

Dissertation

**Impact of ApoB-100 expression on
cognition and brain pathology in
WT and hAPPsl mice**

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis.

Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”

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Abbreviations

ABCA1	ATP-binding cassette transporter type A1
ABCG1	ATP-binding cassette transporter type G1
Abeta	amyloid beta
ACAT	Acetyl-CoA-Acetyltransferases
AD	Alzheimer's disease
AICD	APP intracellular domain
ALT	Alanine-aminotransferase
ApoB	apolipoprotein B
ApoE	apolipoprotein E
APP	Abeta Precursor Protein
ASCVD	atherosclerotic cardiovascular disease
BACE	beta-APP-cleaving-enzyme or beta-secretase
BBB	blood-brain barrier
BHT	butylated Hydroxytoluene
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CAA	cerebral amyloid angiopathy
CAD	coronary artery disease
CBF	cerebral blood flow
CE	cholesterol esters
CFC	Contextual Fear Conditioning
CNS	central nervous system
CSF	cerebrospinal fluid
CYP27A1	Cholesterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
DEA	diethylamine
EOAD	early-onset AD
ER	endoplasmatic reticulum
FA	formic acid
FFA	free fatty acid
FDG-PET	Fluorodesoxyglucose Positron-Emission-Tomography
FTDP-17	Parkinsonism-linked to chromosome 17
GC	Gas Chromatography
GFAP	Glial fibrillary acidic protein
hAPP	human amyloid precursor protein
HDL	high-density lipoprotein
HFD	high fat diet
HMGCoA-R	3-hydroxy-3-methylglutaryl conenzyme A reductase
HPLC	high pressure liquid chromatography
LDL	low-density lipoproteins
LDLR	LDL-receptor
LOAD	late-onset AD
LRP1	LDL-receptor related protein 1
LXR	liver X receptors
MDA	1,1,3,3 Tetramethoxypropane or Malondialdehyde

MSD	Mesoscale Discovery
MT	microtubules
MWM	Morris Water Maze task
ND	normal/standard diet
NFT	Neurofibrillary Tangles
OD	Optical density
OF	Open Field test
PD	Parkinson's Disease
PHF	Paired Helical Filaments
PL	phospholipids
SRE	sterol regulatory element
SREBP	sterol regulated element binding protein
TG	triglycerides
THB	Tissue Homogenization Buffer
TMCS	trimethylchlorosilane
VLDL	very-low-density lipoproteins
WT	wild type/non-transgenic
24S-OHC	24S-hydroxycholesterol
27-OHC	27-hydroxycholesterol

Chapter 1

Zusammenfassung

Die Alzheimer'sche Erkrankung (AD) ist, wie viele andere neurodegenerative Krankheiten, durch Aggregation und Ablagerung bestimmter Proteine gekennzeichnet. Im Fall von AD handelt es sich dabei hauptsächlich um sogenannte extrazelluläre Amyloid Plaques und intrazelluläre neurofibrilläre Bündel.

Im Allgemeinen kann diese Erkrankung in eine erbliche, sehr früh auftretende Form (EOAD), sowie eine spät einsetzende Form (LOAD) unterteilt werden. Während EOAD durch bekannte Mutationen im Amyloid Precursor Protein (APP) Gen sowie in den Genen für Preseniline ausgelöst wird (Tanzi and Bertram, 2001), sind die Ursachen für das Entstehen von LOAD, der weitaus häufiger auftretenden Form, bis heute ungeklärt.

Besonders wegen der bei EOAD Patienten betroffenen Gene, wurde in den letzten Jahren verstärkt auf den Gebieten der Amyloid beta (Abeta) Produktion, Ablagerung und dem Abbau geforscht. Die daraus entstandene und momentan vorherrschende "Amyloid Hypothese" beschreibt ein Ungleichgewicht zwischen Produktion und Abbau von Abeta als Auslöser der gesamten Krankheits-Kaskade (Hardy and Selkoe, 2002). Warum es aber in LOAD Patienten zu einem solchen Ungleichgewicht kommt ist weiterhin ungeklärt.

Immer mehr Forschungsergebnisse deuten darauf hin, dass Hypercholesterinämie und anderer vaskuläre Faktoren, einen großen Einfluss auf die Entstehung und den Fortschritt von LOAD haben (Humpel, 2011). Es wurde mehrfach gezeigt, dass AD und Atherosklerose viele Risikofaktoren teilen, wie etwa Diabetes, Bluthochdruck oder Hyperlipidämie (Skoog et al., 1999). Auch Träger des Apolipoprotein E4 (ApoE4) Alleles haben ein erhöhtes Risiko sowohl an AD als auch an Atherosklerose zu erkranken (Corder et al., 1993).

Neben ApoE wurde aber auch Apolipoprotein B (ApoB) mit AD und Atherosklerose in Verbindung gebracht (Kuo et al., 1998, Martins et al., 2009).

ApoB ist der Hauptproteinbestandteil der Lipoproteine geringer Dichte (low density lipoproteins, LDL), ein wichtiger Transporter von Cholesterin, kommt aber auch in den

Triglycerid-reichen “very-low-density lipoproteins” (VLDL) vor. Interessanterweise haben AD und Atherosklerose Patienten ein sehr ähnliches, pathologisches, Plasmalipid Profil und weisen erhöhte LDL sowie gesenkte HDL Levels auf (Kuo et al., 1998, Sparks et al., 2000). In einer post-mortem Studie an AD Patienten wurde zusätzlich gezeigt, dass LDL, und somit auch ApoB, Levels mit Abeta1-42 Konzentrationen im Gehirn korrelieren (Kuo et al., 1998).

Außerdem konnten verschiedene zerebrovaskuläre Veränderungen in AD Gehirnen festgestellt werden: verminderte Dichte der Mikrovaskulatur, Durchlässigkeit der Blut-Hirn-Schranke (BBB) und zerebrale Amyloid Angiopathie (CAA) (Farkas and Luiten, 2001).

Bis heute ist die Frage ob und wie solche vaskulären Veränderungen zur Pathologie von LOAD beitragen nicht geklärt.

In dieser Studie werden die Effekte einer Überexpression von humanem ApoB, sowohl alleine als auch in Kombination mit der zerebralen Expression des humanen Amyloid Precursor Proteins (hAPP), auf Kognition und AD-Pathologie in Mäusen untersucht.

Die dazu verwendete ApoB-100 Maus-Linie exprimiert das gesamte humane ApoB-100 Gen unter seinem natürlichen Promotor (Bjelik et al., 2006, Callow et al., 1994). Diese Tiere wurden ursprünglich als Mausmodell für Hyperlipidämie und Atherosklerose entwickelt und weisen ein Plasmalipid Profil auf, welches dem eines humanen Patienten näher kommt. Da Mäuse, durch ihr Plasmalipid Profil mit sehr hohen HDL- und kaum messbaren LDL Levels, unter normalen Umständen vor Hypercholesterinämie und Atherosklerose geschützt sind (Breslow, 1996), führt die Expression von zusätzlichem hApoB zu verstärkt atherogenen Bedingungen (Callow et al., 1995). Es wurde bereits gezeigt, dass ApoB-100 Tiere Läsionen in der zerebralen Mikrovaskulatur, Veränderungen des APP Metabolismus sowie plaque-artige Akkumulationen in den Gehirnen aufweisen. (Bereczki et al., 2008, Bjelik et al., 2006, Sule et al., 2009)

Die zweite Mauslinie in dieser Studie, hAPPSl transgene Tiere, sind ein gut beschriebenes Modell der familiären Form von AD. Diese Tiere exprimieren das humane APP751 mit den sogenannten London (V717I) und Swedish (KM670671NL) Mutationen unter der Kontrolle des murinen Thy-1 Promotors (Rockenstein et al., 2001). Mäuse der hAPPSl Linie zeigen sehr starke zerebrale Amyloid Pathologie sowie besonders früh auftretende kognitiven Defizite (Havas et al., 2011).

Im Zuge dieser Studie wurden diese beiden Mauslinien, hAPPSl und ApoB-100, miteinander gekreuzt um einen vaskulären Risikofaktor in das bestehende AD Mausmodell einzubringen. Damit sollte einerseits der Pathologie eines “alten Menschen” näher gekommen, andererseits das Zusammenwirken der beiden Pathologien untersucht werden. Die Effekte der ApoB-100 Überexpression und mögliche Synergismen in doppelt transgenen ApoBxAPP Tieren, auf verschiedene biochemische und histologische Marker sowie das Verhalten dieser Tiere wurden analysiert.

In einer weiterführenden Untersuchung wurde zusätzlich der Einfluss verschiedener Diäten auf die kognitiven Fähigkeiten und AD Pathologie aller Genotypen untersucht.

Die erhaltenen Ergebnisse zeigen, dass alleine die Expression von hApoB-100 zu einer Verschlechterung der kognitiven Fähigkeiten führt, was möglicherweise auf erhöhte Lipid Peroxidation zurück zu führen ist.

In doppelt transgenen ApoBxAPP Tieren konnte keine Verschlechterung der kognitiven Fähigkeiten, durch das zusätzliche hApoB-100, im Vergleich zu einfach-transgenen hAPPSl Tieren festgestellt werden. Trotzdem weisen ApoBxAPP Mäuse mehrere pathophysiologische Veränderungen auf, die sie möglicherweise zu einem vollständigeren AD Maus-Modell machen. Dazu gehören das veränderte, atherogene Plasmalipid Profil, erhöhte Lipid Peroxidation sowie Durchlässigkeit der Blut-Hirn-Schranke.

Die zusätzliche Fütterung mit einer fett-reichen Diät (HFD) scheint einen Einfluss auf den Abtransport von Abeta über die Blut-Hirn-Schranke zu haben, was als erniedrigtes Plasma-Abeta in Kombination mit erhöhten zerebralen Abeta Levels gemessen wurde. Verhaltenstests der Tiere auf HFD lieferten divergente Ergebnisse, welche nur auf eine Beeinträchtigung des emotionalen, nicht aber des räumliches Lernens hin deuten.

Untersuchungen der kortikalen mRNA Expression hoben die Wichtigkeit von APP im zerebralen Lipid Metabolismus hervor und deuten eine mögliche Verbindung zwischen APP, mit den zugehörigen Spaltprodukten wie Abeta, und Veränderungen in Menge und Metabolismus von Cholesterin hin.

Chapter 2

Summary

“Alzheimer’s disease (AD), a progressive neurodegenerative disease, highly associated to accumulation and deposition of amyloid beta (Abeta) peptides, is the most common form of dementia (Burns et al., 2002, Ferri et al., 2005). Generally, the disease can be separated into (i) heritable early-onset AD (EOAD) linked to mutations in the genes for Abeta Precursor Protein (APP) as well as presenilins (Tanzi and Bertram, 2001) and (ii) late-onset AD (LOAD) with unknown origin, which accounts for more than 95% of all AD cases (Harman, 2006).” (Löffler et al. 2013)

Based on the mutations of the genes found to be affected in EOAD patients, the main effort in AD research focused on the investigation of Abeta generation, processing and clearance.

Although the generally accepted ‘amyloid cascade hypothesis’ implies an imbalance between increased production and decreased clearance of Abeta as the initiator of AD (Hardy and Selkoe, 2002), the underlying causes for LOAD remain unclear.

“In the last years increasing evidence suggests that hypercholesterolemia and other vascular factors may contribute to the pathogenesis of LOAD (Humpel, 2011).

Notably, AD, atherosclerosis and especially coronary artery disease (CAD) share many risk factors, such as diabetes, hypertension, hyperlipidemia and the inheritance of an apolipoprotein E4 (ApoE4) allele (Corder et al., 1993, Jarvik et al., 1995, Skoog et al., 1999). In addition to the ApoE genotype, apolipoprotein B (ApoB) is also associated with both CAD as well as AD (Kuo et al., 1998, Martins et al., 2009).

ApoB-100 is known as the primary apolipoprotein of cholesterol-carrying low-density lipoproteins (LDL) and triglyceride-rich very-low-density lipoproteins (VLDL). ApoB-48, a truncated version of ApoB-100, is predominantly found in chylomicrons. CAD and AD patients share a very similar pathological plasma lipid profile, exhibiting increased levels of LDL along with decreased high-density lipoprotein (HDL) levels (Kuo et al., 1998, Sparks et al., 2000).

Intriguingly, a post-mortem study of AD patients showed that LDL and ApoB-100 levels positively correlate with brain Abeta42 levels (Kuo et al., 1998). Additionally, several cerebrovascular abnormalities have been identified in AD brains: decreased microvascular density, an impaired blood-brain barrier and cerebral amyloid angiopathy (CAA) (Farkas and Luiten, 2001).

So far it is still under debate to what extent these pathologies contribute to the development of LOAD and how they influence the time course of progression. In this study the effects of ApoB-100 expression, either alone or in combination with cerebral expression of human amyloid precursor protein (hAPP) and in combination with different diets, on cognitive functions and brain pathology were assessed.

ApoB-100 animals, overexpressing the entire 43 kb human ApoB-100 gene including its natural human promoter (Bjelik et al., 2006, Callow et al., 1994) were originally designed as a mouse-model of hyperlipidemia and atherosclerosis. Since the murine plasma lipid profile has a very low LDL to HDL ratio compared to the human profile, wild type mice are protected against hypercholesterolemia and are usually not affected by atherosclerosis (Breslow, 1996).

It has been shown that overexpression of human ApoB-100 in mice shifted the lipoprotein profile to a more atherogenic phenotype. Accordingly, atherosclerotic lesions were detected at branch points of the aorta, especially as a consequence of a high-cholesterol diet (Callow et al., 1995). Recent publications showed that ApoB-100 overexpressing animals display cerebral microvascular lesions, changes in APP metabolism and some plaque-like accumulations in their brains (Bereczki et al., 2008, Bjelik et al., 2006, Sule et al., 2009).

In contrast, the hAPP^{sl} transgenic mouse line is a well described model of familial AD. These animals are highly overexpressing human APP751 with London (V717I) and Swedish (KM670671NL) mutations under the control of the murine Thy-1 promoter as published for a very similar model (TASD41) by Rockenstein et al. (2001). The hAPP^{sl} mice display severe brain amyloid pathology associated with early onset progressive memory deficits (Havas et al., 2011).

The aim of the present study was to crossbreed hAPP^{sl} with ApoB-100 mice to introduce a vascular risk factor into the existing AD mouse model to better mimic the situation in elderly people and to investigate the interplay between the two pathologies. Effects of ApoB-100

overexpression, but also possible synergisms in ApoBxAPP animals, on several biochemical and histological markers as well as the behavioural phenotype were analyzed in an age dependent manner. “ (Löffler et al., 2013)

In the second part of the study, animals of every genotype were fed either a high-cholesterol or a standard diet for three months and the impact of this additional challenge on cognitive function and AD pathology was evaluated.

Results of this study show that ApoB-100 overexpression induces memory decline and increases cerebral lipid peroxidation and amyloid beta levels compared to WT animals.

While double-transgenic ApoBxAPP animals did not develop more distinct cognitive deficits than single-transgenic hAPPsl littermates, hApoB-100 expression caused additional pathophysiological properties, like high LDL and lowered HDL-cholesterol levels, early lipid peroxidation and blood-brain-barrier (BBB) leakage.

“Thus, our results indicate that ApoBxAPP mice might better reflect the situation of elderly humans than hAPPsl overexpression alone.” (Löffler et al., 2013)

A High-fat diet (HFD) seemed to impact on cerebral Abeta clearance, seen as decreased plasma Abeta together with increased levels of Abeta in the brain. Behavioural testings of HFD fed animals revealed diverse results, pointing towards changes in emotional behaviour, but not in spatial learning.

Investigations of cortical mRNA expression patterns underlined the importance of APP in cerebral lipid metabolism and showed a possible connection between APP (with its proteolytic products) and changes in the amount and metabolism of cholesterol.

Chapter 3

Introduction

3.1 Alzheimer's Disease

3.1.1 Definition, epidemiology and history

In the last years, neurodegenerative diseases like Alzheimer's disease (AD) affected more people than ever before. The increased life expectancy, especially in western countries, leads to an increasing number of persons achieving an age at which neurodegenerative disorders occur more frequently (Selkoe, 2001). Worldwide more than 4 million new dementia cases are diagnosed annually and the total number of patients is estimated to increase up to 80 million people by 2040 (Ferri et al., 2005). Since AD accounts for 60-70 % of all dementia cases, the primary attention of AD research has, therefore, been focused on the characterization of mechanisms that underlie the disease.

Generally the disease manifests in two forms, early-onset AD (EOAD) occurring in persons younger than 65 years and late-onset AD (LOAD) which affects people older than 65 years. Only a small percentage of the patients are affected by EOAD, an inheritable form of AD with a more severe pathology associated with mutations in the genes coding for amyloid precursor protein (APP) or presenilin 1 and 2 (Tanzi and Bertram, 2001).

Although LOAD accounts for more than 95% of all AD cases, the triggering factors remain unknown. Accordingly, no medication exists to treat the underlying causes of the disease. Currently, the available Alzheimer's drugs, like cholinesterase inhibitors, only alleviate the symptoms, temporarily lessen memory problems in about 50% of treated patients. The main risk factor for developing LOAD is age. However, the inheritance of an apolipoprotein e4 allele (APOE e4) is an additional well established risk factor (Corder et al., 1993, Skoog et al., 1999).

At the beginning of the 20th century the psychiatrist Alois Alzheimer was the first to describe this particular disease on the case of Auguste, a 51 year old woman who developed a rapid memory decline and had become disorientated in time and space (Alzheimer et al., 1907). After histological examinations of the affected brain after Augustes death in 1906, Alzheimer described dense deposits in the degenerated limbic and cerebral cortex.

These deposits as well as neuronal loss are nowadays still recognized as typical hallmarks of AD. Many neurodegenerative diseases like Parkinson's Disease (PD), frontotemporal dementia with Parkinsonism-linked-to-chromosome-17 (FTDP-17) and AD share this common feature: aggregation and deposition of abnormally modified and misfolded proteins.

Aggregated forms of the protein alpha-synuclein occur in the substantia nigra of PD patients, so called Lewy-Bodies (Clayton and George, 1999, Giasson et al., 1999). In contrast FTDP-17 (Foster et al., 1997) is characterized by aggregates consisting of the microtubule associated protein Tau, which also plays a crucial role in AD.

3.1.2 Neuropathology of AD

Besides progressive neurodegeneration, the two pathological hallmarks of AD are neuritic plaques and neurofibrillary tangles (NFT).

Neuritic Plaques are extracellular deposits around neuronal cells and principally consist of amyloid beta (Abeta), particularly the 1-40 and 1-42 amino acid peptides (Abeta1-40 and Abeta1-42). These peptides are degradation products of the membrane-bound Amyloid-Precursor-Protein (APP). In this pathological pathway, APP is first cleaved by an enzyme known as BACE (beta-APP-cleaving-enzyme or beta-secretase) and further processed by the enzymatic complex gamma-secretase (Selkoe, 1994). Especially Abeta1-42 is considered to be neurotoxic and seems to play a central role in the formation of these plaques (Younkin, 1995).

The biological function of APP is still not fully resolved. However, also a non-amyloidogenic pathway is known, where APP is cleaved by alpha-secretase into soluble alpha-APP (sAPPalpha) and a shorter C-terminal fragment. After alpha-secretase cleavage, no amyloid peptides are generated (see Fig. 1).

In both pathways an APP intracellular domain (AICD) is released into the cytoplasm after gamma-secretase processing. It is still unclear how these pathways are regulated, however many factors including mutations, hormone status and diet seem to be involved.

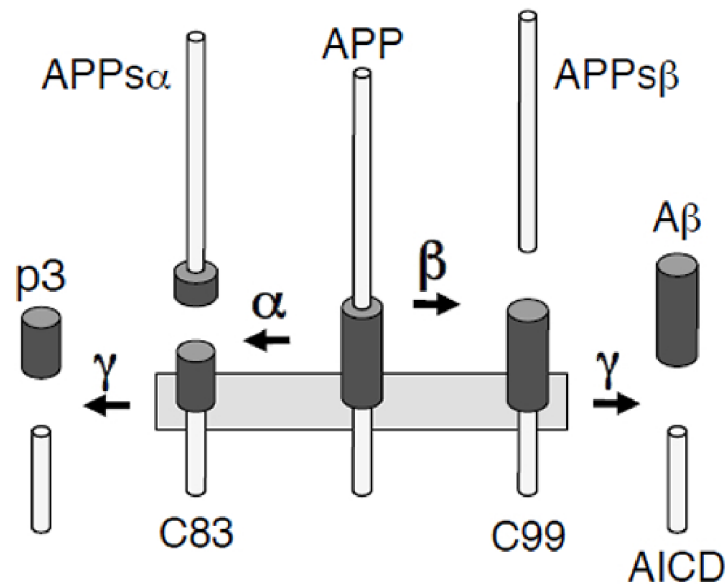


Figure 1: Cleavage products and involved secretases in APP processing. The amyloidogenic cleavage of APP happens via beta- and gamma-secretase (right) while the non-amyloidogenic cleavage is provoked by alpha-secretase (left). APP intracellular domain (AICD) is generated in both pathways (Lichtenthaler, 2012).

Less than 5% of AD patients are affected by inheritable forms of the disease. These patients carry mutated forms of APP or presenilin-1/presenilin-2, which are parts of the enzymatic complex gamma-secretase, all showing disturbances in APP processing. Two specific mutations in APP, the so called “Swedish” and “London” mutations, are described in detail in section 3.3.2.

Neurofibrillary Tangles (NFT) are the second histological hallmark of AD. NFT occur intraneuronally and represent protein aggregates of mainly hyperphosphorylated Tau. Tau is a microtubule-associated protein and it is known to play a role in promoting assembly and stabilization of microtubules (Wang and Liu, 2008). In a non- or low-phosphorylated state, Tau binds to microtubules (MT) and stabilizes them, while phosphorylation of Tau lowers its affinity to bind to MTs. In AD the dynamic transaction of phosphorylation and dephosphorylation of

Tau becomes imbalanced and shifted towards increased phosphorylation. Several serine/threonine kinases are involved in this process of hyperphosphorylation. Abnormally hyperphosphorylated Tau accumulates at first to so called Paired Helical Filaments (PHF), which further aggregate as neurofibrillary tangles. These incidents tend to result in destabilized microtubules and accordingly impair the axonal transport (Wang and Liu, 2008).

Although all processes, especially the underlying causes, are not yet fully understood, neuritic plaques and NFTs together with many other factors, like inflammatory processes (Eikelenboom et al., 2006) and oxidative stress (Akiyama et al., 2000) lead to cortical and hippocampal neuronal and synaptic loss.

Neuropathological stages: AD brains are affected by so called selective neuronal vulnerability, i.e. neurodegeneration that selectively targets subpopulations of neurons, leading to the progressive failure of defined brain systems. Especially acetylcholine-releasing neurons in the basal forebrain degenerate selectively in AD brains (Coyle et al., 1983).

Braak and Braak (1991) distinguished between six neuropathological stages of AD based on NFT occurrence, starting with alterations in the entorhinal cortex. At this early pathological stage (stadium I and II) the patients do not yet display cognitive disturbances. During the “limbic” stages III and IV, the hippocampal formation as well as the temporoparietal cortex are affected. Crucial subsets of pyramidal cells and their projections are particularly disordered.

In the late isocortical stages V and VI, not only sensory association areas but also primary sensory and motor areas are destructed and the full clinical appearance of AD dementia has developed.

3.1.3 Vascular contributions to AD

The majority of AD cases are sporadic forms with unknown origin. In the last 20 years, more and more evidence accumulated that vascular risk factors contribute to the pathogenesis of AD (Humpel, 2011). A high rate of dementias is caused by mixed, AD and vascular, pathology especially in older patients.

Several cerebrovascular abnormalities have been identified in AD brains: decreased microvascular density, thickening of basement membranes, endothelial damage, vessels that express inflammatory markers, impaired glucose transport across the blood-brain barrier (BBB), capillaries with fewer branches, changes in vessel diameter, atherosclerotic plaques or cerebral amyloid angiopathy (Farkas and Luiten, 2001).

The question arose whether these changes are an initial cause for the onset of AD or if these changes occur as a result of the disease in late stages. Nevertheless, all of the described vascular changes have a negative effect on brain pathology and definitely influence normal neuronal function.

Brain microvascular endothelial cells together with astrocytes are the main components of the BBB, which restricts the passage of foreign substances from the bloodstream to protect the brain from infections and maintain a constant environment. It was shown that damage of the cerebral microvasculature leading to an increased BBB permeability plays an important role in AD (Bowman et al., 2007). Capillaries from AD brains were shown to be confronted with high levels of inflammatory mediators, chemokines and cytokines (Grammas et al., 2006), indicating a strong cerebrovascular involvement of neuroinflammation in AD (Zlokovic, 2005). Brain endothelia often degenerate in AD and amyloid deposits accumulate on the outer side of the basement membrane, inducing local neuroinflammatory responses.

Abeta was shown to be a potent vasoconstrictor (Thomas et al., 1996) and high levels may lead to degeneration of smooth muscle cells (Van Nostrand et al., 2001), which contribute to cerebrovascular pathology and hemorrhagic stroke.

Most notably, it has been shown that vascular clearance of Abeta across the BBB is impaired in AD brains. And, in line, Abeta brain depositions and formation of amyloid lesions in capillaries are increased (Deane et al., 2004). A current theory implies, that Abeta plaques in AD patients have vascular origin and are initiated within degenerating blood vessels (Zlokovic, 2005). The fact that Abeta can cause vascular damage and BBB dysfunction again raises the question if changes in APP processing are triggering or downstream events in the time course of the disease.

In addition, decreased cerebral blood flow (CBF) and reduced glucose metabolism in the medial temporal and parietal lobes (FDG-PET), which can be measured years before the onset of AD (Mosconi et al., 2010), argue for a vascular hypothesis in AD. Larger cerebral arteries in AD

brains do not develop amyloid lesions but are often affected by atherosclerotic plaques, which reduce blood supply to the brain (Roher et al., 2004). the possibility exists, that reduced CBF in AD brains due to atherothrombotic lesions create a chronic hypoperfusion state leading to an impaired provision of glucose and oxygen.

It is also interesting that AD and atherosclerosis, especially coronary artery disease (CAD), share many risk factors, such as diabetes, hypertension, dyslipidemia and the possession of at least one ApoE4 allele. Also the very similar plasma lipoprotein profile of CAD and AD patients, with increased levels of total cholesterol and low-density lipoproteins (LDL) along with decreased high-density lipoprotein (HDL) levels underlines the connection between vascular changes, lipid metabolism and LOAD (Jarvik et al., 1995, Sparks et al., 2000).

3.2 Lipid metabolism in the pathogenesis of AD

3.2.1 Lipoproteins

Lipoproteins are lipid transport vesicles that consist of lipid and protein components and are based on the biphasic principle of micelles (Bassett et al., 2000, Martins et al., 2009). To ensure solubility of hydrophobic lipids in an aqueous environment, lipid components of the inner hydrophobic core (esterified cholesterol; CE and triglycerides; TG) are coated with phospholipids (PL) and free cholesterol as surface components. Specific protein components, the apolipoproteins, represent the outermost, hydrophilic layer (Bassett et al., 2000, Martins et al., 2009).

The physiological role of lipoproteins, in plasma as well as in the central nervous system, is on the one hand to supply cells with lipids, on the other hand to remove excess lipids. Other hydrophobic substances like steroid hormones, tocopherols and carotenoids also need lipoproteins as transport vehicles in the blood-circulation (Montine et al., 2002).

Four main classes of lipoproteins can be found in plasma: from the largest (i) chylomicrons, (ii) very low density lipoproteins (VLDL), (iii) low density lipoproteins (LDL) to the smallest (iv) high density lipoproteins (HDL) (Gotto et al., 1986, Ginsberg, 1998) (see Tab. 3.1).

Table 3.1: Overview about major lipoprotein classes.

lipoprotein	approx. size [nm]	major apolipoproteins	physiological function
Chylomicron	<1000	ApoB-48, ApoA-I & II, ApoE	Dietary TG transport
VLDL	50	ApoB-100, ApoC-II, ApoE	TG transport
LDL	20	ApoB-100	Cholesterol transport
HDL	10	ApoA-I & II, ApoE	Reverse cholesterol transport

ApoB48 is the main surface component of chylomicrons, the transporter of dietary TG. VLDLs are the carriers of endogenously produced TGs, secreted from the liver, containing ApoE, ApoC and ApoB. While LDL, with ApoB100 as single protein component, is the main deliverer of

cholesterol from the liver to the periphery, HDL, the “good” cholesterol carrier, is responsible for the reverse cholesterol transport (Rothblat et al., 1986).

High levels of LDL together with low HDL levels are associated with cardiovascular disease and especially atherosclerosis. The latter, characterized by subendothelial accumulation of lipids in arteries, is caused by inflammatory processes in the endothelial cells of the vessel wall in response to retained LDL particles (Ross, 1993).

Lipoproteins in the central nervous system (CNS) are, in contrast to those in the plasma, very dense, similar to HDL, and contain different combinations of apolipoproteins (ApoE, ApoA-I, ApoA-IV, ApoD, ApoH and ApoJ), but mainly ApoE and ApoA-I (Bassett et al., 2000). ApoB is not expressed in the cerebral tissue and thus, does not appear in the brain under physiological conditions (Martins et al., 2009).

