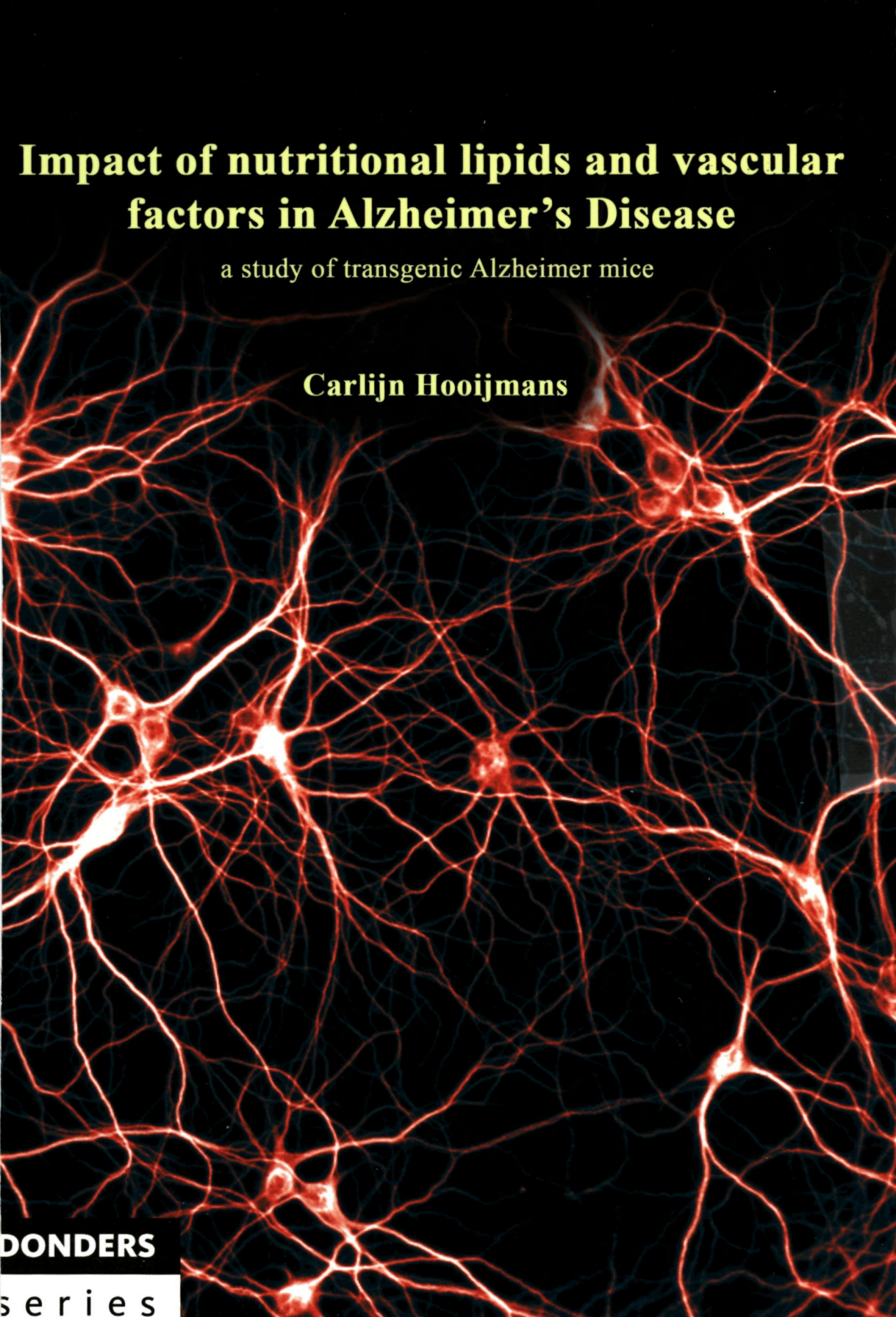
Impact of nutritional lipids and vascular factors in Alzheimer's Disease

a study of transgenic Alzheimer mice

Carlijn Hooijmans



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Impact of nutritional lipids and vascular factors in Alzheimer's Disease

-a study of transgenic Alzheimer mice-

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Promotores:	Prof. dr. M. Ritskes-Hoitinga
	Prof. dr. A. Heerschap
Copromotor:	Dr. A.J. Kiliaan
Manuscriptcommissie:	Dr. R.M.W. de Waal
	Prof. dr. G.J.M. Martens
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Voor "the ones I love"

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Chapter 1

General Introduction

Part of this chapter is based on:
Fatty acids, lipid metabolism and Alzheimer pathology.

Hooijmans CR [a], Kiliaan AJ [ab]
[a] Radboud University Nijmegen Medical Centre, Department of Anatomy and [b] Department of Cognitive Neuroscience, Donders Centre for Neuroscience, Geert Grooteplein noord 21, 6500 HB, Nijmegen, The Netherlands.

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General introduction

Alzheimer's disease (AD) is acknowledged as the most common form of dementia in the elderly. In 1906 Alois Alzheimer, Director of the Cerebral Anatomical Laboratory of the Ludwig-Maximilians University Munich, presented for the first time his observations about amyloid plaques, neurofibrillary tangles, and arteriosclerotic changes, the neuropathological hallmarks of what later was termed AD, as found in the post mortem brain of his 55-year old patient Auguste D(eter). Her illness lasted just 5 years in which she developed the neuropathology and progressive cognitive impairment, hallucinations, delusions, and impaired social functioning.
Hundred years later, in 2006, approximately 26.6 million people worldwide suffered from AD and because of the growing life expectancy we can expect that the global prevalence of Alzheimer's will quadruple by 2050 to more than 100 million, which means that 1 in 85 persons worldwide will be affected by this disease.

Alzheimer's disease pathology

Clinical features

Alzheimer's disease (AD) affects a person's ability to carry out daily activities and is characterized by severe cognitive impairment, memory loss, anomia (problems with word finding), apraxia (problems with complex movements), impaired executive functioning (difficulties with everyday decision making) and personality changes (for example mistrust, aggression and delusions).

Neuropathological features

Neuropathologically AD is characterized by intracellular neurofibrillary tangles extracellular amyloid plaques, cerebrovascular alterations and neurodegeneration.

Plaques and tangles

It took over 70 years from the first description of the amyloid plaques in 1906 until it was discovered in the eighties, that the plaques consist of aggregates of extracellular loads of 8 nm filaments of small peptides called amyloid-β (Aβ) [1]. Early in the course of the disease specific brain regions like the forebrain and medial temporal lobe structures (hippocampus, amygdala and entorhinal cortex) are affected with extracellular lesions characterized by

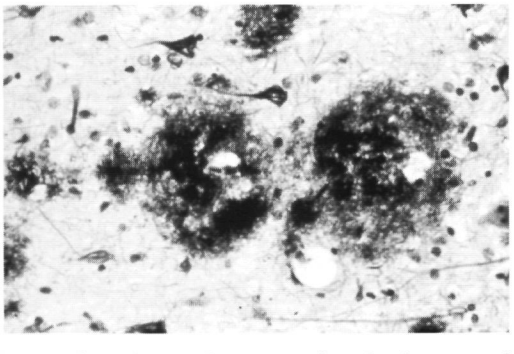

Fig.1 Immunohistochemical staining of senile plaques and tangles

accumulation of Aβ peptide [2,3]. A second lesion described by Alzheimer, are the neurofibrillary tangles (NFT), which are composed of aggregates of abnormally hyperphosphorylated tau protein, occurring both in neuronal cell bodies and in axons and dendrites [4]. The tau protein is normally predominantly involved in the assembly and stabilization of microtubules. It is suggested that NFT's are correlated with cognitive decline in AD [4,5].

Synaptic loss and neurodegeneration.

The amyloid deposits form a good diagnostic marker but do not correlate well with neuronal damage and cognitive decline. Synaptic loss however is an early pathological hallmark of AD which is age dependent and largely independent of amyloid-β [6,7]. Studies in early stage AD patients suggest that the neurodegenerative process initiates in the entorhinal cortex resulting in denervation in the hippocampus followed by loss of synapses in the molecular layer of the dentate gyrus and the nucleus basalis of Meynert [8]. Amyloid plaques formation and development of neurofibrillary tangles and gliosis accompany this process. In comparison it has been found in transgenic mice models of AD that synaptic loss and memory impairment precede amyloid deposits in the limbic system [9]. Also in other studies it has been found that AD is associated with disruption of synaptic contacts in specific cortical and subcortical areas and in early AD first 20% loss synapses in the outer molecular layer of the hippocampal dentate gyrus has been observed [8]. In later stages of AD, also loss in neocortex and even more damaged synapses has been found in the outer molecular layer of the dentate gyrus. Synaptic loss is correlated with memory impairment being a prodromal stage in the process of AD [10] and it is accompanied with abnormal processing of APP and soluble amyloid-β consecutively resulting in plaque formation [9].

Microvascular changes

When Alois Alzheimer presented for the first time his observations as found in the post mortem brain of Auguste D(eter), he described besides amyloid plaques and tangles also some arteriosclerotic changes. Vascular alterations in AD did not receive much attention in the past. However in the last decades vascular factors finally got some renewed attention. For example, AD and vascular dementia share the same risk factors [11,12], and pharmacotherapy improving cerebrovascular insufficiency decreases AD symptoms [13]. There is also evidence showing that cerebral perfusion is decreased in AD patients [14]. Besides haemodynamic changes, various microvascular abnormalities have been observed in AD patients, such as atrophic thin vessels, glomerular loop formations, fragmented vessels and twisted vessels, basement membrane thickening, pericyte degeneration, endothelial cell shape changes and luminal buckling (see ref [15] for review). A decrease in vascular density is also frequently observed [16]. In addition, in 80-90% percent of patients with AD also cerebral amyloid angiopathy (CAA) is noted. CAA is characterized by the deposition of Aβ in the media and adventitia of meningeal and cerebral arteries and arterioles, and sometimes in capillaries and veins. It is suggested that CAA causes severe impairments of de vascular wall [17].

Generation of amyloid-β

Amyloid-β is generated by sequential cleavage of the transmembrane amyloid precursor protein (APP), by groups of enzymes named α-, β- and γ-secretase. The cleavage and processing of APP can be divided into a non-amyloidogenic pathway and an amyloidogenic pathway. In the most common pathway, the non-amyloidogenic pathway, APP is cleaved by the α-secretase, producing a large amino (N)-terminal ectodomain (sAPPα), which is secreted into the extracellular medium. Because this cleavage occurs inside the amyloid-β region, formation of amyloid-β is prohibited. Three enzymes with α-secretase activity have been identified, all belonging to the ADAM family (a disintegrin- and metalloproteinase-family enzyme.

In the amyloidogenic pathway, amyloid-β is generated by an initial cleavage of APP at the N-terminus by a β-secretase (BACE; β-site APP cleaving enzyme) followed by a cleavage in the transmembrane domain by a γ –secretase. The γ-secretase is a complex of enzymes composed of presenilin 1 or 2 (PS1 and PS2). γ-secretase cleaves at positions 40 and 42 of the amyloid-β protein, generating amyloid-β 40 and amyloid-β 42, respectively. Approximately 10% of the residues exist of amyloid-β 42 whereas the predominant amount produced, exists of amyloid-β40. The amyloid-β42 is the principal isoform in amyloid plaques because of its fibrillary nature.

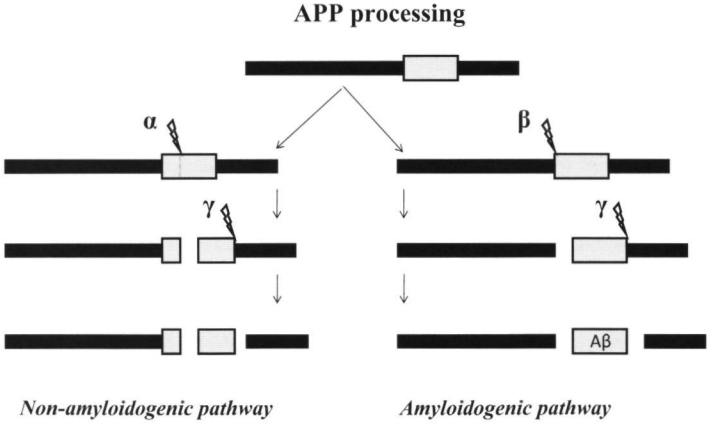

Fig 2. Processing of the Amyloid precursor protein (APP).
Proteolytic cleavage of APP by the α-secretase (such as ADAM10), within the Aβ sequence, precldes formation of amyloidogenic peptides (non amyloidogenic pathway).Cleavage of APP by β (BACE 1 and 2) and subsequently γ-secretases, causes the production of the Aβ peptide (amyloidogenic pathway).

Genetics of Alzheimer's disease

Ageing is the major risk factor for AD, reflected in increasing prevalence with advanced age. Less than 1% of people of 60-64 yrs old have AD whereas 40% of people older than 85 yrs are affected [18]. The cut-off to distinguish between early onset and late onset AD is arbitrarily set at 60-65 years.

The early onset, or presenile Alzheimer's disease, often have a positive family history

which is the second most important risk factor for AD [19] Early-onset AD has so far been linked to mutations in the genes for the amyloid precursor protein (APP) on chromosome 21, presenilin 1 (PS1) on chromosome 14, and presenilin 2 (PS2) on chromosome 1. The mutations in these genes affect the metabolism or stability of amyloid-β, and they are thought to account for 40% of early-onset AD[19].

Late-onset or senile Alzheimer's disease accounts for 95% of all AD cases. The late-onset AD genes discovered until now, act as either risk factors and/or genetic modifiers. A number of potential susceptibility genes have been implicated as genetic risk factors for late-onset AD from which the apolipoprotein E gene is the best known.

The apolipoprotein E gene on chromosome 19 encoding the cholesterol transport protein, apolipoprotein E (ApoE) was identified as risk factor in both familial and sporadic AD in which the ε4 allele of the APOE gene has been found associated with AD [19]. Carriers of one or two ε4 alleles of the APOE gene have a considerably higher risk to get AD on an earlier age of onset [20,21], whereas inheritance of the ε2 allele appears to be protective against AD. The mechanism by which the ApoE4 protein predisposes subjects to AD is not fully revealed. However evidence is accumulating, that E4 enhances amyloid-β aggregation by stimulating amyloidogenic processing of APP and reduces amyloid-β clearance [22], impairs the cerebrovasculature [23,24] or causes neurodegeneration [25].

Risk factors and cause of Alzheimer's disease

Many theories have been proposed about the cause of AD and the "amyloid cascade hypothesis", in which amyloid deposition is seen as the primary pathway leading to neurodegeneration and AD, is one of the most persistent [1]. However this hypothesis is still under debate because of conflicting results about for example the neurotoxicity of amyloid-β deposition in vivo [26] and it has been found that also many non-demented persons show large amounts of AD pathology [4]. Amyloid deposition is not the earliest neuropathological event observed in the disease [2,3] and is not always correlated to neuronal loss [27]. In transgenic mice it has been found that cognitive loss is independent of amyloid-β overexpression and precedes amyloid-β deposition [28].

Nowadays, more and more consensus is reached about vascular disorders being major risk factors for AD leading to the vascular hypothesis [26,29]. Many large epidemiological studies [11,30] have revealed that several risk factors for AD are vascular-related, (table 1) causing impaired cerebral perfusion, indicating that cerebral hypoperfusion may play an important role in the onset of AD [31,32].

Table 1. Risk factors for Alzheimer's disease

Ageing	Diabetes mellitus
Presence of APOEε4 allele	Stroke
Hypertension	Head injury
Congestive heart failure	Menopause
Atrial fibrillation	Lower education
Atherosclerosis	Depression
Smoking	Sedentary lifestyle
Alcoholism	Overweight
High intake of saturated fat	White matter lesions

Vascular risk factors

Major vascular risk factors for AD are hypertension and atherosclerosis. Hypertension is a risk factor for stroke, ischemic white matter lesions, silent infarcts, general atherosclerosis, myocardial infarction and cardiovascular diseases, and often clusters with other vascular risk factors, including diabetes mellitus, obesity and hypercholesterolemia. Hypertension is present in approximately 50% of people above 70 years and predicts both AD and vascular dementia already 20 years before onset of AD and elevated midlife systolic blood pressure of 160 mmHg or above is associated with lower brain weight, white matter lesions and greater numbers of senile plaques in hippocampus and neocortex [33-35] The development of atherosclerosis also takes decades before it manifests itself and it is logical to assume that diminished blood flow to the brain from stenosed or jammed carotid arteries leading to cognitive dysfunction is a process that likely precedes dementia symptoms by many years. Hypertension and atherosclerosis cause blood vessel wall pathology, leading to dysfunction in the blood brain barrier function, hypoperfusion, ischemia in the brain which may initiate the pathological process with cognitive impairment [36,37], ultimately leading to a significant increase in the incidence of AD [34] More and more evidence comes forward that cerebral ischemia plays an important role in development of AD and animal studies suggest that cerebral ischemia in the brain may lead to accumulation of APP and amyloid-β [38]. De la Torre [39] proposed that aging in combination with a vascular risk factor that further decreases cerebral perfusion promotes the Critically Attained Threshold of Cerebral Hypoperfusion (CATCH) which causes oxidative stress, decreased energy metabolism, followed by excessive glutamate production. All these events contribute to progressive cognitive decline synaptic loss, senile plaques, neurofibrillary tangles, tissue atrophy and neurodegeneration [39,40]

Lifestyle and diets

Influencing vascular risk factors via diet and lifestyle may influence AD development Indeed a correlation exists between AD and dietary fat as important contributor to familiar atherosclerosis, and a good example is the link between cholesterol metabolism and development of AD [41-43]. On the contrary, omega-3 fatty acids from fish oil might beneficially influence cardiovascular disease by decreasing blood pressure [44], and atherosclerosis formation [45,46] The strongest evidence of a relation between omega-3 fatty acid intake and vascular disease is the occurrence of coronary heart disease which have been shown in many experiments and clinical trials (for review. Intervention with cholesterol lowering agents or fish oil diets to prevent or reduce atherosclerosis could also lessen the prevalence of AD Recently performed docosahexaenoic acid (DHA) trials show inhibition of the course of AD [47] and several trials on cholesterol lowering statins have shown beneficial effects on inhibition of AD [47-49]

Western diets consist of high saturated fat and in combination with the sedentary lifestyles they have led to a growing incidence of obesity, hypercholesterolemia, and high blood pressure, causing atherosclerosis, coronary artery disease, and diabetes, major risk factors of AD, which are even more aggravated in people carrying the APOE ε4 alleles.

Summarizing we could postulate that a combination of diet, lifestyle, vascular, genetic,

and amyloid related factors, which can enhance each other's contribution in the onset and course of AD will be more likely the cause of the disease than a single factor alone.

Omega-3 fatty acids

The long-chain polyunsaturated fatty acids exist of two key families: omega-3 and omega-6, named after the place of the first double bond in the hydrocarbon chain. Especially docosahexaenoic acid (DHA) is the most abundant (omega-3) fatty acid in the mammalian brain, and its levels in brain membrane lipids can be altered by diet, and with life stage, i.e. increasing during development and decreasing with aging [50]. Very high levels of fatty acids and lipids can be found in the neuronal membrane and the myelin sheath. About 50% of the neuronal membrane is composed of poly unsaturated fatty acids, while in the myelin sheath lipids constitute about 70%. The omega-3 poly unsaturated fatty acids are mainly present in fish, shellfish, and sea mammals and are scarce in land animals and plants, whereas the omega-6 poly unsaturated fatty acids are derived mainly from vegetable oil. The precursors of the omega-3 and omega-6 poly unsaturated fatty acids cannot be synthesized endogenously from carbohydrates and are therefore called essential fatty acids (EFA). The current Western diet is deficient in long-chain omega-3 fatty acids which caused a switch in the ratio of omega-6 to omega-3 intakes from 2 to more than 20 within a century [51]. This has negative consequences because an excess of omega-6, and a deficient amount of omega-3, is stimulating thrombogenesis, lowering immune response, increasing inflammation and decreasing neuronal membrane fluidity and function [52-55].

Omega-3 fatty acids and the brain.

Particularly the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are incorporated into neuronal phospholipids (mostly phosphatidylethanolamine and phosphatidylserine) and in retinal pigment epithelium. Incorporation of omega-3 fatty acids in the neuronal membrane increases fluidity of neuronal membranes and herewith improves neurotransmission and signalling via increased receptor binding and enhancement of the number and affinity of receptors and function of ion channels [56,57]. Omega-3 fatty acids increase membrane fluidity by replacing omega-6 fatty acids and cholesterol from the membrane maintaining an optimal membrane fluidity as obligatory for neurotransmitter binding and signalling within the cell [58]. Large amounts of DHA are necessary during brain development, for neural membrane production used for synaptogenesis, axonal and dendritic outgrowth, in both brain and retina [59,60].

It will be clear from the above evaluated involvement of omega-3 fatty acids in neuronal functions, that they may play a role in cognitive development and memory-related learning via increase of neuroplasticity of nerve membranes, synaptogenesis and improvement of synaptic transmission.

Omega-3 fatty acids and cardiovascular disease.

In developed countries, consumption of the long-chain omega-3 poly unsaturated fatty acids DHA and EPA protects people against cardiovascular disease. These beneficial effects have been explained by the capacity to prevent arrhythmias [61], lowering plasma triacylglycerols [62,63], decreasing blood pressure [44], decreasing platelet aggregation ,

improving vascular reactivity [62,64] and decreasing atherosclerosis [45] and inflammation [54]. Almost 30 years ago studies in Eskimos from Greenland suggested already that ingestion of omega-3 fatty acids protects against cardiovascular diseases. The Eskimos consumed diets very high in fat from seals, whales, and fish, which contained large quantities of the long-chain polyunsaturated fatty acids EPA and DHA [65] In other studies it has been found that eating fish once a week significantly decreased coronary heart disease mortality rates [66]

Omega-3 fatty acids and Alzheimer's Disease

A decreased level of plasma Docosahexaenoic acid (DHA) is associated with cognitive impairment with aging [67,68] and does not seem to be limited to AD patients [69,70]. There are several possible reasons for this decline a decrease in the ability of dietary fatty acids to cross the blood brain barrier due to impaired transport function in aging, or lipid peroxidation caused by enhanced free radicals [71], decreased dietary intake or impaired liver docosahexaenoic acid shuttling to the brain [72] The deficiency affected mostly the cortex and hippocampus, areas which mediate learning and memory

Many animal, epidemiological and clinical studies showed that high DHA consumption is associated with reduced AD risk [73-76] In adult rats, learning and cognitive behavior are related to brain DHA status, which, in turn, is related to the levels of the dietary omega-3 fatty acids [77] In fact administration of DHA seems to improve learning ability in amyloid-β-infused rats [78,79] and inhibit decline in avoidance learning ability in the AD model rats, associated with an increase in the cortico-hippocampal omega-3/omega-6 ratio, and a decrease in neuronal apoptotic products [80] Similarly, recent studies showed that dietary DHA in an aged AD mouse model could be protective against amyloid-β production, deposition in plaques and cerebral amyloid angiopathy [73,81]. In other transgenic AD mouse models DHA also protects against dendritic pathology [82] and prevents neuronal apoptosis induced by soluble amyloid-β peptides [83] , increases synaptic protein and phospholipid densities [84,85] and inhibits degradative endopeptidase activities [86].

Omega-3 fatty acids and membrane fluidity

DHA is able to shift cholesterol from the neuronal membrane, increasing membrane fluidity which favours sequencing of APP via the non/amyloidogenic pathway explaining the inhibition of amyloid-β formation due to DHA supplementation according to the Wolozin theory [87]

Omega-3 fatty acids and clinical studies

Recently a large trial has been performed in which the favorable effects of DHA in AD become visible [47,75] and in the prospective follow-up study from the Framingham Heart Study it has been found that high DHA levels in plasma correlated with reduction in the risk of developing dementia [47,75] Moreover, also other epidemiologic studies suggest neuroprotective consequences of diets enriched in omega-3 fatty acids [88-90].

Cholesterol

The strongest known risk factor in sporadic or late onset AD [20,91], is the genotype for

apolipoprotein E ε4 (ApoE ε4), a major carrier of cholesterol in the central nervous system (CNS) and an important component of very low density lipoproteins (VLDL). Since ApoE is the major cholesterol transporter in the central nervous system a link between cholesterol metabolism and AD is suggested. Subsequently, many genetic studies show associations between AD and other cholesterol related genes such as cholesterol 24-hydroxylase (CYP46A1), ATP-binding cassette transporter A1 (ABCA1), lipoprotein receptor-related protein (LRP) and liver X receptor (all reviewed in [92]). In addition, hypercholesterolemia is an important risk factor for vascular disorders, and a link between hypercholesterolemia, cardiovascular diseases and AD has been suggested [12,33,93-95]. Epidemiological studies have indicated that patients treated for cardiovascular disease with cholesterol lowering therapy (cholesterol synthesis inhibitors; statins) show diminished prevalence of AD [96] and less deterioration of cognitive function [97]. These data indicate that cholesterol is involved in AD pathogenesis, however at present, the mechanism is not completely clear and will be discussed below.

Cholesterol metabolism.

Cholesterol is an essential component of cell membranes and therefore plays an important role in the physical properties of the membrane such as membrane fluidity, membrane permeability and functioning of membrane associated proteins. Cholesterol is also a precursor of bile acids, and the precursor of all steroid hormones Cholesterol can be obtained from the diet or synthesized in the body, mainly in the liver and intestine but also in the brain.
The first step in cholesterol biosynthesis is the conversion of 3 molecules of acetyl-CoA to 3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) in the cytosol followed by the reduction of HMG-CoA to mevalonate. HMG-CoA reductase catalyzes this reaction, and is the rate-limiting step in the cholesterol synthesis pathway, and is an important target for pharmaceutical interventions, such as with statins. Mevalonate can eventually be converted to cholesterol. Cholesterol homeostasis is maintained by strict regulation of synthesis, uptake and catabolism. Low levels of cholesterol induce the generation of transcription factors leading to increased transcription of target genes, such as HMG-CoA reductase.
When cholesterol levels rise, HMG-CoA reductase production is inhibited and thus cholesterol synthesis is reduced. Excessive cholesterol destined for storage is converted to cholesterol esters in the ER by the Acyl-CoA acyltransferase (ACAT) and stored in fat droplets. This process is important because the toxic accumulation of free cholesterol in various cell membrane fractions is herewith prevented. Excessive cholesterol allocated to elimination may be oxidized by CYP7a to bile acids or binds to the ATP-binding cassette transporter ABCA-1 which is responsible for the transportation of intracellular free-cholesterol and phospholipids from the plasma membrane to lipid free high-density lipoproteins (HDL) acceptor molecules and ultimately to the liver [98].

Cholesterol in the brain.

The human brain is the most cholesterol-rich organ of the human body. Almost 25% of the total unesterified cholesterol within the body is present in the brain, especially in the myelin sheath and membranes of neurons and astrocytes. Since cholesterol is necessary for the formation of synapses [99], proper electrical transmission [100] and serves as an important

barrier against sodium leakage [101], cholesterol homeostasis is essential and is tightly regulated by controlling uptake, de novo synthesis, esterification, catabolism (oxidation) and release.

Serum cholesterol is almost not able to pass the blood brain barrier [102] and the brain synthesizes its own cholesterol, mainly by astrocytes. Cholesterol binds to ApoE and may be taken up in neurons via LDL receptors, LDL receptor related protein (LRP), VLDL receptors, and ApoE receptor 2 amongst others [103]. The neurons internalize cholesterol via endosomes, which fuses with lysosomes causing release of free cholesterol. This intracellular free cholesterol functions as a negative feedback loop to HMG-CoA reductase to reduce synthesis but may also be esterified by ACAT for efficient storage [104]. It also serves as an important source for synaptic plasticity and dendritic formation and remodeling [105]. Since there is no degradation mechanism for cholesterol in the brain, cholesterol is released from the brain after oxidation by for example CYP46 (24-hydroxylase) to 24S-hydroxycholesterol (24S-OH) [71,106] which easily passes the blood brain barrier [101].

Cholesterol and amyloid metabolism.

Since cholesterol is such an essential component in the brain and many epidemiological studies have suggested a role for cholesterol in AD development [12,33,93], it is not surprising that cholesterol has an impact on APP processing and subsequently on amyloid-β production (reviewed in [87]). Transgenic mice fed cholesterol containing diets showed increased amounts of neuritic plaques[107-109]. It has been suggested that cholesterol increases the activity of β- and γ-secretases [110,111], which reside in cholesterol rich lipid rafts and via this way increases the generation of amyloid-β. α-secretases, which prevent amyloid-β production by cleaving APP within the amyloid-β domain need cholesterol poor membranes, such as phospholipid rich long chain poly unsaturated fatty acids domains which are more fluid [112]. Altogether, this indicates that a high membrane cholesterol content favors amyloid-β production and low membrane cholesterol content prevents amyloid-β production [87]. Further, cell culture studies showed that reduction of intracellular cholesterol levels in rat hippocampal neurons inhibit the formation of amyloid-β [48]. Cholesterol lowering agents (statins) increase processing of APP through the non-amyloidogenic α-secretase pathway in different cell lines because membrane fluidity increases with lower cholesterol concentrations, [112] and as a consequence decrease the amount of amyloid-β via favored β-secretase APP cleavage [48,111]. Moreover, increasing intracellular cholesterol levels in different cell lines upregulate amyloid-β production [113]. It is suggested that the intracellular cholesterol levels regulate the APP processing, in contrast to total cholesterol levels [114]. Whether cholesterol esters or free cholesterol are responsible for this involvement in APP processing is not completely clear. However, there is a study reporting that an increase in cholesterol esters regulates the generation of amyloid-β [114] and inhibitors of an enzyme catalyzing the formation of cholesterol esters (ACAT inhibitors) down regulated the generation of amyloid-β [114]. It is however unclear how cholesterol esters modulate APP processing. Since the equilibrium between cellular cholesterol and cholesterol esters is maintained by ACAT, this enzyme may be a potential target in AD.

Other processes that regulate cholesterol homeostasis also affect APP processing [49] such as involvement of LRP [115], NPC1 [116], liver X receptor [117,118] or ABCA [117] re-

ceptors. The involvement of these receptors in APP metabolism will be discussed later.
Cholesterol does not only influence amyloid-β metabolism, vice versa amyloid-β metabolism is able to influence cholesterol metabolism. Amyloid-β 42 causes accumulation of cholesterol in hippocampal neurons, and amyloid-β42 is able to inhibit HMG-CoA reductase, and thus cholesterol synthesis. It is therefore possible, as Canevari et al. suggested [41], that changes in amyloid-β40/amyloid-β42 ratio in AD alter membrane lipid composition, alter the activity of membrane associated proteins and subsequently APP processing etc, causing a vicious circle.

ApoE and cholesterol.
Since ApoE is involved in AD development and is the major cholesterol transporter in the central nervous system, playing an important role in the mobilisation and redistribution of cholesterol and other brain lipids in repair, growth and maintenance of nerve cells, a link between cholesterol metabolism and AD is obvious. Nonetheless there is no consensus about the relation between cholesterol metabolism and ApoE4 in AD [119,120].
It is known that APOE4 carriers display high serum cholesterol levels [121,122] and several experimental animal studies have shown an association between elevated serum cholesterol levels with AD and amyloid-β levels [33,123,124]. In addition, other studies have shown that high brain cholesterol levels increase the amount of amyloid-β depositions in animal models [107,109,125]. Therefore, it is possible that ApoE4 affects AD risk by causing high serum cholesterol, leading to increased brain cholesterol levels and ultimately results in increased amyloid-β levels. However, many studies indicate there is no increase in brain cholesterol in APOE4 carriers [126] and brain cholesterol is not influenced by serum cholesterol because cholesterol does almost not cross the blood brain barrier into the central nervous system [102]. Thus, although ApoE4 is involved in brain cholesterol homeostasis it is unlikely to influence total brain cholesterol levels directly. For example a locally increased cholesterol concentration in the neuronal membrane via redistribution of brain cholesterol by ApoE4 provides an enriched environment for amyloid-β production but does not change total brain cholesterol levels.
Since carrying the ApoE4 isoform is a major risk factor for the development of atherosclerosis [122] via hypercholesterolemia, another plausible explanation for the relation between cholesterol levels and ApoE4 in AD may be cerebrovascular diseases. The APOE ε4 allele increases or aggravates serum cholesterol levels causing atherosclerosis and may subsequently cause hypoperfusion of specific brain regions followed by amyloid-β depositions and neuronal degeneration, emphasizing a role for vascular disease in AD.

Cholesterol and statins.
The effect of cholesterol on amyloid-β deposition and metabolism is also fortified by the beneficial role of statins in the development of AD. Statins are HMG-CoA reductase inhibitors, inhibiting cholesterol synthesis by slowing down the enzyme (HMG-CoA reductase) catalyzing the rate-limiting step of the cholesterol synthesis pathway. As mentioned earlier, epidemiological studies have shown that treatment of cardiovascular patients with statins diminish the prevalence of AD [96] (also reviewed in [127]).
It is possible that statins decrease cholesterol levels in the brain via an ApoE dependent

mechanism, since ApoE deficient mice do not benefit from statin treatment [128] A direct effect of statins on brain cholesterol levels is also possible since lipophilic statins are able to cross the blood brain barrier. Surprisingly, hydrophilic statins (such as pravastatin and atorvastatin) also show benefits in decreasing AD risk (Wolozin et al , 2000) This points more towards an indirect effect of statins on AD pathology

It is also possible that statins affect peripheral 27-hydroxy cholesterol levels (27-OHC). 27OHC can cross the blood brain barrier and is taken up by the brain and in this way may influence brain sterol metabolism

Other studies suggested that statins alter the bilayer distribution of cholesterol. Normally, neurons and glia cells display an asymmetrical distribution of cholesterol among the membrane leaflets, whereas the cytofascial leaflet contains the highest amount. Ageing or ApoE4 genotype decreases the amount of cholesterol in the cytofascial leaflet and it is suggested that statins reverse this effect hereby decreasing the production of amyloid-β [72,129] via prevention of APP processing via the amyloidogenic pathway These data suggest that cholesterol distribution and not total cholesterol levels may be important to amyloid-β production in the central nervous system [127].

All together, these findings led to prospective clinical trials of cholesterol-lowering statins in AD patients. However the results of these trials are conflicting [96,130-133]; [97,134-137] and there is insufficient evidence to suggest the use of statins for treatment in patients with AD [138].

Cholesterol and associated genes

The link between cholesterol and AD is also amplified by the finding that many other genes than ApoE are involved in cholesterol metabolism and have been shown to be associated with late onset AD. A few of the most important genes are discussed shortly below

The ATP-binding cassette transporter A1 The ATP binding cassette transporter ABCA1 is responsible for the transportation of intracellular free-cholesterol [98] and is involved in elimination of excess cholesterol. ABCA1 is expressed in neurons, astrocytes and microglia after for example activation of the liver X receptor [139], and an increased expression is accompanied by cholesterol efflux from neurons and glia This affects APP processing, causing a decrease in amyloid-β production.

Individuals with a genetic polymorphism (R219K) in the ABCA1 gene have 30% decreased cholesterol in their cerebrospinal fluid, via modifying brain cholesterol metabolism and a 1.7 year delay in age of onset of AD. A large genetic association study in 1750 individuals of a European population showed an increased risk in developing both late onset and early onset AD with different polymorphisms of ABCA1 [140]

Liver X receptor The liver X receptor is a nuclear receptor which is expressed on neurons, endothelial cells and glia cells [141,142] and following activation induces a variety of genes involved in pathways of cholesterol metabolism, transport and elimination (i e ABCA1, ApoE etc). An important liver X receptor target gene in the brain is ABCA1. Activation of liver X receptor has been shown to stimulate ABCA1 levels and thereby decreasing amyloid-β concentrations and cholesterol levels [139,143,144] In addition, an in

vivo study using a liver X receptor agonist showed lowered hippocampal amyloid-β42 and improved memory in the Tg2576 transgenic mouse [145] and a study from Koldamova et al showed the liver X receptor ligand T0901317 decreases amyloid-β production in vitro and in a mouse model of AD [118]. In summary, liver X receptor may be a potential target for the treatment of AD possibly by influencing cholesterol and lipid metabolism.

Oxysterols. When excess of cholesterol is oxidized by members of the cytochrome p450 family oxysterols are formed. Oxysterols are important in lipid gene expression since they can bind liver X receptor and ultimately regulate the transcription of many lipid genes. The most important oxysterols in this perspective are 24-hydroxy cholesterol (24-OHC) and 27-hydroxy cholesterol (27-OHC). These oxysterols can easily pass the blood brain barrier and in this way can be utilized for further sterol biosynthesis. In the liver they are being further degraded to bile acids.
Oxysterols may accumulate in vascular tissue, and in this way exert toxic effects, such as development of atherosclerotic plaques [146]. In AD cholesterol flux is elevated as shown by increased levels of 24-OHC [147], which may cause vascular problems in AD. Oxysterols may also have effects on amyloid-β formation [148].

Low-density lipoprotein receptor-related protein. Low-density lipoprotein receptor-related protein (LRP) is a low-density lipoprotein predominantly expressed in neurons, but also detected in capillaries and pericytes. LRP is involved in brain lipid and cholesterol distribution, protection against atherosclerosis and neurodegeneration [149]. LRP is able to bind and internalize amyloid-β- ApoE complexes facilitating degradation of secreted amyloid-β[42]. Moreover, LRP is a substrate, competing with APP for both BACE [150] and γ-secretase activity [151]. LRP has also been shown to be involved in amyloid-β transport over the blood brain barrier to the periphery, and may thus be involved in amyloid-β clearance [152]. However it has also been shown that LRP interacts with APP causing APP processing via the amyloidogenic pathway [115,153] and recently it has also been found that after internalization, aggregation of amyloid-β takes place, stimulating amyloid-β deposition.

ACAT. Cellular cholesterol may be stored as either free cholesterol or cholesterol esters. Acyl-coenzyme cholesterol acyltransferase (ACAT) catalyzes the formation of cholesterol esters from free cholesterol and controls the equilibrium between cellular cholesterol and free cholesterol. Cholesterol esters are stored in lipid droplets and excessive accumulation of lipid droplets is a hallmark of early atherosclerosis but accumulation of free cholesterol in various cell membrane fractions is also toxic.
It has been described that an increase in cholesterol esters seems to regulate the generation of amyloid-β [114]. Additionally, mice lacking ACAT1 (i.e showing decreased levels of cholesterol esters) showed dramatically reduced amounts of amyloid-β levels[114] [154].

LDL-receptor. In the brain the LDL-receptor is predominantly expressed in astrocytes, neurons and endothelial cells. This receptor mediates the endocytosis of cholesterol-rich LDL. LDL is directly involved in the development of atherosclerosis, due to accumulation of LDL-cholesterol in the blood. Dysfunction of the LDL-receptor can lead to hypercholes-

terolemia, which in turn is an important risk factor in AD [155] The most important ligand for LDL-receptor is ApoE and since ApoE is a major risk factor in early onset AD, this is subsequently also an important link between LDL-receptor and AD Mulder et al showed that LDL-receptor knock out mice have an impaired spatial memory and a decreased synaptic density in the hippocampus [156]. This is in agreement with another recent study showing that lack of the LDL-receptor aggravates learning deficits and amyloid deposits in Tg2576 mice [157].

Cholesterol and brain vasculature

Even though many possible mechanisms for the involvement of cholesterol in AD pathogenesis are described above, the most important one has been neglected so far, i.e. the involvement of cholesterol on brain vasculature It is well known that cardiovascular risk factors such as hypertension, hyperlipidemia, diabetes mellitus and stroke are important risk factors for the development of AD [40] For example hypercholesterolemic patients have an increased risk of developing AD [12] and the prevalence of AD will be reduced by 70% when these patients are treated with statins [96,132]. Longstanding elevated hypercholesterolemia leads to accelerated atherosclerosis, this can express itself in a number of cardiovascular diseases such as stroke, myocardial infraction etc, but may also change the brains haemodynamics.

