69, T153, T175, T181, S184, S195, S198, S199, S202, T205, S208, T212, S214, T217, T231, S235, S262, S396, S400, T403, S404
GSK-3α	S184, S199, S202, T212, T231, S235, S262, S324, S356, S396, S404, S413
JNK	T153, T175, T181, S202, T205, T212, T217, T231, S235, S356, S396
cdk5	T153, S195, S202, T205, T212, S214, T231, S235, S396, S404
ERK1/2	S46, T50, T69, T153, T175, T181, S199, S202, T212, S396, S404
PKA	S195, S198, S202, T205, T212, S214, S237, S262, S293, S305, S320, S324, S356, S403, S416
AKT	T212
CamkII	S262, S285, S356, S403, S416
P38	S46, T50, T153, T175, T181, S199, S202, T205, T212, T217, S235, T246, S356, S396, S404, S422

Several protein phosphatases have been implicated in dephosphorylation of tau. Of these, only PP1, PP2A and PP2B, have been shown to dephosphorylate abnormally hyperphosphorylated tau, and the activities of PP2A and PP1 have been shown to be compromised in AD brain (Gong et al., 1995; Gong et al., 1993). PP2A is likely to be the major tau phosphatase. PP2A shows a much stronger ability in dephosphorylating NFTs in AD brain than PP1 or PP2B (Wang et al., 1995; Wang et al., 1996). Inhibition of PP2A activity by okadaic acid (OA) was shown to induce tau hyperphosphorylation and hamper the biological activity in cultured neurons (Kim et al., 1999; Tanaka et al., 1998). Acute injection of OA or calyculin acid in the hippocampus of mice induced hyperphosphorylation of tau, as well as deficits in learning and memory (Lee et al., 2000; Sun et al., 2003). Tau hypherphosphorylation was also seen in transgenic mice in which PP2A activity was reduced to 66% (Kins et al., 2001). In addition, chronic inhibition of PP2A induced activation of ERK- and JNK-signalling pathways (Kins et al., 2003).

NEUROINFLAMMATION

Inflammation represents a potential pathogenic factor for many CNS diorders, including chronic neurodegenerative diseases such as AD, PD, NPC and prion disease (Minghetti, 2005). In these disorders, inflammation is characterized by activation of resident cellular elements, in the absence of overt leukocyte infiltration. Microglial cells are central to the inflammation, but astrocytes also

contribute to the process by limiting the area of lesions and releasing local mediators. There is considerable debate regarding the role of microglial activation, whether beneficial or harmful.
The cellular evidence of inflammation in AD consists of the appearance of activated microglia and astrocytes around the amyloid deposits (McGeer and McGeer, 2003a). Damaged neurons, neurites, highly insoluble Aβ deposits and NFTs provide stimuli for inflammation in AD (Akiyama et al., 2000). Even before the appearance of severe cognitive decline, there is significant microglial activation, suggesting that it occurs at the early stages of the disease (Vehmas et al., 2003). Using positron emission tomography (PET), a significant increase in activated microglia was shown in demented patients (Cagnin et al., 2001). Also, increased expression of HLA-DR (major histocompatibility complex class II antigen) was seen in post-mortem brain tissue from AD patients (Overmyer et al., 1999b). Reactive astrocytes observed in the AD brain appear to be walling off the SP. Activated astrocytes are characterized by an increased expression of glial fibrillary acidic protein (GFAP), and hypertrophy (McGeer and McGeer, 2003b). Increased GFAP expression is seen in the AD brain, and astrogliosis was shown to correlate significantly with the number of NFTs in demented patients (Overmyer et al., 1999a). The accumulation of astrocytes around Aβ deposits may indicate a direct role for these cells in the degradation of Aβ. Deficits in astroglial clearance of Aβ, could then participate in the pathogenesis of AD (Wyss-Coray et al., 2003). Studies on inflammation in transgenic mouse models for AD show the occurrence of activated microglia in the vicinity or within amyloid plaques. Most of these mice exhibit amyloid plaques with associated microglial activation. Chronic gliosis is associated with altered APP-processing and may thus initiate or exacerbate pathological changes associated with AD (Bates et al., 2002). Experimentally induced neuroinflammation was shown to increase APP expression and processing, and to result in intracellular accumulation of Aβ (Sheng et al., 2003).

The degeneration of dopaminergic neurons in PD is associated with massive microglial activity (McGeer et al., 1988), and post-mortem examination revealed the presence of activated microglia decades after MPTP drug exposure (Langston et al., 1999). In an animal model for PD, microglial activation was detected before the appearance of a dopaminergic lesion (Czlonkowska et al., 1996). In models for other neurodegenerative disorders, microglial activation was seen even before neuronal damage in cerebral ischemia (Morioka et al., 1991), and in association with both diffuse plaque and PrP^{Sc} deposits in prion disease (Eikelenboom et al., 2002).

Cytokines

Cytokines represent a heterogeneous group of mediators that are involved in activation of the immune system and inflammatory responses. In the CNS, cytokines can be synthesized both by glial cells and neurons (Hopkins and Rothwell, 1995). Only some cytokines have been investigated in AD. Analysis of post-mortem brain specimens, showed a differential expression pattern of cytokines in different clinical stages of dementia, suggesting a correlation with the clinical progression of AD (Luterman et al., 2000). Under normal physiological conditions, the proinflammatory cytokines in the brain are very low. Proinflammatory cytokines that were demonstrated in the AD brain and or CSF and serum samples include interleukin-1 (IL-1), interleukin-6 (IL-6)) and tumour necrosis factor-α (TNF-α).