3.2.2 Cholesterol metabolism in the CNS: synthesis, transport and processing

In the brain, the most cholesterol-rich organ, cholesterol plays a crucial role in maintaining neuronal plasticity as constituent of plasma membranes of neuronal cells and particularly myelin sheaths (Dietschy and Turley, 2001, Muse et al., 2001). Since it was shown that the BBB essentially prevents the exchange of lipids between brain and plasma, almost all CNS cholesterol originates from de novo synthesis (Dietschy and Turley, 2004). The capacity of neurons to synthesize cholesterol is limited, therefore they depend on the supply of cholesterol produced by glial cells, mainly astrocytes (Funfschilling et al., 2007, Saher et al., 2005).

Levels of free cholesterol in the brain are maintained by three mechanisms (i) regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA-R) and therefore production of free cholesterol, (ii) transposition of cholesterol between different cell types by lipoproteins and (iii) conversion of free cholesterol either to cholesterol esters or oxysterols (see Fig. 3.2). The

rate limiting step of cholesterol synthesis is mevalonate production by HMGCoA-R, a very tightly regulated enzyme, which can be inhibited by statins.

(i) Regulation of HMGCoA-R activity is mainly controlled by the abundance of cholesterol in endoplasmatic reticulum (ER) membranes. Cholesterol acts partially as feed-back inhibitor of HMGCoA-R (an ER membrane protein) as well as inducer of rapid degradation of the enzyme (DeBose-Boyd, 2008). Additionally cholesterol and other sterols affect the transcription of the HMGCoA-R gene via a sterol regulatory element (SRE). Activation of transcriptional control occurs through the cleavage of the transcription factor “sterol regulatory element binding protein” (SREBP), which is located within the ER membrane to sense sterols (Wang et al., 1994, Edwards et al., 1977). All in all, high levels of free cholesterol down regulate biosynthesis of new cholesterol.

(ii) If higher amounts of cholesterol are available in a cell, e.g by de novo synthesis in astrocytes or degradation of nerve terminals, it is usually transported to neurons, via an apoE-rich HDL-like particle (Leduc et al., 2010). ATP-binding cassette transporter types A1 and G1 (ABCA1/ABCG1) are responsible for loading cholesterol from cellular pools on apoE, forming a functional HDL-like lipoprotein (Leduc et al., 2010, Hirsch-Reinshagen et al., 2005). These discoidal particles are released into the extracellular space where they migrate towards functional receptors.

Neuronal cells express on their surface mainly the LDL-receptor (LDLR), but also LDL-receptor related protein 1 (LRP1) and other receptors of the LDL-R family. These receptors recognize ApoE and thus are involved in binding and internalization of these particles (Leduc et al., 2010). In neurons, released free cholesterol can be used for synapse formation and proliferation.

Small amounts of these lipoproteins can also be directed via the BBB into the blood-circulation.

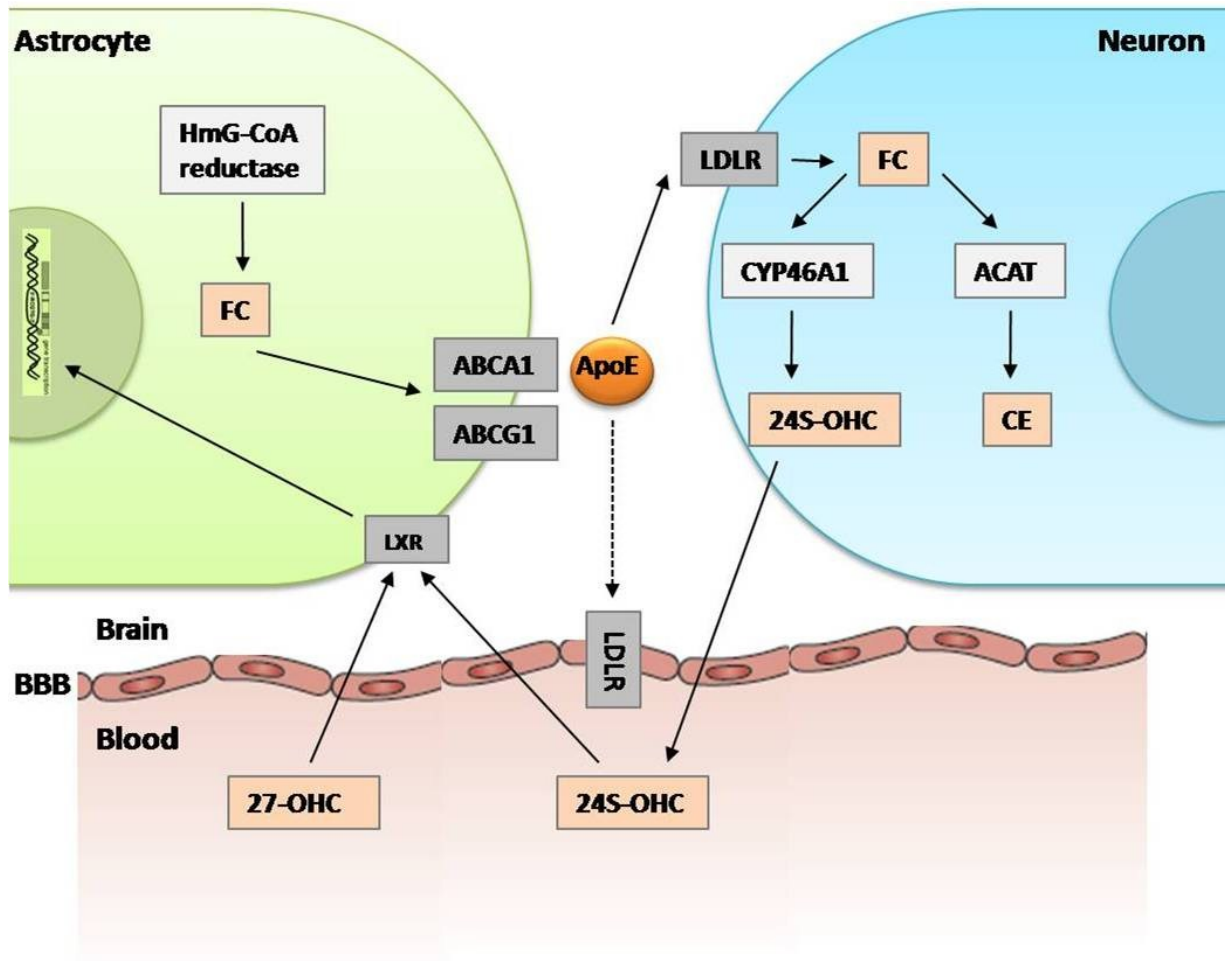


Figure 3.2: Cholesterol metabolism in the CNS. Free cholesterol (FC) is transferred from astrocytes onto ApoE containing HDL-like particles via ABCA1 dependent pathway. Neuronal uptake of FC containing lipoproteins is mediated via LDL-receptor (LDLR) or other receptors of the LDL-receptor family. Excess FC can be converted into cholesterol esters (CE) for storage or oxysterol 24S-OHC for secretion. Oxysterols, 27-OHC and 24S-OHC, are activators of the liver X receptors (LXR) a transcription factor for several lipid metabolism associated genes.

(iii) Beside excretion of excess cellular cholesterol, it can alternatively be converted into derivatives with other chemical properties. For storage free cholesterol is converted by Acetyl-CoA:Acetyltransferases (ACAT) into highly hydrophobic cholesterol esters. Pools of cholesterol esters are limited in the CNS and large intracellular CE accumulations are only found under pathological conditions (Giovannoni et al., 2003).

Conversion of free cholesterol into oxysterols is a pathway for excretion of excess cholesterol from the brain. The most abundant oxysterol produced in the CNS is 24S-hydroxycholesterol (24S-OHC), synthesized by the enzyme CYP46A1, a cytochrome P450 species (Bjorkhem and Meaney, 2004). Unlike cholesterol, 24S-OHC is capable to passively cross the BBB (Bjorkhem et al., 1997). Oxysterols not only represent FC derivatives which can be quickly released from the brain, they are also important activators of the liver X receptors (LXR). These transcription factors regulate, among others, expression of ABCA1, ABCG1 and ApoE (Abildayeva et al., 2006). This leads to upregulation of apoE-mediated cholesterol efflux in the presence of oxysterols.

Another abundant oxysterol in the circulation is 27-OHC, synthesized by sterol 27-hydroxylase (CYP27A1) in peripheral tissue. Whereas 24S-OHC is mainly produced in the brain and is able to traverse the BBB to be removed, 27-OHC has been shown to be transported from the circulation into the brain (Heverin et al., 2005). Since also 27-OHC is able to activate LXRs, this influx of 27-OHC has been proposed to be the possible link between diet induced hypercholesterolemia and CNS changes (see Fig. 3.2).

Taken together, the abundance of FC in the brain is carefully regulated. This underlines the importance of a balanced cholesterol metabolism in the brain, to maintain healthy function. Although the role of cholesterol in neurodegeneration is still ambiguous, there is growing evidence that disturbances in cholesterol homeostasis are involved in several neurodegenerative diseases (Pfrieger, 2003, Ledesma and Dotti, 2006, Shobab et al., 2005).

3.2.3 APP, Aβ and lipid metabolism

Already Alois Alzheimer described a high abundance of ‘lipoid granules’ in AD brains, a first sign of changes in the cerebral lipid homeostasis in the pathology of AD (Foley, 2010).

This important finding fell into oblivion for several decades, but since the ε4 allele of ApoE was discovered to be the strongest genetic risk factor for sporadic AD, a direct link between lipid metabolism and AD was made. Nowadays it is well established, that most lipid

classes are connected to AD pathogenesis. This section will mainly focus on cholesterol and its metabolites and their association to AD.

Cholesterol metabolism and amyloidogenesis

Since APP, BACE1 and subunits of the gamma-secretase are transmembrane proteins, lipid composition of the plasma membrane plays a crucial role in trafficking and proteolysis of APP (Hartmann et al., 2007). Although APP and secretases are found in both raft and non-raft regions of the membrane, raft-associated APP cleavage seems to be shifted to the amyloidogenic pathway (Hartmann et al., 2007, Di Paolo and Kim, 2011). Since lipid rafts are specialized cholesterol-rich membrane domains, it is tempting to speculate that high plasma cholesterol levels increase in raft areas.

Reducing membrane cholesterol levels and concomitantly lipid rafts, through cholesterol extracting compounds, leads to decreased amyloidogenic processing of APP and reduced Abeta levels. Dissociation of BACE1 from lipid rafts and/or decreased activity of BACE1 are responsible for this phenomenon (Simons et al., 1998, Ehehalt et al., 2003, Vetrivel and Thinakaran, 2010). In contrast, high levels of cholesterol were shown to promote co-clustering of APP and BACE1 in raft domains *in vitro*, suggesting increased Abeta levels due to a high cholesterol content (Marquer et al., 2011).

Inhibition of cholesterol synthesis by statins was also shown to reduce Abeta levels *in vitro* and *in vivo* (Fassbender et al., 2001).

These data and the fact that hypercholesterolemia is an early risk factor for the development of amyloid pathology (Kivipelto et al., 2001), promoted investigations to test statins, HMGCoA-R inhibitors, as AD drugs in clinical trials. Early studies indicated a benefit for mild AD patients from statin treatment (Sparks et al., 2005, Simons et al., 2002), although more recent studies found no significant impact of statin treatment on clinical outcome (Feldman et al., 2010).

It has also been shown that intracellular cholesterol storage modulates the generation of Abeta. High intracellular amounts of cholesterol esters, synthesized by ACAT, were found to correlate with high Abeta levels (Puglielli et al., 2001). Pharmacological inhibition, as well as knock out, of ACAT1 reduced Abeta levels and cognitive impairment in AD mouse models (Hutter-Paier et al., 2004, Bryleva et al., 2010). Reduction of ACAT activity also increased cholesterol efflux

from the CNS via 24S-OHC, suggesting a potential role of this metabolite in decreasing Abeta (Bryleva et al., 2010). These data underline the importance of a fine-tuned balance between free and esterified cholesterol and other cholesterol metabolites, although the underlying mechanisms remain still unclear.

Interestingly it has not only been demonstrated that lipids influence Abeta generation, but also conversely, APP processing is able to impact on lipid homeostasis (Grimm et al., 2007). APP knock out as well as gamma-secretase inhibition were found to change membrane fluidity by alteration of the cellular cholesterol content (Grimm et al., 2006, Grimm et al., 2005). Addition of Abeta to APP KO cells downregulated increased cholesterol levels by inhibiting HMGCoA-R (Grimm et al., 2005). Although it is not known whether this regulation happens directly or via another intermediate step, Abeta apparently inhibits cholesterol synthesis. Therefore, some kind of feedback loop between Abeta and cholesterol was proposed, indicating APP as cellular sensor for increased cholesterol levels, leading to higher Abeta formation which in turn down regulates cholesterol synthesis (Grimm et al., 2007).

Besides cholesterol homeostasis, Abeta was also found to be involved in sphingolipid and ganglioside synthesis (Hartmann et al., 2007), which are both found to be decreased in AD brains.

ApoE and Abeta

Apolipoprotein E is a glycoprotein which, in the brain, is primarily secreted by glia cells (Boyles et al., 1985) and responsible for redistribution of lipids in the CNS. This function is important for nerve repair mechanisms. After brain injury, synthesis of ApoE is increased up to 150-fold (Snipes et al., 1986).

Human ApoE is encoded by a single gene with 3 polymorphic alleles, epsilon 2, 3 and 4 (Weisgraber, 1994). The most common allele is epsilon3, but also epsilon4 appears with a frequency of 15% in general population (Vance and Hayashi, 2010). Persons with at least one epsilon4 allele are 3 to 4 times more prone to develop sporadic AD, homozygous carriers even have a 12 fold higher risk (Corder et al., 1993).

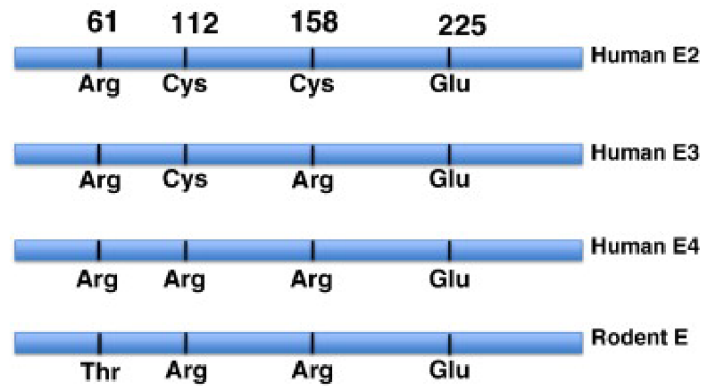


Figure 3.3: “Schematic representation of apo E isoforms in humans and rodents. Humans express 3 major isoforms of apo E: E2, E3 and E4 that differ from each other in a single amino acid. Apo E2 contains Cys at positions 112 and 158, whereas apo E3 contains Cys at position 112 and Arg at position 158. Apo E4 contains Arg at both 112 and 158. A “domain interaction” occurs between Arg-61 and Glu-225 and this occurs more readily for apo E4 than for apo E3 because the interaction is promoted by Arg-112. Rodent apo E is like human apo E4 in that it contains Arg-112. On the other hand, rodent apo E lacks Arg-61 and therefore cannot participate in domain interaction and is functionally like apo E3.” (Vance and Hayashi, 2010)

The three ApoE isoforms differ in amino acids at position 112 and 158 (Fig.3.3). There are two domain interactions recognized, one in the C-terminal part, Glu-255, and one at the N-terminal domain, Arg-61 (Fig.3.3) (Weisgraber, 1994, Dong and Weisgraber, 1996). The interaction with Arg-61 is enhanced in the presence of Arg-112 in ApoE4, which is not found in ApoE2 and 3. Interestingly, mouse ApoE is similar to human ApoE4 at position 112 but lacks Arg-61 (Fig.3.3) (McLean et al., 1983). This lack of Arg-61 inhibits the interaction with Glu-225. Therefore mouse ApoE is functionally similar to human ApoE3 (Vance and Hayashi, 2010).

Since it is well established that APOE epsilon4 carriers are at higher risk for developing sporadic AD, much research effort focused onto the investigation of ApoE in connection to Abeta processing and aggregation.

Indeed, the presence of ApoE4 is associated with increased Abeta accumulation in humans and transgenic mice (Rebeck et al., 1993, Riddell et al., 2008, Bales et al., 2009). Additionally, ApoE mRNA and protein levels have been shown to be increased in AD patients’ brains (Yamada et al., 1995, Yamagata et al., 2001), and ApoE immunoreactivity was found within and around amyloid plaques (Harr et al., 1996, Poirier, 2000).

The presence of ApoE in plaques might be explained by the good Abeta scavenging ability of ApoE. In numerous studies it was shown that ApoE binds Abeta and even promotes Abeta clearance across the BBB via LRP1. ApoE4 was found to be the least and ApoE2 the most effective form promoting this kind of Abeta clearance (Yang et al., 1999, Deane et al., 2004, Deane et al., 2008).

Binding of Abeta depends on the lipidation status of ApoE; the less lipidated the lower is the affinity of ApoE to Abeta (Tokuda et al., 2000). While bound Abeta inhibits further lipidation of ApoE, thus interfering with normal lipid metabolism (Hone et al., 2005).

Why ApoE4 in fact increases amyloid pathology remains undetermined. Interestingly, knock out of murine ApoE in AD mouse models leads to a reduction of amyloid plaques (Bales et al., 1999, Holtzman et al., 2000). These studies show that (i) ApoE is directly involved in Abeta accumulation and that (ii) ApoE4 may increase AD risk by promoting Abeta deposition.

3.3 Mouse models

3.3.1 hApoB-100 transgenic mouse line

The hApoB-100 transgenic mouse line was generated by Bjelik et al (2006) and is a validated model of atherosclerosis. As described in detail in section 3.2.1, ApoB-100, the main apolipoprotein B, is found in VLDL and LDL.

The murine plasma lipid profile is different from that in humans. In rodents, HDL is the major cholesterol-carrying lipoprotein and they show only very low, if any, LDL-cholesterol levels. Due to this low LDL to HDL ratio, mice are protected against hypercholesterolemia and are usually not affected by atherosclerosis (Breslow, 1996). Since there is only one ApoB-100 molecule per LDL particle, both high ApoB and LDL levels are equally associated with cardiovascular disease and atherosclerosis.

Besides diabetes and hypertension, high serum concentrations of LDL in combination with low HDL concentrations are the best known risk factors for developing atherosclerosis in humans.

Atherosclerosis or atherosclerotic cardiovascular disease (ASCVD) manifests as a thickening of the arterial walls as a result of the accumulation of lipids within macrophages. Today it is recognized as a subacute inflammatory condition of vessel walls coming along with macrophage infiltration. Lipid oxidation plays also a crucial role in this process leading to endothelial activation, a higher expression of adhesion molecules and in the end to the formation of foam cells out of macrophages (Libby, 2006).

Genetic background and atherosclerotic pathology

To study the role of ApoB-100 in lipid metabolism as well as in atherogenesis, several investigators have developed mice expressing human apoB-100, which is difficult due to the large size of the apoB gene of >43 kb. The animals used in this study were generated by injecting fertilized oocytes from wild-type female mice (C57/B6 CBA F1) with purified P1-phagemid DNA containing the entire 43 kb human apoB-100 gene with its natural promoter, the 19 kb of the 5' and the 14 kb of the 3' flanking genomic sequences (Bjelik et al., 2006, Callow et al., 1994).

The transgenic F1 generation mice were backcrossed twice with the C57/B6 strain in order to get a more homogenous genetic background.

In humans, apoB-100 mRNA is converted exclusively in the small intestine to ApoB-48, while in rodents apoB mRNA is processed in both the small intestine and the liver.

Human ApoB-100 transgenic animals develop a plasma lipid profile similar to that of humans with heightened atherosclerosis susceptibility, showing an increase in LDL-cholesterol and triglycerides and a decrease in HDL. The strong increase in plasma LDL in this mice is not only due to the pronounced overexpression of hApoB-100, but also due to decreased affinity of human ApoB to the murine LDL-Receptor, which leads to reduced clearance of LDL particles (Corsini et al., 1992). This atherogenic phenotype can be increased by feeding the animals a high-cholesterol diet (Callow et al., 1995). It was also shown that hApoB-100 transgenic animals develop atherosclerotic lesions at branch points of arteries along the aorta and at the root of the aorta, especially when fed a high-cholesterol diet (Callow et al., 1995).

Brain alterations in hApoB-100 animals

In more recent studies, the influence of hApoB-100 overexpression on the cerebral vasculature, brain APP metabolism and neurodegeneration has been investigated.

The effects of a high cholesterol diet and the overexpression of the hApoB-100 gene on the expression and processing of APP in homozygous hApoB-100 mice brains were examined (Bjelik et al., 2006). In this study it was shown that APP mRNA levels were not altered in hApoB-100 transgenic compared to wild type animals. Only in wild type animals, high cholesterol diet increased APP mRNA levels, but the diet had no influence on APP mRNA levels in hApoB-100 mice.

When looking at the soluble and membrane bound fraction of APP protein in cortex samples, Bjelik et al. observed that hApoB-100 overexpression lead to a decreased level of membrane bound APP compared to non transgenic animals.

In contrast, hApoB-100 overexpressing animals on a high cholesterol diet showed significantly increased levels of membrane bound APP compared to hApoB-100 overexpressing animals on a normal diet and non-transgenic animals on either diet. These results indicate that the high-cholesterol diet leads to a stronger response in hApoB-100 mice, but only in terms of protein distribution in the cell and not on mRNA expression level.

A follow-up study of the same group showed for the first time that apoptosis and neurodegeneration is increased in brains of 6-7 months old hApoB-100 animals (Bereczki et al., 2008). In addition, amyloid-plaques were detected in brains of 6-7 months old hApoB-100 mice using an anti-human amyloid (1-17) antibody.

Another study assessed the impact of hApoB-100 overexpression on the cerebral vasculature. This group observed that hApoB-100 overexpression leads to a decrease in cortical microvascular density and to alterations in the lumen diameter (Sule et al., 2009). High cholesterol diet did not aggravate this phenotype.

Although the hApoB-100 transgenic mice were originally designed as a model for atherosclerosis, this transgenic mouse model is an interesting tool to investigate the interplay between lipid metabolism, vascular alterations and sporadic AD.

3.3.2 hAPPsl transgenic mouse line

The hAPPsl transgenic mouse line is a well described model of familial AD and is often used to study the influence of developmental compounds on amyloid production, processing and deposition in preclinical trials. These animals contain a gene construct first described by Rockenstein et al. (2001) and are highly overexpressing human APP751 with London (V717I) and Swedish (KM670/671NL) mutations (see Fig. 3.43.4) under the control of the murine Thymocyte differentiation antigen 1 (Thy-1) promoter.

While the pathogenic mutations at codons 670/671 in APP (“Swedish”) lead to enhanced cleavage at the beta-secretase site and thus to an increased production of both Abeta 1–42 and Abeta 1–40, the mutation at codon 717 (“London”) is modulating the gamma-secretase cleavage

site and thus shifting amyloid secretion to an increased proportion of Abeta 1–42 (Sinha and Lieberburg, 1999).

Production of high levels of Abeta 1–42 results in early plaque formation (Citron et al., 1997), which can also be observed in this hAPP transgenic mouse line (Havas et al., 2011).

661	IKTEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	TVIVITL	720	P05067	A4_HUMAN
661	IKTEEISEVNLDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	TVIIITL	720		A4_HUMAN_mut
661	IKTEEISEVKMDAEFGHDSGFEVRRHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	TVIVITL	720	P12023	A4_MOUSE

Figure 3.4: Comparison of wild type human, mutated human and murine APP between amino acid 661 and 720. Abeta1-42 (yellow), swedish (KM670/671NL) and london (V717I) mutation (purple) and differences between human and murine Abeta (green) are marked.

The murine Thy-1 promoter is known to be active in the central nervous tissue, predominantly in neurons (Kollias et al., 1987). Thy-1 protein is an abundant component of the neuronal surface (Morris, 1985) but can also be found on peripheral T cells and thymocytes in mice (Raff, 1971). Expression of mutant hAPP under the Thy-1 promoter was shown to favor the formation of mature plaques in the hippocampus and neocortex (Andra et al., 1996), whereas other promoters like the PDGF-B promoter seem to be responsible for the production of mainly diffuse plaques (Mucke et al., 2000). It is obvious that the distribution pattern as well as the expression level play a role in the development of different plaque types.

The hAPPsl transgenic mouse line develops dense plaques consisting of amyloid depositions in early age, starting at 3 – 6 months in the frontal cortex (Rockenstein et al., 2001). These plaques can also be intensely labeled with Thioflavine-S and Congo red.

Not only the early development of plaques but also the early appearance of behavioral deficits e.g. in the Morris Water Maze Task and the high levels of particular Abeta 1–42, but also Abeta1–40 and Abeta1–38 in brain samples complete this model of familial AD (Havas et al., 2011).

Chapter 4

Aim of work

In the last few years many publications pointed towards an overlap between cerebrovascular and primary neurodegenerative diseases. It is now well accepted that most of the Alzheimer's Disease (AD) patients also display a certain degree of cerebrovascular lesions. These two pathologies also share several risk factors like hyperlipidemia and hypertension. However, it is still under debate to what extent both pathologies are contributing to the development of the disease and how they influence the time course of progression. Therefore, the creation of relevant animal models might help for a better understanding of the etiopathogenesis of sporadic AD.

By heterozygous crossbreeding of the well established hAPP_{SL} transgenic mouse line with ApoB-100 transgenic mice, a relevant model of human AD should be established. A vascular risk factor, elevated ApoB-100 levels, is introduced into an AD mouse model.

In a first intense basic characterisation, double transgenic ApoBxAPP, as well as all single (ApoB-100 and hAPPsl) and non transgenic littermates were investigated over age in terms of behavior, biochemistry and histology.

Several questions are addressed in this study:

- (i) does the vascular risk factor per se induce changes in APP metabolism or cognitive function?
- (ii) does the vascular risk factor in combination with hAPP overexpression lead to an accelerated development of amyloid pathology and an earlier onset of cognitive dysfunction in double transgenics compared to hAPPsl single transgenic mice?
- (iii) how are lipid metabolism and inflammation, both associated with AD and atherosclerosis, influenced by the different transgenes?

Therefore, I investigated the learning and memory abilities of double transgenic animals in comparison to single transgenic as well as non transgenic littermates using the Morris Water Maze task (MWM) and Contextual Fear Conditioning (CFC). Furthermore, the spontaneous activity and anxiety behavior as well as neurological, motor or sensory differences between the genotypes were examined.

Additionally, specific biochemical markers for APP processing, inflammation, oxidative stress and lipid metabolism were investigated in brain tissue and plasma of these mice. Similar markers were also examined using immunohistological methods. I assessed how the brain pathology in terms of vascular lesions, amyloid depositions and development of inflammatory responses develops.

To further challenge the system and to investigate how additional dietary uptake of cholesterol influences behaviour, APP pathology and cerebral cholesterol metabolism, I designed the second working package as a 3 months-feeding study of young animals from all four genotypes.

12 weeks old mice were assigned to either a high fat (HFD) or a standard diet (ND) group and fed the corresponding diet for three months. At an age of 6 months all animals underwent several behavioural tests for two more weeks, tissue was sampled and investigated subsequently.

This work should give an insight into the crosstalk between vascular risk factors, lipid metabolism and AD-specific pathology leading to new approaches in the development of drugs and a better understanding of the disease.

Chapter 5

Methods

5.1 Animals

By heterozygous crossbreeding of ApoB-100 transgenic (Bjelik et al., 2006) and hAPPsl mice (Hutter-Paier et al., 2004), both on a C57Bl/6J background, four different genotypes were obtained: double transgenic ApoBxAPP, single transgenic ApoB-100, single transgenic hAPPsl and non-transgenic littermates (WT). From every genotype about 10 male and 10 female mice (see Tab. 5.1) were tested for the basic characterization at 4, 6, 9 and 12 months of age.

Tab. 5.1 Number of animals used for basic characterization over age

		female	male
ApoBxAPP	4m	10	8
	6m	11	9
	9m	11	9
	12m	6	9
ApoB	4m	11	8
	6m	10	10
	9m	12	11
	12m	12	11
hAPPsl	4m	10	6
	6m	10	9
	9m	13	9
	12m	6	7
WT	4m	8	9
	6m	11	10
	9m	10	10
	12m	12	13

All animals were housed in individually ventilated cages under a constant light-cycle (12 h light/dark). Room temperature and humidity were kept constant at approximately 24°C and 40 to 70 %, respectively. Chow and normal tap water were available ad libitum.

For the feeding study eight groups were analyzed, starting with n=8 animals per group. All four genotypes were fed either a high fat diet (HFD – D12451 mod) or standard chow (ND) obtained from Ssniff (see Fig. 5.1). With an age of three months animals started to receive their assigned diet and were kept on the diet for three more months until behavioural testing started. Every week animals were weighted to follow their weight gain.

