PhD Thesis

Interactions of simvastatin and apoJ with APP processing and amyloid-β clearance in blood-brain barrier endothelial cells

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 and agreed to publish the data in 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 "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

The major part of investigation presented in this thesis has been summarized and published in:

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ABBREVIATIONS

7DHC: 7-dehydrocholesterol
24(S)OH-C: 24(S)-hydroxycholesterol
Aβ: amyloid-beta peptide
ABCA1: ATP binding cassette transporter A1
AD: Alzheimer’s Disease
ADAM10: activity of disintegrin-like metalloproteinase 10
ApoJ: apolipoprotein J
APP: amyloid precursor protein
BACE1: β-secretase β-site APP cleavage enzyme 1
BBB: blood-brain barrier
CAA: cerebral amyloid angiopathy
CE: cholesterol ester
CNS: central nervous system
CSF: cerebrospinal fluid
CTF: C-terminal fragment
HDL: high-density lipoproteins
HMG-CoA reductase: 3-hydroxy-3-methyl-gutaryl-CoA reductase
HPRT1: hypoxanthine phosphoribosyltransferase 1
ISF: interstitial fluid
LDL: low-density lipoproteins
LRP1: low-density lipoprotein receptor-related protein 1
LXR: liver-X receptor
mBCEC: primary murine brain capillary endothelial cells
MCI: mild cognitive impairment
mm: mus musculus
NEFA: non-esterified fatty acids
pBCEC: primary porcine brain capillary endothelial cells
PGP: P-glycoprotein
PBS: phosphate-buffered saline
PL: phospholipid
PPAR: peroxisome proliferator activated receptor
RAGE: receptor for advanced glycation end products
RT-qPCR: quantitative real-time polymerase chain reaction
RXR: retinoic-X receptor
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
sHSP: small heat shock proteins
SNP: small nucleotide polymorphism
SREBP2: sterol regulatory element binding protein 2
ss: sus scrofa
TEER: transendothelial electrical resistance
TAG: triacylglycerides
VLDL: very low-density lipoprotein
vWF: Von Willebrandt factor
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In apoJ Silencing Versuchen in pBCEC konnten wir eine reduzierte Ansammlung von intrazellulärem APP (59%) und Aβ Oligomeren (56%) feststellen. Inkubation von

Zusammenfassend schlagen wir eine enge und komplexe Interaktion vom zellulären Cholesterinstoffwechsel, apoJ Expression und der APP bzw. Aβ Prozessierung und Eliminierung an der BHS vor.
ABSTRACT

Background: Amyloid-β peptides (Aβ) may accumulate in cerebral capillaries indicating a central role of the blood-brain barrier (BBB) in the pathogenesis of Alzheimer’s disease (AD). Although a relationship between apolipoprotein-, cholesterol- and Aβ metabolism is evident, the interconnecting mechanisms operating in brain capillary endothelial cells (BCEC) are poorly understood. Apolipoprotein (apo)J, also known as clusterin, is present in lipoprotein particles and regulates cholesterol and lipid metabolism of brain which is disturbed in AD. ApoJ expression is increased in AD brains and apoJ binds, prevents fibrillization, and enhances endocytosis of Aβ.

Aims: Our aim was to define the involvement of apoJ and cellular cholesterol homeostasis in amyloid precursor protein (APP) processing/Aβ metabolism at the BBB. Primary porcine (p)BCEC were incubated in the presence and absence of plasma-derived apoJ and modulators of cholesterol metabolism prior to analyses of APP/Aβ and apoJ mRNA and protein expression levels. In vivo studies with aged 3xTg AD and Non-Tg mice (64±4 weeks) and apoJ ko mice treated for 21 days with the HMGCoA-reductase inhibitor simvastatin [40 mg/kg] by oral gavage were aiming to elucidate the role of cellular cholesterol homeostasis and apoJ in APP metabolism at the BBB in greater detail.

Results: RNA interference-mediated silencing of apoJ in pBCEC decreased intracellular APP and Aβ oligomer levels by 59% and 56%, respectively, while the addition of 2 or 20 µg/ml of purified apoJ to pBCEC increased intracellular APP (by 3.3 and 2.4-fold, respectively) and enhanced Aβ clearance across the in vitro model of the BBB. Cerebromicrovascular endothelial cells isolated from 3xTg AD mice revealed 3.4-fold higher expression levels of apoJ as compared to Non-Tg animals. Treatment with simvastatin markedly increased intracellular and secreted apoJ levels in pBCEC, and, in parallel, increased secreted Aβ oligomers, and reduced Aβ uptake and cell-associated Aβ oligomers. In accordance, simvastatin increased protein levels of apoJ in primary murine (m)BCEC of Non-Tg mice by 73%. In mBCEC of
apoJ ko mice, on the other hand, we detected enhanced levels of C-terminal fragments (CTFs) - cleavage products of APP-, which were further increased in response to simvastatin treatment.

**Conclusion:** Our results, so far, suggest a close and complex interaction of cellular cholesterol homeostasis, apoJ, and APP/Aβ processing and clearance at the BBB.
1. Introduction

Parts of this chapter have been literally published in:

1.1. Alzheimer’s disease

Alzheimer’s Disease (AD) was firstly described by the German Psychiatrist Alois Alzheimer in 1907. He performed post mortem studies on the female patient Auguste Deter, who suffered from severe dementia at the early age of 51 (2). During his examinations he found a uniformly atrophic brain with severe neurofibrillary pathology. In addition, he described the existence of unusual deposits in the cortex of the patient, which revealed difficulties during histological staining (3).

AD is a progressive human neurodegenerative disease, which implies memory loss, disturbances in language, and cognitive decline (4). It is the most common form of dementia with about 75% of all dementia cases clinically diagnosed as AD. Currently approximately 50 million people in the world are suffering from dementia, with about 5 million new cases per year (4,5). Due to the aging population it is suggested that this number will quadruple until 2050. For European people aged 65 and older the risk for developing dementia and AD lies at 6.4% and 4.4 %, respectively (4). In addition, it was shown in several European studies that the risk for developing dementia and AD is higher among women as compared to men, whereas North American studies did not find gender differences (4).

In general, two forms of AD are known. The late-onset sporadic form occurs if no family history of AD is obvious (6). Genetic factors and mutations lead to early-and late-onset familial AD (FAD), which takes place in less than 10% of all cases (7).
Especially being a carrier of the apoE4 allele is meant to be a major risk factor for developing FAD.

“Over the past 35 years, extensive research and effort has been put into understanding the mechanisms and biochemical pathways underlying AD. By now, it has been shown that there are two main pathological hallmarks in the development of the disease (8). On the one hand, abnormally phosphorylated tau proteins are responsible for the formation of intracellular neurofibrillary tangles. On the other hand, deposits of differently sized amyloid beta (Aβ) peptides form extracellular amyloid plaques, which are surrounded by dystrophic neurites and microglia (9,10).“


1.2. The blood-brain barrier (BBB)

The BBB was firstly described by Paul Ehrlich and Edwin Goldwins in 1904 as “some sort of structure” that separated the brain from the rest of the body (11). They performed experiments in which they injected water-soluble dyes into the circulation. As a result, the dye stained all organs except for the brain. When they injected tryptan blue directly into the cerebrospinal fluid (CSF), all cell types in the brain were stained, but not in the periphery. Therefore, Ehrlich and Goldwin concluded, that there must be a barrier which separates the central nervous system (CNS) from the periphery. In 1900 this barrier was firstly named bluthirnschränke (blood-brain barrier) by Lewandowsky, who performed penetration studies of potassium ferrocyanide into the brain.

By now we know that the BBB is a highly dynamic and complex barrier, which is present in all organisms with well-developed CNS (Figure 1) (12). The walls of the capillaries are formed by endothelial cells linked with tight and adherens junction
proteins (claudins, occulins, VE-cadherin, β-catenin) (13). The endothelial cells are surrounded by various cell types of the CNS, including neurons, astrocytes, microglia and pericytes (12). Pericytes for examples are associated with endothelial cells and are important during development and for BBB permeability (14). The surface area range of the BBB in an average human adult lays between 12 and 18 m² (12).

As the protein content and composition of the plasma is much higher as compared to the CSF, the BBB is highly selective and prevents many macromolecules from entering the brain (12). Only lipid-soluble non-polar molecules are able to pass the BBB by passive diffusion. Most of the polar molecules need transport proteins and carriers to passage through the BBB. The orientation of these transporters, either to the basolateral (facing the brain side of the BBB) or apical side (facing the blood side of the BBB) of the barrier, indicates the preferential transport of these molecules either from the brain or from the periphery across the BBB (12). Many transporters and receptors are known to be important at the BBB. The ATP-binding cassette (ABC) transporters, for example, in human are a superfamily of proteins containing 48 members divided in 7 sub-families. The significance of ABC transporters at the BBB lies in the efflux of lipid-soluble compounds from the brain, thereby consuming ATP. The ABC transporters ABCA1 and ABCG1 are responsible for cholesterol transport/efflux. P-glycoprotein (Pgp, multidrug resistance protein/MDR, ABCB1) is expressed at the basolateral side of the BBB and clears/removes drugs from the brain (12).
**Figure 1: Scheme depicting the structure of the blood-brain barrier.** Capillary endothelial cells form a tight barrier, which are linked with tight and adherens junctions. Astrocytes, neurons and pericytes are also part of the BBB. Transport across the BBB is highly regulated. [Reproduced from Abbott et al. with permission of Nature Reviews Neuroscience (15)]

"In the last decade, it became increasingly evident, that AD patients reveal biochemical, morphological and functional changes in the cerebrovasculature, which implies a thus far underestimated role of the BBB in pathogenesis of AD (16,17)." [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj J, Pietrzik CU, Wilson MR, Björkhem I, Panzenboeck U. Regulatory effects of simvastatin and apoJ on APP processing and amyloid-β clearance in blood-brain barrier endothelial cells. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60.(1)] Amyloid plaque precursors are found in brain parenchyma and are also present within cerebral blood vessels (18). Such cerebral amyloid angiopathy (CAA) also occurs in vascular dementia (VAD), the second most common form of dementia after AD that is characterized by a cognitive deficit of cerebrovascular origin. It was shown that CAA can cause hypoperfusion which then further stimulates Aβ production (19).

### 1.3. APP (amyloid precursor protein) processing and metabolism in the brain

"Among various hypotheses of AD development, the amyloid cascade hypothesis, suggesting the formation of amyloid plaques from Aβ peptides receives the most attention (20). In this hypothesis, the amyloid precursor protein (APP) is processed..."

Cleavage of the transmembrane protein APP with the caspase-like enzymes β-secretase and γ-secretase leads to the formation of neurotoxic Aβ peptides. Alternatively, APP can be processed via the α-secretase, which results in the secretion of the non-amyloidogenic and neuroprotective sAPPα.

**Figure 2: The amyloid cascade hypothesis.** The amyloid precursor protein (APP) contains cleavage sites for α-, β-, and γ-secretases, which will either lead to the formation of the non-amyloidogenic sAPPα, or to the development of amyloidogenic Aβ peptides [Reproduced from Rajasekhar et al. with permission of Chemical Communications (21)]

For the development of amyloid plaques and also the progression of AD continuous removal of Aβ is crucial (22). Various receptors are involved in the uptake and transport of amyloid beta fragments across the BBB. "Among others, RAGE
(Receptor for advanced glycation end products) and LRP1 (Low-density lipoprotein receptor-related protein-1) appear to be the major receptors responsible for the transport of amyloid beta peptides from the plasma and the brain parenchymal side across the BBB, respectively.**” [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj J, Pietrzik CU, Wilson MR, Björkhem I, Panzenboeck U. Regulatory effects of simvastatin and apoJ on APP processing and amyloid-β clearance in blood-brain barrier endothelial cells. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60.(1)]. Genetic studies in the past years also suggested a link between the expression of LRP1 and CAA (23). Lrp1 consists of an α- (515 kDa) and a transmembrane β- (85 kDa) chain, which can be further processed to form soluble Lrp1 (sLrp1) by metalloproteinases, like ADAM10, and secretases, like BACE1 (24,25). sLrp1 is mainly present in human plasma, and removes 70-90% of plasma Aβ peptides (26). In AD patients, plasma sLrp1 levels are reduced. Therefore, the capacity to bind Aβ is decreased leading to increased levels of free plasma Aβ.

1.4. **Cholesterol metabolism and lipoproteins in the brain**

The brain is the most cholesterol rich organ in the body with approximately 25% of total cholesterol distributed to this part of the body. Almost all (>99.5%) cholesterol is unesterified and the majority of cholesterol is present in myelin sheaths, where it is responsible for insulating axons (27). Most of the cholesterol is produced by local de novo synthesis in the brain, rather than from peripheral cholesterol transported across the BBB (28). In addition, it was shown that the half-life of brain-derived cholesterol is much longer (max. 5 years) as compared to that in the periphery, which will stay only for days (29). Cellular cholesterol synthesis in the CNS is a complex and intense process which starts with the conversion of acetyl-CoA to acetoacetyl-CoA which will be further converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the HMG-CoA synthase (Figure 3). HMG-CoA is then further converted to mevalonic acid (27). This step is described to be the rate-limiting step in cellular cholesterol synthesis and can be inhibited by treatment with the HMG-CoA reductase inhibitors called “Statins”. Cholesterol will then be formed by a series of enzymatic
reactions, which involves the production of cholesterol precursors such as lanosterol, lathosterol, FF-MAS, T-MAS and desmosterol (27).

Since excess cholesterol needs to be removed from the brain, two recycling pathways of cholesterol from the CNS are known. A minor part of cholesterol is recycled via the CSF. The major part of cholesterol is converted to 24OH-cholesterol by CYP46, which then is further transported across the BBB and cleared through the blood. In addition, cholesterol can be hydrolysed by CYP27 to form 27OH-cholesterol. This process mainly occurs in the periphery, rather than in the CNS.
Figure 3: Scheme depicting cellular cholesterol synthesis. Cellular cholesterol synthesis is a complex enzymatic pathway, which involves many steps. By using the HMGCoA reductase inhibitor statin, which interferes with the rate-limiting enzymatic step, cellular cholesterol synthesis can be inhibited.

1.5. **HDL biogenesis at the BBB**

In general, lipoproteins consist of a core formed by triacylglycerides (TAG) and cholesterol esters (CE) which are surrounded by phospholipids and free cholesterol (30). In addition, apoproteins function as surface stabilisators, cofactors for enzymes or ligands for lipoproteins receptors (30). Depending on the size, plasma lipoproteins
can be separated into four classes: chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (30). Lipoproteins detected in the central nervous system (CNS) reveal a density similar to HDL (30,31). HDL is mainly synthesized by neurons and astrocytes in the brain, but it was also shown that BBB endothelial cells are able to synthesize HDL-like particles (29,32). Previous studies by our group revealed that in porcine brain capillary endothelial cells (pBCEC), cholesterol efflux is mediated especially through activation of the ATP binding cassette transporter A1 (ABCA1) and that HDL assembly is mainly localized at the basolateral (brain) side of the BBB (Figure 4). The transcription factors liver-X-receptor (LXR) and retinoic-X-receptor (RXR), or peroxisome proliferator activated receptor (PPAR, comprising PPARα, PPAR β/δ and PPARγ) agonists will thereby stimulate activation of ABCA1. Small nascent HDL-like particles are formed through the incorporation of cholesterol and phospholipids. ApoA-I, together with other apolipoproteins function as acceptors for HDL biogenesis (33). In addition, Chirackal et al. showed that phospholipid transfer protein (PLTP), a glycoprotein involved in lipid and lipoprotein metabolism, also contributes to formation and remodelling of HDL-like particles at the BBB (32).
1.6. **The role of HDL in Alzheimer’s disease**

It was shown by Martins *et al.* that AD patients and Atherosclerosis patients reveal a similar lipoprotein and cholesterol profile (20). It seems that a high level of plasma cholesterol and LDL together with a low amount of HDL significantly correlates with the development and progression of AD. Furthermore, lipoproteins detected in the CSF (and CNS) reveal a density similar to HDL (34,35). Next to its major responsibility to eliminate excess tissue cholesterol through reverse cholesterol transport mediated by ABC transporters, HDL-like particles in the brain are also able to bind Aβ and thereby facilitate its transport across the BBB (36,37). Various studies defined a correlation between cholesterol homeostasis in the brain with Aβ metabolism (38–40). In addition, it was shown that AD patients display higher LDL
cholesterol and lower HDL cholesterol (41). Altogether these findings suggest an important role of the cholesterol metabolism in the progression of AD.

1.7. Statins as therapy for AD

As mentioned above, cellular cholesterol synthesis can be efficiently inhibited by the use of HMGCoA reductase inhibitors called statins, which interfere with the rate-limiting step in cellular cholesterol synthesis. These drugs are usually prescribed to patients with cholesterol-induced atherosclerotic cardiovascular diseases (42). Since it became increasingly evident that plasma cholesterol levels are linked to Aβ deposition and AD pathology, researchers also became increasingly interested in studying the effects of statin treatment during the progression of the disease (42). But it has to be mentioned that not all classes of statins are able to permeate the BBB (42). Thelen et al. performed studies in which they showed that only simvastatin was able to cross the BBB, as compared to others such as pravastatin (42). Simvastatin is simply the most lipophilic statin which facilitates transport across the BBB, while statins, which are hydrophilic or less lipophilic are not able to cross the BBB.