IL-6

The levels of IL-6 in the normal brain are low or undetectable (Wilson et al., 2002). However, in Down's syndrome and AD patients, both CSF and serum levels of IL-6 were shown to be increased, correlating with the severity of dementia (Kalman et al., 1997). The presence of IL-6 in SP in the AD brain was shown by immunohistochemistry, while plaques in non-demented elderly subjects did not exhibit IL-6 immunoreactivity (Bauer et al., 1991; Strauss et al., 1992). Increased expression of IL-6 was predominantly found in diffuse plaques, suggesting that IL-6 induction may be an early event in AD pathology (Fiebich et al., 1996; Hull et al., 1996). The expression of IL-6 was shown to be subjected to an age-related increase. Elevated plasma levels of IL-6 have been correlated with modest decline in cognitive function (Weaver et al., 2002). Transgenic overexpresson of IL-6 in astrocytes resulted several abnormalities, including astrogliosis, neurodegeneration of hippocampal neurons, reduction in dendritic arborisation, angiogenesis and induction of acute phase proteins (Campbell et al., 1993).

IL-1

Low levels of IL-1 expression have been documented in microglia, astrocytes, neurons and endothelium (Wilson et al., 2002). There is abundant data in support of a role of IL-1 in AD. Increased expression of IL-1 has been found in the brain of AD and Down's syndrome patients (Griffin et al., 1989), with a localization in activated microglia associated with SP and immediately adjacent to neurons bearing NTFs (Griffin et al., 1995; Sheng et al., 1997).

Stimulation with IL-1 has been shown to increase APP expression (Goldgaber et al., 1989). Also, chronic exposure to IL-1 by impregnated pellets in the rat brain caused elevated levels of tau mRNA and tau immunoreactivity in neuronal cell bodies (Sheng et al., 2000). Microglial overexpression of IL-1 is responsible for increased neuronal expression and activity of acetylcholinesterase (AChE) (Li et al., 2000). Caspase-1, initially named interleukin-1β converting enzyme (ICE) cleaves inactive proIL-1β to the mature IL-1β, that is responsible for most of the IL-1 activity in AD. The levels and activity of caspase-1 were found to be increased in the AD brain compared to controls (Zhu et al., 1999).

Several lines of evidence implicate IL-1 in the pathogenesis of cerebral ischemia, although its pathophysiological role has not been fully elucidated (Touzani et al., 1999). In an animal model of ischemia, increased expression of caspase-1 was shown in hippocampal microglia. Other studies showed changes in the expression of caspase-1 in CA1 pyramidal neurons (Bhat et al., 1996).

Increased expression of IL-1 in microglial cells can be seen in the acute phase following head injury (Griffin et al., 1994). In acute stage of multiple sclerosis, elevated levels of IL-1 have been found in CSF, suggesting its role in myelin destruction (Hauser et al., 1990). In PD, the concentration of several cytokines, including TNF-α, IL-1β and IL-6 has been found to be elevated in the CSF and in the striatum (Mogi et al., 1994a; Mogi et al., 1994b; Muller et al., 1998).

AIMS

The presence of the E4 isoform of apoE and a HC intake in diet are both important risk factors for neurodegeneration, especially for AD. ApoE4 has been suggested to have a lack of function as compared to other apoE isoforms. We used apoE KO and WT mice on HC or normal diet to study the impact of apoE deficiency and HC intake on different neurodegenerative features, specifically:

- the effects on gliosis and on the expression of the antioxidant enzyme NQO1 in the brain (paper I).
- the effects on the expression of IL-6 and caspase-1 in the brain (paper II).
- the effects on tau hyperphosphorylation (paper III).
- In paper IV, we investigated the effects of long-term hypercholesterolemia on brain gene expression.

MATERIALS AND METHODS

ANIMALS AND DIETS

Five to six weeks old C57BL/6 WT and apoE KO mice were purchased from Möllegärd laboratory Denmark, and B&K Sweden, respectively. The animals were housed in groups under ordinary conditions with 12 hours cycle light/dark. The mice were divided into two groups, one group given a regular chow diet, and the other group were on high HC diet (0.15%) (Lactamine, Sweden) for nine months.

IMMUNOHISTOCHEMISTRY

All animals were anesthetized with sodium chloral hydrate (40 mg/100 g, intraperitoneally) and perfused transcardially with 20 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.4, followed by 60 ml 4% paraformaldehyde (PF) in 0.1 M PBS buffer, pH 7.4, for 10 min. After perfusion, the animals were decapitated and brains were dissected out and fixed by immersion in the same fixative for 8 h. The brains were then transferred to a 10% sucrose (pH 7.2) solution and kept at +4°C until sectioning in the cryostat. Mice brains were coronally sectioned at 14 μm at four different levels (i.e. at 0.74, 0.02, -1.82 and -2.80 mm from Bregma) according to the Franklin & Paxinos mouse atlas, and placed on Polysine ™ microscope slides (Manzel-Gläser, Germany). The slides were kept at -20ºC until immunostaining. Table II shows the different primary antibodies used in immunohistochemistry. Staining was carried out using the ABC-method. Briefly,14 μm sections were air-dried at room temperature (RT) for 15 min, followed by rehydration in PBS (0.01 M, pH 7.4) for 5 min. The slides were then exposed to 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, followed by washing with PBS and incubated with 1% normal bovine serum in PBS/0.3%Triton X-100 for 1 h. Sections were washed with PBS and then incubated with primary antibodies over night at 4-8ºC in a humidified chamber. Subsequently, the slides were washed with PBS and incubated with the biotinylated secondary antibodies for 30 min at RT, and then with avidin-biotin-peroxidase complex (Vectastain®Elite, Vector Laboratories, code no: PK-6100) for 30 min. Diaminobenzidine was used as chromogenic substrate (10 min) followed by washing with running tap water (10 min). For a negative control, slides were treated in the same manner except for replacement of primary antibodies with the respective nonimmune serum.