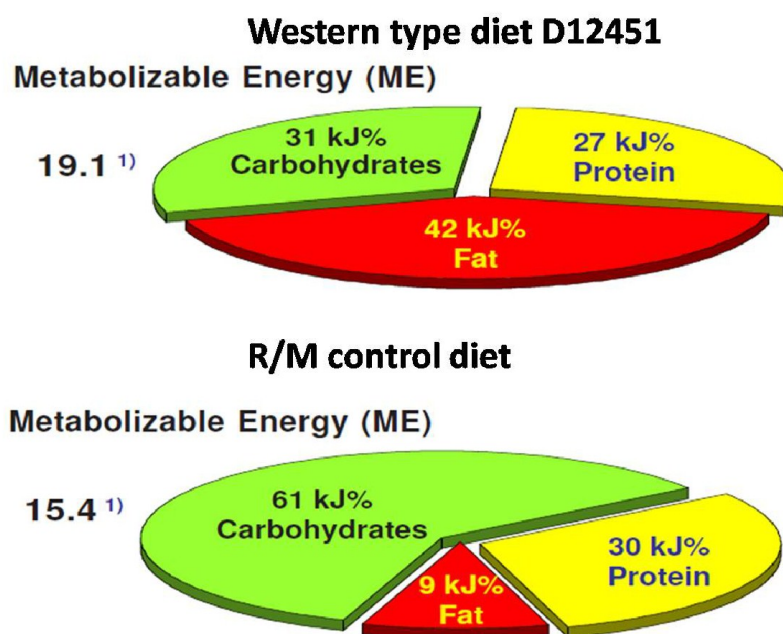


Figure 5.1: Composition of the different diets. 1) Metabolizable energy calculated according to the pig formula, Annex 4 of the German feed regulation.

Animal studies conformed to the Austrian guidelines for the care and use of laboratory animals and were approved by the EU legislation 2010/63/EU.

Four to five hours before tissue sampling, chow was taken out of the cages to be able to obtain fasted plasma.

From every tested mouse, blood and brain tissue were sampled after sedation by standard inhalation-anaesthesia (Isofluran). Each mouse was placed in dorsal position, thorax was opened and a 26-gauge needle attached to a 1 cc syringe was inserted into the right cardiac ventricular chamber.

Blood was collected into heparin coated vials and consequently used to obtain plasma. Therefore the blood was then centrifuged for 10 min at 1000 x g and the supernatant collected as plasma.

Following blood sampling, mice were transcardially perfused with physiological (0.9 %) saline. Thereafter, brains were removed and hemisected.

The left hemisphere was subdivided in cortex, hippocampus, cerebellum and the residual brain parts, immediately frozen on dry ice and stored at -80°C until biochemical examination.

The right hemispheres of all mice were fixed by immersion in freshly produced 4 % paraformaldehyde/ PBS (pH 7.4) at room temperature for 1 h, followed by 24 h incubation in 15 % sucrose solution for cryo-conservation.

Hemispheres were stored at -80°C until cutting and histological processing.

5.2 Behavioral Testings

5.2.1 Irwin Testing Battery

To test and survey general health, each mouse undergoes an Irwin test prior to any behavioral investigation. Using this test battery, physical characteristics and conspicuities, sensorimotor reflexes and motor abilities are checked.

The collected physical characteristics are body weight, body temperature, existence of whiskers, constitution of the fur and the eyes as well as individual remarks (e.g. bites or other lesions).

Whiskers-orienting, righting, ear twitch, eye blink and pupillary reflexes are evaluated as well. Furthermore, the wire suspension time is calculated and the vertical pole test is carried out.

The righting reflex is evaluated by turning the mouse onto its back. Normal mice immediately turn themselves over, to right themselves onto all four feet.

Other basic neurological reflexes measured are the eye blink, ear twitch and whisker-orienting responses to a light brush.

The pupillary reflex is a simple measurement to evaluate visual abilities via the response to light. The beam of a small flashlight is directed to the eye. Pupil contraction occurs immediately, followed by pupil dilation when the light is removed.

With the calculated wire suspension time, it is possible to detect neuromuscular abnormalities, measures of motor strength. For this test, a wire cage lid is used; duct tape is placed around the perimeter to prevent the mouse from walking off the edge. The animal is placed on the top of the cage lid. The lid is lightly shaken three times to force the mouse to grip the wires and then the lid is turned upside down.

The lid is then held at a height of 30 cm above a soft underlay, high enough to prevent the mouse from jumping down, but not high enough to cause harm in the event of a fall.

The latency to fall down is quantified. A 60-second cut-off time is used although a normal mouse can hang upside down for several minutes.

The vertical pole test is another measure of motor coordination and balance. A wooden pole (2 cm diameter) is wrapped with cohesive tape for improved traction. The mouse is then placed in the center of the pole, which is held in horizontal position. The pole is lifted to a vertical position. Latency to fall off the pole is the dependent variable.

Normal mice will stay on the pole and may walk up or down the length of the pole. Deficits in motor coordination and balance are detected by falling off before reaching a 45° angle.

5.2.2 Open Field Task

The psychomotoric activity as well as the explorative behavior of each animal are examined in the Open Field Test (Walsh and Cummins, 1976).

Every single mouse is put into a transparent box and its horizontal and vertical movements are detected for 5 minutes.

The overall path length and number or time of rearings give clues about possible motor deficits of the mice. Thigmotaxis is evaluated by analyzing the abidance in the border areas of the box. Also hyperactivity can be measured in the Open Field.

5.2.3 Contextual Fear Conditioning

The Contextual Fear Conditioning (CFC) Task is performed to assess emotional learning and memory and it is conducted in an automated box provided by TSE-Systems, Germany.

On the first day the animals are placed in a transparent box, disinfected with ethanol and equipped with a metallic grid on the floor (see Fig.5.2). After 5 seconds a 2 seconds foot shock [0.5 mA; unconditioned stimulus (US)] is administered.



Figure 5.2: Automated box provided by TSE-Systems for the Contextual fear conditioning task.

Rodents are removed from the chamber 30 seconds later. During the whole phase the freezing behavior is recorded by the automated system via a light beam grid.

Freezing is defined as lack of movement, except that required for respiration.

On the second day rodents are returned to the same box in which training occurred (context), and freezing behavior is again recorded by the automated system. The freezing threshold is set to 5 seconds for all experiments in this study. At the end of the 5 minutes context test, rodents are returned to their home cages.

Low freezing times are associated with an impairment in emotional memory.

5.2.4 Morris Water Maze Task

The Morris Water Maze Task (MWM) is the most widely used test to assess spatial learning in rodents. The animals are trained to find a hidden platform in an open swimming arena using distal cues. This test is relatively unsusceptible from activity or body mass differences, which makes it a robust and reliable test strongly related to hippocampal and striatal lesions.

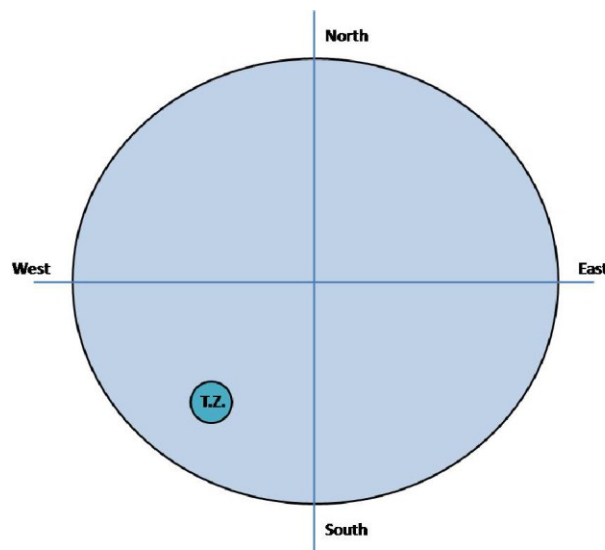


Figure 5.3: Schematic diagram of the Morris Water Maze. T.Z. is target zone

The protocol used in this study contained four training days, with three trials per day and one probe trial 1.5-2 hours after the last trial on the fourth day. Before starting the test the water temperature is set to 22°C and the light intensity is dimmed to 40-60 lux. The pool is filled until the platform is approximately 5 mm beneath the water surface.

Investigated animals are allocated to swimming groups of a size between 5 and 9 mice, so that one mouse has 8 to 10 min time between each trial for grooming and drying.

On each training day the mouse is put in the starting area three times (1st trial: **north**, 2nd trial: **south**, 3rd trial: **east** - see Fig. 5.3) facing the wall. The trial is automatically over when the mouse finds the target and remains in the target zone for more than two seconds or the trial time is over.

If the mouse does not find the target within 60 seconds, it is placed onto the target. In both cases the mouse is allowed to spend about 10 seconds on the target to memorize its position.

The probe trial is performed about two hours after the last trial on the fourth training day. The platform is removed before start and it is tested how often the animal crosses the target zone and also the abidance in the target quadrant is measured within 30 sec. Starting position for the probe trial is North.

The last test performed is the so called visual test in wich probable visual deficits are assessed. Here the mice are supposed to find a marked platform in the middle of the pool starting from South. If mice do not find the platform within 1 min they are excluded from the evaluation.

5.3 Biochemical Methods

5.3.1 Homogenization of Frozen Brain Samples

Reagents and supplies

- **Tissue Homogenization Buffer (THB)** (250 mM Sucrose, 1 mM EDTA, 1 mM EGTA, 20 mM Tris pH 7.4)
42.8 g Sucrose in 300 ml
10 ml Tris [1 M, pH 7.4]
5 ml EDTA [100 mM]
12.5 ml EGTA [40mM]
ad A. bidest up to 500 ml, store at 4°C
- Protease Inhibitor Cocktail 100x (Calbiochem)
- Tissue Ruptor (Qiagen)

Protocol

The frozen tissue samples were weighted and Tissue Homogenization Buffer (THB) including 1x protease inhibitor (Calbiochem) was added.

For cortex samples, 1 ml THB per 100 mg tissue were added, for hippocampal samples 2.5 ml were used for 100 mg tissue. The tissue was homogenized with the Tissue Ruptor (Qiagen) for 20 sec using the highest level.

5.3.2 Extraction of Non-Plaque Associated Proteins

Reagents and supplies

- **DEA solution** (0.4% DEA, 100mM NaCl)
200 µl Diethylamine
1 ml 5 M NaCl

ad A. bidest up to 50 ml; store at 4°C

- **Tris** (0.5 M, pH 6.8)

3.03 g Tris

ad A. bidest up to 50 ml and adjust pH with HCl

- Tissue Ruptor (Qiagen)

Protocol

For extraction of non-plaque associated proteins, 100 µl of the THB homogenate were mixed with 100 µl diethylamine (DEA) solution. The mixture was centrifuged for 1 h at 74200xg, 4°C. 170 µl of the supernatant were transferred to a 1.5 ml Eppendorf tube and neutralized with 17 µl 0.5 M Tris, pH 6.8.

Aliquots were stored at -20°C until further use.

5.3.3 Extraction of Deposited Proteins

Reagents and supplies

- Sonicator Bandelin Sonoplus GM70
- Formic acid
- **FA Neutralization Solution** (1 M tris, 0.5 M Na_2HPO_4 , 0.05% NaN_3)

60.67 g Tris

44.5 g Na_2HPO_4

2.5 ml Sodium Azide (10%)

ad A. bidest up to 500 ml ; store at RT

- Tissue Ruptor (Qiagen)

Protocol

For extraction of deposited proteins, 100 µl of the THB homogenate were mixed with 220 µl cold formic acid (FA) and sonificated for 1 min on ice. 300 µl of this solution were transferred to a centrifugation tube and centrifuged for 1 h at 74200xg, 4°C.

After centrifugation, 210 µl of the supernatant were transferred to a fresh tube and mixed with 4 ml FA Neutralization Solution.

Aliquots were stored at -20°C until further use.

5.3.4 Determination of Protein Concentration

Reagents and supplies

- Pierce BCA Protein Assay Kit (Cat No. 23225)
- 96well plate
- µQuant Platephotometer

Protocol

The Pierce BCA Protein Assay Kit was used for all protein determinations. Before starting the BCA reaction standards of diluted albumine (BSA) between 2000 µg/ml and 25 µg/ml were prepared according to the instruction manual. Then 25 µl of the standards and samples were pipetted into wells of a 96 well plate and 200 µl working reagent (50:1 Reagent A:B) were added.

The plate was shaken for 30 sec on a plate shaker and then incubated for 30 min at 37°C. After incubation the absorbance was measured at 562 nm with µQuant photometer.

5.3.5 Lipid Peroxidation Assay

Reagents and supplies

- **5% SDS solution**
5 g SDS in 100 g A. bidest

- **20% Acetic Acid pH 3,5**

20 ml Acetic Acid 100 %

pH adjusted to 3.5 with NaOH

add A. bidest up to 100 ml

- **1.33% Thiobabituric Acid (TBA)**

dissolve 133 mg TBA in 1 ml DMSO

store at -20°C until use

ad A. bidest up to 10 ml before use

- **n-Butanol/Pyridin (15:1)**

35 ml n-Butanol

2.5 ml Pyridin

- **0.5 M BHT Solution (100x)**

5.5 g butylated Hydroxytoluene

50 ml Acetonitril

- Protease Inhibitor Cocktail 100x (Calbiochem)

- 1,1,3,3 Tetramethoxypropane (MDA) 10 mM stock

- Sonicator Bandelin Sonoplus GM70

- µQuant photometer

Protocol

For the Lipid Peroxidation Assay the samples were prepared by mixing 50 µl of the THB homogenates with 50 µl 5% SDS solution including 1x protease inhibitor as well as 1x BHT solution and sonificated for 5 sec. The standards were a mixture of 50 µl THB solution with 50 µl 5% SDS and before starting the assay, 2 µl of the MDA working solutions (Tab. 5.2) were added.

The assay was started by adding 55 µl 1.33% TBA and 95 µl 20% acetic acid pH 3.5 to 100 µl of the prepared samples or standards. After 1 h incubation in a water bath at 95°C, 250 µl

n-Butanol/Pyridin were added and the solutions were mixed by gently inverting the tubes two times.

Afterwards the solution was centrifuged for 10 min at 4000xg to separate the phases. 200 µl of the upper organic phase were then transferred to one well of a 96 well plate and absorbance was measured at 535 nm.

This assay was also used for plasma samples. Therefore 1 µl BHT was added to 100 µl plasma and the assay was carried out as described above.

Tab.5.2 Preparation of MDA standards

STD conc. [µM]	Working solution conc. [mM]	dilution
20	1	20 µl 10 mM MDA + 180 µl d.H ₂ O
10	0.5	40 µl 1 mM MDA + 40 µl d.H ₂ O
5	0.25	40 µl 0.5 mM MDA + 40 µl d.H ₂ O
2.5	0.125	20 µl 0.25 mM MDA + 20 µl d.H ₂ O
1	0.05	10 µl 0.25 mM MDA + 40 µl d.H ₂ O

5.3.6 Plasma Parameter Measurements

Reagents and supplies

- Fluitest ® CHOL (Cat.No 4241)
- Fluitest ® TG (Cat.No 5741)
- Fluitest ® CHOL-HDL direct (Cat.No 4101)
- Fluitest ® CHOL-LDL direct (Cat.No 4102)
- Fluitest ® GLU (Cat.No 4341)
- Fluitest ® GPT/ALAT (Cat.No 1186)

Protocol

For the determination of total cholesterol, triglycerides and HDL-cholesterol Fluitest ® CHOL, Fluitest ® TG and Fluitest ® HDL direct (Analyticon) assays, respectively were used. Plasma of all animals was diluted 1:2 with 0.2 % NaCl solution and the assays were carried out according to the supplied manuals.

LDL cholesterol content was measured with Fluitest ® LDL direct (Analyticon) assay, as well as calculated with Friedewald equation ($\text{LDL-chol} = \text{total chol} - \text{HDL chol} - (\text{TG}/5)$).

Also glucose and alanine-aminotransferase (ALT) levels were also measured with Fluitest assays from Analyticon according to the manual with endpoint and substrate start methode, respectively.

5.3.7 Brain Lipid Extraction

Tissue (rest brain and cortex) of 9 months old animals was pulverized on liquid nitrogen and weighted. After addition of 1 ml dH₂O and 2 ml chloroform/methanol (2:1 v/v), samples were vortexed shortly and put onto a rotating wheel for 40 min. Afterwards samples were centrifuged for 15 min at 2000xg, 4°C.

The lower phase was transferred into a fresh glass vial and dried under nitrogen atmosphere. Samples were stored at -70°C until further processing.

Lipid extract was resuspended in 1 ml chloroform before use and aliquots again dried under nitrogen atmosphere once needed.

5.3.8 Brain Cholesterol Measurement with Enzymatic Detection

Reagents and supplies

- BioVision Cholesterol Quantitation Kit (Cat No.K603-100)

Protocol

For the determination of brain cholesterol levels an 100 µl aliquot of extracted lipids was dissolved in 100 µl Cholesterol Assay Buffer (BioVision).

The assay was carried out twice, once for total and once for free cholesterol (without esterase) according to the manual using fluorometric detection.

5.3.9 Brain Cholesterol Measurement with Gas Chromatography (GC)

Reagents and supplies

- 5- α -cholestan
- acetone
- BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide)
- TMCS (trimethylchlorosilane)
- toluol
- GC Trace Ultra

Protocol

For GC measurements a 20 μ l / 100 μ l (rest brain/cortex) chloroform-aliquot was mixed with 15 μ g 5- α -cholestan as internal standard. Samples were dried under nitrogen stream and afterwards 80 μ l acetone and 20 μ l BSTFA 1% TMCS were added and derivatized for 15 min in a sand bath.

Afterwards samples were again dried under nitrogen and resuspended in 50 μ l toluol. 1 μ l of this suspension was then analyzed with GC Trace Ultra.

Column: Ultra2 25 m x 0.32 mm

Injection temp.: 280°C

Detector (F.I.D.) temp.: 300°C

Time program: Initial: 220°C, 1 min; R1: 260°C, 10°C/min; R2: 285°C, 1°C/min; final: 295°C, 10 min.

Equilibration time: 0.3 min; Column head pressure: 100 kPa const.

5.3.10 Measurement of Fatty Acids in Brain Lipid Extract

Reagents and supplies

- C15 FFA
- BF_3 -COOH complex
- hexan
- toluol
- GC Trace Ultra

Protocol

A 250 μl chloroform-aliquot was dried under nitrogen stream and afterwards 100 μg C15 FFA as internal standard as well as 400 μl toluol and 1 ml BF_3 -COOH complex were added and derivatized in a sand bath at 110°C for 1 h.

For extraction, 2 ml dH_2O as well as 200 μl hexan were added to the sample and vortexed. After centrifugation at 4°C, 2000xg, the hexan-toluol phase was transferred into a fresh glass vial and 50 μl of it were dried under nitrogen stream.

Samples were resuspended in 50 μl toluol and 1 μl of it was analyzed with GC Trace Ultra

Column: WCOT fused silica 25 m x 0.32 mm, 0.32 μm FFAP-CB

Injection temp.: 230°C

Detector (F.I.D.) temp.: 250°C

Time program: Initial: 150°C R1: 215°C, 2.5°C/min; 10 min; R2: 10°C/min; final: 230°C, 12.5 min.

Equilibration time: 0.3 min; Column head pressure: 60 kPa const.

5.3.11 Alpha-tocopherol Measurements

Reagents and supplies

- DL-alpha-tocopherol-standard
- hexan
- methanol
- photometer
- HPLC water alliance system

Protocol

For preparation of standards, 100 mg DL-alpha-tocopherol-standard were resuspended in 4.6 ml hexan and concentration was determined with photometer at 292 nm. Afterwards standards were diluted with methanol from the stock at concentrations between 100 and 11 ng/20 µl.

A 250 µl chloroform-aliquot was dried under nitrogen stream and resuspended in 100 µl methanol. 60 µl of this sample were analyzed with HPLC.

Column: BDS Hypersil C18 150 x 4.6 mm; 5 µM

Flow: 1 ml/min

Mobile phase: methanol

Detector: Fluorescence Ex 292/ Em 335 nm; gain 1000.

5.3.12 Abeta1-38, Abeta1-40 and Abeta1-42 Measurements

Cortical and hippocampal samples (DEA and FA fraction) of all groups were analyzed for Abeta1-38, Abeta1-40 and Abeta1-42 with MSD® 96-well MULTI-SPOT® 4G8 Abeta Triplex Assay (MesoScale Discovery; see Fig.5.4).

The immune assay was carried out according to the manual and plates were analyzed on the Sector Imager. The assay detects human and rodent Abeta species.

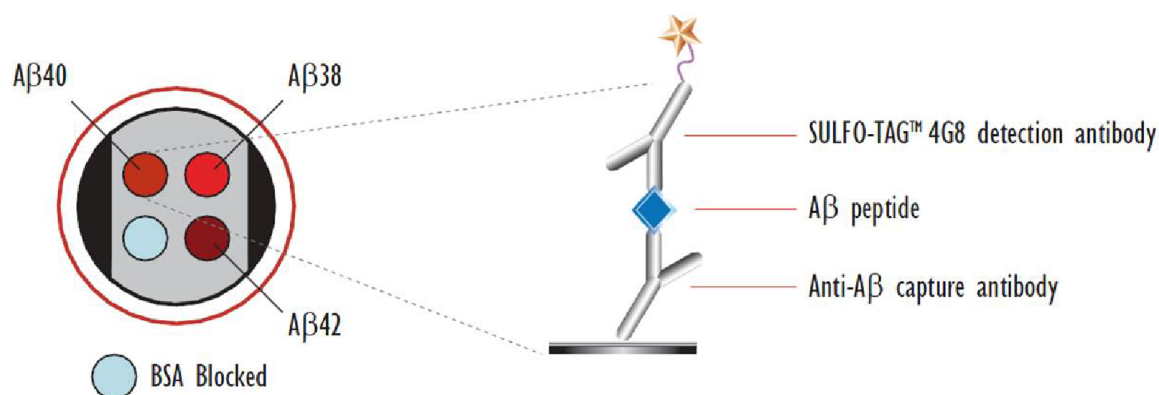


Figure 5.4: Principle of MSD ® 96-Well MULTI-SPOT ® Alzheimer's Disease Assay: The principle of MSD-ISA measurement is similar to common ELISA technique, but detection happens via electrochemiluminescence. Per well more than one parameter can be measured. <http://www.mesoscale.com>

5.3.13 Measurement of Inflammation Markers

Cortical and hippocampal DEA samples, as well as plasma, of animals from all groups were analyzed for IL-1 β , IL-12p70, IFN- γ , IL-6, KC/GRO, IL-10 and TNF-alpha with MSD® 96-Well MULTI-SPOT® Mouse Proinflammatory 7-plex ultrasensitive assay (Mesoscale Discovery). The immune assay was carried out according to the manual and plates were analyzed on the Sector Imager.

5.3.14 SDS-PAGE

Reagents and supplies

- Rainbow coloured protein molecular weight marker, Amersham (RPN 800/ RPN755E)
- Mini-PROTEAN 3 Cell Electrophoresis Module, BioRad (165-3301)
- Minipuls 3 pump (Gilson)
- Hoefer SG15 chamber
- **Separation Gel Buffer** (1.5M Tris, 0.4 % SDS, pH 8.8)

45.4 g Tris

dissolved in 100 ml 1M HCl and adjusted pH to 8.8

1 g SDS

add A. bidest to 250 ml

- **Comb Gel Buffer** (0.5M Tris, 0.4 % SDS, pH 6.8)

15 g Tris

dissolved in 100 ml 1M HCl and adjusted pH to 6.8

1 g SDS

add A. bidest to 250 ml

- Ammonium peroxodisulfate (10 %)
- **10 x TGS Runningbuffer** (2M Glycine; 0,25M Tris; 1% SDS)

30 g Tris

144 glycine

10 g SDS

add A. bidest to 1000 ml

- **5 x Sample Buffer** (0.3M Tris, 10 % SDS, 0.6M DTT, 50 % Glycerol, 0.02 % Bromphenol blue)

3.6 g Tris

dissolved in 30 ml 1M A. bidest and adjusted pH to 6.8

10 g SDS

9.25 g DTT

57 ml glycerol 87 %

20 mg Bromphenolblue

add A. bidest to 100 ml

Protocol

SDS Page is a common electrophoresis method to separate protein samples according to their size.

According to the needed density of the separation gel the components were mixed as described in Tab. 5.1, pouring the solution about 0.5 cm below the comb between the biozym 1.5 mm glass plates. For gradient gels (4-20 %) the two separate PAA solutions were poured into Hoefer SG15 chamber and mixed via Minipuls 3 pump directly into the glass plates. While polymerizing the gel was covered with 1 ml water.

After about 20-30 min, the water was poured off and the comb gel was prepared (see Tab.5.1), poured onto the separation gel and the combs were inserted immediately. After about 40-50 min the gel was ready to use.

Table 5.1: Ingredients for different PAA Gels The volumes are calculated for 2 gels with 1.5mm thickness

4% separation gel	10% separation gel	20% separation gel	comb gel	
2.1ml	5.3ml	10.6ml	0.75ml	acrylamid solution
4.0ml	4.0ml	4.0ml	-	separation gel buffer
-	-	-	1.25ml	comb gel buffer
9.9ml	6.7ml	1.4ml	3.0ml	A. bidest
20µl	20µl	20µl	10µl	TEMED
50µl	50µl	50µl	25µl	APS 10%

Samples have to be prepared with 5x Sample Buffer (e.g. 10-50 µg total Protein in 20 µl + 5 µl 5x Sample Buffer) and boiled for about 10 min to break up secondary and tertiary structures.

The gels were put into the Mini-PROTEAN 3 Cell Electrophoresis Module, the chamber was filled up with 1x TGS Buffer and the samples and standards were loaded.

In this study all gels were run at a constant voltage of 150 V for 60-80 min. After electrophoresis the gels were used for western blotting.

5.3.15 Western Blotting

Reagents and supplies

- **TBS - Stock Solution** (1.5M NaCl, 0.5M Tris, pH7.4)

60.55 g Tris

87.66 g NaCl

dissolved in 600 ml A. bidest and adjusted pH to 7.4

add A. bidest to 1000 ml

- TBS - Working Solution (100 ml TBS Stock Solution 900 ml Aqua bidest)

- **Anodebuffer I** (0.3M Tris, 10% Methanol, pH10.4)

36.34 g Tris

dissolved in 600 ml A. bidest and adjusted pH to 10.4

add 100 ml methanol

add A. bidest to 1000 ml

- **Anodebuffer II** (25mM Tris, 10% Methanol, pH10.4)

3.03 g Tris

dissolved in 600 ml A. bidest and adjusted pH to 10.4

add 100 ml methanol

add A. bidest to 1000 ml

- **Cathodebuffer** (25mM Tris, 10% Methanol, 40mM Glycine, pH9.4)

3.03 g Tris

3g glycine

dissolved in 600 ml A. bidest and adjusted pH to 7.4

add 100 ml methanol

add A. bidest to 1000 ml

Protocol

For transfer, the semi-dry blotting unit was prepared by rinsing the anode and cathode with tap water.

For each gel, 6 pieces of extra thick blotting paper and one nitrocellulose membrane were cut to the same size as the gel (see Fig.5.5).

Two pieces of blotting paper were put into the anode buffer I, one piece of blotting paper and the membrane into the anode puffer II and three pieces of blotting paper and the gel into the cathode buffer. The membrane was also pre-wetted in anode buffer II whereas the gel was put into cathode buffer. The stack was assembled, starting with the blotting paper from the anode buffer I, then the paper from anode buffer II was added, then the membrane, the gel and finally on top the blotting paper from the cathode buffer.

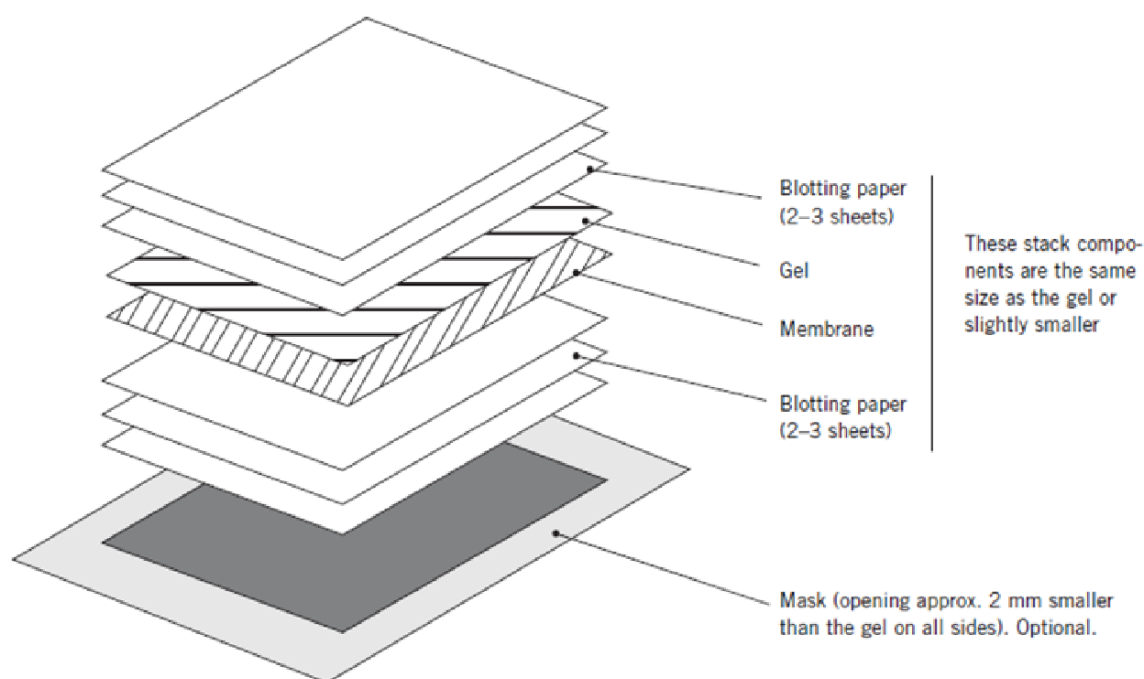


Figure 5.5: Transfer stack for semi-dry blotting The lower two blotting papers are soaked with Anode buffer I, the blotting paper under the membrane as well as the membrane itself are soaked with Anode Buffer II and the gel as well as all three blotting papers on the top are soaked with Cathode buffer.