When comparing clinical studies where they used statins for the purpose of AD prevention, the results obtained were quite controversial. In 2002, for instance, a German clinic trial was performed with the outcome that simvastatin lowered both cholesterol and Aβ levels in the CSF of AD patients (43). Between 2003 and 2007 the Alzheimer’s Disease Cooperative Study conducted a randomized double-blind trial where they treated mild to moderate AD patients for 18 months with simvastatin or placebo control (44). Here, on the other hand, no beneficial effects of statin treatment were reported. Further, during the ESPRIT study, which was conducted between 2005 and 2009, people with elevated risk for AD development were treated with simvastatin or placebo control (45): thereby improved cognitive performance was reported, albeit with no significant effects observed in CSF Aβ or tau levels. A recent study performed by Zissimopoulos et al. reported that the risk of AD development in response to statin treatment varies in its efficiency depending on sex and race.
differences (46). In detail, they found that statin treatment, especially treatment with simvastatin, in white women is more efficient as compared to black men (46).

To sum up, until now, the exact mechanisms how statins work within the CNS have not yet been identified, and further research needs to focus on this topic.

1.8. The role of apolipoproteins in the brain

Except for its presence in plasma, apolipoproteins also occur in other parts of the body, as for example in the CNS. HDL-like particles in the brain mainly consist of apolipoprotein (apo)E, apoA-I, apoA-IV, apoD, and apoJ (34). Besides its function in lipid transport and association with HDL-like particles in the CNS, apolipoproteins play an important role for the development and during the progression of AD. Especially, apoE seems to be linked to the disease. Humans carrying the apoE4 allele are more prone to develop this form of dementia as compared to apoE2 carriers. In addition, it was shown that apoD is also expressed in the brain and that its expression levels are increased during aging and also in affected brain regions of AD patients. Li et al. reported that loss of apoD in APP-PS1 amyloidogenice mice resulted in increased production of amyloid plaques (47). Further, expression of transgenic apoD reduced hippocampal plaque load significantly. Therefore, a protective role of apoD during AD pathogenesis is suggested. In addition, apoD was described as a lipid antioxidant (47).

1.9. ApoJ (clusterin) in the brain

**simvastatin and apoJ on APP processing and amyloid-β clearance in blood-brain barrier endothelial cells.** Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60. (1)]. It is a 75-80 kDa heterodimeric glycoprotein linked by five disulfide bonds (51). In addition to its function in lipid trafficking, apoJ acts as a chaperone with broad substrate specificity. *In vitro* studies performed by Poon *et al.* revealed that its chaperone activity is described by an ATP-independent mechanism (51). The chaperone action is similar to that of small heat shock proteins (sHSP), which are only produced under stress conditions and which play important roles in stabilizing partly unfolded proteins (51).

Although highest expression levels of apoJ were detected in brain, only a few studies have investigated its physiological behaviour in the CNS so far. Apart from its function in lipid and cholesterol transport and trafficking in the brain, and a revealed chaperone function (52), the physiological roles and biochemical properties of apoJ are poorly understood (53). “Two independent genome-wide association studies identified gene encoding apoJ in addition to the well-established apoE4 gene, as a novel susceptibility locus for late-onset AD (37,38).” In the past years it has become increasingly evident that small nucleotide polymorphisms (SNPs) in the CLU gene show an association with mild cognitive impairment (MCI) and AD progression (56). Meta-analysis revealed an association for 2 SNPs at the CLU locus, namely rs9331888 and rs11136000 (56). In addition, it was shown that carriers of the rs11136000 allele suffer of a more rapid cognitive decline to MCI or AD as compared to non-carriers (56). Further, cognitively normal carriers of the rs11136000 allele reveal increased regional cerebral blood flow in brain areas essential for memory processes (56). To sum up, these data indicate a strong involvement of genetic variability in the progression of MCI to AD.

ApoJ expression was reported to be increased in AD, especially in neuritic plaques and cerebrovascular deposits (49). “A recent study reported increased plasma apoJ levels in cerebral amyloid angiopathy (CAA) patients as compared to AD patients or controls (57). ApoJ is able to avidly bind Aβ, prevent their fibrillization, and enhances Aβ endocytosis by glial cells (Figure 5A) (49). Importantly, Zlokovic *et al.* revealed that upon binding to apoJ, Aβ peptides can be cleared across the BBB via binding to

However, some studies showed that depending on the concentration of present Aβ in the brain, the protective role of apoJ in Aβ plaque formation can be reversed (Figure 5B) (53). If the stoichiometric ratio of apoJ to Aβ is high, apoJ appears to establish a beneficial effect on plaque formation. If, however the ratio is low, apoJ acts pro-amyloidogenic and can even induce cytotoxic and oxidative stress, which ultimately leads to cell death (53). Although the mechanism still remains unclear, it has been reported that apoJ is able to induce formation of small diffusible Aβ peptides (53). These soluble oligomeric Aβ peptides are associated with neurotoxicity, as they are able to bind to trypsin-sensitive domains at the cell surface which cause neuronal death, especially in the hippocampus (53). Memory loss during the early phases of AD is often caused by these diffusible, non-aggregating Aβ peptides (53). Therefore, some researchers suggest a potential harmful role of apoJ, as apoJ may trigger formation of these small Aβ peptides. In addition, it was shown that Aβ can induce expression of the canonical wnt antagonist Dickkopf-1 (Dkk-1), which further leads to so called Aβ-induced apoptosis (58). Killick et al. wanted to assess the role of apoJ in the Aβ/Dkk1 neurotoxic pathway, since apoJ is regulated by wnt. In their studies they described an apoJ dependent induction of Dkk1 (58), which further promotes Aβ toxicity.

To sum up the current knowledge, it was shown that apoJ is able to bind to Aβ peptides but the exact role and underlying mechanism(s) of apoJ actions during the progression of AD remains unclear and further research has to be performed to elucidate the importance of this chaperone in greater detail.
Figure 5: Role of apoJ in Aβ metabolism. (A) ApoJ, also known as clusterin, was shown to bind to Aβ peptides, that it can be taken up by astrocytes, neurons and glia cells, and that it can be cleared across the BBB [Reproduced from Yu et al. with permission of Molecular Neurobiology (59)]. (B) In addition to its beneficial effects during AD (preventing Aβ aggregation and clearance of Aβ trough the BBB), apoJ can enhance toxic oligomer formation and promotes the Aβ-Dickkopf1 (Dkk1)-wnt pathway, which leads to cell death. [Reproduced from Li et al. with permission of Neuroscience Bulletin (53)]
### 1.10. Rationale and aims

Increasing evidence suggests that the BBB plays an important role in the pathogenesis of AD, especially in the processing and clearance of Aβ plaques. Our laboratory works with primary porcine brain capillary endothelial cells (pBCEC), which were reported to express all key mRNAs that are involved in AβPP processing, i.e. α-, β-, and γ-secretases, that synthesize APP, sAPPα, and Aβ. In addition, inhibition of *de novo* cholesterol biosynthesis by LXR ligands, the HMGCR inhibitor simvastatin, or exogenously supplied cholesterol are effectors of APP processing in pBCEC. These findings indicate an important role of both systemic and cellular cholesterol metabolism and of the BBB in pathogenesis, progression as well as prevention of AD (39).

**During this study we addressed the following aims:**

1. To examine the effects of modulated cholesterol metabolism on APP processing/ Aβ metabolism and apoJ levels *in vitro* using pBCEC. Therefore, pBCEC were incubated with natural LXR agonists (24(S)OH-cholesterol, 27OH-cholesterol), the synthetic LXR agonist TO901317, the HMGCoA reductase inhibitor simvastatin, or cholesterol for 24 h in serum-free conditions.

2. To examine the effects of apoJ on APP/Aβ and cholesterol metabolism at the BBB *in vitro* using pBCEC.

3. To examine the interactions of apoJ, cellular cholesterol synthesis and Aβ metabolism *in vivo* using murine (m)BCEC isolated from 3xTg AD model mice, apoJ ko mice or Non-Tg controls after simvastatin treatment.

4. To determine gene expression of apoJ and apoD in CYP46tg, CYP27tg, and in the BBB-deficient mouse model PDGF(-/-).
2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and solutions used for cell culture experiments

Purchased chemicals are listed in Table 1. 75cm² cell culture flasks, 6- and 12- well plates were obtained from Greiner Bio-One. Transwell 6- and 12-well plates were obtained from Corning Life Sciences. Self-made buffers and solutions are listed below.

*Table 1: Chemicals/solutions used for cell culture experiments*

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 -hydroxycholesterol</td>
<td>Avanti Polar lipids</td>
</tr>
<tr>
<td>TO 901317</td>
<td>Cayman Chemicals</td>
</tr>
<tr>
<td>Medium 199 (1x)</td>
<td>Life technologies</td>
</tr>
<tr>
<td>Minimum Essential Medium (MEM, 10x)</td>
<td>Life technologies</td>
</tr>
<tr>
<td>DMEM/ F-12 (Ham)</td>
<td>Life technologies</td>
</tr>
<tr>
<td>Dispase</td>
<td>Life technologies</td>
</tr>
<tr>
<td>Pure apoJ (clusterin) protein</td>
<td>Mark Wilson, University of Wollongong, Australia</td>
</tr>
<tr>
<td>24 (S)- hydroxycholesterol</td>
<td>Medical Isotopes</td>
</tr>
<tr>
<td>Collagen G from bovine calf skin</td>
<td>M&amp;B Stricker</td>
</tr>
<tr>
<td>Penicillin/ streptomycin/ gentamycin</td>
<td>PAA Laboratories</td>
</tr>
<tr>
<td>Trypsin- EDTA</td>
<td>PAA Laboratories</td>
</tr>
<tr>
<td>Horse serum</td>
<td>PAA Laboratories</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>PAA Laboratories</td>
</tr>
<tr>
<td>Collagenase/dispsase</td>
<td>Roche</td>
</tr>
<tr>
<td>Amyloid β protein fragment 1-40</td>
<td>Sigma- Aldrich</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>Sigma- Aldrich</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Sigma- Aldrich</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Sigma- Aldrich</td>
</tr>
<tr>
<td>Percoll® pH 8.5- 9.5</td>
<td>Sigma- Aldrich</td>
</tr>
<tr>
<td>Dextran</td>
<td>VWR</td>
</tr>
</tbody>
</table>

**1x PBS, pH 7.38-7.42, 5 liters**
- 40 g NaCl
- 1.5 g KCl
- 0.1 g KH₂PO₄
- 0.457 g Na₂HPO₄ + 2 H₂O
- 10 g glucose or dextrose

Fill up to 5 liters with d₂H₂O, filter the solution and autoclave it.

**Collagenase/dispase solution**
Sterile-filter (0.2 µm pore size) 100 mg collagenase/dispase and 10 mg plating medium A and store 350 µl aliquots at – 20 °C.

**Dextran solution**
- 200 g dextran
- 2.4 g NaHCO₃
- 109.1 ml MEM (10x)

Fill up to 1.2 liters with d₂H₂O, stir overnight at 4°C, determine and adjust density to 1.0612. Autoclave solution.

**Percoll® biphase**

<table>
<thead>
<tr>
<th>1.03 g/ml density</th>
<th>1.07 g/ml density</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 ml PBS, 1x</td>
<td>20 ml PBS, 1x</td>
</tr>
<tr>
<td>9 ml Percoll® pH 8.5- 9.5</td>
<td>27 ml Percoll® pH 8.5- 9.5</td>
</tr>
<tr>
<td>1 ml MEM (10x)</td>
<td>3 ml MEM (10x)</td>
</tr>
</tbody>
</table>
# Hydrocortisone solution (50 µg/ml)
- 1 ml
- 19 ml medium
- 1 mg hydrocortisone

Prepare 2 ml aliquots and store at – 20 °C

# Collagen G solution (60 µg/ml and 120 µg/ml)
Mix 450 µl (for 60 µg/ml) or 900 µl (for 120 µg/ml) of Collagen G stock solution (4 mg/ml) with 30 ml PBS, 1x.

# Preparation medium
- 500 ml medium M199 (1x)
- 1% penicillin/streptomycin
- 1% gentamycin
- 1 mM L-glutamine

# Plating medium A
- 500 ml medium M199 (1x)
- 10% horse serum
- 1% penicillin/streptomycin
- 1% gentamycin
- 1 mM L-glutamine

# Plating medium B
- 500 ml medium M199 (1x)
- 10% horse serum
- 1% penicillin/ streptomycin
- 1 mM L-glutamine
Medium serum free (SF)
- 500 ml medium M199 (1x)
- 1% penicillin/ streptomycin
- 1 mM L-glutamine

DMEM/Ham’s F12 medium
- 1% penicillin/ streptomycin
- 0.7 mM L-glutamine
- 0.4% hydrocortisone stock solution [50 µg/ml]

24(S)/27-hydroxycholesterol stock solution
Add 621 µl ethanol absolute to 5 mg hydroxycholesterol to obtain a 20 mM stock solution. Store the stock at -20°C.

TO901317 stock solution
Mix 1 ml ethanol absolute with 50 mg TO901317 and further dilute the solution with dilution factor 10 to obtain a 10 mM stock. Store the stock at -20°C.

Cholesterol stock solution
Mix 1 ml ethanol absolute with 7.74 mg cholesterol to obtain a 20 mM stock. Store the stock at -20°C.

Simvastatin stock solution
Mix 100 µl ethanol absolute with 1 mg simvastatin. Then add 150 µl of 0.1 N NaOH and incubate at 50°C for 2 h. Adjust pH to 7.0 (with HCl) and the final concentration to 4 mg/ml. Store stock solution at -20°C.

Hydrocortisone stock solution [50 µg/ml]
Dissolve 1 mg hydrocortisone in 1 ml ethanol and 19 ml DMEM/ Ham’s F12 Medium. Store aliquots at -20°C.
CBC buffer pH 9.0
- 0.5 M Na$_2$CO$_3$
- 0.5 M NaHCO$_3$

Fluorescent labelling of Aβ$^{1-40}$
100 µl of Aβ$^{1-40}$ [1mg/ml] was labelled fluorescently while incubation with 3 µl Alexa Flur 488 5-TFP for 1 h at 37°C in 18 µl carbonatebicarbonate (CBC) buffer. Unbound fluorescent dye was removed via size-exclusion on a PD-10 column. Elution was performed with 1 ml PBS buffer.

2.1.2 Chemicals and solutions used for silencing, RNA isolation, cDNA synthesis and q RT-PCR

All products and reagents used for RNA silencing, isolation and RT-qPCR are listed in Table 2. Pre-validated primers for quantitative RT-PCR were purchased from Qiagen or self-designed and obtained from Life technologies (Table 3 and Table 4). Small interfering RNA used for silencing apoJ was purchased from Microsynth and are listed in Table 5. Self-made buffers are listed below.

Table 2: Materials used for RNA isolation, cDNA synthesis and RT-qPCR

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>iQ SYBR® green supermix</td>
<td>Biorad</td>
</tr>
<tr>
<td>Hard-Shell® Low-profile thin-wall 96-well skirted PCR plates</td>
<td>Biorad</td>
</tr>
<tr>
<td>Microseal ‘B’ Adhesive seals for PCR plates</td>
<td>Biorad</td>
</tr>
<tr>
<td>0.2 ml PCR tubes</td>
<td>Biorad</td>
</tr>
<tr>
<td>C-1000™ thermal cycler</td>
<td>Biorad</td>
</tr>
<tr>
<td>Icycler iQ™, Real-time PCR detection system</td>
<td>Biorad</td>
</tr>
<tr>
<td>Product Description</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Molecular imager ChemiDoc XRS System</td>
<td>Biorad</td>
</tr>
<tr>
<td>Power Pac HC, power supply</td>
<td>Biorad</td>
</tr>
<tr>
<td>Biozym LE agarose</td>
<td>Biozym</td>
</tr>
<tr>
<td>OneTouch filter-tips, 10 µl</td>
<td>Biozym</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>ON target plus siRNA, 5 nmol</td>
<td>Dharmacon</td>
</tr>
<tr>
<td>HE 33 Mini submarine electrophoresis unit</td>
<td>Hoefer® Inc.</td>
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<tr>
<td>High capacity reverse transcriptase kit</td>
<td>Life technologies</td>
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<tr>
<td>Ethidiumbromide (10 mg/ml)</td>
<td>Life technologies</td>
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<tr>
<td>Taqman gene expression master mix</td>
<td>Life technologies</td>
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<tr>
<td>TriReagent RT</td>
<td>Molecular Research Center, Inc</td>
</tr>
<tr>
<td>2-Log DNA ladder</td>
<td>New England laboratories</td>
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<tr>
<td>Gel loading dye, blue (6x)</td>
<td>New England laboratories</td>
</tr>
<tr>
<td>PrimeFect™ siRNA transfection reagent</td>
<td>Lonza</td>
</tr>
<tr>
<td>PrimeFect™ diluent</td>
<td>Lonza</td>
</tr>
<tr>
<td>NanoDrop® ND-1000 UV-Vis spectrophotometer</td>
<td>Peqlab</td>
</tr>
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</table>