There are studies describing altered haemodynamics in AD [158-160], and this in combination with high serum cholesterol being a risk factor may suggest an association between those two. Therefore, we and others suggest that high serum cholesterol may cause AD via brain hypoperfusion since cholesterol does almost not cross the blood brain barrier [102] and there is no evidence that AD patients have a disrupted blood brain barrier causing cholesterol transport into the brain. Therefore, it can be hypothesized that high cholesterol intake impairs the vasculature causing atherosclerosis, hypoperfusion of vulnerable brain areas, ultimately leading to an overproduction of amyloid-β.

The increased amyloid-β production may also result in cerebral amyloid angiopathy further exacerbating cerebrovascular degeneration [161-163] De la Torre proposes that haemodynamic changes occur first in the AD development [40] which ultimately causes AD pathology. The hypothesis that brain circulation and vasculature are important in AD development is also fortified by that fact that statins diminish the prevalence of AD, since statins diminish serum cholesterol levels and may thereby diminish blood vessel obstruction [164] Altogether, changes in brain vasculature and heamodynamics are still underexposed in AD, but may be a key regulator in AD pathogenesis

Outline thesis

Although the cause of late onset AD is not yet clear and the amyloid hypothesis is still the most accepted hypothesis, more and more evidence accumulates showing the involvement of the vasculature and cerebral hemodynamics in sporadic AD.

We propose that AD pathology starts with impairment of the peripheral and consequently cerebral vasculature due to a combination of ageing, lifestyle, dietary factors and genetic background, which enhance each other in the onset and course of the disease Dietary fac-

tors such as cholesterol and DHA may influence the course of AD at many different levels, and might be important tools in developing preventive strategies against AD and vascular disease.

The aims of this thesis were to

- Investigate the mechanism by which cholesterol and DHA influence AD development.
- Determine whether changes in cerebral circulation precede changes in Aβ deposition or vice versa.
- Investigate what may cause the changes in cerebral hemodynamics, and determine what the microvascular consequences are.

In order to achieve the above mentioned aims we used a double transgenic APPswe/PS-1dE9 Alzheimer mouse model. In this mouse model, APP695 harboring a human Aβ domain with mutations K595N and M596L linked to the Swedish familial AD pedigrees, and human PS1 with a deletion of exon 9, are expressed and controlled by independent mouse prion protein elements. This mouse line has rapid age-dependent accumulation of Aβ into extracellular plaques starting from 4-6 months of age. Table 2 gives an overview of the most important techniques used in this thesis.

Table2: techniques used in this thesis.

Technique	Determination of
Immunohistochemistry	amount of Aβ and GLUT-1
Contrast enhanced magnetic resonance imaging (MRI)	Cerebral blood volume (CBV)
Magnetic resonance spectroscopy (MRS)	Cerebral blood flow (CBF)
Gas chromatography-tandem mass spectrometry (GC-MS/MS)	Sterol levels in brain tissue and serum
Gas chromatography - flame ionization detector (GC-FID)	Fatty acid levels in brain tissue
Liquid chromatography-tandem mass spectrometry (LC-MS/MS)	AdoMet and AdoHcy levels in brain tissue
Open Field, Morris water maze, 12 circular hole board	Behavior and cognition

Chapter 1.

Chapter 1 reviewed the literature about AD pathology and the role of dietary factors such as DHA and cholesterol in AD.

Chapter 2.

Since different epidemiological studies have shown that cholesterol and DHA intake influence AD development we studied the long-term effects of these dietary lipids on Aβ pathology and the cerebral circulation in the brains of double transgenic APPswe/PS1dE9 mice.

Chapter 3.

Many different researchers underline the importance of vascular factors in AD. It is even suggested that changes in cerebral perfusion are a primary trigger in AD development. In addition both DHA and cholesterol may affect the degenerative processes in AD by influencing Aβ metabolism indirectly via the vasculature. In this study we therefore investigated the effects of cholesterol and DHA containing diets in young and old APPswe/PS1dE9 mice and wild type mice in order to investigate whether changes in hemodynamics precede changes in Aβ deposition and cognitive impairment or vice versa.

Chapter 4.
In chapter 1 and 2 an impaired cerebral perfusion has been shown (i.e decreased relative cerebral blood volume (rCBV)) in APPswe/PS1dE9 mice. Here we investigated whether alterations in capillary density might be the cause of this decreased rCBV.
In addition, because impaired cerebral perfusion may cause a hypometabolic state which probably has microvascular consequences, we determined the expression of glucose transporter type-1 (GLUT-1), a transporter responsible for glucose transfer over the blood brain barrier We also investigated the relationship between Aβ, GLUT-1 and hippocampal atrophy in the brains of young and old APPswe/PS1dE9 mice.

Chapter 5.
Until recently hyperhomocysteinemia was believed to be a risk factor for cardiovascular disorders and leading to cerebral hypoperfusion and accelerating AD development. However, this mechanism is now under debate, and it might be possible that other factors of the Hcy cycle, such as s-Adenosylmethionine (AdoMet) and s-aenosylhomocysteine (AdoHcy) play a more important role in vascular disease and subsequently AD development Therefore in this study we investigated AdoMet and AdoHcy levels in the brain tissue of young and old APPswe/Ps1dE9 mice In addition we investigated whether dietary factors such as cholesterol and DHA alter AdoMet and AdoHcy levels, since it could be hypothesized that these nutritional components alter vascular health via alterations in AdoMet and AdoHcy levels and subsequently the progression of AD development.

Chapter 6.
In this chapter, we summarize and discuss the data presented in this thesis and we propose future plans.

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Chapter 2

Changes in cerebral blood volume and amyloid pathology in aged Alzheimer APP/PS1 mice on a docosahexaenoic acid (DHA) diet or cholesterol enriched Typical Western Diet (TWD)

Hooijmans CR [a], Rutters F [a], Dederen PJ [a], Gambarota G [b], Veltien A [b], van Groen T [c], Broersen LM [d], Lütjohann D [e], Heerschap A [b], Tanila H [f], Kiliaan AJ [a]

[a] Radboud University Nijmegen Medical Centre, Department of Anatomy, Geert Grooteplein noord 21 6500 HB, Nijmegen, The Netherlands. [b] Radboud University Nijmegen Medical Centre, Department of Radiology, Geert Grooteplein zuid 10, 6500 HB, Nijmegen, The Netherlands. [c] University of Alabama at Birmingham, Department of Cell Biology, AL 35294-0006, Birmingham, USA .[d] Numico Research, PO Box 7005, 6700 CA, Wageningen, The Netherlands. [e] University of Bonn, Department of Clinical Pharmacology, Sigmund-Freud-Strasse 25, 33105 Bonn, Germany. [f] A.I.Virtanen Institute, University of Kuopio, and Dept. Neurology, Kuopio, University Hospital, Kuopio, Finland

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Abstract

High dietary cholesterol and low dietary docosahexaenoic acid (DHA) intake are risk factors for Alzheimer's disease (AD). However, it is unclear how these components influence the course of the disease. We investigated the effects of dietary lipids on amyloid-β deposition and blood circulation in the brains of 18-month-old APP/PS1 mice. Starting at 6 months of age, mice were fed a regular rodent chow, a Typical Western Diet (TWD) containing 1% cholesterol, or a diet with a high (0.5%) level of DHA for 12 months. Relative cerebral blood volume (rCBV) and flow (CBF) were determined with 2H MR spectroscopy and gradient echo contrast enhanced MRI. Deposition of amyloid-β was visualised in fixed brain tissue with immunohistochemistry. The TWD diet increased plaque burden in the dentate gyrus of the hippocampus, but did not significantly reduce rCBV. In contrast, the DHA-enriched diet increased rCBV without changing blood flow indicating a larger circulation in the brain probably due to vasodilatation and decreased the amount of vascular amyloid-β deposition. Together, our results indicate that the long-term intake of dietary lipids can impact both brain circulation and amyloid-β deposition, and support the involvement of haemodynamic changes in the development of AD.

Introduction

Worldwide, 15 million people suffer from Alzheimer's disease (AD). This number will continue to grow as a result of our aging population. Our western population is not only getting older, but has also developed a less healthy lifestyle with high cholesterol and high caloric diets, resulting in overweight with its associated disorders. Hypercholesterolemia for example is an important risk factor in vascular disorders, and a link between vascular risk factors, hypercholesterolemia and AD has been suggested [1-5]. Epidemiological studies have indicated that cholesterol lowering statins diminish the prevalence of AD [6] and cause less deterioration of cognitive functions [7]. Identification of the cholesterol transporter apolipoprotein E4 as a major genetic risk factor for hypercholesterolemia, vascular dementia and sporadic AD [8-10], also reinforces the relationship between cholesterol and AD. Further support for the link between high cholesterol intake and AD come from experimental animal studies. In cell culture studies, reduction of intracellular cholesterol levels in rat hippocampal neurons inhibits the formation of amyloid-β (Aβ) [11] and animal studies with double transgenic (amyloid precursor protein (APP) - Presenilin (PS)) mice have shown that high dietary cholesterol increases Aβ accumulation [12] and cholesterol lowering agents increase processing of APP through the non-amyloidogenic α-secretase pathway in different cell lines via increased membrane fluidity [13].

Membrane fluidity can be influenced by alterations in membrane phospholipid and cholesterol content [14-16], which in turn, can modulate the activity of membrane bound enzymes [17-20] such as the main enzymes cleaving the transmembrane protein APP to generate Aβ (β- and γ- secretase). It has been suggested that these secretases require cholesterol-rich lipid domains (lipid rafts) within the membrane [21,22], whereas the non-amyloidogenic pathway by α-secretases needs cholesterol poor membranes, such as phospholipid domains which are more fluid [15,23].

Important components of membrane phospholipids that contribute to membrane fluidity are the omega-3 Long Chain Poly-Unsaturated Fatty Acids (n3 LCPUFAs), like docosahexaenoic acid (DHA) and the omega-6 (n6) LCPUFAs (such as arachidonic acid). Further, increased DHA levels in membranes are known to augment membrane fluidity [24]. Epidemiological studies show that sufficient DHA intake reduces the risk of developing AD [25-27]. In support of this notion, two recent experimental studies reported decreased brain Aβ levels in APP transgenic mice after dietary DHA supplementation [28,29]. In addition to its influence on Aβ metabolism, DHA has also a positive effect on vascular health [30-32].

Taken together, these data indicate that high dietary cholesterol levels or low DHA levels are risk factors in AD. However, it remains unclear how these dietary factors may influence the development of AD. It can be hypothesized that high cholesterol intake impairs the vasculature causing hypoperfusion of vulnerable brain areas, ultimately leading to an overproduction of Aβ. The increased Aβ production may also result in cerebral amyloid angiopathy (CAA) further exacerbating cerebrovascular degeneration. [33-35]. In contrast, high intake of DHA may have a protective effect by improving vascular health and accor-

dingly cerebral perfusion.
In a recently published study of our collaborators [29] it was found that a Typical Western Diet (TWD) containing 1% cholesterol increases hippocampal Aβ levels in the double transgenic APP/PS1 mice at 10 months of age. In contrast, a DHA-enriched diet decreased hippocampal Aβ levels of the same mice compared to the TWD group However, although these in vivo dietary effects could be detected with ELISA, immunohistochemically determined Aβ plaque load was unaffected These findings raise the obvious question whether life-long dietary factors may have an even stronger impact on Aβ pathology.

In the present study we used the same APP/PS1 transgenic mice and the same effective dietary manipulations as Oksman et al. to study the long-term effects of dietary lipids on Aβ accumulation and brain circulation This mouse line has rapid age-dependent accumulation of Aβ into extracellular plaques but relatively little vascular deposition, which allows the assessment of dietary lipids on both parenchymal Aβ accumulation and vascular function. We started the dietary manipulation at 6 months of age and continued up to 18 months To address the diet effects on brain circulation we measured relative cerebral blood volume (rCBV) and cerebral blood flow (CBF). Both parameters have been shown to be affected in AD [36-38] We used 2H MR spectroscopy with the freely diffusible tracer deuterium oxide, and susceptibility enhanced MRI, a technique that has been widely used in experimental stroke studies in rats but never applied to APP transgenic mice before In addition immunohistochemical analysis of neuropathology and a chemical assessment of sterol and fatty acid profiles of the brain were performed The findings indicate that dietary lipids can impact both brain circulation and deposition of Aβ in plaques and blood vessel walls.

Materials and Methods

Animals and diets

The APPswe/PS1dE9 founders were obtained from Johns Hopkins University, Baltimore, MD, USA (D Borchelt and J Jankowsky, Dept. Pathology) and a colony was established at the University of Kuopio Finland. In short, mice were created by co-injection of chimeric mouse/human APPswe (mouse APP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transfected genes co-integrated and co-segregate as a single locus [39]. This line was originally maintained in a hybrid background by backcrossing to C3HeJ x C57BL6/J F1 mice (so-called pseudo F2 stage). For the present work, the breeder mice were backcrossed to C57BL6/J for 5-6 generations to obtain mice for the current study
Male APP/PS1 transgenic mice were assigned to different diet groups, which differ in the composition of the 5% fat added in the diets (table 1) The diets started at the age of 6 months and were maintained for 12 months Before assigning the mice to the different experimental groups the mice were weighed
One group of transgenic mice was fed a Typical Western Diet (TWD(tg), n=9), containing 1% cholesterol, a high percentage of saturated fatty acids (44%) and long chain poly unsaturated fatty acid (LCPUFA) ratio of n6/n3=23) The second group of transgenic mice

(DHA(tg) n=8) received a DHA diet that was supplemented with 0.50% of n3 (PUFA) DHA, low in saturated fatty acids (19%), and LCPUFA ratio of n6/n3=3). The third transgenic (STD(tg) n=8) and the fourth wild type C57BL6/j (STD(wt) n=12) group received a control diet (Standard), containing 5% soy oil (low in saturated fatty acids (15%), LCPUFA ratio n6/n3=8)).

Table 1. Sources and contents of fatty acids in the experimental diets

	Dietary Groups		
diet components	Standard	TWD	DHA
% soya oil	5.00	0.60	2.00
% coconut oil	-	1.72	0.50
% corn oil	-	1.68	1.79
% DHA 70EE	-	-	0.50
% cholesterol	-	1.00	-
diet content			
% total n3	7	1	14
% total n6	52	31	40
ratio n6/n3	8	23	3
% SFA	15	44	19
% MUFA	22	18	19
% PUFA	59	32	54

SFA= saturated fatty acids, MUFA= Mono unsaturated fatty acids, PUFA= poly unsaturated fatty acids

Throughout the experiments the animals were housed individually in a controlled environment, food and water were available ad libitum. One DHA(tg) mouse died during the experiment and was not included in the analysis.
The experiments were performed according to Dutch and Finnish federal regulations for animal protection and were approved by the Veterinary Authority Radboud University Nijmegen. At 16 months the mice were transported to Radboud University Nijmegen, the Netherlands and at 18 months the experiments were performed.

MR measurements / experimental protocol

To study differences in brain haemodynamics, relative cerebral blood volume (rCBV) and cerebral blood flow (CBF) were determined in our experimental diet groups at 18 months of age. The CBF and the rCBV of the entire brain, were determined by MR Spectroscopy (MRS) in combination with administration of deuterium oxide as contrast agent, which can freely diffuse across the vascular membranes. To verify the MRS results and to obtain an indication about region differences, the rCBV was also determined in a subgroup of animals with a susceptibility-induced contrast MRI technique using Ultra Small Particles of Iron Oxide (USPIO) as blood-pooled contrast agent.

Before starting with the MR measurements, the mice were weighed. Following anesthesia, all mice received an intravenous tail vein catheter for bolus injections of deuterium

oxide or USPIO During the MR experiments mice were anesthetized with 1.9% isoflurane (Abott, Cham, Switzerland) in a mixture of oxygen and N2O (1 2) through a nose cone The composition of the mixture was monitored continuously using a gas analyzer (Datascope, Multinex) The body temperature was maintained at 37 2 ± 1 2 °C using a heated water pad and monitored with a rectal fluoroptic temperature probe Breathing of the animal was monitored using an optical respiratory gating apparatus (Sirecust 401, Siemens) MR measurements were performed on a 7 Tesla/200 mm horizontal bore MR spectrometer (Magnex Scientific, Abingdon, England) interfaced to a S M I.S console (Surrey Medical Imaging Systems, Surrey, England) The MR acquisition parameters were optimized in normal wild-type mice matched in weight and age to the animals participating in the present study.

^{2}H MR Spectroscopy and data analysis

Deuterium Oxide (D_2O)

Cerebral blood flow (CBF) and relative cerebral blood volume (rCBV) of the entire brain were estimated by ^{2}H MR spectroscopy using the freely diffusible tracer D_2O. Enrichment of the blood with D_2O (D_2O 99.8% enriched; Merck, Darmstadt, Germany) was achieved by intravenous bolus injection into the tail vein of 0 3 ml D_2O in saline within 4 seconds in all mice (STD(wt) n=6, STD(tg) n=6, DHA(tg) n=7, TWD(tg) n=8). An in-house-built ^{2}H elliptical shaped (15 x 11 mm) surface head coil, which was tuned to D_2O frequency (46 MHz), was used for excitation and signal reception The 2H MR spectra were obtained using a pulse-acquire sequence (flip angle of 90°, repetition time (TR) of 1000 ms, the array size of 100, and 2 number of averages per measurement). Since every measurement contained 2 dummy scans, each measurement took 4 seconds, the total measurement time was 400 seconds. After Fourier transformation the amplitude of the D_2O signal was monitored and evaluated as a function of time

The concentration of D_2O in the brain as a function of time is given in arbitrary units (a u). The rCBV was estimated by calculating the surface area under the bolus peak of each curve. A tangent line was drawn from 380 till 200 seconds (along the almost horizontal part of the MR curve) and extrapolated till the crossing with the increasing slope of the D_2O curve (Fig 1). Thereafter the surface area of the bolus peak could be determined [40,41]. The rCBF was determined by using a nonlinear least squares fit of the data between 28 and 68 sec (Fig 1) to a two-parameter single-exponential of the form: y=Ae-t/T [42] T is the exponential time constant governing the rate of blood flow washout of the deuterium label from the brain tissue. T can be related to blood flow per unit volume (or mass) of tissue through the central volume principle of tracer kinitics (CBF= λ/T) [43,44]. The coefficient λ is the ratio of the water weight of a unit mass (100g) of brain tissue to the water weight of a unit volume of blood, in this case 0.90 g/ml. [45] Thereafter peak height and time to peak (TTP) was determined (Fig 1). In 2 TWD(tg) and 2 DHA(tg) mice the rCBV measurement failed due to technical errors These mice are not included in further analysis of the rCBV.

MR imaging using USPIO

In order to verify our MRS results a second technique to measure rCBV, contrast enhanced MRI, in combination with the administration of a superparamagnetic blood-pool contrast

agent (USPIO), was performed on the four groups of mice (STD(wt) n=6, STD(tg) n=3, DHA(tg) n=3, TWD(tg) n=3). The USPIO contrast agent provides a valuable tool to characterize tissue vascularity since it remains intravascular for a prolonged period of time and highly enhances the transverse water proton MR relaxation rates (R2*). Previous studies have shown that magnetic susceptibility effects caused by USPIO can be used to assess relative blood volume within tissues [46,47]. In particular, the enhancement in the transverse relaxation rate (ΔR2*) after administration of USPIO is proportional to the tissue blood volume.

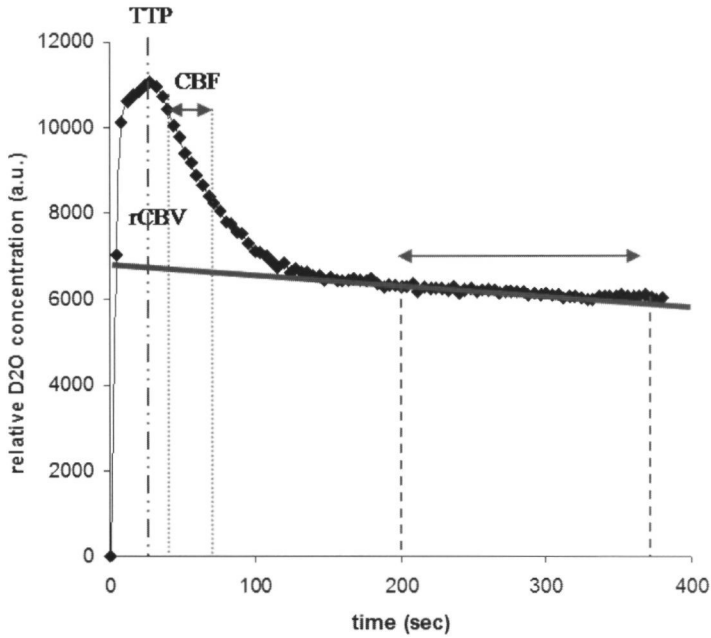

Fig 1. rCBV and CBF are determined along the D2O uptake graph in time.
A tangent line was drawn from 380 back to 200 seconds and prolonged till the crossing with the D2O curve. Thereafter the surface area under the peak (AUP) of the curve could be determined. CBF was determined by unraveling the two-parameter single exponential of the form Y=Ae –t/T between 28 and 68 seconds. TTP is the time used to reach a peak concentration of deuterium. rCBV= relative cerebral blood volume, CBF= cerebral blood flow, AUP= area under peak, TTP= time to peak.

An elliptical shaped (15 x 11 mm) surface coil was used for excitation and signal reception. The image acquisition protocol started with three gradient-echo scout images for slice positioning. Thereafter, multislice gradient-echo imaging was performed prior to and 1 minute after administration of a bolus injection with USPIO (AMI-277, Sinerem®, Guerbet Laboratories, France, 170 μg Fe/mouse). Imaging parameters were: field of view (FOV) of 25 mm x 25 mm, matrix size of 256 x 256, slice thickness of 1 mm, echo time (TE) of 7 ms, repetition time (TR) of 2000 ms and 2 averages per image, resulting in a total scan time of 17 minutes per multislice gradient-echo series. For each animal, pixel-by-pixel ΔR2* maps were obtained from the formula: $\Delta R2^* = (1/TE)\log(S_o^{bef}/S_o^{aft})$, where TE is the echo time, and S_o the signal amplitude pre-USPIO (S_o^{bef}) and post-USPIO (S_o^{aft}), in the gradient

echo images. The mean ΔR2* were calculated by drawing a Region of Interest (ROI) on the ΔR2* maps and averaging the values of all pixels within the ROI. To assess the blood volume, regions of interest (ROIs) that included the entire hippocampus, cerebral cortex (all cortical areas above the corpus callosum) and the entire brain, were drawn on the images. These ROIs are based on the mouse brain atlas of Franklin and Paxinos 1997 [48] in image 8 about –2.46 posterior to bregma. The change in ΔR2* in the hippocampus, cerebral cortex and entire brain is proportional to the rCBV in these regions. In some regions, ΔR2* was very low and due to image noise, negative values could be obtained in some regions. ΔR2* was then assumed to be zero. All algorithms were implemented in Matlab (Mathworks, Natick, MA, USA). Due to technical failures 1 STD(tg) and 1 DHA(tg) animal were omitted from further analysis.

Because we ran into a power problem in the detection of differences in CBV between transgenic (STD tg) and wildtype (STD wt) mice on control diet, we executed CBV measurements with identical susceptibility-induced contrast MRI techniques as used in this study in a larger group of 15-month-old mice within the framework of another experiment. The animals were kept under exactly the same conditions. One group of APP/PS1 transgenic mice (STD(tg) n=8) and a wildtype control group (STD(wt) n=13) received the same control diet (Standard) for the same length of time as described in section 2.1.

Serum sterol analysis

Directly after completion of the MR experiments, mice were anaesthetized with Nembutal (60mg/kg i.p.) (Ceva Santa Animals BV, Maassluis) and blood samples were collected via heart punction. Serum cholesterol levels were determined by gas-liquid chromatography-flame ionization detection (GC-FID) as described previously [49].

Immunohistochemistry

All mice used in the MRS experiment (STD(wt) n=6, STD(tg) n=6, DHA(tg) n=7, TWD(tg) n=8) were transcardially perfused (after blood sampling via heart punction) starting with a 0.1 M phosphate buffered saline (PBS) followed by Somogyi's fixative (4% paraformaldehyde, 0.05% glutaraldehyde and 0.2 % picric acid in 0.1 M phosphate buffer, PB). Following transcardial perfusion fixation, mice were decapitated and brains were dissected from the skull. The entire brain, without the spinal cord, was weighed and thereafter post fixed for 15 hours at 4 °C in Somogi's fixative. The brain tissue was then cryoprotected by immersion in 30% sucrose in PB at 4 °C. Series of 40 µm coronal sections were cut through the brain using a sliding mircrotome (Microm HM 440, Walldorf, Germany). The Aβ load was visualized using WO-2 antibody (mouse anti-human Aβ4-10, T Hartmann, Heidelberg, Germany). Immunohistochemistry was performed using standard free-floating labeling procedures. Briefly, first the sections were pretreated with sodium citrate solution at 85 °C for 30 minutes. Monoclonal mouse anti-Aβ4-10 (1:20.000) was used as primary antibody. The sections were incubated overnight at room temperature on a shaker table. Following incubation the sections were rinsed thoroughly with PBS and transferred to the solution containing the secondary antibody; donkey-anti-mouse biotin 1:1500 (Jackson Immuno research). After 90 minutes of incubation the section were rinsed three times and transferred to a solution containing Vector ABC-elite 1:800 (Vector laboratories, Burlinga-

me) for again 90 minutes. Visualization of Aβ plaques and CAA was achieved by incubation with DAB-Ni solution. All stained sections were mounted on gelatin-coated slides and dehydrated in alcohol series, cleared with xylol and mounted in Entellan.

Quantification
To determine the amount of Aβ in the parenchyma of the frontal cortex (prelimbic area (PLA)), anterior cingulate gyrus (ACg 1 and 2) and in the hippocampus (CA1, CA3 and dentate gyrus (DG)), appropriate slices were digitized and quantified, using a Zeiss Axioskop microscope equipped with hardware and software of Microbrightfield, (Williston, USA). These cortical regions were chosen because of their large amyloid load in humans and transgenic mice and their importance in memory and hippocampal functioning [50,51]. Quantitative analyses were done with a computer-assisted analysis system (Stereo Investigator) using Cavalieri's probe. A contour was drawn along the borders of the hippocampal subregions. In the ACg and PLA a square box was placed, within the borders of intended brain areas.
To determine the amount of Aβ in the vasculature, a contour was placed in the frontal cortex (PLA), anterior cingulate gyrus (ACg 1 and 2) and in the hippocampus, respectively containing the middle prefrontal branches of the anterior cerebral artery, the anterior cerebral artery and the vessels in the hippocampal fissue. Brain regions were based on the mouse brain atlas of Franklin and Paxinos 1997 [48] and quantified in three sections, with 200 µm distance between the sections. ACg1 and 2 were quantified at level +1.10 up to +0.86 anterior to bregma, PLA was quantified at +1.98 upto +1.78 anterior to bregma. The hippocampus was quantified at –2.18 up to –2.46 posterior to bregma.
All measurements were performed double blind by two investigators, Aβ load was defined as the percentage of area covered by Aβ.

Preparation of brain homogenates and sterol analysis
A subgroup of mice (which were used in the USPIO experiment; STD(wt) n=6, STD(tg) n=3, DHA(tg) n=3, TWD(tg) n=3) was sacrificed to determine brain sterol levels. The brains were snap-frozen in liquid nitrogen and were kept frozen at -80˚C until homogenization. Thereafter the brains were homogenized and sterols were determined as described elsewhere [52,53].

Phospholipid and fatty acid analysis of brain homogenates
Fatty acid analyses were performed with a part of the homogenate (described above). Total lipid was extracted from brain homogenates by methanol and chloroform. Subsequently, samples were centrifuged at 3000 rpm for 10 min and the lower phase (chloroform and lipids) was removed. Chloroform was added to the upper phase, samples were centrifuged again at 3000 rpm for 10 min and the lower phase was combined with the first one. The chloroform fractions were dried in a SpeedVac® and the lipid extracts were dissolved in 125 µl chloroform. The phospholipids were separated from total lipid by SPE columns (Bond Elut NH2, Varian). The separation of the phospholipids from the brain homogenates was accomplished by a gradient of two mobile phases: 1) hexane: iso-propanole: acetic acid, 2) iso-propanole: water: acetic acid. Both mobile phases were supplied with 0.08%

of triethylamine. The gradient changed linearly from 95:5 to 10:90 within 28 min, and 1 min after reaching the final ratio the gradient was changed back to the original composition at a flow rate of 1 ml/min. Peaks were identified by comparison with phospholipid standards obtained (3-sn-phosphatidic acid sodium salt, 3-sn-phosphatidyl-l-serine from bovine brain, 3-sn-phosphatidylethanolamine from bovine brain, L-α-phosphatidylcholine 99% from bovine brain, L-α-phosphatidylinositol ammonium salt from bovine liver and sphingomyelin from bovine brain) from Sigma. Quantification of the phospholipids from the brain homogenates was performed by HPLC-LSD with a LiChrospher Diol-100 column (250x4.6 mm, 5 µm, Merck).

To determine the fatty acid profile of each phospholipid, the phospholipid extract was methylated by adding 0.5 ml 10% BF3. The samples were heated at 100 °C for 60 min, and 2 ml hexane and 1 ml sodium hydroxide were added. After vortexing and centrifuging the samples for 5 min at 3000 rpm, the lower phase was vortexed and centrifuged with 2 ml hexane for 5 min at 300 rpm. Top phases of both steps were combined and hexane was evaporated from the samples by means of a SpeedVac®. The fatty acids were dissolved in 125 µl iso-octane and analyzed on a GC-FID with a CP-SIL88 column (50 m x 0.25 mm id. 0.22 µm film thicknesses). The n6/n3 ratio was calculated as a sum of analyzed n6 FAs divided by the sum of n3 FA.

Statistical analysis

Data are expressed as mean ± SEM and were analyzed with SPSS for windows 12.0 software (SPSS Inc. Chicago, IL, USA). Genotype differences were determined by comparing transgenic and wild type mice on STD diet using an independent t-test, whereas diet effects among the three diet groups of transgenic mice were determined using the One-way ANOVA. The One-way ANOVA was also used to evaluate regional differences in Aβ load. If the overall analysis revealed a significant difference, the separate groups were analyzed post hoc by using Tukey's HSD test. Statistical significance was set at $p \leq 0.05$.

Results

Body and brain weight

Mice were weighed before assigning them to the different diet groups. The body weight did not differ between the groups neither at the start (STD(wt) 32.1 ±1.0; STD(tg) 31.9 ±1.0; TWD(tg) 32.2 ±1.2; DHA(tg) 32.4 ±1.1) nor at the end of the experiment (STD(wt) 40.7 ± 1.7; STD(tg) 43.5 ±2.5; TWD(tg) 41.0 ± 2.0; DHA(tg) 45.0 ± 2.4). Thus, body weight was not affected by genotype (p=0.36) or dietary manipulations (p=0.56). Also brain weight did not differ between the genotypes (p=0.71) or diet groups (p=0.19; STD(wt) 0.46 ±0.01 STD(tg) 0.47 ±0.01, TWD(tg) 0.47 ±0.01, DHA(tg) 0.49 ±0.01).

No change in CBF but increased rCBV in animals fed DHA-enriched diet.

The parameters that were derived from the D2O uptake graph in time (Table 2) show that genotype or diet interference did not significantly influence CBF (t-test; p=0.43 and ANOVA; p=0.45). However, the time to the reach the peak concentration of deuterium (TTP)

Table 2. Curve parameters along the D_2O uptake graph in time.

	Genotype effects		Dietary effects			
			t-test			ANOVA
	STD(wt)	STD(tg)	p value	TWD(tg)	DHA(tg)	p-value
CBF (ml/100gr/min)	36 ± 8	34 ± 7	0.43	32 ± 8	41 ± 8	0.45
TTP (sec)	17 ± 4.9	19 ± 3.6	0.75	18 ± 2.7	30 ± 3.7 [a*. b]	0.040
Peak Height (a.u.)	11414 ± 903	10538 ± 430	0.40	10441 ± 900	11290 ± 1011	0.24
Curve Height at 20 sec (a.u)	10738 ± 851	9918 ± 567	0.44	10158 ± 825	10830 ± 973	0.29
Curve Height at 32 sec (a.u.)	10235 ± 990	9567 ± 731	0.58	9178 ± 731	10953 ± 1023 [b]	0.044

*Curve parameters; cerebral blood flow (CBF), time to peak (TTP), peak height and curve height, were determined along the D2O uptake graph in time. STD(wt) n=6, STD(tg) n=6, DHA(tg) n=5, TWD(tg) n=6 were used for analysis. Values represent mean ± SEM. a= different from standard(tg), b=different from TWD(tg), c=different from DHA(tg) and *= trend p<0.07*

was longer in DHA(tg) mice compared to TWD(tg) ($p=0.04$) and STD(tg) mice ($p=0.07$). Peak height (at TTP) and curve height at 20 s, which was the mean TTP for STD(wt), STD(tg) and TWD(tg) did not differ between the groups (genotype effect; $p>0.40$ and diet effect $p>0.24$). In contrast, peak height at 32 s (TTP for DHA(tg)) was increased (ANOVA $p=0.04$) in DHA(tg) mice compared to TWD(tg) ($p=0.04$) and STD(tg) ($p=0.09$). Altogether, a prolonged TTP in combination with a comparable peak height at 20 s and an increased peak height at 32 s shows a significant expanded peak surface area, which provides evidence for a significant increased rCBV in DHA(tg) mice [40,41].

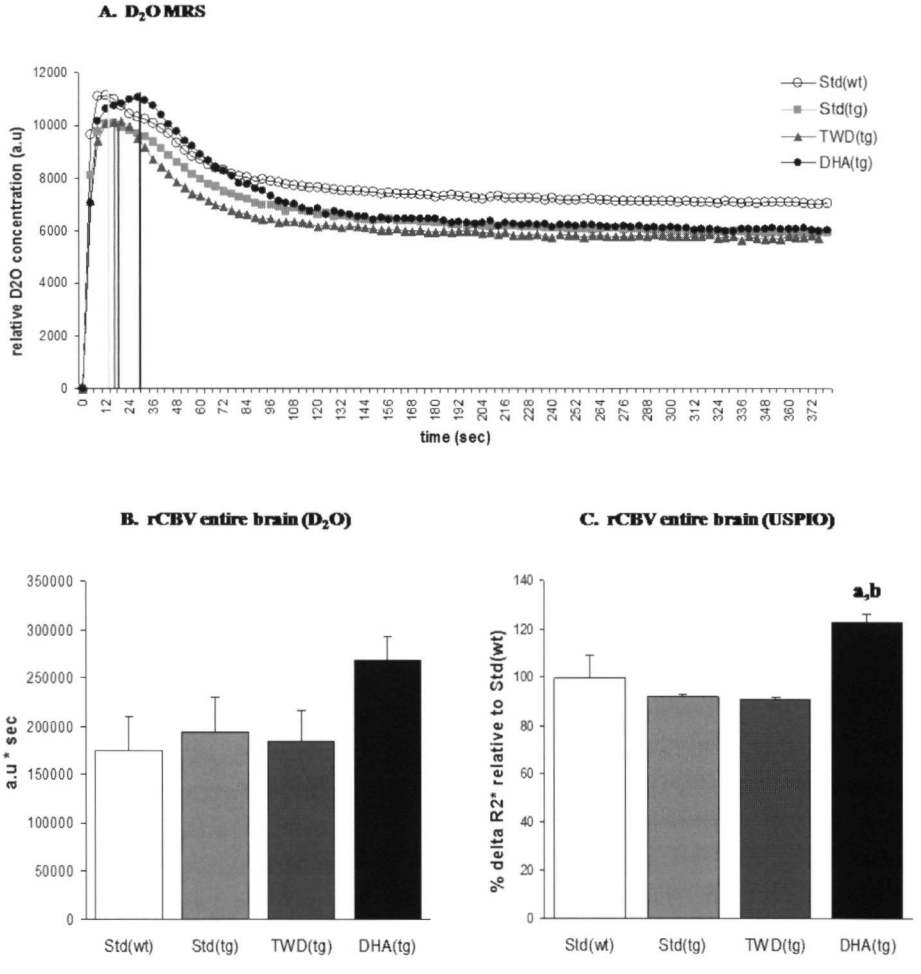

Fig 2. Relative cerebral blood volume (rCBV).
A: Cerebral uptake of D2O after bolus injection. Each experimental group contained 6 mice except DHA group, which contains 5 transgenic mice. a.u.= arbitrary units. Vertical lines represent time to peak (TTP). B: D2O tracer bolus peak area. rCBV of the whole brain is determined by estimating the area under the peak of each D2O spectrum in STD(wt), STD(tg), TWD(tg) and DHA(tg) fed animals. Values represent the mean and SEM. C: Cerebral changes in R2 relaxation upon administration of USPIO in STD(wt), STD(tg) TWD(tg) and DHA(tg) fed animals. The change in ΔR2* is proportional to the rCBV. a= different from STD(tg) (p=0.001), b= different from TWD(tg) (p=0.001).*

Comparable results were also obtained by a second method, in which the area under the peak of each curve was directly measured by drawing a tangent line between 200 and 380 s (Fig.1). The rCBV in the DHA(tg) group was higher compared to the STD(tg) (38%) and TWD(tg) (43%) group (Fig.2a and b). However, the increase in rCBV in DHA(tg) animals was not significant (ANOVA; p=0.11). No differences in rCBV were observed between the STD(wt) and STD(tg).
To further explore possible regional differences in CBV, which cannot be determined with the MRS method and to support our MRS results, a subgroup of animals (not used for MRS) were exposed to a susceptibility-induced contrast-imaging technique.

In correspondence with the MRS experiments, no differences were observed in rCBV between the transgenic and wild type animals fed the STD diet ($p<0.7$) in any of the regions. However in the large group of 15-month-old wildtype and APP/PS1 transgenic mice a 28% decrease in the cortical rCBV of APP/PS1 transgenic mice compared to their wild

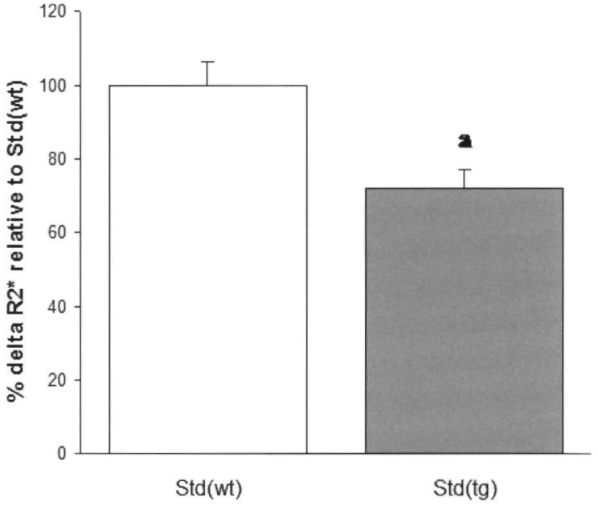

Fig 3. Relative cerebral blood volume (rCBV) in 15 month old APP/PS1 and wildtype mice. Cerebral changes in R2 relaxation upon administration of USPIO in 15 month old STD(wt) and STD(tg) animals. The change in ΔR2* is proportional to the rCBV. a= different from STD(wt) (p=0.017).*

type controls (p=0.017) was observed (Fig.3). A decrease of rCBV was also detected in the hippocampus of these 15-month-old mice and was of the same magnitude (29%), although not significant (p=0.18).
Further, a non significant decrease in the rCBV of TWD(tg) mice compared to STD(tg) mice in the cortex (mean STD(tg) 91% ± 3.6, mean TWD(tg) 78% ± 5.6) could be observed and transgenic animals on DHA diet, (DHA(tg)), showed a significantly higher delta R2* (rCBV) in the entire brain (ANOVA p=0.001) and especially in the neocortex (ANOVA; p= 0.003) compared to TWD(tg) (both $p<0.007$) and STD(tg) mice (both $p<0.002$) (Fig.2c). Together, these data indicate that the increase in the entire brain blood volume in DHA(tg) mice is probably caused by an increase in cortical blood volume.