The transfer was performed at a constant amperage of 1 mA/cm^2 for 1 hour, that means for one membrane (7 cm* 9 cm) an amperage of 63 mA was used.

Membranes were blocked in 5 % non-fat dried milk in TBS.

Primary antibodies were diluted in 5 % non-fat dried milk in TBS according to the manual and membranes were incubated for 1-2 hours on a shaker. Afterwards, the membrane was washed in TBS for 3 x 10 minutes at room temperature.

The corresponding HRP labeled secondary antibody was diluted in 5 % non-fat dried milk in TBS. The membrane was incubated in diluted secondary antibody for 1 hour at room temperature on an orbital shaker. Afterwards, the membrane was again washed in TBS for 3 x 10 minutes at room temperature and rinsed once with aqua bidest before detection.

Detection was done with ECL substrate (ECL plus or WesternBright ECL Spray) according to the manual.

5.3.16 RNA Extraction and Reverse Transcription

Reagents and supplies

- RNeasy Lipid Tissue (Qiagen Cat. No 74804)
- QuantiTect Reverse Transcription Kit (Qiagen Cat. No 205313)
- filter tips

Protocol

RNA was extracted from a ~5 mg part of the left frontal cortex with RNeasy ® Lipid Tissue Mini Kit (Qiagen) according to the manual. Amount and quality of RNA was determined with Nanodrop Photometer and 1 µg of each RNA sample was used for Reverse Transcription with QuantiTect Kit (Qiagen) according to the manual. cDNA was stored at -20°C until further use.

5.3.17 Quantitative Real Time PCR

Reagents and supplies

- ssoAdvabced SYBR Grren (Biorad)
- QuantiTect Primer Assays (Qiagen)
- Opticon 2 Real Time cycler
- filter tips

Protocol

Prepared cDNA was diluted 1:10 (5ng/μl) for all analyses. QuantiTect Primer Assays for Hmg-CoA-R (QT01037848), ACAT2 (QT01771441) and BACE1 (QT00493948) and self designed primer sets for ACAT1, ApoE, ApoA1, LDL-R, LRP1, Cyp46a1, ABCA1, mAPP and HPRT, as housekeeping gene, (see Tab.5.2) were used with a SYBR Green Supermix for qReal Time PCR.

For each sample, a 25 μl reaction was set up according to the manual with 2.5 μl (12.5 ng) cDNA. All samples were analyzed in duplicates with a real-time cycler.

The so called delta-delta c(t) calculation was used to normalize amounts of cDNA in relation to the housekeeping gene in the first step ($\Delta c(t) = c(t)_{\text{target gene}} - c(t)_{\text{HKG}}$) and to detect differences between groups in the second step ($\Delta\Delta c(t) = \Delta c(t)_{\text{transgenics}} - \Delta c(t)_{\text{WT}}$).

Relative expression differences are then calculated with the formula $\text{ratio} = 2^{(-\Delta\Delta c(t))}$ and are expressed as x-fold change to WT group.

Table 5.2: Primer sets for mRNA expression analysis with qPCR

mHPRT	CGTCATGCOGACCCGCAGTC	212bp
	CCTTGAGCACACAGAGGGCCAC	
mABCA1	TGTGCTGCOGTACGAAGCCG	213bp
	TCCCCGAAGGCCCGTCTGTT	
mApoE	GATCCCCTGCTCAGACCCTGGA	137bp
	GCTCTCCCTCGGCTAGGCATC	
mLDL-R	GCCGGAGTTGCAGCAGAAGAC	282bp
	TCCTGGGAGCACGTCTTGGGG	
hAPP	GCATTTCCAGGAGAAAGTGG	324bp
	ATGCGCTCATAAATCACACG	
mLXRa	CTCAATGCCTGATGTTTCTCCT	150bp
	TCCAACCCTATCCCTAAAGCAA	

5.3.18 Evans Blue Test

Reagents and supplies

- Evans Blue (Sigma Aldrich)
- PBS (Lonza)
- N, N-dimethylformamide (Sigma Aldrich)

Protocol

Two 10 months old mice of every genotype received a single i.p. injection of 50 µg/g Evans Blue dye in PBS. Three hours after the application animals were sacrificed.

Therefore animals were anesthetized and perfused with PBS. After perfusion the livers as well as left brain hemispheres were dissected, weighted and prepared for Evans Blue Assay. For measurement of Evans blue leakage, liver, plasma and brain were homogenized in 5 volumes N, N-dimethylformamide, then incubated for 40 hours at 56°C and centrifuged at 20,000xg for 20 min.

The Evans Blue level was determined in 200 µl of the supernatants by reading absorbance at 620 nm using a plate reader. Additionally, a standard curve between 100-1 µg/ml Evans Blue in N, N-dimethylformamide was prepared and measured together with the samples.

Optical density (OD) values of brain and liver homogenates were normalized for plasma values.

Right brain hemispheres were cut sagittally as described in section 5.4.1 and directly analyzed with a Zeiss microscope using excitation at 620 nm, emission at 680 nm.

5.4 Histological Methods

5.4.1 Cryocutting

Reagents and supplies

- Polysine Object slides (Lactan)
- O.C.T. tissue freezing medium (Leica)
- Cryotome

Protocol

Cryo-conserved brains were embedded in O.C.T. tissue freezing medium and cut sagittally from the medial to the lateral side.

The brain was divided into 12 layers, each containing 30 slices á 10 µm, according to the mouse brain atlas.

From these 10 µm slices always 7 slices per layer were collected on object slides, whereas the other 23 slices were discarded. Thus receiving 12x7 slices per brain which were stored at -20°C until further use.

5.4.2 Immunohistochemistry

Five 10-µm-thick mounted sections deriving from five different medio-lateral levels (L2, 4, 6, 8 and 11) per animal were stained. All used antibodies and dilutions are displayed in Tab 5.5.

In brief, for quantitation of plaque load, sections were stained with an anti-amyloid fibrils LOC antibody recognizing amyloid fibrils and fibrillar oligomers, but not monomers.

Region size of hippocampus and cortex was measured and immunoreactive area within each region was quantified using automated image analysis software (Image Pro Plus, version 6.2).

Distribution of ApoB-100 in the brain was visualized by staining with an antibody detecting both human and murine ApoB.

For investigating inflammatory processes, GFAP-IHC for astrocytes as well as CD11b-IHC for microglia was performed and quantified, taking account of the outlined region size, with Image Pro Plus.

To detect cerebral amyloid angiopathy 5 slices per animal were co-stained with 6E10 and anti-SMA antibodies. Twelve defined regions/arteries per slice were outlined and vessel size (SMA) as well as intravascular-amyloidogenic area (6E10) were measured.

Tab. 5.5 Antibodies used for Immunohistochemistry

name	purpose	company	dilution
Rabbit Anti-Amyloid Fibrils LOC	fibrillar Abeta	Millipore AB2287	1:500
Sheep to anti-Apolipoprotein B	human and murine ApoB	abcam ab20737	1:500
Rabbit Anti-Glial Fibrillary Acidic Protein (GFAP)	Inflammation	Dako Z0334	1:500
Rat Anti Mouse CD11b		Serotec MCA711	1:1000
Rabbit anti- alpha smooth muscle actin (SMA)	Cerebral amyloid angiopathy	abcam ab5694	1:1000
Mouse Human Amyloid Beta Protein, 6E10		Signet 9320-02	1:1000

Chapter 6

Results

6.1 Characterization over age

This study consists of two parts. The first section, described here, deals with a basic characterization of all four genotypes over age, without any intervention. Results of this working package have already been published in large parts (Löffler et al., 2013).

The impact of genotype, age and sex on all investigated parameters was statistically evaluated. Since sex differences were not revealed for most markers, results are displayed for a mixed population except as noted otherwise.

6.1.1 Plasma lipid profile

Pathological plasma lipid profiles, especially high LDL to HDL ratios, are well described risk factors for CAD but are currently attracting more attention regarding other age-related diseases. Plasma lipid profiles were assessed in all genotypes at 6 months of age.

“Compared to WT animals, LDL cholesterol levels were increased up to 3.5 fold in both ApoB-100 overexpressing genotypes, ApoBxAPP and ApoB-100 (Fig. 6.1 A).

In contrast, HDL cholesterol (Fig. 6.1 B) was significantly decreased, to 68 % and 75 % in ApoBxAPP and ApoB-100 animals, respectively compared to WT littermates. Additionally, plasma triglyceride levels, but also total cholesterol, were significantly increased in ApoBxAPP and ApoB-100 animals compared to WT mice (Fig. 6.1 C,D).” (Löffler et al., 2013)

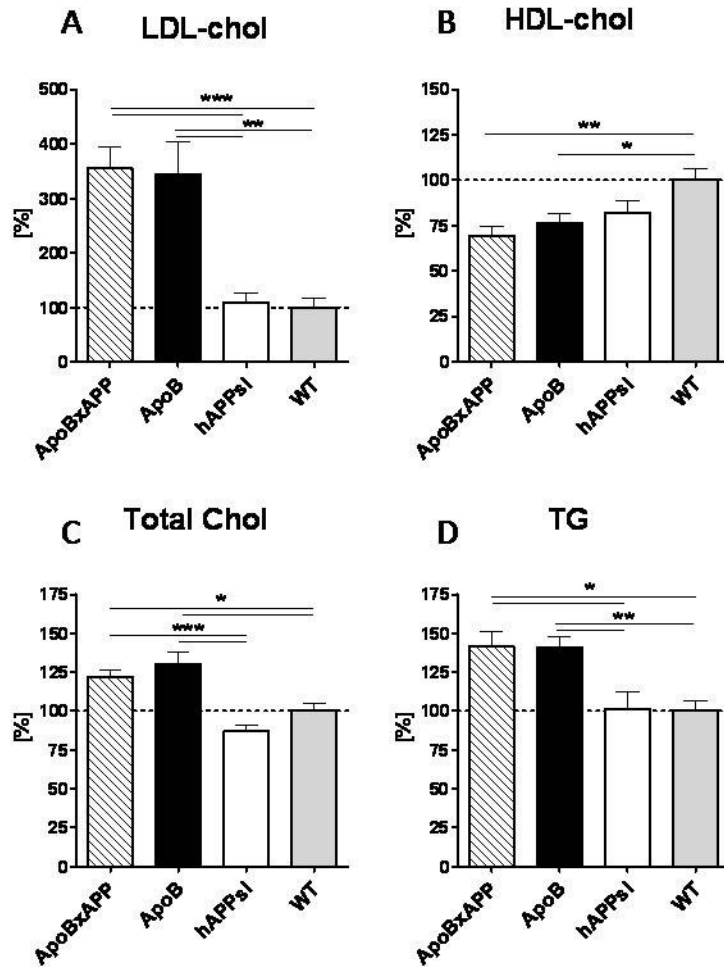


Figure 6.1: hApoB-100 overexpression leads to an atherogenic plasma lipid profile. (A) Plasma LDL-, (B) HDL- and (C) total cholesterol as well as (D) triglyceride levels of 6 months old animals; n=18 per group; Statistical analysis: One-way-ANOVA followed by Bonferroni post-test (Löffler et al. 2013).

APPs1 single transgenic animals did not show any significant difference to WT mice regarding LDL-cholesterol and triglyceride levels, but a tendency to reduced HDL- and total cholesterol levels was observed (Fig. 6.1)

The corresponding plasma lipid profiles did not significantly change over age.

6.1.2 Brain lipids

As described in more detail in section 3.2.2, the balance between free and esterified cholesterol seems to play a crucial role in AD pathology.

In 9 months old animals free, cholesterol and cholesteryl esters were quantified using cortical and rest-brain tissue, representing grey and white matter, respectively.

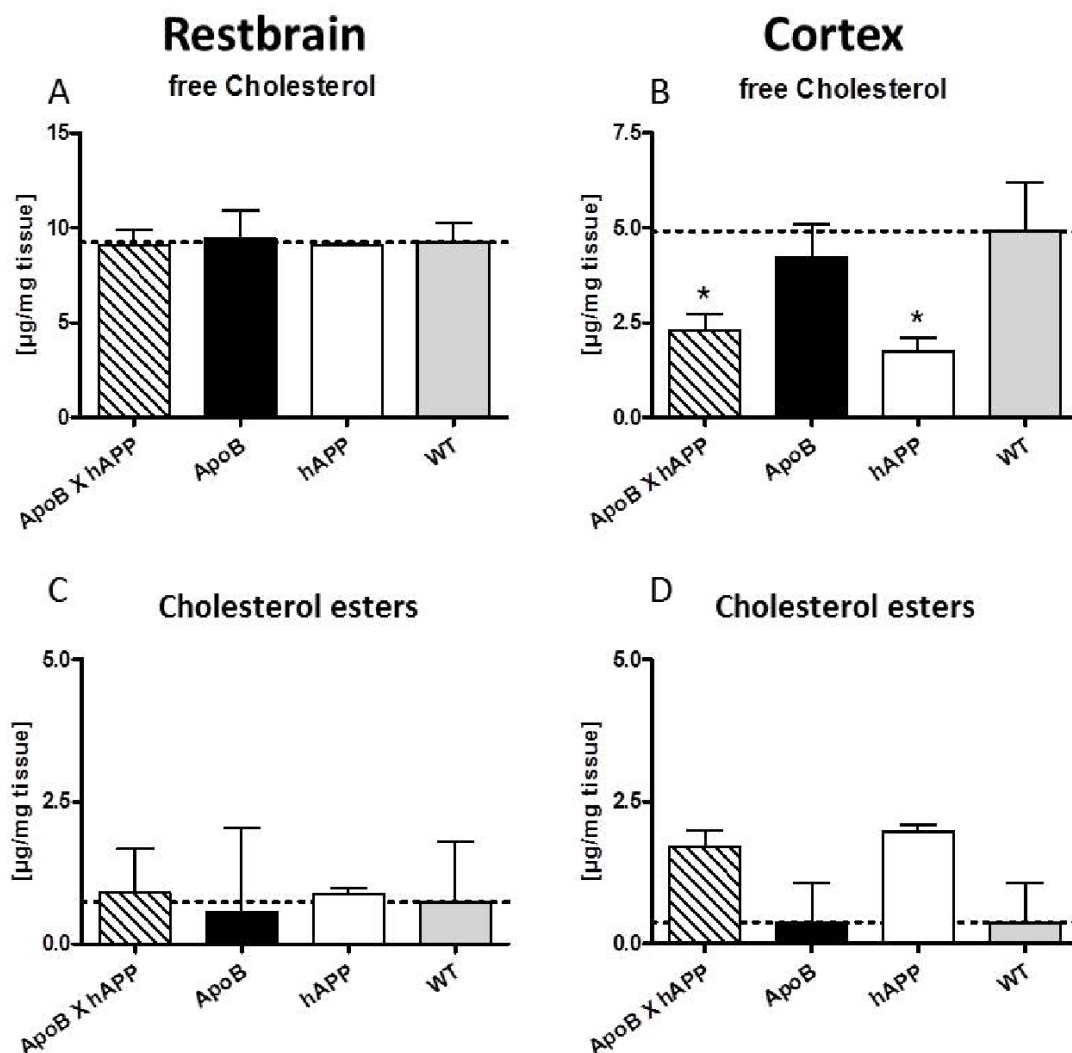


Figure 6.2: Assessment of brain cholesterol levels. Free cholesterol in restbrain (A) and cortex (B) of all four genotypes at an age of 9 months. Also esterified cholesterol was measured in restbrain (C) and cortices (D) of the same animals. n=5 per group; Statistical analysis: One-way-ANOVA followed by Bonferroni post-test.

In both APP overexpressing genotypes, ApoBxAPP and hAPPsl, a shift towards decreased free and increased esterified cholesterol levels was observed, in cortical but not in restbrain samples (Fig. 6.2 A,B). Free cholesterol levels in cortices of hAPP overexpressors were almost half of those in WT brains (Fig. 6.2 B) .

Significantly higher levels of free cholesterol in the restbrain compared to the cortex are expected since the restbrain mainly consists of white matter, which contains high amounts of cholesterol in the myelin sheaths (Fig. 6.2 A,B).

Only trace amounts of cholesteryl esters were detected in WT cortices, with levels around 0.3 $\mu\text{g}/\text{mg}$ tissue, but increased to more than 2 $\mu\text{g}/\text{mg}$ tissue in ApoBxAPP and hAPPsl animals. This trend is obvious, but the difference did not reach statistical significance due to the high standard deviation (Fig. 6.2 D). In the restbrain, no difference in cholesteryl ester levels between the genotypes was observed (Fig. 6.2 C).

Since changes in fatty acid composition are associated with AD (Chan et al., 2012), also the composition of fatty acids in the same brain extracts as used for cholesterol determination was evaluated. These analyses did not reveal any differences, neither in total amounts of fatty acids (data not shown), nor in the distribution of fatty acid species (see Fig.6.3) between genotypes.

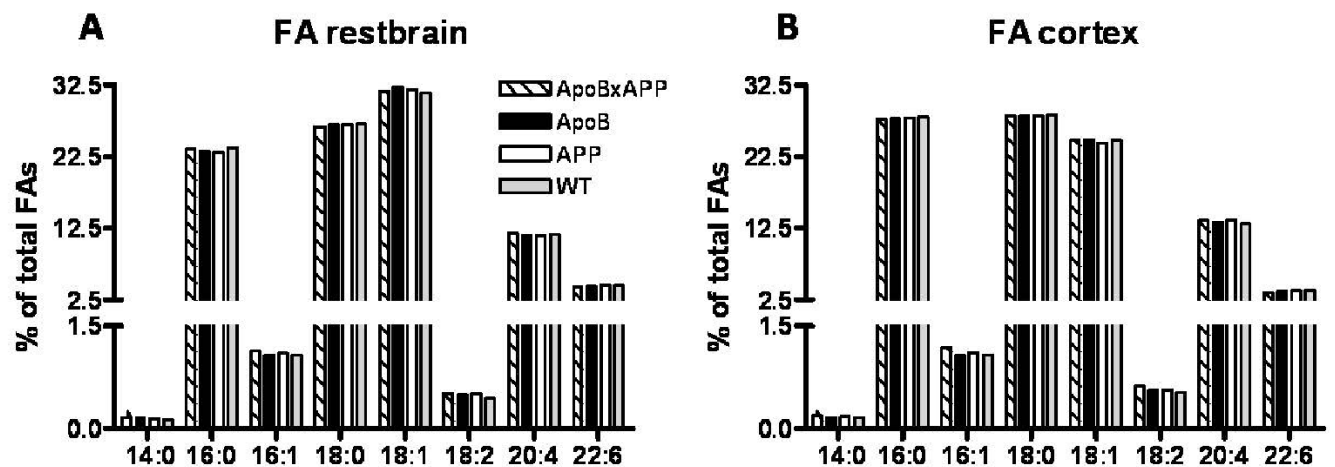


Figure 6.3: Distribution of fatty acids from lipid extracts of restbrains (A) and cortices (B) of 9 months old animals. n=5 per genotype; Statistical analysis: One-way-ANOVA followed by Bonferroni post-test.

6.1.3 Lipid peroxidation

Increased oxidative stress is proposed to play a key role in the development of atherosclerosis as well as AD. Therefore, malondialdehyde (MDA) levels were assessed in cortex and hippocampus of all genotypes over age.

In the cortex, MDA values in ApoBxAPP and ApoB-100 mice were significantly increased at an age of 6 months (Fig. 6.4 A), while this effect was already observed in hippocampal samples of 4-months old mice (Fig. 6.4 B).

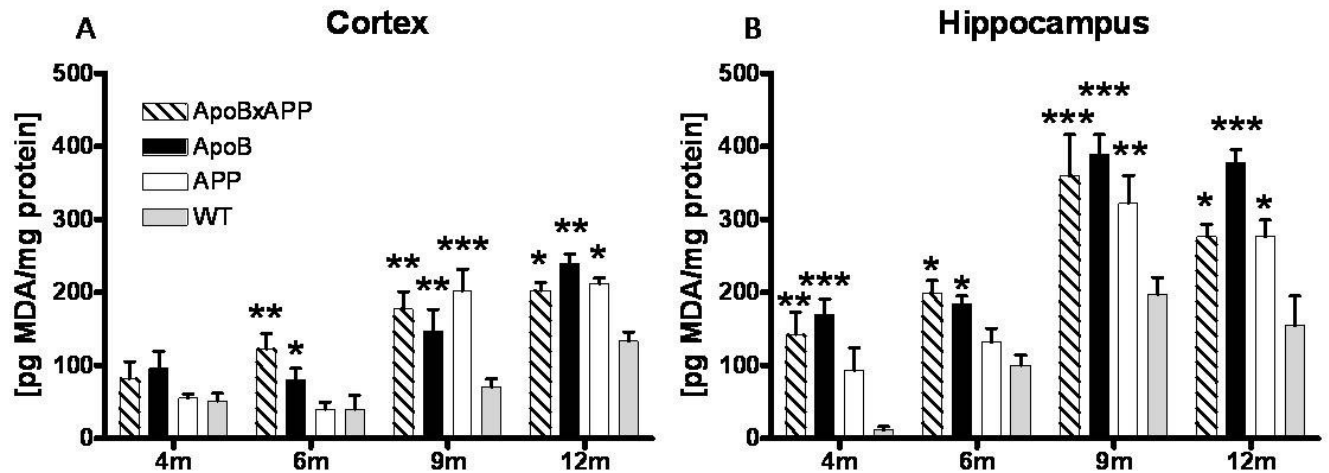


Figure 6.4: Increased cerebral lipid peroxidation shown as malondialdehyde (MDA) levels in cortical (C) and hippocampal (D) samples. n=15 per group; Statistical analysis: Two-way-ANOVA followed by Bonferroni post-test (Löffler et al. 2013).

At 9 months of age, an increase in MDA levels was also measured in cortical and hippocampal samples of hAPPs1 single transgenic animals. In summary, MDA levels in both cortex and hippocampus of all three transgenic mouse models were significantly elevated at 9 and 12 months of age compared to WT animals.

To evaluate the possibility that increased MDA levels are due to decreased levels of antioxidants like alpha-tocopherol, measurements of alpha-tocopherol levels in restbrain and cortex of 9 months old animals were performed. Unexpectedly, these data did not reveal any difference

between genotypes (Fig.6.5) and thus, the reason for increased cerebral lipid peroxidation products is currently unclear.

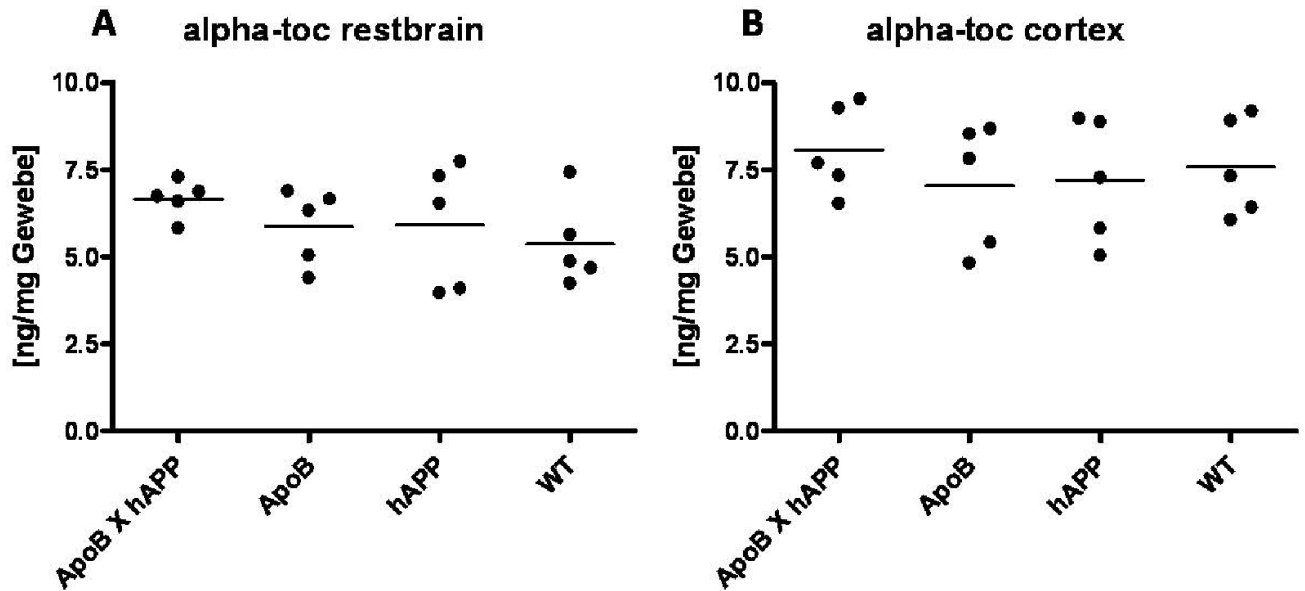


Figure 6.5: Alpha-tocopherol levels in (A) restbrain and (B) cortex of 9 months old animals. n=5 per group; Statistical analysis: Two-way-ANOVA followed by Bonferroni post-test.

6.1.4 Amyloid pathology

Amyloid pathology is one of the major hallmarks of AD. To test whether additional ApoB-100 overexpression and therefore changes in the plasma lipid profile impact on cerebral Abeta metabolism, several measurements dealing with amyloid pathology were carried out.

Abeta quantitation in cortical and hippocampal homogenate fractions of 6, 9 and 12 months old animals were carried out using an assay which detects human and rodent Abeta. These Abeta species are differing at three amino acids close to their N-terminus (see Fig. 6.6), while capture antibodies of this assay bind to the C-terminus.

In contrast to ApoBxAPP and hAPPSl mice, which display both, murine and human Abeta species, ApoB-100 and WT animals only generate murine Abeta peptides from endogenous APP.

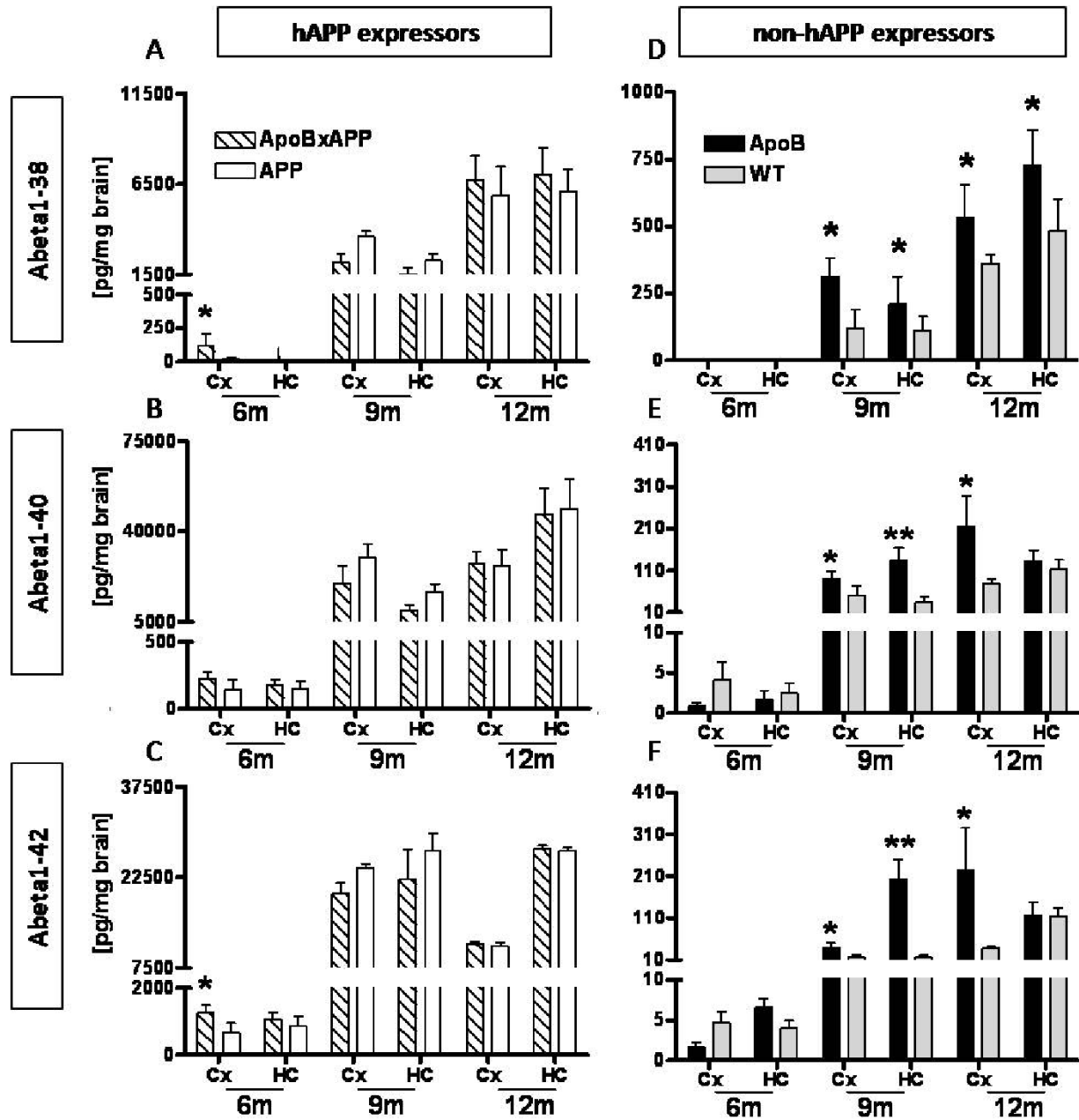


Figure 6.6: Influence of hApoB-100 overexpression on insoluble Abeta levels (FA fraction) in cortex (Cx) and hippocampus (HC) of hAPP expressors, ApoBxAPP and hAPPSl (A-C) as well as non-hAPP expressors, ApoB and WT (D-F). n=10 per group. Statistical analysis: Two-way-ANOVA with Bonferroni post-test.