**Table 3: Primers used for RT-qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’-3’</th>
<th>Amplicon length (bp)</th>
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<tbody>
<tr>
<td></td>
<td>Reverse Primer 3’-5’</td>
<td></td>
</tr>
<tr>
<td>ssADAM10</td>
<td>F, AGCAACATCTGGGGACAAAAC</td>
<td>219</td>
</tr>
<tr>
<td></td>
<td>R, CTTCCCTCTGGTTGATTTGC</td>
<td></td>
</tr>
<tr>
<td>ssAPOA-I</td>
<td>F, GAGATTATGTGCCCAGTTTG</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>R, CTGCCCAAGTTGCCCAGAG</td>
<td></td>
</tr>
<tr>
<td>ssAPP</td>
<td>F, CGTGGGAGTTTAGCTGCTTC</td>
<td>122</td>
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<tr>
<td></td>
<td>R, TCAAATGCAATCGTGGAAAA</td>
<td></td>
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<tr>
<td>ssAPOJ</td>
<td>F, CCTTCTCGACATGATCCAC</td>
<td>282</td>
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<tr>
<td></td>
<td>R, AGAGCTTGCTGACTTTCTCC</td>
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<tr>
<td>ssBACE1</td>
<td>F, TGGACTGCCTCATGTTGTG</td>
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</tr>
<tr>
<td></td>
<td>R, GTGACCAAGTGAACCCACCG</td>
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<tr>
<td>ssHPRT1</td>
<td>F, AGGACCTCTCGAAGTGTTGG</td>
<td>247</td>
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<tr>
<td>ssLRP1</td>
<td>F, GCAGATGTATCAACATCAACTGG</td>
<td>98</td>
</tr>
<tr>
<td>ssLRP2</td>
<td>F, CATGTTTGTTGTCAGCCATTC</td>
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<tr>
<td>ssLRP2</td>
<td>R, TCGTCACCTCCATCTCCACA</td>
<td></td>
</tr>
<tr>
<td>ssPGP (MDR-1)</td>
<td>F, GAATTGGCCAGATAACAGCACC</td>
<td>123</td>
</tr>
<tr>
<td>ssRAGE</td>
<td>F, TCAAAAACATCACAGCCCGGA</td>
<td>129</td>
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<tr>
<td>mmLRP1</td>
<td>F, CCGCATCCTTCTCAGTGACA</td>
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<td>mmLRP1</td>
<td>R, ACAGAGCCACATTTTCCAC</td>
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<td>mmRAGE</td>
<td>F, GGGAGGCCTGGAGTAGTAG</td>
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<td>mmRAGE</td>
<td>R, ATTCAGCTCTGACGTTCTT</td>
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<tr>
<td>mmAPP</td>
<td>F, TCCGAGAGGTGTGCTCTGAA</td>
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<td>mmAPP</td>
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<tr>
<td>ssHMGR</td>
<td>F, CTTGTTCACCGGCACAGTCG</td>
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<td>ssHMGR</td>
<td>R, GACAGCCAGAAGGAGAGCCA</td>
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<tr>
<td>ssABCA1</td>
<td>F, GCCATTCTCCGGCGCAAC</td>
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<td>ssABCA1</td>
<td>R, GGCTTCAGCGCGGTGAT</td>
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<tr>
<td>ssSREBP2</td>
<td>F, GCTTCTCCCTACCTCCATC</td>
<td>151</td>
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<tr>
<td>ssSREBP2</td>
<td>R, GAGAGGCACAGGAAGGTA</td>
<td></td>
</tr>
<tr>
<td>mmHPRT1, mmLRP2</td>
<td>QuantiTect Primer Assay (Qiagen)</td>
<td></td>
</tr>
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</table>

**Table 4: Taqman primers used for q RT-PCR**

<table>
<thead>
<tr>
<th>Taqman Primer Assay</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusterin (apoJ), pig specific</td>
<td>Life technologies</td>
</tr>
<tr>
<td>HPRT1, pig specific</td>
<td>Life technologies</td>
</tr>
<tr>
<td>AGER, pig specific</td>
<td>Life technologies</td>
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Table 5: siRNA used for apoJ silencing

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoJ_1</td>
<td>5’- ACA GAU AAA GAC CCU AAU ATT -3’</td>
<td>Microsynth</td>
</tr>
<tr>
<td>ApoJ_2</td>
<td>5’- AUU UCC CGU GUG CCA CU A ATT -3’</td>
<td>Microsynth</td>
</tr>
<tr>
<td>ApoJ_3</td>
<td>5’- UUC CCU AUC ACU UCC CUG ATT -3’</td>
<td>Microsynth</td>
</tr>
</tbody>
</table>

TAE buffer (50x)

- 2 M Tris
- 0.05 M EDTA
- 57.1 ml glacial acid

Fill up to 1 liter with d₂H₂O, filter the solution, adjust pH to 8.5 and store in the dark at 4°C

2.1.3 Chemicals and solutions used for protein isolation, SDS-PAGE, and western blotting

All purchased products and solutions used for protein precipitation, isolation and immunoblotting are listed in Table 6. Self-made buffers and solutions are listed below.

Table 6: Materials used for protein isolation and immunoblotting

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>XT sample buffer (4x)</td>
<td>Biorad</td>
</tr>
<tr>
<td>XT sample reducing agent (20x)</td>
<td>Biorad</td>
</tr>
<tr>
<td>Blotting-Grade blocker, nonfat dry milk</td>
<td>Biorad</td>
</tr>
<tr>
<td>Clarity western ECL substrate</td>
<td>Biorad</td>
</tr>
<tr>
<td>Equipment/Chemical</td>
<td>Supplier</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>IKA® model MS1 minishaker</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Combi-shaker KL 2</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Microcentrifuge, Qualitron DW-41</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Chemicals</td>
<td>Carl Roth or Sigma Aldrich</td>
</tr>
<tr>
<td>Concentrator plus</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>IKA® Model MS1 minishaker</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Combi-shaker KL 2</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Microcentrifuge, Qualitron DW-41</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>ELMA Transsonic 460, ultrasonic unit</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>PVDF membrane, Hybond-P</td>
<td>GE healthcare</td>
</tr>
<tr>
<td>NuPage® Novex 4-12% Bis-Tris midi gel</td>
<td>Life technologies</td>
</tr>
<tr>
<td>NuPage® MES SDS running buffer (20x)</td>
<td>Life technologies</td>
</tr>
<tr>
<td>NuPage® MOPS SDS running buffer (20x)</td>
<td>Life technologies</td>
</tr>
<tr>
<td>Pierce® BCA protein assay kit</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Bovine serum albumine, 2 mg/ml</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>CL- Xposure film (12.5 x 17.5 cm)</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Cell proliferation reagent WST-1</td>
<td>Roche</td>
</tr>
<tr>
<td>Sunrise photometer with Magellan software</td>
<td>Tecan</td>
</tr>
<tr>
<td>Centrifuge Sigma 3K15 with angle rotor 12154-H</td>
<td>Sigma</td>
</tr>
<tr>
<td>Protease Inhibitor cocktail tablets</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Magnetic stirrer with heating, MR 3001, Heidolph</td>
<td>VWR</td>
</tr>
<tr>
<td>Ratek RSM7, Rotary suspension mixer</td>
<td>VWR</td>
</tr>
</tbody>
</table>
Protein lysis buffer

- 50 mM Tris, pH 7.5
- 10 mM EDTA
- 1% Triton-X-100
- 1 tablet of protease inhibitor cocktail

Fill up to 10 ml with d$_2$H$_2$O and store in aliquots at -20°C

Running buffer (10x, pH 8.3)

- 30.3 g Tris
- 144.0 g glycine
- 10.0 g SDS

Transfer buffer (1x, 2.4 l)

- 240 ml running buffer, 10x
- 480 ml methanol

Fill up to 2.4 ml with d$_2$H$_2$O and store at 4°C

TBS-TT (1x, 5 liters)

- 100 ml Tris, 1 M, pH 7.5
- 250 ml NaCl, 5 M
- 10 ml Triton-X-100
- 2.5 ml TWEEN 20

Fill up to 5 l with d$_2$H$_2$O and store at 4°C.

Blocking solution

Dissolve 5% blotting-grade blocker in 1x TBS-TT.

2.1.4 Antibodies

Primary and secondary antibodies used for immunoblotting were dissolved in 5% milk powder in TBST-T and are described in Table 7.
Table 7: Antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Working dilution</th>
<th>Company</th>
<th>Protein detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibody</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit-anti-b-actin</td>
<td>1:5000</td>
<td>Sigma Aldrich</td>
<td>β-Actin</td>
</tr>
<tr>
<td>Rabbit-anti-β-amyloid precursor protein</td>
<td>1:1500</td>
<td>Invitrogen</td>
<td>full-length APP</td>
</tr>
<tr>
<td>Rabbit-anti-APP/CTF</td>
<td>1:4000</td>
<td>Sigma Aldrich</td>
<td>APP/ CTF</td>
</tr>
<tr>
<td>Rabbit-anti-amyloid oligomer</td>
<td>1:10 000</td>
<td>Millipore</td>
<td>Aβ oligomers</td>
</tr>
<tr>
<td>Mouse-anti-beta-amyloid 1-16 (6E10)</td>
<td>1:1000</td>
<td>Biolegend</td>
<td>sAPPα</td>
</tr>
<tr>
<td>Rabbit-anti-clusterin</td>
<td>1:800</td>
<td>Cloud-Clone Corp.</td>
<td>apoJ/ clusterin in pBCEC</td>
</tr>
<tr>
<td>Rabbit-anti-clusterin/apoJ</td>
<td>1:2000</td>
<td>Santa Cruz Biotechnology</td>
<td>apoJ/ clusterin in mouse</td>
</tr>
<tr>
<td>Rabbit-anti-LRP1 (1704)</td>
<td>1:10 000</td>
<td>AG Pietrzik</td>
<td>LRP1-β-chain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Johanes-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gutenberg-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>University, Mainz,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Germany</td>
<td></td>
</tr>
<tr>
<td>Rabbit-anti-LRP1 (B411E2)</td>
<td>1:1000</td>
<td>Prof. Ernst Malle,</td>
<td>LRP1-α-β-chain, soluble LRP1 (sLRP1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medical University</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>of Graz</td>
<td></td>
</tr>
<tr>
<td>Rabbit-anti-apoA-I</td>
<td>1:2500</td>
<td>Prof. Ernst Malle,</td>
<td>secreted apoA-I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medical University</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>of Graz</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary antibody</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-rabbit IgG-HRP</td>
<td>1:10 000</td>
<td>Santa Cruz Biotechnology</td>
<td></td>
</tr>
<tr>
<td>Goat-anti-mouse-HRP</td>
<td>1:5000</td>
<td>Sigma Aldrich</td>
<td></td>
</tr>
</tbody>
</table>
2.1.5 Materials used for immunocytochemistry

All reagents and products used for immunocytochemistry are listed in Table 8.

Table 8: Reagents and products used for immunocytochemistry

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration/ dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid beta (1-40) fragment – Alexa Fluor 488</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lab-Tek chamber slides</td>
<td></td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Hoechst staining</td>
<td>1:1000 dilution in PBS</td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-human Zo-1/TJP1</td>
<td>1.0 µg/ml</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Mouse anti-human VE-cadherin (F-8)</td>
<td>0.4 µg/ml</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>1st antibody LRP1 (B411E2) [1.68 µg/ml]</td>
<td>3.25 µg/ml in TBS</td>
<td>AG Pietrzik</td>
</tr>
<tr>
<td>2nd antibody (Alexa Fluor 488 goat @ mouse)</td>
<td>1:1000 in TBS</td>
<td>AG Pietrzik</td>
</tr>
<tr>
<td>Donkey anti-rabbit Cy-3 (red)</td>
<td>2.0 µg/ml</td>
<td>Jackson Immuno Lab</td>
</tr>
<tr>
<td>Donkey anti-mouse Dylight 488 (green)</td>
<td>1.67 µg/ml</td>
<td>Jackson Immuno Lab</td>
</tr>
<tr>
<td>Vectashield ® Mounting medium with DAPI</td>
<td></td>
<td>Vector Laboratories</td>
</tr>
</tbody>
</table>

2.1.6 Materials used for Aβ transport and uptake studies

All products used for Aβ transport and uptake experiments are listed in Table 9.
Table 9: Materials and Reagents used for Aβ transport studies

<table>
<thead>
<tr>
<th>Material/Reagent</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma 5500B</td>
<td>Beckman</td>
</tr>
<tr>
<td>Ultima Gold scintillation cocktail</td>
<td>New England Nuclear</td>
</tr>
<tr>
<td>Tri-Carb 2100 TR liquid scintillation Analyzer</td>
<td>Packard</td>
</tr>
<tr>
<td>^14^C-sucrose</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>^125^I-Aβ_{1-40}</td>
<td>Phoenix Peptides</td>
</tr>
</tbody>
</table>
2.2 **Methods**

2.2.1 **Isolation of primary porcine brain capillary endothelial cells (pBCEC)**

Method has been literally published in:

“pBCEC were isolated from 3 hemispheres of freshly slaughtered pigs from the local abattoir (39,60). Forceps were used to remove meninges and capillaries and a scalpel and a cutter with rolling plates was used to mince the gray and white matter of the brain cortex. To isolate the capillaries, dispase (70 mg/brain) was mixed with 40 ml preparation medium and incubated at 37°C in the water bath for 1 h with gentle stirring. After the incubation time, 150 ml dextran solution was added and the suspension was centrifuged (6 800xg, 10 min, 4°C). The pellet was resuspended in Medium A and the capillaries were disrupted mechanically by filtering the suspension through a nylon mesh and enzymatically by adding 350 µl collagenase/ dispase. The suspension was carefully pipetted onto a percoll bi-phase gradient (15 ml of 1.07 g/ml percoll solution on bottom, 20 ml of 1.03 g/ml percoll solution on top) and centrifuged (1300xg, 10 min, RT) in a swinging bucket rotor. Endothelial cells were isolated from the interphase and washed once with Medium A. Cells were plated on to collagen coated 75 ccm cell culture flasks in Medium A and incubated at 37°C in humified air containing 5% CO₂. After 26 h incubation time, endothelial cells were washed twice with 1xPBS and cultured in Medium B until confluency. Confluent cells were trypsinized and split on to coated 6-well plates and 12-well plates (60 µg/ml collagen) or transwells (120 µg/ml collagen) for further experiments.”
2.2.2 Isolation of plasma-derived apoJ

Method has been literally published in:

“We received exogenous apoJ from Prof. Mark R Wilson from the University of Wollongong, Australia. ApoJ was isolated from human plasma as described by Dabbs and Wilson (61). In brief, plasma was prepared by centrifuging human blood containing 10 mM sodium citrate. After addition of Complete® protease inhibitor, debris was removed with a GFC glass fibre filter (MicroAnalytic Products Inc., Mountain Lakes, NJ, USA) and a 0.45 µm cellulose nitrate filter. ApoJ was isolated from the plasma using a G7 anti-CLU monoclonal antibody column (62) connected to an Econo pump system (Biorad). ApoJ bound to the column was eluted using 2 M GdHCl in PBS and dialysed against 20 mM MES, pH 6.0. A 1 ml HiTrap™ SPXL cation exchange column (GE healthchcare) was used to collect apoJ from the dialysed fraction.”

2.2.3 Lipid extraction and GC-MS analysis

Method has been literally published in:

“pBCEC were cultured in 75cm² flasks until confluent and incubated for 24 h in the absence or presence of 5 µM simvastatin, 2 and 20 µg/ml exogenous apoJ in SF conditions. For examining cellular cholesterol synthesis/deuterium enrichment
pBCEC were additionally incubated for 24h in the presence of 10% deuterium in serum-free medium. Cellular lipids were extracted according to Folch’s method using Folch’s solution (chloroform:methanol= 2:1 (v/v)) for 30 min at room temperature. Total cholesterol levels, cholesterol precursors and deuterium enrichment was determined using combined gas chromatography-mass spectrometry (GC-MS) analysis as described previously (63). In brief, after lipid extraction certain amounts of the extract were hydrolyzed in 1 M NaOH in ethanol and incubated in water bath for 1 h at 65°C. Cyclohexane was used to extract the organic phase which then was evaporated and silylated by treating with pyridine/ hexamethyldisilazane/ chlorotrimethylesilane (3:2:1, v/v/v) at 60°C for 30 min. After evaporation of the solvent hexane was added and then transferred into special glass vials. Cholesterol levels and cholesterol precursors were measured using GC-MS and deuterium-labeled internal standards (d6-cholesterol, d4-lathosterol and d6-sitosterol). GC-MS analysis was performed as described previously on a Hewlett Packard 6890 Series Plus gas chromatograph using a gas chromatograph column (64). Measurement of deuterium in cellular cholesterol was done as described by Diraison et al. (65).”

### 2.2.4 Transwell studies

Method has been literally published in:

“In order to determine polarized secretion of proteins either to the basolateral or apical compartment of the BBB, transwell studies were performed. Upon confluence, pBCEC were washed with 1x PBS and incubated in DMEM/ Ham’s F12 medium containing 1% P/S, 0.25% glutamine and 500 nM hydrocortisone (Sigma Aldrich), added for inducing tight junctions. Additionally, cholesterol [100 µM] and the HMGCo-A reductase inhibitor simvastatin [5 µM] were added to determine the effect of a changed cellular cholesterol metabolism. Increasing transendothelial electrical
resistance (TEERs) of pBCEC and hence, formation of tight junctions—significant for an intact BBB—was measured by using an Endohm tissue resistance chamber and the Evohm ohmmeter (World Precision Instruments).“

2.2.5 Using small interfering RNA to silence apoJ in pBCEC

Upon reaching 50-60% confluency in 12-well plates, pBCEC were ready for silencing. Therefore, 250 µl of PrimeFect diluent were mixed with 5 µl of PrimeFect reagent and incubated for 15 min at RT. Then, 25 nM and 50 nM of the already prepared mix of 3 different apoJ siRNAs were added to the suspension and incubated for 15 min at RT. In between, cells were washed once with 1xPBS and 350 µl medium A was added to each well. After the incubation time, 250 µl of the “transfection suspension” was added slowly and drop wise to each well and incubated for 28-30 h at 37°C. In parallel, silenced pBCEC were incubated with 5 µM simvastatin for 24 h, and/or 50 ng/µl Aβ_{1-40} for additional 1 h in serum-free conditions. Mock (using no siRNA) and scramble (using non-coding siRNA with minimal unintended off-target effects) were used as negative controls. After the incubation period RT-qPCR and western blot analysis was performed using scramble as non-targeting control for normalization (1).

