A study of biomolecules that influence α-cleavage of APP in a cell culture model

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A study of biomolecules that influence α-cleavage of APP in a cell culture model

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presented by
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Abstract

The Alzheimer’s disease (AD) hallmark peptide Aβ is generated after β-cleavage of its precursor protein APP and its production is precluded by α-cleavage. Hence, measuring the concentrations of the soluble cleavage products sAPPα and sAPPβ produced after α- or β-cleavage, respectively, indicates changes in APP cleavage. Previous reports suggested biomolecules such as fatty acids and cortisol to influence APP cleavage. We aimed at verifying those findings in a cell culture model.

Growth medium of SH-SY5Y cells was supplemented with sera from different species origin (human, bovine or equine), saturated and unsaturated fatty acids, i.e. trans-vaccenic acid (VA), elaidic acid (EA), docosahexaenoic acid (DHA) and stearic acid (SA) or hormones and growth factors (cortisol and retinoic acid). The concentrations of the APP cleavage products sAPPα and sAPPβ were measured by ELISA and compared to concentration of samples from not supplemented cells. APP, ADAM10 (the major α-secretase) and BACE1 (β-secretase) levels were estimated by Westernblot analysis.

Providing cells with fatty acids, VA and DHA, respectively, led to an increased concentration of sAPPα, while SA supplementation decreased the concentration of sAPPα. However, the cleavage ratio remained unaffected for all fatty acids. We found a clear effect of serum origin on the sAPPα/sAPPβ ratio: human or equine serum significantly increased this ratio compared to foetal calf serum. Moreover, heat-inactivation of foetal calf serum significantly reduced sAPPα concentration and the sAPPα/sAPPβ ratio, while inactivation of human serum had the opposite effect. Experiments with dialysed sera showed that at least one of the factors responsible for the different effects of serum origins on sAPP levels had a molecular weight of more than 12 kDa.
Therefore, this factor might be a protein differing in its expression levels in the three investigated sera. Identification of this factor could help elucidating the control mechanism for APP cleavage. The effects seen for cortisol supplementation ranged from clearly increasing to slightly decreasing ratios with high inter-experimental variations.

Developing new drugs against AD, tight junctions need to be considered, as they represent the molecular basis for the barrier effect in epithelia preventing e.g. the targeting of drug molecules with high molecular weight that are not able to partition through cell membranes to the brain. Proteins of the claudin family were shown to play a pivotal role in establishing a tight barrier at cell junctions with claudin-1 being the most common and providing the tightest junctions. We aimed at setting up an assay to test the strength of the interaction between extracellular loop domains of claudin. This test would allow screening for molecules able to reversibly open tight junctions, opening new strategies to target hydrophilic molecules or macromolecules to the brain. We aimed at generating the extracellular loop domain of claudin-1 by purification from mammalian cell culture, by solid phase peptide synthesis or by cloning and expression in bacteria. However, none of the strategies led to successful generation of the desired loop domain.
Zusammenfassung


Wenn Zellmedium mit Fettsäuren angereichert wurde, führte dies bei VA und DHA jeweils zu einer erhöhten Konzentration von sAPPα, während die Konzentration von sAPPα bei SA abnahm. Das sAPPα/sAPPβ-Verhältnis blieb jedoch für alle Fettsäuren unbeeinflusst. Wir fanden einen deutlichen Einfluss von unterschiedlichen Seren auf das sAPPα/sAPPβ-Verhältnis: Menschen- oder Pferdeserum erhöhten dieses Verhältnis signifikant im Vergleich zu fötalem Kälberserum. Hitzeinaktivierung von fötalem Kälberserum führte ausserdem zu signifikant reduzierter sAPPα-Konzentration und einem signifikant reduzierten sAPPα/sAPPβ-Verhältnis,
während Inaktivierung von humanem Serum das Gegenteil bewirkte. Experimente mit dialysiertem Serum zeigten, dass mindestens einer der Faktoren, welcher für die unterschiedlichen Auswirkungen der untersuchten Seren auf die sAPP-Konzentrationen verantwortlich ist, ein Molekulargewicht von mehr als 12 kDa haben muss. Daher könnte dieser Faktor ein Protein sein, welches in den drei untersuchten Seren unterschiedlich stark exprimiert wird. Die Identifizierung dieses Faktors könnte bei der Aufklärung der Kontrollmechanismen für die APP-Spaltung hilfreich sein. Die Behandlung mit Cortisol führte zu stark schwankenden Ergebnissen, welche von deutlich steigenden bis zu leicht abnehmenden Verhältnissen reichten.