“Analysis of plaque-associated Abeta (FA-fraction), from ApoBxAPP and hAPPsl mice showed, due to hAPP overexpression, profoundly increased levels of all Abeta peptides compared to endogenous Abeta in WT animals (Fig. 6.6) . At 12 months of age Abeta values increased in the hippocampus by 15x, 300x and 100x for Abeta1-38, Abeta1-40 and Abeta1-42, respectively.

To allow visualization of ApoB-100 induced differences between hAPP expressors (ApoBxAPP versus hAPPsl) as well as non-hAPP transgenic mice (ApoB-100 vs. WT), these genotypes were compared separately (Fig.6.6).” (Löffler et al., 2013)

Comparison of cerebral Abeta levels of ApoBxAPP and hAPPsl mice revealed significant differences only in the cortex of 6 months old animals. At this age, Abeta1-38 and Abeta1-42 levels were significantly higher in cortical samples of ApoBxAPP animals. This difference vanished in older animals (Fig. 6.6 A-C).

A direct impact of hApoB-100 expression on endogenous Abeta levels was observed when comparing ApoB-100 to WT animals. Intriguingly, all three investigated Abeta species were significantly increased in brains of ApoB-100 animals compared to WT mice, starting with an age of 9 months (Fig. 6.6 D-F). This difference persisted up to 12 months and indicates that overexpression of ApoB-100 has a direct effect on the abundance of all three Abeta peptides in both investigated brain regions.

In 12 months-old hAPP overexpressing animals, ApoBxAPP as well as hAPPsl, the Abeta1-40 peptide was the most abundant Abeta species, followed by Abeta1-42, while WT mice hardly displayed murine Abeta1-40 and Abeta1-42 up to an age of 9 months. WT as well as ApoB-100 animals mainly produced murine Abeta1-38 at higher ages.

The same trends were obtained for soluble Abeta measurements (DEA-Fraction), but soluble Abeta levels were about 50x lower than insoluble species of the FA fraction (data not shown).

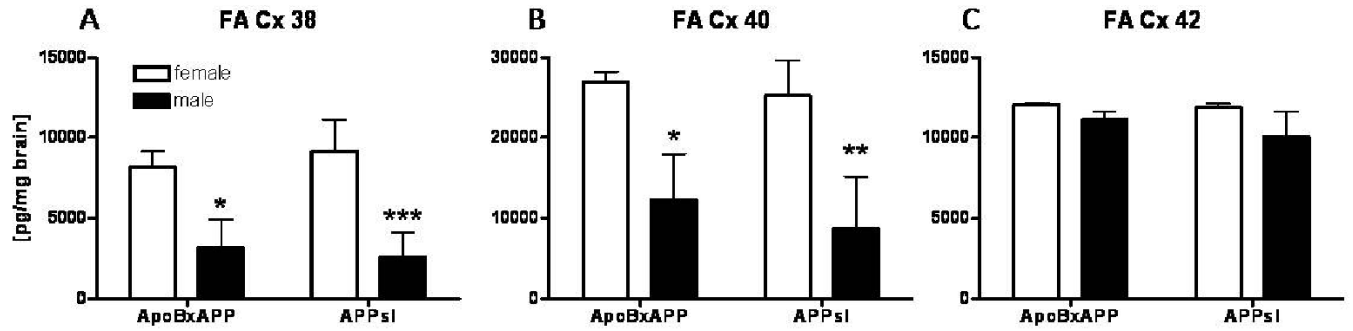


Figure 6.7: Sex differences in 12 months old hAPP expressors ApoBxAPP and hAPPsl. (A) Abeta1-38, (B) Abeta1-40 and (C) Abeta1-42 in cortical FA fraction. 5 male and 5 female animals per genotype

“Sex differences were detected in hAPP expressors (ApoBxAPP and hAPPsl) only at an age of 12 months. When evaluated separately, 12 months old male ApoBxAPP and hAPPsl mice displayed significantly lower Abeta1-38 and Abeta1-40, but not Abeta1-42 levels compared to females (Fig. 6.7). ApoB and WT mice displayed no sex differences regarding Abeta levels.” (Löffler et al., 2013)

Additional immunohistochemical detection (Fig. 6.8) of fibrillar Abeta in cortex and hippocampus underlined the above described results obtained from MSD measurements. Abeta peptides rapidly aggregate to form oligomers and fibrils which in the end leads to deposition of amyloid plaques (Walsh et al., 2002). Therefore detection of fibrillar Abeta displays both, dense plaques as well as intra- and extracellular pre-stages.

Again no difference between ApoBxAPP and hAPPsl mice could be detected (Fig. 6.8 E). But, similar to MSD measurements, a strong increase of immunoreactivity over age was detected, revealing a profound plaque burden at 12 months of age (Fig. 6.8 C,E). In ApoB-100 and WT animals no plaque formation was detected (Fig. 6.8 D).

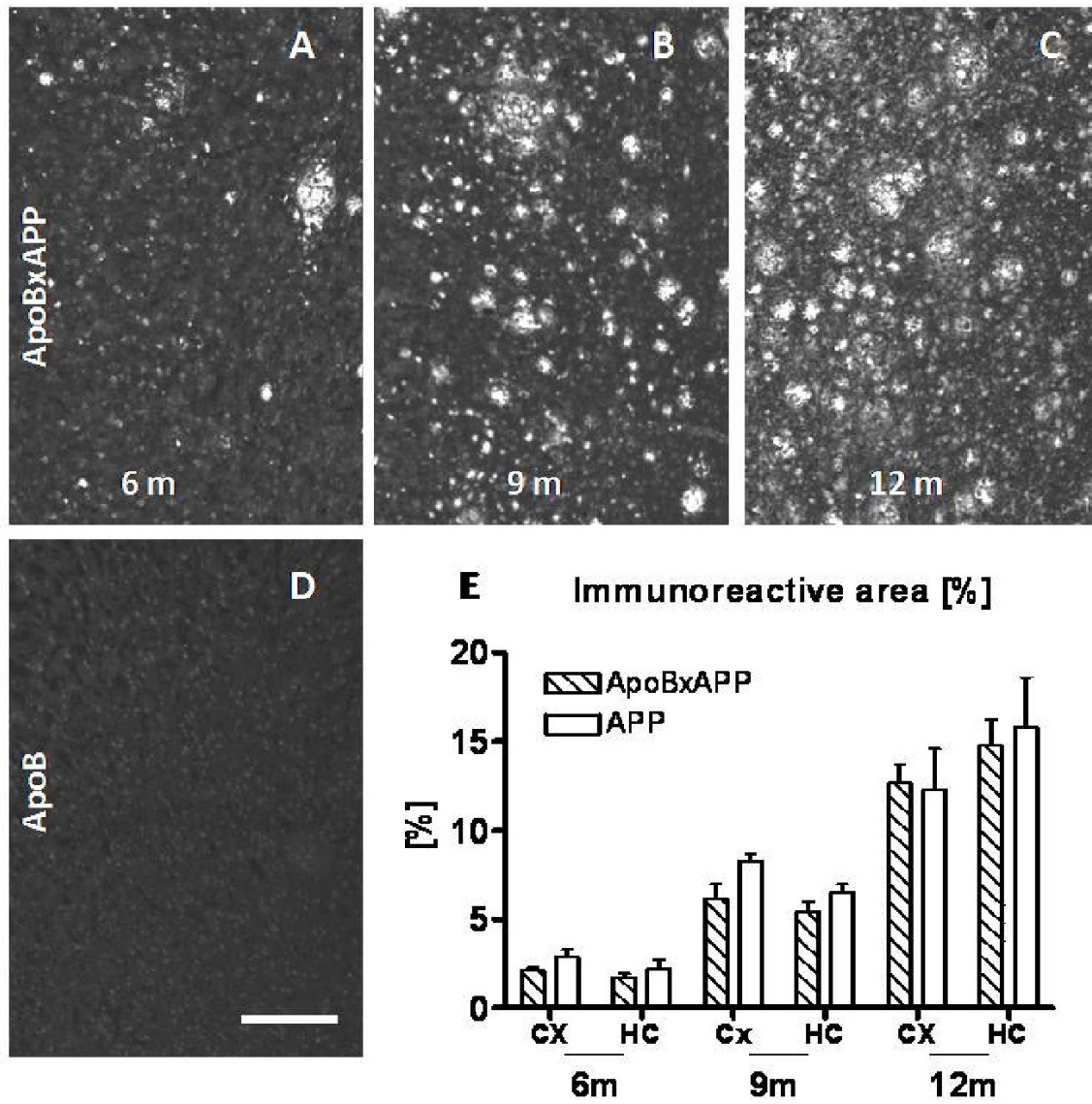


Figure 6.8: Immunohistochemical detection and evaluation of amyloid plaques. Representative pictures of LOC antibody, detecting fibrillar Abeta, on brain slices of (A) 6, (B) 9 and (C) 12 months old ApoBxAPP animals and a (D) 12 months old ApoB transgenic mouse; scale bar 100 μ M. (E) Evaluation of LOC immunoreactive area, representing plaque burden over age in ApoBxAPP and hAPPsl mice. n=6 per group; Statistical analysis: Two-way-ANOVA with Bonferroni post-test.

Cerebral amyloid angiopathy (CAA), the presence of Abeta in vessel walls, was assessed in 12 months old animals by co-staining with smooth-muscle actin, a marker for arteries, and 6E10, an antibody detecting human Abeta species. In the course of this measurement also vessel area was assessed.

Considerable manifestation of CAA was found in both, ApoBxAPP and APPsl mice compared to WT animals. But again no significant difference between hAPP overexpressors, ApoBxAPP and hAPPsl, could be detected. Surprisingly, a tendency to a decreased amount of CAAs in double transgenics could be observed (Fig. 6.9 A), indicating that additional ApoB-100 overexpression slightly ameliorates CAA pathology.

Vessel sizes only differed between WT and hAPPsl mice. Increased vessels diameters were observed in hAPPsl animals (Fig. 6.9 B), while in ApoBxAPP mice this phenotype could not be detected.

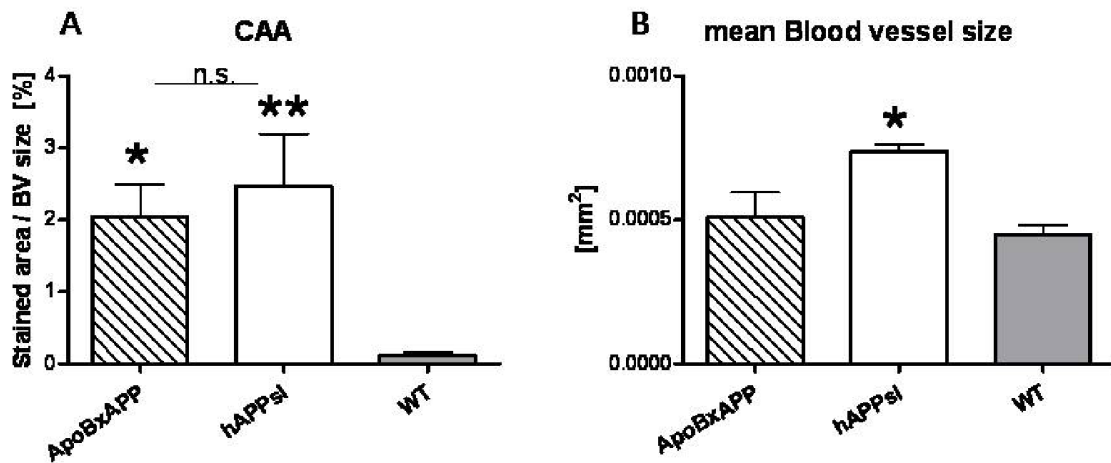


Figure 6.9: Evaluation of (A) cerebral amyloid angiopathy, as immunoreactive area per blood vessel size, and (B) vessel size in ApoBxAPP and hAPPsl mice compared to WT littermates at 12 months of age. n=5 per group; Statistical analysis: One-way-ANOVA with Bonferroni post-test.

6.1.5 Blood brain barrier changes

Impairment of the blood brain barrier (BBB) was found in AD patients brains (Farkas and Luiten, 2001). Besides the above mentioned aggregation of Abeta in cerebral vessels (CAAs), disturbance of BBB transporters or a complete break-down and leakage of the BBB are additionally described in AD.

Since hApoB-100 expression is controlled by its natural promoter, ApoB should only be expressed in liver and intestine, while cerebral ApoB-100 immunoreactivity should develop exclusively due to damage in the cerebral vasculature. Expression of hApoB-100 in brains of transgenic animals was excluded by mRNA expression analysis.

Immunohistochemical analyses of brain slices for ApoB abundance with an antibody detecting both, murine and human ApoB was carried out for all four genotypes. hApoB overexpressors, ApoBxAPP and ApoB-100, showed strong accumulation of ApoB in the cerebral vasculature already at an age of 6 months.

Especially in leptomeningeal vessels in the hippocampal area, high ApoB immunoreactivity was detected (Fig. 6.10 A). In deeper vessels, most likely representing venules and capillaries, accumulation and even extravasation of ApoB into brain tissue was discovered (Fig. 6.10 C, E). In contrast, WT and hAPPsl mice showed almost no cerebral ApoB immunoreactivity (Fig. 6.10 B, D, F).

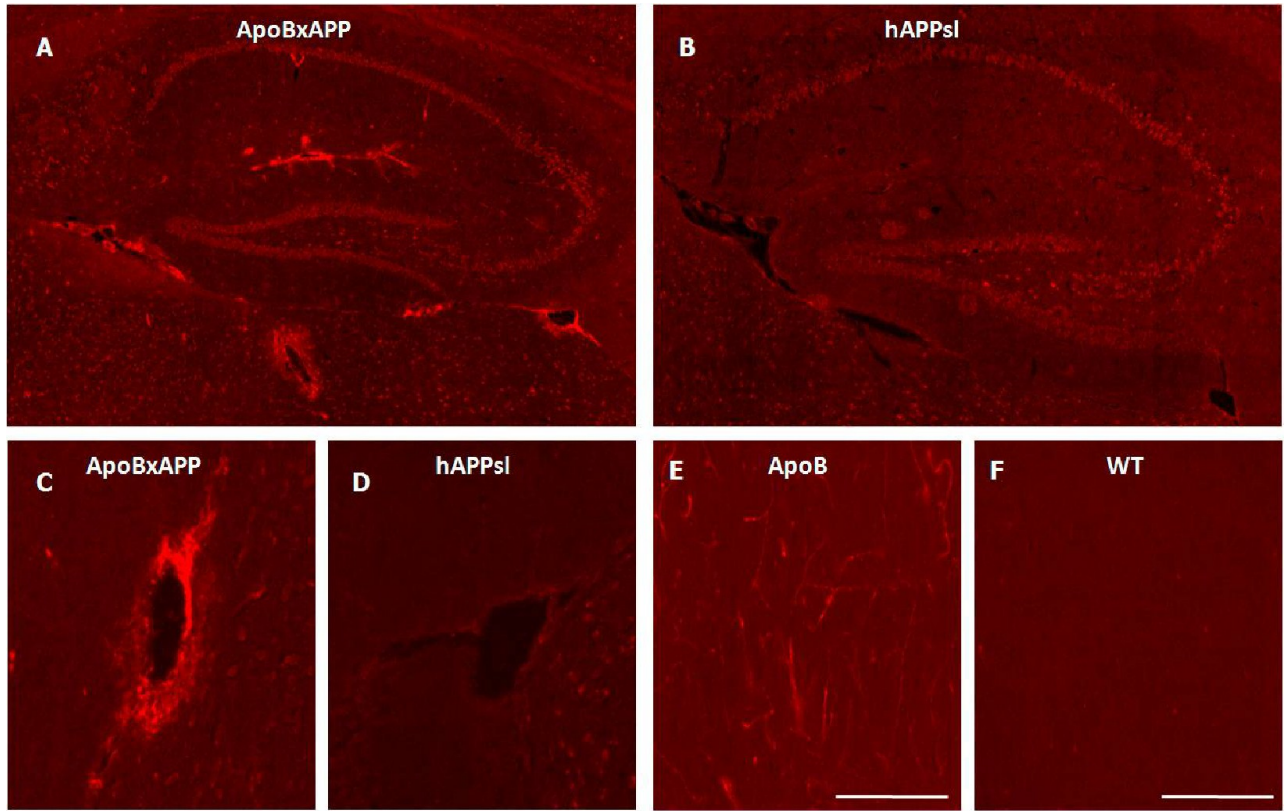


Figure 6.10: Cerebral accumulation and extravasation of ApoB in leptomeningeal vessels in the hippocampal area of 6 months old ApoBxAPP (A) and hAPPsl (B) animals. Accumulated ApoB in deep vessels, of ApoBxAPP (C) brains in contrast to hAPPsl mice (D). ApoB-immunoreactivity of capillaries in the thalamic region of ApoB (E) and WT (F) animals. Scale bars: 100 μ m. (Löffler et al. 2013)

In order to show whether ApoB extravasation was due to BBB damage, an Evans Blue test was carried out with 10 months-old animals (Fig. 6.11).

Significant differences were only obtained for ApoB and hAPPsl animals compared to WT mice. Unfortunately a high standard deviation averted statistical significance for ApoBxAPP mice (Fig. 6.11 A). However, histological analysis of animal brains showed leakage of the BBB in all three transgenic genotypes compared to WT controls (Fig. 6.11 B-E)). Interestingly, the dye was found to be highly enriched in plaque areas in both hAPP overexpressors, ApoBxAPP and hAPPsl.

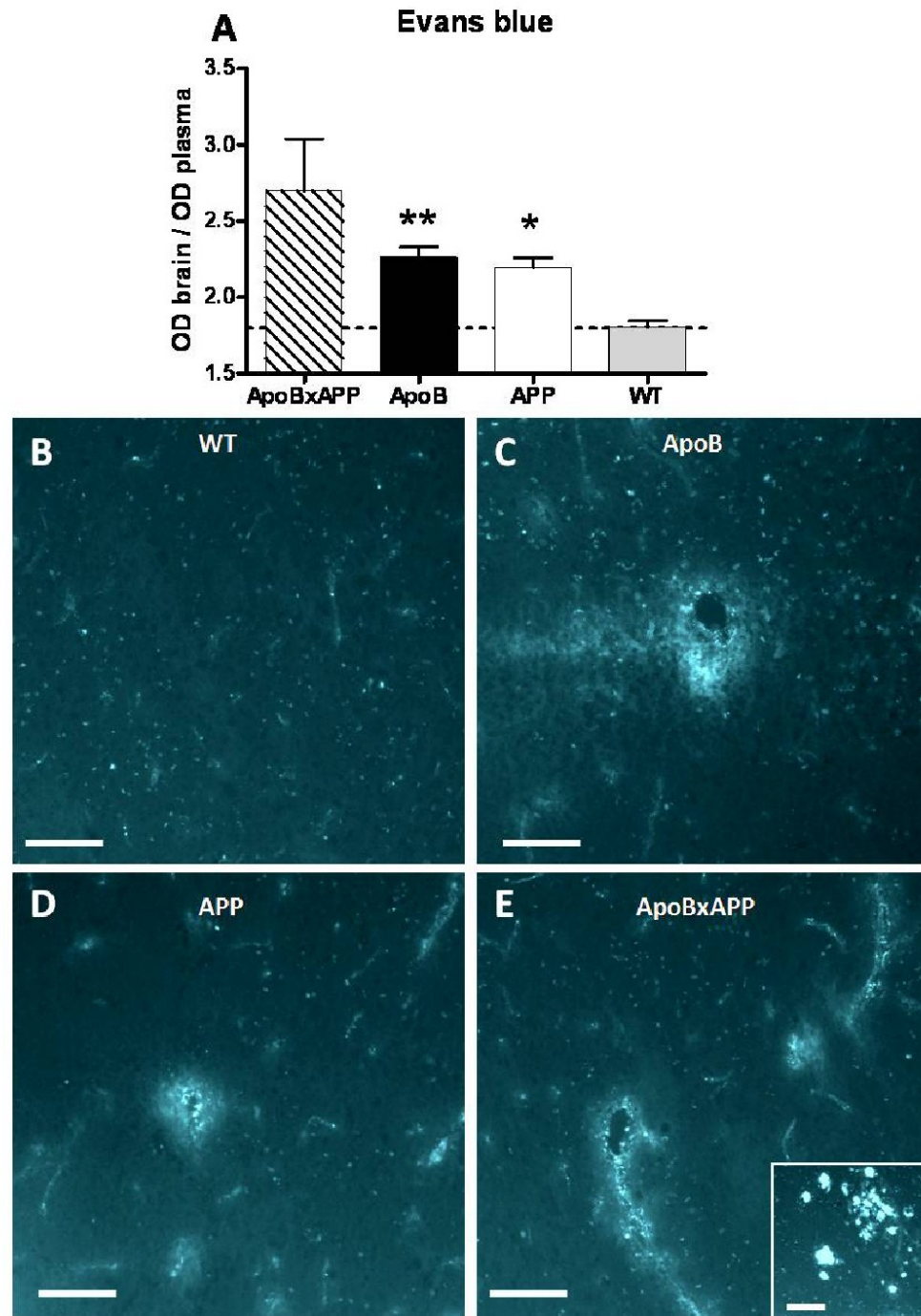


Figure 6.11: Evans blue test. (A) Results of quantitative biochemical assessment of cerebral Evans Blue levels. Representative pictures of the lateral preoptic area from Evans Blue treated (B) WT, (C) ApoB, (D) hAPPsl and (E) ApoBxAPP animals. Insert (E) Evans Blue highly enriched in plaques. Scale bars: 100 μ m

6.1.6 Inflammation

The inflammation status of brains from all genotypes was analyzed by quantitative immunohistochemical detection of Glial Fibrillary Acidic Protein (GFAP), a marker for activated astrocytes and CD11b, involved in adhesive interactions of microglia.

“In the cortical region of double transgenic ApoBxAPP and single transgenic hAPPsl mice strong GFAP immunoreactivity was detected at 9 and 12 months of age (Fig. 6.12 A). Notably, increased GFAP immunoreactivity in the hippocampus was only detected in 12 months old double transgenic animals (Fig 6.12 B; K-N).

Signs of astrogliosis in the corpus callosum were only found in old ApoBxAPP mice (Fig. 6.12 C). At 12 months of age, an increase in activated microglia could be observed in all investigated brain regions of ApoBxAPP and hAPPsl mice (Fig. 6.12 D-F; G-N).

The only statistically significant difference in CD11b immunoreactivity between these two genotypes was detected in the cortex at 6 months of age (Fig. 6.12 D). Here, the additional ApoB overexpression in ApoBxAPP mice led to increased microglia activation in the cortex in contrast to 6 months old hAPPsl mice and non transgenic littermates.” (Löffler et al., 2013)

Activated astrocytes and microglia in the cortex were predominantly found on and around amyloid plaques as previously described for other hAPP overexpressing mice (Rockenstein et al., 2001).

Unfortunately, further characterization of the inflammation status of all four genotypes failed. The approach to quantitate several inflammation markers in plasma and brain homogenates using MSD® 96-Well MULTI-SPOT® Mouse Proinflammatory 7-plex was unsuccessful, since most of the measured parameters were under or around the evaluated detection limits.

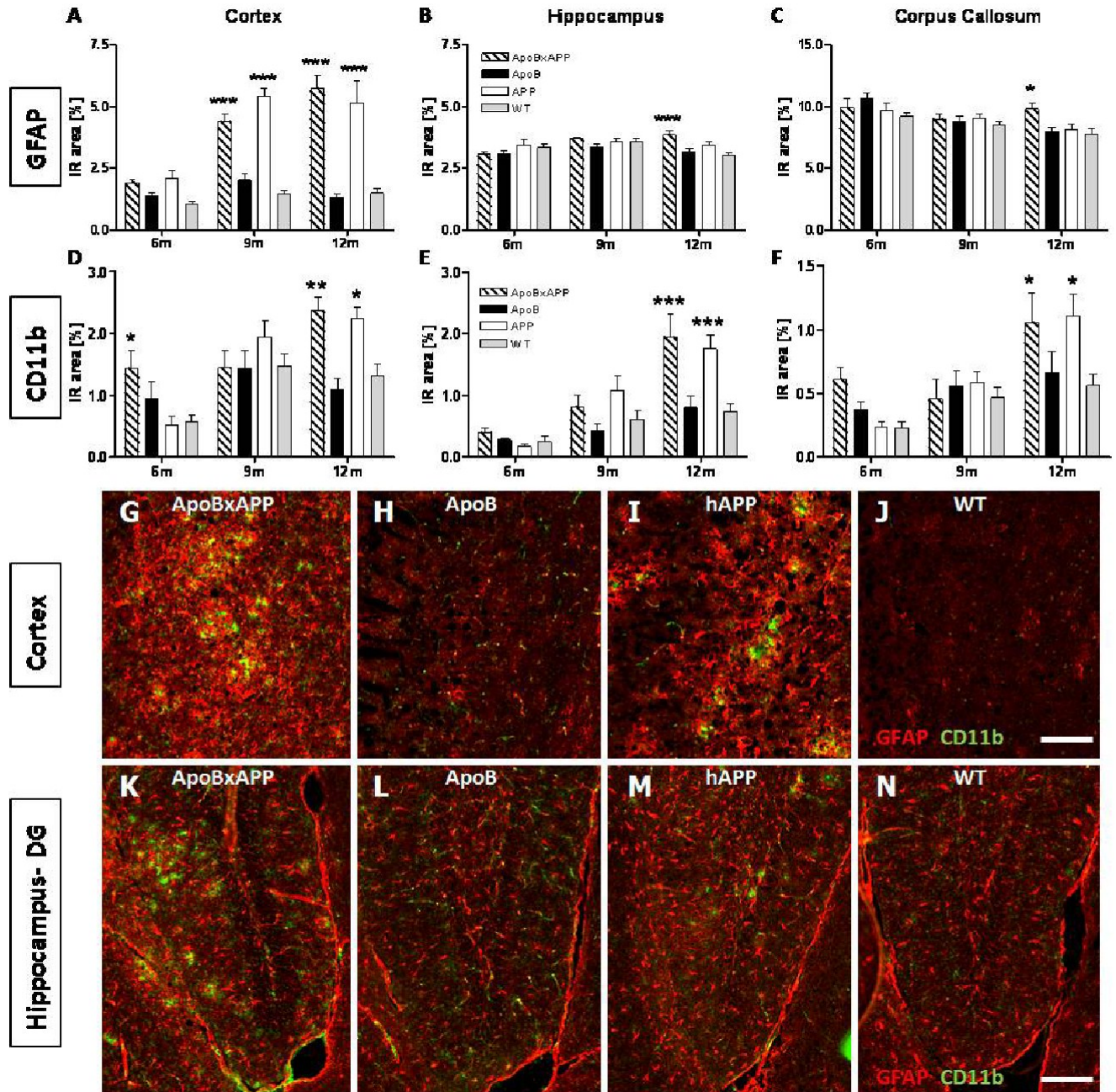


Figure 6.12: Immunohistochemical detection of activated astrocytes and microglia by GFAP and CD11b staining. Quantitative evaluation of GFAP immunoreactive area in the cortex (A), hippocampus (B) and corpus callosum (C). Percentage of CD11b immunoreactive area in cortex (D), hippocampus (E) and corpus callosum (F). Statistical analysis: Two-way-ANOVA with Bonferroni post-test. N=6 per group. Representative pictures of cortex (G-J) and dentate gyrus, DG (K-L) of 12 months old ApoBxAPP (G;K), ApoB (H;L), hAPPsl (I;M) and WT animals (J;N). Red: GFAP, green: CD11b. Scale bars: 100μm. (Löffler et al. 2013)

6.1.7 Behavioural testing

In order to analyze differences in behaviour and memory performance between all genotypes, diverse behavioural tests were applied to 4, 6, 9 and 12 months old animals of both sexes.