Typical western diet increases parenchymal plaque burden in the dentate gyrus of the hippocampus

We quantified the Aβ plaque load (total Aβ) of the transgenic mice in frontal cortex (prelimbic area (PL) and anterior cingulate gyrus (ACg)) and hippocampus (CA1, CA3 and dentate gyrus (DG)) in three serial sections per region. The Aβ plaque load showed significant regional variation, such that an overall higher Aβ plaque load was demonstrated in the DG compared to all other hippocampal and cortical regions (ANOVA $p<0.001$). Aβ plaques covered 21% of the DG, while corresponding ratio was 12% in CA1, 8% in CA3, 7% in PL and 13% in ACg.

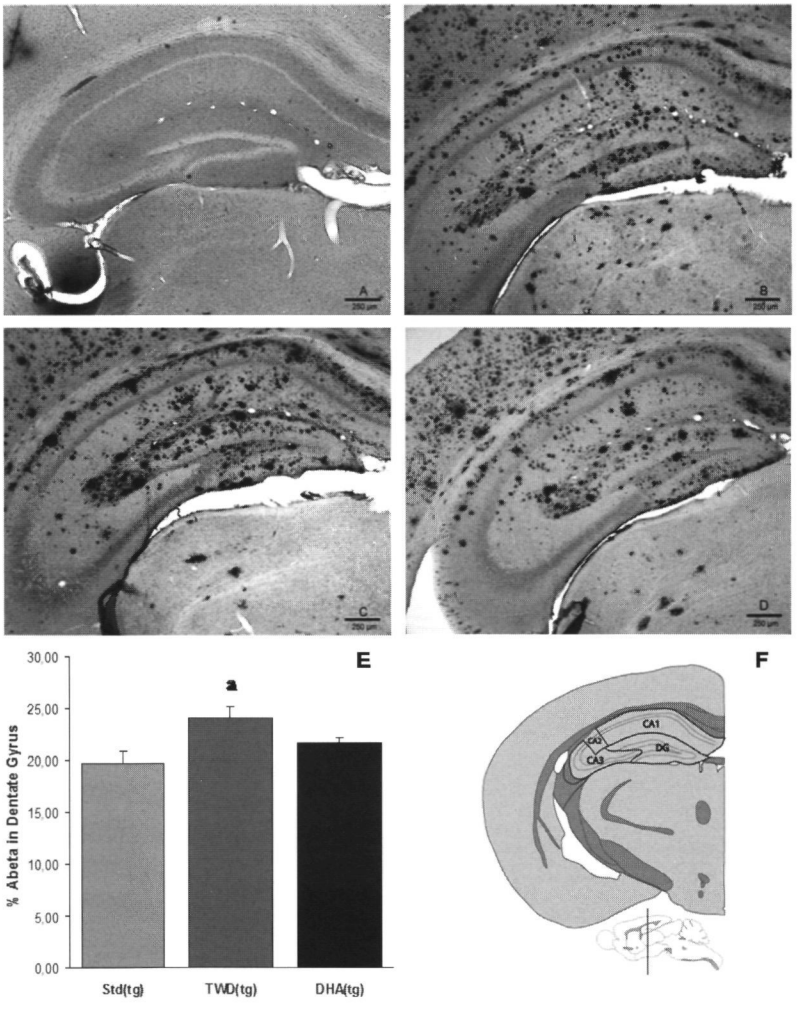

Fig 4. Parenchymal β-amyloid pathology in the brains of APP/PS1 mice.
Photomicrographs demonstrate the differences in β-amyloid load in the dentate gyrus of the hippocampus stained with WO-2 antibody (mouse anti-human Aβ5-8). Bar = 250μm A: STD(wt) B: STD(tg), C: TWD(tg), D: DHA(tg). E: diet affect on plaque burden in STD(tg), TWD(tg) and DHA(tg). Values represent mean ± SEM. a=different from STD(tg) (p=0.012). Figure F shows the location of the dentate gyrus of the hippocampus.

These results indicate that in these mice the dentate gyrus is most vulnerable to Aβ deposition. In addition, the ANOVA revealed an overall diet effect in the DG (p=0.013); i.e., significantly more plaques were detected in the DG (Fig.4) of TWD(tg) animals compared to STD(tg) (p=0.011), and a similar trend was observed between TWD(tg) and DHA(tg) groups (p=0.10). The DHA(tg) mice did not differ from STD(tg) mice. No significant differences between the diet groups were observed in other brain areas.

a DHA-enriched diet decreases Aβ deposition in the vasculature of the cingulate gyrus

Apart from parenchymal Aβ plaque load, we also determined the Aβ deposition in the vasculature of the PL, ACg and hippocampus, respectively containing the middle prefrontal branches of the anterior cerebral artery, the anterior cerebral artery and the vessels in the hippocampal fissure. No dietary effects were found in the amount of vascular Aβ in the hippocampus and PL area. However, in the anterior cerebral artery of the cingulate gyrus, the amount of vascular amyloid-β was decreased in DHA(tg) mice compared to STD(tg) (p=0.003) and TWD(tg) mice (p=0.002) (Fig.5).

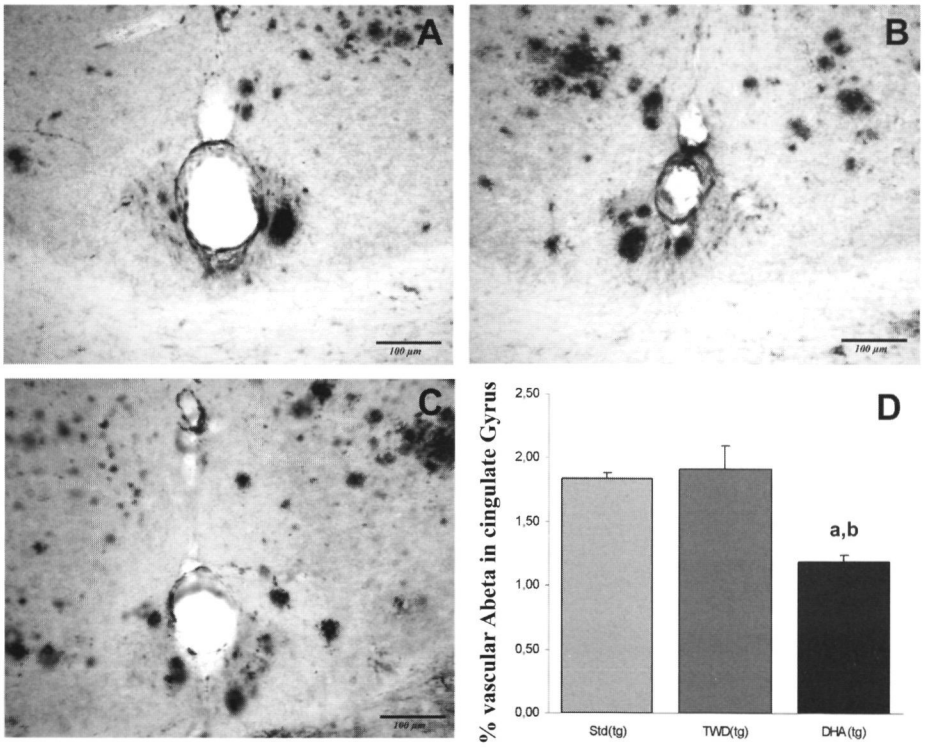

Fig 5. Vascular β-amyloid pathology in the brains of APP/PS1 mice.
Photomicrographs demonstrate the differences in vascular β-amyloid pathology in the cingulate gyrus stained with WO-2 antibody (mouse anti-human Aβ5-8). Bar = 250μm.
A: STD(tg) B: TWD(tg), C: DHA(tg). D: diet affect on vascular β-amyloid pathology in STD(tg), TWD(tg) and DHA(tg). Values represent mean ± SEM. a=different from STD(tg) (p=0.003) and b=different from TWD(tg) (p=0.002).

High dietary cholesterol does not influence the cholesterol content in the brain of APP/PS1 mice but does increase serum cholesterol levels

Table 3 summarizes the serum and brain sterol levels in different diet groups. No differences were observed in serum sterol levels between wildtype and STD(tg) mice. However, Serum cholesterol levels were significantly increased in TWD(tg) mice compared to STD(tg) and DHA(tg) mice (both $p<0.001$). Further, lathosterol levels, a main cholesterol precursor in the de novo synthesis pathway, was significantly decreased in TWD(tg) mice compared to STD(tg) ($p=0\ 009$) and in addition, the cholesterol elimination rate from the brain measured by 24S-OH-cholesterol levels, was increased in TWD(tg) mice compared to STD(tg) ($p<0.001$) and DHA(tg) mice ($p=0.001$). The diets did not directly affect brain cholesterol levels, which was not surprising as most cholesterol in the brain is metabolically quite inert in the myelin sheath and serum cholesterol crosses the blood brain barrier in minimal amounts. Further, neither absolute nor relative levels of lathosterol differed between the groups. In addition, the diets did not affect cholesterol elimination rate from the brain (24S-OH-cholesterol levels). In contrast, transgenic mice compared to wild type mice did show increased brain cholesterol levels (ANOVA $p=0\ 01$) However the "de novo" synthesis or cholesterol elimination rate did not differ between the genotypes (no changes in lathosterol or 24s-OH-cholesterol levels).

Dietary manipulation shifts the balance between n3 and n6 fatty acids in the brain of APP/PS1 transgenic mice

The relative concentrations of different fatty acids in the brain of 18-month-old APP/PS1 mice are shown in Table 4 Notably, animals fed with the diet containing DHA show a general increase in n3 fatty acids (1.2%) and a decrease (1.5%) in n6 fatty acids in brain tissue compared to STD(tg) and TWD(tg). This means a pronounced shift in the n6/n3 ratio in favour of n3. The change in n6 content was mainly caused by the decrease in arachidonic acid (20 4*n*6, ($p<0,001$), while the change in n3 content stemmed from an increase in DHA (22 6n3, $p<0.001$) content. The brains of TWD(tg) mice had significantly higher *n*6 FA content than STD(tg) mice. This increase was mainly caused by an increase in arachidonic acid ($p<0.03$) in TWD(tg) as compared with the other two transgenic groups. These changes clearly indicate that dietary manipulations lasting 12 months are able to influence the composition of fatty acids in the phospholipid fraction of the mouse brain.

Table 3
Serum and brain concentration of sterols

	Transgenic effects		*t*-Test	Dietary effects		ANOVA
	STD(wt)	STD(tg)	*p*-value	TWD(tg)	DHA(tg)	*p*-value
Serum sterols						
Cholesterol (mg/dl)	104.3±6.88	108.8±9.88	0.59	239.4±32.84[a,c]	81.3±10.76	0.00
Lathosterol (mg/dl)	0.018±0.001	0.023±0.003	0.95	0.013±0.001[a]	0.017±0.003	0.01
24s-OH-cholesterol (ng/ml)	19.4±0.81	21.1±1.18	0.87	52.9±7.42[a,c]	18.57±0.65	0.00
Brain sterols						
Cholesterol (μg/mg)	52.2±0.10	56.7±0.59	0.01	55.7±6.09	55.3±2.64	0.93
Lathosterol (ng/mg)	54.2±3.52	49.0±2.15	0.28	44.5±4.97	39.5±2.61	0.14
24s-OH-cholesterol (ng/mg)	197.1±18.6	210.8±5.9	0.39	237.6±25.7	230.5±14.3	0.34

Different sterols were determined in serum (STD(wt) $n=12$, STD(tg) $n=8$, TWD(tg) $n=9$, DHA(tg) $n=8$) and brains (STD(wt) $n=3$, STD(tg) $n=6$, TWD(tg) $n=3$, DHA(tg) $n=3$) of wild type and APP/PS1 mice. Values represent mean±SEM. [a]Different from standard(tg), [b]different from TWD(tg), [c]different from DHA(tg).

Table 4
Relative fatty acid composition (%) of the phospholipid fraction of brain homogenates of 18-month-old APP/PS1 mice on various diets

Brain fatty acids	Genotype effects		*t*-Test	Dietary effects		ANOVA
	STD(wt)	STD(tg)	*p*-Value	TWD(tg)	DHA(tg)	*p*-Value
SFA	40.79±0.46	40.81±0,24	0.94	41.83±0.19	41.89±0.69	0.12
MUFA	26.46±0.74	26.85±0,25	0.22	25.49±0.35	26.31±0.70	0.26
PUFA	21.97±0.54	21.61±0,17	0.45	22.64±0.32	21.73±0.24	0.13
∑*n*-3	10.72±0.39	10.41±0,11	0.35	10.63±0.10	11.80±0.15[a,b]	0.002
∑*n*-6	11.25±0.20	11.09±0,11	0.21	11.94±0.29[a,c]	9.81±0.35[a,b]	0.000
Ratio *n*-6/*n*-3	1.01±0.04	1.02±0,01	0.42	1.10±0.02	0.81±0.04[a,b]	0.000
22:6 *n*-3 (DHA)	10.60±0.41	10.28±0,10	0.34	10.53±0.10	11.55±0.14[a,b]	0.004
20:4 *n*-6 (AA)	7.35±0.20	7.28±0,10	0.74	7.77±0.17[a,c]	6.39±0.12[a,b]	0.001

Brain fatty acids were determined in STD(wt) $n=3$, STD(tg) $n=6$, TWD(tg) $n=3$ and DHA(tg) $n=3$. Values represent mean±SEM. [a]Different from standard(tg), [b]different from TWD(tg) and [c]different from DHA(tg).

Discussion

In this study we investigated the long-term effects of dietary cholesterol and docosahexaenoic acid (DHA) in the development of AD-like pathology and brain circulation in transgenic APP/PS1 mice. Both cholesterol and DHA may affect the degenerative processes in AD by influencing the Aβ metabolism of via indirect effects on vasculature. High serum cholesterol may contribute to the development of AD via hypoperfusion of the brain leading to the elevated production of Aβ [33,34,54] followed by cerebrovascular degeneration and cerebral amyloid angiopathy (CAA), whereas DHA may have a protective effect by maintaining vascular health [55-57]. We demonstrated that the typical western diet (TWD), containing 1% cholesterol, increased plaque burden in the dentate gyrus of the hippocampus of 18-month-old APP/PS1 double transgenic Alzheimer mice, but did not significantly affect rCBV or CBF. In contrast, the DHA enriched diet significantly increased the relative cerebral blood volume (rCBV), indicating a larger circulation in the brain compared to STD(tg) and TWD(tg), without changing the cerebral blood flow (CBF) and decreased the vascular amyloid deposition in the anterior cingulate gyrus.

Several studies show that AD patients and elderly people in general have a diminished CBF [58-60] and CBV [37,38]. However, our 18-month-old mice did not show significant differences in rCBV and CBF between transgenic and wild type mice on a standard diet. This observation may be explained by a large age-related decrease of rCBV and CBF in both genotypes, which may mask a smaller genotype difference or by the fact that our 18-month-old experimental groups were to small to notice small differences in rCBV. Namely, the additional results from the much larger group of 15-month-old mice, show a significant decrease in rCBV in STD(tg) mice compared to STD(wt). These data confirm that our APP/PS1 mouse model is a good model to represent AD related hypoperfusion. Furthermore, it could be hypothesized that dietary changes influence haemodynamics and thereby affect the cognitive state and AD development. For example our transgenic animals that were fed a DHA enriched diet displayed an increase in rCBV compared to both the STD(tg) and TWD(tg) groups. Theoretically, this increase of rCBV may be caused by vasodilatation or genesis of new capillaries. Vasculogenesis is an unlikely explanation for the finding, because anti-angiogenic effects of DHA have been reported [61,62]. Vasodilatation is in our view a more likely explanation, since CBF did not change, and a mismatch between CBF and CBV has been interpreted as compensatory vasodilatation [63]. In support of this notion, intake of n3 PUFAs (to which family DHA belongs) in human subjects has been shown to affect endothelial function and improve flow-mediated dilatation of the brachial artery [57] and enhance nitric oxide mediated vasodilatation in the microcirculation of the forearm [64]. Furthermore, transgenic mice overexpressing APP have an impairment in cerebrovascular responses mediated by the release of vasoactive factors from brain endothelial cells, such as nitric oxide (NO), while simultaneous overexpression of superoxide dismutase restores their responses, implicating reactive oxygen species in the mechanisms of the endothelial dysfunction [65]. It is of interest in this context that DHA has been reported to increase glutathione peroxidase levels in aortic endothelial cells and cerebrum of aged rats, although it is generally known to enhance susceptibility of membranes to lipid peroxidation due to its several double bonds [66,67]. Apart from an increased rCBV, pro-

bably due to vasodilatation in these aged APP/PS1 transgenic mice fed a DHA containing diet, we also expected less hypometabolic and degenerating neurons [68] resulting in a better cognitive status [29,30]. Oksman et al. have already shown in the same APP/PS1 mice that a diet supplemented with DHA increased exploratory behaviour [29] and indirectly consolidates the influence of rCBV on behaviour, since Aβ deposition did not change in DHA(tg) mice.

The TWD diet, however, did not significantly decrease the rCBV in aged APP/PS1 transgenic mice compared to STD(tg) and DHA(tg) groups. We did find a decrease in the rCBV of TWD(tg) mice compared to STD(tg) mice in the cortex, although not significant. This finding is rather surprising since it could be expected that chronic high cholesterol intake causes the development of atherosclerosis, as shown in studies in C57BL/6J and transgenic Alzheimer mice models [69,70], resulting in a decreased CBV, and high cholesterol in combination with aging is known to further decrease CBF [71]. Accordingly, our latest results with 15-month-old APP/PS1 and wild type littermates fed the same TWD diet show a significant decrease in rCBV compared to STD mice (not shown). The experimental groups used in that experiment are larger which could explain why significant difference could be found in the 15 and not in the 18-month-old APP/PS1 mice.

In accordance with Refolo, who reported that high dietary cholesterol increases Abeta accumulation and accelerates AD-related pathology [12], the brains of our TWD(tg) mice also did show increased Aβ levels in the DG of the hippocampus, indicating that Aβ deposition can be modulated by diet-induced hypercholesterolemia. Altogether, the results showing a decrease in rCBV in combination with the increased amount of Aβ deposition in the DG in mice fed a cholesterol enriched diet, do support the hypoperfusion theory, in which high serum cholesterol may contribute to the development of AD via hypoperfusion of the brain leading to the elevated production of Aβ [33,34,54].

It is possible that there is an increase of cholesterol in the cerebral vasculature which causes vascular damage or mild atherosclerosis leading to increased Aβ deposition. Although we did not find significant increases in total brain cholesterol level in TWD(tg) animals, an increased vascular cholesterol level is still possible. However, the intravascular compartment, representing just a small volume fraction of the total brain (3-5%), could not be measured separately. Increased amyloid plaque load with no significant changes in cerebral circulation may also suggest that high dietary intake of cholesterol directly influences Aβ processing in the brain. This finding is consistent with the recent report of Oksman et al. (2006), showing that addition of SFA and cholesterol containing oils (as opposed to fish-oil) increased Aβ production in transfected cells. Notably, the effect of high SFA and cholesterol containing oils on Aβ deposition in the brain was regionally specific. This is consistent with the fact that the DG, which responded significantly to TWD, was also more vulnerable for Aβ depositions than other areas in these APP/PS1 mice. In addition, our Aβ analysis also showed the systematic finding that the DG is, in all transgenic mice more vulnerable for Aβ depositions compared to all other brain areas. This is in accordance with the AD syndrome in which Aβ depositions are first present in the hippocampus and especially the DG [72-74]. The DG is the first area that is affected in AD [74,75], even before the accumulation of Aβ plaques [75] and this may explain why a TWD diet influences the amount of Aβ only in the DG, and not other brain regions. Another explanation for this finding that

only the hippocampus shows differences in abeta load, is that we may face the interesting possibility that abeta load in the cortex has already reached the ceiling and is therefore more insensitive to small manipulations, whereas hippocampal Aβ is in a more dynamic state since this brain area maintains neurogenesis throughout the lifespan of rodents.

In mice fed a DHA-enriched diet we expected, besides of an increased rCBV, a decreased Aβ load. Firstly, it has been hypothesized that hypoperfusion of the brain per se leads to increased Aβ load [76,77]. Therefore, a more healthy circulation is expected to lead to decreased Aβ load levels. Secondly, it has been suggested that increased incorporation of DHA in neuronal membranes increases membrane fluidity, resulting in increased APP processing via the non-amyloidogenic α-secretase pathway instead of the amyloidogenic pathway [13,15]. Indeed, Lim et al. (2005) reported lower Aβ levels as measured by ELISA and reduced amyloid pathology in the hippocampus and parietal cortex of APPswe mice, which had been fed a DHA diet from 18 until 23 months [28]. This is partially consistent with the study by Oksman et al. [29], who also reported lower Aβ levels in ELISA assays in APP/PS1 mice receiving a DHA enriched diet. However, DHA supplementation did not affect the immunohistochemically measured amyloid plaque burden either at the age of 10 months [29] or at 18 months (present study) in the parenchyma. The DHA effects in Lim's study [28] may be due to the fact that their control group received a DHA depleting diet and our mice a control diet containing a small amount of linolenic acid which may be converted to DHA. In our study we therefore investigated effects of supplementation with DHA instead of replenishment of depleted DHA as in the Lim study. The results of our phospholipid and fatty acid analysis of the brain homogenates show no difference in DHA content nor n6 and n3 LCPUFA content in the brains of the transgenic mice compared to wildtype mice, showing that transgenicity does not cause DHA depletion. The animals used in the Oksman study [29] were 10 months of age and plaque burden is much less in this mouse compared to the 18-month-old animals in our study; it therefore may be difficult to bring about effects on that age.

Nevertheless, we did find a significant decrease in the amount of vascular Aβ in the cingulate gyrus tissue surrounding the longitudinal fissure and in leptomeningeal arteries (mainly anterior cerebral artery), suggesting that not only replenishment of a DHA deficit (Lim et al., 2005) but also DHA supplementation may be warranted in aged people at risk for AD. This result in combination with the increased rCBV fortifies the hypothesis that high intake of DHA may have a protective effect by improving vascular health and thus the perfusion of the brain ultimately leading to a decreased amount of Aβ. Another beneficial effect of DHA supplementation may be a reduced microglia activation as found by Oksman (Oksman et al., 2005) in the same APP/PS1 mice receiving exactly the same DHA enriched diet as used in our study which may indicate dampening of the neuroinflammatory response resulting in neuroprotection.

According to the changes in Aβ load and rCBV due to different diets, alterations in the fatty acid and sterol profile of cholesterol, its precursor lathosterol or its main metabolite 24S-OH-cholesterol in brain tissue, were also expected. However, no differences in SFA and cholesterol levels in the brain were found. Lütjohann et al. (2002) has also shown no significant changes in brain cholesterol levels in APP transgenic animals of different age groups (3, 6, 9, 12 and 18 months) on cholesterol diets compared to wild types, indicating two

separate cholesterol mechanisms for brain and body [78]. In addition, the observation that cholesterol levels do not change in brain homogenates due to dietary cholesterol supplementation in mice may indicate that membrane fluidity is not influenced by administration of dietary cholesterol. This statement questions Wolozin's theory in which he hypothesizes that high cholesterol in neuronal membranes increases amyloid-β (Aβ) production [15], and is furthermore an indirect ratification for the hypoperfusion theory [33-35].
Fatty acid analysis did show differences in brain tissue composition of TWD(tg) and DHA(tg) animals. TWD(tg) mice showed a small increase in brain levels of the n6 LCPUFA arachidonic acid (AA) compared to STD(tg) mice, and the ratio of n6/n3 PUFAs in DHA(tg) animals was shifted in favour of n3, which is in line with the DHA diet containing more n3 PUFA than the other diets (Table1). This indicates a direct influence of dietary FA on brain, demonstrating exchange of LCPUFA across the blood brain barrier [79]. Moreover, the relative increase of n3 FAs over n6 FAs may imply increased membrane fluidity [80]. Gray matter is composed of of approximately 30-40% of DHA, which is almost 17% of the total weight of fatty acids in the brain. Because of this huge abundance of DHA in the brain, it can be expected that a significant increase or decrease of DHA in the brain, will greatly influence membrane fluidity. In previous studies, increased DHA concentrations in neuronal membranes have lead to changes in the physical properties of membranes, enzyme activities, receptors, transport and cellular interactions [81-83]. These physiological changes are also accompanied by improved learning in behavioural studies [84,85]. More specifically, an optimal n6/n3 ratio can also influence the level of performance in learning and memory tasks, and has been shown to improve spatial learning in rats after a 4-week treatment with oils low in n6/n3 ratio [85]. Another study on fish-oil-depleted young rats demonstrated improved maze learning after 10-week DHA supplementation, which further correlated positively with the hippocampal and cortical DHA/AA ratio [84].
Consequently, one should expect an improved cognitive state in DHA(tg) mice compared to, for example, TWD(tg) and STD(tg) animals. Our parallel study in Finland [29] performed the behavioural studies with our mice when they were 10 months of age, and showed that a diet supplemented with DHA increased exploratory behaviour. This increased explorative behaviour suggests more active and less anxious mice, whearas in AD patients, anxiety, agitation and restlessness often occur [86]. Therefore an increase in explorative behaviour may suggest less AD related problems. Altogether, it could be hypothesized that a DHA containing diet fed to aged APP/PS1 mice may influence cognition via increased membrane fluidity, but as stated above, also has effects via the circulation, since the DHA diet increased rCBV.

In conclusion, our results show that that long-term intake of dietary lipids can impact brain circulation, neuronal membrane composition and the deposition of Aβ plaques in the brain. This study adds to the accumulating evidence that the classic dietary risk factors for cardiovascular disease, high amounts of saturated fatty acids and cholesterol, promote amyloid deposition in the brain. Whereas DHA supplementation has effects on brain circulation, it increased cerebral blood volume probably due to vasodilatation, and decreases the amount of vascular Aβ, fortifing the hypothesis that high intake of DHA may have a protective effect by improving vascular health and thus the perfusion of the brain ultimately leading to

a decreased amount of Aβ. The present study also demonstrates that the recent advances in MR imaging methods can be applied to genetically manipulated mice modelling AD. Future studies should utilize even more challenging imaging techniques such as arterial spin labelling to explore the exact contribution and regional differences of the cerebral circulation in the pathogenesis of AD.

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Chapter 3

DHA and cholesterol containing diets influence Alzheimer-like pathology, cognition and cerebral vasculature in APPswe/PS1dE9 mice

Hooijmans CR [a], Van der Zee CEEM [b], Dederen PJ [ag], Brouwer KM [a], Reijmer YD [a], van Groen T [c], Broersen LM [d], Lütjohann D [e], Heerschap A [f], Kiliaan AJ [ag]

[a] Radboud University Nijmegen Medical Centre, Department of Anatomy and [g] Department of Cognitive Neuroscience, Donders Centre for Neuroscience, Geert Grooteplein noord 21, 6500 HB, Nijmegen, The Netherlands. [b] Radboud University Nijmegen Medical Centre, Department of Cell Biology, NCMLS, Geert Grooteplein zuid 26, 6525 GA, Nijmegen, The Netherlands; [c] University of Alabama at Birmingham, Department of Cell Biology, AL 35294-0006, Birmingham, USA. [d] Danone Research, PO Box 7005, 6700 CA, Wageningen, The Netherlands [e] University of Bonn, Institute of Clinical Chemistry and Pharmacology, Sigmund-Freud-Strasse 25, 53105 Bonn, Germany. [f] Radboud University Nijmegen Medical Centre, Department of Radiology, Geert Grooteplein zuid 10, 6500 HB, Nijmegen, The Netherlands.

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Abstract

Both cholesterol and docosahexanoic acid (DHA) may affect degenerative processes in Alzheimer's Disease (AD) by influencing Aβ metabolism indirectly via the vasculature. We investigated whether a DHA-enriched diet or cholesterol-containing Typical Western Diet (TWD) alter behavior and cognition, cerebral hemodynamics (relative cerebral blood volume (rCBV)) and Aβ deposition in 8 and 15-month-old APPswe/PS1dE9 mice. In addition we investigated whether changes in rCBV precede changes in Aβ deposition or vice versa. Mice were fed either a regular rodent chow, a TWD diet, or a DHA-containing diet until 8 and 15 months of age. Behavior, learning and memory were investigated, and rCBV was measured using contrast-enhanced MRI. The Aβ load was visualized immunohistochemically. We demonstrate that a DHA-enriched diet altered rCBV in 8-month-old APP/PS1 and wild type mice In 15-month-old APP/PS1 mice DHA supplementation improved spatial memory, decreased the amount of Aβ and slightly increased rCBV, indicating that a DHA-enriched diet can diminish AD-like pathology. In contrast, a TWD diet decreased the rCBV in 15-month-old mice. The present data indicate that long-term dietary interventions can change AD-like pathology in APP/PS1 mice In addition, the effects of the tested diets on vascular parameters were observed before effects on Aβ load were noted. These data underline the importance of vascular factors in the APP/PS1 mouse model of AD pathology.

Introduction

The cause of Alzheimer's disease (AD) is still largely unknown despite many years of extensive research. Since AD is characterized by the presence of neurofibrillary tangles and amyloid-β (Aβ) containing aggregates in neuritic plaques and cerebral blood vessel walls, it has been suggested accumulation of Aβ in the brain is the primary influence driving AD pathogenesis (the amyloid-β hypothesis) [1]. However, gene mutations in presenilin 1 (PS1), presenilin 2 (PS2), or amyloid precursor protein (APP) responsible for increased production of Aβ [2], are only responsible for approximately 5% of all cases of AD worldwide. Therefore, it could be suggested that beside Aβ, other AD risk factors such as cardiovascular-, lifestyle- or environmental factors, play an important role in the development of AD [3-5].

There are indications that modification of lifestyle factors, such as nutrition, alter the risk of developing AD later in life [4,6]. For example, high serum cholesterol, which can be caused by dietary intake, is an important risk factor in AD [7,8]. The role of cholesterol in AD is also strengthened by epidemiological studies that associate cholesterol lowering statins with diminished prevalence of AD [9] and with less deterioration of cognitive functions [10]. Identification of the cholesterol transporter apolipoprotein E4 as a major genetic risk factor for hypercholesterolemia, vascular dementia and sporadic AD [11-13], has reinforced the relationship between cholesterol and AD. Further support for the link between high cholesterol intake and AD pathology comes from cell culture and experimental animal studies. For example, reduction of cellular cholesterol levels in rat hippocampal neurons reduce the formation of Aβ [14], whereas high dietary cholesterol intake in double transgenic AD mice increases Aβ accumulation [15,16]. In addition, a study in guinea pigs showed that lowering whole body cholesterol with statins decreases Aβ formation [17].

Another lipid diet factor influencing the risk of AD is the omega-3 long chain poly-unsaturated fatty acid (n3 LCPUFA) docosahexaenoic acid (DHA). The Framingham Heart study showed that people with high plasma DHA levels have a decreased risk of developing dementia, and AD in particular [18]. Also other epidemiological studies have indicated that sufficient DHA intake reduces the risk of developing AD [19-21]. Several studies have shown that dietary intake of n3 PUFA may reduce cognitive decline [22,23], and a recent trial by Freund-Levi et al showed positive effects of DHA supplementation on cognition in patients with very mild AD [24]. Recent experimental studies in APP transgenic mice reported decreased brain Aβ □ levels after dietary DHA supplementation [25,26]. It has been proposed that DHA supplementation increases the amount of the neuronal sorting protein LR11 which regulates APP processing together with a subsequent decrease in Aβ production [27]. In addition, Calon et al showed that DHA supplementation to DHA depleted tg2576 AD mice improved memory acquisition [28] Taken together, the above-mentioned data show that high cholesterol levels or low plasma DHA levels are risk factors for AD. Moreover, cholesterol and DHA are also involved in cardiovascular diseases such as hypertension and atherosclerosis [29]. It could therefore be suggested that cholesterol and DHA may also exert their effects on the development of AD by influencing the peripheral and cerebral vasculature. A recent study performed in our lab, showed that a cholesterol enriched Typical Western Diet (TWD) and a DHA diet, indeed, influenced cerebral hemodynamics

such as the cerebral blood volume (CBV) in 18-month-old double transgenic APP/PS1 mice [15] This is in line with studies showing that CBV is affected in AD [30] In addition a study from Niwa et al showed that in Tg2576 Alzheimer mice cerebral blood flow (CBF) was reduced throughout the brain prior to Aβ deposition, suggesting that cerebrovascular abnormalities are early events in the pathogenesis of Alzheimer's disease [31]
Our study also showed that the cholesterol enriched TWD diet increased plaque burden in the hippocampus, whereas the DHA enriched diet decreased the amount of Aß deposits in cerebral blood vessel walls in the 18-month-old double transgenic APP/PS1 mice [15]
In the current study we investigate whether changes in relative cerebral blood volume (rCBV), determined using susceptibility enhanced MRI, precede changes in Aß deposition in these APP/PS1 mice or vice versa We also tested the effects of DHA and TWD diets on cognition, rCBV, the amount of Aβ depositions and the sterol and fatty acid profiles of the brain, from 8 and 15-month-old APP/PS1 mice We used similar diets as in our former study [15] Explorative behavior was determined with an open field test, and spatial learning and memory with both the Morris water maze (MWM), and the 12 circular hole board in 8 and 15-month-old wild type and double transgenic APPswe/PS1dE9 mice

Materials and methods

Animals and diets

The APPswe/PS1dE9 founders were obtained from Johns Hopkins University, Baltimore, MD, USA (D Borchelt and J Jankowsky, Dept of Pathology) and a colony was established at the Radboud University Nijmegen Medical Centre, The Netherlands In short, mice were created by co-injection of chimeric mouse/human APPswe (mouse APP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements The two transfected genes co-integrate and co-segregate as a single locus [32] This line was originally maintained on a hybrid background by backcrossing to C3HeJ x C57BL6/J F1 mice (so-called pseudo F2 stage) For the present work, the breeder mice were backcrossed to C57BL6/J for 7 generations to obtain mice for the current study
Male APP/PS1 transgenic mice and their wild type littermates were assigned to different diet groups, which varied only with respect to the composition of the 5% fat in the diets (table 1)
Apart from that, the remaining components in all diet groups are the same as used in standard rodent chow, with for example, wheypowder, barley, wheat etcetera and a vitamin/mineral mix containing vitamin E in a concentration of 30 mg/kg in each diet group Feeding the diets started when the mice reached the age of 2 months and was maintained until 8 and 15 months of age
Transgenic mice and their wild type littermates were fed either a Typical Western Diet (TWD), containing 1% cholesterol, a high percentage of saturated fatty acids (SFA), a low percentage of long chain poly unsaturated fatty acids (LCPUFAs) and a high n6/n3 LCPUFA ratio, or a DHA diet containing 0 4% DHA, a low percentage SFA, a high percentage LCPUFA and a low n6/n3 LCPUFA ratio, or a standard control diet (STD), with interme-

Table 1. Sources and contents of fatty acids in the experimental diets

	Dietary Groups		
Source	Standard	TWD	DHA
% soya oil	2.9	0.6	2.0
% coconut oil	1.6	1.7	0.5
% corn oil	-	1.2	-
% fish oil	-	-	1.5
% sunflower oil	0.3	0.6	-
% linseed oil	0.3	-	0.3
% cholesterol	-	1.0	-
Contents			
total n3 (g/kg)	2.2	0.5	7.1
DHA	-	-	3.5
EPA	-	-	0.8
total n6 (g/kg)	16	11	18
ratio n6/n3	7.5	22.5	2.5
% SFA	38	53	20
% MUFA	23	23	24
% PUFA	38	23	55

SFA= saturated fatty acids, MUFA= Mono unsaturated fatty acids, PUFA= poly unsaturated fatty acids

Table 2. Number of mice used in each experimental group

8-month-old		In vivo		Post mortum	
Groups	total	behaviour	rCBV	IHC	FA/sterols
STD(wt)	12	10	9	7	5
STD(tg)	13	11	9	7	6
TWD(wt)	13	10	10	7	6
TWD(tg)	15	13	12	9	6
DHA(wt)	13	11	9	7	6
DHA(tg)	14	12	10	7	7

15-month-old		In vivo		Post mortum	
Groups	total	behaviour	rCBV	IHC	FA/sterols
STD(wt)	16	14	10	6	10
STD(tg)	15	10	9	7	8
TWD(wt)	15	12	9	6	9
TWD(tg)	14	9	8	6	8
DHA(wt)	15	13	10	6	9
DHA(tg)	10	7	7	7	3

rCBV= relative cerebral blood volume, IHC= immunohistochemistry, FA= fatty acids

diate values for SFA and LCPUFA content and the n6/n3 LCPUFA ratio.
In total 165 mice were used, table 2 describes the number of mice used in each experiment. The majority of the mice were used for behavioral analyses, and subsequently for relative cerebral blood volume measurements. Mice from MRI experiments during which technical errors occurred were excluded from further analyses of rCBV (Table 2). The largest part of the mice used for rCBV measurements were subsequently sacrificed (transcardially perfused) and their brains processed for immunohistochemistry. The remaining mice were sacrificed to determine brain sterol and fatty acid levels.
Throughout the experiments the animals were housed individually in a controlled environment, with room temperature at 21°C, and an artificial 12:12h light:dark cycle (lights on at 07:00 a.m.) Food and water were available ad libitum. The experiments were performed according to Dutch federal regulations for animal protection and were approved by the Veterinary Authority of the Radboud University Nijmegen Medical Centre. All testing was performed on mice of 8 and 15 months of age between 8:00 a.m. and 17:00 p.m.

Body and brain weight

The body weights of the mice were determined one week before the start of the behavioral tests. The entire brain without the spinal cord was dissected and weighed directly after completing all experiments, respectively at 8 and 15 months of age. Brain weights are expressed as a percentage of the total body weight.

Behavioral analysis

Behavioral testing was performed in 8 and 15 months-old mice in the following order: First open field, then Morris water maze (MWM), followed by reversal MWM and finally the 12 circular hole board.

Open field

To analyze explorative behaviour mice were placed individually in a square open field (L: 50 cm, W: 50 cm, H: 40 cm) with white Plexiglas walls, and were videotaped for 30 min. The durations (s) of walking, wall leaning, rearing, sitting and grooming were scored and later analyzed in three blocks of 10 min.These open field parameters were defined as follow: walking, movement of its hind paws and forepaws with a minimal distance of 1 cm, wall leaning, standing on its hind legs, the mouse places one or two forepaws against the wall, rearing, the mouse stands upright on the hind legs, while the forepaws are not touching any surface, sitting, no movements of the hind paws and forepaws for a duration of 2 s, and grooming, washing (parts of) its body. In addition, moving latency, the time before starting to move after being placed in the open field, and the total number of faeces during the 30 min were recorded.
By applying a computer-assisted walking pattern analysis [33] the videotaped sessions were further used to calculate the total walking distance during the 30 min observation, the walking pattern and the time mice spent in the border respectively the centre of the open field.

Morris water maze (MWM)

To investigate spatial learning abilities, mice were tested in the Morris MWM. In short, mice were placed in a pool (120 cm diameter) filled with water (21–22 °C; made opaque by the addition of milk powder) at different starting positions and trained to find the submerged platform by using distant visual cues in the room. On the four walls in the test room spatial cues were present at a distance of 0.5 m. To facilitate video camera tracing of the mouse, a strip of white tape (width 15 cm) was taped along the top/inside perimeter of the pool to prevent dark reflection. The 8 cm diameter round platform was submerged 1 cm below the water surface and placed in the middle of the NE quadrant. The latency (sec) to find the platform and the time spent (sec) in the training quadrant and the number of crossings of the former platform location (platform crossings) were measured. During all trials the researcher was present and always located at the same location in the room.