Table II

Antibody	Raised in	Dilutions	Company
GFAP	Cow	1:2000	Dako, Cytomation AB
NQO1	Rabbit	1:2000	Gift from Dr. C. Lind
F4/80	Rat	1:100	Serotec Ltd, Scandinavia
IL-6	Rabbit	1:300	PierceBiotechnology, USA
Caspase-1	Rabbit	1:300	Pfizer, UK
MP33	Rat	1:50	Gift from Dr.R. Mebius
AT8	Mouse	1:500	Innogenetics, Belgium
S100 A8	Goat	1:100	Santa Cruz Biotechnology

CELL COUNTING

In papers I and II, all immunostained slides were were coded and analyzed in a Nikon Eclipse E800 microscope. The density of astrocytes containing GFAP or NQO1 (number of cells/mm^2) was assessed in a 0.5x0.5 mm grid at 20x magnification. The number of microglia labelled for IL-6, caspase-1 or F4/80, was assessed in a 0.25x0.25 mm grid at 40x magnification. GFAP- and NQO1-positive cells were counted in four areas in each section, i.e. two in the hippocampus and two in the cerebral cortex, as these areas are more prone to neurodegeneration in AD and stroke. The cells labelled for IL-6, caspase-1 or F4/80 were counted in one CA1 area in the hippocampus and inn two areas in cortex.

The processed sections were photographed with Open Lab™ v.2 (Improvision®, UK) software coupled to a Hamamatsu digital camera (C4742-95, Japan) and Nikon Eclipse E800 microscope**.**

WESTERN BLOTTING

Mice were sacrificed by cervical dislocation, brains immediately removed, and the hippocampus, cerebral cortex and cerebellum of one hemisphere dissected out, immediately collected in dry ice and stored at –80°C. Before the analysis, the tissue was thawed on wet ice, cut into pieces and homogenized (10 % wt/vol) at 4°C in a lysis buffer (40 mM Tris-HCl, pH 6.8, 1% NP-40, 2 nM Okadaic acid, 1 mM Na_3VO_4, 50 mM NaF, 20 mM β-glycerophosphate, 1 mM PMSF and Sigma protease inhibitor cocktail 1:500) by sonication (Soniprep 150,MSE Ltd, Crawley, Sussex, UK). The supernatants were stored at -20°C until use. The BSA protein assay kit (Pierce, Rockford, IL) was used to quantify total protein levels the in brain tissue extracts. Expression of different

proteins were analysed by separating equivalent amounts of total protein (40 μg/lane) by SDS-PAGE, using 10% polyacrylamide gels. The proteins were transferred to a Protran nitrocellulose membrane (Schleicher & Schuell, Germany) by trans-blot electrophoretic transfer for 8 hours at a constant current of 125 mA. After blocking for 1 h using 5% (w/v) BSA or 5% dried milk in Tris-buffered solution containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight with primary antibodies at concentrations specified in Table III. The membranes were subsequently washed and incubated for 1 h with HRP-linked, secondary antibodies (Amersham Biosciences, Little Chalfont, England, 1:2000). Bound secondary antibodies were detected by ECL detection systems (Amersham Biosciences, Little Chalfont, England) and exposed to hyperfilm MP (Amersham Biosciences, Little Chalfont, England). Some of the immunoblots were stripped using stipping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM mercaptoethanol) at 50° C for 30 min and reblotted with other antibodies. Relative density of the immunoreactive bands was calculated from the average optical density (OD) multiplied by the area of the selected band (NIH Image 1.62 software).

Table III

Antibody	Specificity	Dilution	Company	Address
AT-8 Tau	P-tau (S202/T205)	1:1000	Innogenetics	Zwijnaarde, Belgium
AT-100 Tau	P-tau (T212/S214)	1:500	Innogenetics	Zwijnaarde, Belgium
AT-180 Tau	P-tau (T231)	1:1000	Innogenetics	Zwijnaarde, Belgium
Tau	Total tau	1.1000	Dako	Glostrup, Denmark
CamkII	Total CamkII	1.1000	Cell Signaling Technology	Beverly, MA, USA
CamkII-P	P (Thr286)	1:1000	Cell Signaling Technology	Beverly, MA, USA
cdk5	Total cdk5	1:1000	Santa Cruz Biotechnology	California, USA
cdk5-p35	Neuron specific activator of cdk5	1:1000	Santa Cruz Biotechnology	California, USA
ERK	Total ERK	1:1000	BD transduction laboratories	San Diego, CA, USA
ERK-P (1/2)	P (Thr202/Tyr204) P (Thr183/Tyr185)	1.1000	Cell Signaling Technology	Beverly, MA, USA
GSK-3	Total GSK-3	1:1000	BD transduction laboratories	San Diego, CA, USA
GSK-3β (Ser9)	P-GSK-3β (Ser9)	1:1000	QCB-Biosource international	Camarillo, CA, USA
GSK-3 (Tyr-216)	P-GSK-3α/β (Tyr216)	1.1000	QCB-Biosource international	Camarillo, CA, USA
JNK	Total JNK	1:1000	Cell Signaling Technology	Beverly, MA, USA
JNK-P	P-JNK (Thr183/Tyr185)	1:1000	Cell Signaling Technology	Beverly, MA, USA
P38	Total p38	1:1000	Cell Signaling Technology	Beverly, MA, USA
P38-P	P-p38 (Thr180/Tyr182)	1.1000	Cell Signaling Technology	Beverly, MA, USA
AKT-P	P-AKT (Ser473)	1:1000	Cell Signaling Technology	Beverly, MA, USA

PROTEIN KINASE ACTIVITIES ASSAYS

The activity of a kinase depends on its phosphorylation state. As an indication of the activity, we calculated the ratios between the levels of the active phosphorylated form and the total levels for each kinase. The levels were determined by immunoblotting, with the exception of PKA. The

PKA activitiy was assayed in brain tissue homogenates using a commercial kit (Pierce, Rockford, IL, USA), with kemptide as substrate. The reaction was initiated by adding 5-10 μg of total protein. Incubation was performed for 50 min at 30°C and the reaction stopped by boiling the samples for 5 min. Twenty μl of the reaction mixture were applied onto Spin Zyme ™ separation units containing affinity membranes and the unreacted kemptide removed by elution. Quantitation of both non-phosphorylated and phosphorylated kemptide was determined by measuring relative absorbance unit by spectrophotometer at 570 nm.