Before starting with the examination of cognitive performance, all animals underwent the Irwin test and Open Field task to determine the general health status as well as changes in activity and anxiety behavior, respectively.

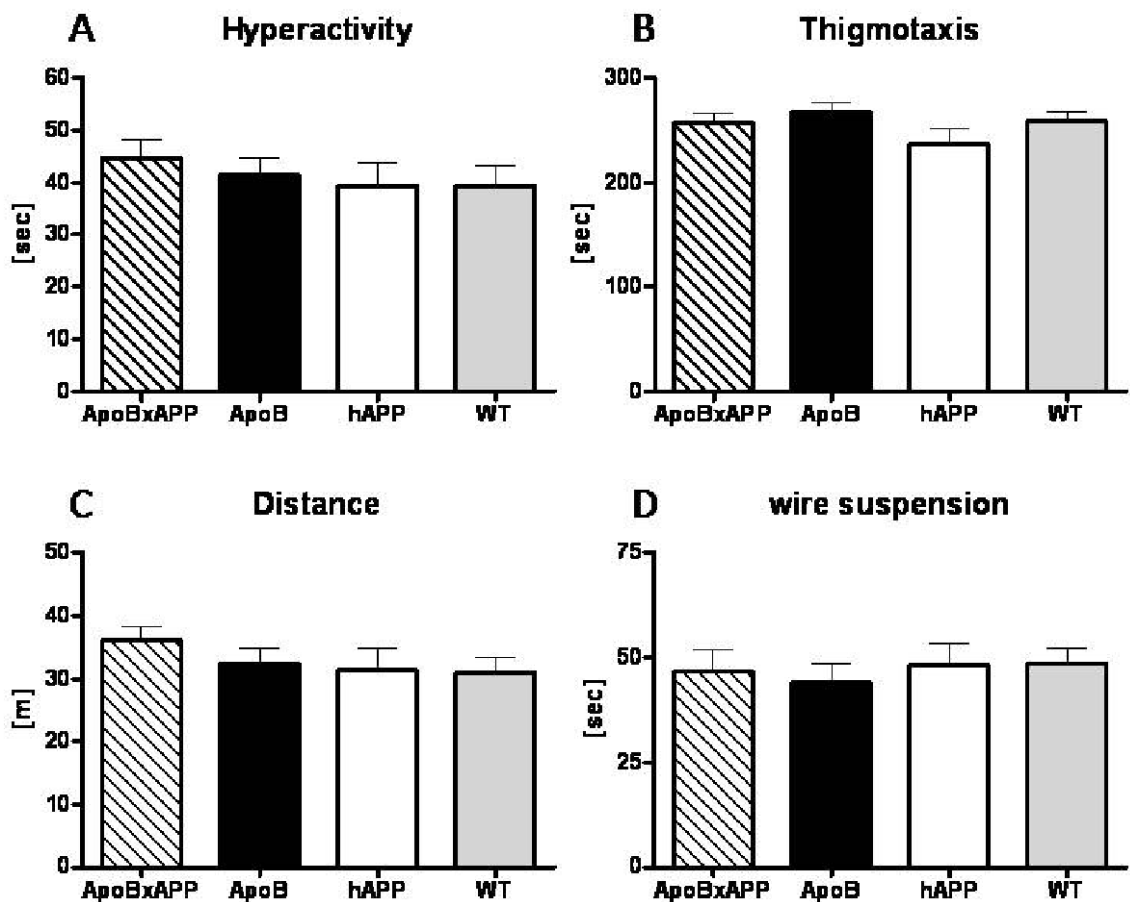


Figure 6.13: Open Field and Irwin parameters in all four genotypes. (A) Hyperactivity, (B) thigmotaxis and (C) distance obtained from Open Field testing as well as (D) wire suspension time from Irwin test. Graphs display results from 12 months old animals. n=15 to 25 per group; Statistical analysis: One-way-ANOVA with Bonferroni post-test.

As a result, no differences between genotypes and sexes could be detected, despite expected differences in the body weight between males and females (data not shown). In terms of hyperactivity, distance, thigmotaxis and wire suspension time, all genotypes performed similarly (Fig.6.13). This outcome did not significantly change over age.

“According to these results, bias of cognitive tests by disturbed motor abilities, anxious behaviour or hyperactivity could be excluded.

Two cognitive tests were carried out with animals of all genotypes and both sexes to determine learning and memory abilities.

The Morris water maze task (MWM) was used to assess spatial learning and memory function related to hippocampal conditions. This test showed that ApoBxAPP as well as hAPPsl animals display early learning deficits at an age of 6 months as illustrated by increased escape latencies compared to wild type animals (Fig. 6.14 B).

These significant differences compared to non transgenic animals were even more pronounced in 9 and 12 months old mice (Fig. 6.14 C, D).

At an age of 6 months, ApoB-100 single transgenic mice did not show any difference compared to wild type animals in the MWM. However, 12 months old ApoB-100 mice showed significantly increased escape latencies on the last training day (Fig. 6.14 D) as well as reduced abidance in the target quadrant during the probe trial (Fig. 6.14 E). This finding is unexpected and underlines the impact of ApoB-100 overexpression on cognitive function.

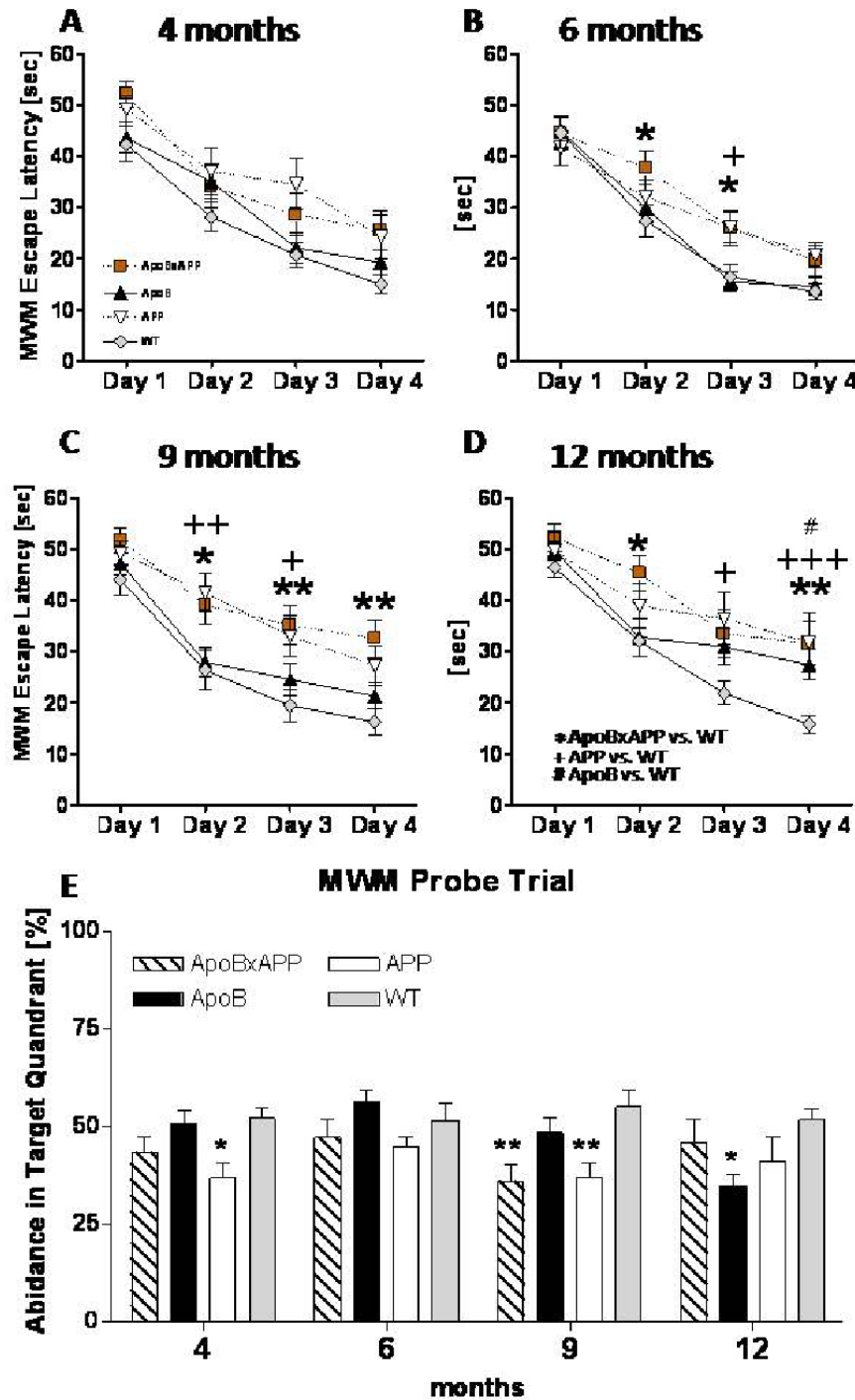


Figure 6.14: Assessment of spatial learning. Morris water maze (MWM) learning curves showing escape latencies during 4 testing days of 4 (A), 6 (B), 9 (C) and 12 months (D) old animals. (E) Abundance in the target quadrant during MWM probe trial. 4m ApoBxAPP n=18, ApoB n=19, APP n=16, WT n=17; 6m ApoBxAPP n=18, ApoB n=20, APP n=19, WT n=21; 9m ApoBxAPP n=20, ApoB n=22, APP n=21, WT n=19; 12m ApoBxAPP n=14, ApoB n=23, APP n=13, WT n=22; Statistical analyses: Two-way-ANOVA with Bonferroni post-test compared to WT. (Löffler et al. 2013)

The second cognitive test was the contextual fear conditioning task (CFC), detecting differences in emotional learning. In the CFC, ApoBxAPP and hAPPsl mice also showed first significant memory deficits at an age of 6 months, displayed by a reduction of the freezing time (Fig. 6.15) compared to WT animals. This phenotype was stable for both genotypes at 9 and 12 months of age.” (Löffler et al., 2013)

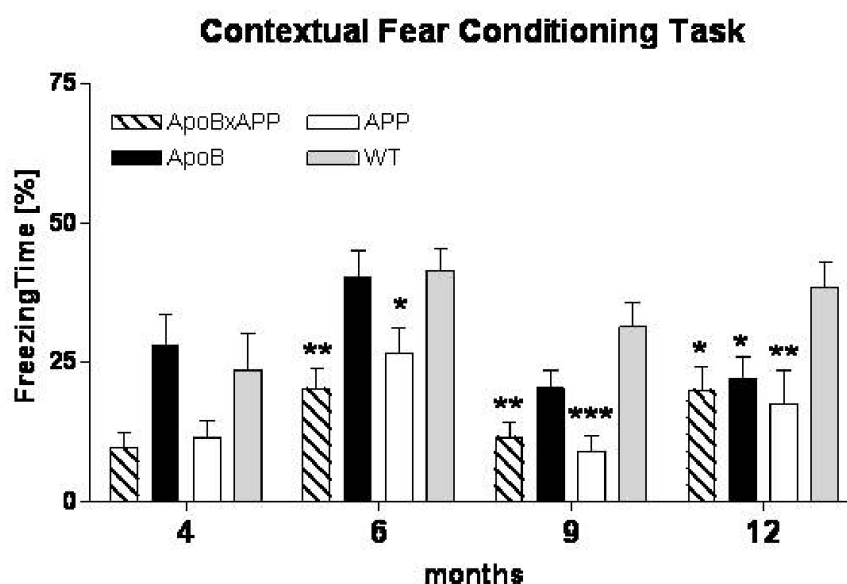


Figure 6.15: Assessment of emotional learning over age. Freezing times obtained from Contextual Fear Conditioning Task. 4m ApoBxAPP n=18, ApoB n=19, APP n=16, WT n=17; 6m ApoBxAPP n=18, ApoB n=20, APP n=19, WT n=21; 9m ApoBxAPP n=20, ApoB n=22, APP n=21, WT n=19; 12m ApoBxAPP n=14, ApoB n=23, APP n=13, WT n=22; Statistical analyses: Two-way-ANOVA with Bonferroni post-test compared to WT. (Löffler et al. 2013)

Most notably, ApoB-100 animals, showed significant differences in emotional learning compared to WT mice at an age of 12 months (Fig. 6.15), which is in line with MWM findings.

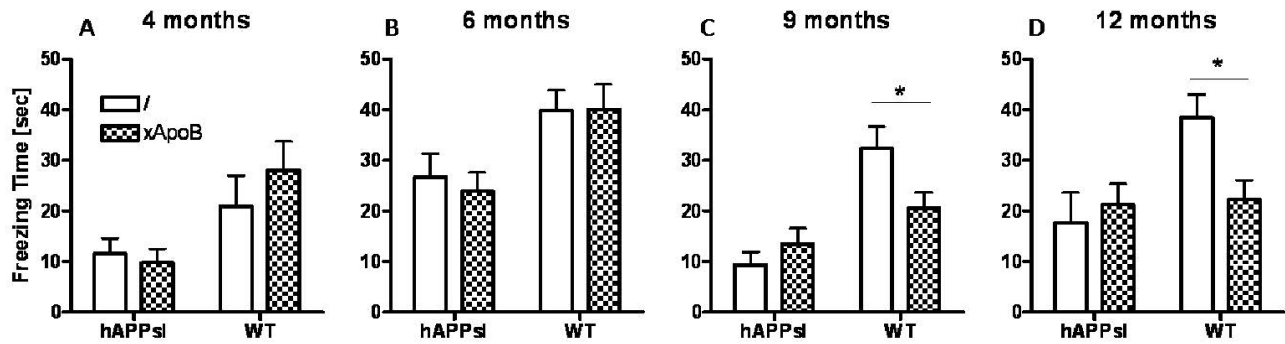


Figure 6.16: Influence of ApoB100 overexpression on emotional learning. Data from 4 (A), 6 (B), 9 (C) and 12 (D) months old animals were analyzed with 2-way ANOVA for impact of additional ApoB100 overexpression on CFC Freezing Time.

Further statistical analysis of the same data showed the direct impact of additional ApoB100 overexpression in hAPPsl as well as WT mice on CFC Freezing times (Fig. 6.16). These results indicate that already at 9 months of age ApoB100 alone leads to changes in emotional learning compared to WT littermates (Fig. 6.16 C), whereas additional ApoB100 overexpression in hAPPsl mice does not lead to changes in the behavioral phenotype.

6.1.8 Cerebral lipid peroxidation correlates with memory function

“To investigate a potential functional link between behavioural data and several biochemical parameters, correlation analyses were carried out. Correlation of cortical and hippocampal MDA levels of all 9 months old animals with their corresponding freezing times obtained from the CFC, revealed significant correlation of these variables (Fig. 6.17 A).

This data show that increased MDA levels are associated with a restricted memory performance.

Correlations containing Abeta values were done separately for hAPP-expressors (ApoBxAPP, hAPPsl) and non-hAPP-expressors (ApoB-100 and WT) due to the remarkable differences of Abeta levels between these groups.

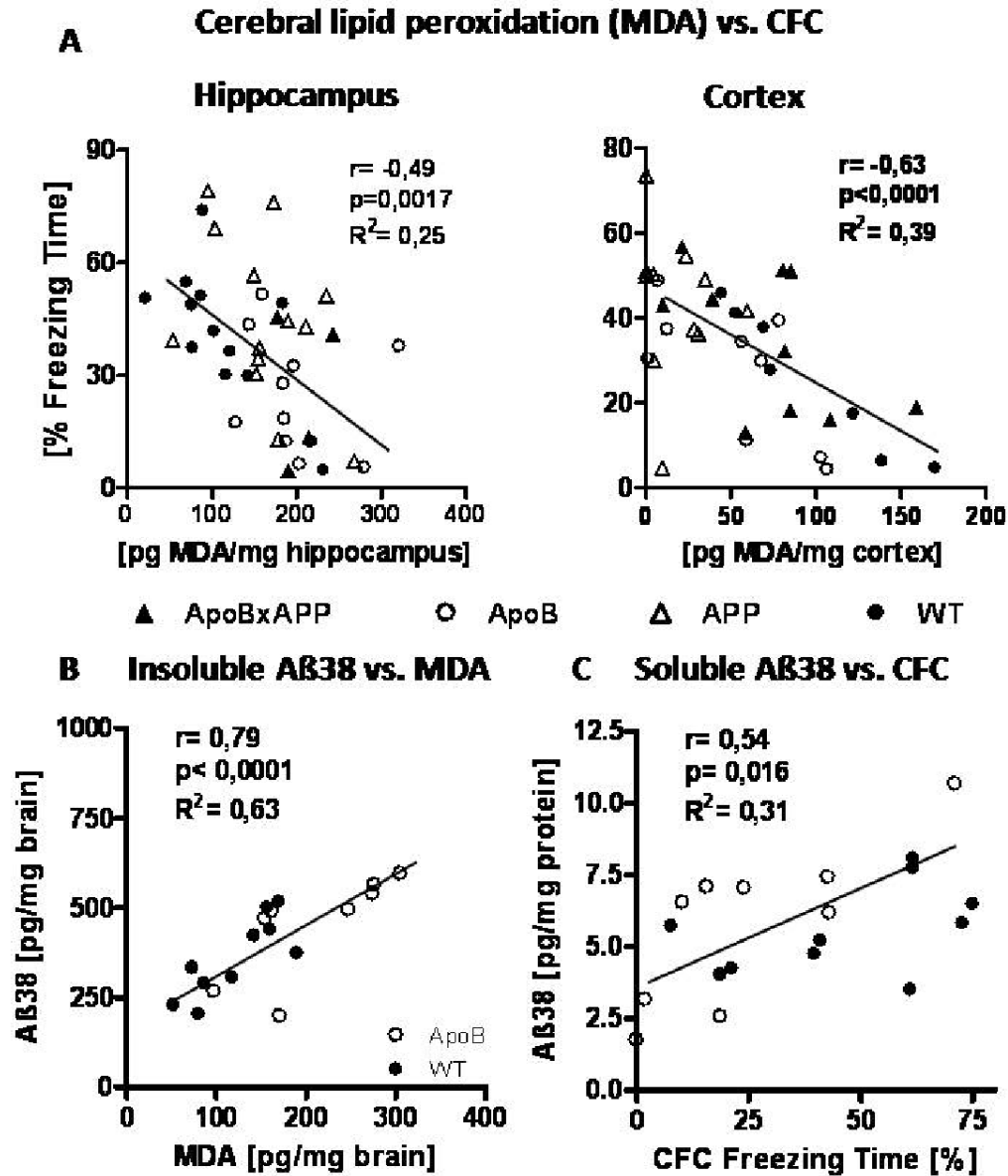


Figure 6.17: Correlation analyses of behavioural and biochemical data. (A) Negative correlation between cerebral malondialdehyde (MDA) levels and corresponding CFC freezing times of all genotypes at 9 months of age; $n=42$. (B) Insoluble Abeta1-38 correlates with dedicated cortical MDA levels in 12 months old non-hAPP expressors, ApoB-100 and WT (pooled); $n=22$ (C) Cortical soluble Abeta1-38 correlates with corresponding CFC freezing time of 12 months old non-hAPP expressors, ApoB-100 and WT (pooled); $n=22$. Statistical analyses: Pearson correlation. (Löffler et al. 2013)

Correlation analysis of cerebral Abeta values obtained from ApoB-100 and WT animals to several relating behavioural or biochemical variables revealed two major findings both connected to Abeta1-38 in 12 months old animals:

- (i) A strong proportional link was found between increased insoluble Abeta1-38 and MDA levels especially in the cortex (Fig. 6.17 B), pointing to an association between Abeta aggregation and lipid peroxidation.
- (ii) A correlation between the CFC freezing time and soluble Abeta1-38 in the DEA fraction became apparent (Fig. 6.17 C).

High levels of soluble Abeta1-38 correlate with better memory performance shown as increased freezing time. The same correlation analyses for ApoBxAPP and hAPPsl mice did not reveal any significance.” (Löffler et al., 2013)

6.2 Challenging the system - a high fat diet study

The first part of my work dealt with a basic characterization of all four genotypes over age, on a chow diet basis.

To challenge the system and see how additional dietary uptake of cholesterol and other lipids influences behaviour, APP pathology and cerebral cholesterol metabolism, I investigated this parameters following a 3 months-feeding study of young animals from all four genotypes. 12 weeks old mice were assigned to a high fat (HFD) and a standard diet (ND) group and fed the corresponding diet for three months. At an age of 6 months all animals underwent several behavioural tests for two more weeks, tissue was sampled and investigated subsequently.

6.2.1 Response to high fat diet

Regarding weight gain due to HFD feeding, ApoB-100 mice responded best with more than 15 g weight gain over three months of feeding and total body weights around 40 g (Fig. 6.18). WT mice gained significantly more weight when fed HF compared to ND fed WT mice, while mice expressing hAPP, ApoBxAPP and hAPPsl, did not gain significantly more weight (Fig. 6.18 B).

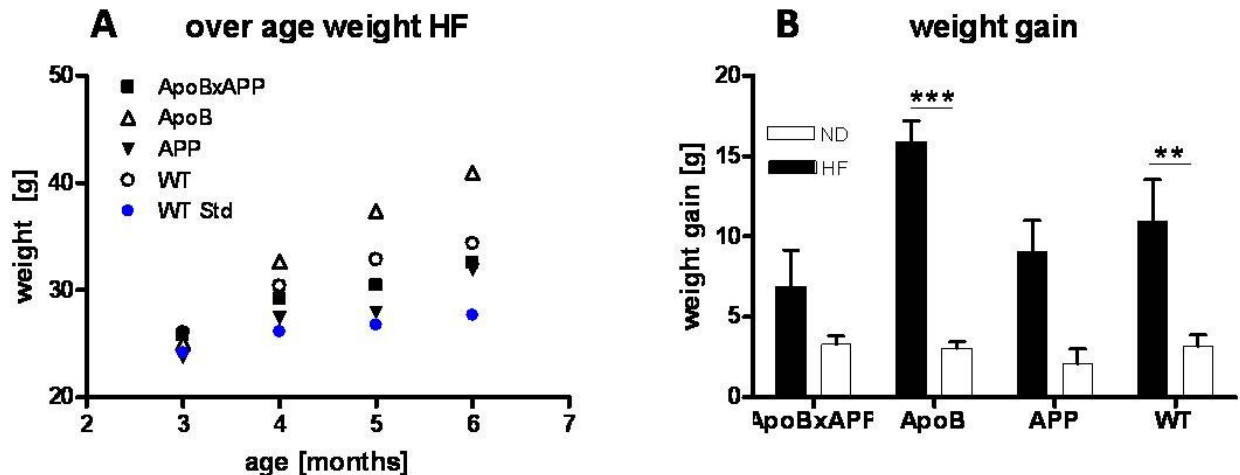


Figure 6.18: Response to high fat diet. (A) Average body weights of all genotypes on HFD compared to WT mice on ND over time. (B) Comparison of final weight gains due to different diets for every genotype. Statistical analysis: Two-way-ANOVA with Bonferroni post-test. HFD (n=8 per genotype) and ND (n=7 per genotype).

Also food consumption was assessed during this study, but since we used standard and no real metabolic cages, we only obtained approximate values. Nevertheless a tendency to increased food consumption was observed for both hAPP overexpressors on ND, ApoBxAPP and hAPPsl (data not shown).

6.2.2 Plasma parameters

Several plasma parameters were measured in all four genotypes, both on ND and HFD. While all lipid parameters, like total cholesterol, triglycerides, HDL- and LDL-cholesterol, increased due to HFD (Fig. 6.19 A-D), Glucose levels showed a tendency to decrease on HFD (Fig. 6.19 E).

As a marker for liver damage, alanine aminotransferase (ALT) levels were determined. Only ApoB-100 mice revealed significantly increased ALT plasma reactivity as response to HFD compared to WT littermates, which was not found for any other genotype (Fig. 6.19 F).

The response on all cholesterol related parameters (total cholesterol, LDL- and HDL-cholesterol) was found to be weaker as soon as hAPP was expressed (Fig. 6.19 A,B,C), which can be observed when comparing ApoBxAPP with ApoB and accordingly hAPPsl with WT mice.

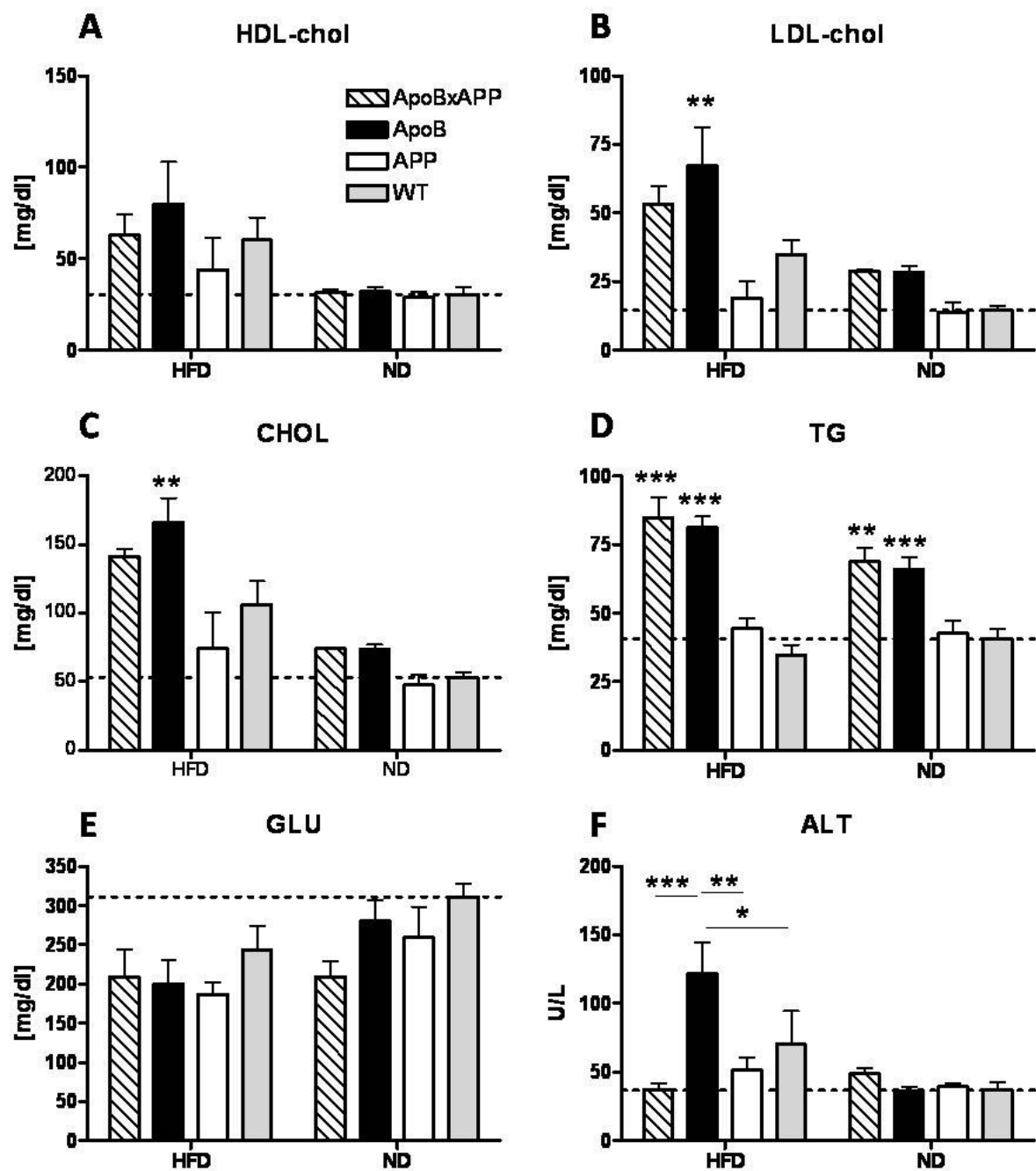


Figure 6.19: Plasma parameter after feeding experiment. Results of all measurements evaluating (A) total cholesterol, (B) triglyceride, (C) HDL-cholesterol, (D) LDL-cholesterol, (E) glucose and (F) alanine aminotransferase levels in plasma of HF (n=8 per genotype) and ND (n=7 per genotype) fed animals. Statistical analysis: Two-way-ANOVA with Bonferroni post-test.

6.2.3 Abeta levels

Three Abeta species, Abeta1-38, 1-40 and 1-42, were measured in the plasma and cortex of all four genotypes on both diets.

In the plasma, Abeta1-40 was the most abundant Abeta species in hAPP expressors as well as non-hAPP expressors, while Abeta1-38 was below detection limit.

HFD led to a profound decrease of Abeta1-40 levels in ApoBxAPP and hAPPsl mice at 48%. ApoB-100 mice also showed a tendency towards reduced plasma Abeta1-40 levels (Fig. 6.20 A), while this effect was not observed in WT mice.