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List of Abbreviations

Aβ ....................... amyloid β

AChEI ..................... acetylcholinesterase inhibitor

AD .......................... Alzheimer’s disease

ADAM ........................ A disintegrin and metalloproteinase

AICD ........................ APP intracellular domain

ALP ........................ alkaline phosphatase

APH-1 ........................ anterior pharynx defective 1

APOE ........................ apolipoprotein E

APP ........................ amyloid precursor protein

APS ........................ ammonium persulfate

BACE1 ...................... β-site APP-cleaving enzyme

BBS1 ........................ anti-β-site antibodies

BDNF ........................ brain-derived neurotrophic factor

BSA ........................ bovine serum albumine

Cdk5 ........................ cyclin-dependent kinase 5

CI ........................... confidence interval

CNS .......................... central nervous system

CSF ............................ cerebrospinal fluid

CTF ............................ C-terminal fragment
LIST OF ABBREVIATIONS

DHA .................... docosahexaenoic acid
DMEM .................. Dulbecco’s modified eaque medium
DOPE .................. dioleoyl-phosphatidyl-ethanolamine
DPBS .................. Dulbecco’s PBS
EA ..................... elaidic acid
EBSS ................... Earl’s balanced salt solution
ECL ..................... electrogenerated chemiluminescence
EGCg ................... epigallocatechin-3-gallate
EGTA ................... ethylene glycol tetraacetic acid
EIA ...................... enzyme immunoassay
ELISA .................. enzyme-linked immunosorbent assay
ER ...................... endoplasmic reticulum
ESM .................... equine serum supplemented medium
FCS ...................... foetal calf serum
FDA ...................... food and drug administration
FSM .................... FCS supplemented medium
GPCR .................. G protein-coupled receptor
GSM ..................... γ-secretase modulator
HATU .................. 2-(1H-9-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES .................. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LIST OF ABBREVIATIONS

HPA axis .......... hypothalamic-pituitary-adrenal axis
HPLC ............... high-performance liquid chromatography
HRP ................. horseradish peroxidase
HSD ................. honestly significant difference
HSM ................ human serum supplemented medium
IgG ................ immunoglobulin G
IP .................... immunoprecipitation
IVIg ................ intravenous immunoglobulin
LA ................... linoleic acid
LMT ................ leuco-methylthioninium
MCI ................... mild cognitive impairment
MDCK cells ....... Madin Darby canine kidney cells
MS ................... mass spectrometry
MTC ................ methylthioninium chloride
MW ................... molecular weight
NCT ................ nicastrin
NFT ................ neurofibrillary tangles
NGF ................ nerve growth factor
NGXsc ............... NGX series compounds
NMDA ............... N-methyl D-aspartic
OG ................... octylglucopyranoside
LIST OF ABBREVIATIONS

OR .................. odds ratio
PBS .................. phosphate buffered saline
PEG .................. polyethylene glycol
PEN .................. presenilin enhancer
PKC .................. protein kinase C
POPC ................. palmitoyl-oleoyl-phosphatidyl-choline
POPE ................. palmitoyl-oleoyl-phosphatidyl-ethanolamine
PS1 .................. presenilin 1 (protein)
PS2 .................. presenilin 2 (protein)
PSEN1 ................ presenilin 1 (gene)
PSEN2 ................ presenilin 2 (gene)
PUFA ................ poly unsaturated fatty acid
RA .................. retinoic acid
RIPA ................ radioimmunoprecipitation assay
SA .................. stearic acid
sAPP ................ soluble, extracellular fragment of APP
sAPPα .............. soluble extracellular fragment of APP after α-cleavage
sAPPβ .............. soluble extracellular fragment of APP after β-cleavage
SDS ................ sodium dodecyl sulfate
SDS-PAGE ......... sodium dodecylsulfate polyacrylamide gel electrophoresis
SPPS ................ solid phase peptide synthesis
LIST OF ABBREVIATIONS