PROTEIN PHOSPHATASE ACTIVITY ASSAYS

Activities of the protein phosphatases PP2A, PP2B and PP1 were measured using a RediPlate™ 96 EnzCheck® Serine/Threonine Phosphatase Assay Kit from Invitrogen (Leiden, The Netherlands) according to the manufacturer's instructions. This kit uses a specific buffer for each phosphatase and a phosphate substrate (DiFMUP) that upon cleavage results in a fluorescent product DiFMU. The fluorescence signal was measured by a CytoFluor 4000 (PerSeptive Biosystems), the excitation and emission wavelengths were being 345 and 490 nm, respectively. From each brain, a sample of 32.25 μg of total protein was used. Briefly, part of the cerebral cortex was thawed on ice, cut into pieces and and homogenized (10% wt/vol) at 4°C (in modified RIPA buffer containing, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF and protease inhibitor cocktail 1:500, Sigma-Aldrich, Saint Louis, MO, USA) by sonication (Soniprep 150, MSE Ltd, Crawley, Sussex, UK). Homogenization the samples were centrifuged at 11,000 rpm for 10 min at 4°C and supernatants stored at -20°C until use. The BSA protein assay kit was used to quantify the total protein in the brain tissue extracts. Fifty μl of appropriate reaction buffer were added to solubilize the substrate in the microplate wells before adding the sample (50 μl of brain homogenate containing 33.25 μg of protein). After incubation at room temperature for 30 min, fluorescence signal was measured.

RNA ISOLATION AND MICROARRAY ANALYSIS

Total RNA was isolated from the right cerebral hemisphere of each brain using the Qiagen RNeasy lipid tissue mini kit (Qiagen, USA) according to the manufacturer's instructions. The quality of the RNA samples was ascertained on a denaturing agarose gel. Additionally, total RNA was isolated from a pool containing the cerebellum from all animals. This pool sample was used

as a reference for the individual animal samples in the cDNA microarrays. Ten mg of total RNA from each sample were used in the microarrays. Fluorescence-labelled cDNA probes were synthesized by oligo DT-primed reverse transcription (RT) reaction by using Superscript II (Life Technologies Inc) in the presence of cyanine-labelled nucleotides (Cy3-UTP for LNCaP-FGC and Cy5- UTP for LNCaP-r, Amersham Pharmacia Biotech). Cy3 and Cy5–labelled cDNA probes were combined and purified by using Microcon (Millipore). The final volume was adjusted to 25 μl with hybridization buffer consisting of 5×SSC, 0.2%SDS, 10 μg poly A RNA and 10 μg yeast tRNA. After heating at 100ºC for 2 min, the probes were added to the array and covered with a 22x22 mm cover slip (Grace Biolabs, OR). The array chip was placed in a sealed hybridization chamber (Corning, NY) and the hybridization took place at 65ºC for 15-18 h. The array chip was then washed and immediately scanned by using the GMS418 scanner (Affymetrix). Each hybridization was performed four independent times, one of which was performed in a dye swoop manner.

STATISTICAL ANALYSIS

Cell counting data

Data obtained from cell counting were first converted to logarithmic scale and then analysed by two-factorial analysis of variance (ANOVA), followed by analysis of the statistical differences using the Tukey *post hoc* test with STATISTICA (version 6.0).

Biochemical data

Data from western blotting, PKA-activity assay and phosphatase activity assays, were analyzed by ANOVA followed by Fisher's PLSD *post hoc* test. A p-value < 0.05 was considered statistically significant.

Microarray data

The Cy3 and Cy5 images were superimposed and analyzed by using the GenePix Pro software (Axon Instruments, CA). Automatic and manual flagging were used to localize absent or very week spots (less than 1.6 times above the background level), which were excluded from analysis. The value of the signal from each spot was calculated as the average intensity minus the background. Using "LOWESS" normalization method in the SMA-package, normalization was performed between the two fluorescent images. SMA is an odd-on library written in the public domain statical language R. The LOWESS algorithm performs a local fit to the data in an intensity-dependent

manner. The intensity value for each spot is normalized based on data distribution in the immediate neighbourhood of the spot's intensity. The significance of the expression ratios of the transcript and its mean value were calculated using the significance analysis of microarray (SAM) software. SAM is a statistical technique for finding genes with significantly altered expression in a set of microarray experiments. SAM assigns a score to each transcript on the basis of the changes in gene expression relative to the standard deviation of the multiple measurements. Thereby, SAM allows us to detect differentially regulated genes taking into account the percentage of a gene differentially regulated by chance, so called false discovery rate (FDR). To each of the genes in the array a q-value was assigned. This value is similar to the commonly used p-value and indicates the lowest FDR at which the gene is considered significant. In this, study q-values below 5% were regarded as significant.