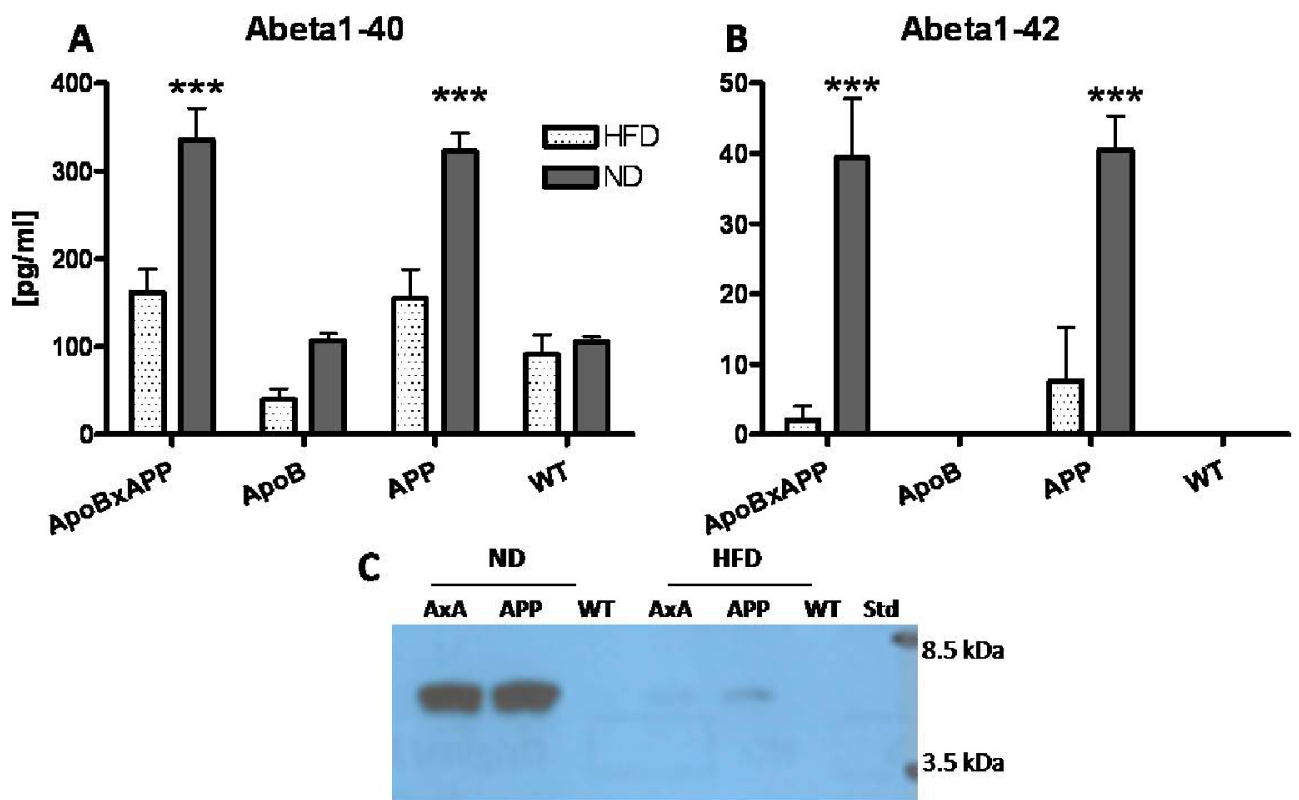


Figure 6.20: Abeta levels in plasma. Abeta1-40 (A) and 1-42 (B) levels in plasma of HF and ND fed animals. n=6 per group; Statistical analyses: Two-way-ANOVA with Bonferroni post-test. (C) Western blot with an antibody detecting only human Abeta (6E10) of 20µl plasma. AxA= ApoBxAPP; Std = RPN755E

The HFD effect on plasma values of Abeta1-42 was even more pronounced. Abeta1-42 peptides could only be measured in ApoBxAPP and hAPPsl mice, but not in ApoB-100 or WT animals. Again, this Abeta species was drastically reduced in the plasma of hAPPsl mice fed HFD and was almost undetectable in ApoBxAPP animals (Fig. 6.20 B).

Western blot analysis was performed to confirm this result. Using an antibody which detects only human Abeta, this profound reduction of plasma Abeta species on HFD could be substantiated (Fig. 6.20 C), indicating that HFD has a direct and profound impact on APP metabolism.

Cerebral insoluble Abeta species (FA fraction) increased in the brains of HFD-fed ApoBxAPP and hAPPsl animals (Fig 6.21 A-C) and a sex-specific difference was clearly observed. Intriguingly, female ApoBxAPP mice responded much stronger to the HFD and developed significantly higher Abeta levels compared to males (Fig 6.21 E, F).

Endogenous insoluble Abeta1-40 levels in brains of ApoB and WT mice showed a tendency to higher levels as a consequence of HFD, but lacked statistical significance (Fig. 6.21 D). Neither Abeta1-38 nor Abeta1-42 could be measured in ApoB-100 and WT mice brains.

Levels of soluble Abeta from the DEA fraction did not significantly differ between diets in ApoBxAPP and hAPPsl mice (Fig. 6.21 G-I). Soluble endogenous Abeta in ApoB and WT animals was below detection limit.

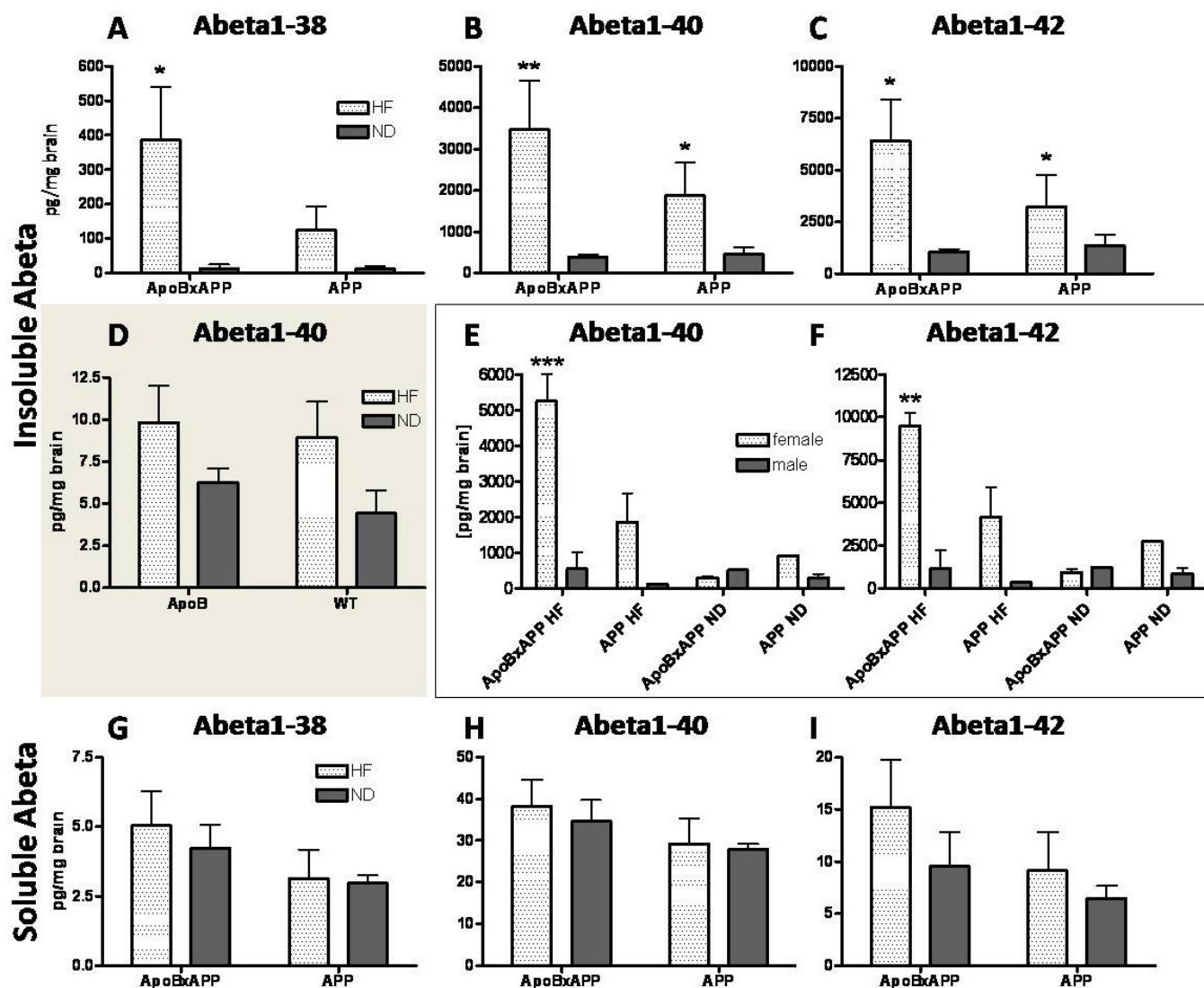


Figure 6.21: Cerebral Abeta levels. Abeta1-38 (A), 1-40 (B) and 1-42 (C) levels in cortical, insoluble fraction of both hAPP overexpressors on HF and ND. (D) Endogenous Abeta1-40 levels in cortical samples of ApoB and WT mice on both diets. Gender differences for Abeta 1-40 (E) and Abeta1-42 (F) in hAPP overexpressors due to HF diet. Soluble (G) Abeta1-38, (H) 1-40 and (I) 1-42 levels from DEA fraction in ApoBxAPP and hAPPsl animals n=6 per group; Statistical analyses: Two-way-ANOVA with Bonferroni post-test.

6.2.4 Behavioural testings

In order to analyze differences in behavior and memory performance between all genotypes the same testing battery as for the basic characterization, including Irwin test, Open Field (OF), Morris Water Maze (MWM) and Contextual Fear Conditioning task (CFC), was applied to all animals on both diets.

The Irwin test revealed only differences in body weight, which is closer described in section 6.2.1, but not in any other measured parameter (data not shown).

The Open Field Test was used to assess activity and anxiety of the animals. Measured parameters indicating changes in activity did not reveal any significant difference (Fig. 6.22 A).

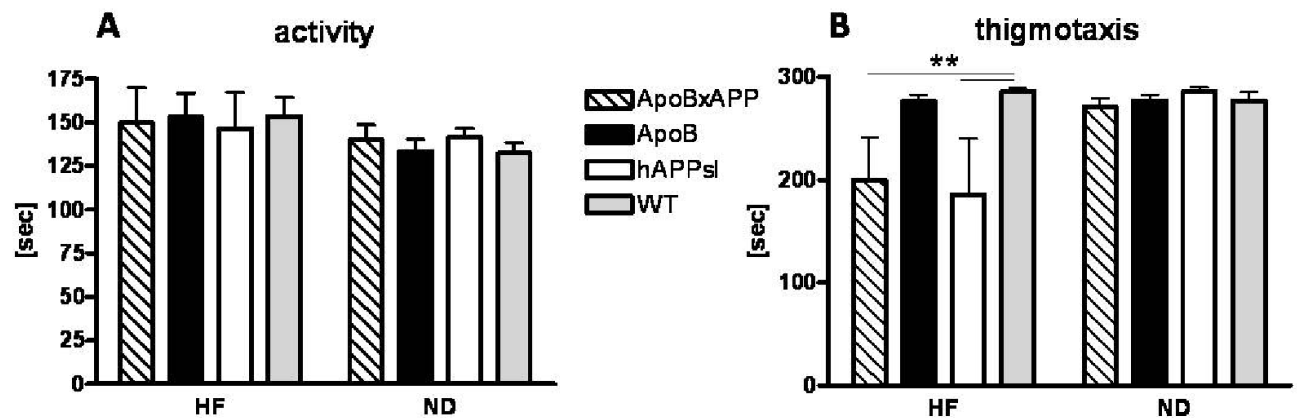


Figure 6.22: Impact of HFD on activity and anxiety behavior. Activity (A) and thigmotaxis (B) from Open Field measurements of HF (n=8 per genotype) and ND (n=7 per genotype) fed animals. Statistical analyses: Two-way-ANOVA with Bonferroni post-test.

Also the covered distance and rearing behavior did not differ between genotypes (data not shown). Thus, also motor disabilities could be excluded.

Only the time spent close to walls (thigmotaxis), an indication for anxiety-like behavior, was significantly decreased due to HFD in hAPP overexpressing mice, ApoBxAPP and hAPPsl (Fig.

6.22 B). Abundance in the middle, the counterpart to thigmotaxis, was increased in these animals as expected (data not shown).

Therefore, ApoBxAPP and hAPPsl mice on HFD seem to be less anxious.

Two cognitive tests were carried out with all animals to determine the effect of feeding on learning and memory abilities.

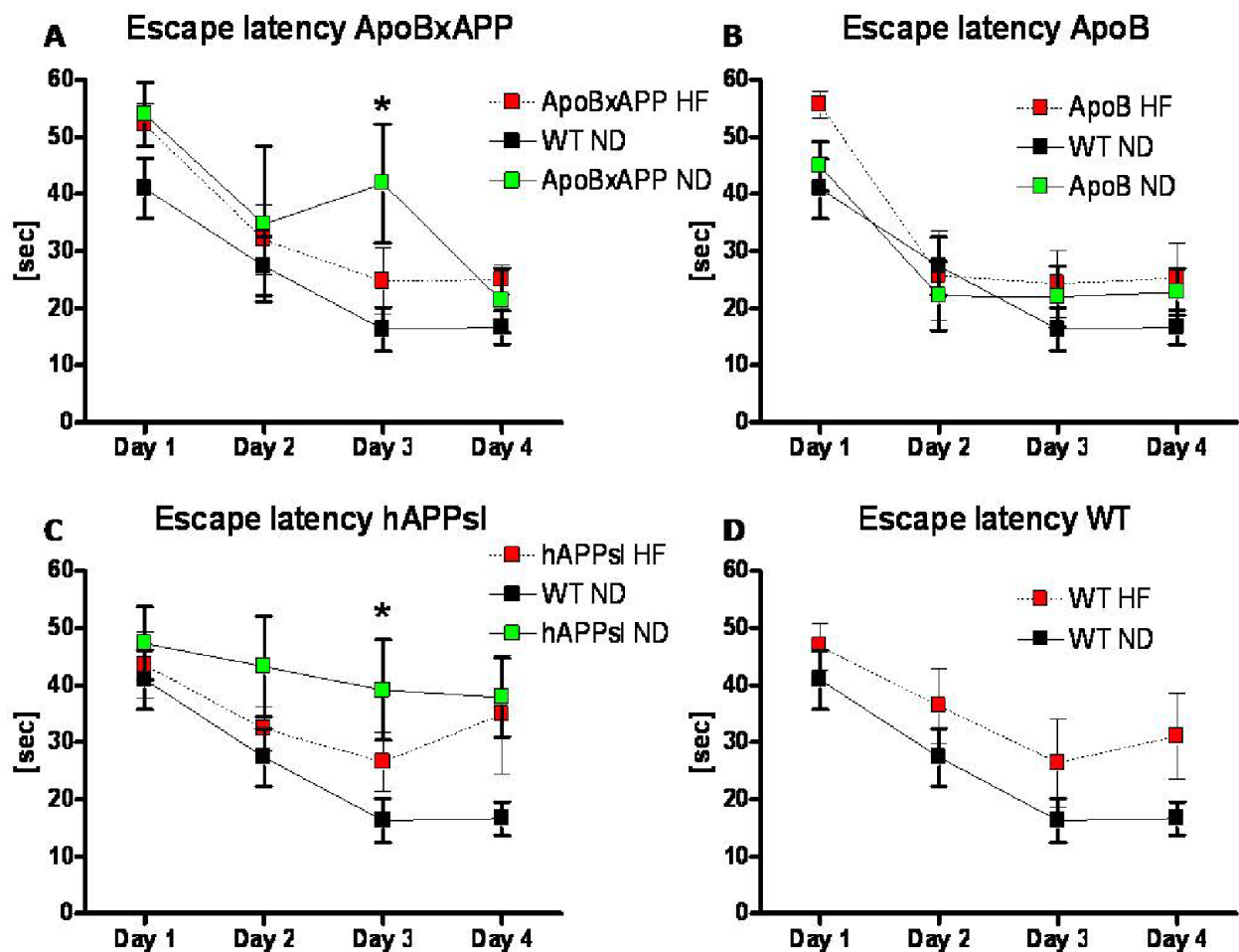


Figure 6.23: Spatial learning in HFD and ND fed animals. Morris water maze (MWM) learning curves showing escape latencies during 4 testing days of ApoBxAPP mice on HF and ND versus WT on ND (A), ApoB mice on HF and ND versus WT on ND (B), hAPP mice on HF and ND versus WT on ND (C) and WT mice on HF and ND (D). Statistical analyses: Two-way-ANOVA with Bonferroni post-test.

The MWM showed, as expected, reduced memory performance of ApoBxAPP and hAPPsl mice compared to WT control, but showed no negative impact of HFD (Fig. 6.23 A-D).

ApoBxAPP and hAPPsl mice on HFD even showed a tendency to perform better than the same genotype on ND (Fig. 6.23 A, C). ApoB single transgenic animals did not show any difference to WT controls, neither on ND nor on HFD (Fig. 6.23 B). Also WT mice did not perform significantly different on the two diets, although a slight tendency to a worsened memory performance could be observed (Fig. 6.23 D), lacking significance.

The second cognitive test was the so-called contextual fear conditioning task (CFC) detecting differences in emotional learning.

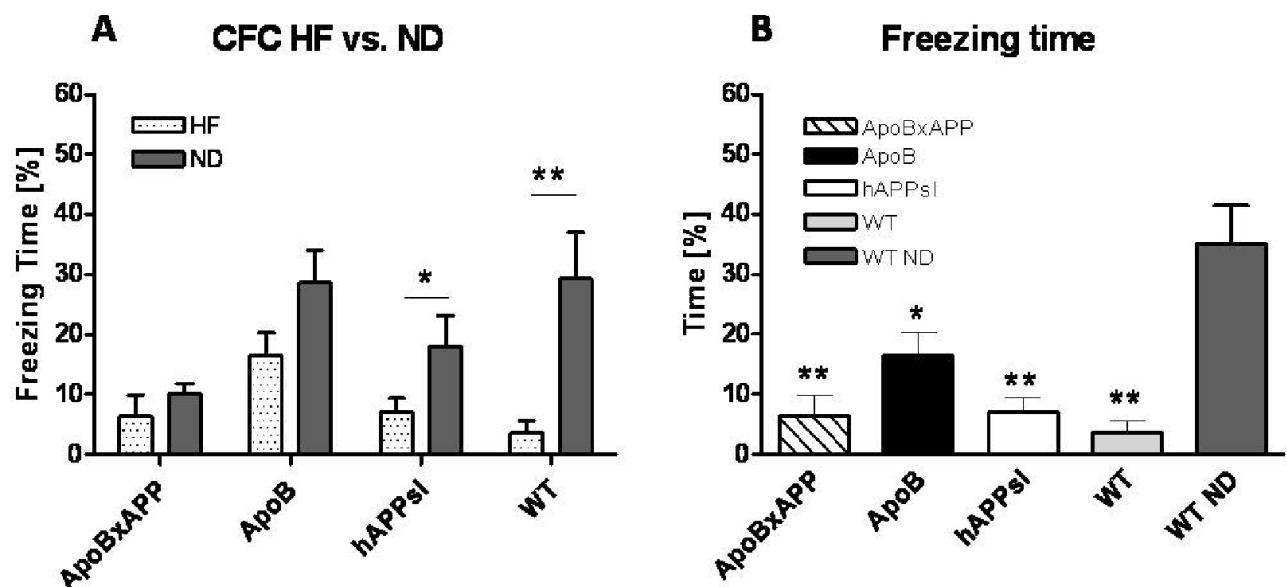


Figure 6.24: Contextual Fear Conditioning task. Influence of HFD on freezing times during testing day (A). Comparison of freezing times obtained from all genotypes on HF (n=8 per genotype) to baseline, WT on ND (n=7) (B). Statistical analyses: Two-way-ANOVA with Bonferroni post-test

Results for this test differed from MWM results. Here a negative impact of HFD could be detected in every genotype, most prominent in WT mice (Fig. 6.24 A). For ApoBxAPP and ApoB mice HFD induced a slight but not significant reduction of freezing time.

Nevertheless, every genotype on HFD showed a significantly reduced freezing time compared to WT mice on ND (Fig. 6.24 B).

6.2.5 mRNA expression analysis

Expression analysis of several genes related to cholesterol metabolism, cholesterol transport and APP processing were carried out with SYBR Green quantitative mRNA RT-PCR in the cortex and liver of all animals.

In the cortex, mRNA levels of the rate limiting enzyme in cholesterol synthesis, Hmg-CoA-reductase, were found to be increased in both genotypes overexpressing hAPP, ApoBxAPP and hAPPsl, and on HFD compared to WT (Fig. 6.25 A).

mRNA levels of Cholesterol 24-hydroxylase (Cyp46A1), an enzyme converting cholesterol to 24S-hydroxycholesterol, a mechanism of lowering brain cholesterol levels, was only found to be increased in ApoB mice brains on HFD (Fig. 6.25 B).

Acetyl-CoA acetyltransferases (ACAT) are enzymes converting free cholesterol into cholesterol esters. ACAT2 mRNA levels were significantly increased in ApoBxAPP and hAPPsl cortices compared to WT on HFD (Fig. 6.25 C), whereas ACAT1 was only found to be increased in ApoB single transgenic animals (Fig. 6.25 D).

Also mRNA expression levels of several cholesterol transport associated proteins like ApoE, LDL-R, LRP1 and ABCA1 were analyzed. While ApoE was found to be significantly increased in ApoB brains on HFD compared to WT mice, APP and ApoBxAPP mice showed a tendency for about 20% reduced levels on HFD compared to WT littermates (Fig 6.25 E).

LDL-receptor mRNA levels were found to be significantly increased in ApoBxAPP and hAPPsl cortices on HFD compared to WT (Fig. 6.25F). Already when fed ND a tendency to increased LDL-R levels was found in those animals.

Levels of ABCA1 and LRP1 mRNA did not differ between genotypes and/or diets (data not shown).

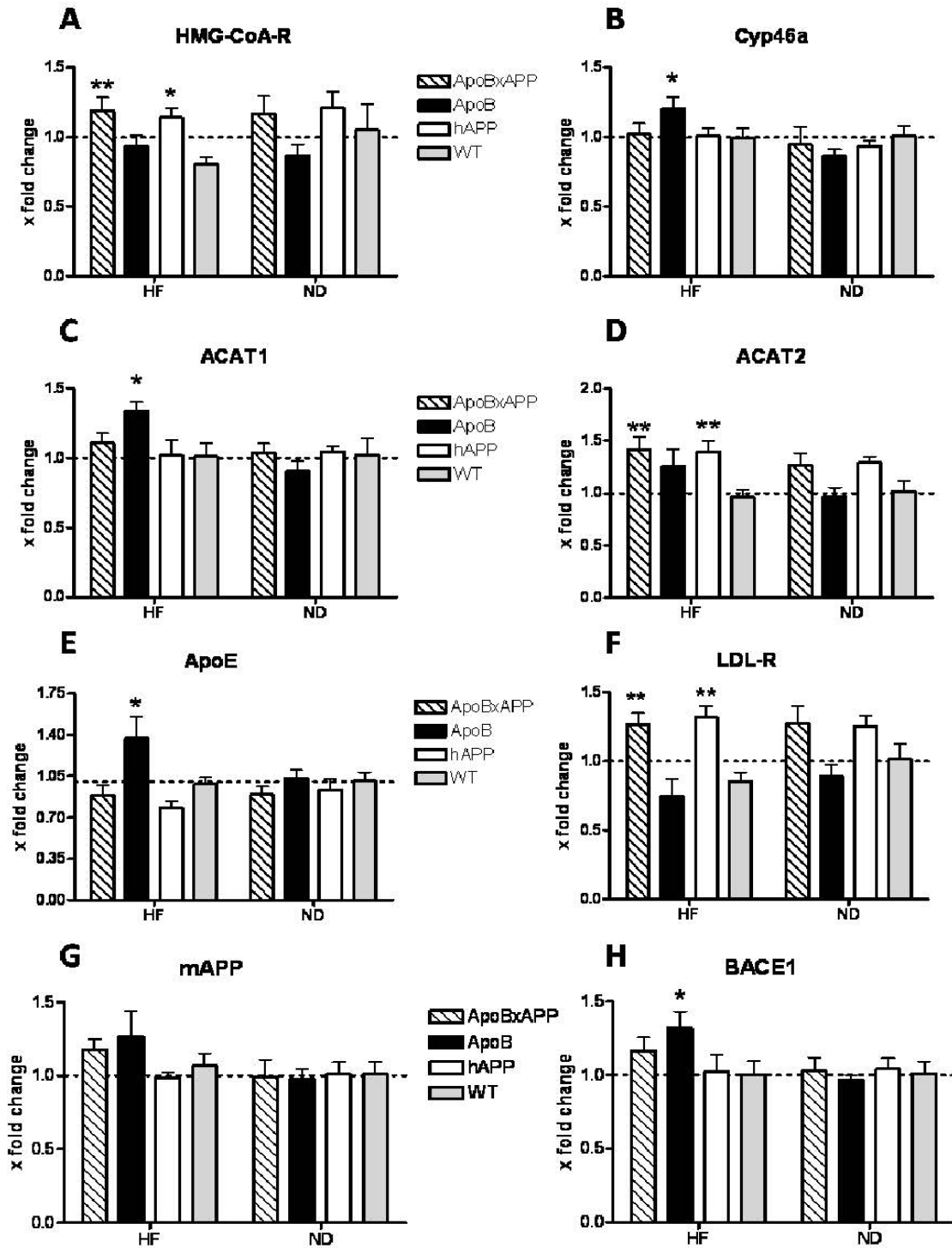


Figure 6.25: mRNA expression levels in the cortex of all animals shown as x-fold change to levels in corresponding WT mice. HMG-CoA-Reductase (A), Cholesterol 24-hydroxylase (B), Acetyl-CoA-Acetyltransferase 1 (C), Acetyl-CoA-Acetyltransferase 2 (D), Apolipoprotein E (E), LDL-Receptor (F), murine APP (G) and beta-secretase (H) mRNA levels; n=6 per group. Statistical analyses: Two-way-ANOVA with Bonferroni post-test compared to WT.

Beta-secretase (BACE1), the enzyme responsible for amyloidogenic cleavage of APP, as well as murine APP mRNA levels were also assessed. While mAPP mRNA levels only showed a tendency to be increased in both ApoB overexpressing genotypes on HFD, ApoBxAPP and ApoB-100, compared to WT (Fig. 6.25 G), this increase was found to be significant for BACE1 mRNA in ApoB-100 cortices (Fig. 6.25 H).

In the liver, mRNA expression of ApoE obtained results similar to those from the brain. On HFD, levels of ApoE mRNA were found to be significantly increased in ApoB mice compared to WT, whereas ApoBxAPP and hAPPsl animals showed a tendency to reduced ApoE mRNA levels (Fig. 6.26 A).

The increase of LDL-R mRNA due to hAPP overexpression, which was observed in the brain, was only detected in livers of ND fed mice. Surprisingly, on HFD all four genotypes showed a reduction of LDL-R mRNA (Fig. 6.26 B).

This reduction of mRNA levels due to HFD feeding was also observed for ACAT1 and 2 (Fig. 6.26 C,D). On ND, levels of ACAT 1 and 2 mRNAs were found to be increased in ApoB mice compared to WT controls, which was only significant for ACAT2 (Fig. 6.26 D). While in ApoBxAPP and hAPPsl brains ACAT mRNA was shown to be increased (Fig. 6.26 C), in the liver a tendency to reduced levels was observed (Fig. 6.26 C,D).