TACE .................. tumour necrosis factor-α-converting enzyme
TBS .................. tris-buffered saline
TEER ................. trans-epithelial electric resistance
TEMED ............... tetramethylethlenediamine
TGN .................. trans-Golgi network
THF .................. tetrahydrofuran
TMB .................. tetramethylbenzidine
TNF-α ................. tumour necrosis factor-α
TPK I ................ Tau protein kinase system I
TPK II ............... Tau protein kinase system II
T/TBS ................. tris-buffered saline with Tween20
VA ................... trans-vaccenic acid
1. Introduction

1.1. The history of Alzheimer’s Disease research

When Alois Alzheimer first described a new disease of his patient Auguste Deter in 1907 [Alzheimer 1987], he did not know that this at his time considered a rare disease once would become the most frequent form of dementia. He followed his patient from 1901 until her death in 1906. In the five following years a dozen further cases were published all describing the same disease pattern and in 1910, Emil Kraepelin termed the disease after Alois Alzheimer [Kraepelin 1910]. Alois Alzheimer was the first to describe a dementia that was accompanied by detectable physiological changes in the affected brain: congo-red stainable fibrils and plaques revealed by silver stain [Alzheimer 1987]. Electron microscopy allowed to localise the fibrils as intracellular neurofibrillary tangles (NFT) [Kidd 1963]. It took over half a decade until it became clear that Alzheimer’s disease (AD) was not as rare as previously thought. Modern techniques allowed to further characterise the two hallmarks NFTs and amyloid plaques and the general ageing of the world’s population led to an increased number of "cases" to study. An overview of the history of AD is shown in Table 1.1 and details have been reviewed by Cipriani et al. [Cipriani, Dolciotti et al. 2011].

Two different hypotheses were established, claiming either the NFTs (see Section 1.3) or the amyloid plaques (see Section 1.4) and their respective origins the main culprits of brain degradation. The next piece of the puzzle was the finding that inheriting the ε4 allele of the apolipoprotein E gene (APOE) leads to excess amyloid accumulation in the brain even before AD symptoms arise [Wisniewski and Frangione 1992]. APOE4 still is the major known genetic risk factor for the sporadic form of AD, also termed late-onset AD. Currently known genetic risk factors in AD are discussed in Section 1.9.
Table 1.1: The history of Alzheimer’s Disease from 1900 to 2004. Taken from [Toledo 2005].