RESULTS AND DISCUSSION

PAPER I

An increasing body of evidence supports the idea that inflammatory processes play a critical role in the pathogenesis of AD and other neurodegenerative disorders. In this study, we analyzed the effects of HC diet and apoE deficiency on gliosis and on a marker for oxidative stress. ApoE KO and WT mice were given a ND or HC diet for 9 months. Analysis of GFAP- immunoreactivity showed labelled astrocytes in all of the experimental groups. The number of GFAP labelled cells and intensity of the GFAP signal was lowest in the WT mice on ND, and highest in apoE KO mice on HC diet, with no apparent differences between the four areas analyzed. In both areas of hippocampus and in the area including the corpus callosum and adjacent cerebral cortex (cc/cortex) a significant interaction was found between lack of apoE expression and the HC diet. This interaction was not observed in the cortical area. In all areas, GFAP expression was significantly higher in animals on HC diet (both apoE KO and WT) as compared to animals on ND. In apoE KO mice on HC diet, the astrocytes were hypertrophied with large cell body and thick processes.

Microglia were identified with a cell surface marker, an anti-F4/80 antibody. In all areas analyzed, there was a significant increase in the number of microglial cells in mice on HC diet, irrespective of genotype (Fig. 4). An antibody against antioxidant enzyme NQO1 was used as a marker for oxidative stress (Fig. 5). NQO1-immunoreactivity was seen in the cell body of astrocytes and in the proximal part of their processes. Some endothelial cells in the cerebral blood vessels were also positive for NQO1 labelling. HC diet and the lack of apoE expression resulted in a synergistic in increase in NQO1-labelling in three (two cortical and one hippocampal) of the four areas analyzed ($p < 0.0005$). In the other hippocampal area analyzed, HC diet also resulted in a higher density of NQO1-labelled cells in the WT mice ($p < 0.05$).

These findings suggest that both apoE-deficiency and hypercholesterolemia are associated with changes in glial reactivity. Activated astrocytes and microglia have been demonstrated to be associated with neuritic plaques, studies on postmortem on AD brain (Overmyer et al., 1999a; Overmyer et al., 1999b). Chronic gliosis is a trigger for altered APP-processing *in vivo* (Bates et al., 2002), and several cytokines and proinflammatory and cytotoxic factors have been suggested to potentiate amyloidosis and neuronal cell death (Del Bo et al., 1995; Forloni et al., 1992; Mrak et al., 1995). In support of this idea, microglial activation, astrocytosis and intraneuronal Aβ

accumulation were the major immunohistochemical features observed in the brain of rabbits on a HC diet. Furthermore studies on transgenic mice have shown that hypercholesterolemia accelerates the deposition of Aβ (Refolo et al., 2000; Refolo et al., 2001).

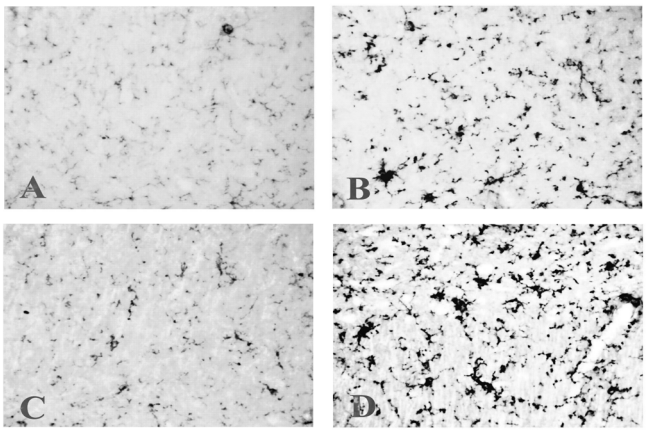

Fig. 4: Microglial cells in WT (A, B) and apoE KO (C, D) mice on ND (A, C) and HC diet (B, D), labelled with the F4/80 antibody at 40x magnification in hippocampus.

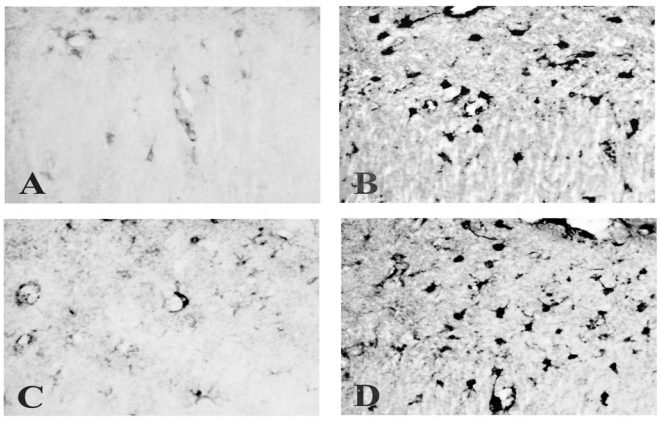

Fig. 5: NQO1 labelled cells in WT (A, B) and apoE KO (C, D) mice on ND (A, C) and HC diet (B, D), at 40x magnification in hippocampus.

The antioxidant enzyme NQO1 was previously described in different cell types in the rat brain (Schultzberg et al., 1988). NQO1 has been shown to occur in astrocytes and in neurites surrounding the SP in the AD brain. (SantaCruz et al., 2004; Wang et al., 2000). In our studies, there was a marked increase in the expression of NQO1 in astrocytes upon HC diet, indicating an

upregulation of antioxidant systems in response to hypercholesterolemia. This effect was potentiated by the lack of apoE, where as apoE deficiency did not result in a significant change in mice on ND.

These data, together with some previous reports (Prasad and Kalra, 1993), support the notion that hypercholesterolemia may be an additional potentiating factor, or even one of several possible initiating factors, in a vicious circle of glial reactions, cytokine release and oxidative stress leading to neurodegeneration.