Acquisition. 8 and 15 months-old mice performed four acquisition trials (maximal swimming time 120 s; 30 s on the platform; inter-trial interval 60 min) per day during 4 consecutive days. Starting positions were: S, N, E, W. After the 2 min swim the mice were placed back in their home cage. A paper towel was available inside the cage for additional drying.

Probe. All mice performed a single probe trial at 60 min following the last trial on day 4, in which the platform was removed from the swimming pool. Mice were allowed to swim for 120 s and the time spent swimming and searching in the NE quadrant (where the platform had been located) was recorded using a video-tracking computer analysis program made by D. Heeren (Radboud University Nijmegen).

Reverse Morris water maze

On the day after the standard MWM probe trial, a simplified reversal MWM session was performed in which the platform location was changed to SW [34]. In this procedure, earlier locations need to be encoded in the long-term memory. Memory retrieval needs to be selective for the most recently learned location, introducing an episodic like component in the spatial memory task [34]. Acquisition and probe sessions were performed similar to the standard MWM sessions, except that starting positions were: E, W, S, N for acquisition and the target quadrant was SW.

Circular hole board

Spatial learning ability of the mice was also tested in the circular hole board (dry maze) similar to a previously described method [35,36] with some minor changes. In short, mice were placed in the middle of the 12-circular hole board and were trained to find the escape tunnel to their home cage, at a fixed location, by using distant visual cues in the room. The spatial cues were present on the four walls in the test room at a distance of 0.5 m. The circular hole board consists of a large, white circular board (110 cm diameter) containing 12 equally spaced holes (4 cm diameter) at a distance of 9 cm from the rim. The maze was affixed at 90 cm above the floor by a tripod. The holes can be open (an open escape tunnel (s-shaped)) or closed (a closed tube (5 cm depth)). Only in close proximity to the hole, the mouse can recognize whether the hole is open or closed. The open escape tunnel ends in the home cage of the animal, which is placed underneath the circular hole board. To cor-

rect for possible odor cues, two additional cages with used bedding material were placed underneath the board.

Habituation and handling During the pre-training, an s-shaped tube is placed inside the home cage for the mice to explore (similar tubes are used during the acquisition) The mice were handled and habituated to the testing procedure by placing them individually inside an open cylinder positioned around a hole with an escape tunnel, allowing the mice to enter the escape tunnel freely (within 120 s) during two handling sessions per day for 3 days. When animals did not enter freely, they were gently guided into the tunnel. Also, before starting the acquisition trials all mice were individually placed once on the board for 5 min with all holes closed

Acquisition The mouse was placed in an open cylinder in the center of the board with 11 holes closed and one hole connected to an escape tunnel, at a fixed position in respect to the spatial cues, leading to the home cage. After 30 s the cylinder was lifted and the mouse could start to search the escape tunnel for maximally 2 min. During the acquisition trials (two trials per day for 7 days) a hole visit was scored when the animal was in contact with the hole (closed or open) Two successive visits to the same hole were counted separately if the animal visited another hole in between, or moved away over a distance of at least his own body length The time to find the escape tunnel in the NW quadrant was registered as the escape latency (s), a visit to a closed hole was registered as an error The return to the home cage via the escape tunnel is the reward, which serves as a positive motivation to learn about the location of the open escape hole. After each trial the board and escape tunnel were cleaned and turned in such a way that another hole became the escape hole at the fixed NW position

Probe Each mouse was allowed to explore the hole board, now with all holes closed During the 2 min probe trial the number of holes visited in the NW, NE, SE, SW quadrants (each containing three holes) was scored using a video-tracking computer analysis program made by D. Heeren (Radboud University Nijmegen). In addition, the percentage of hole visits was calculated for each quadrant

Relative cerebral blood volume measurements

To assess differences in brain vasculature, relative cerebral blood volume (rCBV) was determined in 8 and 15-month-old mice with a susceptibility-induced contrast MRI technique using Ultra Small Particles of Iron Oxide (USPIO) as blood-pooled contrast agent.

Before starting with the MR measurements, the mice were weighed Following anesthesia, all mice received an intravenous tail vein catheter for bolus injections of USPIO During the MR experiments mice were anesthetized with 1 9% isoflurane (Abott, Cham, Switzerland) in a mixture of oxygen and N2O (1:2) through a nose cone The composition of the mixture was monitored continuously using a gas analyzer (Datascope, Multinex) The body temperature was maintained at 37.2 ± 1 2 °C using a heated water pad and monitored with a rectal fluoroptic temperature probe. Breathing of the animal was monitored using an optical respiratory gating apparatus (Sirecust 401, Siemens) MR measurements were performed on a 7 Tesla/200 mm horizontal bore MR spectrometer (Magnex Scientific, Abingdon, England) interfaced to a S M I.S. console (Surrey Medical Imaging Systems, Surrey, England) The MR acquisition parameters were optimized in normal wild-type

mice matched in weight and age to the animals participating in the present study.
The USPIO contrast agent provides a valuable tool to characterize tissue vascularity since it remains intravascular for a prolonged period of time and highly enhances the transverse water proton MR relaxation rate (R2*). Previous studies have shown that magnetic susceptibility effects caused by USPIO can be used to assess relative blood volume within tissues [37,38]. In particular, the enhancement in the transverse relaxation rate (ΔR2*) after administration of USPIO, is proportional to the tissue blood volume.
An elliptical shaped (15 x 11 mm) surface coil was used for excitation and signal reception. The image acquisition protocol started with three gradient-echo scout images for slice positioning. Thereafter, multislice gradient-echo imaging was performed prior to and 1 minute after administration of a bolus injection with USPIO (AMI-277, Sinerem®, Guerbet Laboratories, France, 170 μg Fe/mouse). Imaging parameters were: field of view (FOV) of 25 mm x 25 mm, matrix size of 256 x 256, slice thickness of 1 mm, echo time (TE) of 7 ms, repetition time (TR) of 2000 ms and 2 averages per image, resulting in a total scan time of 17 minutes per multislice gradient-echo series. For each animal, pixel-by-pixel ΔR2* maps were obtained from the formula: $\Delta R2^* = (1/TE)\log(S_0^{bef}/S_0^{aft})$, where TE is the echo time, and S_0 the signal amplitude pre-USPIO (S_0^{bef}) and post-USPIO (S_0^{aft}), in the gradient echo images. The mean ΔR2* were calculated by drawing a Region of Interest (ROI) on the ΔR2* maps and averaging the values of all pixels within the ROI. To assess blood volume, regions of interest (ROIs) that included the entire hippocampus, prefrontal cortex, cerebral cortex (all cortical areas above the corpus callosum), diencephalon and the entire brain, were drawn on the images. These ROIs are based on the mouse brain atlas of Franklin and Paxinos 1997 [39]. The change in ΔR2* in the hippocampus, cerebral cortex and entire brain is proportional to the rCBV in these regions. In some regions, ΔR2* was not different from image noise and therefore assumed to be zero. All algorithms were implemented in Matlab (Mathworks, Natick, MA, USA).

Immunohistochemistry

Mice (Table 2) were transcardially perfused (after blood sampling via heart puncture) starting with a 0.1 M phosphate buffered saline (PBS) followed by Somogyi's fixative (4% paraformaldehyde, 0.05% glutaraldehyde and 0.2 % picric acid in 0.1 M phosphate buffer, PB, pH=7.2). Following transcardial perfusion fixation, mice were decapitated and brains were removed from the skull. The entire brain, without the spinal cord, was weighed, thereafter postfixed for 15 hours at 4 °C in Somogi's fixative and subsequently stored in PB at 4° C. Before cutting, the brain tissue was cryoprotected by immersion in 30% sucrose in PB at 4 °C. Series of 40 μm coronal sections were cut through the brain using a sliding microtome (Microm HM 440, Walldorf, Germany) equipped with a object table for freeze sectioning at –60° C. The Aβ load was visualized using WO-2 antibody (mouse anti-human Aβ4-10, T Hartmann, Heidelberg, Germany). Immunohistochemistry was performed using standard free-floating labeling procedures. Briefly, after blocking the brain sections against endogenous peroxidase with 0.3% H2O2 in PB the sections were pretreated with 0.05M sodium citrate solution at 85 °C for 30 minutes. The following steps are carried out in Tris buffered saline with 0.5% Triton-X-100 (TBS-T) Monoclonal mouse anti-Aβ4-10 (1:20.000) was used as primary antibody. The sections were incubated overnight at room

temperature on a shaker table Sections were then rinsed thoroughly with TBS-T and transferred to the solution containing the secondary antibody; donkey-anti-mouse biotin 1 1500 (Jackson Immuno research). After 90 minutes of incubation the section were rinsed three times and transferred to a solution containing Vector ABC-elite 1 800 (Vector laboratories, Burlingame) for another 90 minutes After rinsing with TBS-T, visualization of Aβ was achieved by incubation with 3-3'diaminobenzidine tetra hydrochloride with Ammonium nickel sulphate as an intensifier (DAB-Ni solution) After subsequent washing with PB, all stained sections were mounted on gelatin-coated slides, dried overnight in a stove at 37°C, dehydrated in alcohol series, cleared with xylol and mounted in Entellan

Quantification

To determine the amount of Aβ in the parenchyma of the frontal cortex (prelimbic area (PLA)), anterior cingulate gyrus (ACg) and in the hippocampus (CA1, CA3 and dentate gyrus (DG)), appropriate sections were digitized and quantified, using a Zeiss Axioskop microscope equipped with hardware and software of Microbrightfield, (Williston, USA). These cortical regions were chosen because of their large amyloid load in humans and transgenic mice and their importance in learning and memory [40,41]. Quantitative analyses were done with a computer-assisted analysis system (Stereo Investigator) using Cavalieri's probe A contour was drawn along the borders of the hippocampal subregions. In the ACg and PLA a square box was placed, within the borders of intended brain areas To determine the amount of Aβ in the vasculature, a contour was PLA, ACg and in the hippocampus, respectively containing the middle prefrontal branches of the anterior cerebral artery, the anterior cerebral artery and the vessels in the hippocampal fissure. Brain regions were based on the mouse brain atlas of Franklin and Paxinos 1997 [39] and quantified in three sections, with 200 μm distance between the sections The ACg was quantified at level +1.10 up to +0.86 anterior to bregma, PLA was quantified at +1.98 upto +1.78 anterior to bregma. The hippocampus was quantified at –2 18 up to –2.46 posterior to bregma
All measurements were performed double blind by two investigators. Aβ load was defined as the percentage of area covered by Aβ

Hippocampal atrophy

In the mice used for immunohistochemistry (Table 2), hippocampal atrophy was measured. As a measure for hippocampal atrophy, the thickness of the outer molecular layer of the dentate gyrus (DG) was measured in two sections, with 200 μm distance in between using a Zeiss Axioskop microscope, equipped with hardware and software of Microbrightfield (Williston, USA). The width of the outer molecular layer of the DG has been measured since this layer is subject of age related loss of synapses [42,43] partly originating from perforant path afferents from the entorhinal cortex which are affected in AD.

Serum sterol analysis

Directly after completion of the behavioral experiments, mice were anaesthetized with Nembutal (60 mg/kg, i p) (Ceva Santa Animals BV, Maassluis) and blood samples were collected via heart puncture Serum cholesterol levels and the cholesterol precursor lathosterol and its oxidative brain specific metabolite 24S-hydroxycholesterol were measured by

gas-chromatography-mass spectrometry-selected ionmonitoring (GC-MS-SIM) as described in detail previously [44-46].
Preparation of brain sterol extracts GC-MS analysis. A subgroup of mice was sacrificed to determine brain sterol levels (Table 2). The brains were snap frozen in liquid nitrogen and were kept frozen at -80°C. Sterols were extracted overnight by chloroform/methanol trimethylsilylated prior to GC-MS-SIM analysis [44,46].

Brain fatty acid analysis
Fatty acid analyses were performed with a part of the homogenate (described above). Total lipid was extracted from brain homogenates by methanol and chloroform. Subsequently, samples were centrifuged at 3000 rpm for 10 min and the lower phase (chloroform and lipids) was removed. Chloroform was added to the upper phase, samples were centrifuged again at 3000 rpm for 10 min and the lower phase was combined with the first one. The chloroform fractions were dried in a SpeedVac® and 2 ml methanol and 40 µl concentrated sulfuric acid were added to the dried extract. The samples were heated at 100 °C for 60 min, and 2 ml hexane and 0.5 ml 2.5 M sodium hydroxide solution were added. After vortexing and centrifuging the samples for 5 min at 3000 rpm, the upper layer was collected and evaporated in a SpeedVac®. The fatty acids were dissolved in 125 µl iso-octane and analyzed on a GC-FID with a CP-SIL88 column (50 m x 0.25 mm id. 0.22 µm film thicknesses). The n6/n3 ratio was calculated as a sum of analyzed n6 FAs divided by the sum of n3 FA.

Statistical analyses
Data are expressed as mean ± SEM and were analyzed with SPSS for windows 12.0 software (SPSS Inc. Chicago, IL, USA). The repeated measures ANOVA was used for the open field parameters (with the repeated measure: time) and the acquisition phases of the MWM, reversal MWM and circular hole board (with the repeated measure: trial block). Multivariate ANOVA's were conducted to analyze possible differences between the two genotypes and the different diet groups in the probe trials of the MWM, the reversal MWM and the circular hole board. MANOVA's were also applied to body and brain weights and serum cholesterol levels. The between group factors were Genotype and Diet. If interactions between genotype and diet (between-group-factors) were present, the data were splitted for the concerning factor and thereafter analyzed with the MANOVA. To evaluate regional differences in Aβ load, a one-way ANOVA was used. If no interactions between the genotype and diet were present and overall analysis revealed a significant difference, the separate groups were analyzed post hoc by using Tukey's HSD test. For clarity reasons, F-values are only displayed for the behavioral studies. Statistical significance was set at $p \leq 0.05$.

Results

Body and brain weight
All mice were weighed one week before starting the behavioral test battery. Body weight was not affected by genotype or diet at either 8-months or 15-months. Overall mean body weight was 32.4 ± 0.4 g in the 8-month-old mice and 44.0 ± 0.6 g in the 15-month-old group. Relative brain weight was not affected by genotype or diet in both 8- and 15-month-

old mice. Overall mean brain weight was 1.60 ± 0.03 % of total body weight in the 8-month-old mice and 1.17 ± 0.03 % in the 15-month-old group. Absolute brain weights did not differ between the age groups either.

Behavioral Testing

Open field

In the open field locomotion activity and active exploration parameters (walking, sitting, wall leaning, rearing) and grooming are scored for 30 minutes divided in three blocks of 10 minutes.

8-month-old mice

During the 30 min of observation, both in wild type and APP/PS1 mice the time spent walking ($F(2,120)=66.70$, $p<0.001$) and wall leaning ($F(2,120)=26.48$, $p<0.05$) decreased (Fig. 1A,D), whereas sitting ($F(2,120)=44.53$, $p<0.001$), rearing ($F(2,120)=26.48$, $p<0.001$) and grooming ($F(2,120)=6.83$, $p<0.01$) increased (Fig. 1B,C and E).

8-month-old APP/PS1 mice were more active in the open field than wild type mice (Fig 1): APP/PS1 mice walked more (Fig. 1A: $F(1,60)=23.61$, $p<0.001$), traveled a longer distance ($F(1,60)=29.66$, $p<0.001$) and sat less (Fig. 1B: $F(1,60)=18.71$, $p<0.001$) than wild type mice. APP/PS1 mice spent less time rearing (Fig. 1C: $F(1,60)=14.30$, $p<0.001$), and more time leaning (Fig. 1D: $F(1,60)=6.36$, $p<0.05$) than wild type mice. No differences were observed in the time spent grooming (Fig. 1E) or the frequency of defecation (Fig. 1F) between the genotypes. Dietary intervention for 6 months, with either the DHA enriched or the Typical Western Diet (TWD) did not alter any of the open field parameters.

15-month-old mice

Like at 8 months, the time spent walking (Fig. 2A: $F(2,118)=60.86$; $p<0.001$) decreased whereas the time spent sitting (Fig. 2B: $F(2,118)=15.61$; $p<0.001$), rearing (Fig.2C: $F(2,118)=42.56$; $p<0.001$) and grooming (Fig. 2E $F(2,118)=7.06$; $p<0.001$) increased over time in both wild type and APP/PS1 mice. However, the increase in rearing over time was significantly less in APP/PS1 mice compared to wild type mice ($F(2,118)=1.76$; $p=0.05$), indicating less habituation to the new situation.

Compared to wild type mice, APP/PS1 mice spent less time sitting (Fig 2B; $F(1,59)=5.48$; $p<0.05$), more time walking ($F(1,59)=7.11$; $p<0.01$), and they traveled a longer distance ($F(1,57)=25.72$; $p<0.001$) and spent less time in the centre of the open field ($F(1,57)=4.73$; $p<0.05$). Furthermore, APP/PS1 mice showed less rearing (Fig 2C; $F(1,59)=9.90$; $p<0.01$) and more wall leaning behavior (Fig 2D; $F(1,59)=25.13$; $p<0.001$). Grooming was not affected by genotype. Finally, APP/PS1 mice showed increased frequency of defecation during the testing phase (Fig 2F, $F(1,57)=6.33$; $p<0.01$), indicating a higher anxiety level. Altogether, these data show increased activity and anxiety in transgenic APP/PS1 mice compared to wild type mice. No behavioral differences regarding all open field parameters were found between the diet groups.

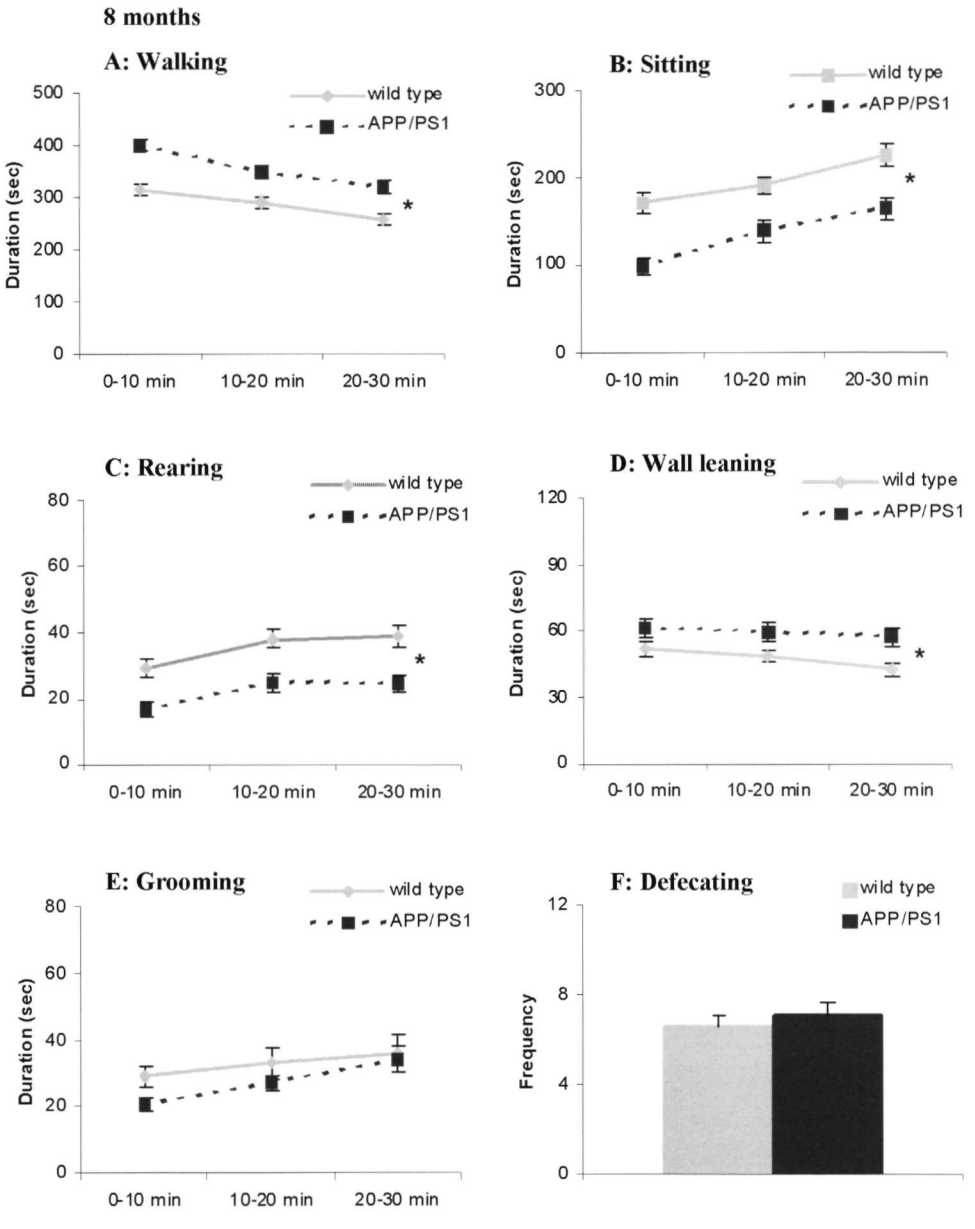

Fig.1. Open Field behaviour in 8-month-old APP/PS1 and wild type mice.
*Different open field parameters were measured within a 30 min period, and scored in three 10 min trial blocks. A) duration of walking, B) duration of sitting, C) duration of rearing; i.e. standing on the hind paws, and exploring the environment, D) duration of wall leaning, E) duration of grooming and F) frequency of defecating. APP/PS1 mice spent more time walking ($p<0.001$) and wall leaning ($p<0.05$), and spent less time sitting ($p<0.001$) and rearing ($p<0.001$) compared to wild type mice. Values represent the mean and SEM. * $p<0.05$.*

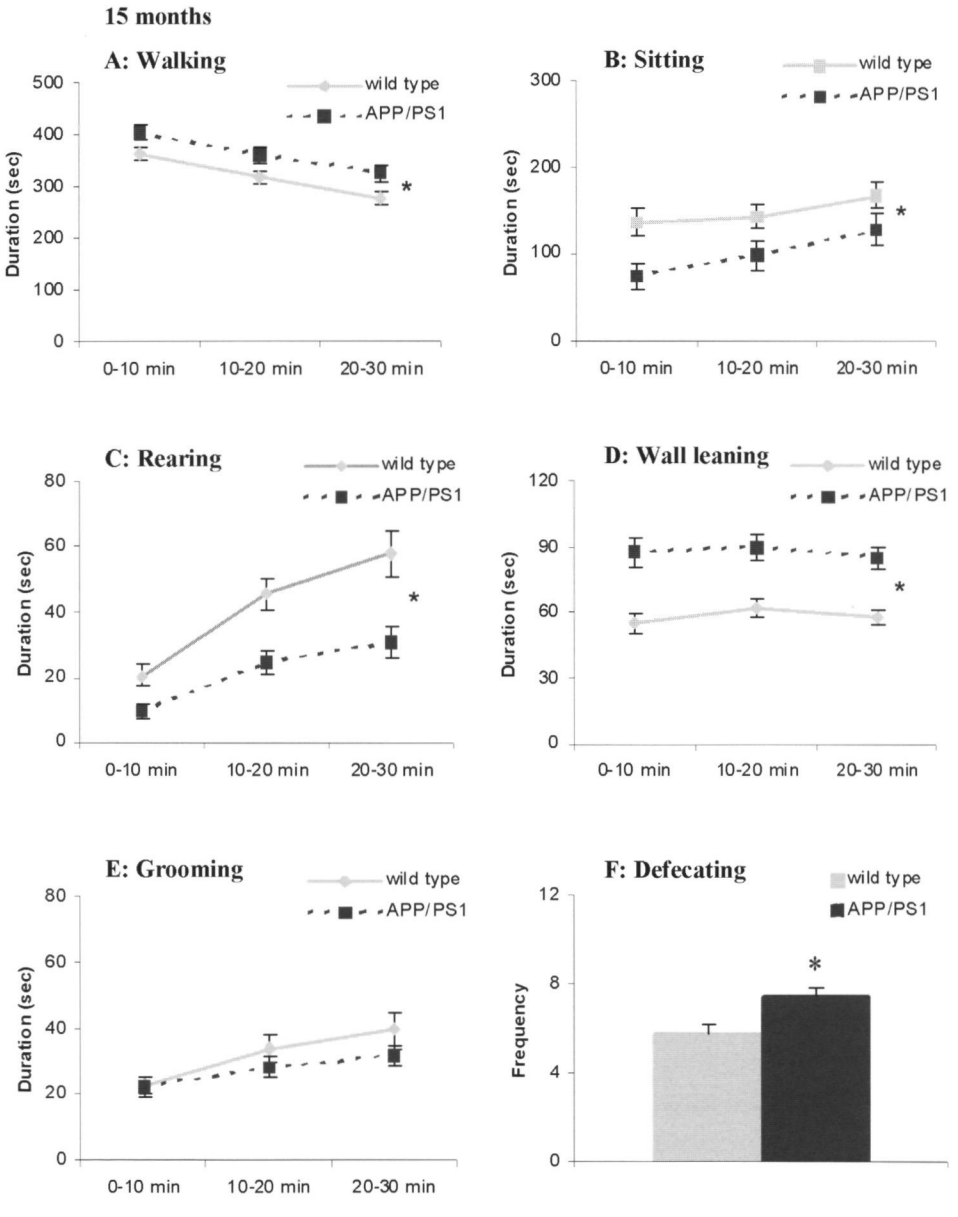

Fig. 2. Open Field behaviour in 15-month-old APP/PS1 and wild type mice.
*Different open field parameters were measured within a 30 min period, and scored in three 10 min trial blocks. A) duration of walking, B) duration of sitting, C) duration of rearing; i.e. standing on the hind paws, and exploring the environment, D) duration of wall leaning, E) duration of grooming and F) frequency of defecating. APP/PS1 mice spent more time walking ($p<0.01$) and wall leaning ($p<0.001$) and defecated more ($p<0.02$), and spent less time sitting ($p<0.02$) and rearing ($p<0.003$) compared to wild type mice. Values represent the mean and SEM. * $p<0.05$.*

Morris water maze (MWM)

The Morris water maze is designed to test spatial learning by training the mice to find a hidden platform (acquisition phase). Spatial memory is tested in a trial in which the platform is removed from the maze (probe trial) directly following the acquisition phase.

8-month-old mice

Both APP/PS1 and wild type mice showed a decrease in escape latency during training (Fig. 3A: $F(7,420)=39.00$, $p<0.001$). Escape latencies did not differ between the wild type and the APP/PS1 mice (Fig. 3A), indicating that spatial learning was not affected by genotype in 8-month-old mice. No effects of diets were found.

Wild type mice tended to spend more time in the platform quadrant (NE) during the probe trial than APP/PS1 mice (Fig 3B: $F(1,60)=3.73$, $p=0.058$), but neither group deviated from 25% chance performance (wild type 26.7 ± 1.4%, APP/PS1 23.1 ± 1.2%) indicating no memorization of the platform location . No effects of diets on probe test performance were noted.

15-month-old mice

Both wild type and APP/PS1 animals learned to find the platform during acquisition, as indicated by a decrease in latency over trials (Fig. 3C: $F(7,385)=23.31$; $p<0.001$). However, APP/PS1 mice needed more time to find the platform than the wild types ($F(1,55)=6.2$; $p<0.05$). Diet did not change MWM acquisition.

During the probe trial, APP/PS1 mice spent less time in the NE target quadrant compared to their wild type littermates (Fig. 3D: $F(1,50)=22.45$; $p<0.001$) irrespectively of diet. Since APP/PS1 mice spent only 20.9 ± 1.1% in the target quadrant, which is less than the 25% chance level, these results indicate that APP/PS1 mice did not remember the location of the platform at all. The number of platform crossings was also lower in APP/PS1 mice compared to wild types (Wild type 5.2 ± 0.4, APP/PS1 3.6 ± 0.5; $F(1,50)=4.53$; $p<0.05$), reflecting impaired spatial memory as well.

Interestingly, transgenic animals fed a DHA diet, spent more time in the target quadrant during the probe trial compared to transgenic mice fed STD diet ($F(2,24)=4.27$; $p<0.05$; fig 3F) indicating improved memory due to DHA. This dietary effect was not found in wild type animals. No TWD effects were found.

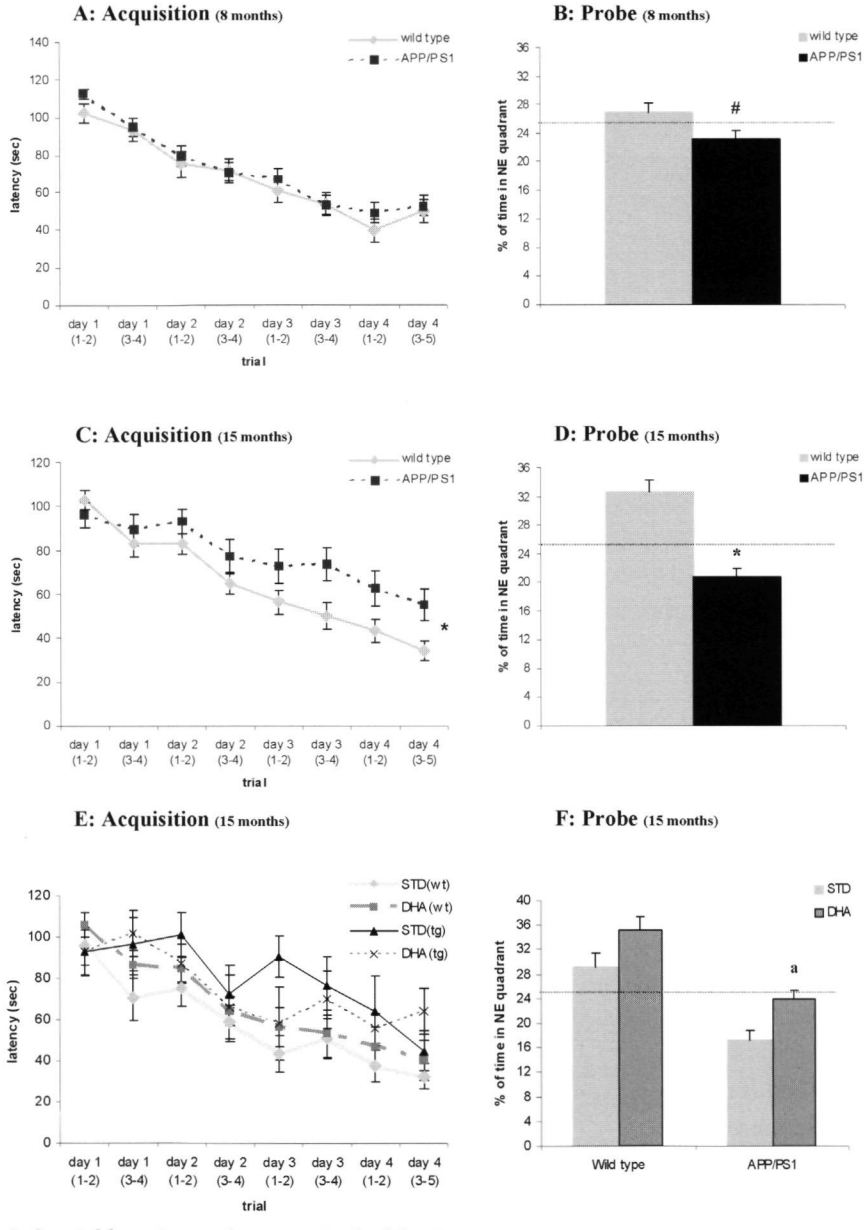

Fig. 3. Spatial learning and memory in the Morris water maze.
*Spatial learning was measured in a 4 day acquisition phase (A, C and E) by determine the latency to find a hidden platform. The spatial memory was tested in the probe phase (B, D and F) in which the % of time spent in the north east (NE) quadrant (i.e. where formerly the platform had been located), was measured. A) acquisition phase of 8-month-old wild type and APP/PS1 mice, B) probe trial of 8-month-old wild type and APP/PS1 mice. C) acquisition phase of 15-month-old wild type and APP/PS1 mice, D) probe trial of 15-month-old wild type and APP/PS1 mice. E) DHA effects in the acquisition phase of 15-month-old wild type and APP/PS1, F) DHA effects in the probe trial of 15-month-old APP/PS1 mice. Values represent the mean and SEM. # trend $p=0.058$, * $p<0.05$, a=different from STD(tg).*

Reverse Morris water maze

In the reverse Morris water maze, mice have to learn to find a novel position for the hidden platform. This task is considered to be a test for new learning abilities, in which a previous successful strategy must be inhibited and a new strategy should be developed.

8-month-old mice

The wild type animals showed a significant decrease in escape latency over the time in the acquisition phase (Fig. 4A: $F(3,90)=7.46$; $p<0.001$), indicating spatial learning (Fig. 4A). The APP/PS1 mice in contrast, did not show a significant decrease in escape latencies (Fig.4A),

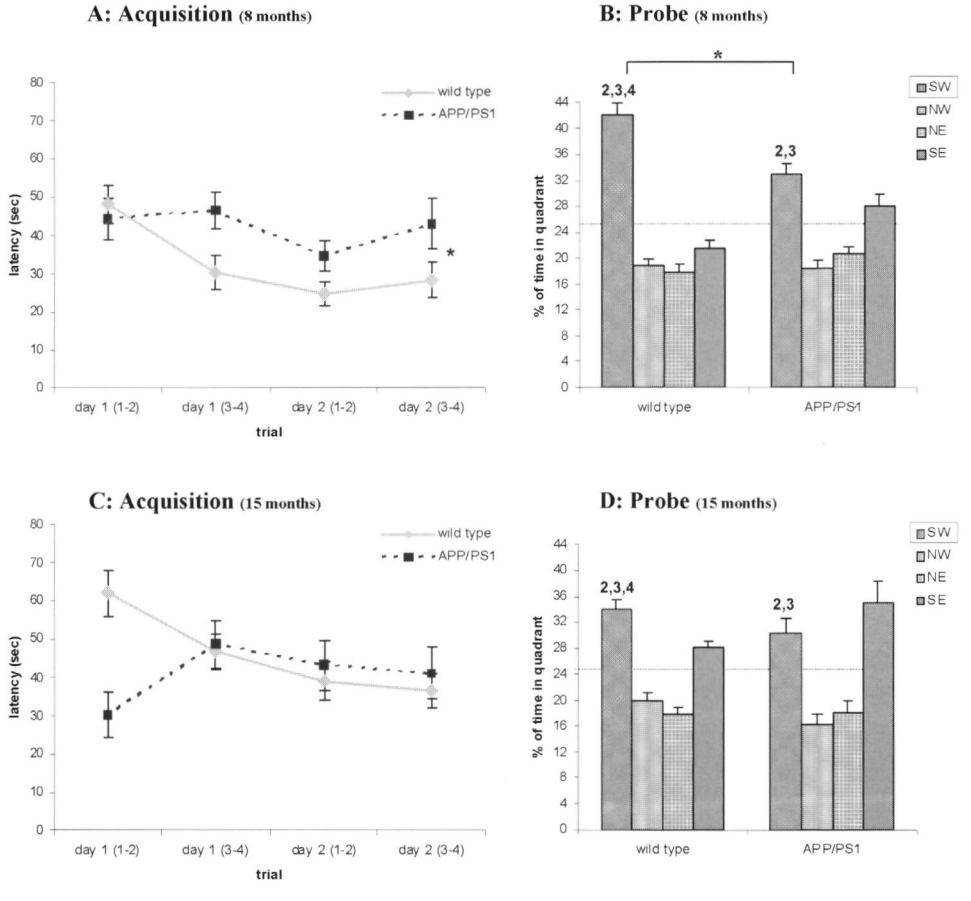

Fig. 4. Spatial learning and memory with an extra episodic memory component in the reversal Morris water maze.

*Spatial learning and memory with an extra episodic memory component in the reversal Morris water maze. Spatial learning was measured in a 2 day acquisition phase in 8-month-old mice (A) and 15-month-old mice (C), by determine the latency to find a hidden platform in the south-west (SW) quadrant. The spatial memory was tested in the probe phase in both 8-month-old mice (B) and 15-month-old mice (D), in which the % of time spent in the quadrant where the platform formerly was located and the other quadrants was measured. Values represent the mean and SEM. * $p<0.05$, 2=different form north-west (NW) quadrant, 3= different form north-east (NE) quadrant, 4= different from south-east (SE) quadrant.*

indicating that these mice had problems learning to find the new SW platform location, and showed indeed longer escape latencies compared to wild type mice ($F(1,60)=3.90$; $p<0.05$). Analyses of the probe test revealed that wild type mice spent significantly more time swimming and searching in the "right" SW quadrant compared to APP/PS1 mice ($F(1,60)=16.43$; $p<0.001$), indicating a better new learning ability. Although both groups of mice spent more than 25% of their time in the SW quadrant, indicating that both groups remembered the new platform location, the wild type mice performed better. No dietary effects were found in the probe test.

15-month-old mice

In the acquisition phase, wild type mice showed decreased latencies over time ($F(3,108)=7.53$; $p<0001$ (Fig 4C). In contrast, escape latencies tended to increase in APP/PS1 mice (Fig. 4C; $F(3.57)=2.74$ $p=0.05$), due to unexpected low latencies at the initial trials. In spite of that, there was no difference in average escape latencies between the APP/PS1 and wild type animals. In addition, like in the 8-month old mice, no significant dietary effects were found.

The probe trial (Fig. 4D) revealed that both wild types and APP/PS1 mice learnt to locate the platform, since they spent more time than the 25% chance level in the SW target quadrant. There was no significant difference in spatial memory between wild type and APP/PS1 mice in time spent in the SW quadrant. Nevertheless, APP/PS1 mice did perform a bit worse, since they showed a preference for both SW and SE, indicating that they did not learn the new task as good as the wild types. Similar to the 8-month old mice, no significant dietary effects were found.

The 12 circular hole board

In order to confirm spatial learning and memory ability without swimming related stress factors, the 12 circular hole board was performed.

8-month-old mice

Latencies to find the escape hole decreased during the acquisition trials irrespective of genotype (Fig. 5A; $F(6,35)=15.81$; $p<0.001$). Spatial learning acquisition did not reveal differences between the genotypes. In the probe test wild type mice spent 36.4 ± 2.0 % of their time in the target quadrant, which is significantly more compared to the other three quadrants (NW-SE $p<0.001$, NW-SW $p<0.001$, NW-NE $p<0.001$), indicating that wild type mice memorized the location of the platform. In contrast, APP/PS1 mice spent only 29.4 ± 2.3 % of their time the NW quadrant, which was not significantly different from the 25% chance level (Fig. 5B).

More importantly, compared to wild type mice, the APP/PS1 mice spent significantly less time in the target quadrant ($F(1,59)=5.09$; $p<0.05$). This result was fortified by the number of visits ($F(1,59)=3.96$, $p<0.05$) with wild type mice visiting 33.8 ± 1.6% holes in the NW quadrant, compared to the 28.7 ± 1.9% of the visits to holes in the NW quadrant made by APP/PS1 mice. Both findings point to impaired spatial memory in APP/PS1 mice. Diets did not affect 12CHB performance in either the acquisition or the probe phase.

15-month-old mice

Both transgenic and wild type animals showed decreasing escape latencies during the acquisition of the task (Fig. 5C: $F(6,120)=9.03$ $p<0.001$ and $F(6,120)=15.48$ $p<0.001$), but

the mean latency was higher in APP/PS1 mice ($F(1,56)=12.61$ $p<0.001$), indicating impaired spatial learning in APP/PS1 mice. In the probe test (Fig. 5D) wild type mice spent significant more time in the NW-quadrant compared to the three remaining quadrants (all $p<0.001$) showing good spatial memory. This was in contrast to APP/PS1 mice which did not spent significantly more time in the NW-quadrant, indicating that APP/PS1 mice did not memorize the location of the escape hole. More importantly, APP/PS1 mice spent significantly less time in the correct quadrant compared to the wild types ($F(1,56)=4.69$ $p<0.05$). No diet effects were detected in the acquisition or probe trial in the 15-month-old mice.