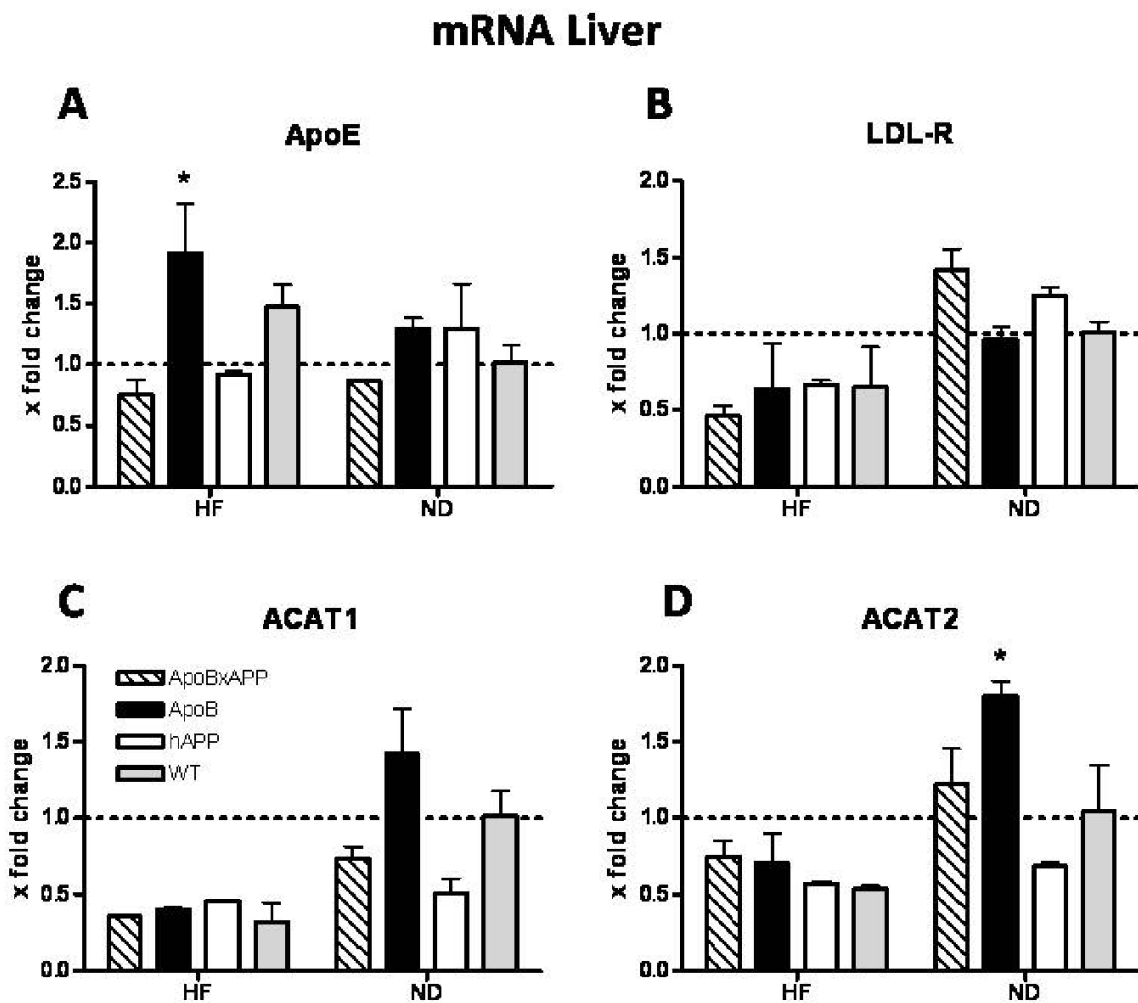


Figure 6.26: mRNA expression levels in the liver of all animals shown as x-fold change to levels in corresponding WT mice. Apolipoprotein E (A), Cholesterol LDL-Receptor (B), Acetyl-CoA–Acetyltransferase 1 (C) and Acetyl-CoA–Acetyltransferase 2 (D) mRNA levels; n=3 per group. Statistical analyses: Two-way-ANOVA with Bonferroni post-test compared to WT.

Chapter 7

Discussion

7.1 Characterization over age

There are major differences between humans and mice regarding their plasma lipid profiles. Whereas HDL is the major and LDL the minor cholesterol-carrying lipoprotein in mice, humans display considerably higher LDL levels together with low amounts of HDL. High LDL levels are a major, independent, risk factor for the development of atherosclerosis. Due to their low LDL to HDL ratio, wild type mice are usually not affected by atherosclerosis and other cardiovascular pathologies, when kept on a chow diet (Breslow, 1996).

By overexpression of ApoB-100, the murine plasma lipid profile is shifted towards a human-like, more atherogenic status, i.e. high LDL and lowered HDL-cholesterol levels (Berezki et al., 2008; Bjelik et al., 2006).

In addition to the well described changes in the plasma lipid profile, the present study shows that hApoB-100 overexpression also plays a crucial role in initiating cerebral lipid peroxidation. In the hippocampus, elevated MDA levels were detected in ApoBxAPP and ApoB-100 mice as early as 4 months after birth which further increased over age.

It is generally accepted that oxidative modifications of LDL are one of the earliest events in atherosclerosis. Oxidized LDL displays a variety of alterations within both the lipid and the ApoB protein moieties (Bonomini et al., 2008). Most notably, the current results demonstrate that these peroxidation-events occur in ApoBxAPP and ApoB-100 mice brains and are associated with cognitive decline.

A synergistic effect of additional hAPP overexpression on MDA levels could not be observed in ApoBxAPP animals. Single transgenic hAPPsl animals also displayed increased MDA levels, however, at later time points (see Fig. 7.1).

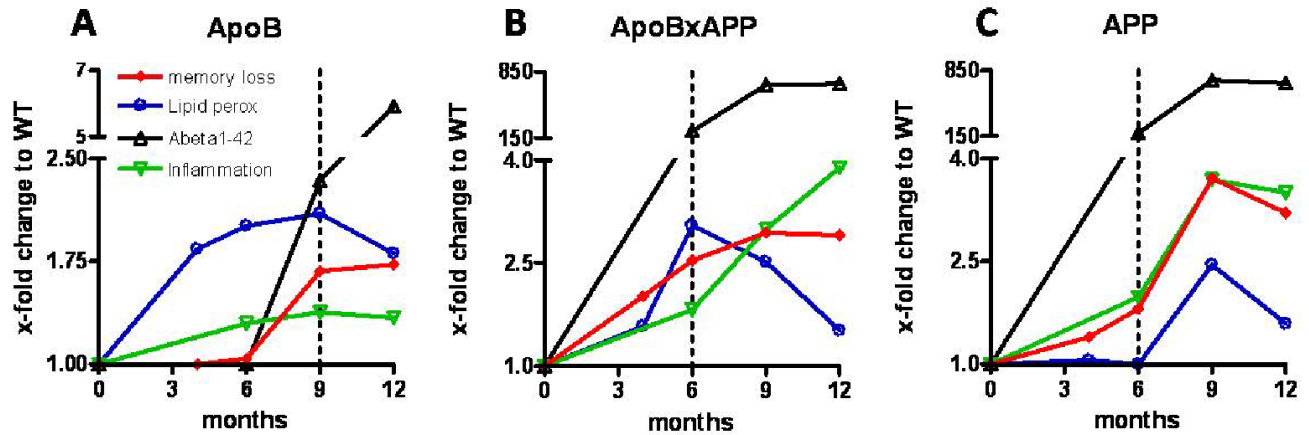


Figure 7.1: Overview about development of different parameters in (A) ApoB, (B) ApoBxAPP and (C) hAPPsl mice. The graphs display the “x-fold change” to WT values. Dotted line represents onset of first significant memory loss/cognitive impairment.

In ApoB-100 mice, lipid peroxidation was detectable before onset of memory decline and increased Abeta levels, whereas in hAPPsl mice onset of lipid peroxidation appeared after manifestation of cognitive deficits (Fig.7.1 A). “This may indicate that lipid peroxidation plays an initial role in memory decline of ApoB-100 animals, but is rather a downstream effect in hAPPsl transgenic mice.” (Löffler et al., 2013)

In LOAD patients brains, lipid peroxidation was described as an early event (Markesbery et al., 2005; Yao et al., 2003). It is therefore tempting to speculate that ApoBxAPP mice represent a better model than hAPPsl single-transgenic animals regarding oxidative events.

One possibility for oxidative processes is a reduction in the amounts of antioxidants and may be due to the reduction of HDL which is a main transporter of these substances. However, this assumption could be excluded, since no differences in the amounts of alpha-tocopherol in brains of all genotypes could be observed. Therefore it may be that the higher amount of oxidizable LDL in the circulation as such is responsible for the increase in lipid peroxidation. Alternatively, other antioxidative mechanisms, like activity of PON (paraoxonase) or SOD (superoxide dismutase), could be impaired. The characterization of such processes goes, however, beyond the scope of this work.

ApoB-100 overexpression had no impact on any measured cerebral lipid component. Neither cholesterol levels nor distribution of fatty acids differed between ApoB transgenics and WT mice.

Interestingly, APP overexpression in hAPPsl single-transgenics as well as ApoBxAPP double transgenics led to significant changes in proportions of free and esterified cholesterol. As discussed in detail in section 7.2, increased levels of ACAT mRNA in the same genotypes are in line with this result of increased cortical cholesteryl ester levels.

In AD research, the current paradigm to explain neuronal dysfunction, neuronal loss and cognitive decline is the 'amyloid cascade hypothesis' (Hardy and Selkoe, 2002; Kowalska, 2004). Abeta peptides are the main component found in plaques, were shown to be neurotoxic, particularly in an oligomerized form (Walsh et al., 2002) and are believed to be the elicitor of the whole cascade of neurodegeneration.

Previous studies (Rockenstein et al 2001, Havas et al. 2011) have demonstrated that overexpression of hAPPsl led to a profound increase of Abeta secretion and also to a shift from Abeta1-38 towards more toxic Abeta species, Abeta1-40 and Abeta1-42.

In this work we observed, that 12 months old female ApoBxAPP and hAPPsl mice showed significantly increased levels of Abeta1-38 and Abeta1-40 compared to males, which is in line with findings from Havas et al. (2011). Male mice seem to reach a plateau after 9 months, whereas in female brains Abeta accumulation and/or production proceeded further. That this sex effect was not mirrored by any behavioural test is most likely due to the unphysiologically high levels of Abeta. It might be that increased Abeta levels are only relevant for cognitive changes until reaching a certain amount and every additional accumulation of Abeta does not further change the phenotype. In line with this theory is the fact that 20 fold lower Abeta levels in 6 months old ApoBxAPP and hAPPsl mice already led to a significant behavioural phenotype.

Similarly, also in human AD patients alterations in Abeta markers are one of the earliest detectable changes, but seem to be plateaued by the mild cognitive impairment (MCI) stage (Frisoni et al., 2010).

This hypothetical ‘ceiling effect’ may also be causative for the lack of correlation between Abeta levels of ApoBxAPP and hAPPsl mice and the corresponding behavioural data. Also a potential effect of additional ApoB-100 overexpression on Abeta levels in hAPPsl mice is probably not detectable since Abeta levels in hAPP expressors are already highly increased (Löffler et al., 2013).

Comparison of non-hAPP expressors, i.e. ApoB-100 and WT animals, revealed a reproducible and profound impact of ApoB-100 overexpression on endogenous Abeta levels. Although plaques were not observed in ApoB-100 animals brains up to an age of 12 months, all three analyzed Abeta species were significantly increased compared to WT animals starting with an age of 9 months.

Interestingly, Abeta1-38 was not only elevated in young ApoBxAPP animals compared to hAPPsl single transgenics, but was also the most abundant species found in ApoB-100 as well as WT brains. This finding of high endogenous Abeta1-38 levels points towards a possible important physiological role of Abeta1-38. Also in human cerebrospinal fluid (CSF) Abeta1-38 was found to be, besides Abeta1-40, the most prominent Abeta species (Maddalena et al., 2004). Additionally, in cell culture experiments it was shown that increased generation of Abeta1-38 is accompanied by a decrease of the more toxic Abeta1-42 peptides, especially when treated with anti-inflammatory drugs (De Strooper and König, 2001, Weggen et al., 2001).

In the present study we observed a significant correlation between insoluble Abeta1-38 and MDA levels in 12 months old ApoB-100 and WT mice. It is therefore tempting to assume increased aggregation of Abeta1-38 is connected to increased lipid peroxidation - either due to an identical underlying cause or one factor being causal for the other.

The fact that elevated MDA levels in ApoB-100 mice were already detected before the raise of insoluble Abeta1-38 supports the hypothesis that increased lipid peroxidation causes increased aggregation of Abeta1-38, but not vice versa. Correlation between high levels of soluble, non-aggregated Abeta1-38 and impaired memory (high CFC freezing times) even points towards a possible protective role of soluble Abeta1-38, probably as scavenger against compounds inducing oxidative stress. Abeta1-38 as cleavage product of APP has not been intensely investigated in earlier studies. However, monomeric Abeta1-40 was previously shown

to have antioxidative properties against metal-induced oxidative damage (Zou et al., 2002) and to inhibit oxidation of CSF lipoproteins and plasma LDL (Kontush et al., 2001).

Therefore, findings of the present study point for the first time towards a similar antioxidative effect of soluble Abeta1-38, but this hypothesis needs further evaluation.

An explanation why correlation analyses between Abeta and other markers in hAPP overexpressors, ApoBxAPP and hAPPsl, did not reveal any significance may be that hAPP expression is driven by a different promoter (Thy1) or, alternatively, may be caused by differential processing of human mutated APP in contrast to the endogenous murine APP. HFD feeding led to the expected changes of the plasma lipid profile resulting in more atherogenic conditions in all genotypes.

Cerebral Abeta clearance seemed to be influenced by HFD, especially in hAPP overexpressors, ApoBxAPP and hAPPsl. Observation of decreased plasma Abeta, together with increased levels of Abeta in the brains of these animals, highly emphasize this hypothesis. Whether there are changes in the cerebral vasculature due to HFD and/or ApoB overexpression will be subject of further investigations via immunohistochemistry. Behavioural testings revealed diverse results, indicating changes in emotional behaviour, but not in spatial learning due to HFD.

Cortical mRNA expression patterns under ND and HFD conditions emphasized the importance of APP in cerebral lipid metabolism, pointing towards a possible connection between APP overexpression and changes in the amount and distribution of cholesterol.

Although hApoB-100 is not expressed in brain, overexpression led to profound changes in the histological appearance of cerebral vessels.

It is important to note that accumulation and extravasation of ApoB-100, a 515 kDa protein, was found in cerebral vessels of ApoBxAPP and ApoB animals. This finding may indicate a disturbance of the blood-brain-barrier (BBB). To further investigate this contingency an Evans Blue test was carried out. Interestingly, this test displayed BBB leakage not only in ApoB-100 overexpressors, but in all three transgenic mouse models, including hAPPsl mice, compared to WT littermates. Since BBB breakdown is a well described AD-pathology (Bowman et al., 2007), this finding reflects the importance of the hAPPsl transgenic mice as a model for AD.

Changes in the lumen of arteries due to ApoB-100 expression could not be detected. Interestingly, a previous study from another group showed, that old ApoB-100 mice exhibit severe changes in the capillary network, which was detected as decreased density and increased capillary lumen diameter (Sule et al., 2009). The authors hypothesized impaired angiogenesis being responsible for the reduced number of capillaries and the concomitant dilation of capillary lumens as a complementary and compensatory mechanism to maintain blood supply.

In the present study the oldest group of 12 months old ApoB-100 mice was probably too young to observe these changes of the vessel lumen.

CAA, accumulations of Abeta in vessel walls, was detected in both hAPP overexpressors, ApoBxAPP and hAPPsl, but again no significant difference between these two genotypes could be observed. Surprisingly, when additional ApoB-100 was overexpressed the animals showed HFD feeding led to the expected changes of the plasma lipid profile resulting in more atherogenic conditions in all genotypes.

Cerebral Abeta clearance seemed to be influenced by HFD, especially in hAPP overexpressors, ApoBxAPP and hAPPsl. Observation of decreased plasma Abeta, together with increased levels of Abeta in the brains of these animals, highly emphasize this hypothesis. Whether there are changes in the cerebral vasculature due to HFD and/or ApoB overexpression will be subject of further investigations via immunohistochemistry. Behavioural testings revealed diverse results, indicating changes in emotional behaviour, but not in spatial learning due to HFD.

Cortical mRNA expression patterns under ND and HFD conditions emphasized the importance of APP in cerebral lipid metabolism, pointing towards a possible connection between APP overexpression and changes in the amount and distribution of cholesterol. It showed a tendency to less CAA symptoms. The reason for that could be the strong accumulation of plasma-derived ApoB in the vasculature, impeding further Abeta accumulation.

ApoB accumulation was found in all kinds of vessels, according to their shape veins were also affected at a high rate, which suggests a possible impairment in cerebral clearance of metabolites.

In general, ApoB-100 overexpression seems to induce remarkable alterations in shape and, most likely, also function of the cerebral vasculature, partially comparable to those found in human AD patients (Farkas and Luiten, 2001; Miyakawa, 2010).

In the present study an increase in activated astrocytes in the corpus callosum was found only in double transgenic ApoBxAPP animals. Notably, increased GFAP immunoreactivity in the hippocampus and corpus callosum was the unique parameter which pointed towards synergistic effects of both transgenes in ApoBxAPP mice, since it could not be detected in any of the single transgenic groups.

A major outcome of the present study is that hApoB-100 overexpression by itself induces cognitive decline. As shown with two independent cognitive tests, the CFC and MWM tasks, restricted memory performance of ApoB-100 mice was observed in older animals.

Correlation between CFC freezing time and cerebral lipid peroxidation levels suggests that oxidative stress is an important triggering factor for cognitive decline. Additionally, it is tempting to assume that the described changes in the vasculature also contribute to the decline in memory performance of ApoB-100 mice.

A picture arises, in which changes in the peripheral lipid metabolism seem to induce severe oxidative stress and accordingly perceptible alterations of the cerebral vasculature, adversely affecting physiological brain function.

7.2 Challenging the system - a high fat diet study

Although the BBB prevents most of lipid exchange between CNS and periphery, it was already shown in 1994 that dietary cholesterol uptake impacts on cerebral A β production in rabbits (Sparks et al., 1994). Several investigations in AD mouse models pointed towards a direct connection between dietary cholesterol, plaque load and amyloidogenic cleavage of APP in the brain (Refolo et al., 2000, Shie et al., 2002, Oksman et al., 2006).

To investigate how the increase in dietary cholesterol, together with an additional change in the plasma lipoprotein composition in mice, caused by hApoB-100 overexpression, influences AD pathology, cerebral lipid metabolism and other parameters, a high-fat feeding study was conducted. Animals of every genotype, ApoBxAPP, ApoB-100, hAPPsl and WT, received either a standard chow or high fat diet for 3 months.

Liver damage due to HFD was exclusively found in ApoB-100 transgenic animals via measurement of alanine amino transferase (ALT). Expectedly, liver histology, on the other hand, demonstrated in all genotypes on HFD the presence of high amounts of fat in and around the liver. Probably, this steatosis further exacerbated only in ApoB-100 transgenic animals into a more pathologic status, because lipid parameters and body weight were most affected by HFD in these mice.

While hApoB-100 overexpression led to enhanced weight gain and increased total and LDL-cholesterol as well as triglyceride levels in the plasma, hAPP overexpression, either alone or in combination with hApoB-100, partially counteracted these effects. ApoBxAPP and hAPPsl animals had a reduced body weight and total cholesterol levels even on HFD compared to ApoB and WT mice, respectively. Also when kept on ND, hAPPsl mice show a tendency to reduced body weight and lowered cholesterol levels in the plasma, compared to WT mice. That these observed effects are due to decreased food consumption or increased activity of hAPP overexpressing mice can be rejected, since we observed a tendency to even increased food consumption of hAPP overexpressors and no difference in spontaneous activity, measured during the Open field test.

Our finding that hAPP overexpression causes a drop in plasma cholesterol levels is novel and deserves further investigation. The systemic influence of APP on body weight has been published previously in another APP transgenic mouse model (Pugh et al., 2007).

Especially, since APP overexpression in this mouse model is driven by the mThy1 promoter and therefore restricted to neuronal tissue, the impact on the peripheral metabolism is highly interesting. The current understanding implies that the main proteolytic peptide of APP, amyloid beta (Abeta), is released from the brain into the plasma (Podlisny et al., 1990), mainly via ApoE and LRP1/LDL-R mediated clearance (Kang et al., 2000, Shibata et al., 2000, Prior et al., 2000).

In this study also plasma Abeta levels were assessed with an assay detecting both, human and murine isoforms. Due to the HFD, a profound decrease of plasma Abeta1-40 in hAPPsl and ApoBxAPP mice as well as a slight reduction in ApoB mice, was measured. Abeta1-42 was only found in the plasma of hAPP overexpressors, ApoBxAPP and hAPPsl, when kept on chow diet and again a drastic reduction was revealed as a consequence of HFD.

Together with the high increase of all insoluble Abeta species in the brain of HFD animals, impaired clearance of Abeta from the brain due to HFD feeding can be assumed. Since overexpression of human APP is restricted to neuronal tissue, all of the human Abeta in the plasma must be brain-derived. Therefore, a reduction of human Abeta in the plasma of transgenic animals, as visualized by western blot analysis, is in line with this theory of 'impaired clearance' caused by HFD.

Interestingly, MWM results did not reflect this increase of Abeta in the brains of HFD fed animals. No change of spatial learning or memory function could be observed in the HFD groups.

Divergent results were obtained from the second cognitive test, the contextual fear conditioning task. In this behavioural assay, emotional learning is examined, mainly related to the amygdala-hippocampus complex. We observed, that all genotypes on HFD performed worse than on ND. Especially WT animals were highly affected, but in general, every genotype on HFD showed a significantly reduced freezing time compared to WT mice on ND.

Interestingly, a second parameter related to emotional behavior revealed an impact of HFD. Thigmotaxis, a measure of anxiety-like behavior in the OF, was significantly decreased in hAPP overexpressors on HFD.

Obviously, HFD mainly affects emotional behavior, but has no measureable impact on spatial learning. While the latter mainly involves the hippocampus and several neocortical areas (D'Hooge and De Deyn, 2001), emotional learning is related to the amygdala-hippocampus complex (Kim and Jung, 2006). There, the amygdala receives its input from the hippocampus (Maren, 2001). Therefore, damage in the hippocampus would affect both spatial and emotional learning, while changes in the amygdala only impact on emotional learning.

It is likely that the reduced anxiety in HFD fed ApoBxAPP and hAPPsl mice is responsible for their slightly “better” MWM performance compared to ND mice of the same genotype, since these mice may overcome the water induced shock faster than the other, more anxious, animals and are less afraid of entering the central areas of the maze.

On the other hand, it may also be that reduced anxiety on HFD influences the outcome of the contextual fear conditioning task of HFD fed ApoBxAPP and hAPPsl mice. In this task less freezing time is interpreted as worsened memorization of the bad stimulus, but in this case it could also be that lowered freezing time is just a consequence of reduced anxiety.

Nevertheless, these results imply an impact of HFD on emotional learning at least in WT animals, since WT mice on HFD displayed no changes in thigmotaxis but performed worse in the CFC.

To study a potential crosstalk between APP and cholesterol pathways, mRNA expression of several genes related to cholesterol metabolism, cholesterol transport and APP processing, was analyzed.

Several previous studies found strong connections between APP processing and lipid, especially cholesterol metabolism. It is not only commonly accepted that the lipid composition of cell membranes plays a crucial role in the occurrence of amyloidogenic APP cleavage (Hartmann et al., 2007, Marquer et al., 2011) , but also vice versa, APP and its proteolytic products directly impact on lipid metabolism (Grimm et al., 2007, Grimm et al., 2012).

Most notably, Grimm et al. (2005) showed that cells lacking APP/APLP2 or a functional gamma-secretase exhibit an increase in cellular cholesterol levels, which can be reversed by

treatment with Abeta1-40. Therefore, a regulatory feedback loop between Abeta and cholesterol was proposed, indicating APP as cellular sensor for increased cholesterol levels, leading to higher Abeta formation which in turn down regulates cholesterol synthesis (Grimm et al., 2007). Results of the present study strongly support this hypothesis. In both mouse models overexpressing mutated hAPP, which leads to highly increased amyloidogenic cleavage and therefore to high Abeta levels, decreased levels of free cholesterol were detected in the cortex (section 6.1.26.1.2). Additionally, a higher expression level of HMGCoA-R mRNA was observed in these mice, indicating a possible adaptive mechanism to maintain the supply of cholesterol by boosting HMGCoA-R expression. In line, high levels of LDL-R mRNA were found in the cortices of hAPP overexpressing mice, compared to WT littermates, indicative of the low free cholesterol level of the respective cells. Taken together we show at the molecular level that a strong overexpression of APP in mice down regulates levels of free cholesterol and leads to a mRNA expression pattern reflecting insufficient supply of free cholesterol in the cortex.

Taking in account that also ACAT mRNA levels are increased in ApoBxAPP as well as hAPPsl mice brains, it is feasible that hAPP overexpression and/or excess levels of a proteolytic product of APP is causative of this effect. An increase in ACAT further enhances a decrease in free cholesterol as a consequence of cholesteryl ester (CE) production. Indeed, a strong tendency to increased cholesteryl esters abundance, in cortices of 9 months old ApoBxAPP and hAPPsl mice on ND, was obvious in the basic characterization (section 6.1.26.1.2). In previous studies inhibition of ACAT was tested as an AD drug and it was shown that ACAT inhibitors can reverse brain amyloid pathology in hAPPsl mice (Huttunen et al., 2010). These experiments demonstrated that ACAT inhibition has an influence on APP metabolism and trafficking (Huttunen et al., 2009, Huttunen et al., 2010). Conversely, our data may indicate that an additional feedback loop between APP and ACAT exists. Thus, high levels of ACAT, and consequently CEs, are associated with high Abeta processing/deposition and vice versa.

From our results we conclude that increased APP metabolism may induces a pathway upregulating several mechanisms of reducing free cholesterol levels, (i) on the one hand by inhibiting cholesterol synthesis(Grimm et al. 2005), (ii) on the other hand by upregulating CE production.

Additionally the above mentioned results for decreased body weights and plasma cholesterol levels in hAPP overexpressing animals might be related to this hypothesis of APP as “free cholesterol sensor” with cholesterol down regulating functions. Speculatively, this hypothesis could also explain the risk factor of hypercholesterolemia in humans. The chronic oversupply of cholesterol over years may lead to upregulation of the APP/Abeta related cholesterol down regulation pathway and therefore to increased production of Abeta.

The results obtained for ApoB-100 transgenic animals on HFD further complete this picture. These mice represent healthy, non-demented animals, chronically exposed to high plasma cholesterol levels.

Compared to ApoB-100 animals on ND, in ApoB-100 mice HMGCoA-R mRNA levels are not altered due to HFD, but, in turn, Cyp46A1 as well as ACAT1 mRNA levels are increased, both mechanisms for removal of excessive free cholesterol.

ApoE mRNA is upregulated in ApoB-100 mice on HFD compared to WT mice on HFD as well as ApoB-100 mice on ND. Since ApoE is the most important apolipoprotein in the brain, responsible for distributing free cholesterol in the CNS, an increase of this apolipoprotein may additionally indicate an excess of free cholesterol in ApoB-100 brains on HFD. This represents a quite distinct phenotype, different from that observed for hAPP overexpressors.

Interestingly, only in ApoB-100 single transgenic animals on HFD, mRNA levels of murine APP as well as BACE1 were altered compared to WT mice on HFD. This increase in both, APP as well as the amyloidogenic APP cleavage enzyme BACE1, is in line with the above proposed hypothesis of APP cleavage as inducer of cholesterol down-regulating mechanisms. Since in brains of ApoB-100 animals on HFD several other mechanisms for removal of excessive free cholesterol seem to be upregulated (Cyp46A1, ACAT, ApoE), the increase in APP and BACE1 levels may represent an additional pathway of reducing free cholesterol levels. These findings underline the importance of a balanced lipid metabolism in the onset and progression of AD.

A probable limitation of our mRNA expression analysis is that just the mRNA levels in the total cortex have been investigated. We can currently not distinguish between mRNA levels of

different cell types. Especially regarding cholesterol metabolism, there are major differences between neurons and glial cells, e.g. astrocytes, since neurons depend on the supply of free cholesterol which is mainly synthesized by astrocytes.

In the liver, mRNA expression of most investigated genes, more precisely ACAT1, ACAT2 and LDL-R, was down regulated on HFD. Usually, high levels of plasma-cholesterol are expected to induce upregulation of all mechanisms involved in removal of excessive cholesterol, like ACATs. While down regulation of LDL-R mRNA was expected, the reduction in ACAT1/2 remain obscure.

Notably, double transgenic ApoBxAPP mice mainly display the same mRNA expression pattern as single transgenic hAPPsl mice, in both the brain as well as in the liver, but have almost no similarity to the mRNA expression profile of ApoB-100 single transgenics. This is an additional indicator of the strong impact of APP and its metabolites on cerebral cholesterol metabolism.

In summary, we describe a hitherto unrecognized effect of APP overexpression on brain cholesterol metabolism on the molecular level. Future experiments will be carried out to confirm these results on protein levels and to distinguish between different cerebral cell types.

Chapter 8

Conclusion

The fact that hApoB-100 overexpression by itself induces memory decline in older animals indicates the importance of a balanced (peripheral) lipid metabolism and an intact vascular network to maintain cognitive functions.

In our study, no plaque formation could be detected in ApoB-100 single transgenic animals. However, a direct impact of hApoB-100 overexpression on cerebral Abeta levels was observed in single-transgenic mice compared to wild-type (WT) littermates.

In double transgenic ApoBxAPP animals, no synergisms between the two transgenes were detected. These mice did not develop more distinct behavioural deficits or Alzheimer's-disease-pathology than single-transgenic hAPPsl littermates. But additional pathophysiological features, obtained by hApoB-100 expression, like a more atherogenic plasma lipid profile, increased cerebral lipid peroxidation, blood-brain-barrier (BBB) leakage and astrogliosis in the hippocampus indicate that this combination reflects the situation of elderly humans better than hAPPsl overexpression alone.

High-fat diet (HFD) feeding led to the expected changes of the plasma lipid profile resulting in more atherogenic conditions in all genotypes.

Cerebral Abeta clearance seemed to be influenced by HFD, especially in hAPP overexpressors, ApoBxAPP and hAPPsl. The observation of decreased plasma Abeta, together with increased levels of Abeta in the brains of these animals, highly emphasize this hypothesis. Whether there are changes in the cerebral vasculature due to HFD and/or ApoB overexpression will be subject of further investigations. Behavioural testing revealed divergent results, indicating changes in emotional behaviour, but not in spatial learning as a consequence of HFD.

Cortical mRNA expression patterns under normal diet (ND) and HFD conditions argue for an important role of APP in cerebral lipid metabolism, pointing towards a possible connection between APP overexpression and changes in the amount and distribution of cerebral cholesterol. These findings are novel and deserve further investigation.

Chapter 9

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