PAPER II

Cytokines represent a heterogeneous group of mediators that are involved in activation of the immune system and inflammatory responses. The expression of cytokines is increased in microglial cells in AD brain and other age related neurodegenerative disorders. In this study, we used our model of investigation to examine the effects of HC diet and apoE deficiency on the proinflammatory cytokine IL-6 and on caspase-1, which is responsible for converting inactive pro-IL-1β into active and mature IL-1β expression. Three different brain areas, one hippocampal and two cortical, were analysed by immunohistochemistry. We found that the majority of the IL-6 positive cells were very small and showed multiple short processes. However, in animal fed with HC diet, a few IL-6 positive cells, located near the medial sulcus and the lateral part of the cortex, were round in shape and did have no processes. In general, cells with IL-6 immunoreactivity were diffusely distributed all over the hippocampus, except in the pyramidal cell layers of CA1, CA2 and CA3. HC diet induced a significant increase in IL-6 expression in the hippocampus and the dorsal part of the cerebral cortex in both WT and apoE KO animals. No significant interaction between apoE deficiency and HC diet was found.

Caspase-1 expression was found only in cell bodies and not in the processes. In apoE KO mice on HC diet, some positive cells were round in shape and larger in size. In all experimental groups, the caspase-1 immunoreactivity was more abundant in the dorsal part of the cortex, near the median sulcus, than in other parts of the brain. Numerous positively stained cells were also found in the corpus callosum. HC diet increased the intensity and the cell number of caspase-1 positive cells in cortical areas, independently of the presence or absence of apoE. In the hippocampus of apoE KO mice, the density of caspase-1 positive cells was increased by the HC diet.

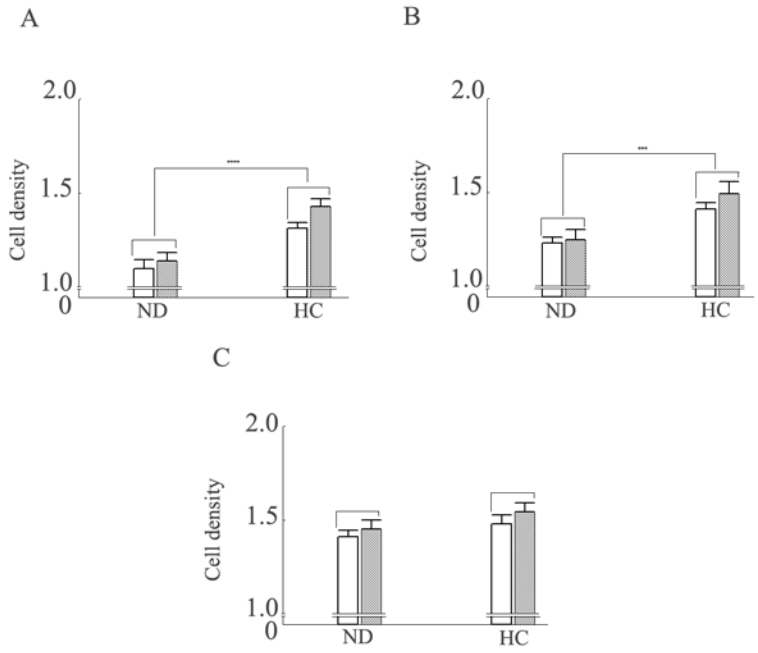

Fig. 6: Density of IL-6 positive cells in (A) the hippocampus area (B) cortex area 1 and (C) lateral cortical area. White and shaded bars indicate WT and apoE KO mice respectively. Data are expressed as log of cells/mm^2. HC = High cholesterol, ND = Normal diet, * ($p < 0.001$) and **** ($p < 0.0001$) and n = 8.**

In paper II we found that chronic exposure to HC diet increases brain expression of some important proinflammatory cytokines, such as IL-6 and Caspase-1, independently of the apoE functionality. These findings suggest that high blood cholesterol levels facilitate pro-inflammatory mechanisms in brain. The levels of IL-6 in the CNS, under normal physiological conditions are very low (Wilson et al., 2002), but increase with age (Ershler and Keller, 2000). Some harmful conditions such as hypoxia and oxidative stress were shown to induce IL-6 expression in astrocytes (Maeda et al., 1994). In AD, IL-6 is present in the microglia (Bauer et al., 1991; Strauss et al., 1992), and is increased in the serum and CSF of AD patients (Kalman et al., 1997). Factors responsible of IL-6 synthesis in AD brains are still not known, however there is evidence that Aь may stimulate microglial activation, leading to increased synthesis and release of proinflammatory cytokines (Araujo and Cotman, 1992; Lindberg et al., 2005). In addition, the fact that the increase in IL-6 correlates with the severity of dementia in AD (Kalman et al., 1997),

further suggests that this proinflammatory cytokine has an important role in the development of the disease. If this is the case, our findings in paper II could indicate that the induction of IL-6 expression may be one of the mechanisms by which high blood cholesterol increases the risk for AD, and that this mechanism would be independent of deficits in the function of apoE.