2.2.6 Isolation of RNA and RT-qPCR

Method has been literally published in:

“pBCEC were split onto 12-well plates and treated with 24OH-cholesterol [10 µM], 27OH-cholesterol [10 µM], TO 901317 [2 µM], cholesterol [100 µM], simvastatin [5 µM], exogenous apoJ [2 µg/ml or 20 µg/ml] or different concentrations of soluble Aβ_{1-40} [0.25 µg/ml, 0.5 µg/ml or 2 µg/ml]. Additionally, pBCECs were treated with a combination of plasma-derived apoJ [2 µg/ml] and Aβ_{1-40} [0.5 µg/ml] after 30 min
preincubation at 37°C. Cells from 3 wells were pooled and resuspended in 1000 µl TriReagent RT. To reach a phase separation 50 µl BAN were added, incubated for 5 min at RT and centrifuged (12 000xg, 15 min, 4°C). The aqueous phase of the suspension was isolated and mixed with isopropanol 1:1 (v/v). After an incubation period of 10 min at RT and one centrifugation step (12 000xg, 8 min, 4°C), the pellet was washed once with 75% ethanol. The RNA pellet was air dried and resuspended in 40 µl RNase free water. To dissolve the RNA, the samples were incubated for 10 min in the thermocycler (55°C, 300 rpm) before checking the concentration and the purity in the Nanodrop.”

For cDNA synthesis the “High Capacity Reverse Transcriptase Kit” by Life technologies was used. On average 500 ng RNA were reversed to receive a final concentration of 5 ng/µl of cDNA. Relative gene expression was analysed by using either Sybr Green Master Mix (Biorad) or Taqman Gene Expression Master Mix (Life technologies). Primers used throughout the study are listed in Table 3 and Table 4. The relative gene expression was either quantified by using the standard-curve method or the \( \Delta \Delta Ct \) method described by Livak and Schmittgen (66,67).

2.2.7 Isolation of intracellular and secreted proteins

Method has been literally published in:

“pBCEC were split into 6-well plates and incubated with serum-free medium containing, vehicle (ethanol), 24OH-cholesterol [10 µM], 27OH-cholesterol [10 µM], TO 901317 [2 µM], cholesterol [100 µM], simvastatin [5 µM], exogenous apoJ [2 µg/ml or 20 µg/ml] or different concentrations of soluble Aβ_{(1-40)} [0.25 µg/ml, 0.5 µg/ml or 2 µg/ml]. Additionally, pBCECs were treated with a combination of plasma-derived
apoJ [2µg/ml] and Aβ(1-40) [0.5 µg/ml] after 30 min preincubation at 37°C. Intracellular and secreted proteins were isolated after an incubation period of 1 h and 24 h.

To isolate secreted proteins, the medium was collected and centrifuged at 10 000 rpm for 10 min at 4°C. Proteins were precipitated by adding 3% (v/v) trichloroacetic acid. After an incubation time of 1 h the suspension was centrifuged (10 000xg, 10 min, 4°C), the pellet was washed twice with ice cold acetone and dissolved in 1x sample buffer (Biorad) and 1x reducing agent.”

After removing the medium from the 6-well plates, cells were washed twice with cold 1x PBS and lysed in protein lysis buffer [40 µl/well]. Cell lysates were transferred to a fresh 1.5 ml tube, vortexed and sonicated in a water bath sonicator for 2x 3 min. To remove residual DNA the suspension was centrifuged (12 000 rpm, 10 min, 4°C) and the supernatant was transferred to a fresh tube.

After determining the protein concentration by using a BCA assay (Thermo Scientific), 15 µg of the proteins were mixed with 1x loading buffer, 1x reducing agent and filled up with ddH₂O up to 20 µl.

### 2.2.8 SDS-PAGE and western blotting

Method has been literally published in:

“Before loading protein samples onto a NuPage® Novex 4-12% Bis- Tris Midi Gel (Thermo Scientific) proteins were denatured at 95°C for 5 min in the thermocycler. Proteins were separated by SDS-PAGE using the method described by Laemmli (68). Western blot was performed according to Haid and Suissa (16) using a 0.45 µm PVDF membrane (GE healthcare) as blotting membrane. After blotting the membrane was blocked with 5% non-fat dry milk (Biorad) in TBS-TT.” The membrane was probed overnight at 4°C with primary antibodies in 5% milk powder in TBS-TT. Antibodies used throughout the studies are listed in Table 7.
After incubation of the blot with the adequate secondary antibody the membrane was detected in the dark room using Clarity Western ECL Substrate (Biorad) for detecting proteins of interest. Films were scanned and bands densitometrically analyzed using Image J software (version 1.47v).

2.2.9 Bace activity assay


“The activity of the β- secretase BACE1 was examined using the “Beta Secretase Activity Assay Kit (fluorometric)” from Abcam. In brief, pBCEC were treated with vehicle control (ethanol) or the HMGCo-A reductase inhibitor simvastatin [5µM] followed by treatment with lysis buffer and subsequent determination of the activity using 100 µg of protein. Activity was measured fluorescently with a fluorimeter (FlexStation II, Molecular Devices; Ex= 345 nm/Em: 500 nm).“

2.2.10 Detection of Aβ40 peptides by ELISA

pBCEC cultures were grown in 75 cm² cell culture flasks and treated for 24 h with vehicle control, 5 µM simvastatin, or 20 µg/ml apoJ in serum-free medium. Aβ40 ELISA (Invitrogen, Cat no: KHB3481) was performed with aliquots of intracellular protein (30 µg) and of the supernatant according to manufacturer’s protocol.

2.2.11 Immunhistochemistry

pBCEC were split into collagen coated [120 µg/ml] chamber slides and after reaching 70% confluence cells were treated with Alexa Fluor 488- Aβ(1-40) [0.5 µg/ml] in serum-free medium for 45 min. For nuclei staining 20 µl Hoechst staining (1:1000 dilution in
PBS) was added to the medium and incubated for 15 min. Cells were washed twice with 1x PBS and fixated in acetone for 5 min.

2.2.12 Single/ double labelled immunofluorescence staining of pBCEC

Method has been literally published in:

“Cerebrovascular endothelial cells were cultured on Lab-Tek chamber slides (Thermo Fisher Scientific, NY, USA) and were fixated with 4% paraformaldehyde (PFA) for 10 min. After fixation, cells were washed in TBST and blocked with donkey serum for 30 min before primary antibodies incubation for 1 h (rabbit anti-human Zo-1/ TJP1 1.0µg/ml (Thermo Fisher Scientific, IL, USA), mouse anti-human VE-cadherin (F-8) 0.4µg/ml) (Santa Cruz Biotechnology, Inc., TX, USA) and anti-rabbit Lrp1 (1704)-obtained from Prof. Claus Pietrzik. All incubation steps were performed in a dark moist chamber at room temperature. After 5 min of TBST wash, secondary antibodies were applied: donkey anti-rabbit Cy-3 (red) 2.0µg/ml (Jackson Immuno Lab, Inc., West Grove, PA, USA) and donkey anti-mouse Dylight 488 (green) 1.67µg/ml (Jackson Immuno Lab, Inc., West Grove, PA, USA) for 30 min. After rinsing in TBST, DAPI was added to the slides for 20 min as a nuclei counter stain. Sections were rinsed again with TBST before mounting with Vectashield mounting medium (Vector Lab, Inc., Burlingame, CA, USA). Normal rabbit immunoglobulin fraction (Millipore Corp., Temecular, Ca, USA) and mouse immunoglobulin fraction (CedarlaneLab, Burlington, NC, USA) were used as negative controls To acquire and analyze computerized images of sections and cells, a Leica DM4000 B microscope (Leica Cambridge Ltd) equipped with Leica DFC 320 Video camera (Leica Cambridge Ltd) was used.”
2.2.13 Aβ transport studies

pBCEC were split onto collagen coated [120 µg/ml] transwells, incubated in DMEM/ Ham’s F12 medium containing 1% P/S, 0.25% glutamine and 500 nM hydrocortisone (Sigma Aldrich), and increasing transendothelial electrical resistance (TEERs) was measured by using an Endohm tissue resistance chamber and the Evohm ohmmeter (World Precision Instruments). To test the influence of a depleted cellular cholesterol metabolism on Aβ\textsubscript{(1-40)} transport, pBCEC were treated with 5 µM simvastatin prior to transcytosis experiment. In an additional experiment, 2 µg/ml apoJ were added to the basolateral compartment to examine the role of apoJ in Aβ transport. Aβ transport assay was performed by adding 0.3 nM \textsuperscript{125}I-Aβ\textsubscript{(1-40)} and 100 nM \textsuperscript{14}C-Sucrose as non-diffusion control to the basolateral compartment. Transport of \textsuperscript{125}I-Aβ\textsubscript{(1-40)} was studied for 2 h from the brain to the blood. From each input and apical compartment (acceptor) 400 µl and 50 µl were taken to examine transport of \textsuperscript{125}I- Aβ\textsubscript{(1-40)} and \textsuperscript{14}C- sucrose, respectively. To investigate transport of intact \textsuperscript{125}I- Aβ\textsubscript{(1-40)} 400µl probes were subjected to TCA precipitation and pellet was measured on a γ-counter. 50 µl of input and acceptor were counted on a β-counter with 5ml Ultima Gold scintillation cocktail to examine passive diffusion of \textsuperscript{14}C-sucrose across the BBB. Transcytosis quotient (TQ) was calculated as followed:

\[
Aβ \text{ TQ} = \frac{[\text{125I}] - Aβ \text{ acceptor}}{[\text{125I}] - Aβ \text{ input}} \times \frac{[\text{14C}] - \text{sucrose acceptor}}{[\text{14C}] - \text{sucrose input}}
\]

2.2.14 Aβ uptake studies

Method has been literally published in:
“pBCEC were grown in collagen-coated 24-well plates until confluent and incubated with vehicle control (ethanol) or simvastatin [5 µM] for 24 h. Aβ uptake assay was performed by adding 0.3 nM of $[^{125}\text{I}]-\text{A}\beta_{(1-40)}$ and incubating at 37°C for 22 h. Alternatively, for examining uptake of Aβ$_{(1-40)}$ in combination to treatment with purified apoJ [2 µg/ml or 20 µg/ml], Alexa Fluor 488 (AF488) labelled Aβ$_{(1-40)}$ was used and pBCEC were incubated at 37°C for 3 h and 24 h. To allow complex formation apoJ was incubated with AF488-Aβ$_{(1-40)}$ at 37°C for 30 min prior to cell treatment. Cells were washed twice with ice-cold PBS and medium and PBS were collected and subjected to TCA precipitation (30%TCA). Cells were lysed by adding 0.3 N NaOH and subsequently subjected to protein precipitation with 100% TCA. Total cellular protein concentration was quantified using the Qubit fluorometer (Quant-IT protein assay kit, Invitrogen). Uptake of $^{125}\text{I}$-Aβ$_{(1-40)}$ was calculated as the percentage of cpm/mg cell protein in the pellet of cell lysates relative to the pellet of supernatants. Uptake of AF488-Aβ$_{(1-40)}$ was determined using a fluorescent plate reader.”

### 2.2.15 Cytotoxicity Assay

Method has been literally published in:


“Cytotoxicity assay was performed using cell proliferation reagent WST1 by Roche. In brief, pBCEC were seeded onto collagen coated [60 µg/ml] 96 well plates and upon reaching confluency cells were treated for 24h with increasing concentrations of Aβ$_{(1-40)}$ [0.25 µg/ml, 0.5 µg/ml and 2 µg/ml]. Absorbance of the formazan product was measured at 450 nm and 650 nm was used as reference wavelength.”
2.2.16 Animal studies and isolation of mBCEC

Parts of the method have been literally published in:

Animal studies were performed with ethical approval of the Austrian Department of Science, Research and Economy (approval number 66010/0052-WF/V/3b/2015). Female 3xTg AD, apoJ ko mice and non-transgenic (Non-Tg) C57/Bl6 mice were maintained on a 12-h light/12-h dark cycle in a temperature-controlled environment with free access to chow diet (Ssniff, Germany). Aged mice (Non-Tg: 58±11 weeks, 3xTg AD: 64±4 weeks) were gavaged for 21 days with vehicle only or 40 mg/kg simvastatin in 0.2% agarose in PBS. “Mice were sacrificed and murine brain capillary endothelial cells (mBCEC) were isolated from a pool of 2 hemispheres (Non-Tg +/- sim: n=2, 4; 3xTg +/- sim: n=8). In brief, hemispheres were washed in PBS (containing 2% penicillin/streptomycin) and the olfactory bulb was removed. Scalpel and douncer were used to mince the grey and the white matter of the cortex. To isolate the capillaries, the homogenate was mixed with dispase (0.01 g /2 hemispheres) in 5 ml MCDB131 medium (containing 2% FBS, 1% L-glutamine and 10% penicillin/streptomycin) and incubated at 37°C in the water bath for 1 h. Disruption of the capillaries followed by endothelial cell isolation using Percoll biphasic gradient was performed as described above for pBCEC (32).”

2.2.17 Statistics

Results are reported as means ± SD unless stated otherwise. All experiments were performed at least three or more times. Statistical significances (*p< 0.05: **p<0.01; ***p< 0.001) were determined by analysis for variance (ANOVA) or two-tailed Student’s t-test performed with Prism software (Graphpad version 6, CA, USA).
3. Results

3.1 Simvastatin and apoJ modulate cholesterol metabolism in pBCEC

Data presented in this chapter have been published in:


Statins are effective drugs in lowering plasma cholesterol levels in coronary heart disease patients (69). Since it was shown in previous studies that plasma cholesterol levels are linked to AD pathology (42), we were interested to examine the effect of statin treatment on Aβ metabolism. In addition, it was reported that the BBB has a thus far underestimated importance during the progression of AD, as it is responsible for clearing Aβ peptides from the brain (16,17). To study the effects of a modified cellular cholesterol metabolism on amyloidosis at the BBB, our research group uses a well-established primary cell culture model, namely primary porcine brain capillary endothelial cells (pBCEC).

In initial experiments we were interested to investigate the influence of simvastatin treatment on pBCEC cholesterol metabolism and levels, using GC-MS analysis. Additionally, we treated pBCEC with plasma-derived apoJ. GC-MS analysis was performed from cellular lipid extracts of pBCEC, which were treated for 24 h with vehicle control (0.5% ethanol), 5 µM simvastatin, 2 and 20 µg/ml exogenous apoJ in serum-free conditions. We found that after an incubation period of 24 h overall cholesterol content remained unchanged in response to all conditions (Figure 6A). In another GC-MS experiment we incubated pBCEC in presence and absence of simvastatin [5 µM] or apoJ [2 and 20 µg/ml] in SF medium containing 10% D2O.
Newly synthesized cholesterol was determined by measuring incorporation of deuterium in the cholesterol molecule. Here, we found that simvastatin treatment led to a reduction of deuterium enrichment by 73.5±6.5% (Figure 6B). Treatment with 2 and 20 µg/ml apoJ, on the other hand, did not reveal significant differences in deuterium enrichment. In addition to the above experiments, we measured levels of cholesterol precursors (Figure 6C). Here we detected a significant reduction in levels of desmosterol (72.4%), lathosterol (72.0%), lanosterol (81.2%), FF-MAS (85.1%), and T-MAS (69.6%) in response to simvastatin treatment. Treatment with 2 µg/ml apoJ, on the other hand, did not significantly alter levels of cholesterol precursors.

This indicates that simvastatin inhibits HMGCoA reductase thereby causing a strong decline in total cellular cholesterol synthesis and cholesterol precursors, albeit total cellular cholesterol levels remain unchanged after 24 h of treatment. ApoJ treatment, on the other hand, does not directly influence cellular cholesterol metabolism.
Figure 6: Influence of simvastatin and apoJ on cholesterol metabolism. Porcine BCEC were treated for 24 h with simvastatin [5 µM], 2, or 20 µg/ml apoJ. Cellular lipids were extracted using Folch’s solvent and subjected to GC-MS analysis as described in Material and Methods. Data represent mean±SD from 4 independent experiments. *p<0.05; **p<0.01.

3.2 Simvastatin influences APP processing pathways

Data presented in this chapter have been published in:


To study the role of an altered cellular cholesterol metabolism on mRNA and protein levels of APP and APP processing enzymes, pBCEC were treated for 24 h with vehicle (0.5% ethanol), the HMGCO-A reductase inhibitor simvastatin [5 µM], or cholesterol [100 µM]. We observed a significant increase in relative APP mRNA levels (78±24.7%) in response to treatment with simvastatin (Figure 7). Simvastatin treatment also revealed a significantly higher transcription of α-secretase (ADAM10)
(84±13.4%), the enzyme responsible for cleavage of the transmembrane protein APP to yield the beneficial cleavage product sAPPα. Furthermore, we observed a significant reduction in BACE1 (β-secretase) mRNA levels by 53±28.7%. Cholesterol treatment did not affect mRNA levels of APP, ADAM10, or BACE1.

In order to investigate APP and APP processing in response to simvastatin and cholesterol treatment at the protein level, we performed western blot analysis. We detected higher protein amounts of full-length APP (2.1±0.60-fold) and C-terminal fragments (CTF) (3.4-fold)-cleavage products of APP- in response to simvastatin (Figure 8). The combined treatment of cholesterol and simvastatin diminished effects seen with simvastatin alone from 2.1±0.60-fold to 1.5±0.34-fold in cellular APP protein levels, whereas CTF levels changed back to control levels.