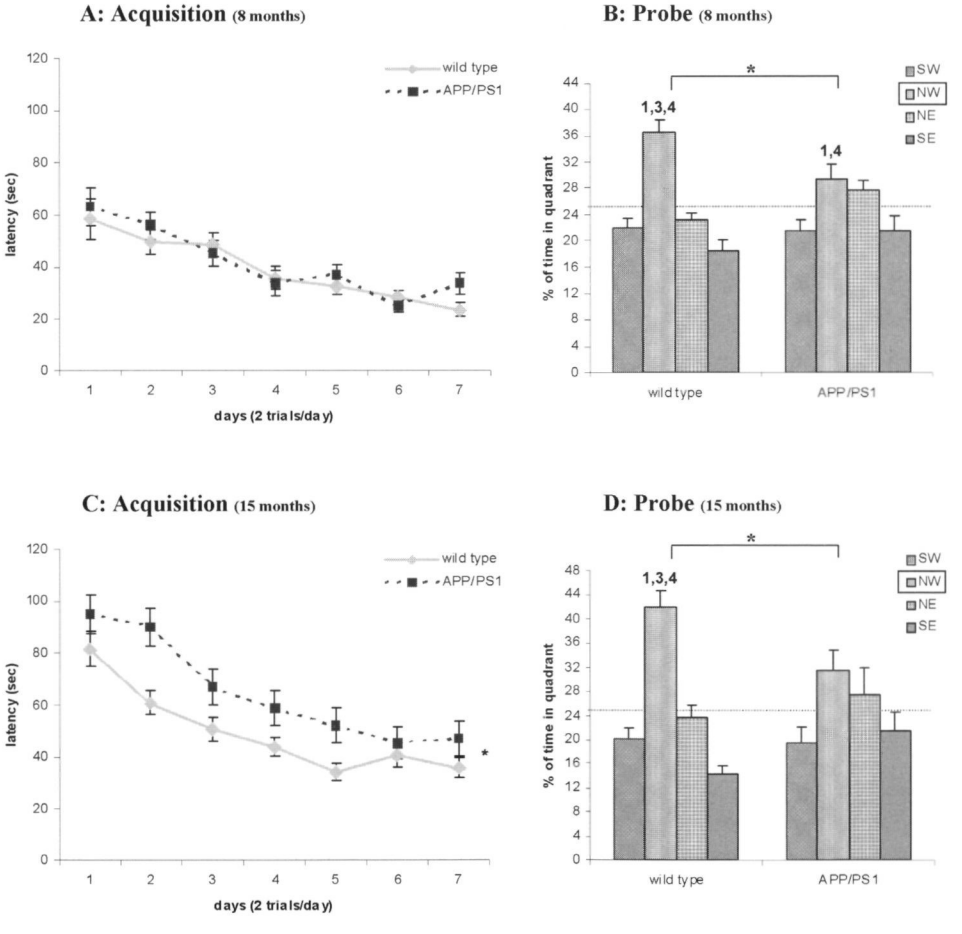

*Fig. 5. Spatial learning and memory in the 12 circular hole board test. Spatial learning was measured in a 7 day acquisition phase in 8-month-old mice (A) and 15-month-old mice (C) by determining the latency to find a hidden platform in the north-west (NW) quadrant. The spatial memory or memory retention was tested in the probe phase in both 8-month-old mice (B) and 15-month-old mice (D), in which the % of time spent in the quadrant where the platform formerly was located and the other quadrants was measured. Values represent the mean and SEM. * $p<0.05$, 1=different form south west (SW), 3= different form north-east (NE) quadrant, 4= different from south-east (SE) quadrant.*

Relative cerebral blood volume (rCBV)

To explore regional differences in rCBV in the brain, a susceptibility-induced contrast MR imaging technique was used.

8-month-old mice

In 8-month-old mice no significant differences were found in rCBV in either the diencephalon, prefrontal cortex or the hippocampus between wild type and APP/PS1 mice. In the cerebral cortex no differences were observed in rCBV between STD(wt) and STD(tg) mice (Fig. 6A).

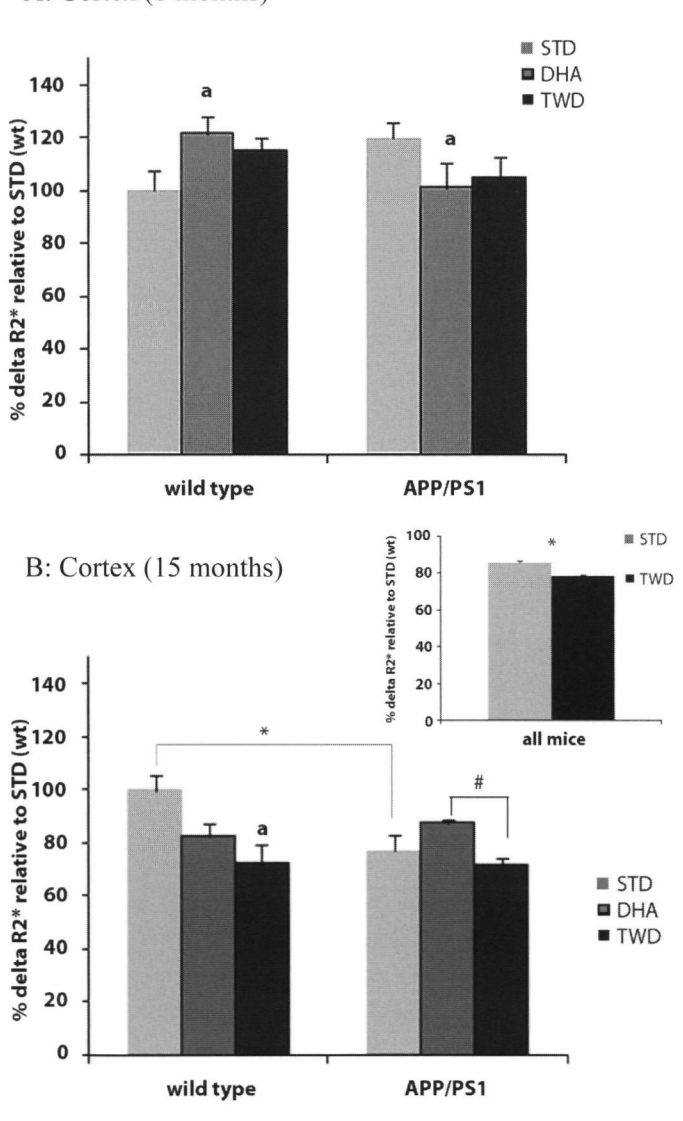

Fig. 6. Relative cerebral blood volume (rCBV) in the brains of wild type and APP/PS1 mice. Changes in ΔR2 relaxation after injection of ultra small particles of iron oxide (USPIO) was measured in A) 8-month-old mice, and B) 15-month-old mice. The change in ΔR2* is proportional to rCBV. Values represent the mean and SEM. * $p<0.05$, a= different from STD fed mice, $p<0.05$.*

However, diet effects were observed in the cerebral cortex. Wild type mice fed the DHA diet showed an increased rCBV compared to mice fed the STD diet (mean DHA(wt) 127,3 ± 3.5%, mean STD(wt) 100.0 ± 7.2%, $p<0.01$). APP/PS1 mice fed the DHA diet in contrast, showed a significant decreased rCBV compared to APP/PS1 mice fed the STD diet (mean DHA(tg) 88.3 ± 2.3%, mean STD(tg) 119.6 ± 5.8%, $p<0.05$). Dietary intervention with either the DHA enriched or the TWD did not alter rCBV in any of the other brain regions.

15-month-old mice

15-month-old APP/PS1 mice fed the STD diet showed a significant decrease in rCBV compared to their wild type littermates in the cerebral cortex (Fig. 6B; $p<0.05$). The hippocampus showed a comparable decrease in rCBV as found in the cortex (29%), although not significant. Furthermore, some diet effects were observed. Mice fed the TWD diet showed a significant decreased rCBV compared to mice fed the STD diet ($p<0.05$). This effect was most pronounced in the wild type mice ($p<0.01$). Also a small DHA diet effect was seen in APP/PS1 mice. DHA(tg) mice showed a 14% bigger rCBV compared to TWD(tg) mice ($p=0.058$). No dietary effects were found in any of the other brain regions (hippocampus, diencephalon and prefrontal cortex).

Amyloid-β load

In cortical regions (prelimbic area (PLA), anterior cingulate gyrus (ACg)) and in hippocampal regions (CA1, CA3 and dentate gyrus (DG)) both the parenchymal and vascular Amyloid-β (Aβ) load was determined. The brains of all mice were immunohistochemically stained with WO-2 antibody (mouse anti-human Aβ4-10). Wild type mice showed no immunoreactivity with this antibody, while Aβ deposits in transgenic mice were intensively stained.

8-month-old mice

8-month-old APP/PS1 mice showed some regional variation (Fig. 7A; $p<0.001$) in the Aβ plaque burden in the parenchyma, such that the DG showed a significantly higher Aβ plaque load ($p<0.001$) compared to all other regions, directly followed by the cingulate gyrus. ($p<0.002$). In the DG approximately 4.7% of the total area was covered with plaques, compared to 2.3% in the ACg, 0.77% in the CA1, 0.5% in the CA3 and 1.22% in the PLA. No diet effects could be observed on vascular or parenchymal Aβ.

15-month-old mice

In 15-month-old APP/PS1 mice regional variation in Aβ plaque load was observed as well (Fig 7A; $p<0.001$). Again, the DG showed the highest amount of Aβ plaques compared to the GC ($p<0.01$), the CA3 ($p<0.001$), CA1 ($p<0.001$) and PLA area ($p<0.001$).

Aβ depositions covered 21.3% of the DG compared to 18.0 % in the ACg, 10.5% in the CA1, 9.3% in the PLA and 3.7% in the CA3. These results indicate that the DG, directly followed by ACg, is most vulnerable to Aβ dposition. In addition an overall main diet effect was observed in the ACg (Fig. 7B; $p<0.05$); where DHA decreased the Aβ load compared to STD (Fig 7D,E and F; $p<0.05$). The TWD diet seemed to decrease the amount of parenchymal Aβ as well, but this effect was not significant.

The DHA diet also diminished the amount of vascular Aβ in the PLA compared to mice fed the TWD diet (Fig. 7C, G and H and I; $p<0.05$). All together, these results indicate that the DHA diet diminished the amount of Aβ.

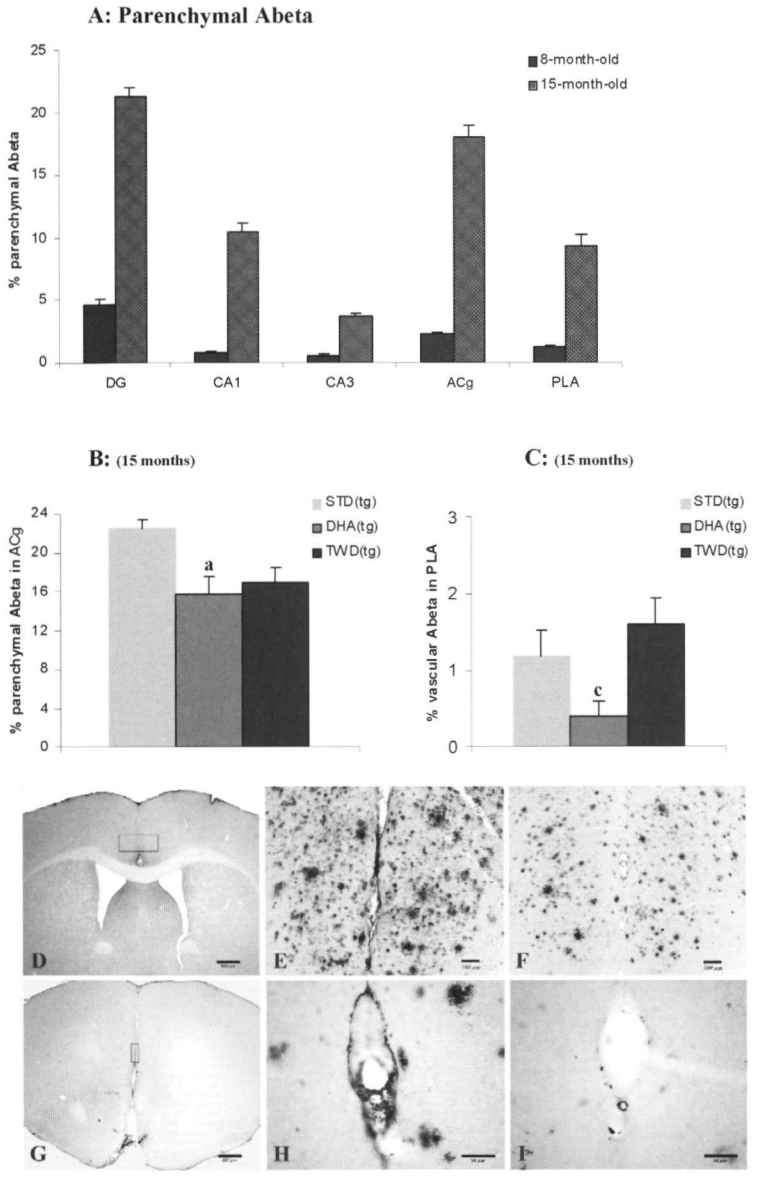

Fig. 7. β-amyloid pathology in the brains of APP/PS1 mice.
A) regional distribution of Aβ in the cerebral parenchyma in 8 and 15-month old APPPS1 mice. The dentate gyrus (DG) exposes the highest Aβ load compared to all other areas (CA1, CA3, cingulate gyrus (ACg), prelimbic area (PLA)) in both the 8 and 15-month old mice. B) the DHA containing diet decreases the amount of parenchymal Aβ load in the cingulate gyrus of 15-month-old APP/PS1 mice. C) the DHA containing diet decreases the amount of vascular Aβ in the prelimbic area (PLA) of 15-month-old APP/PS1. Values represent the mean and SEM. a= different from STD fed mice, c=different from TWD fed mice, $p<0.05$.D,E,F) Representative examples of parenchymal amyloid load in the cingulate gyrus of 15-month-old mice (D= Overview, 2.5x; E=STD(tg), 10x and F=DHA(tg), 10x) G,H,I) Representative examples of the vascular amyloid load in the PLA (G=overview, 2.5x; H=STD(tg), 40x and I=DHA(tg), 40x).

Atrophy

To determine whether atrophy of the hippocampus occurs in APP/PS1 mice the width of the outer molecular layer of the dentate gyrus (DG) was measured.

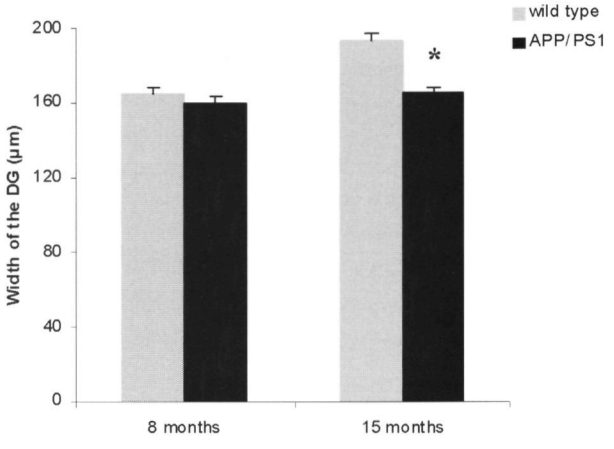

*Fig. 8. Width of the outer molecular layer of the DG, as a measure of hippocampal atrophy. Values represent the mean and SEM. * $p<0.05$.*

8-month-old mice

In 8-month-old mice we did not observe differences in the width of the outer molecular layer of the DG between wild type and APP/PS1 mice (Fig. 8). Also no effects of either a TWD or DHA diet were observed.

15-month-old mice

15-month-old APP/PS1 mice however, showed a 15% thinner outer molecular layer of the DG compared to their age matched wild type littermates (Fig. 8). No diet effects were observed.

Serum and brain sterols

8-month-old mice

As shown in Table 3, brain and serum sterol levels did not change due to genotype. However, serum cholesterol levels did increase due to the cholesterol containing TWD diet in both wild type and APP/PS1 mice ($p<0.01$).

In addition, serum lathosterol, a main cholesterol precursor in the "de novo" synthesis pathway, significantly decreased in TWD fed mice ($p<0.001$), and the cholesterol elimination rate from the brain, measured by serum 24S-OH-cholesterol levels decreased in mice fed the TWD diet ($p<0.001$). Further, the TWD diet did not alter brain lathosterol, 24S-OH-cholesterol or cholesterol levels.

The finding that brain cholesterol did not change, indicates that there is not much transport of cholesterol across the blood brain barrier. The DHA diet also did not alter brain cholesterol levels, but did reduce brain lathosterol levels ($p<0.05$), indicating diminished cholesterol de novo synthesis in DHA fed mice.

15-month-old mice

In 15-month-old mice no genotype effects were found in serum lathosterol nor 24S-OH-cholesterol levels (Table 3). Serum cholesterol levels did not differ between the genotypes as well, except for mice fed a TWD diet. Mice fed a TWD diet showed an interaction between diet and genotype for serum cholesterol levels ($p=0.05$) and a split file test revealed that this interaction is present because of a genotype effect ($p=0.05$). Like at 8 months the TWD diet, increases the serum cholesterol in both wild type and APP/PS1 mice ($p<0.001$). Thus, a TWD diet increases serum cholesterol, but the effect is significant larger in APP/PS1 mice compared to wild types. Also serum 24S-OH-cholesterol levels increase due to a TWD diet ($p<0.001$), but no interaction between genotype and diet could be observed. The cholesterol synthesis precursor lathosterol, decreased as a result of a TWD diet in serum of both wild types and APP/PS1 mice ($p<0.001$).

The brain cholesterol concentration decreased in 15-month-old APP/PS1 mice compared to wild type mice ($p<0.005$). No changes in brain sterol levels due to the TWD or DHA diet were observed in 15-month-old mice.

Table 3. Serum and brain concentration of sterols

	Serum			Brain		
	Cholesterol (mg/dl)	Lathosterol (mg/dl)	24s-OH-chol. (ng/ml)	Cholesterol (µg/mg)	Lathosterol (ng/mg)(ng/mg)	24s-OH-chol.
8-month-old						
Wild type:						
STD(wt)	116 40±12.59	0.023±0.003	23 40±1.54	68.80±2 64	91.70±5.37	227.18±8.90
DHA(wt)	99.33+7.60	0.022+0.001	21.00±0.58	68.98±0.67	80.22±7 57 [a]	222.38±10.83
TWD(wt)	158.00±8.13 [ab]	0.016±0.002 [ab]	35 83±2.39 [ab]	71.55±1.88	94.11±7.64	234 05±5.17
APP/PS1:						
STD(tg)	107.33±13.56	0.027±0.005	23.17±2.06	71.70±3 33	96.82+7.93	233 30±12.79
DHA(tg)	91.83+9.17	0.020±0.002	19.50±6.20	70.38±1.53	77 30±2 58 [d]	234.80±5.24
TWD(tg)	147.20±12.28 [de]	0 014+0.002 [de]	35.80+4.28 [de]	72.72±2.17	87 04±2 63	238 30±8.57
MANOVA:						
Genotype	**0.30**	**0.93**	**0.74**	**0.84**	**0.43**	**0.81**
Diet	**0.007**	**0.001**	**0.001**	**0.71**	**0.05**	**0.61**
15-month-old						
Wild type:						
STD(wt)	104.25+6.90	0 018±0.003	19.44±0.80	79.99±1.81	83 42±4.05	234.35+4.63
DHA(wt)	88 18±6 92	0 017+0.001	18.36+0.93	77.70±1.49	74.46±4.25	231.99±4.39
TWD(wt)	163.57+21.25 [ab]	0 010±0.001 [ab]	37.50±4.56 [ab]	81.67±1.73	77.52+5.74	233.02±5 37
APP/PS1:						
STD(tg)	108 80+9 86	0 023±0 003	21 30±1 17	75.93±1.44	73.99±5.56	238.36+4.09
DHA(tg)	81.20±10.74	0.017+0 003	18.60+0.51	72.44±0.24	63.48±12.76	237 04±10.14
TWD(tg)	239.44+32 80 [de]	0.010±0.001 [de]	52.88±7.40 [de]	76.60±1.66	79.13±6.12	238.14+4.16
MANOVA						
Genotype	**0.11***	**0.11**	**0.07**	**0.004**	**0.22**	**0.30**
Diet	**0.001**	**0.001**	**0.001**	**0.16**	**0.27**	**0.94**

a=different from STD(wt), b=different from DHA(wt), d=different from STD(tg), e=different from DHA(tg).). * indicates the presence of an interaction between genotype and diet (p=0.05). This interaction is present because of a genotype effect ($p=0.05$) in mice fed the TWD diet.

Brain fatty acids

8-month-old mice

The relative concentrations of different fatty acids (FA) in brain tissue of 8 and 15-month-old mice are shown in Table 4. APP/PS1 and wild type mice fed the DHA containing diet showed a shift in the balance between n3 and n6 fatty. The relative amount of n3 FA in DHA fed mice increased compared to the STD and TWD groups ($p<0.001$), whereas the relative amount of n6 decreased ($p<0.001$) resulting in a pronounced shift in the n3/n6 ratio in DHA fed mice in favor of the n3 ($p<0.001$). The reduction in the relative n6 content was mainly caused by the decrease in arachidonic acid (20:4n6, ($p<0.001$), while the higher n3 content originated from an increase in relative DHA (22:6n3, $p<0.001$) content.

15-month-old mice

In 15-month-old mice (Table 4), a similar shift in the balance between n3 and n6 fatty acids in DHA fed mice was observed. Again, the amount of n3 fatty acids in DHA fed mice increased compared to the STD and TWD groups ($p<0.001$), whereas the amount of n6 decreased ($p<0.001$).

In contrast to the 8-month-old group, 15-month-old APP/PS1 mice showed a significant increase in n6 fatty acids ($p<0.05$) irrespective of diet, and consequently a decreased n3/n6 ratio ($p<0.05$).

Table 4. Relative fatty acid composition % of the lipid fraction of brain homogenates of 8 and 15/month-old wild type and APP/PS1 mice on various diets

	SFA	MUFA	PUFA	Σn3	Σn6	n3/n6	DHA	AA
8-month-old								
Wild type:								
STD(wt)	39.60±0.97	25.37±0.43	25.87±0.61	12.93±0.54	12.68±0.17	1.04±0.04	12.20±0.51	8.64±0.11
DHA(wt)	40.75±0.79	24.52±0.57	26.73±0.31	15.45±0.23^{ac}	11.14±0.14^{ac}	1.40±0.03^{ac}	14.66±0.2 ^{ac}	7.65±0.08^{ac}
TWD(wt)	39.63±1.08	25.28±0.49	26.31±0.49	13.08±0.31	13.15±0.21	0.98±0.02	12.29±0.29	8.90±0.17
APP/PS1								
STD(tg)	40.35±0.70	23.85±0.89	27.00±0.69	13.82±0.29	13.08±0.42	1.05±0.02	13.16±0.38	8.87±0.41
DHA(tg)	40.22±0.84	25.41±0.62	26.17±0.50	15.08±0.30^{df}	11.00±0.25^{df}	1.39±0.03^{df}	14.26±0.26^{df}	7.50±0.26^{df}
TWD(tg)	40.81±0.61	24.60±0.12	26.41±0.32	13.04±0.25	13.25±0.09	0.97±0.02	12.30±0.25	8.96±0.15
MANOVA:								
Genotype	**0.62**	**0.36**	**0.60**	**0.54**	**0.77**	**0.54**	**0.47**	**0.80**
Diet	**0.92**	**0.81**	**0.80**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
15-month-old								
Wild type:								
STD(wt)	39.56±0.29	24.65±0.39	25.46±0.28	13.08±0.11	12.38±0.19	1.06±0.01	12.34±0.14	8.58±0.16
DHA(wt)	40.30±0.41	23.89±0.53	25.76±0.39	14.81±0.29^{ac}	10.94±0.12^{ac}	1.35±0.02^{ac}	14.24±0.32^{ac}	7.60±0.10^{ac}
TWD(wt)	40.16±0.67	24.45±0.70	25.45±0.44	12.88±0.23	12.58±0.22	1.02±0.01	12.13±0.30	8.62±0.21
APP/PS1								
STD(tg)	39.64±0.62	23.86±0.59	25.49±0.38	12.97±0.19	12.51±0.21	1.04±0.01	12.32±0.20	8.59±0.17
DHA(tg)	40.32±0.19	24.17±0.33	25.77±0.26	14.76±0.17^{df}	11.01±0.20^{df}	1.34±0.03^{df}	14.14±0.18^{df}	7.54±0.15^{df}
TWD(tg)	40.36±0.26	23.45±0.39	26.59±0.34	13.11±0.20	13.48±0.17	0.97±0.01	12.47±0.22	9.26±0.13
MANOVA:								
Genotype	**0.82**	**0.31**	**0.25**	**0.89**	**0.04**	**0.02**	**0.75**	**0.19**
Diet	**0.29**	**0.84**	**0.32**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**

a=different form STD(wt), c=different from TWD(wt), d=different from STD(tg), f=different from TWD(tg).

Discussion

The present data show that the DHA-enriched diet altered rCBV in 8-month-old APP/PS1 and wild type mice. In addition, 15-month-old APP/PS1 mice fed DHA-enriched diet for more than a year showed improved spatial memory, decreased parenchymal and vascular Aβ in the brain and a slightly increased relative cerebral blood volume (rCBV), indicating that a DHA-enriched diet may diminish aggravation of Alzheimer-like pathology. In contrast, a TWD diet may increase AD development by decreasing the rCBV in 15-month-old mice, and increasing the amount of Aß depositions at a later stage as shown in our previous study [15]. Furthermore, the effects of the tested diets on vascular parameters were observed before effects on Aβ load were noted, indicating that the DHA and TWD diets may influence AD-like pathology by initially changing the cerebral circulation.

Genotype

The most obvious and first appearing clinical sign of late onset AD is memory impairment. A mouse model featuring this characteristic early in the disease development would be very useful in AD research. In this study APP/PS1 mice already show cognitive impairment at the age of 8 months in combination with amyloid-β deposition and increased activity and decreased explorative behavior compared to their wild type littermates. The increased activity is a specific characteristic of many APP transgenic mice [47-49] and may be explained as either a results of elevated anxiety levels [49,50] or impaired habituation learning [47,48],[51]. In our view increased anxiety is most likely, since the 8-month-old APP/PS1 mice showed also less rearing (exploring the environment), but more importantly, at the age of 15 months the APP/PS1 mice spend less time in the centre of the open field accompanied by increased defecating (i.e. anxious mice prefer the border of the field and defecate more [52,53]). Increased anxiety and restlessness, as noticed as hyperactivity in mice, also occur in AD patients [54,55].

At the age of 8 months, APP/PS1 mice also showed impaired spatial reversal learning. The reversal task requires selective memory retrieval of the newly learned location of the platform, and contains therefore a extra episodic component [56]. Since episodic memory impairment is a major characteristic in early AD [57-59] our results in APP/PS1 mice resemble the problems that are present in early AD patients.

Initial spatial memory (i.e. without the extra episodic component) in contrast, is diminished in both 8 and 15-month-old mice. However, at 15 months of age, differences in cognition are more aggravated; i.e. compared to the 8-month-old mice, spatial learning is impaired in both the MWM and the 12 circular hole board, and in the probe of the MWM a significant decrease in spatial memory in 15-month-old APP/PS1 mice is present as well. Further, we noticed that transgenic mice do not show learning in the reverse MWM acquisition phase but did perform better in the first 2 acquisition trials compared to their wild type littermates. Because the MWM results show that APP/PS1 mice have a preference for both the SE and SW quadrants, and the platform was moved to the SW quadrant in the reversal task, the better performance of the APP/PS1 mice in the first 2 trials might be a coincidence. This preference probably does not interfere with our MWM results, since despite the preferences to the SE and SW quadrants, the mice still learned to locate the platform in the NE

quadrant. The preferences for the above mentioned quadrants will likely only have made the spatial learning task a bit harder.

Besides cognitive impairment, we also found a relative increase in cerebral omega-6 fatty acid content, which is thought to increase the risk for AD in man [60,61], and decreased brain cholesterol levels in the 15-month-old APP/PS1 mice compared to their wild type littermates. It could be suggested that the decrease in cholesterol levels are a consequence of increased brain export as indicated by a trend toward a increase in serum 24s oxysterol levels in 15-month-old APP/PS1 mice. Either way, these observations and literature in AD raises questions about the use of cholesterol lowering statins. There are studies showing that brain cholesterol levels are also decreased in AD patients [62,63] and reviewed in [64]), and serum cholesterol levels might thus not reflect the cholesterol status of the brain. Further lowering of brain cholesterol with lipophilic statins, which cross the blood-brain barrier, might even accelerate disease development, despite helping some aspects of AD pathogenesis (APP processing, farnosylation, etc.). The decrease in cholesterol levels may also be caused by Aβ related oxidation [65,66]. Oxidation of cholesterol plays an important role in atherogenesis and is indicated as a pathogenic event in AD [65,66]. Thus, increased cholesterol oxidation can cause increased atherosclerosis, which in turn may induce hypoperfusion in the brain, aggravating AD development. Indeed the rCBV in the 15-month-old APP/PS1 mice decreased compared to the wild type mice.

Various studies in elderly and AD patients have shown that both cerebral blood flow (CBF) and CBV are diminished [15]. It has been shown that CBF diminishes with age [67,68] and that the effects are even more pronounced in AD patients, especially in the parietal and temporal cortices [69]. In our 15-month-old mice, the rCBV did alter in these regions as well, but in addition shows a slight decrease in the hippocampus. Together, these results indicate that the APP/PS1 mouse model is a good model to study cognitive impairment but also AD related hypoperfusion of the brain.

A reduction in rCBV may be caused by a reduction in capillaries, however, previous research with 18-month-old APP/PS1 mice has shown no changes in capillary density [70]. Another study has shown a reduction in capillary segments, in the white matter of APP/PS1 mice [71] and we in contrast have found differences in CBV only in the gray matter.

A second possibility is that a reduction in rCBV in APP/PS1 mice is caused by enhanced vasoconstriction, possibly mediated by Aβ deposition in the vasculature [72,73]. Vascular Aβ has previously been suggested to have a vasoactive role through the production of free radicals [72,74]. The effect of Aβ on vasoregulation was also strengthened by the observation that Aβ interacts with endothelial cells in the rat aorta causing increased production of superoxide radicals leading to enhanced contraction [75]. Enhanced vasoconstriction may cause hypoperfusion of specific brain regions [76] leading to cerebrovascular dysfunction and subsequent neuronal loss and degeneration [76-78]. Hence, these vasoactive mechanisms can be an important factor in the pathogenesis of AD.

We have also determined the Aβ load and the amount of hippocampal atrophy in APP/PS1 mice. From 8 up to 15 months of age the amount of Aβ increased fivefold in the DG. The DG contains the highest amount of Aβ in both the 8 and 15-month-old APP/PS1 mice compared to all other brain regions. This sensitivity of the DG is similar to human AD pathology where Aβ depositions appear first in the hippocampus and especially the DG [79-81].

The DG is also the area first affected with neuronal dysfunction [81,82] It is therefore not surprising that we also find hippocampal atrophy in the brains of 15-month-old APP/PS1 transgenic mice The sensitivity of the DG for AD pathology is also fortified by the observation that between 8 and 15 months of age, the width of the outer molecular layer of the DG increases in wild type mice (i e probably due to growth), whereas APP/PS1 mice do not show this increase, which may indicate atrophy in this area or less growth

Taken together, we observe the highest amount of Aβ levels in the DG of the hippocampus in both 8 and 15-month old mice, a decreased rCBV in the cortex and hippocampus, and hippocampal atrophy in 15 month-old mice These results indicate that in the APP/PS1 mice the hippocampus is heavily affected, which support our finding that spatial learning and memory are also impaired since they are both dependent on hippocampal functioning

It is however unclear, which parameter (i e rCBV, Aβ or hippocampal atrophy) is responsible for the cognitive impairment and it is likely that all contribute to cognitive impairment together Many researchers have indeed shown that AD should not be seen as a single nosological entity [83] However, Aβ deposition must be involved, since at 8 months of age both Aβ deposition and cognitive impairment are already present at the same time, without changes in rCBV or atrophy

This conclusion indirectly reinforces the amyloid hypothesis in which accumulation of Aβ in the brain is suggested to be the primary influence driving AD pathogenesis [1] However, it has also been shown that cerebral hypoperfusion, due to for example occlusion of the carotid arteries of rats, impairs spatial learning and memory as well [84-87] All together, it could be hypothesized that aggravation of the disease only happens when changes in the brain circulation develop which are followed by cell death and hippocampal atrophy

Diet effects

Both wild type and APP/PS1 mice received a TWD diet containing 1% of cholesterol, a DHA enriched diet, or a control/ STD diet Besides these obvious components such as cholesterol and DHA, the experimental diets slightly differ from each other in the amount of SFA and n6/n3 ratio

However, a study from Oksman (Oksman et al, 2006), performed with the same mice showed that a diet with comparable high concentrations of SFA and increased n6/n3 ratio did not influence behavior, cognition or the amount of Aβ deposition compared to their standard diet which has exactly the same composition as our STD diet In addition, conversion of linolenic acid from soy oil into DHA, and the natural occurrence of cholesterol in the different oils is minimal Our DHA-enriched diet also contains a small amount of the omega-3 fatty acid Eicosapentaenoic acid (EPA)

DHA is the most important omega-3 fatty acid in brain tissue, whereas both DHA and EPA are known for their effects on the vasculature Because of the large amount of DHA compared to EPA (almost 5 1) it is most likely that the effects are due to DHA instead of EPA Thus, the largest differences between the t experimental diets lay in the cholesterol and DHA content, which suggests that these dietary ingredients are responsible for the observed differences due to the experimental diets in our mice

TWD Diet

The hypothesis that the rCBV is important in aggravating or declining symptoms of AD is strengthened by the effects of diets found in this study. During the use of a cholesterol containing diet, the rCBV is the first parameter changing, and this seems also to be the case in mice fed the DHA diet (Table 5).

15-month-old mice fed the cholesterol containing TWD diet, show increased serum cholesterol levels, and decreased rCBV. This decrease in rCBV, likely causing hypoperfusion of the brain, may be the result of by an aggravation of the vasoconstriction induced by Aβ depositions [72,73,75]. Nevertheless, it can also be caused by atherosclerosis. It has been shown that AD patients show atherosclerosis in both intracranial and extracranial vessels [88,89]. Atherosclerosis is suggested to be a primary event in the development of AD [88,89], and can therefore be the trigger for aggravation of AD symptoms. However, it is also possible that atherosclerosis and Alzheimer's disease are independent but convergent disease processes [90].

In addition, our previous research in 18-month-old mice fed similar TWD diets for a year [13] shows that rCBV decreased alongside an increased Aβ deposition in the DG. These results together with the decrease in rCBV in 15-month-old mice fed TWD as found in this study, indicate that a TWD diet first changed the cerebral circulation before altering the histologically detectable Aβ load. Theoretically it is still possible that small oligomeric Aß aggregates are involved in initiating AD pathogenesis.

However, a study from Bennett et al [91], showed that also smaller biochemical detected Aß fragments increase as a consequence of an impaired circulation, and indirectly strengthens our conclusion that vascular abnormalities precede Aß deposition.

A relation between high serum cholesterol levels and cognition, is found by Cao et al. [92] who have shown that hypercholesterolemic Tg2576 mice show more spatial learning deficits than normal transgenic Tg2576 mice after the manifestation of Aβ deposition [92]. This finding in combination with our results indicates that a TWD diet possibly aggravates AD pathology already at 15 months, but may alter cognition at a later age (Table 5).

Besides this major conclusion , we also noticed a larger effect of a TWD diet on plasma cholesterol levels in 15-month-old APP/PS1 mice compared to the age matched wild types. We did not find larger effects of a TWD diet on lathosterol and 24-OH cholesterol levels in transgenic animals which could have explained the difference found in serum cholesterol levels via an affected brain cholesterol metabolism. Also literature [64,93] does not report increased serum cholesterol levels in AD patients compared to healthy subjects. We therefore have no other explanation further than that it might be an epiphenomenon of the animal model used.

Table 5

Genotype effects:		**APP/PS1 vs Wild type**		
		8-month-old	**15-month-old**	**18-month-old ***
Open Field	Activity	↑	↑	
	Exploration	↓	↓	
Water maze	Learning	ND	↓	
	Memory	↓	↓	
Reverse water maze	New learning	↓	NS / ↓	
	Memory	↓	↓	
12 circular hole board	Learning	ND	↓	
	Memory	↓	↓	
rCBV		ND	↓	NS / ↓
Atrophy		ND	↑	↑
Diet effects		**DHA(tg) vs STD(tg)**		
		8-month-old	**15-month-old**	**18-month-old ***
Behavior	Activity	ND	ND	
	Exploration	ND	ND	
	Learning	ND	ND	
	Memory	ND	↑	
rCBV		↑(wt) ↓	↑ / NS	↑
Abeta load	Parenchymal	ND	↓	ND
	Vascular	ND	↓	↓
Atrophy		ND	ND	ND
Diet effects		**TWD(tg) vs STD(tg)**		
		8-month-old	**15-month-old**	**18-month-old ***
Behavior	Activity	ND	ND	
	Exploration	ND	ND	
	Learning	ND	ND	
	Memory	ND	ND	
rCBV		ND	↓	NS / ↓
Abeta load	Parenchymal	ND	ND	↑
	Vascular	ND	ND	ND
Atrophy		ND	ND	ND

ND= not different, NS=not significant,*= results obtained from Hooijmans CR, et al., Neurobiol Dis 2007.

DHA Diet

The effect of DHA supplementation upon the order of events in the currently used model is a bit less clear since spatial memory was improved, the amount of vascular and parenchymal Aβ decreased, and the rCBV slightly increased in APP/PS1 mice at 15 months of age. The sequence of events is therefore not clear. However, in 8-month-old mice, there are some changes in rCBV present. Although it is unclear why the rCBV reacts oppositely on DHA diet in the 2 different genotypes, it is clear that DHA may affect the rCBV at a very early age, at which diet-induced changes in Aβ load were not yet detected.

Our finding that a DHA containing diet improves spatial memory in APP/PS1 mice, is confirmed by others in both mice [28,94] and rats [95]. However, there are also some reports showing no effects of DHA on behavior or cognition [26,96]. Both research groups did not find any differences in spatial learning and memory, but this could be explained in the Oksman case by the fact that they studied the mice at a much younger age, i.e. 10 months, and it could be suggested that DHA benefits take a longer supplementation time to establish their effects, than the 4 months used in Oksman's study. In addition, studies in which a DHA-enriched diet was supplemented might have suffered from oxidation of DHA in the food aliquots, which may have accounted for a reported lack of efficacy. In order to minimize oxidation of DHA in our study, the experimental diets were stored at −20 °C in 2-day supply aliquots to make sure that all mice received fresh food without oxidated DHA.

In addition, several studies in AD patients have already shown that DHA intake may improve cognition. A study from Kotani et al showed an improvement in immediate memory and attention scores after 90 days of supplementation of DHA in subjects with mild cognitive dysfunction [22]. Furthermore a dose response relation was noted between intake of n3 fatty acids and cognitive decline, showing less cognitive decline with a higher n3 FA intake [19,23]. Also a recent study by Freund-Levi et al has shown some positive effects on cognition in patients with very mild AD [24].

The fact that the improvement in spatial memory due to DHA intake was most pronounced in the APP/PS1 animals indicates that a diseased brain profits more from a DHA containing diet than a normal healthy brain. It could be suggested that the replenishment of DHA, to DHA poor neuronal membranes such as in AD [97,98], are the cause for this improvement. Indeed there is a study which shows that DHA supplementation protects from NMDA receptor subunit loss [99]. In addition, a few other reports show that DHA, and some other n3 PUFA's, in neuronal membranes increase membrane fluidity and thereby improve neurotransmission and signaling via increased receptor binding and the affinity of receptors and function of ion channels [100-102].