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1864</td>
<td>Dr. Alois Alzheimer is born in Bavaria.</td>
</tr>
<tr>
<td>1901-1906</td>
<td>Dr. Alzheimer observes the 51 year old female Auguste Deter of Frankfurt, Germany.</td>
</tr>
<tr>
<td>1906</td>
<td>Dr. Alzheimer publishes histological changes associated with AD.</td>
</tr>
<tr>
<td>1910</td>
<td>Emil Kraepelin names &quot;Alzheimer’s Disease&quot;.</td>
</tr>
<tr>
<td>1910-1960s</td>
<td>AD remains a little known disease.</td>
</tr>
<tr>
<td>1976</td>
<td>Biochemical changes in brain are associated with AD.</td>
</tr>
<tr>
<td>1979</td>
<td>Alzheimer’s Association is founded.</td>
</tr>
<tr>
<td>1980s</td>
<td>AD becomes a social movement. Research on biochemistry of toxic proteins of plaques and tangles.</td>
</tr>
<tr>
<td>1987</td>
<td>The famous actress Rita Hayworth dies of AD.</td>
</tr>
<tr>
<td>1993</td>
<td>Cognex, a cholinesterase inhibitor, is the first FDA approved drug to treat AD.</td>
</tr>
<tr>
<td>1994</td>
<td>A possible effect of estrogens on AD is postulated.</td>
</tr>
<tr>
<td>1996</td>
<td>Aricept (acetylcholinesterase inhibitor, AChEI) is FDA approved.</td>
</tr>
<tr>
<td>1997</td>
<td>Effects of antioxidants on AD are studied.</td>
</tr>
<tr>
<td>1999</td>
<td>Genetic mutations are linked to programmed cell death of neurons. Techniques leading towards direct genetic manipulation for treatment of AD are developed. The first anti-AD vaccine is tested.</td>
</tr>
<tr>
<td>2000</td>
<td>Exelon (AChEI) is FDA approved to treat AD. Brain imaging is used to study AD.</td>
</tr>
<tr>
<td>2001</td>
<td>Razadyne (previously Reminyl, an AChEI) is FDA approved.</td>
</tr>
<tr>
<td>2002</td>
<td>Clinical trials of anti-AD vaccine are conducted.</td>
</tr>
<tr>
<td>2003</td>
<td>Namenda (first NMDA receptor antagonist) is FDA approved.</td>
</tr>
<tr>
<td>2004</td>
<td>Ronald Reagan, US president from 1981 to 1989, dies of AD. Diabetes is linked with increased risk of AD.</td>
</tr>
</tbody>
</table>
1.2.  **Prevalence and economic costs of Alzheimer’s disease**

AD is nowadays the most common form of dementia in the elderly. In the course of the next 25 years, AD is estimated to evolve to the world’s health care problem number one. Age is the most important non-genetic risk factor for AD. Elevated life expectancy will increase the number of elderly people affected by AD. Caring for more and more AD patients will become a major hurdle for the western health care systems.

In 2006, Brookmeyer et al. estimated the worldwide prevalence of AD to more than 25 million of patients [Brookmeyer, Johnson et al. 2007]. In Table 1.2 the estimated prevalence of AD for 2006 is shown as well as a forecast on AD prevalence progression. They claimed that the global burden of AD will increase to fourfold by the year 2050 [Brookmeyer, Johnson et al. 2007]. The recent report "Alzheimer’s disease facts and figures 2010" from the Alzheimer’s Association estimates that 5.3 million Americans are affected by AD. About 5.1 millions of these patients are aged older than 65 years and suffer from sporadic AD. The 200’000 younger affected people are early-onset AD patients. Thus, 13% of all Americans aged 65 years or older suffer from AD [Alzheimer's Association 2010]. This number can presumably be adopted for all western countries. A study by Kraft et al. estimated the total of Swiss AD patients in 2007 to 103’000 representing about 10% of the population older than 65 years [Kraft, Marti et al. 2010].

The effects of AD are devastating for the affected patients, but also for their relatives. Patients diagnosed with AD may still have several years to live, but they continuously lose the abilities needed to manage a simple daily life. Thus, in later stages patients are completely dependent on care for even the
simplest tasks. They need intensive care from their relatives or from a professional caregiver.

Wimo and Winblad estimated the worldwide direct cost of all dementia to about 156 billion dollar in 2003 [Wimo, Jonsson et al. 2006]. This estimate excludes all informal care costs, which represent another huge economic burden and are difficult to determine due to lack of methodologies and basic data [Wimo and Winblad 2008]. The total cost of dementia and AD in particular has therefore to be considered enormous. Increasing prevalence with a prognosis of fourfold increase of AD by 2050 leading to an enormous problem of western health policies and might make AD the biggest health economic challenge in the next few decades. There is an urgent need for effective treatments and, therefore, it is important to understand the mechanism leading to AD.