In addition, pBCEC were treated for 24 h with simvastatin [5 µM], cholesterol [100 µM], or a combination of these compounds, to examine secretion levels of sAPPα, the non-amyloidogenic APP processing product. Here we found increased protein...
levels of sAPPα by 41±0.25%, 17±0.12% and 33±0.19% in response to treatment with simvastatin, cholesterol and a combined treatment with cholesterol and simvastatin, respectively (Figure 8C).
Figure 8: Simvastatin increases APP, CTF and sAPPα protein levels in pBCEC. Cells were treated for 24h in presence of vehicle control (0.5% ethanol), simvastatin [5µM], cholesterol [100 µM] and a combination with simvastatin [5 µM]/cholesterol [100 µM]. (A,B) Immunoblot analysis of intracellular protein was performed using β-actin as loading control and rabbit-anti-APP/CTF to detect APP and CTF. Data represent mean±SD from 4 independent experiments performed in triplicates. (C) Media were collected, TCA-precipitated and subjected to western blot analysis was performed. Samples were normalized to total cellular protein and anti-beta-amyloid 1-16 (6E10) antibody was used for detecting sAPPα. Data represent mean±SD form 3 independent experiments performed in triplicates. *p<0.05; **p<0.01; # indicates significance (p<0.05) of simvastatin [5 µM] as compared to combined treatment with simvastatin [5 µM] and cholesterol [100 µM]. [Reproduced from Zandi-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

In order to also analyse the amyloidogenic pathway of APP processing in response to simvastatin treatment of pBCEC, we performed a BACE activity assay provided by Abcam. Smvastatin reduced total Bace activity by 14±3.3% in pBCEC (Figure 9A). Functionality of Bace activity assay was assessed by using a pBCEC control sample, a positive (active β secretase) and a negative (active β secretase plus β secretase inhibitor) control provided by Abcam.
Taken this results section together, we conclude that simvastatin clearly shifts APP processing towards the non-amyloidogenic pathway and the observed effects are due to depletion of cellular cholesterol levels, rather than through non-cholesterol dependent “pleiotropic” effects of simvastatin.

![Figure 9: Simvastatin reduces BACE1 activity.](image)

Figure 9: Simvastatin reduces BACE1 activity. (A) pBCECs were treated with simvastatin [5µM] or vehicle control (ethanol) for 24 h and cell lysates were subjected to protein determination. Bace activity assay was performed with 100 µg protein. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)] (B) Functionality of Bace1 activity assay was assessed with cell lysates (100 µg protein) of control sample from pBCECS, active β secretase and β secretase inhibitor (provided by Abcam). Data represent mean±SD from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01; ***p<0.001.

Additionally, we detected alterations in intracellular and secreted Aβ oligomer levels in response to treatment with simvastatin: relative protein amounts of secreted Aβ oligomers were reduced (by 50±11.8%), whereas secreted Aβ peptides were increased by 51±11.4 % (Figure 10 A,B). In an uptake assay using radiolabelled Aβ1-40 we found a moderate decrease in uptake of Aβ1-40 to pBCEC by 17±8.5%, whereas
transport studies revealed no significant changes in response to simvastatin treatment (Figure 10C, Figure 35A)
Figure 10: Simvastatin reduces cell associated Aβ oligomers and increases secreted Aβ peptides. pBCECs were incubated in the presence and absence of simvastatin [5 µM] for 24 h. (A) Protein concentration of cell lysates was measured by BCA assay and subsequent subjected to western blot analysis. (B) Supernatants were undertaken TCA precipitation and western blot analysis was performed using A11 rabbit-anti-amyloid oligomer antibody for detecting Aβ oligomers. Data represent mean±SEM from 4 independent experiments performed in triplicates. (C) pBCEC were cultured in the presence or absence of vehicle control or simvastatin [5µM]. Uptake assay was performed by incubating cells with $[^{125}]$I-Aβ(1-40) [0.3 nM] for 22 h. Data shown represent cpm/mg protein and mean±SD from 3 independent experiments performed in triplicates. *p<0.05. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

Additionally, we performed an ELISA to analyse total levels of Aβ1-40 within the cells and released to the culture media, and to examine effects of simvastatin and apoJ treatments (Figure 11). We found that a higher amount of Aβ1-40 was cell-associated (4.3±0.45 pg/µg cell protein) than present in the supernatants (10.6±6.57 pg/ 100 µg cell protein). Although ELISA assay did not reveal significant changes, we observed a shift towards less cell-associated Aβ1-40, and increased Aβ1-40 in the cell culture media in response to simvastatin treatment.
Figure 11: Influence of simvastatin and apoJ on cellular and secreted Aβ\textsubscript{1-40} levels. pBCEC were incubated with vehicle control, simvastatin [5 µM], or apoJ [20 µg/ml]. Aβ40 ELISA was performed from intracellular (A) and secreted (B) proteins as described in the manufacturer’s protocol. Data shown represent mean±SD from 4 independent experiments.

3.3 ApoJ mRNA and protein expression levels are increased upon simvastatin treatment

Data presented in this chapter have been published in:


In order to investigate the role of modulators of human cholesterol metabolism on mRNA and protein levels of apoJ, pBCEC were treated for 24 h with vehicle (0.5% ethanol), the natural LXR ligands 24O(S)H-cholesterol [10µM] or 27OH-cholesterol [10µM], the HMGCO-A reductase inhibitor simvastatin [5 µM], cholesterol [100 µM],
or the synthetic LXR ligand TO901317 [2 µM]. We observed a 2.1-fold increase in apoJ mRNA levels and a 2.3-fold increase in intracellular protein levels in response to treatment with simvastatin (Figure 12). No significant change in either mRNA expression or intracellular protein levels in response to treatment with cholesterol or LXR agonists were detected.

Figure 12: Simvastatin increases apoJ mRNA expression and protein level in pBCEC. Cells were treated for 24h in the absence (control) or presence of 10 µM 24OH-cholesterol (24OH-C), 10 µM 27OH-cholesterol (27OH-C), 5 µM simvastatin, 100 µM cholesterol or 2 µM TO. (A) mRNA expression analysis was performed as described in Materials and Methods. Relative gene expression was calculated with standard curve method using HPRT1 as house-keeping gene. Data represent mean±SEM from 4 independent experiments in triplicates. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)] (B) Western blot analysis was performed with cell lysates using β-actin for normalization and rabbit-anti-clusterin antibody for detecting apoJ. Data represent mean±SEM from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01; ***p<0.001.

In order to examine whether the increase in apoJ mRNA levels was due to a reduced cellular cholesterol synthesis or other “pleiotropic” effects of simvastatin, pBCEC were treated for 24 h with vehicle control, simvastatin, cholesterol, and a combination
of simvastatin and cholesterol (Figure 13). Here, we detected a 2.3±0.77-fold increase in cellular apoJ protein levels in response with simvastatin, but combined treatment with cholesterol and simvastatin did not change apoJ protein levels. Therefore, we conclude that effects observed with simvastatin treatment are caused by depletion of cholesterol, rather than through cholesterol-independent pleiotropic effects.

Figure 13: Simvastatin, but not cholesterol induces apoJ protein expression in pBCEC. pBCEC were incubated 24h in the absence (vehicle control) or presence of simvastatin [5µM], cholesterol [100 µM], or a combination of cholesterol and simvastatin. Western blot analysis of intracellular apoJ was performed using rabbit-anti-clusterin antibody for detecting apoJ. Data represent mean±SD from 4 independent experiments performed in triplicates. *p<0.05; **p<0.01. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
3.4 **Simvastatin influences secretion of apoJ at the BBB**

Data presented in this chapter have been published in:


To examine the secretion levels towards the apical (mimicking the blood-side of the BBB) and basolateral (mimicking the brain parenchymal side of the BBB) side of polarized pBCEC, cells were seeded onto transwell filters and treated with simvastatin [5 µM] or cholesterol [100µM]. After an incubation period of 24 h, secreted proteins were TCA-precipitated from the medium and subjected to immunoblotting. Secretion of apoJ to both the basolateral and apical compartments was observed, albeit with a ~3.2 fold higher amount of apoJ detected at the basolateral side (Figure 14A). Furthermore, we found a 1.8 fold higher secretion of apoJ towards the basolateral compartment in response to simvastatin treatment as compared to vehicle control (Figure 14B). Cholesterol treatment revealed no significant effects.
Figure 14: apoJ is primarily secreted towards the basolateral compartment of the BBB and its secretion is influenced by simvastatin. (A) pBCEC were incubated on multiwell transwell filters to detect either apical (“blood compartment”) or basolateral (“brain compartment”) secretion of apoJ. (B) pBCEC were treated for 24 h with simvastatin [5µM] or cholesterol [100 µM] in serum-free medium and western blot analysis was performed from proteins precipitated from basolateral media. (A,B) Apical (1.5 ml) and basolateral (2.6 ml) media were collected, TCA-precipitated, and aliquots corresponding to 1 ml of supernatant and normalized to total cell cellular protein contents were subjected to immunoblot analysis using SDS-PAGE (4-12%) and PVDF membranes. The antibody rabbit-anti-clusterin was used to detect secreted apoJ protein levels. All data represent mean±SD from 4 independent experiments performed in triplicates. *p<0.05; **p<0.01. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

3.5 **Simvastatin treatment does not influence apoA-I expression**

In order to examine whether simvastatin also influences the expression and secretion of the major apolipoprotein detected in HDL particles, namely apoA-I, western blot analysis was performed (Figure 15). We did not detect significant changes and,
therefore, conclude that a reduction in cellular cholesterol levels due to simvastatin treatment solely changes expression of apoJ, but not of apoA-I.

Figure 15: Simvastatin does not alter secreted apoA-I protein levels in pBCEC. pBCEC were treated for 24 h in the presence and absence of simvastatin. For detecting secreted apoA-I (28 kDa) supernatants were collected, TCA-precipitated and subjected to western blot analysis. Samples were normalized to total cellular protein. Data represent mean±SD from 3 independent experiments performed in duplicates.

3.6 Small interfering RNA reduces apoJ mRNA and protein expression

Data presented in this chapter have been published in:

processing and amyloid-β clearance in blood-brain barrier endothelial cells. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60 (1).

To investigate the influence of reduced levels of apoJ on genes involved in APP processing and cholesterol metabolism, apoJ was silenced using small interfering RNA. We observed a ~50% reduction in apoJ mRNA levels using 25 nM or 50 nM of siRNA (Figure 16A). Furthermore, western blot analysis revealed a clear reduction of apoJ cellular protein levels using 25 nM siRNA (30%) and an even more pronounced reduction of protein levels when using 50 nM siRNA (60%) (Figure 16B). As the effect of apoJ silencing was more prominent with 50 nM siRNA further studies were conducted with this concentration.

![Figure 16: small interfering RNA used for silencing of apoJ.](image)

(A) Silenced cells were harvested for gene expression analysis and RT-qPCR was performed as described in Materials and Methods using Taqman probes and ΔΔCt method. Relative apoJ mRNA expression was normalized to scrambled RNA as non-targeting control. Data represent mean±SD of 3 independent experiments.

(B) Western blot analysis of apoJ intracellular protein was performed as described in Materials and Methods. *p<0.05; **p<0.01. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
To examine the effects of apoJ silencing on genes involved in the amyloid pathway, mRNA expression of APP, α-secretase ADAM10, and β-secretase BACE1 was determined (Figure 17). As we observed significant changes in the expression profile of apoJ, APP and ADAM 10 in response to simvastatin treatment, we also analysed HMG-CoA reductase (HMGR) expression level. However, no alterations were observed in mRNA expression levels of APP, ADAM10, BACE1, nor HMGR in response to silencing of apoJ. Western blot analysis, on the other hand, revealed a ~59% reduction of intracellular APP protein levels in response to apoJ silencing (Figure 18). Moreover, western blot analysis of silenced apoJ showed a reduction in intracellular Aβ oligomer levels by ~56% (Figure 19), whereas extracellular Aβ oligomer levels were unaltered.

Figure 17: apoJ silencing does not influence mRNA levels of apoJ, HMGCo-A reductase, APP, and APP processing enzymes. Relative gene expression analysis of APP, ADAM10, BACE1 and HMG-CoA reductase (HMGR) was performed using ΔΔCt method and non-targeting control (NTC) for normalization. HPRT1 was used as house-keeping gene. Data represent mean ± SD from 3 independent experiments performed in triplicates.
Figure 18: apoJ silencing decreases full-length APP levels. Porcine BCEC were treated with a mix of 50 nM siRNA to knock down gene expression of apoJ. Western blot analysis of full-length APP was performed using rabbit-anti-amyloid precursor protein antibody and β-actin for normalization. Data represent mean±SD from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
Figure 19: Influence of apoJ silencing on Aβ oligomer levels. ApoJ was genetically inactivated in pBCEC using 50 nM siRNA. (A) Western blot analysis for intracellular Aβ octamer levels (~32 kDa) was performed using β-actin for normalization and using A11 rabbit-anti-amyloid oligomer antibody for detecting Aβ oligomers. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)] (B) Supernatants were undertaken TCA precipitation and using A11 rabbit-anti-amyloid oligomer antibody for detecting Aβ oligomers. Samples were normalized to total cellular protein. Data represent mean±SD from 4 independent experiments performed in triplicates. *p<0.05.

3.7 ApoJ treatment increases APP protein levels in pBCEC

Data presented in this chapter have been published in:


The influence of exogenous, purified plasma apoJ on APP processing and genes involved in cholesterol metabolism was examined by incubating pBCEC with 2 µg/ml of apoJ, 20 µg/ml apoJ (matching approximate concentrations detected in the CSF
and plasma, respectively (70)), and a combination of apoJ [20 µg/ml] and simvastatin [5 µM]. Treatment with purified apoJ alone did not affect apoJ, APP, ADAM10 or BACE1 mRNA expression (Figure 20). Further, we detected a 1.7-fold increase in ADAM10 mRNA in response to treatment with apoJ/simvastatin. No detectable changes were observed in genes involved in cholesterol biosynthesis like HMGR, SREBP2, and ABCA1 in response to treatment with apoJ. Nevertheless, we observed a significant decrease (58.2%) in ABCA1 mRNA expression in response to apoJ/simvastatin. Additionally, an increase in gene expression of HMG-CoA reductase and SREBP2 in response to combined treatment with apoJ [20 µg/ml] and simvastatin [5 µM] was detected. These effects were obviously elicited by simvastatin but not by apoJ.

Figure 20: Effects of exogenous apoJ on mRNA levels of genes involved in amyloid pathway and cholesterol pathway. pBCEC were treated with simvastatin [5µM], apoJ [2 µg/ml], apoJ [20 µg/ml apoJ] or apoJ [20 µg/ml]/ simvastatin [5 µM]. After 24h of incubation, cells were harvested and RNA isolation and RT-qPCR was performed using ΔΔCt method and HPRT1 as house-keeping gene. Data represent mean±SD from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
Western blotting of intracellular proteins revealed a 3.3-fold and 2.4-fold higher protein amount of full-length APP in response to 2 or 20 µg/ml apoJ treatment, respectively (Figure 21). A combination of 20 µg/ml apoJ and 5 µM simvastatin increased protein of full-length APP up to 2.6-fold. Protein levels of cellular and secreted Aβ peptides were not changed by apoJ or simvastatin treatments (Figure 22). Taken these results together, we strongly suggest an importance of apoJ in APP production and Aβ transport in cerebrovascular endothelial cells. As modulation of apoJ levels in pBCEC did not reveal significant changes of gene transcription, we conclude that the action of apoJ is mainly based at the posttranslational level.

![Figure 21: Treatment with plasma-derived apoJ increases protein level of full-length APP in pBCEC.](image)

**Figure 21: Treatment with plasma-derived apoJ increases protein level of full-length APP in pBCEC.** pBCEC were treated with apoJ [2 µg/ml], apoJ [20 µg/ml], or apoJ [20 µg/ml]/simvastatin [5 µM] for 24 h. Cell lysates of 15 µg protein were subjected to western blot analysis. Bands were evaluated densitometrically using β-actin for normalization and rabbit-anti-β- amyloid precursor protein antibody was used to detect full-length APP. Data represent mean±SD from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandi-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
Figure 22: Influence of exogenous apoJ treatment on Aβ oligomer levels. pBCEC were treated with plasma-derived, purified apoJ [2 µg/ml], apoJ [20 µg/ml] or apoJ [20 µg/ml]/simvastatin [5 µM] for 24 h. Western blotting was performed using A11 rabbit-anti-amyloid oligomer antibody for detecting Aβ oligomers. Bands were evaluated densitometrically. Data represent mean±SD from 4 independent experiments performed in triplicates.

3.8 Aβ(1-40) is taken up by pBCECs

Uptake of Aβ(1-40) in pBCEC was examined by microscopy after incubating cells with Alexa Fluor 488 labelled Aβ(1-40) for 45 min (see Material and Methods). Nucleus staining was performed by incubating living cells for 15 min with Hoechst dye. As can be seen from Figure 23A, a clear uptake and storage of fluorescently labelled Aβ fragments in defined areas within the cells is evident. Experiments performed at 37°C and 4°C for 5 h revealed a clear difference in the uptake of AF488-Aβ(1-40). At 37°C, distinct fluorescently labelled patches within the cells were visible, whereas incubation at 4°C revealed diffuse and background staining (Figure 23B).
Figure 23: Uptake of fluorescently labelled Aβ(1-40) by pBCEC. pBCEC were seeded on collagen G coated chamber slides and uptake of fluorescently labelled Aβ(1-40) was visualized by fluorescence microscopy using a Leica DM4000B microscope (Leica Cambridge Ltd) equipped with Leica DFC 320 Video Camera (Leica Cambridge Ltd) (see Materials and Methods).
3.9 **Aβ_{(1-40)} treatment increases expression of receptors involved in Aβ transport**

Data presented in this chapter have been published in:


To examine the importance and influence of receptors known to be involved in Aβ transport at the BBB, pBCEC were treated with increasing concentrations of Aβ_{(1-40)} for 1 h. We found a dose-dependent increase in LRP1 mRNA levels in response to treatment with 25 ng/ml, 50 ng/ml, or 200 ng/ml Aβ_{(1-40)} (Figure 24A). Treatment with 25 ng/ml increased LRP1 mRNA expression 1.5±0.28-fold, whereas 50 ng/ml led to a 1.9±0.25-fold and treatment with 200 ng/ml led to even higher mRNA expression (by 2.2±0.24-fold) of LRP1 as compared to control. With the combined treatment of Aβ_{(1-40)} [50 ng/ml]/ apoJ [2 µg/ml] LRP1 mRNA expression was as high as observed with control samples. Messenger RNA levels of receptor for advanced glycation end products (RAGE), low-density lipoprotein receptor-related protein 1 (LRP2), and P-glycoprotein (PGP) were solely increased in response to treatment with 200 ng/ml Aβ_{(1-40)}, by 2.1±0.55-fold, 2.3±0.48-fold and 1.6±0.22-fold as compared to controls, respectively.