PAPER III

Hyperphosphorylation of tau, leading to the formation of PHFs, dystrophic neurites and NFTs is a characteristic feature of several neurodegenerative diseases, including AD. This neurofibrillary pathology causes the loss of axonal integrity and eventual decline in connectivity and synapses. In Paper III, we have investigated the effects of HC diet and apoE deficiency on tau phosphorylation. We found that HC diet potently and invariably induced tau hyperphosphorylation in the brain of apoE KO mice. By immunohistochemistry, we detected abundant intraneuronal inclusions of phospho-tau in the cytoplasm of neurons in the cerebral cortex, especially in retrosplenial and lateral parts, of all apoE KO mice on HC diet. Additionally, some animals of this group had abundant phospho-tau immunoreactivity in the dentate gyrus of the hippocampus (particularly in the polymorphic layer). Homogenates prepared from the cerebral cortex were tested by immunoblotting, with the AT8 (that recognises tau phosphorylated at Ser-202) and two additional anti-phosphorylated tau antibodies, AT180 (recognising tau phosphorylated at Thr-231) and AT100 (recognising tau phosphorylated at Thr-212 and Ser-214). All three antibodies detected markedly increased phospho-tau levels in all apoE KO mice fed on HC diet. Previous reports showed conflicting results regarding the effects of apoE deficiency on tau phosphorylation. Genis et al showed increased phopho-tau levels in brain homogenates from apoE KO mice on ND (Genis et al., 1995). This was not seen by others (Mercken and Brion, 1995). In our study on a total number of 12 animals per group, only two female apoE KO mice on ND, showed increased tau phosphorylation. Bi et al. also reported the presence of AT-8 immunolabelled cells in some, but not all, apoE KO mice analysed (Bi et al., 2001). The explanation for these contrasting results remains to be found. We also observed increased tau hyperphosphorylation in one female WT mouse on HC diet. Therefore, it is tempting to speculate that females may be more susceptible to tau hyperphosphorylation upon HC diet or upon deficiency of apoE, and that apoE deficiency may increase neuronal vulnerability to hyperphosphorylation of tau, but not be the direct cause.

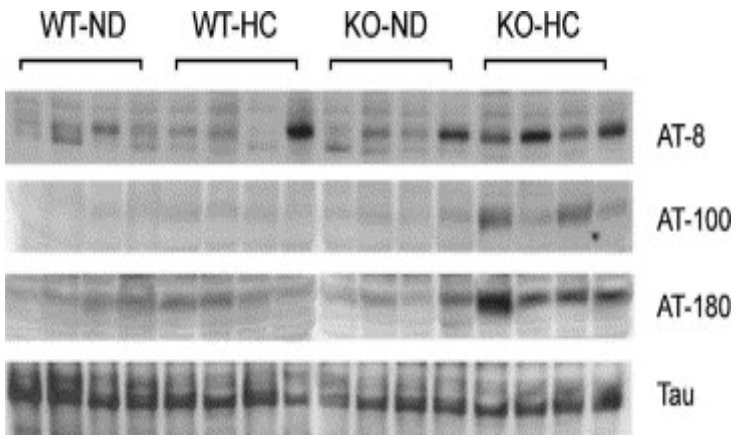

Fig. 7: Immunoblots of the cerebral cortex homogenates from WT and apoE KO mice on ND or HC diet using anti-P-tau antibodies (AT-8, AT-100 and AT-180) and the antibody recognising total tau protein.

The phosphorylation state of tau is a balance between the activity of several kinases and phosphatases. In order to explore the mechanism by which tau is hyperphosphorylated in apoE KO animals on HC diet, we investigated the activities of the major kinases and phosphatases responsible for the phosphorylation state of tau. We found that apoE KO mice on HC diet had significantly increased activities of cdk5, GSK-3β, JNK and p38 kinases. Independently of the diet, apoE KO animals also had significantly increased CamkII and PKA activities. This increase was intensified in apoE KO mice on HC. HC diet did not change the activity of any of the kinases investigated in WT animals. The activities of Akt and ERK1/2 and of the protein phosphatases PP1, PP2A and PP2B, did not differ between the groups of investigation. Our results suggest that the synergistic effects of apoE deficiency and HC intake on tau hyperphosphorylation, are mediated by an increase in tau phosphorylation, rather than by a decrease in tau dephosphorylation.

Biochemical studies have shown that apoE deficiency is associated with enhanced lipid peroxidation and chronic oxidative stress in the brain (Montine et al., 1999; Ramassamy et al., 2001). A clear relation has been described between tau hyperphosphorylation and oxidative stress, possibly by upregulation of several tau kinases (Gomez-Ramos et al., 2003; Lovell et al., 2004). Our results from paper I demonstrated that HC diet induced enhanced oxidative stress in brains from apoE KO mice. In view of these observations, it can be proposed that an enhanced oxidative stress with a subsequent upregulation of tau kinases upon HC diet, mediates the increased tau hyperphosphorylation seen in apoE KO mice on HC diet.

In humans, the presence of the E4 isoform of apoE, confers higher risk for several tauopathies including AD (Josephs et al., 2004). Increased phosphorylation of tau was reported in apoE4 knock-in mice as compared to apoE3 knock-in mice (Kobayashi et al., 2003). Futhermore increased tau phosphorylation in mice with transgenic expression of apoE4 or apoE4 fragments in neurons was associated with activation of ERK (Harris et al., 2004). Very little is known about role of apoE4 in the hyperphosphorylation of tau and the development of NFTs. It has been hypothesized that lack of function provided by apoE4, as compared to apoE3, in binding and protecting tau from hyperphosphorylation, could be the reason (Strittmatter et al., 1994). In paper III, we demonstrated that the combination of apoE deficiency and HC intake resulted in a drastically intraneuronal accumulation of hyperphosphorylated tau, probably by increasing the activity of several tau kinases. Our results suggest that the interaction between dietary and genetic factors might be relevant for the development of tauopathies in humans.