It has also been suggested that DHA improves cognition by influencing the neuronal membrane fluidity thereby shifting APP processing to the non-amyloidogenic pathway [103]. Indeed, both 8 and 15-month-old mice fed a DHA diet showed a pronounced shift of the ratio n3/n6 in favor of the n3 FA. This indicates an exchange of LCPUFAs across the blood brain barrier [104], incorporation in neuronal membranes and possibly a subsequent increase in neuronal membrane fluidity. However it has also been shown in other studies that DHA is able to improve vascular conditions via lowering plasma triaglycerol [105,106], vascular reactivity [105,107], and decreasing atherosclerosis [108,109]). These positive effects on the circulation may improve age, and AD related hypoperfusion in the brain and

in this way may contribute to improvement of cognition

Indeed in our study rCBV is slightly increased in 15-month-old DHA(tg) mice, and these effects are even more pronounced in 18-month-old mice [15]. Theoretically, this increase of rCBV may be caused by vasodilatation or genesis of new capillaries Vasculogenesis may be an unlikely explanation for the finding, because anti-angiogenic effects of DHA in for example tumors have been reported [110,111]. In addition, unpublished results from our lab showed no changes in capillary density in 15 and 18-month-old mice fed the DHA diet Vasodilatation is in our view a more likely explanation, since CBF did not change, and a mismatch between CBF and CBV has been interpreted as compensatory vasodilatation [112]. In support of this notion, intake of n3 PUFAs in human subjects has been shown to affect endothelial function and improve flow-mediated dilatation of the brachial artery [107] and enhance nitric oxide mediated vasodilatation in the microcirculation of the forearm [113]

In line with the hypothesis that DHA will be beneficial to prevent AD via improving vascular conditions, is the decreased amount of vascular Aβ due to DHA. This decrease in vascular Aβ load is not a surprise, since hypoperfusion induces increased amounts of Aβ [69,91,114,115], and thus theoretically, an improved perfusion could decrease the amount of Aβ Furthermore, there is also research showing the vasoactive role of Aβ [72-75], causing vasoconstriction, and this again underscores the involvement of DHA in improving vascular conditions

In addition, another experimental study also reported lower Aβ levels as measured by ELISA and reduced amyloid pathology in the hippocampus and parietal cortex of single transgenic APPswe mice which had been fed a DHA diet from 18 until 23 months [25] The difference with our study is that in Lim's study [25] most of the DHA effects result from a comparison between a 0 6% DHA diet with a DHA depleting diet (<0 01% DHA), and our control group received a standard diet containing a small amount of alpha-linolenic acid from soy oil which may be converted to DHA Thus, in our study we investigated effects of supplementation with DHA instead of replenishment of depleted DHA as in the Lim et al. study Nevertheless, both supplementation and replenishment of depleted DHA seem to have diminishing effects on Aβ plaque load

The results of our phospholipid and fatty acid analysis of the brain homogenates show no difference in DHA content nor n6 and n3 LCPUFA content in the brains of the transgenic mice compared to wild-type mice, showing that transgenicity does not cause DHA depletion In conclusion, our results show that the APP/PS1 mouse model is a valid model to study Alzheimer's Disease, because behavior and cognition become impaired at early age and Aβ deposition increases fast, especially in the hippocampal region, during ageing. The changes in the cerebral hemodynamics and the observed hippocampal atrophy observed in this mice provides in a good model to study AD and related hypoperfusion.

In addition, a cholesterol containing Typical Western diet aggravates AD–like pathology by impairing the cerebral circulation (i e rCBV) at 15 months and subsequently increasing Aβ deposition at 18 months [13]. A DHA containing diet seems to inhibit development of AD-like pathology, improving vascular health and decreasing parenchymal Aβ load Therefore, the present data indicate that dietary lipids cause alterations in cerebral brain hemodynamics before changing the histologically detectable Aβ load in the brain, and we have

shown that these changes influence the course of the disease. These data further underline the importance of vascular factors in the APP/PS1 mouse model of AD pathology.

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Chapter 4

Amyloid beta deposition is related to decreased glucose transporter-1 levels and hippocampal atrophy in brains of aged APP/PS1 mice

Hooijmans CR [a], Graven C [a], Dederen PJ [a], Tanila H [b], van Groen T [c], Kiliaan AJ [a].

[a] Radboud University Nijmegen Medical Centre, Department of Anatomy, Geert Grooteplein noord 21 6500 HB Nijmegen, The Netherlands. [b] A.I. Virtanen Institute, University of Kuopio, and Department of Neurology, Kuopio University Hospital, Kuopio, Finland. [c] University of Alabama at Birmingham, department of Cell Biology, AL 35294-0006, Birmingham, USA.

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Abstract

The amount of the glucose transporter type-1 (GLUT-1) is decreased in the hippocampus and cerebral cortex of AD patients In this study we therefore wanted to investigate the causal relationship between amyloid-β (Aß), GLUT-1 and hippocampal atrophy in the brains of young (8-months) and old (18-months) APP/PS1 mice Methods: Aß and GLUT-1 were visualized immunohistochemically. Aß load, GLUT-1 amount, capillary density and GLUT-1 amount per capillary density were determined in cortical and hippocampal areas using computer-assisted analysis systems Hippocampal atrophy was determined by calculating the width of the outer molecular layer of the dentate gyrus (DG) Results In18-month-old APP/PS1 mice we found a reduced GLUT-1 amount in the hippocampus but no differences in capillary density. The DG of these mice contained the highest level of Aβ in combination with hippocampal atrophy, and a reduced GLUT-1 amount per capillary density. At 8-months no differences were observed. The highest Aβ deposition was found in the DG, although fourfold less compared to 18-month-old mice. Conclusions: We conclude that the GLUT-1 amount and capillary density in both wild type and transgenic mice decrease due to ageing Further, a decreased amount of GLUT-1 is caused by decreased GLUT-1 amount/capillary density and not due to a reduced capillary density. We suggest that Aβ load in the hippocampus precedes the reduction of GLUT-1 A certain level of Aß must be reached in the hippocampus, before it affects GLUT-1 amount/capillary density leading to further impairment of energy metabolism and hippocampal atrophy

Introduction

Alzheimer's disease (AD) is a multifactorial neurodegenerative disease characterized by the presence of amyloid-β (Aβ) peptide containing plaques, and neurofibrillary tangles (NFT). Aβ accumulates in the brain either as neuritic plaques or as vascular deposits that cause cerebral amyloid angiopathy (CAA). Aβ is derived through the proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ secretases (see ref [1] for review). Aβ has been suggested to be a major contributor to the neurodegenerative processes in AD in which the entorhinal cortex, hippocampus and neocortex of AD patients are predominantly affected [2-5].

However, accumulated evidence suggests that vascular risk factors play an important role in AD. For example, AD and vascular dementia share the same risk factors [6,7], and pharmacotherapy improving cerebrovascular insufficiency decreases AD symptoms [8]. There is also evidence showing that cerebral perfusion is decreased in AD patients [9]. Cerebral blood flow diminishes with age [10,11]; however, in AD these perfusion/haemodynamic effects are even more pronounced and especially the parietal and temporal cortices are consistently shown to be affected [9].

Besides haemodynamic changes, various microvascular pathologies have been observed in AD patients, such as basement membrane thickening, pericyte degeneration, endothelial cell shape changes and luminal buckling (see ref [12] for review). Furthermore, a decrease in vascular density is also frequently observed [13]. Capillary degeneration in ageing rats can be induced by mild chronic cerebral hypoperfusion [14], and this in turn may trigger cognitive and degenerative changes [15,16]. These findings support the CATCH hypothesis of de la Torre [17], positing that advanced age, in combination with a vascular risk factor converges to create a critical attained threshold of cerebral hypoperfusion (CATCH). This further triggers brain microcirculatory disturbances and hypoperfusion. These changes may generate a chain of events leading to the progressive evolution of brain metabolic, cognitive and tissue pathology that characterize Alzheimer's disease [17].

The CATCH hypothesis is in line with findings of reduced glucose metabolism in AD, which is most pronounced in the temporal and parietal cortex [18,19]. Glucose transporter type 1 (GLUT-1) is selectively expressed at high levels in the capillary endothelium of the brain and is responsible for the transfer of glucose across the blood brain barrier [20]. Immunolabeling and binding experiments using GLUT-1 as a marker for vascular endothelial cells have revealed decreased amount of GLUT-1 transporter in the hippocampus and cerebral cortex of AD patients [21-23]. This finding indicates diminished glucose transport, reflecting decreased glucose metabolism and probably a hypometabolic state.

Hypoperfusion may cause endothelial degeneration in cerebral small vessels leading to Aβ deposition [9,24] and ultimately to loss of GLUT-1 transporters. Several studies have shown that cerebral ischemia results in progressive accumulation of Aβ [25,26]. In addition, a recent study from Kouznetsova et al. reported a diminished capillary density in areas with high Aβ depositions [27]. In that study, capillary density was determined by using the percentage of brain area covered by GLUT-1 immunoreactivity as a measure. However, in our view, a decreased percentage of the area covered by GLUT-1 immunoreactivity could

also mean that GLUT-1 transporter amount/capillary density is decreased in the vicinity of Aβ deposits rather than capillary density per se. This view has been supported by some other studies as well [28,29].

The present study set out to determine Aβ deposition, capillary density, the amount of GLUT-1 in total and in relation to capillary surface area (GLUT-1 amount/capillary density) in different brain regions in young (8-month-old) and old (18-month-old) double transgenic AD mice expressing the human mutated APPswe and PS1dE9 genes [30]. In addition the degree of tissue atrophy in the hippocampus was determined.
We hypothesize that an increased amount of Aβ reduces the GLUT-1 amount/capillary density in the hippocampus of AD mice, and as a consequence contributes to hippocampal atrophy via decreased glucose availability.

Materials and Methods

Animals

The APPswe/PS1dE9 breeder mice were obtained from Johns Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. Pathology), and a colony was established at the University of Kuopio Finland (experiment 1). Mice from the colony of the University of Kuopio were used to set-up our own colony at the Radboud University Nijmegen Medical Centre. For experiment 2 mice were used from the latter colony. In short, mice were created by co-injection of chimeric mouse/human APPswe (mouse APP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transvected genes co-integrated and co-segregate as a single locus [30]. This line was originally maintained in a hybrid background by backcrossing to C3HeJ x C57BL6/J F1 mice (so-called pseudo F2 stage). For the present work, the breeder mice were backcrossed to C57BL6/J for 5-6 generations to obtain mice for the current study. Age matched non transgenic littermates served as controls. Throughout the experiments the animals (experiment 1: wild type n=10 and APP/PS1 n=8, experiment 2: wild type n=7 and APP/PS1 n=7) were housed individually in a controlled environment, food and water were available ad libitum. Experiments were performed according to Dutch and Finnish federal regulations for animal protection and were approved by the Veterinary Authority Radboud University Nijmegen. At 16 months the mice used in experiment 1 were transported to Radboud University Nijmegen, the Netherlands. At 8 respectively 18 months of age the experiments were performed.

Immunohistochemical procedures

All mice were weighed directly before starting the immunohistochemical procedures at 8 respectively 18 months of age. Subsequently, mice were anesthetized with Nembutal (60mg/kg i.p.) (Ceva Santa Animals BV, Maassluis, the Netherlands) and transcardially perfused with a 0.1 M phosphate buffered saline (PBS) followed by Somogyi's fixative (4% paraformaldehyde, 0.05% glutaraldehyde and 0.2 % picric acid in 0.1 M phosphate buffer,

PB). Following transcardial perfusion fixation mice were decapitated and brains were dissected from the skull. The entire brain without spinal cord was weighed and thereafter post fixated for 15 hours at 4°C in Somogi's fixative. The brain tissue was then cryoprotected by immersion in 30% sucrose in PB at 4°C. Six series of 40 µm coronal sections were cut through the brain using a sliding microtome (Microm HM 440, Walldorf, Germany). Immunohistochemistry was performed using standard free-floating labeling procedures on series 1 and 2. All sections were stained in one session to minimize differences in staining intensity.

Amyloid-β

The Aβ load was visualized using WO-2 antibody (mouse anti-human Aβ4-10, kind gift of K. Beyreuther, Centre for Molecular Biology, University of Heidelberg, Germany). Briefly, the sections were pretreated with sodium citrate solution at 85°C for 30 minutes. Monoclonal mouse anti-human Aβ4-10 (1:20.000) was used as primary antibody. The sections were incubated overnight at room temperature on a shaker table. Following incubation the sections were rinsed thoroughly with PBS and transferred to the solution containing the secondary antibody; donkey-anti-mouse biotin 1:1500 (Jackson Immuno research). After 90 minutes of incubation the section were rinsed three times and transferred to a solution containing Vector ABC-elite 1:800 (Vector laboratories, Burlingame, CA, USA) for again 90 minutes. Visualization of Aβ plaques was achieved by incubation with DAB-Ni solution. All stained sections were mounted on gelatin-coated slides and dehydrated in alcohol series, cleared with xylol and mounted in Entellan.

GLUT-1

The GLUT-1 amount was visualized using GLUT-1 antibody (rabbit anti GLUT-1 transporter, Chemicon AB 1340, Chemicon International, Inc., Temecula, CA, USA). In short, the sections were pretreated for 30 minutes with 0.3% H2O2 in 0.1 M PBS. Thereafter, the sections were rinsed in PBS and pre-incubated in 0.1M PBS containing 1% bovine serum albumin (BSA) and 0.3% triton-X-100 (PBS-BT) followed by a 18h incubation with rabbit anti-GLUT-1 AB 1340 (1:10,000, Chemicon International, Inc., Temecula, CA). Following incubation the sections were rinsed thoroughly with PBS and transferred to the solution containing the secondary antibody; donkey anti rabbit biotin 1:1500 (Jackson Immuno research). After 90 minutes of incubation the sections were rinsed three times and transferred to a solution containing Vector ABC-elite 1:800 (Vector laboratories, Burlingame, CA, USA) for another 90 minutes. Visualization of the GLUT-1 amount was achieved by incubation with DAB-Ni solution. All stained sections were mounted on gelatin-coated slides and dehydrated in alcohol series, cleared with xylol and mounted in Entellan.

Quantification

To determine the amount of Aβ, GLUT-1 and capillary density in the hippocampus, CA1, CA3 and dentate gyrus (DG) (exp 1 and 2), in the frontal cortex (prelimbic area (PLA)) and anterior cingulate gyrus (ACg 1 and 2) (exp 1), the appropriate slices were digitized using a Zeiss Axioskop microscope, equipped with hardware and software of Microbrightfield, (Williston, VT, USA). Brain regions were based on the mouse brain atlas of Franklin and

Paxinos 1997 [55] and quantified in three sections (with 200 μm distance between the sections) for each region. ACg1 and 2 were quantified at level +1.10 up to +0.86 anterior to bregma, PLA was quantified at +1.98 upto +1.78 anterior to bregma. The hippocampus was quantified at –2.18 up to –2.46 posterior to bregma. The cortical regions were chosen because of their large amyloid load in humans and transgenic mice and their importance in memory [56,57].

Amyloid-β

Quantitative analyses were performed with a computer-assisted analysis system (Stereo Investigator, Microbrightfield (Williston, USA).) using Cavalieri's probe. A contour was drawn along the borders of the hippocampal subregions (CA1, CA3 and DG). In the ACg and PLA a contour was placed, largely within the borders of the particular cortical areas. Aβ load was defined as the percentage of area covered by Aβ. All measurements were performed double blind by two investigators, in three sections with 200-μm distance in between.

GLUT-1

Capillary density was determined with a computer-assisted analysis system (Stereo Investigator) using Cavalieri's probe and was defined as percentage of the area covered by GLUT-1 immunoreactivity as compared to the total area of the region measured. The capillary density was determined within a square contour, touching the anatomical borders of respectively the CA1, CA3, DG, ACg and PLA.

The total amount of GLUT-1 was estimated exactly in the same area as the capillary density, with stereo investigator collecting luminance information. The amount of GLUT-1 was measured as the luminance intensity of an entire region and was corrected for the background luminance in areas with no GLUT-1 expression. To avoid changes in exposure, which might affects measurements, intensity measurements of all sections were performed in one session.

The ratio between the GLUT-1 amount and the capillary density was measured by dividing the total GLUT-1 amount by capillary density.

Hippocampal atrophy

As a measure for hippocampal atrophy, the thickness of the outer molecular layer of the dentate gyrus (DG) was measured in two sections, with 200 μm distance in between (Fig2) using a Zeiss Axioskop microscope, equipped with hardware and software of Microbrightfield (Williston, USA). The width of the outer molecular layer of the DG was measured since this layer is subject of age-related loss of LTP and synapses in both animals models and humans [45,46], partly originating from primary perforant path afferents from the entorhinal cortex which are affected in AD.

Statistical analysis

Data are expressed as mean ± SEM and were analyzed with an independent t-test, except for regional differences in amyloid-β load, which was quantified by using the one-way ANOVA. If the overall analysis revealed a significant difference, the separate groups were analyzed post hoc by using Tukey HSD. Correlation analyses were done with the bivariate Pearson's correlation method. Statistical significance was established at $p \leq 0.05$.

Results

Experiment 1: 18-month-old mice

Body and brain weight

Mice were weighed at two different time points during the follow-up. The body weight did not differ between the groups at 6 months of age (p=0.42, wild type 32.1 ±1.0, APP/PS1 31.9 ±1.0) nor at 18 months of age (p=0.36, wild type 40.7 ± 1.7, APP/PS1 43.5 ±2.5). Brain weight was determined only at the end of the experiment (18 months of age). Brain weight did not differ (p=0.39) between the genotypes (wild type 0.46 ±0.01, APP/PS1 0.47 ±0.01). Thus, body and brain weight were not affected by genotype.

The dentate gyrus of the hippocampus demonstrates the highest amount of amyloid-β

The brains of all mice were immunohistochemically stained with WO-2 antibody (mouse anti-human $^{A\beta 4\text{-}10}$). Wild type mice showed no immunoreactivity with this antibody, while Aβ deposits in transgenic mice were intensively stained.

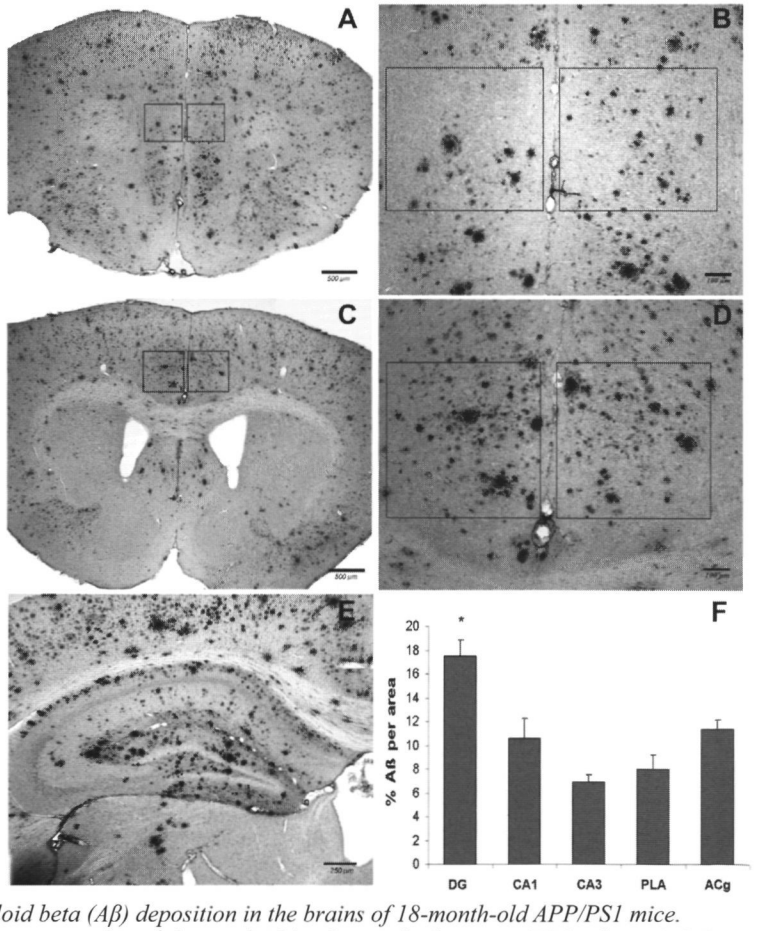

*Fig 1. Amyloid beta (Aβ) deposition in the brains of 18-month-old APP/PS1 mice. Representative examples of the amyloid load in prelimbic area (PLA, photo A, 2.5x –and B 10x), anterior cingulated gyrus (ACg, photo C 2.5x –and D 10x) and hippocampus (photo E 5x) stained with WO-2 antibody (mouse anti human Aβ 4-10). F: dentate gyrus (DG) exposes the highest amount of Aβ compared to all other areas. Values represent mean ± SEM. * p<0.05.*

There was a significant regional variation in Aβ plaque burden, such that cortical and hippocampal areas showed many plaques (Fig 1) while the cerebellum, thalamic nuclei, hypothalamic nuclei and other diencephalon structures were relatively free of plaques. We measured % of section area covered with Aβ depositions in the hippocampus (DG, CA1 and CA3 regions), frontal cortex (prelimbic area (PL) and anterior cingulate gyrus (ACg1 and 2) and showed that the dentate gyrus expressed the highest amount of Aβ compared to all other areas (ANOVA $p<0.001$) (Fig 1f). Aβ depositions covered 17.5% of the DG, which is significantly more than the 10.6% load in the CA1 ($p=0.006$), 6.91% in the CA3 ($p<0.001$), 7.99% in the PLA ($p<0.001$) and 11.34% in the ACg ($p=0.014$). These results indicate that in 18-month-old mice the dentate gyrus is most vulnerable to Aβ depositions.

Total amount of GLUT-1 is decreased in the hippocampus of APP/PS1 mice

GLUT-1 is an active glucose transporter and is primarily localized in brain vascular endothelial cells. Quantification of the immunohistochemical staining (Fig 2) revealed that in APP/PS1 mice, and especially in the DG and CA3 regions, the GLUT-1 amount was significantly decreased in comparison to wild type mice (DG: $p=0.023$, CA3: $p=0.006$). Additionally, a trend towards decreased GLUT-1 amount was observed in the CA1 region ($p=0.064$). In contrast, no difference in the amount of GLUT-1 transporter was found in the cortical areas PLA ($p=0.55$) and ACg ($p=0.16$).

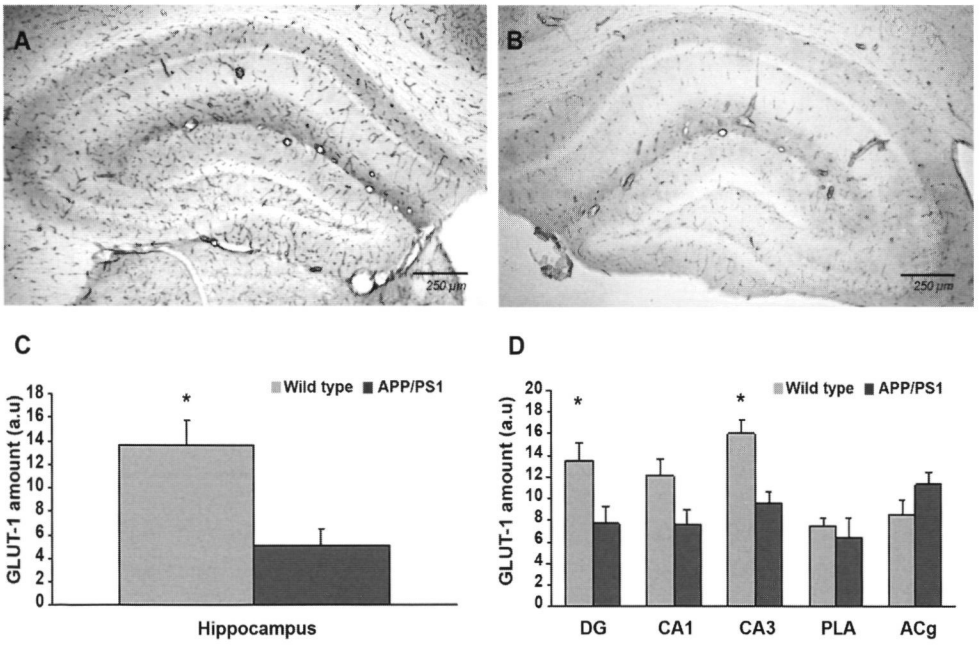

*Fig 2. Total amount of GLUT-1 is decreased in the hippocampus of 18-month-old APP/PS1 mice. Characteristic photomicrographs of labeled glucose transporter type 1 (GLUT-1) in the hippocampus of wild type (photo A) or APP/PS1 (photo B) mice showing a decreased GLUT-1 amount in the entire hippocampus (C) and especially in the DG and CA3 region (D). DG dentate gyrus, PLA prelimbic area, ACg anterior cingulated gyrus. Values represent mean ± SEM. * $p<0.05$. a.u. = arbitrary units.*

Capillary density is not changed in APP/PS1 mice

In order to reveal the underlying cause for the decrease in total GLUT-1 amount, the distribution of brain capillaries in the mouse brain (capillary density) was measured in hippocampal (DG, CA1 and CA3) and cortical (ACg and PLA) areas. Although the capillary density appeared slightly lower in CA1 and CA3 areas in APP/PS1 mice, the statistical analysis a showed no significant differences in capillary density between transgenic and wild type mice in any of the observed regions ($p>0.14$ Fig 3a). Nonetheless, a 4-fold higher capillary density was observed in cortical areas (approximately 20%) compared to hippocampal areas (approximately 5-6%) in both genotypes.

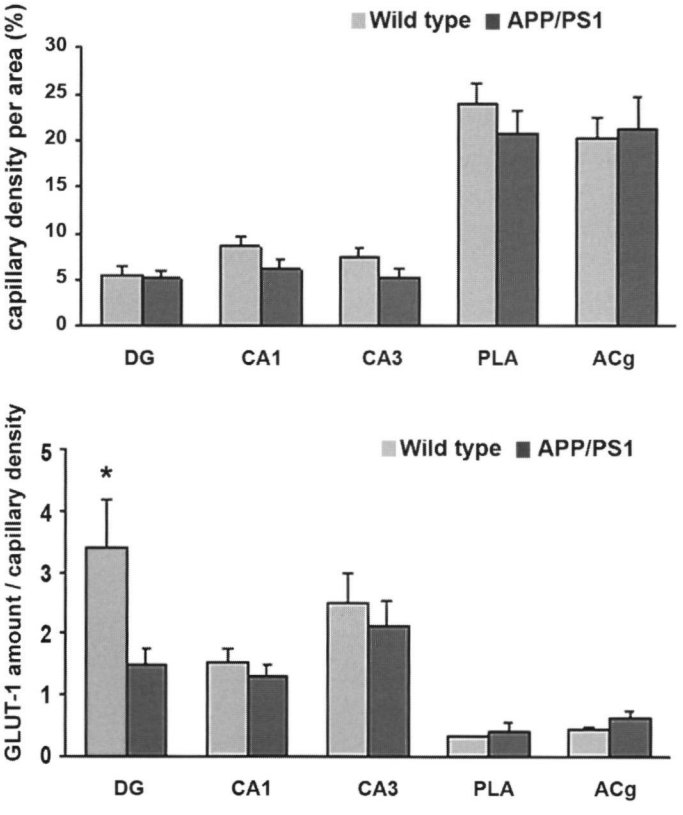

Fig 3. Decreased GLUT-1 amount/capillary density, and no changes in capillary density in the DG of 18-month-old APP/PS1 mice.

*A: Capillary density in hippocampal (DG, CA1, CA3) and cortical (PLA, ACg) areas in wild type and APP/PS1 transgenic mice. B: GLUT-1 expression, calculated as the ratio between GLUT-1 amount and capillary density, in hippocampal and cortical areas. DG dentate gyrus, PLA prelimbic area, ACg anterior cingulated gyrus. Values represent mean ± SEM. * $p<0.05$.*

GLUT-1 amount/capillary density is decreased in the dentate gyrus of APP/PS1 mice

To further assess the decrease in the GLUT-1 amount, we also measured the amount of GLUT-1 per capillary density by dividing the total GLUT-1 immunoreactivity by the capillary density. This parameter was 56 % lower in the DG of APP/PS1 mice compared to

wild type controls (p=0.042, Fig 3b). The GLUT-1 amount/capillary density did not differ in other areas (p>0.18), indicating a major vulnerability of the DG 18-month-old APP/PS1 mice.

Atrophy in the dentate gyrus of aged APP/PS1 mice

A reduced width of the outer molecular layer in the dentate gyrus was used as a measure for hippocampus atrophy. At the age of 18 months, APP/PS1 transgenic mice had significantly thinner molecular layer than the wild type mice (Fig 4 p=0.015). The reduction in the width (wild type: 189 ± 4 μm, and APP/PS1: 175 ± 2 μm) was 8 %.

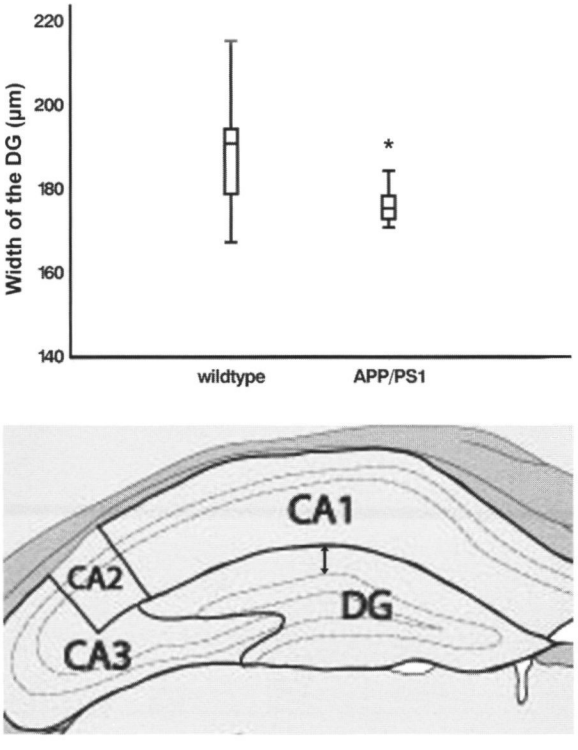

Fig 4. Decreased width of the dentate gyrus in 18-month-old APP/PS1 mice.
*A: Width of the outer molecular layer of the DG, as a measure of hippocampal atrophy, in wild type and APP/PS1 transgenic mice. B: Figure showing the location of the DG and the outer molecular layer within the DG of the hippocampus. DG dentate gyrus. Values represent mean ± SEM. * p<0.05.*

Correlation between GLUT-1 amount/capillary density and hippocampal atrophy

A bivariate Pearson correlation between hippocampal atrophy and GLUT-1 amount/capillary density (Fig 5) showed a moderate to good relation between GLUT-1 amount/capillary density and hippocampal atrophy (r =0.65 and p=0.013).

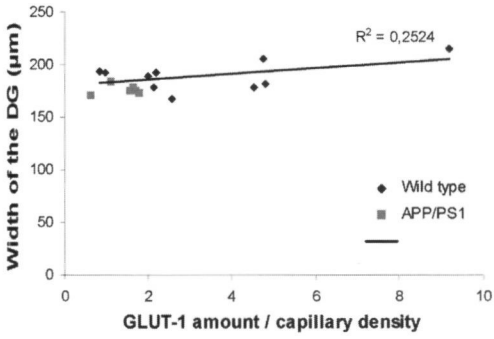

Fig 5. Correlation between GLUT-1 amount per capillary and hippocampal atrophy in 18-month-old mice The line depicts the linear regression (r=0.61, p=0.013).

Experiment 2: 8-month-old mice

In order to examine whether Aβ precedes the observed differences in GLUT-1 amount/capillary density and hippocampal atrophy in 18-month-old mice, we performed an additional experiment in which we measured Aβ deposits, GLUT-1 amount/capillary density and hippocampal atrophy in the hippocampus of 8-month-old APP/PS1 and wild type mice.

Body and brain weight

Body and brain weighed were determined at the end of the experiments. The body weight and brain weight did not differ between the groups (p=0.12, wild type 30.54 ±0.75, APP/PS1 32.46 ±1.20 respectively p=0.16 wild type 0.49 ± 0.02, APP/PS1 0.52 ±0.01)
Thus, body and brain weight were not affected by genotype in 8-month-old mice.

The dentate gyrus of the hippocampus demonstrates the highest amount of amyloid-β

There was a significant regional variation in Aβ plaque burden, such that cortical and hippocampal areas showed many plaques (Fig 1) while the cerebellum, thalamic nuclei, hypothalamic nuclei and other diencephalon structures were relatively free of plaques. The dentate gyrus expressed the highest amount of Aβ compared to the other hippocampal areas (ANOVA $p<0.001$) (Fig 6a). Aβ depositions covered 4.1% of the DG, which is less than one fourth of the amount observed in 18-month-old mice, which was 17.5%. However, the amount of Aβ depositions in the DG was significantly higher compared to the Aβ load in the CA1 (0.77%, $p<0.001$) and the CA3 (0.66%, $p<0.001$). These results indicate that also in 8-month-old mice the dentate gyrus is most vulnerable to Aβ depositions.

Total amount of GLUT-1, capillary density and GLUT-1 amount/capillary density are not changed in 8-month-old mice.

Analysis of the GLUT-1 staining in the hippocampus revealed no differences in any of the regions in the amount of GLUT-1 (Fig 6b; DG p=0.37, CA1 p=0.68 and CA3 p=0.26), capillary density (Fig 6c; DG p=0.96, CA1 p=0.69 and CA3 p=0.95) or GLUT-1 amount/capillary density (Fig 6d; DG p=0.30, CA1 p=0.95 and CA3 p=0.09).

No atrophy in the dentate gyrus of young APP/PS1 mice

At the age of 8 months, APP/PS1 transgenic mice did not have a significantly thinner molecular layer compared to the wild type mice (Fig 6e; p=0.50).

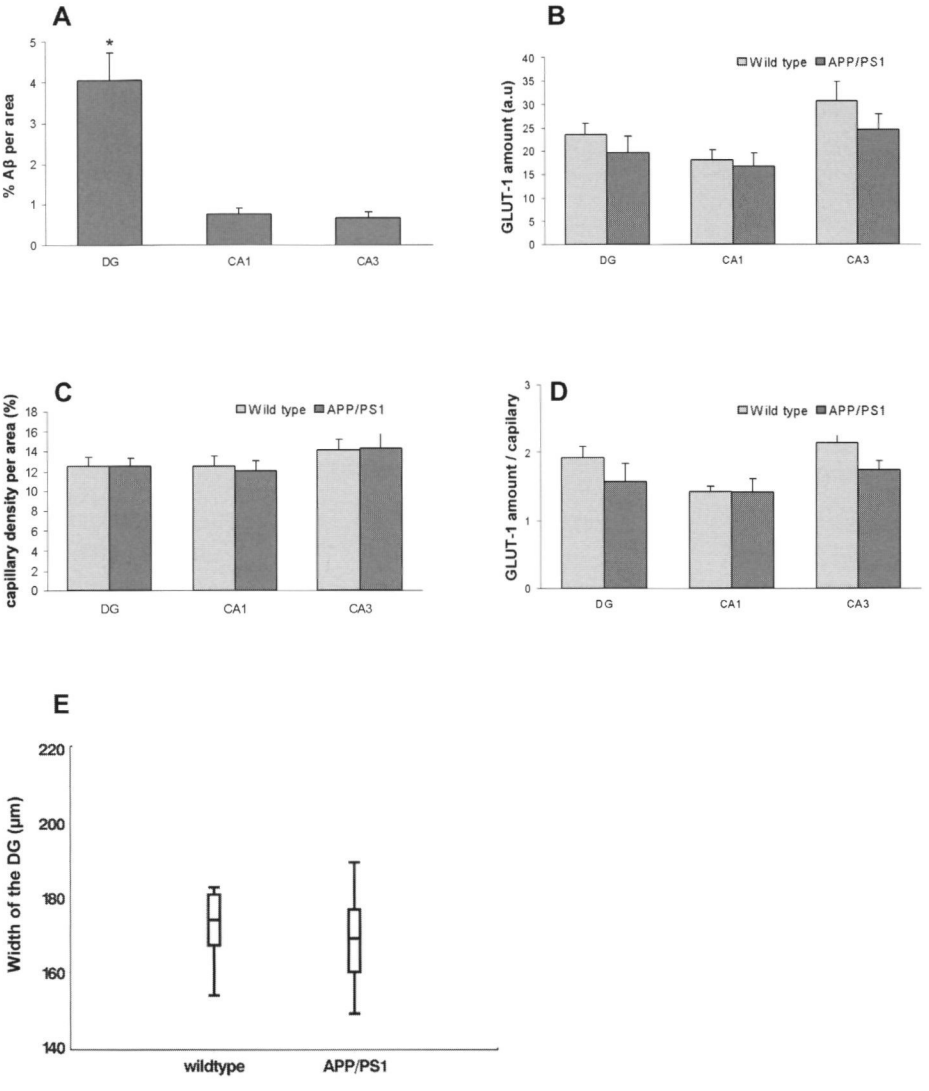

Fig 6. 8-month-old mice
*A: The dentate gyrus (DG) exposes the highest amount of Aβ compared to other hippocampal areas. Values represent mean ± SEM. * $p<0.05$.B: Total amount of GLUT-1 in hippocampal (DG, CA1 and CA3) areas. C: Capillary density in hippocampal (DG, CA1, CA3) areas. D: GLUT-1 expression, calculated as the ratio between GLUT-1 amount and capillary density, in hippocampal (DG, CA1 and CA3) areas. E: Width of the outer molecular layer of the DG, as a measure of hippocampal atrophy, in wild type and APP/PS1 transgenic mice.*

Effects of ageing

Ageing causes a decrease in GLUT-1 and capillary density

In order to investigate the effects of ageing, differences between 8 and 18-month-old mice were determined for both the wild type and transgenic group. As mentioned earlier, the Aβ load significantly increased during ageing in APP/PS1 mice in all hippocampal areas

(Table 1; $p<0.01$). The width of the outer molecular layer in the dentate gyrus was increased in both groups, however, this increase was only significant in the wild type mice ($p=0.02$), indicating a diminished growth of the hippocampus in transgenic mice probably due to neurodegeneration. Both wild type and transgenic mice showed a significant decrease in GLUT-1 amount and capillary density in the DG, CA1 and CA3 region during ageing (Table 1). No differences were observed in the ratio GLUT-1 amount/capillary density due to ageing.

Discussion

In this study we investigated the relation between Aß deposition, amount of GLUT-1 transporter and capillaries, hippocampal atrophy and the effect of ageing on these parameters in 8 and 18-month-old double transgenic APPswe PS1dE9 (APP/PS1) mice GLUT-1 is a marker for vascular endothelial cells in the brain and has been shown to be reduced in AD patients [21-23]. Our results show a reduced amount of the GLUT-1 immunoreactivity in the hippocampus of 18-month-old APP/PS1 but no decrease in the capillary density compared to age matched wild type mice. Therefore, these findings point towards a decreased GLUT-1 amount/capillary density in this transgenic AD mouse model. Furthermore, amyloid deposition (Aβ), atrophy and a reduced GLUT-1 amount/capillary density are all concentrated in the dentate gyrus (DG) of 18-month-old mice. 8-month-old mice also show an increased amount of amyloid deposition in the DG, although much less compared to 18-month-old mice but do not show any differences in GLUT-1 immunoreactivity nor hippocampal atrophy. Therefore, we suggest that Aβ deposition in the hippocampus precedes the reduction of GLUT-1 but the amount of Aβ must be high enough to affect GLUT-1 immunoreactivity. Further, ageing causes a decrease in GLUT-1 amount and in capillary density in both wild type and APP/PS1 mice. However, the decrease in GLUT-1 amount is more pronounced in transgenic mice.