To examine whether increasing concentrations of Aβ_{(1-40)} are cytotoxic, pBCEC were treated with 25, 50, and 200 ng/ml for 24 h and subjected to cell proliferation assay with WST-1 reagent provided by Roche. No cytotoxic effects were detected with the various Aβ_{(1-40)} concentrations used (Figure 24B).
Figure 24: (A) Effects of Aβ(1-40) on transcription of receptors known to be involved in amyloid transport. pBCEC were treated for 1 h with different concentrations of Aβ(1-40) [25 ng/ml, 50 ng/ml, 200 ng/ml] or a combination of Aβ(1-40) [50 ng/ml]/ apoJ [2 µg/ml]. RNA was isolated, reverse transcribed and RT-qPCR was performed using ΔΔCt method and HPRT1 as housekeeping gene. Data represent mean±SD from 4 independent experiments performed in triplicates. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)] (B) Treatment with increasing concentrations of Aβ(1-40) is not cytotoxic for pBCEC. Cell viability test was performed as described in Material and Methods using Cell Proliferation Reagent WST-1 by Roche. *p<0.05; **p<0.01; ***p<0.001.
3.10 Aβ_{(1-40)} treatment leads to sLRP1 protein secretion

Data presented in this chapter have been published in:


To examine the effect of increasing concentrations of Aβ_{(1-40)} on LRP1 protein expression, antibodies which recognize two different epitopes were used. Antibody Lrp1 (B411E2) recognizes α- and β- chain (pre-mature) of LRP1, whereas LRP1 (1704) recognizes the cytoplasmic domain of LRP1. We observed that Aβ_{(1-40)} treatment had no significant effect on the cytoplasmic domain of LRP1, whereas treatment with 50 ng/ml, 200 ng/ml or a combination of Aβ and apoJ led to a reduction of LRP1 levels by 39±3.1%, 29±11.4% and 48±8.6%, respectively (Figure 25). Along with reduced levels of LRP1 α-chain, we detected 1.6±0.20-fold and 2.4±0.92-fold higher levels of secreted, soluble LRP1 (sLRP1) in the supernatants in response to treatment with 50 ng/ml and 200 ng/ml Aβ_{(1-40)}, respectively, as compared to untreated pBCEC (Figure 25C). Combined treatment with 50 ng/ml Aβ_{(1-40)} and apoJ [2 µg/ml] revealed significant increases (by 3.1±1.08-fold) in sLRP1 protein expression levels.
[caption on next page]
**Figure 25: Effects of Aβ(1-40) on LRP1 mRNA and protein expression.** pBCEC were treated for 1 h with different concentrations of Aβ(1-40) [25 ng/ml, 50 ng/ml, 200 ng/ml] or a combination of Aβ(1-40) [50 ng/ml]/apoJ [2 µg/ml]. Cell lysates of 15 µg protein were subjected to western blot analysis. Bands were evaluated densitometrically using β-actin as loading control. Data represent mean±SD from 3 independent experiments performed in duplicates. *p<0.05; **p<0.01. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

### 3.11 Influence of Aβ(1-40) on ADAM10 and BACE1 expression

Data presented in this chapter have been published in:


In RT-qPCR analysis we found that mRNA levels of ADAM10 and BACE1, the two APP processing enzymes also known to cleave membrane bound LRP1 to yield soluble LRP1 (27,28), were significantly increased in response to treatment with Aβ(1-40) (Figure 26). Treatment with 50 ng/ml or 200 ng/ml Aβ(1-40) increased relative gene expression of ADAM10 by 62±24.2% and 76±30.0%, respectively. Relative mRNA levels of BACE1 increased by 34±12.2% and 64±18.9% in response to 50 ng/ml and 200 ng/ml Aβ(1-40), respectively. Lower concentrations (25 ng/ml) of Aβ(1-40), nor a combined treatment of Aβ(1-40) and apoJ did influence BACE1 and ADAM10 mRNA levels.

“In summary these results strongly suggest that exposure of porcine cerebrovascular endothelial cells to Aβ(1-40) induces release of LRP1 from the cell surface, potentially mediated by ADAM10 and/or BACE1 and that the observed differences in Aβ(1-40) transport in presence of apoJ are the result of an sLRP1-dependent mechanism.” [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj...

**Figure 26: Effects of Aβ_{1-40} on BACE1 and ADAM10.** pBCEC were treated for 1 h with increasing concentrations of Aβ_{1-40} [0.25 µg/ml, 0.5 µg/ml, 2 µg/ml] or a combination of Aβ_{1-40} [0.5 µg/ml] and apoJ [2 µg/ml] and RNA isolation and cDNA synthesis was performed as described in Material and Methods. RT-qPCR was performed using ΔΔCt method and HPRT1 as house-keeping gene. Data represent mean±SD from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

**3.12 Aβ_{1-40} treatment influences apoJ expression in pBCEC**

Data presented in this chapter have in part been published in:

We studied the alterations of apoJ expression caused by amyloid peptides in capillary endothelial cells by treatment with Aβ(1-40) for 1 h and 24 h, respectively. Gene transcription analysis revealed an increase in apoJ mRNA by 37±9.7% and 35±13.9% in response to treatment with 50 ng/ml and 200 ng/ml Aβ(1-40), respectively (Figure 27A). Further, we found a dose-dependent decrease in cellular apoJ protein levels (Figure 27C). Treatment with 25 ng/ml, 50 ng/ml, or 200 ng/ml Aβ(1-40) for 1 h led to a reduction in apoJ levels by 32±9.0%, 54±9.3%, and 46±9.5%, respectively. In addition, the combined treatment with Aβ and apoJ decreased cellular apoJ protein levels by 65.6±10.6%. Compared to Aβ treatment for 1 h, 24 h incubation time revealed a less pronounced reduction in cellular apoJ protein levels: 50 ng/ml and 200 ng/ml of Aβ(1-40) showed a reduction by 25±17% and 29±10.6%, respectively. As we found no detectable apoJ protein signal in the supernatants after 1 h incubation time, pBCEC were treated with increasing concentrations of Aβ(1-40) for 24 h (Figure 27B). We observed a dose dependent 1.5±0.20-fold and 2.5±0.39-fold increase in secreted apoJ protein levels in response to treatment with 50 ng/ml and 200 ng/ml Aβ(1-40), respectively.
Figure 27: Effects of Aβ\textsubscript{(1-40)} on apoJ protein and gene expression levels. pBCECs were treated with different concentrations of Aβ\textsubscript{(1-40)} [0.25 µg/ml, 0.5 µg/ml, 2 µg/ml] or a combination of apoJ [2µg/ml] and Aβ\textsubscript{(1-40)} for 1 h (A,B,C) and for 24 h (C). (A) Gene expression analysis was performed as described in Material and Methods using ΔΔCt method and normalization to HPRT1. (B, C) Cell lysates of 15 µg protein and supernatants were collected and subjected to western blot analysis using rabbit-anti-clusterin antibody for detecting apoJ. Bands were evaluated densitometrically. Data represent mean±SD from 4 (A,B) and 5 (C) independent experiments. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

To test whether the addition of apoJ prevents Aβ from binding to pBCEC and subsequent uptake, we performed uptake assays using fluorescently (Alexa Fluor 488) labelled Aβ\textsubscript{(1-40)}. A 3 h treatment with 2 and 20 µg/ml purified apoJ led to a dose-dependent reduction in uptake of Alexa Fluor488 labelled Aβ\textsubscript{(1-40)} by 40±17.6% and 59±11.0%, respectively (Figure 28). An incubation time of 24 h led to an even more significant reduction in the uptake by 28±9.3% and 64±10.6%, respectively, confirming our hypothesis. These results together suggest us that enhanced transcription and secretion of apoJ in response to Aβ\textsubscript{(1-40)} may serve to counteract the Aβ challenge and protect the cells by scavenging extracellular Aβ\textsubscript{(1-40)}. 
Figure 28: ApoJ inhibits uptake of Aβ(1-40) by pBCEC. Uptake assay was performed using Alexa Fluor 488 (AF488) labelled Aβ(1-40), pBCEC were incubated with AF488- Aβ(1-40) in the presence or absence of 2µg/ml and 20 µg/ml purified apoJ for 3h and 24h. Cells were lysed with 0.3 N NaOH and uptake was measured fluorescently using a fluorescent plate reader. Data represent mean±SD from 3 independent experiments performed in quadruplicates. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

In addition to examine effects of Aβ(1-40) treatment on apoJ, we were also interested to examine the effects on apoAI which represents a major apolipoprotein in brain associated with HDL-like particles. Interestingly, treatment with 0.5 µg/ml and 2 µg/ml Aβ(1-40) led to a 1.5-fold and 1.4-fold increase in apoAI mRNA expression, respectively (Figure 29).
Figure 29: Increased APOA-I gene expression levels in response to Aβ(1-40) treatment. pBCEC were treated for 24 h with increasing concentrations of Aβ(1-40) [0.25 µg/ml, 0.5 µg/ml, 2 µg/ml] or a combination of Aβ(1-40) [0.5 µg/ml] apoJ [2 µg/ml] and RNA isolation and cDNA synthesis was performed as described in Material and Methods. RT-qPCR was performed using ΔΔCt method and HPRT1 as house-keeping gene. Data represent mean±SD from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01.

3.13 Simvastatin and apoJ influence expression of receptors involved in Aβ transport

Data presented in this chapter have been published in:


To investigate the influence of an impeded cellular cholesterol synthesis on receptors expressed at the BBB, pBCEC were treated for 24 h with simvastatin [5 µM] and subjected to gene expression analysis (Figure 30A). RT-qPCR showed that
simvastatin increased mRNA levels of LRP1, RAGE and PGP by 1.4-fold (SEM=0.08), 2.8-fold (SEM=0.21) and 1.5-fold (SEM=0.09), respectively. Gene expression of LRP2, however, revealed no significant change in response to simvastatin treatment.

Additionally, we were interested to examine the influence of apoJ treatment on receptors involved in Aβ transport. We found that treatment with 2 or 20 µg/ml reduced RAGE gene expression by 65% (SEM=19.8%) and 54% (SEM=10.1%), respectively (Figure 30B). The addition of 20 µg/ml of purified apoJ decreased mRNA levels of LRP2 by 61% (SEM=14.3%). Further, we found a 1.6-fold (SEM=0.11) increase of PGP in response to the combined treatment of pBCEC with apoJ [2µg/ml] and simvastatin [5µM]. The latter observed effect was most probably caused by simvastatin treatment.
Figure 30: Effects of simvastatin and apoJ on gene expression of receptors involved in Aβ transport. pBCEC were treated with simvastatin [5µM], apoJ [2 µg/ml; 20 µg/ml] or with combined treatment with simvastatin [5 µM]/apoJ [2 µg/ml]. RNA was isolated, reverse transcribed and RT-qPCR was performed using ΔΔCt method and HPRT1 as house-keeping gene. Data represent mean±SEM from 5 independent experiments. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

Next, during immunoblot analysis we focused on LRP1 and confirmed an increase in protein levels by 77% (SEM=23.4%) in response to simvastatin treatment (Figure 31A). In response to 2 µg/ml and 20 µg/ml apoJ treatment we detected an increase in LRP1 protein levels by 41% (SEM=10.5%) and 47% (SEM=17.1%), respectively.

“By immunofluorescent staining we also observed higher expression of LRP1 in response to simvastatin treatment (Figure 31B). Treatment with 20 µg/ml apoJ led to an even more pronounced increase in Lrp1 expression levels. Taken these data together, we conclude that both simvastatin and treatment with apoJ induce levels of receptors expressed at the BBB, confirmed for LRP1 at the protein level.” [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj J,

**Figure 31: Simvastatin and apoJ influence LRP1 protein levels.** Porcine BCEC were treated for 24 h in the absence (0.5% ethanol as vehicle control) and presence of simvastatin [5 µM] or apoJ [2 and 20 µg/ml]. (A) Western blot was performed from intracellular protein using anti-Lrp1 (1704) antibody. Data represent mean±SEM from 4 independent experiments performed in triplicates. (B) pBCEC were splitted on collagen G coated FlexiPerm Slides, treated with 5 µM simvastatin and 20 µg/ml apoJ and subjected to immunofluorescence microscopy using anti-rabbit-Lrp1 (1704) (red) as described in the Methods section. Cell nuclei were stained with DAPI (blue). Magnification: 20x *p<0.05; **p<0.01. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
3.14 Effects of simvastatin and Aβ treatment in apoJ silenced pBCEC

Data presented in this chapter have been published in:


In order to examine potential effects of simvastatin and Aβ treatment on APP, LRP1, ADAM10, and BACE1 when apoJ expression is suppressed, we performed apoJ silencing studies in combination with simvastatin and Aβ_{(1-40)} treatment in pBCEC. We detected significant silencing effects by up to 79±14.7% with all conditions applied (Figure 32). However, we observed significantly reduced apoJ silencing efficiency (by 50±26.0%-fold) when pBCEC were treated with simvastatin.

![Figure 32: Effects of simvastatin and Aβ treatment in apoJ silenced pBCEC.](image)

ApoJ was silenced by incubating pBCEC for 30 h with 50 nM siRNA. Scrambled siRNA was used as non-targeting control (NTC). In parallel, pBCEC were treated with simvastatin [5 µM] or Aβ_{(1-40)} [50 ng/ml] and silencing
studies were performed as described in Methods. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)]

In addition, relative gene expression levels of APP increased by 1.9±0.62-fold in response to simvastatin treatment in apoJ silenced cells (Figure 33A); APP mRNA levels in apoJ silenced pBCEC treated with 50 ng/ml Aβ(1-40) increased to very similar extent, by 1.9±0.47-fold, when cells were treated with simvastatin. Simvastatin treatment also increased LRP1, ADAM10, and BACE1 mRNA levels by 1.6±0.42-, 2.6±0.95-, and 2.0±0.51-fold, respectively in apoJ silenced pBCEC (Figure 33B). Treatment with Aβ(1-40), on the other hand, did not reveal significant changes in apoJ, APP, LRP1, ADAM10, nor BACE1 gene expression levels.
Figure 33: Effects of simvastatin and Aβ treatment on apoJ silenced pBCEC. ApoJ was silenced by incubating pBCEC for 30 h with 50 nM siRNA. Scrambled siRNA was used as non-targeting control (NTC). In parallel, pBCEC were treated with simvastatin [5 µM] and Aβ(1-40) [50 ng/ml] was performed as described in Methods. (A-D) Total RNA was isolated, reverse-transcribed, and qPCR analysis was performed with CFX 96 real time system (Bio-Rad) using SYBR Green technology. mRNA expression levels were calculated using ΔΔCt method and HPRT1 as reference gene. Data represent mean±SD from 4 independent experiments performed in triplicates. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

In addition, we were interested to examine potential effects of apoJ silencing in combination with simvastatin [5µM] and Aβ(1-40) [50 ng/ml] treatment on CTF protein levels (Figure 34). Again, we observed an increase by 3.3±2.12-fold in response to simvastatin treatment in apoJ silenced pBCEC. Treatment with Aβ(1-40) [50 ng/ml] did not reveal significant changes.

“Although these results altogether have to be interpreted with caution, since treatment with simvastatin appears to inhibit silencing efficiency of apoJ, we conclude that overall simvastatin effects are maintained under apoJ silencing conditions in cerebrovascular endothelial cells.” [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj J, Pietrzik CU, Wilson MR, Björkhem I, Panzenboeck U. Regulatory effects of simvastatin and apoJ on APP processing and
amyloid-β clearance in blood-brain barrier endothelial cells. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60 (1).

Figure 34: Effects of simvastatin and Aβ treatment on apoJ silenced pBCEC. Western blot analysis was performed using extracted cell lysates of apoJ silenced pBCEC and rabbit anti- amyloid precursor protein, C-terminal antibody was used to detect C-terminal fragments (CTF) Data represent mean±SD from 3 independent experiments performed in triplicates. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
3.15 Influence of simvastatin and apoJ on Aβ transport

Data presented in this chapter have been published in:


To examine effects of inhibiting cholesterol biosynthesis and of the presence of increased apoJ on Aβ transport across the BBB, pBCEC were grown on transwell filters, treated with simvastatin or apoJ, and $^{125}$I-Aβ$_{(1-40)}$ was added to the basolateral compartment in order to examine transcytosis of radiolabelled Aβ peptides from the basolateral to the apical compartment. These experiments showed that apoJ increases the transcytosis rate of $^{125}$I-Aβ$_{(1-40)}$ by 21% (SEM=3.4%) (Figure 35B). Simvastatin, on the other hand, did not reveal significant changes in transcytosis (Figure 35A). In addition, we found that in total a mean of 23.6±10.33% $^{125}$I-Aβ$_{(1-40)}$ was transported from the basolateral to the apical compartment, comparing to only 4.6±2.80% transport of $^{14}$C-sucrose, used as control to monitor paracellular passage and thus tightness of the barrier (Figure 35C).