PAPER IV

The mechanisms by which HC in blood increase the risk for neurodegenerative disorders such as AD remain puzzling, since blood cholesterol is segregated from the CNS by the BBB. To investigate the effects of HC diet on the brain, WT mice on HC diet were analyzed with regard to changes in brain gene expression using cDNA microarrays. We found that HC diet induced mild changes. Among the 15,000 genes examined in our study, significant changes were observed in 74 genes (53 up-regulated and 21 down-regulated). The gene expression changes ranged from ratios 2.70 to 0.46. The genes were assigned functional categories by using the Gene Ontology (GO) system (www.geneontology.org) (Table IV)

Table IV

Functional Category	Percentage of genes with altered expression
Transcriptions	23
Signalling	21.6
Apoptosis or oxidative stress	16.2
Proteolysis and ubiquitin cycle	9.5
Cholesterol metabolism	8.1
Inflammation and defense	6.7
Others	14.8

Some of the genes altered by the HC diet were already known to be involved in AD neuropathology. The expression of angiotensin-converting enzyme (ACE) was increased. This enzyme is involved in blood pressure regulation by converting angiotensin I to angiotensin II, and ACE inhibitors are widely used as antihypertensive drugs (Sica and Elliott, 2001). In human CNS, ACE has been detected in the choroid plexus, basal ganglia, substantia nigra, the cerebral cortex, hippocampus and it is a normal constituent in CSF (MacGregor et al., 1995; Savaskan et al., 2001). Increased intensity and density of the ACE staining was observed in AD brain cortex as compared to controls (Barnes et al., 1991), and ACE polymorphisms are suggested as a risk factor for AD (Farrer et al., 2000). Another vascular regulatory molecule, the neurohypophyseal hormone arginine-vasopressin (AVP), was upregulated in mice fed HC diet. AVP is involved in the control of vasoconstriction and increased levels have been in the temporal lobe of the cortex, where as reduced levels were seen in the cerebellum of AD and Down's syndrome subjects (Labudova et al., 1998).

The regulatory subunit of PP2A was down-regulated by the HC diet. PP2A is likely to be the major tau phosphatase, since it has a much stronger ability in dephosphorylating NFTs in the AD brain than PP1 or PP2B (Wang et al., 1995; Wang et al., 1996). Inhibition of PP2A activity by okadaic acid (OA) was found to induce tau hyperphosphorylation and hampered its biological activity in cultured neurons (Kim et al., 1999; Tanaka et al., 1998). All PP2A holoenzymes consist of a catalytic subunit (C), structural scaffolding subunit (A) and regulatory subunit (B). The PP2A regulatory subunits are categorized into several distinct families that generate a diversity of holoenzymes. Besides regulating phosphatase activity, the B subunits are thought to be responsible for the substrate specificity and targeting of PP2A (Sontag, 2001). Downregulation

of PP2A by the HC diet may be one of the mechanisms for the aberrant tau hyperphosphorylation in several neurodegenerative disorders.

We have also found that long-term HC diet results increased expression of NPC1 in the brain. NPC1 is mutated in about 95% of all NPC cases, and NPC2 is mutated in the remaining 5%. NPC is a dementia-causing autosomal recessive disorder and mutations in the NPC genes result in considerable intraneuronal cholesterol accumulation and tau hyperphosphorylation (Distl et al., 2001).The altered expression of NPC1 and other molecules involved in brain cholesterol homeostasis by the HC diet opens the question whether blood cholesterol modulates brain cholesterol levels indirectly.

Expression levels of the DNA primase p58 and p49 subunits were also increased by the HC diet. Both subunits have been shown to be up-regulated in neurons treated with Aβ, and also to be essential for the mechanism behind its toxicity (Copani et al., 2002).
The microglial Ca^{2+} binding protein S100A8 was highly upregulated by the HC diet. S100A8 is not expressed by resident microglia in normal conditions, but only in various inflammatory conditions, including cerebral malaria and focal cerebral ischemia (Postler et al., 1997; Schluesener et al., 1998).

The findings that long-term hypercholesterolemia altered the expression of these and other genes provide new insight into the molecular mechanism by which a HC diet enhances the risk for AD and other neurodegenerative disorders. Confirmational and functional studies of these results are currently ongoing.

CONCLUSIONS

Long-term HC diet induced glial activation and increased oxidative stress in the brain. These effects were, in some areas, enhanced by the absence of apoE (Paper I).

Long-term HC diet increased the expression of the proinflammatory cytokine IL-6 and of caspase-1 in the brain (Paper II). These findings were found to be independent of the presence or absence of apoE.

HC diet induced intraneuronal tau hyperphosphorylation in apoE deficient mice (Paper III).

Long-term HC diet induced mild changes in the gene expression in the mouse brain (Paper IV). Interestingly, some of the altered genes were ealier shown to be involved in neurodegenerative disorders, especially in AD.

The present studies the hypothesis that HC diet and apoE deficiency participate in neurodegenerative processes by influencing several signalling pathways. These include increasing inflammation, oxidative stress and tau hyperphosphorylation. The results also suggest that different risk factors could synergistically collaborate in the development of a neurodegenerative disorder. This may be relevant for understanding their role in neurodegenerative diseases.

FUTURE PERSPECTIVES

During the past decade, a tremendous effort has been made in understanding the pathogenesis of neurodegenerative diseases, especially of AD. A large proportion this effort has been focussed on the genetics, with the identification of several disease-causing mutations in a small number of families.

The majority of cases do not, however, have a known genetic deterministic cause. Investigation of the factors leading to these "sporadic" cases remains a vital task for understanding the disease pathogenesis. Both genetic (such as apoE4) and environmental risk factors are important for neurodegeneration. Often the same molecules (such as apoE4) confer risk to different neurodegenerative disorders. Investigation these factors, and possible synergistic effects, is essential to comprehend the processes ending in neurodegeneration.

Many basic questions concerning apoE4 still remain to be solved. Why does apoE4 increase the vulnerability to several neurodegenerative disorders? What is different in apoE4 carriers that never develop a disease?

How HC levels in the plasma, which is segregated from the CNS, influence neurodegeneration? Are different cholesterol metabolites (such as 27-hydroxycholesterol) responsible? Is this association due to microvascular changes?

Neurodegenerative disorders are often complex multifactorial syndromes, therefore unlikely to be cure by a single, directed therapy. Characterization of the risk factors involved and the mechanisms by which they influence, the disease will provide clues for multitarget strategies.

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