Nowadays AD is generally considered to have mixed underlying pathology, in which vascular risk factors combined with amyloid accumulation and age-related degenerative changes cause cognitive impairment. Several studies in recent years have shown haemodynamic and microvascular pathologies in AD [9,10,12], combined with impaired glucose utilization. A consistent finding in positron emission tomography (PET) studies has been regional decreases in glucose utilization, which are most pronounced in the parietal and temporal cortex [21,23,31]. Our findings of a decreased GLUT-1 amount in the hippocampus of 18-month-old APP/PS1 mice compared to wild type mice is in accord with these PET studies, as well as with studies reporting reductions of hippocampal GLUT-1 in AD patients [21,22,27]. Several other studies had also reported reductions in hippocampal GLUT-1 in AD [21,22,27], however it is also reported that during normal ageing glucose utilization diminishes [32,33]. This finding is strengthened by our findings in which both wild type and APP/PS1 mice showed a significant decrease in GLUT-1 amount in time. The decrease in GLUT-1 amount is larger in transgenic mice, causing a significant difference between wild types and transgenic mice at 18 months of age. Nevertheless, investigators disagree about the cause of this decline. A decreased GLUT-1 amount may be caused by a

reduced capillary density or may result from decreased GLUT-1 amount/capillary density. In our study the capillary density remained unchanged, so the results point to a decrease in GLUT-1 amount/capillary.

Since the GLUT-1 antibody was used to measure capillary density and the amount of GLUT-1, it could theoretically have been the case that because of a decreased amount of GLUT-1 a decreased number of vessels could be observed. However, since we found a decreased amount of GLUT-1 in the hippocampus but no changes in these areas occupied with blood vessels (capillary density), these data confirm the reliability of the use of the GLUT-1 antibody to determine capillary density.

Although capillary density did not differ between wild type and APP/PS1 mice at 8 and 18 month of age, ageing did cause a decrease in the density of the capillaries in both wild type and transgenic mice. It could therefore be suggested that ageing on its own triggers the development of a hypoperfused state causing an energy crisis, which could be devastating in transgenic mice since they also showed a larger decrease in GLUT-1 amount, ultimately causing cognitive impairment and other AD pathology.

In 18-month-old mice we found the highest Aβ plaque load in the DG of the hippocampus compared to all measured brain regions. This was also the only brain region with a decreased GLUT-1 amount per capillary density, suggesting that Aβ plaque load and GLUT-1 amount/capillary density may be causally related. In our 8-month-old-transgenic mice we also observed the highest amount of amyloid deposition in the DG, however a fourfold less compared to 18-month-old mice, but we did not find any differences in GLUT-1 immunoreactivity. This indicates that the amount of Aβ in the DG of 8-month-old mice was not sufficient to cause damage to GLUT-1 (i.e. Aβ load needs to reach a threshold before causing damage to endothelial cells and GLUT-1 expression). In the CA3 region another process may be involved, since the amount of GLUT-1 in the CA3 of 18-month-old mice is decreased, while the capillary density and GLUT-1 amount/capillary density are not significantly altered, the, although very weak, trend towards a decreased capillary density in the CA3 region may explain the cause of the decreased GLUT-1 amount. Since parenchymal Aβ could not be the cause of the decreased GLUT-1 amount in the CA3 of these 18-month-old mice, it will be interesting to measure the amount of soluble Aβ and examine whether there is a causal role for soluble Aβ in reducing the GLUT-1 amount in this area.

The notion that Aβ has harmful effects on the cerebral vasculature is also supported by previous studies showing a relation between Aβ plaque load and GLUT-1in a Tg2576 AD mouse [27,34]. This is also consistent with other studies describing the toxicity of Aβ for vascular endothelial cells and GLUT-1 transporters [35-38]. The exact mechanism whereby Aβ reduced GLUT-1 amounts is unknown but it likely involve inhibition of its synthesis or possibly even increase of its degradation.

A decreased amount of GLUT-1 may also affect cognitive deficits associated with AD. For instance, the levels of acetyl-CoA, a product of the glycolytic pathway required for the synthesis of acetylcholine, a neurotransmitter involved in learning and memory will be decreased when glucose supply is diminished. Further, impaired cholinergic neurotransmission can contribute to Aβ deposition since it increases amyloid precursor protein expression and reduces its non-amyloidogenic cleavage by the α-secretase pathway [39-41].

Besides the huge amount of Aβ and the decrease in GLUT-1 in the capillaries in the DG in

these 18-month-old APP/PS1 mice, we also observed atrophy in this brain area, indicating a specific sensitivity of the DG for AD pathology. This observation is also fortified by the finding that between 8 and 18 months of age the width of the outer molecular layer of the DG increases in wild type mice, probably due to growing, whereas transgenic mice do not show a significant increase, indicating atrophy in this area. This sensitivity of the DG is in accordance with human AD pathology in which Aβ depositions appear first in the hippocampus and especially in the DG [42-44]. The DG is also the area first affected in AD with neuronal dysfunction [44,45], even before the accumulation of Aβ plaques [45]. The width of the outer molecular layer of the DG was measured since this layer is subject of age-related loss of synapses [46,47] originating from perforant path afferents from the entorhinal cortex which is affected in AD [45,46,48] Furthermore, loss of synapses in perforant path afferents correlated with memory impairment in aged mice and rats [49,50] and humans [46,48]. In addition, a spatial relation between Aβ depositions and synapse loss has been reported in the hippocampus and entorhinal cortex of APP mutant Tg 2676 mice [49], suggesting a causal role for Aβ in AD associated neurodegeneration.

Although the present findings point to close relationship between Aβ depositions, decreased GLUT-1 amount per capillary density, and tissue atrophy in the hippocampal DG subregion in transgenic APP/PS1 mice, the chronological order of these findings is not clear. We think it is unlikely that a decrease in GLUT-1 amount/capillary density is the primary event in AD and that a reduced availability of glucose to the brain initiates Aβ deposition or neuronal damage and brain atrophy, since we did not find differences in GLUT-1 amount/capillary density in our 8-month-old APP/PS1 mice, while Aβ deposition in the DG was already present. It could therefore be hypothesized that Aβ deposition leads to a decreased GLUT-1 amount/capillary density causing a hypometabolic state, which is followed by atrophy of the DG. However it is also possible that Aβ deposition may lead to neuronal damage and atrophy, followed by a decreased demand for glucose, and a decreased GLUT-1 amount /capillary density as a consequence. These hypotheses are also strengthened by the measured significant correlation between hippocampal atrophy and GLUT-1 amount/capillary density.
However the first hypothesis is most likely since a reduced cerebral glucose uptake by individuals genetically at risk for AD supports the notion that glucose deprivation precedes neuronal degeneration. [51,52]. Furthermore, other studies have shown that reduction in the cerebral glucose metabolism represent a true loss in functional activity, and is not caused by brain atrophy [53], indicating that a decreased GLUT-1 amount/capillary density may precede neuronal atrophy and is not caused by atrophy. Further support to this notion comes from a study demonstrating that Aβ peptide impaired glucose transport in neurons, which may lead to neurodegeneration [54].

In conclusion, the present findings indicate that ageing causes a decrease in GLUT-1 amount and capillary density in the hippocampus of both wild type and transgenic mice and the decrease in GLUT-1 amount is more pronounced in APP/PS1 mice. Further a reduced total amount of GLUT-1 in the hippocampus of 18-month-old APP/PS1 mice results from a decrease in GLUT-1 amount/capillary density and not from a reduced capillary density.

We suggest that Aβ load in the hippocampus precedes the reduction of GLUT-1 and when the Aß load in the hippocampus reaches a sufficient amount, this may lead to a decreased GLUT-1 amount/capillary density which further impairs energy metabolism followed by hippocampal atrophy.

Acknowledgements

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Chapter 5

S-adenosylmethionine and s-adenosylhomocysteine levels in the aging brain of APP/PS1 Alzheimer mice

Hooijmans CR [a], Blom HJ [c], Oppenraaij-Emmerzaal D [c], Ritskes-Hoitinga M [d], Kiliaan AJ [a,b]

[a] Radboud University Nijmegen Medical Centre, Department of Anatomy and [b] Department of Cognitive Neuroscience, Donders Centre for Neuroscience, 6500 HB, Nijmegen, The Netherlands. [c] Radboud University Nijmegen Medical Centre, Laboratory of Pediatrics and Neurology 6500 HB, Nijmegen, The Netherlands. [d] Radboud University Nijmegen Medical Centre, Central Animal Laboratory, 6500 HB, Nijmegen, The Netherlands.

Submitted

Abstract

Hyperhomocysteinemia and factors of homocysteine metabolism, s-adenosylhomocysteine (AdoHcy) and s-adenosylmethionine (AdoMet), may play a role in Alzheimer disease (AD). With liquid-chromatography tandem-mass-spectrometry AdoMet and AdoHcy were determined in brains of 8 and 15-month-old APP/PS1 Alzheimer mice, and the possible role of these methylation metabolites in AD brains investigated. AdoMet brain levels did not differ at 8-months, but were significantly lower in 15-month-old wildtype mice compared to APP/PS1 mice. AdoHcy levels did not differ between genotypes. These findings indicate that alterations in AdoMet are a consequence of AD rather than a cause. During aging, AdoMet levels were only decreased in wildtype mice whereas AdoHcy levels diminished in both genotypes. In conclusion AdoMet levels in APP/PS1 mice are not decreased during aging, probably by less demand due to neurodegeneration. No effect of cholesterol or DHA enriched diets on AdoMet or AdoHcy levels were found.

Introduction

The most pronounced pathological features in the human Alzheimer (AD) brain are amyloid-β (Aβ) depositions, intracellular tangles and neurodegeneration. Although the cause of the disease is largely unknown, the Aβ protein is seen as one of the major contributors [1].

Aβ is produced by cleavage of the amyloid precursor protein (APP) by the β and γ secretases, BACE and Presenilin (PS). Missense mutations in either APP or the γ-secretase complex (PS1 and PS2) , cause overproduction of Aβ, and early onset AD. Early onset AD represents only 5% of all AD cases and therefore explains only a small part of the cause of the disease. In addition, many studies show that Aβ depositions do not correlate well with neuronal damage and cognitive decline. Therefore, it is suggested that beside Aβ, other risk factors play an important role in the development of AD. Nowadays more and more consensus is reached about vascular disorders being major risk factors for AD. Hyperhomocysteinemia for example, was until recently believed to be a risk factor for cardiovascular disorders [2] and associated with an increased risk of AD [3,4]. It could therefore be suggested that hyperhomocysteinemia, cardiovascular disorders and AD are interrelated with each other. However, this is currently under debate because of recent homocysteine-lowering intervention studies, showing no relation between cardiovascular disease and hyperhomocysteinemia [5,6]. In addition it is unclear whether lowering of homocysteine (Hcy) by folic acid intake, can diminish AD prevalence or improve cognition [7,8], because recent large randomized Hcy lowering intervention trials did not show beneficial effect of lowering Hcy with folate and vitamin B12 on cognitive function [7]. It could therefore be suggested that hyperhomocysteinemia itself is not causing AD, and that other factors of the Hcy metabolism, such as s-adenosylhomocysteine (AdoHcy) and s-adenosylmethionine (AdoMet), probably play a more important role in vascular disease [9] and AD development [10-12].

AdoMet is a biological methyl donor and is a product of the conversion of methionine and ATP. Transfer of the methylgroup from AdoMet leads to the formation of AdoHcy which in turn inhibits cellular methylation and eventually will be hydrolyzed into Hcy. Methylation is required in many essential biological processes, such as gene expression, regulation of protein function and neurotransmitter synthesis, and altered methylation patterns might play a role in AD [10-12].

Indeed, there are studies showing a role for AdoMet and AdoHcy in AD [11-13]. It is suggested that DNA methylation for example, is involved in amyloid precursor protein (APP) processing and Aβ production through the regulation of Presenilin1 (PS1) expression. Exogenous AdoMet may silence β and γ secretases, and thereby reducing Aβ production [13]. Other studies have shown decreased AdoMet and AdoHcy levels in the cerebrospinal fluid (CSF) of AD patients compared to age matched healthy volunteers [11,12].

Until now, most studies determined AdoMet and AdoHcy levels in cerebrospinal fluid (CSF) of AD patients [14]. However, AdoMet and AdoHcy remain mostly intracellular and do not pass membranes easily, and it could therefore be possible that AdoMet and AdoHcy levels in brain tissue comprise a more reliable reflection of AD processes than CSF or serum levels which are a 100-fold lower.

There is only one study describing AdoMet levels in brain tissue of AD patients [12]. That study reported severely decreased AdoMet levels. Although the authors carefully matched with respect to postmortem time since AdoMet levels decrease with more than 60% and AdoHcy levels increase with 80% within 15 hours after death [12], it is not certain whether these changes are equal for all AD subjects and age-matched controls. Therefore, the results of this study might be difficult to interpret. In addition, it is still unknown whether alterations in AdoMet and AdoHcy levels are cause or consequence of AD. We therefore investigated AdoMet and AdoHcy levels in brain tissue of 8 and 15-month-old wild type and APP/PS1 double transgenic Alzheimer mice

In addition we investigated the effect of aging and the effects of cholesterol and docosahexanoic acid (DHA) containing diets on AdoMet and AdoHcy levels. These nutritional components influence vascular health [17,18] and the risk of developing AD [15-17], and it could be hypothesized that AdoMet and AdoHcy levels may be influenced subsequently.

Materials and Methods

Animals and diets.

The APPswe/PS1dE9 founders were obtained from Johns Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology) and a breeding colony was established at the Radboud University Nijmegen Medical Centre, The Netherlands. Male APP/PS1 transgenic mice and their wild type littermates were randomly assigned to different diet groups. The number of animals per group was decided on the basis of availability. The experimental diets differed with respect to the composition of the 5% fat in the diets [16] but are all isocaloric. Feeding the diets started at 2 months of age.

Transgenic mice and their wild type littermates were fed either a Typical Western Diet (TWD), containing 1% cholesterol, a high percentage of saturated fatty acids (SFA=53%), and long chain poly unsaturated fatty acid (LCPUFA) ratio of n6/n3 = 22.5, or a DHA diet containing 0 4% DHA, a low percentage SFA (20%), and a lc-PUFA ratio of n6/n3=2.5, or a standard control diet (STD), with 38% SFA and lc-PUFA ratio of n6/n3= 7.5.

In total 82 mice were used, table 1 describes the number of mice used in each experiment. The mice were selected upon availability from another existing study performed in our own lab (submitted).

Throughout the experiments the animals were housed individually in a controlled environment (the mice were housed in the central animal facility with the temperature controlled at 21°C, and an artificial 12:12 h light:dark cycle (lights on at 07.00 am)), with some cage enrichment, consisting of an Iglo and some nesting material.

Food and water were available ad libitum. The experiments were performed according to Dutch regulations of the Animal Experimentation Act and the EC Directive 86/609 and were ethically approved by the Ethical Review Committee of the Radboud University Nijmegen Medical Centre.

Table1. Number of male mice used in each diet group

Groups	8-month-old	15-month-old
STD(wt)	n=5	n=10
STD(tg)	n=6	n=8
TWD(wt)	n=6	n=9
TWD(tg)	n=6	n=8
DHA(wt)	n=5	n=9
DHA(tg)	n=7	n=3

STD= standard diet, TWD= Typical Western Diet, containing 1% cholesterol, DHA= docosahexanoic acid diet, containing 0.4% of DHA. Wt=wild type mice, Tg= transgenic APP/PS1 mice.

Brain sample collection and storage.

Directly following anesthesia with Nembutal (60 mg/kg, i.p.) (Ceva Santa Animals BV, Maassluis), all mice were weighed and thereafter decapitated and the brains were removed from the skull. The entire brain, without the spinal cord was thereafter dissected into 3 smaller pieces, of which the frontal part of the brain was used for this experiment. All pieces were snap frozen in liquid nitrogen and were kept frozen at -80°C.

An 40 mg tissue piece, containing the frontal part of the brain was prepared by sonification in 800 µl cold PBS, and thereafter centrifuged at 4°C at 14000 rpm for 5 min. The supernatant was removed and diluted 8 times. One part was used for HPLC tandem MS measurements, and another part for determination of the protein amount in the sample using the Lowry method [18].

S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) measurements.

As previously described by Gellekink et al [19], AdoMet and AdoHcy levels in brain tissue were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS).

In short, after addition of the internal standards AdoMet-d3 and AdoHcy-d5 to the pretreated tissue sample(see above), solid phase extraction (SPE) columns containing phenyl boronic acid (PBA) were used to bind AdoMet and AdoHcy and their internal standards and to deproteinize the samples.

AdoMet and AdoHcy were eluted in 0.1N formic acid and measured with the LC tandem mass spectrometer (MS/MS)(Quatro LC from Micromass), in the positive-ion mode.

Statistics.

Linear regression analysis was used to verify the linearity of the calibration curves. In order to analyze the possible differences between the two genotypes and the different diet groups a Multivariate ANOVA was conducted with the between group factors diet and genotype. In order to investigate effects of aging in the 2 different genotypes a Multivariate ANOVA was used with the between group factors age and genotype.

Results

No changes in bodyweight were found between the genotypes, nor the two different age groups nor diet groups.

Genotype effects:

No changes in AdoMet and AdoHcy levels, and consequently in the ratio between AdoMet and AdoHcy levels (methylation index), were observed between 8-month-old wild type and APP/PS1 mice (Fig. 1a, b and c).

In addition, also in 15-month-old mice no changes were observed in AdoHcy levels between the genotypes (Fig. 1b). However we did observe a significant decrease in AdoMet levels in 15-month-old wild type mice compared to APP/PS1 mice (Fig. 1a; $F(1,45)=7.54$, $p<0.01$). Consequently, the AdoMet/AdoHcy ratio is increased in the APP/PS1 mice (Fig. 1c; $((1,45)=7.17$, $p<0.01$).

Aging effects

AdoHcy levels significantly decrease during aging in both wildtype and APP/PS1 mice ($F(1,79)=9.50$, $p<0.01$). Due to an interaction between genotype and age in AdoMet levels and the ratio between AdoMet and AdoHcy levels (methylation index), the effects of aging in wild type and APP/PS1 mice were determined separately for these parameters.

A significant decrease in AdoMet levels during aging was only found in wild type mice ($F(1,42)=8.09$, $p<0.01$) and not in APP/PS1 mice.

Thus, AdoHcy decreased in both wild type and APP/PS1 mice during aging, whereas AdoMet only decreased in wild type mice. Consequently, the AdoMet/AdoHcy ratio (methylation index) in wildtype mice did not alter due to aging, but was significantly increased in APP/PS1 mice with aging (Fig. 1f; $F(1,35)=6.61$, $p=0.01$).

Diet effects

Neither a cholesterol nor a DHA containing diet did alter AdoMet or AdoHcy levels in brain tissue of 8 and 15-month-old APP/PS1 and wild type mice as compared to the standard diet (data not shown).

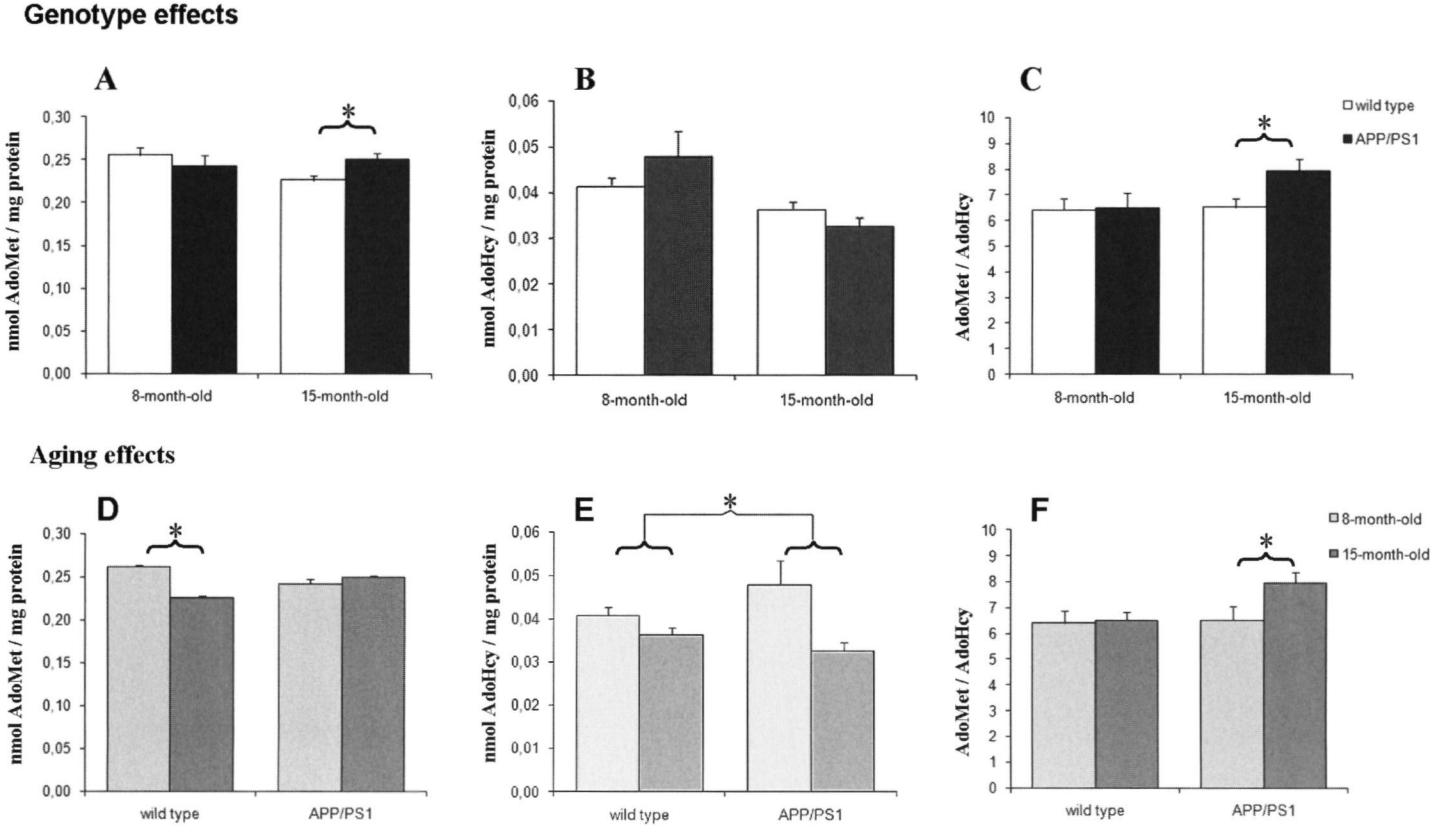

*Fig.1. Levels of s-adensylmethionine (AdoMet; A,D) and s-adenosylhomocysteine (AdoHcy; B,E) and the methylation index (AdoMet/ AdoHcy; C,F) in brain tissue of 8 and 15-month-old APP/PS1 mice and their wild type littermates. Values represent mean and SEM. * $p<0.01$.*

Discussion

In this study we showed that AdoMet tissue levels did not differ between 8-month-old APP/PS1 mice and wild type mice, but are decreased in 15-month-old wild type mice compared to APP/PS1 mice Moreover, tissue levels of AdoHcy decreased in both wild type and APP/PS1 mice during aging, whereas AdoMet only decreases in wild type mice. Because AdoMet and AdoHcy brain levels in (young) 8-month-old mice are unchanged, in contrast to the (old) 15-month-old mice, these results indicate that alterations in AdoMet are a consequence of AD rather than a cause In addition, we showed that a cholesterol containing or DHA enriched diet did not affect AdoMet or AdoHcy levels in brain tissue of APP/PS1 and wild type mice.

This is the first study describing AdoMet and AdoHcy levels in brain tissue of aging mice Stramentinoli et al [20] described, decreased AdoMet levels [20] in brain tissue of aging rats In addition they measured the synthesis of AdoMet by adenosyl transferase and AdoMet utilization by COMT, and they concluded that a decrease in AdoMet levels is due to increased utilization during aging Increased utilization may also be the explanation for the decrease in AdoMet and AdoHcy in our wild type mice It could be suggested that in the APP/PS1 mice, AdoMet levels do not decrease because of less demand caused by neurodegeneration In a subgroup of littermates of the 15-month-old mice used in this study, we observed hippocampal atrophy in APP/PS1 mice compared to wild type mice (not shown), which may express neurodegeneration

If the above mentioned hypothesis is valid, and AdoMet remains unaltered in aged APP/PS1 mice because of less demand for methylation due to neurodegeneration, AdoHcy levels will also be expected to be decreased or unchanged. Indeed, AdoHcy levels are similar in the 15-month-old wild type mice and APP/PS1 mice

All together, the fact that AdoMet levels in APP/PS1 mice are not decreased during aging (in contrast to the wild type mice) is probably a consequence of neurodegeneration rather than a cause for AD.

The finding that AdoMet levels do not differ between the genotypes in 8-month-old mice, but are significantly different in 15-month-old APP/PS1 mice compared to their wild type littermates also indicate that alterations in AdoMet levels are a consequence of AD rather than a cause

In this experiment we have only measured AdoMet and AdoHcy levels, it would however be of great value to measure in future studies more components from the methylation cycle such as homocysteine itself and some of the enzymes involved in the synthesis or utilization of AdoMet, to validate our conclusions

The finding that cholesterol or DHA containing diets do not alter AdoMet or AdoHcy levels in brain tissue of either wild type or APP/PS1 mice, but in contrast do have effects on AD pathology and cerebral hemodynamics [15-17], suggests that AdoMet and AdoHcy are not directly involved in cerebral vascular diseases (which is in agreement with other studies [5,6]), and are no important key players in the mechanisms explaining the effects of cholesterol and DHA in AD pathology

Main Conclusions

We measured AdoMet and AdoHcy levels in brain tissue of 8 and 15-month-old wild type and APP/PS1 mice, to investigate whether there is a role for these metabolites of methylation in the AD brain. The finding that AdoMet levels do not differ between the genotypes in (young) 8-month-old mice, but are different in (older) 15-month-old APP/PS1 mice compared to their wild type littermates, suggests that alterations in AdoMet are a consequence of AD rather than a cause, and possibly the result of neurodegeneration.

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Chapter 6

Summarizing discussion
and concluding remarks

The possibility that the risk for Alzheimer's disease (AD) can be reduced by diet or lifestyle is of great importance and suggests a preventative treatment in Alzheimer's disease. Lipid diets and lipid metabolism play a role in both Alzheimer's disease and cardiovascular disease. In addition a link between cardiovascular disease and Alzheimer has been suggested [1-6]. It could therefore be hypothesized that dietary lipids influence AD progression via effects on the vasculature.
Epidemiological studies have repeatedly shown that dietary intake of DHA decreases, and high serum cholesterol levels increase the risk of AD [3,7-11]. In this thesis we therefore focus on the effect of docosahexanoic acid (DHA), an omega-3 long chain polyunsaturated fatty acid from fish oil, and cholesterol containing diets, on cerebral hemodynamics in transgenic APP/PS1 Alzheimer mice.
In the first part of this chapter I will give a summary of our most important findings. This summary will contain a description of the mechanism by which cholesterol and DHA containing diets may influence the course of AD followed by a description of some of the consequences of AD development. In the second part of this chapter I will provide a general discussion with concluding remarks and some future perspectives.

DHA in AD development

Many animal, epidemiological and clinical studies have shown that high DHA consumption is associated with reduced risk of AD [7,9,11-14]. The underlying mechanisms however are still largely unknown. It is found that DHA decreases the amount of Aβ deposition in plaques and cerebral amyloid angiopathy [13-15], protects against dendritic pathology [16], prevents neuronal apoptosis induced by soluble amyloid-β peptide [17], increases synaptic protein and phospholipid densities [18,19] and inhibits degradative endopeptidase activities [20]. However the results of the experiments described in this thesis **(chapter 2 and chapter 3)** show that changes in the cerebral circulation may play a crucial role.
We have shown in APP/PS1 mice, that a DHA containing diet (which also contains a small amount of EPA) is able to alter the relative cerebral blood volume (rCBV; a possible measure for cerebral hemodynamics) already at 8-month of age, whereas diet induced changes in Aβ plaque load were not yet detected **(chapter 3)**. This indicates that a DHA diet may cause alterations in cerebral hemodynamics in APP/PS1 mice before changes in the Aβ plaque load occur.
In 15-month-old APP/PS1 mice fed the DHA containing diet both changes in Aβ plaque load and rCBV occurred simultaneously. The rCBV slightly increased, probably caused by vasodilatation, and vascular and parenchymal Aβ plaque load decreased. But more importantly we also observed improved spatial memory in these 15-month-old mice fed DHA **(chapter 3)**. 18-month-old mice fed the DHA diet only showed increased rCBV combined with a decreased vascular Aβ load **(chapter 2)**. No changes in parenchymal Aβ levels were found, but this could be due to the extensive Aβ pathology in 18-month-old APP/PS1 mice, and it may therefore be difficult to induce diet effects on that age.
Nonetheless we conclude that DHA intake inhibits AD pathology by decreasing the amount of Aβ, and improves cognition and accordingly delays progression of AD development. We hypothesize that this delay in progression of AD due to DHA intake is

caused by improvement of vascular health, since the AD related changes in this APP/PS1 mouse model fed a DHA containing diet show that alterations in cerebral hemodynamics occur before Aβ plaque load diminishes (Table and Fig. 1)**(chapter 3)**.

Table 1. Summary of the main effects of a DHA and TWD diet in APP/PS1 mice

Effects of DHA diet in APP/PS1 mice				Effects of TWD diet in APP/PS1 mice			
Age (months)	**Memory**	**Aβ**	**rCBV**	**Age (months)**	**Memory**	**Aβ**	**rCBV**
8	-	-	↑/↓	**8**	-	-	-
15	↑	↓	↑*	**15**	-	-	↓
18	ND	↓	↑	**18**	ND	↑	↓

*Table 1. Summary of the main effects of a DHA and TWD diet in APP/PS1 mice. ND= not determined, ↑=increased, ↓=decreased, *=tendency.*

In addition, DHA may also have influenced the neuronal membrane fluidity, since the ratio between omega-3 and omega-6 fatty acids was increased in mice fed a DHA diet, which is an indicator for increased membrane fluidity. According to Wolozin's theory [6] it is hypothesized that rigid neuronal membranes produce more Aβ, and consequently more fluid membranes might diminish the amount of Aβ deposition (Fig 1).

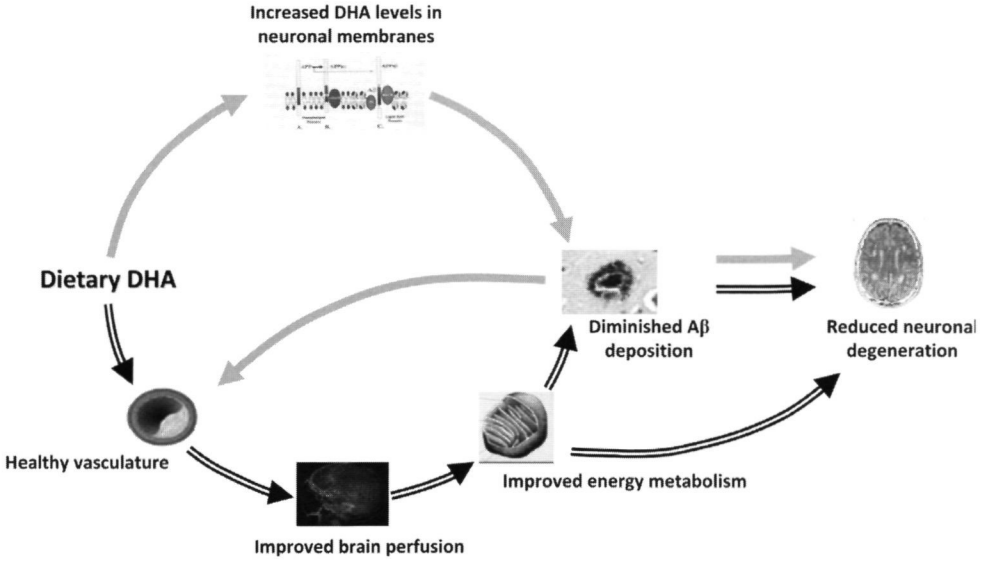

Fig 1: Schematic overview of the two main hypothesis involved in the mechanism by which high dietary DHA levels influence AD development. Grey arrows refer to the membrane fluidity hypothesis of Wolozin [18]. Black arrows refer to the hypoperfusion hypothesis [17,22-23].

Cholesterol in AD development

In contrast to the protective effects of DHA in AD, other dietary lipids increase the risk of developing AD. For example, a high cholesterol containing diet, causing hypercholesterolemia is an important risk factor for vascular disorders, and a link between hypercholesterolemia, cardiovascular diseases and AD has been suggested [3,8,10,21,22]. At present, the mechanism by which cholesterol increases the risk of AD, is not completely clear. The results of the studies presented in **chapter 2 and 3** however show, that like DHA, also cholesterol affects AD development via the cerebral circulation. In 8-month-old APP/PS1 mice no effects of a typical western diet (TWD) containing 1% cholesterol were noticed on Aβ deposition, rCBV or cognition, whereas in 15-month-old mice, decreased rCBV levels were found **(chapter 3)**. During further aging, also the parenchymal Aβ levels increased **(chapter 2)**. All together the results of the experiments in **chapter 2 and 3** indicate that a TWD diet first changes the cerebral circulation before altering the Aβ load (table 1).

In addition, the observation that cholesterol levels do not change in brain homogenates from mice fed high a cholesterol-enriched diet, may indicate that cholesterol is not incorporated in brain tissue as a result of cholesterol intake. Membrane fluidity is therefore not influenced by administration of dietary cholesterol. Wolozin's theory in which he hypothesizes that high cholesterol in neuronal membranes increases amyloid-β (Aβ) production [6] does not seem applicable here. In the hypoperfusion theory (Fig. 2) it is proposed that hypoperfusion of specific brain areas due to for example atherosclerosis is a primary event, and ultimately leads to a lowered energy metabolism (energy crisis), an overproduction of Aβ and cognitive impairment [23-27]. Since we found a decreased rCBV before changes in Aβ load occurred in TWD fed mice, the hypoperfusion hypothesis seems much more likely **(Chapter 2 and 3)**.

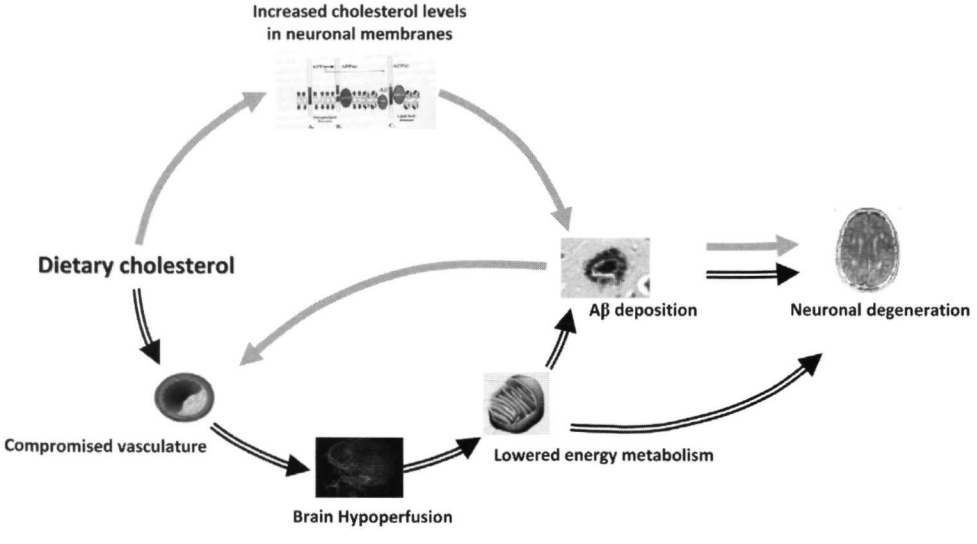

Fig 2: Schematic overview of the two main hypothesis involved in the mechanism by which high dietary cholesterol levels influence AD development. Grey arrows refer to the membrane fluidity hypothesis of Wolozin [18]. Black arrows refer to the hypoperfusion hypothesis [17,22-23].

AD as a vascular disorder

Thus, changes in the cerebral circulation due to cholesterol and DHA containing diets seem to precede changes in Aβ plaque load. Since environmental factors such as lifestyle and diet are important factors in AD development these findings suggest that vascular impairment plays a larger role in AD than always assumed.

The importance of vascular factors in AD emerge from the results described in **chapter 2 and 3**. In **chapter 2** a decreased rCBV was noticed in 18-month-old APP/PS1 mice compared to age matched wild type littermates. In addition **chapter 3** shows that 8-month-old mice show mild Aβ pathology and cognitive impairment, which is aggravated in 15-month-old mice. These 15-month-old mice also display decreased rCBV and hippocampal atrophy **(chapter 3)**.

Although it is largely unclear in which order changes in Aβ load, rCBV and hippocampal atrophy occur and cause cognitive impairment, it is likely that they contribute to neurodegeneration and ultimately cognitive impairment together. This fortifies the hypothesis that AD is not a single nosological entity. However, Aβ deposition must be involved in cognitive impairment in this mouse model, since at 8 months of age both Aβ deposition and cognitive impairment are already present at the same time, without changes in rCBV or atrophy. We therefore suggest that aggravation of AD only happens when changes in the brain circulation develop, which are subsequently followed by cell death and hippocampal atrophy **(chapter 3)**.

Consequences of AD

Besides alterations in cerebral hemodynamics, Aβ plaque load and atrophy in the brains of AD patients, a reduced glucose metabolism and loss of the glucose transporter-1 (GLUT-1) [28,29] is also frequently mentioned. It could be that loss of GLUT-1 is caused by a decrease in capillary density, and this decrease in capillary density might also explain the observed decrease in rCBV in APP/PS1 mice. However, the experiments described in chapter 4 show that there is a decreased GLUT-1 amount but no decrease in capillary density in APP/PS1 mice compared to wild type littermates. This indirectly confirms that the decrease in rCBV **(chapter 3)** in APP/PS1 mice must be due to vasoconstriction, and that a decreased GLUT-1 amount is probably caused by a decreased GLUT-1 amount per capillary (expression), which indeed was the case **(chapter 4)**. The decrease in GLUT-1 expression could be cause or consequence of AD. However, we showed that a certain amount of Aβ deposition must be reached before it affects GLUT-1 expression, demonstrating that reduced glucose metabolism is probably a consequence of amyloid deposition **(chapter 4)**. A reduced glucose metabolism might be partly responsible for the lowered energy metabolism/energy crisis in AD brain regions (Fig 1 and 2). Accordingly it could be suggested that an energy crisis is an essential step in AD pathogenesis (Fig 3). For instance, it has already been shown that hypoperfusion causes an energy crisis which ultimately leads to an overproduction of Aβ and cognitive impairment [24], furthermore a decreased GLUT-1 expression due to Aβ also indicates the presence of an energy crisis and in this way creates a virtuous circle (Fig 3).

Besides an altered glucose metabolism, the homocysteine (Hcy) metabolism seems also to

be involved AD [30,31]. However, since recently performed large Hcy lowering intervention studies did not show beneficial effects on AD development, it is suggested that other factors of the Hcy metabolism may be involved. In chapter 5 we therefore determined brain s-adenosylmethionine (AdoMet) and s-adenosylhomocysteine (AdoHcy) levels in the brains of 8 and 15-month-old wild type and APP/PS1 mice. AdoMet and AdoHcy are involved in all biological methylation processes in the body and also play an important role in vascular disease [32] and AD [33-35]. The results presented in **chapter 5** show that during aging in wild type mice AdoMet and AdoHcy levels decrease. Although APP/PS1 mice also show a decrease in AdoHcy levels during aging, no decrease in AdoMet levels could be observed. The absence of a decrease in AdoMet levels in APP/PS1mice during aging compared to the presence of a decrease in wild type mice, causes a significant difference between the 2 genotypes at 15 months of age. However, since differences in AdoMet levels are not present in 8-month-old mice, it could be concluded that they are a consequence of AD rather than a cause, and probably the result of neurodegeneration **(chapter 5)**.