“Tightness of the endothelial barrier was also assessed by measuring TEER values (Figure 35D). TEER values rose from day 1 to day 2 after plating pBCEC on transwell filters from 96±9.2 to 142±18.6 ohm/cm$^2$. After induction of tight junction formation by addition of hydrocortisone [550 nM], TEER values increased by 2.3-fold reaching 332±24.1 ohm/cm$^2$. Treatment of pBCEC did not affect barrier tightness. Tight junction formation in pBCEC incubated in presence or absence of hydrocortisone [550 nM] was also examined by immunofluorescence microscopy (Figure 35E). Tight junctions were visualized by anti-ZO-1 antibody and anti-VE-cadherin antibody. We observed a clear formation of tight junctions in pBCEC with and without induction with hydrocortisone. After incubation with hydrocortisone, pBCEC formed a more compact and organized barrier, as indicated also by TEER (Figure 35D). These results together suggest a role of apoJ in Aβ transport across cerebrovascular endothelial cells.” [literally published in: Zandl-Lang M, Fanaee-
3.16 **Simvastatin influences APP processing and apoJ protein expression in 3xTg AD mice**

Data presented in this chapter have in part been published in:

processing and amyloid-β clearance in blood-brain barrier endothelial cells. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60 (1).

“In order to investigate in vivo effects of simvastatin on APP processing and apoJ expression at the BBB in a whole animal model, 3xTg AD and Non-Tg mice were gavaged with vehicle or 40 mg/kg simvastatin in 0.2% agarose in PBS for 21 days. 3xTg AD mice are characterized by an overexpression of APP, presinilin1 and MAPT (microtubule-associated protein tau) (71) and are prone to cerebrovascular/BBB dysfunction (47,48). We performed the studies with aged Non-tg (58.4±10.98 weeks) and 3xTg AD (63.7±3.00 weeks) mice.” [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj J, Pietrzik CU, Wilson MR, Björkhem I, Panzenboeck U. Regulatory effects of simvastatin and apoJ on APP processing and amyloid-β clearance in blood-brain barrier endothelial cells. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60 (1).]

Body weights of the mice were examined on a regular basis. 3xTg AD mice revealed higher body weights (32.1±4.54 g) as compared to Non-Tg controls (38.1±6.26 g), but simvastatin treatment did not lead to significant changes (Figure 36). Plasma lipids were measured before and after 21 days of treatment with simvastatin: total cholesterol, free cholesterol, HDL and non-esterified fatty acids (NEFA) remained unchanged in 3xTg and Non-Tg animals before and after simvastatin treatment. TAG, however, raised from 59.7±11.37 mg/dl to 86.1±15.89 mg/dl in vehicle treated and from 50.4±11.63 mg/dl to 91.1±12.49 mg/dl in simvastatin treated 3xTg AD mice. TAG levels remained unchanged in Non-tg control mice. Moreover, we detected a significant increase in free fatty acids (FFA; from 12.0±1.48 mg/dl to 21.8±4.30 mg/dl), when Non-Tg mice were gavaged with simvastatin.
A

B

C

D

E

F
Figure 36: Body parameters of Non-Tg and 3xTg AD model mice. Non-Tg and 3xTg AD mice were gavaged for 21 days with 40 mg/kg simvastatin in 0.2% agarose in PBS. Plasma lipid parameters were assessed enzymatically with commercially available kits (DiaSys, Holzheim, Germany) according to manufacturer's instructions. Body parameters were assessed as described in Material and Methods. *p<0.05. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

In order to examine mRNA levels of the apolipoproteins apoJ and apoE in murine cerebrovascular endothelial cells (mBCEC), RT-qPCR was performed (Figure 37). In mBCEC of Non-tg animals apoJ levels were increased by 2.3±0.25-fold when animals were treated with simvastatin. ApoE mRNA levels were increased in mBCEC of 3xTg AD mice by 2.2±0.26-fold as compared to non-tg control mice. In addition, we analysed gene expression of APP and APP processing enzymes in mBCEC. ADAM10 and BACE1 mRNA levels were increased in mBCEC of 3xTg AD mice by 2.2±0.31-fold and 2.8-fold (SEM=0.34) as compared to Non-TG control animals, respectively, indicating higher APP processing. We also examined gene expression of Aβ transporters expressed at the BBB, and observed 3.5±0.54-fold higher mRNA levels of LRP1 in mBCEC of simvastatin treated 3xTg AD mice as compared to mBCEC of Non-Tg controls (Figure 37D). Further, treatment with 40 mg/kg simvastatin by oral gavage for 3 weeks increased LRP1 mRNA expression in mBCEC of 3xTg animals by 2.4±0.54-fold. In addition, mRNA levels of LRP2 in mBCEC of simvastatin treated 3xTg AD mice were increased by up to 3.5±0.55-fold as compared to Non-tg animals and 2.4±0.54-fold relative to vehicle control treated
3xTg AD mice, whereas the expression profile of RAGE revealed unchanged. Furthermore, we focused on genes involved in cellular cholesterol metabolism. We found, that mBCEC of 3xTg AD mice revealed 2.3±0.32-fold higher mRNA expression of HMGR as compared to those of Non-Tg controls. Analysis of CYP46a, the enzyme responsible for converting cholesterol to 24(S)OH-cholesterol, also revealed by 2.2±0.19-fold enhanced mRNA levels mBCEC of 3xTg AD as compared to Non-Tg control animals. In addition, simvastatin treatment increased CYP46a mRNA levels in mBCEC of Non-Tg animals by 2.0-fold (SEM=0.35).
Figure 37: Increased mRNA levels of genes involved in amyloid processing and cholesterol metabolism in mBCEC of aged 3xTg AD mice. Aged 3xTg AD mice and non-tg controls were gavaged for 21 days with 40 mg/kg simvastatin and vehicle control (0.2% agarose in PBS). mBCEC were isolated as described in Materials and Methods. RNA was isolated, reverse-transcribed and RT-qPCR was performed using ΔΔCt method and HPRT1 as house-keeping gene. Data represent mean±SD. *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

In addition to performing RT-qPCR with mBCEC we also focused on whole brain gene expression analysis. We detected up to 1.8-fold (SEM=0.18) higher APP mRNA levels in brain homogenates of 3xTg animals as compared to Non-Tg controls (Figure 38). Also, gene expression of the γ-secretase presenilin 1 (PSEN1) was increased in 3xTg AD mice by 1.9-fold (SEM=0.12). In addition, simvastatin caused an 8.6-fold (SEM=1.71) increase in cerebral PSEN1 mRNA levels. Surprisingly, we could not detect any significant changes in BACE1 gene expression in whole mice brain.
Figure 38: Gene expression levels of APP and PSEN1 are increased in whole brains of 3xTg AD mice. mRNA analysis of aged 3xTg AD and non-Tg mice, treated for 21 days with simvastatin [40 mg/kg] and vehicle control, was performed of cDNA from whole brain. RNA isolation, cDNA synthesis and RT-qPCR analysis was performed as described in Material and Methods using HPRT1 as housekeeping gene. Data represent mean±SEM. *p<0.05; **p<0.01. [Reproduced from Zandl-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].

Western blot analysis of mBCEC revealed a 1.7-fold increase in C-terminal fragments (CTF)- cleavage products of APP- in 3xTg AD mice compared to non-tg controls, whereas simvastatin caused a reduction in CTF levels by 50% in mBCEC of these mice, indicating decreased APP processing to occur in response to simvastatin treatment (Figure 39). Similar to results obtained from gene expression analysis, cellular apoJ protein levels were increased by 42% in mBCEC of Non-tg animals. In addition, we detected 2.5-fold higher apoJ protein levels in mBCEC of 3xTg AD mice compared to Non-Tg control mice.
Figure 39: Simvastatin (40 mg/kg) treatment *in vivo* influences protein levels of CTF and apoJ. Female 3xTg AD mice and Non-Tg mice (aged 12 months and older) were daily gavaged for 21 days with 40 mg/kg simvastatin in 0.2% agarose in PBS. Western blot analysis of C-terminal fragment (CTF) and cellular apoJ levels in 3xTg AD mice as compared to Non-Tg mice and to simvastatin treatment was performed with mBCEC using β-Actin as loading control and for normalization. All data represent mean±SD in mBCEC of a pool of hemispheres (Non-Tg mice: n=4; 3xTg AD mice: n=8). *p<0.05; **p<0.01; ***p<0.001. [Reproduced from Zandi-Lang et al. with permission from BBA-Molecular and Cell Biology of Lipids (1)].
3.17 Influence of simvastatin treatment in apoJ ko mice

To examine the influence of simvastatin treatment on BBB and cerebral APP/Ab and cholesterol metabolism in apoJ ko mice, we gavaged apoJ ko mice (25.5±5.11 weeks) and C57/Bl6 mice (26.6±2.19 weeks) for 21 days with 40 mg/kg simvastatin in 0.2% agarose (Figure 40). The average weight of the mice at the beginning of the experiment was 25.4±3.45 g for C57/Bl6 mice and 27.0±4.32 g for apoJ ko mice. During the course of the study, animals lost weight, albeit not significantly. When we determined plasma parameters we found that simvastatin increased cholesterol from 41.9±10.47 mg/dl to 60.1±10.29 mg/dl in the apoJ ko mice. Also, TAG levels were higher in apoJ ko mice (117.0±32.65 mg/dl) as compared to controls (69.3±22.84 mg/dl). Surprisingly, treatment with vehicle control reduced TAG levels in apoJ ko mice to 82.3±14.07 mg/dl. Simvastatin treatment did not elicit significant changes. HDL and NEFA levels remained unchanged in apoJ ko and C57/Bl6 mice before and after treatment with 40 mg/kg simvastatin or vehicle control.
Figure 40: Body and lipid parameters of C57/Bl6 control and apoJ ko mice. C57/Bl6 and apoJ ko were gavaged for 21 days with 40 mg/kg simvastatin and vehicle control (0.2% agarose in PBS). Plasma lipid parameters were assessed enzymatically with commercially available kits (DiaSys, Holzheim, Germany) according to manufacturer’s instructions. Data represent mean±SD. (C57/Bl6 – sim: n=8; C57/Bl6 +sim, apoJ ko mice: n=7) *p<0.05; **p<0.01.
In order to examine the role of APP processing in cerebrovascular endothelial cells of apoJ ko mice treated with simvastatin or vehicle control, we performed RT-qPCR analysis of mBCEC (Figure 41). APP gene expression decreased in both, mBCEC of C57/Bl6 (by 45.0%; SEM=3.81%) and of apoJ ko (by 35.9%; SEM=2.45%) mice after treatment with 40 mg/kg simvastatin for 21 days. We did not find significant differences in mBCEC APP mRNA levels between apoJ ko and control animals. ADAM10 mRNA levels were reduced by 41.5±12.32% in mBCEC of apoJ ko mice as compared to those of C57/Bl6 mice. In addition, we found that gene expression of BACE1 in mBCEC was decreased by 2.9-fold (SEM=0.045) in apoJ ko mice after 21 days of treatment with simvastatin as compared to control mice.

In summary these results tell us, that APP processing at the BBB is moderately decreased in apoJ ko versus wild-type mice, and simvastatin is able to reduce APP mRNA expression in mBCEC isolated from these animals.
Figure 41: Reduced mRNA levels of APP (processing enzymes) in response to simvastatin treatment in mBCEC of apoJ ko mice. Gene expression analysis was performed of mBCEC of C57/Bl6 and apoJ ko treated with simvastatin [40 mg/kg] and vehicle control for 21 days. Data represent mean±SEM. (C57/Bl6 –sim: n=8; C57/Bl6 +sim, apoJ ko mice: n=7) *p<0.05; **p<0.01.

Western blot analysis of mBCEC further revealed, that protein levels of CTF are increased by 2.0±0.09-fold in apoJ ko animals as compared to controls and that simvastatin treatment leads to an even higher secretion of CTF in apoJ ko mice (by 3.6±0.03-fold) and control animals (by 1.4±0.31-fold) (Figure 42). When we performed immunoblot analysis of whole brains in these animals, we found neither significant differences in CTF levels in C57/Bl6 brains, nor in apoJ ko mice brains (Figure 43). Simvastatin treatment of apoJ ko mice, on the other hand, reduced CTF levels by 47.4±13.02% as compared to vehicle controls. Further, treatment with 40 mg/kg simvastatin did not change APP and Aβ protein levels in whole brain protein of apoJ ko and C57/Bl6 mice.
Figure 42: CTF levels are changed in mBCEC of apoJ ko mice and after simvastatin treatment. C57/Bl6 and apoJ ko were gavaged for 21 days with 40 mg/kg simvastatin. Murine BCEC were isolated and western blotting of mBCEC proteins was performed as described in Material and Methods. Data represent mean±SD. (C57/Bl6 –sim: n=8; C57/Bl6 +sim, apoJ ko mice: n=7) *p<0.05; **p<0.01; ***p<0.001.
Figure 43: Reduced CTF protein levels in whole brains of simvastatin treated apoJ ko mice. C57/Bi6 and apoJ ko were gavaged for 21 days with 40 mg/kg simvastatin. Whole brain protein was isolated as described in Material and Methods. Western blot analysis was performed using mouse-anti amyloid precursor protein C-terminal and A11 rabbit anti-amyloid oligomer antibody and using β-actin as loading control. Data represent mean±SD. (C57/Bi6 –sim: n=8; C57/Bi6 +sim, apoJ ko mice: n=7) *p<0.05
3.18 Gene expression of apoJ and apoD in CYP27tg, CYP46tg and PDGF (-/-) mice

We examined relative mRNA expression levels of apoJ and apoD in brains of mouse models with altered cholesterol metabolism (CYP27tg or CYP46tg), and a disrupted BBB (Pgdfrb<sup>ret/ret</sup>, (74) (Figure 44). Interestingly, no significant differences in apoJ mRNA levels in any of these mouse models were detected. In addition, no difference between male and female mice was evident. ApoD, on the other hand, revealed a moderate but increased mRNA expression in brains of CYP46tg (by 1.21±0.137-fold) and in <i>Pgdfrb<sup>ret/ret</sup></i> (by 1.43±0.209-fold). In CYP27tg mice brain we were not able to detect significant changes in males nor females.
Figure 44: mRNA analysis of cerebral apoJ and apoD in 3 mouse models. RT-qPCR was performed from murine brain. RNA was isolated, reverse transcribed and mRNA analysis was performed as indicated in Material and Methods. Data represent mean±SD. n=4. *p<0.05
4. Discussion

Parts of this chapter have been literally published in:

The main aim of this PhD thesis was to study the effects and the relations between apoJ and cholesterol metabolism on APP processing at the BBB. Therefore, primary porcine brain capillary endothelial cells (pBCEC) were used as an in vitro model for our studies. Increasing evidence suggesting a correlation between cholesterol metabolism and amyloid-beta metabolism in the brain has been reported (39,75). It is indicated that increasing cholesterol levels increase Aβ abundance. Furthermore, it was shown that AD patients reveal significantly higher LDL cholesterol and significantly reduced HDL cholesterol. In addition, HDL-like particles present in the brain can facilitate Aβ clearance across the BBB (19).

The key findings of the present investigations are that (i) simvastatin shifts APP processing towards the non-amyloidogenic pathway in pBCEC, (ii) inhibition of cholesterol synthesis by simvastatin upregulates apoJ in pBCEC, (iii) both, simvastatin and apoJ increase expression of APP and reduce Aβ uptake in pBCEC, (iv) increased Aβ and simvastatin induce Lrp1 and apoJ levels in vitro and in vivo, and that (v) thereby, clearance of Aβ across the BBB is facilitated.

4.1 Influence of cholesterol metabolism on APP processing in brain capillary endothelial cells

In initial experiments we determined whether the natural LXR agonists 24(S)OH-cholesterol and 27OH-cholesterol, the synthetic LXR agonist TO901317, the HMG-
CoA reductase inhibitor simvastatin, and cholesterol - and thus regulators of cholesterol biosynthesis - influence mRNA and protein expression of APP, APP processing enzymes, and apoJ (Figure 7-9). We observed increased APP and ADAM10 mRNA expression in response to 5 µM simvastatin treatment, whereas simvastatin decreased relative gene expression of the Aβ peptide forming β-secretase BACE1 (Figure 7). Other modulators of cholesterol metabolism did not alter expression levels of the genes analysed. Western blot analysis confirmed the increase of APP in response to simvastatin. Also, protein levels of C-terminal fragments (CTF), fragments which are formed in response to APP processing, were increased. In accordance to the in vitro results obtained, we found increased CTF protein levels in mBCEC of apoJ ko and C57/Bl6 control mice (Figure 42). Using an enzyme activity assay we detected a clear reduction in BACE1 activity in response to simvastatin treatment of pBCEC. In addition, we found an increase in the beneficial cleavage product sAPPα in response to simvastatin treatment of pBCEC. „Barrett et al. identified a cholesterol-binding domain within the APP transmembrane domain (77). Due to the fact that the cholesterol-binding site is immediately adjacent to the α-secretase cleaving site, it is hypothesized that binding of cholesterol may drive APP processing towards the amyloidogenic pathway. A reduction and redistribution of cellular cholesterol could account for this effect observed in statin treated pBCEC. Also, dysregulation of cholesterol trafficking was reported to interfere with APP metabolism since α-/β-secretase processing occurs within cholesterol rich lipid rafts in the membrane (78,79). Therefore, cholesterol depletion decreases membrane fluidity and thus leads to enhanced α-secretase activity (80). Taking these results together with the fact that lower cellular cholesterol content due to simvastatin treatment enhances gene expression of APP and the α-secretase ADAM10, we conclude that simvastatin helps to direct APP towards the neuroprotective, non-amyloidogenic APP secretion pathway.“ [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj J, Pietrzik CU, Wilson MR, Björkhem I, Panzenboeck U. Regulatory effects of simvastatin and apoJ on APP processing and amyloid-β clearance in blood-brain barrier endothelial cells. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60. (1)]. This is in accordance with earlier studies performed by our group where we showed that simvastatin treatment affects
APP production and processing at the BBB in vitro (39). It was also shown in neurons, that depending on the membrane cholesterol content, activities of α- and β-secretases can be altered (81). Cordy et al., for instance, reported that Aβ generation in a neuronal cell line depends on lipid rafts. Aging induced changes in cholesterol and lipid distribution within cell membranes lead to higher activities of BACE and other amyloidogenic enzymes in lipid rafts, which in consequence leads to higher Aβ production (82). Moreover, Simons et al. demonstrated that in cultured hippocampal neurons, cholesterol depletion due to combined treatment with lovastatin and β-cyclodextrin inhibit Aβ production (43). In addition, several in vivo studies reported decreased Aβ loads in response to statin treatment (83,84). When we performed animal studies, we found increased levels of CTF in mBCEC of 3xTg AD mice, which were then reduced in response to treatment with simvastatin (Figure 39). This indicates that simvastatin reduces APP processing in cerebrovascular endothelial cells and thus reduced Aβ reduction.

As it was shown that simvastatin exhibits, in addition to its main property to inhibit cellular cholesterol synthesis, other non-lipid associated or “pleiotropic” effects, we treated pBCEC with a combination of cholesterol and simvastatin. Thereby, we have clearly demonstrated that simultaneous treatment with simvastatin and cholesterol diminished effects observed with simvastatin treatment solely (Figure 8). Therefore, we conclude that simvastatin’s cholesterol lowering effect is responsible for an increase in APP, CTF and sAPPα.

### 4.2 Simvastatin’s effect on Aβ peptide levels

During immunoblot analyses we further detected increased amounts of secreted Aβ oligomers and decreased cellular Aβ oligomers in response to simvastatin treatment (Figure 10). During uptake studies, we then observed reduced uptake of Aβ by cerebrovascular endothelial cells in response to simvastatin. We, therefore, speculate that reduced cholesterol levels/synthesis in the cell and especially a depletion of cholesterol in the membrane may exert effects on Aβ metabolism, processing and transport. Transport studies were performed to elucidate the role of simvastatin in Aβ transport across the BBB and revealed that simvastatin did not alter
transport of Aβ from the basolateral compartment. We, therefore, conclude that simvastatin’s influence in reducing cell-associated oligomers may be beneficial for pBCEC. Nevertheless, oligomers/Aβ in the supernatants are increased. Depending on the fate of these Aβ oligomers, it is important to mention that they may add an amyloidogenic component to simvastatin’s effect.

4.3 Regulation of apoJ secretion at the BBB

Next, we studied potential effects of modulators of the cellular cholesterol metabolism influence on apoJ expression and secretion. In initial in vitro experiments we incubated pBCEC with the LXR agonists, 24(S)OH-cholesterol, 27OH-cholesterol and TO901317, the HMGCoA reductase inhibitor simvastatin and cholesterol. In RT-qPCR and western blot analyses we found an increase in apoJ mRNA and cellular protein level solely to treatment with simvastatin (Figure 12). Other modulators of the cellular cholesterol metabolism did not alter apoJ expression levels significantly. In order to exclude pleiotropic effects of simvastatin we repeated western blot analysis with a combination of simvastatin and cholesterol (Figure 13). As also observed with APP and CTF protein levels, combined treatment diminished effects observed with simvastatin treatment alone. We therefore confirmed that the cholesterol reducing effects of simvastatin causes an observed change in apoJ levels, rather than cholesterol-independent “pleiotropic” effects.

In transwell studies we determined polarized secretion of apoJ to the basolateral and apical compartment of the in vitro BBB (Figure 14). We detected predominant secretion of apoJ to the basolateral side. Conform with previous findings in our laboratory, sAPPα was also highly secreted to the basolateral side (39). In addition, we observed that also other apolipoproteins, such as apoA-I and apoM, and ABCA1 are localized at the brain compartment of the BBB (40,85). Furthermore, PLTP, a protein involved in HDL remodelling and which is able to reduce Aβ oligomer levels in pBCEC, is also mainly secreted to the basolateral compartment of the BBB (32). Hence, we conclude that APP processing and its modulation by apoJ mainly occurs at the basolateral side of the BBB.
Additionally, we found that simvastatin treatment leads to higher secretion levels of apoJ towards the basolateral compartment compared to vehicle control. In accordance with *in vitro* studies we detected increased apoJ protein levels in cerebrovascular endothelial cells isolated from mice in response to administration of 40 mg/kg simvastatin for 21 days (Figure 39). These data indicate that simvastatin, a well-described HMGCoA-reductase inhibitor, has the potential to increase apoJ synthesis and secretion pathways in BBB endothelial cells.

In addition, during *in vivo* studies we observed increased apoJ protein levels in cerebrovascular endothelial cells isolated from 3xTg AD mice as compared to Non-tg controls. Interestingly, it was shown by Montanola et al. that patients suffering of CAA also reveal an increased apoJ plasma profile (57), indicating an important role of apoJ in the cerebrovascular.

### 4.4 Role of apoJ in APP metabolism

In subsequent studies, we examined the effects of exogenous, purified plasma apoJ treatment as well as of apoJ silencing in pBCEC on mRNA and protein expression levels of genes involved in amyloid and cholesterol metabolism. ApoJ at 2 or 20 µg/ml did not alter apoJ, APP, ADAM10, or BACE-1 gene expression (Figure 20). Treatment of pBCEC with simvastatin alone or simvastatin plus apoJ enhanced apoJ, APP, and ADAM10 gene expression, and reduced mRNA levels of ABCA1, responsible for the biogenesis of HDL-like particles at the BBB. Further, simvastatin or simvastatin plus apoJ induced gene expression of HMG-CoA reductase and of SREBP-2, a key protein responsible for cholesterol regulation, uptake and transport. We, thus conclude that the addition of apoJ to the culture medium has no effect on transcription of cholesterol metabolic genes, APP, and APP processing genes analyzed.

During silencing studies, however, we observed that a reduction of apoJ using small interfering RNA lead to decreased full-length APP protein levels in pBCEC (Figure 18). Treatment with exogenous apoJ [2 µg/ml and 20 µg/ml], on the other hand,
significantly enhanced APP protein levels (Figure 21). Given the fact that treatment and silencing of apoJ did not affect APP mRNA but protein levels, we postulate a close connection and a prominent role of apoJ in APP processing in the cerebrovasculature on the posttranslational level.

In addition, we performed Aβ uptake and transport studies in order to examine the role of apoJ in uptake of Aβ<sub>1-40</sub> by cerebrovascular endothelial cells and its clearance from the brain across the BBB to the peripheral blood flow. We detected that apoJ facilitates transport of Aβ from the basolateral to the apical compartment (Figure 35); however, uptake by pBCEC was reduced (Figure 28). It was also reported in previous studies that apoJ can facilitate Aβ transport across well-established BBB models (75,86). In accordance with our studies on pBCEC are also previous studies using other brain cells: thus, apoJ blocks binding, uptake and degradation of Aβ<sub>1-42</sub> in rat microglia (87). Mulder et al. reported that apoJ reduced uptake of Aβ oligomers and fibrils in primary human adult microglia (88). In primary human astrocytes, on the other hand, only uptake of Aβ oligomers was impaired, whereas Aβ fibrils uptake remained unchanged (88).

To further elucidate a correlation of amyloid peptides and apoJ expression and secretion, pBCEC were incubated for 1 h and 24 h with increasing concentrations of Aβ<sub>1-40</sub>. We found that Aβ<sub>1-40</sub> increased apoJ mRNA and secreted protein levels, whereas cellular apoJ levels were reduced after 1 h and to a lesser extent after 24 h treatment (Figure 27). As it was already known that apoJ tends to colocalize with amyloid plaques and that it is able to bind to Aβ, the observed results propose that upon treatment with amyloid peptides more apoJ is translated and secreted to the extracellular space to bind Aβ<sub>1-40</sub> peptides (89,90). In contrast, Wang et al. demonstrated that treatment with Aβ<sub>1-42</sub> led to overexpression of intracellular apoJ, but levels of secreted apoJ remained unchanged in primary hippocampal neurons (91). We therefore hypothesize that apoJ is regulated differently in different brain cell types in response to Aβ treatment.
4.5 **Aβ changes expression of receptors expressed at BBB**

“In subsequent studies we examined the uptake of Aβ\(_{(1-40)}\) by cerebrovascular endothelial cells. We found that pBCEC successfully take up Aβ\(_{(1-40)}\) and are able to store the peptides within the cell (Figure 23). In addition, we observed that Aβ\(_{(1-40)}\) enhanced gene expression levels of RAGE, PGP, and LRP2, but most pronouncedly of LRP1 in pBCEC. Also, mRNA levels of LRP1, but not of RAGE, were increased in mBCEC isolated from 3xTg AD mice as compared to Non-tg controls (Figure 37). It is known that many receptors at the BBB are involved in the uptake of amyloid fragments, albeit with LRP1 as the most important receptor to clear amyloid peptides from the brain and efflux them to the plasma (26). In order to further examine the role of Lrp1 in amyloid beta metabolism, western blot analysis was performed. We observed a reduction in signals for LRP1 α- and β- chain, whereas the cytoplasmic domain of LRP1 revealed unchanged to Aβ\(_{(1-40)}\) treatment (Figure 25). Western blots performed with secreted proteins showed an increase in soluble LRP1 in response to 1 h treatment with Aβ\(_{(1-40)}\). In further experiments we found that gene expression of both, the α-secretase ADAM10 and the β-secretase BACE1 are increased in response to treatment with Aβ\(_{(1-40)}\) (Figure 26). It was reported that ADAM10, which belongs to the group of metalloproteinases, and BACE reveal in addition to its function in APP processing, also a role in Lrp1 shedding, which leads to the production of sLrp1 (24,25). Therefore, we conclude, that in pBCEC clearance and uptake of Aβ is activated through other mechanisms and receptors rather than membrane bound Lrp1.” [literally published in: Zandl-Lang M, Fanaee-Danesh E, Sun Y, Albrecher NM, Gali CC, Čančar I, Kober A, Tam-Amersdorfer C, Stracke A, Storck SE, Saeed A, Stefulj J, Pietrzik CU, Wilson MR, Björkhem I, Panzenboeck U. *Regulatory effects of simvastatin and apoJ on APP processing and amyloid-β clearance in blood-brain barrier endothelial cells*. Biochim Biophys Acta. 2017 Sep 20;1863(1):40–60. (1)]

Surprisingly, we observed that combined treatment with Aβ\(_{(1-40)}\) and apoJ did not increase gene expression of receptors expressed at the BBB. Neither gene expression of Lrp1, RAGE, PGP, nor Lrp2, a receptor known to be specific for apoJ, was changed in response to combined treatment (Figure 24). We therefore conclude
that apoJ binds and scavenges Aβ in the extracellular space and thereby uptake and toxicity is reduced in pBCEC.

4.6 Influence of simvastatin and apoJ on receptors expressed at the BBB in pBCEC

During gene expression analyses we further determined the role of simvastatin and also of apoJ on BBB transporters/receptors. We found that simvastatin increases mRNA levels of LRP1 and PGP, albeit with the highest increase found in RAGE (Figure 30). ApoJ treatment, on the other hand, did not alter gene expression of Lrp1 and PGP, and even decreased RAGE and LRP2 mRNA levels. In immunoblot analysis and immunofluorescence, however, we detected a clear increase in Lrp1 expression. In addition, simvastatin increased protein levels of Lrp1. During in vivo studies, we also observed that simvastatin administration increased LRP1 mRNA levels in mBCEC isolated from 3xTg AD mice and Non-tg control animals. In addition, simvastatin increased gene expression of LRP2 in mBCEC from 3xTg AD model mice (Figure 37).

4.7 Effect of simvastatin treatment in apoJ ko mice

Since it was shown that apoJ plays an important role in the clearance of Aβ from the brain and the pathogenesis of AD (86,92) and we also confirmed a correlation of apoJ and APP metabolism at the blood-brain interface we performed studies on apoJ ko mice.

First of all, we examined plasma lipid parameters of apoJ ko and C57/Bl6 mice (Figure 40). Interestingly, plasma triglycerides levels of apoJ ko mice were increased as compared to C57/Bl6 control mice. To our surprise, simvastatin treatment reduced plasma triglycerides levels in apoJ ko mice, whereas HDL levels and also other lipid parameters, such as NEFA and cholesterol, remained unchanged.
When we performed gene expression analysis of mBCEC we found that mRNA levels of the α-secretase ADAM10 and the β-secretase BACE were reduced in apoJ ko mice as compared to C57/Bl6 mice indicating lower APP processing (Figure 41). Controversially, we detected increased CTF levels in mBCEC of apoJ ko mice (Figure 42). Interestingly, gene expression of APP was decreased in response to simvastatin treatment in apoJ ko and control animals, whereas simvastatin increased CTF protein levels in these animals. In contrast to results obtained from mBCEC, CTF levels from whole brain samples were reduced in response to simvastatin treatment. When examining APP and APP processing products in whole brains of apoJ ko and C57/Bl6 mice, we found no significant alterations neither in APP, Aβ nor in CTF protein levels (Figure 43).

4.8 Role of apoJ and apoD in cholesterol metabolism at the BBB

As mentioned above, the BBB is essential for cholesterol metabolism in the brain. In collaboration with Prof. Björkhem from the Karolinska Institute in Stockholm we aimed to assess expression levels of apoJ and apoD, both apolipoproteins known to play a role in AD, in brain and BBB cholesterol metabolism.

As it was shown that cholesterol is able to pass the BBB after conversion to 24(S)OH-cholesterol, which is formed by 24S-hydroxylase, an enzyme also known as CYP46A (93), we performed gene expression analysis of brains from CYP46tg mice. In addition, RT-qPCR was performed with CYP27tg mice, which reveal increased production of 27-hydroxylase, an enzyme responsible for converting cholesterol to 27OH-cholesterol (94). Hudry et al. showed that injecting an adenovirus associated vector encoding CYP46A1 into the cortex and hippocampus reduced amyloid deposits in an AD mouse model (93). Further, Maioli et al. documented improved cognitive performance in spatial learning in the Morris water maze test of CYP46tg mice as compared to non-tg controls (93). Since CYP46A1 seems to be important in memory function, we examined whether gene expression of apoJ and apoD is altered in the brain of CYP46tg mice. In addition, RT-qPCR was performed on cDNA obtained from RNA isolated from brain homogenates of CYP27tg mice. Surprisingly,
however, we detected no alterations in apoJ mRNA levels in CYP46tg or CYP27tg mouse brain compared to non-tg controls (Figure 44). Also, gene expression of apoD was not changed in CYP27tg mice, whereas mRNA levels were moderately increased in CYP46tg animals.

In addition, we examined the consequences of a leaking BBB on gene expression of the two apolipoproteins expressed in the brain. We, therefore, used a pericyte-deficient mouse model (Pdgfb -/-)), which was shown to have increased permeability of the BBB (63). It was shown that this mouse model revealed increased flux of cholesterol from the circulation to the brain (63). Therefore, a down-regulation of cholesterol synthesis was expected. Instead Saeed et al. reported that cholesterol synthesis was increased by 60%. 24(S)OH-cholesterol, which efficiently suppresses cholesterol synthesis, was reduced in the brain, whereas 24(S)OH-cholesterol was increased in the periphery (63). When we examined gene expression of apoJ and apoD of whole brains, we found that, similar to results obtained from CYP46tg and CYP27tg mice, apoJ mRNA levels were unaltered, whereas apoD mRNA levels were moderately increased. We therefore conclude that apoJ’s role in the brain is more important if amyloid deposits are already present, where it can bind to Aβ peptides and helps to clear them across the BBB.
4.9 **Conclusion**

We clearly demonstrated that simvastatin increases expression of APP, ADAM10 and sAPPα, and reduces gene expression and activity of BACE1 by inhibiting cellular cholesterol synthesis in BCEC (Figure 45). Thereby, APP is processed towards the non-amyloidogenic pathway in BBB endothelial cells. Further, simvastatin decreased cellular levels of Aβ leaving secreted Aβ peptide levels increased. In line, by performing uptake studies we found that simvastatin inhibits uptake of Aβ_{(1-40)} peptides by pBCEC.

Further, simvastatin treatment increased apoJ expression both *in vitro* and *in vivo*, which was shown to be primarily secreted to the basolateral side of the BBB. We, therefore, suggest a predominant secretion of apoJ from BBB endothelial cells to the brain parenchymal side, where it can bind to Aβ and also helps in amyloid clearance across the BBB. Studies showing increased secretion of apoJ to the cell surroundings in response to treatment with Aβ_{(1-40)} confirm this hypothesis. In addition, we show that apoJ facilitates clearance of Aβ peptides from the brain to the periphery across the BBB. Furthermore, Aβ_{(1-40)} treatment increases mRNA levels of receptors expressed at the BBB, with the most significant increase observed for Lrp1 levels. Interestingly, we found that upon Aβ treatment Lrp1 is primarily secreted to form sLrp1. We, therefore, conclude that Aβ is cleared across the BBB through other mechanisms or receptors, rather than membrane bound Lrp1. Moreover, we clearly demonstrated that simvastatin and, therefore, reduced cellular cholesterol synthesis is also able to regulate Lrp1 mRNA levels at the BBB *in vivo* using 3xTg AD mice and non-Tg control.

Further, we detected a direct relationship between apoJ and APP levels. Treatment with exogenous apoJ increased APP protein levels, whereas apoJ silencing decreased APP levels and also cellular Aβ protein levels.

To sum up, we suggest a close and complex relationship of statin treatment, apoJ and amyloid processing at the blood-brain interface. Future *in vitro* and *in vivo*
studies are aiming to elucidate and understand these mechanisms at the BBB in greater detail.

Figure 45: Proposed model of actions of simvastatin and apoJ in APP metabolism at the BBB. [Reproduced from Zandl-Lang et al. with permission of BBA-Molecular and Cell Biology of Lipids (1)].
5. References


92. Miners JS, Clarke P, Love S. Clusterin Levels are Increased in Alzheimer’s Disease and Influence the Regional Distribution of Aβ. Brain Pathol Zurich Switz. 2016 Jun 1;