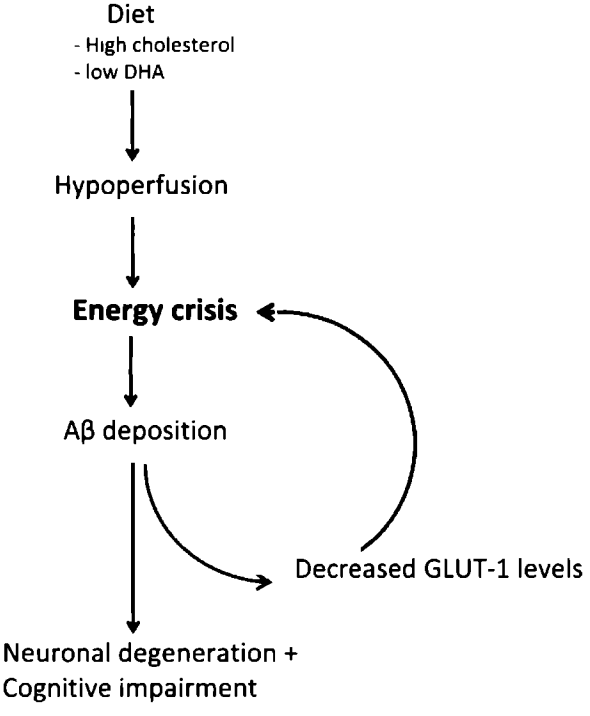

Fig 3: Schematic overview of the proposed sequence of events in the development of AD

Concluding remarks

Although chapter 1 to 5 provide many novel insights in AD pathology, it should be kept in mind that all experiments are performed in a mouse model for AD. The APP/PS1 mouse used in this thesis is an Aβ-producing-model, in which the only difference with wild types are the presence of transfected human mutated genes encoding for the amyloid precursor protein (APP) and the presenilin 1 (PS1) resulting in the production of human Aβ. We investigated the effects of dietary intervention and the role of hemodynamic alterations

in AD development, and showed that hemodynamic alteration due to cholesterol or DHA containing diets occur before changes in Aβ plaque load are present. Whether there is a causal role for hemodynamic alterations in AD development, regardless of diet interventions, is still unclear and no major objective in this thesis. However, it would have been impossible to investigate this with our APP/PS1 mouse model due to its enormous promoter driven production of Aβ. We nevertheless showed that in young (8-month-old) APP/PS1 mice compared to wild type mice already some Aβ pathology combined with mild cognitive impairment was present, which became aggravated in older (15-month-old) mice and accompanied with a decrease in rCBV. Therefore, based on these results we can already conclude that changes in hemodynamics play a major role in progression and aggravation of AD.

In order to give a definitive answer on the question whether changes in the hemodynamics play a causal role in the development of AD we suggest to utilize another experimental model. The use of AD patients or other volunteers is not an option, because these kind of studies will be very time consuming and expensive. For example, the patient should be included in the study already many years before clinical onset and followed until death in order to confirm AD pathology. In addition, many expensive hemodynamic measurements and cognition tests should be performed over time. Thus, an experimental animal model, which expresses idiopathically/spontaneously some Aβ pathology and presents cognitive decline during aging with a relatively short lifespan would be preferable.

For example, aged non human primates, such as the squirrel monkey or the rhesus monkeys, develop age associated behavioural and brain abnormalities similar to those that occur in individuals with AD [36,37]. The squirrel monkeys develop more cerebrovascular Aβ in the natural course of aging [36], whereas the rhesus monkey develops more abundant parenchymal Aβ [37]. In addition, they develop hypertension and atherosclerosis [38-40]. Thus in order to find out whether hemodynamic alterations in spontaneously arising AD could be causal, future research should focus on models for idiopathic AD such as the rhesus or squirrel monkey. With MR methods, comparable as those described in this thesis, hemodynamic parameters such as the CBV and CBF could be visualized and quantified repeatedly during aging. However, other parameters such as blood pressure (bp) should be determined as well, since bp is much easier to determine in monkeys compared to mice, and many studies show a relation between dementia and high bp in mid-life or low bp in late-life (reviewed in [41]).

Nonetheless, experimental research in mice is much cheaper, faster and less complicated due to less ethical concerns compared to experiment with monkeys, and it is therefore more likely to be continued in our laboratory. It would however be very valuable to use positron emission tomography (PET) scans with the radiotracer Pittsburgh Compound-B (PiB) or other MRI techniques [42] instead of immunohistochemical studies, for visualizing Aβ in vivo, in order to minimize animal use. In addition, it would be interesting to use arterial spin labeling (ASL) techniques for determination of the cerebral blood flow in specific brain regions. This technique provides more accurate CBF values compared to our used MRS technique, but was not available at the time. MRS techniques instead should be used to get a greater insight in energy- and glucose metabolism, and may help to further confirm the importance of an energy crisis partly due to hypoperfusion in AD (Fig. 2).

Anyhow, this thesis provides evidence on the importance of cerebral hemodynamic which implicates that AD prevention should focus more on keeping hemodynamic parameters in a normal range already during early-life. Dietary intervention may be a important tool herein. **Chapter 2 and 3** for example showed that cholesterol or DHA containing diets first alter rCBV before changes in Aβ plaque load or cognition were noted. The mechanism by which cholesterol and DHA influence the rCBV in AD development is still unknown, and future research should focus on this mechanism.
Unraveling this mechanism may have important consequences for AD treatment, because therapeutic intervention directly upon this mechanism may improve AD treatment significantly.

Nonetheless this thesis already shows that dietary intervention has a great impact on AD development. A DHA containing diet improves spatial memory and decreases parenchymal and vascular Aβ load. A high cholesterol containing diet however aggravates AD pathology. From these results it could be concluded that consuming DHA, and keeping serum cholesterol levels low, will be beneficial for AD patients. However, the work described in this thesis suggests that a "healthy diet" (DHA rich and cholesterol poor) should already be started early in life. This also needs to be confirmed in clinical intervention studies. Until now it has been shown to be very difficult in human studies to observe the same major effects as found in our experimental animal study. Most clinical studies supplement for a rather short time [9,43,44], and start too late i.e. supplement to already cognitive impaired patients or in aged persons [9,11,43,44]. From the data described in this thesis it could be suggested that a healthy diet is of most benefit in young/ postpuberal individuals.

Altogether, AD is a multi-factorial disease in which vascular factors play a dominant role already early in the course of the disease. This suggests, that treatment of vascular disorders might be an important target for prevention of AD.
In addition , the possibility that the risk for AD can be reduced by preventative strategies like diet is of great importance and promises to be a successful strategy in the treatment of AD and cardiovascular disease. In the ideal situation, long-term clinical studies, in which individuals are supplemented with experimental diets starting from young adult age, are necessary to confirm the importance of a "healthy" diet (DHA rich and cholesterol poor) in delaying AD onset and improving cognitive function. However, this kind of research is for many individual projects too expensive and too much time consuming, and theoretically could only be performed in a large collaboration and subsidized by different governments. Thus for the time being we should focus on experimental animal models resembling the human situation as much as possible.

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Chapter 7

Nederlandse samenvatting

De ziekte van Alzheimer (AD) is de meest voorkomende oorzaak van dementie. Op het moment lijden ongeveer 130.000 Nederlanders aan de ziekte van Alzheimer. Het grootste risico om de ziekte van Alzheimer te ontwikkelen is toenemende leeftijd. De gemiddelde levensverwachting neemt nog steeds toe in onze westerse samenleving en dus zal ook het aantal Alzheimerpatiënten toenemen. In 2050 zullen meer dan 300.000 Nederlanders Alzheimerpatiënt zijn. Dat is één op 57 Nederlanders.
Tijdens deze ziekte sterven geleidelijk zenuwcellen af (neurodegeneratie) in specifieke gebieden van het brein zoals de hippocampus (een gebied in de hersenen dat betrokken is bij het opslaan van nieuwe herinneringen die betrekking hebben op feiten en gebeurtenissen) en de cortex. Ook zijn er kluwens van draadvormige eiwitten (tangles) in de hersencellen aanwezig die het functioneren van de zenuwcel onmogelijk maken. Tevens worden er eiwitophopingen, voornamelijk bestaand uit het eitwit β-amyloid (Aβ), buiten de zenuwcellen (seniele plaques) en in de wand van de bloedvaten (cerebrale amyloid pathologie) gevonden.
De oorzaak van de ziekte is nog steeds grotendeels onbekend maar steeds meer onderzoek laat zien dat voeding een belangrijke rol speelt bij het ontstaan en het beloop van de ziekte van Alzheimer. Het is bijvoorbeeld gebleken dat mensen met een hoge cholesterolbloedspiegel, een hoger risico hebben op de ontwikkeling van AD. En mutaties in genen die betrokken zijn bij het cholesterol metabolisme lijken ook geassocieerd te zijn met de ontwikkeling van AD.
Het gebruik van visolie (ofwel omega-3 vetzuren zoals DHA en EPA) daarentegen lijkt het risico op de ontwikkeling van AD juist te verlagen en het geheugen verbeteren. Verder is uit dierexperimenteel onderzoek gebleken dat het vetzuur DHA uit visolie de vorming van Aβ sterk tegengaat. In hoofdstuk 1 van dit proefschrift zijn de belangrijkste effecten van cholesterol en DHA op de ontwikkeling van AD weergegeven.
Kortom, cholesterol en DHA beïnvloeden het risico op de ontwikkeling van AD, maar tot op heden is het onderliggende mechanisme nog onbekend. Het wordt gesuggereerd dat voeding de ontwikkeling van AD beïnvloedt door de doorbloeding van de hersenen te veranderen. Cholesterol kan bijvoorbeeld zorgen voor aderverkalking (atherosclerose), waardoor er minder zuurstof en voedingstoffen via het bloed in de hersenen aankomen (verminderde doorbloeding van de hersenen), de hersencellen noodlijdend worden en bijvoorbeeld het Aβ eiwit gaan vormen. Uiteindelijk zou dit alles dan leiden tot neurodegeneratie en cognitieve achteruitgang (figuur 1).
Onderzoek naar omega-3 vetzuren heeft juist aangetoond dat het gebruik van visolie (DHA en EPA) de vasculaire gezondheid ten goede komt. Het kan dus worden gesuggereerd dat omega-3 juist de doorbloeding van de hersenen verbetert, en op deze manier Aβ depositie tegengaat en cognitieve achteruitgang afremt.

DHA en cholesterol in de ontwikkeling van Alzheimer

In **hoofdstuk 2 en 3** van dit proefschrift is gekeken op welke manier cholesterol en DHA de ontwikkeling van de ziekte van Alzheimer beïnvloeden, en of veranderingen in de cerebrale doorbloeding hieraan ten grondslag kunnen liggen. In figuur 1 en 2 wordt een schematisch overzicht gegeven van de 2 meest waarschijnlijke hypotheses die de rol van cho-

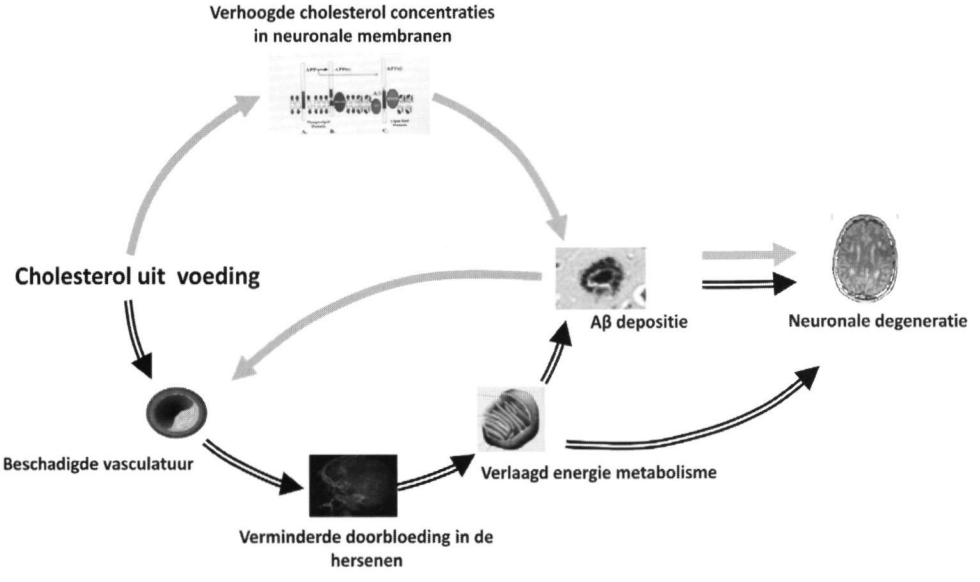

Fig 1: Schematisch overzicht van de twee belangrijkste hypothesen die de effecten van cholesterol op neuronale degeneratie en cognitieve achteruitgang kunnen verklaren. De grijze pijlen geven de membraan vloeibaarheid-hypothese weer (Wolozin theorie). Zwarte pijlen geven de hypoperfusie theorie weer (gebaseerd op een theorie van Farkas, 2001 en de la Torre, 2002).

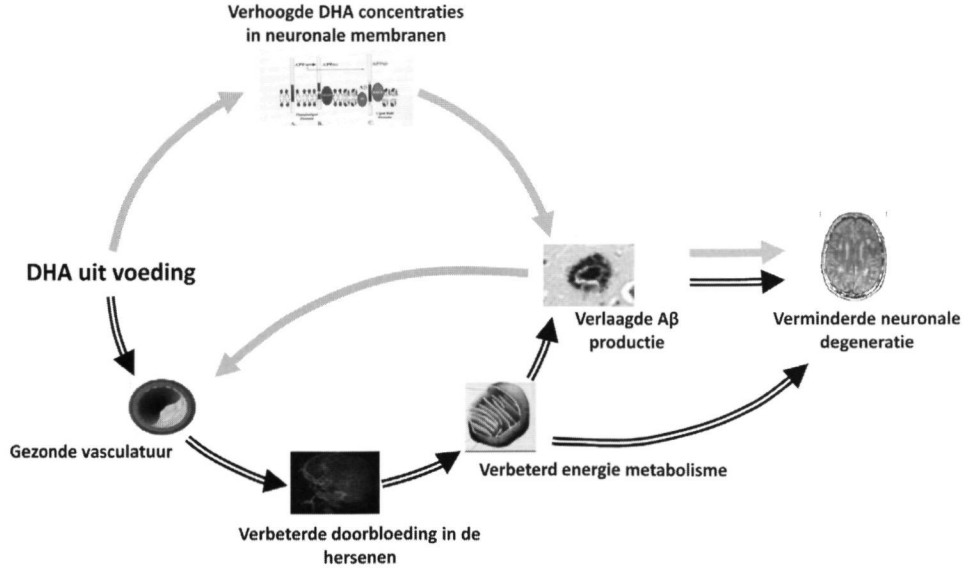

Fig 2: Schematisch overzicht van de twee belangrijkste hypothesen die de effecten van DHA op neuronale degeneratie en cognitieve achteruitgang kunnen verklaren. De grijze pijlen geven de membraan vloeibaarheid-hypothese weer (Wolozin theorie). Zwarte pijlen geven de hypoperfusie theorie weer (gebaseerd op een theorie van Farkas, 2001 en de la Torre, 2002).

lesterol en DHA op de ontwikkeling van AD zouden kunnen verklaren In dit proefschrift werd gebruik gemaakt van dubbel transgene Alzheimer muizen (APPswe/PS1dE9) Dit zijn muizen die 2 gemuteerde humane genen bezitten (APPswe en PS1dE9), die verantwoordelijk zijn voor de vorming van het Aβ proteine. Deze muizen vormen al op jonge leeftijd Aβ depositie in het brein en vertonen cognitieve achteruitgang Alle muizen kregen een cholesterol verrijkt typisch westers dieet (TWD), een DHA verrijkt dieet (waar ook een kleine hoeveelheid EPA inzit), of een standaard (STD) controle dieet.

DHA

In hoofdstuk 2 lieten wij zien dat een DHA verrijkt dieet in 18 maanden oude APPswe/PS1dE9 muizen, de cerebrale doorbloeding verandert (de relatieve cerebrale bloedvolumes (rCBV) verhoogt) en de hoeveelheid Aβ verlaagt Vervolgens wilden we weten welk van deze mechanismen als eerste verandert om oorzaak en gevolg te achterhalen

In hoofdstuk 3 hebben we daarom gebruikt gemaakt van 2 jongere groepen muizen (8 en 15 maanden oud) en lieten we zien dat veranderingen in de doorbloeding als eerste optreden onder invloed van een DHA bevattend dieet Tevens hebben we vastgesteld dat een DHA dieet de cognitie verbetert in APPswe/PS1dE9 muizen.

Kortom, een DHA verrijkt dieet in APPswe/PS1dE9 muizen lijkt AD pathologie te verminderen door de Aβ levels te verlagen, en de cognitie te verbeteren. Wij hypothetiseren dat een verbetering van de vasculaire gezondheid hieraan ten grondslag ligt.

Cholesterol

In hoofdstuk 2 en 3 laten we tevens zien dat ook een cholesterol verrijkt dieet (typisch westers dieet; TWD) de ontwikkeling van de ziekte van Alzheimer lijkt te beinvloeden via de doorbloeding.

In 15 maanden oude muizen zien we een verlaging van de rCBV, die in 18 maanden oude dieren wordt gevolgd door een verhoging van het aantal Aβ plaques Tevens verandert de hoeveelheid cholesterol in het brein weefsel niet onder invloed van een cholesterol dieet Dit impliceert dat de "theorie van Wolozin", waarin hij hypothetiseert dat cholesterol in neuronale membranen de hoeveelheid Aβ zou verhogen en vervolgens cognitieve achteruitgang zou veroorzaken, hier niet van toepassing is, maar juist de hypoperfusie theorie (Fig 1)

De ziekte van Alzheimer als vasculaire aandoening?

De resultaten van de dieet experimenten lieten dus duidelijk zien dat de doorbloeding een belangrijke rol speelt in de ontwikkeling van AD, aangezien bij zowel het DHA en cholesterol dieet de rCBV als eerste veranderde Ook tussen de transgene APPswe/PS1dE9 en de wild type (controle) muizen zien we verschillen in de doorbloeding. Dit impliceert dat ook hier een veranderde doorbloeding van invloed kan zijn op AD ontwikkeling In hoofdstuk 3 lieten we zien dat 15 maanden oude APPswe/PS1dE9 muizen een lagere rCBV hebben dan de wild type dieren, dit duidt erop dat er een hypoperfusie optreedt.

Echter, in jongere (8 maanden oud) dieren werd al cognitieve achteruitgang en Aβ pathologie gezien, zonder veranderingen in doorbloeding Dit zou kunnen betekenen dat in dit

muismodel veranderingen in de doorbloeding niet de causale factor is.
Maar omdat in 15 maanden oude AD muizen de Aβ pathologie en cognitieve achteruitgang verergert in de aanwezigheid van een verlaagde rCBV, kunnen we suggereren dat verheviging van de ziekte van Alzheimer alleen optreedt wanneer er een verminderde doorbloeding (hypoperfusie) ontstaat.
Om definitief een antwoord te geven op de vraag of Alzheimer primair een vasculaire aandoening is, dient meer onderzoek uitgevoerd te worden. Het zou dan verstandig zijn ook een diermodel te gerbuiken waarin AD spontaan ontstaat en dat niet is gebaseerd op een niet fysiologische overproductie van Aβ door een promotor.

Gevolgen van AD pathologie
Naast veranderingen in de cerebrale doorbloeding, de hoeveelheid Aβ plaques en neurodegeneratie in de hersenen van AD patiënten, wordt er ook regelmatig een verlaagd glucose metabolisme en een verlies van glucose transporters (GLUT-1) waargenomen. Het is dus mogelijk dat een verlaging van GLUT-1 wordt veroorzaakt door een verminderde bloedvat dichtheid.

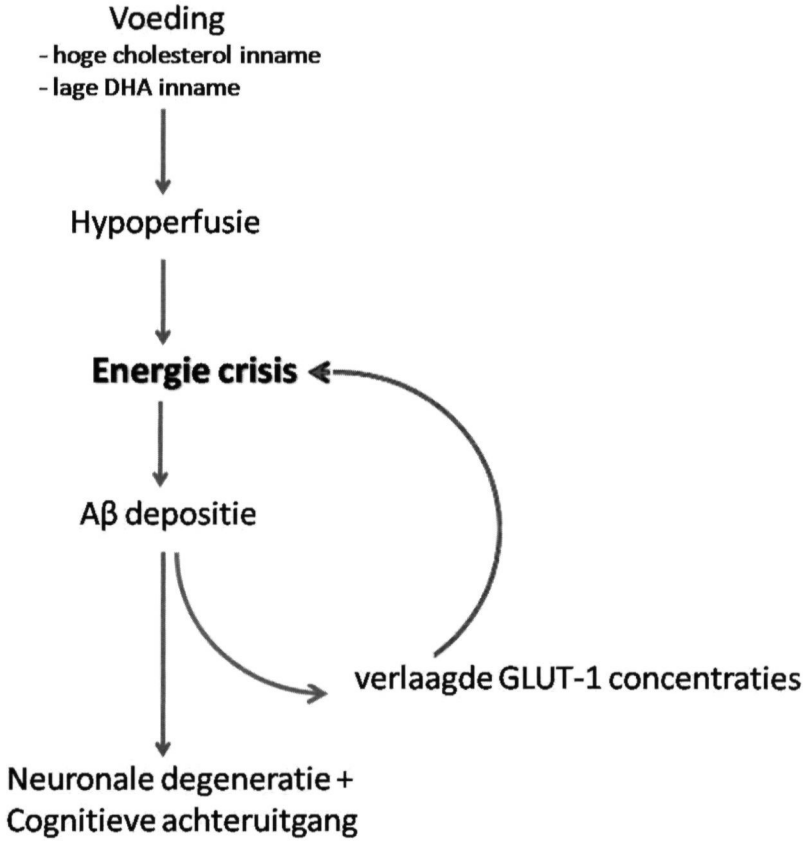

Fig 3: Schematisch overzicht van de gesuggereerde opeenvolging van gebeurtenissen in de ontwikkeling van de ziekte van Alzheimer.

Dit zou dan direct de verklaring kunnen zijn voor de gevonden verlaging in rCBV. Echter, de resultaten in hoofdstuk 4 laten zien dat er geen verlaging is van het aantal bloedvaten, en impliceert dus dat de verlaging in rCBV wordt veroorzaakt door vasoconstrictie (samenknijpen van de bloedvaten), en de verlaging van de GLUT-1 wordt veroorzaakt door een verlaging van de hoeveelheid GLUT-1 per oppervlakte bloedvat. Inderdaad vonden we dat er een verminderde hoeveelheid GLUT-1 per bloedvat was, maar de oorzaak hiervan bleef nog onbekend. Uiteindelijk lieten we zien dat er een bepaalde hoeveelheid Aβ aanwezig moet zijn in de buurt van de bloedvaten voordat de hoeveelheid GLUT-1 per bloedvat vermindert, en dat de verlaging van GLUT-1 niet simpel een gevolg is van neurodegeneratie. Hieruit kan worden geconcludeerd dat een verlaging van de GLUT-1 hoeveelheid per bloedvat een gevolg/ consequentie is van amyloidpathologie.
Vervolgens zou een verlaagd glucosemetabolisme kunnen leiden tot een energiecrisis in neuronale cellen door een tekort aan voedingstoffen (Fig 3). Het is dus mogelijk dat het ontstaan van een energiecrisis een essentiële stap is in AD. We hebben bijvoorbeeld ook al laten zien in hoofdstuk 3 dat hypoperfusie wellicht een energiecrisis veroorzaakt en wordt gevolgd door een overproductie van Aβ en uiteindelijk cognitieve achteruitgang. Kortom, wij suggereren dat het ontstaan van een energiecrisis door enerzijds hypoperfusie, en anderzijds een afname aan GLUT-1 transporters een sleutelrol speelt in AD pathogenese (Fig 3).

In **hoofdstuk 5** tonen we dat naast een verlaagd glucose metabolisme ook het homocysteinemetabolisme een rol lijkt te spelen in de ontwikkeling van AD.
Homocysteine (Hcy) is een afbraakproduct van de menselijke eiwitstofwisseling, en lijkt een risicofactor te zijn voor het ontstaan van hart- en vaatziekten en via deze weg ook voor de ontwikkeling van AD. Toch bleek uit studies waarin Hcy verlaagd werd geen verbetering in cognitie op te treden. Dit impliceert dat wellicht een andere factor dan Hcy een rol speelt bij de ontwikkeling van AD. In hoofdstuk 5 bestudeerden wij 2 andere factoren van het Hcy metabolisme, namelijk s-adenosylmethionine (AdoMet) en s-adenosylhomocysteine (AdoHcy). Beiden metabolieten zijn betrokken bij alle methyleringsprocessen in het lichaam. Methylering is een belangrijk biochemisch proces in het lichaam waarbij een methylgroep (CH3) wordt overgedragen aan een ander molecuul. Als dit proces niet goed verloopt kan dat consequenties hebben voor bijvoorbeeld gen expressie, regulatie van eiwit functie en de synthese van neurotransmitters. Wij laten zien dat er bij veroudering in wild type muizen een afname optreedt in de hoeveelheid AdoMet en AdoHcy. In transgene AD muizen is er geen afname in AdoMet te zien, waarschijnlijk door neurodegeneratie. Dit leidt tot een verschil in AdoMet levels tussen de wild type en transgene AD muizen op een leeftijd van 15 maanden. Aangezien dit verschil niet aanwezig is in 8 maanden oude dieren, kan er worden geconcludeerd dat de veranderde AdoMet levels in AD muizen een gevolg zijn van AD in plaats van een oorzaak, maar wel kunnen bijdragen aan het ziekteproces.

Slotopmerkingen

De ziekte van Alzheimer is een multifactoriële ziekte waarin vasculaire factoren een essentiële rol lijken te spelen al vroeg in de ontwikkeling van de ziekte. Behandeling van vasculaire aandoeningen zou dus ook kunnen bijdragen aan de remming van de ontwikke-

ling van de ziekte van Alzheimer.
De bevinding dat interventie in het voedingspatroon het risico en de ernst van de ziekte van Alzheimer kan beïnvloeden, lijkt een succesvolle strategie te kunnen worden in de preventie van AD.
De eventuele bescherming van DHA tegen cognitieve achteruitgang, en het verergeren van AD pathologie door hoog serumcholesterol zal nog bevestigd moeten worden in langdurige klinische interventiestudies. Tot op heden zijn de "ideale" interventiestudies, waarin vanaf jonge leeftijd gesuppleerd wordt met bijvoorbeeld DHA en hemodynamische en cognitieve parameters regelmatig bepaald worden, niet haalbaar door enorme kosten. Dus voorlopig zullen we ons zeker moeten focussen op diermodellen die de humane situatie zo veel mogelijk nabootsen en op verschillende nieuwe geavanceerde technieken die ook in het klinisch onderzoek gebruikt worden.

Chapter 8

List of abbreviations
List of publications
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List of frequently used abbreviations

AA	arachidonic Acid
Aβ	amyloid-β
ABCA1	ATP-binding cassette transporter A1
ACAT	acyl-CoA acyltransferase
ACg	anterior cingulate gysrus
AD	Alzheimer's Disease
AdoHcy	s-adenosylhomocysteine
AdoMet	s-adenosylmethionine
ApoE	apolipoprotein E
APP	amyloid precursor protein
AUP	area under peak
BBB	blood brain barrier
BSA	bovine serum albumin
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CAA	Cerebral Amyloid Angiopathy
CATCH	critically attained threshold of cerebral hypoperfusion
CBF	cerebral blood flow
CBV	cerebral blood volume
CNS	central nervous system
CSF	cerebrospinal fluid
D2O	deuterium oxide
DAB	diaminobenzidine
DG	dentate gyrus
DHA	docosahexaenoic acid
EFA	essential fatty acids
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas-chromatography-mass spectrometry
GLUT1	glucose transporter-1
HDL	high density lipoprotein
I.P.	intra peritoneal
LC-MS	liquid-chromatography-mass spectrometry
LCPUFA	long chain poly unsaturated fatty acids
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LRP	low density lipoprotein receptor related protein
LXR	liver-x-receptor
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MUFA	mono unsaturated fatty acids
n3	omega-3 fatty acids
n6	omega-6 fatty acids

PB	phosphate buffer
PBS	phosphate buffered saline
PLA	prelimbic area
PS1	presenilin 1
PUFA	poly unsaturated fatty acids
ROI	region of interest
STD	standard
TTP	time to peak
TWD	typical western diet
USPIO	ultra small particles of iron oxide

List of publications

Original papers

Hooijmans CR, Rutters F, Dederen PJ, Gambarota G, Veltien A, van Groen T, Broersen LM, Lütjohann D, Heerschap A, Tanila H, Kiliaan AJ. Changes in cerebral blood volume and amyloid pathology in aged Alzheimer APP/PS1 mice on a docosahexaenoic acid (DHA) diet or cholesterol enriched Typical Western Diet (TWD). Neurobiol Dis. 2007 Oct;28(1):16-29.

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Hooijmans CR, Blom HJ, Oppenraaij-Emmerzaal D, Ritskes-Hoitinga M, Kiliaan AJ. S-adenosylmethionine and s-adenosylhomocysteine levels in the aging brain of APP/PS1 Alzheimer mice (Submitted).

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Hooijmans CR, Doedée AMCM, Mutsaers MPC, Dederen PJ, Kuipers A, Broersen LM, Kiliaan AJ. The effects of cholesterol and DHA containing diets on cognition, β-amyloid deposition, atrophy and amount of synapses in 15-month-old APP/PS1 mice (In preparation).

Abstracts

Hooijmans CR, Rutters F, Dederen PJ, Heerschap A, Veltien A, Gambarota A, Tanila H, van Groen T, Broersen LM, Kiliaan AJ. Relationship between cholesterol, cerebrovascular degeneration and beta amyloid deposition. *poster at the 4th Endo-Neuro-Psycho Meeting 2005, Doorwerth, the Netherlands*

Hooijmans CR, Rutters F, Dederen PJ, Heerschap A, Veltien A, Gambarota A, Tanila H, van Groen T, Broersen LM, Kiliaan AJ. Relationship between cholesterol, cerebro-vascular degeneration and β amyloid deposition. A vascular hypothesis. *Poster at the 2nd Congress of The International Society for Vascular Behavioural and Cognitive Disorders 2005, Florence, Italy.*

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Kiliaan AJ. GLUT-1 transporter density in the brains of APP/PS1 mice. *Poster at the 5th Endo-Neuro-Psycho Meeting 2006, Doorwerth, the Netherlands.*

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Hooijmans CR, Rutters F, Dederen PJ, Heerschap A, Tanila H, van Groen T, Broersen LM, Kiliaan AJ Changes in Cerebral Hemodynamics and Alzheimer Pathology in Aged APP/PS1 Mice Supplemented with Docosahexanoic Acid (DHA) and Cholesterol Enriched Diets. *Poster at the Alzheimer's Association International Conference on Alzheimer's Disease 2006, Madrid, Spain.*

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Hengeveld, YA, **Hooijmans CR**, Kuijper FJ, Kuesters B, Roubos EW, Kiliaan AJ, Kozicz LT. Decreased activity of Einger-westphal urocortin1 system in a mouse model for Alzheimer's disease. *Poster at the 6th Endo-Neuro-Psycho Meeting 2007, Doorwerth, the Netherlands.*

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Reijmer YD, *Hooijmans CR*, Basten E, van der Zee CEEM, Heerschap A, van Groen T, Broersen LM, Tanila H, Kiliaan AJ. Dietary lipids influence spatial memory, cerebral blood volume and amyloid pathology in the APP/PS1 mouse model of Alzheimer's disease. *Poster at the fifth International Congress on Vascular Dementia 2007, Budapest, Hungary.*

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Hooijmans CR, Dederen PJ, Reijmer YD, Brouwer KM, van der Zee, CEEM, van Groen T, Broersen LM, Lütjohann D, Heerschap A, Kiliaan AJ. DHA and cholesterol diets influence Alzheimer pathology and cognition via the cerebral vasculature in APPswe/PS1dE9 mice. *Poster at the 7th Endo-Neuro-Psycho Meeting 2008, Doorwerth, the Netherlands.*

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Hooijmans CR, Dederen PJ, Reijmer YD, Brouwer KM, van der Zee, CEEM, van Groen T, Broersen LM, Lütjohann D, Heerschap A, Kiliaan AJ. DHA and cholesterol diets influence Alzheimer pathology and cognition via the cerebral vasculature in APPswe/PS1dE9 mice. *Poster at the Alzheimer's Association International Conference on Alzheimer's Disease 2008, Chicago, USA.*

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Hooijmans CR, Rutters F, Dederen PJ, Gambarota G, Veltien A, van Groen T, Broersen LM, Lütjohann D, Heerschap A, Tanila H, Kiliaan AJ. Changes in cerebral hemodynamic and Alzheimer pathology in aged APP/PS1 mice supplemented with DHA and cholesterol enriched diets. *Oral presentation at the 5th Endo-Neuro-Psycho Meeting 2006, Doorwerth, the Netherlands.*

Hooijmans CR, van der Zee CEEM, Dederen PJ, Reijmer Y, Brouwer K, van Groen T, Broersen LM, Lütjohann D, Heerschap A, Kiliaan AJ. The effects of dietary cholesterol and DHA on hemodynamic parameters and Alzheimer pathology in APP/PS1 mice. *Oral presentation at the 6th Endo-Neuro-Psycho Meeting 2006, Doorwerth, the Netherlands.*

Hooijmans CR, van der Zee CEEM, Dederen PJ, van Groen T, Broersen LM, Lütjohann D, Heerschap A, Kiliaan AJ. The effects of dietary cholesterol and DHA on Cerebral vasculature, Alzheimer pathology and cognition in APP/PS1 mice: "Resolving the chicken and egg issue". *Oral presentation at the annual Maikammer lipid meeting, Maikammer, Germany.*

Hooijmans CR, van der Zee CEEM, Dederen PJ, van Groen T, Broersen LM, Lütjohann D, Heerschap A, Kiliaan AJ. The role of high cholesterol and fish oil in the development of AD. *Nominated oral presentation at Alzheimer Centrum Nijmegen, 2008, Nijmegen, the Netherlands.*

Curriculum vitae

Carlijn Regina Hooijmans werd geboren op 16 maart 1981 in Leidschendam. Na het behalen van haar VWO diploma aan het Adelbert College te Wassenaar is Carlijn in 1999 begonnen met de studie Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit van Nijmegen. Al gauw werd het duidelijk dat haar interesse vooral uitging naar het ontstaan en beloop van ziekten bij de mens, en daarom koos zij voor het hoofdvak Pathobiologie. Haar brede interesse zorgde er voor dat zij de kans pakte om haar studie uit te breiden van 4 naar 5 jaar, en zij koos voor 3 bijvakken (Neurologie, Hematologie en Oncologie) in plaats van 1 bijvak. Gedurende haar studie voltooide zij 2 stages. Tijdens haar eerste stage van 6 maanden op de afdeling Psycho-Neuro-Farmacologie, werd de rol van dopamine receptoren in de nucleus accumbens in het anti-parkinson effect van SKF83959 bestudeerd. Zij werd hierbij begeleid door M.M. Verheij en Prof. Dr. A.R. Cools. Haar tweede stage van 6 maanden vond plaats op de afdeling Reumatologie van het Nijmegen Centre for Molecular Life Sciences en werd begeleid door Drs. K. Nabbe en Dr. P.L.E.M van Lent. Zij heeft daar onderzoek gedaan naar de rol van zuurstofradicalen in Fcγ-receptor aangestuurde activatie van macrofagen. Gedurende haar studententijd heeft zij eerstejaars studenten begeleid, en is zij voorzitter geweest van de reiscommissie van de Medische Faculteit Vereniging Nijmegen. Tevens is zij student-lid geweest van de opleidingscommissie Biomedische Wetenschappen onder leiding van Dr J.G.M. Kooloos en later Prof. Dr. J.L. Willems. In augustus 2004 behaalde Carlijn haar doctoraal diploma.
Op 1 september 2004 is zij begonnen aan haar promotie onderzoek, beschreven in dit proefschrift, op de afdeling Anatomie van de Radboud Universiteit Nijmegen, onder begeleiding van Dr. A.J. Kiliaan. Tijdens haar promotie onderzoek heeft zij vele studenten en stagiaires begeleid en presenteerde ze haar werk op nationale en internationale congressen.
Sinds 1 oktober 2008 is zij werkzaam in het Centraal Dieren Laboratorium Nijmegen als post-doc onderzoeker, onder leiding van Prof. Dr. M.J. Ritskes-Hoitinga en Dr. Ir. B. Savenije, met als kerntaak het ontwikkelen van richtlijnen voor meta-analyse van dierproeven ten behoeve van betere wetenschap en de implementatie van de 3V's (Vervanging, Verfijning en Vermindering van proefdiergebruik).

Naast haar universitaire loopbaan is Carlijn tevens betrokken bij ontwikkelingssamenwerking. Vanaf september 2001 is zij jaarlijks werkzaam als vrijwilliger in "Open Arms Malawi", en draagt zij bij aan de verzorging van HIV weeskinderen. Tevens heeft zij Open Arms in Nederland geregistreerd als Algemeen Nut beogende Instelling en is zij verantwoordelijk voor de fondsenwerving van dit project in Nederland.

Dankwoord

Nu, na 4 jaar hard werken en veel plezier, het laatste (en waarschijnlijk het meest gelezen) gedeelte van dit proefschrift. Aangezien er vele mensen betrokken zijn geweest bij de totstandkoming van dit proefschrift wil ik hen hier bedanken voor wat zij, op wat voor manier dan ook, hebben bijgedragen.
Maar het waren natuurlijk niet alleen mensen die mij geholpen hebben, want zonder mijn muisjes op typisch westerse (hamburger) en visrijke voeding, waren de nieuwe bevindingen van dit proefschrift niet aan het licht gekomen en was er überhaupt geen proefschrift.

Dit gezegd hebbende, wil ik nu overgaan tot het bedanken van allereerst mijn co-promotor, Amanda en promotoren Arend en Merel.
Amanda, zonder jou was er geen proefschrift. Door jouw enorme passie voor onderzoek heb je ook mijn onderzoeksvuur aangestoken. Ik hoop dan ook in mijn toekomst in het onderzoek werkzaam te blijven, en ooit ook zelf zo'n mooi project te kunnen opstarten en begeleiden. Ik heb heel veel van je geleerd, en wens je alle succes toe in de toekomst.
Na 2,5 jaar kreeg ik eindelijk een definitieve promotor. Merel, ik heb de werkbesprekingen als zeer prettig ervaren en waardeer enorm je enthousiasme en de rust die je uitstraalt en vervolgens ook weet over te brengen. Ik ben erg blij dat ik jouw team op het CDL mocht komen versterken.
Arend, bedankt voor de kennis die je mij hebt bijgebracht met betrekking tot de bijzondere wereld van de "magnetische resonantie". Tevens ben ik erg blij met al die uren dat ik heb mogen meten in het CDL waar ik ook enorm veel van heb geleerd. Maar dit had niet zo leuk en leerzaam geweest zonder de mentale en technische ondersteuning van de mensen uit de radiologie-groep. Bob en Andor bedankt daarvoor, en ook voor alle lekkere appeltaarten, moorkoppen en gezelligheid.

Maar er zijn natuurlijk nog veel meer mensen die bedankt moeten worden. Jos, jouw hulp bij het maken van goede tiff-files, uitleg over de immunohistochemie en het gebruik van de Neurolucida hebben ervoor gezorgd dat ik vele manuscripten heb kunnen schrijven en illustreren. Bovendien heb ik genoten van alle koffiepauzes waarin de belangrijke zaken van de werkvloer en het leven werden besproken.
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Al dacht ik mijn bureau (en directe omgeving daarvan) al erg gezellig gemaakt te hebben

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