### Table 1.2: Worldwide prevalence of AD in 2006 and 2050. Numbers in millions. From [Brookmeyer, Johnson et al. 2007].

<table>
<thead>
<tr>
<th>Region</th>
<th>initial stage of disease</th>
<th>late stage of disease</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2006</td>
<td>Prediction 2050</td>
<td></td>
</tr>
<tr>
<td>Europe</td>
<td>4.0</td>
<td>9.0</td>
<td>16.5</td>
</tr>
<tr>
<td>North America</td>
<td>1.7</td>
<td>4.8</td>
<td>8.9</td>
</tr>
<tr>
<td>World</td>
<td>15.0</td>
<td>58.8</td>
<td>106.2</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>7.5</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>4.0</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>47.5</td>
<td>59.1</td>
</tr>
</tbody>
</table>
1.3. **Neurofibrillary tangles and tau protein**

In 1986, the microtubule associated protein tau was suggested as the main constituent of the NFTs [Grundke-Iqbal, Iqbal et al. 1986] which was confirmed by sequencing two years later [Goedert, Wischik et al. 1988; Goedert, Spillantini et al. 1989]. In the tau hypothesis, claiming tau to be the main culprit of brain degeneration in AD, hyperphosphorylated tau begins to pair with other threads of tau. Eventually, these tau threads form NFTs inside nerve cell bodies. Those tangles consist of paired helical filaments. They are built of two left handed helices [Wischik, Crowther et al. 1985] of transversely oriented subunits with three to four domains each [Crowther and Wischik 1985; Goedert, Spillantini et al. 1989]. Those domains were identified as tandemly repeated microtubule-binding domains.

Tau containing three or four microtubule-binding domains is widely distributed in the cerebral cortex and hippocampus with exclusive neuronal distribution and no expression in glia cells. In the cortex, tau is mainly expressed in the pyramidal cells. In the hippocampus, the three domain protein in addition is expressed in granule cells [Goedert, Spillantini et al. 1989].

Both isoforms containing three or four microtubule-binding domains can be elongated by insertion of 29 or 58 amino acids in the amino-terminal region of tau [Goedert, Spillantini et al. 1989]. Hence, in humans, tau consists of at least six isoforms [Goedert, Wischik et al. 1988; Goedert, Spillantini et al. 1989; Goedert, Spillantini et al. 1989] produced by alternative splicing from a single gene. The function of those different isoforms is not known. Although they are expressed in a stage and cell-type specific manner, no difference in expression could be found for AD patients compared to healthy individuals and, in addition, multiple isoforms have been shown to be involved in NFT
formation ([Goedert, Spillantini et al. 1989], reviewed by [Goedert, Crowther et al. 1991]).

Tau is concentrated in the axons of neurons. When hyperphosphorylated, tau forms NFTs and the microtubules disintegrate, resulting in the collapse of the neuron’s transport system [Iqbal, Alonso Adel et al. 2005]. This hyperphosphorylation of tau – a hallmark of AD – can occur at various residues. Notably, the site of phosphorylation might have a great influence on protein behaviour. An overview to commonly used biomarkers for AD including some of the differently phosphorylated tau proteins is given in Section 1.10. Two tau protein kinase systems are known to be involved in tau phosphorylation: tau protein kinase systems I and II. A comprehensive review of those two systems can be found in [Imahori and Uchida 1997].

The tau protein kinase system I (TPK I) phosphorylates tau at serine 199, threonine 231, serine 396 and serine 413 (numbering according to the longest human tau) [Ishiguro, Omori et al. 1992]. TPK I does not phosphorylate completely dephosphorylated tau, but instead needs already phosphorylated tau. Interestingly, only tau proteins with TPK I phosphorylation are involved in tangle formation [Ishiguro, Takamatsu et al. 1992].

The tau protein kinase system II (TPK II) consists of the cyclin-dependent kinase 5 (cdk5) [Kobayashi, Ishiguro et al. 1993] and its activator p35 [Uchida, Ishiguro et al. 1994]. The proline directed kinase phosphorylates tau at serine 202, threonine 205, serine 235 and serine 404 (numbering according to the longest human tau) [Ishiguro, Omori et al. 1991]. In contrast to TPK I, this protein kinase system is able to phosphorylate completely dephosphorylated as well as already phosphorylated tau. It does not seem to have a direct relation to tau involved in paired helical filament formation, reviewed by [Imahori and Uchida 1997]. Nevertheless, phosphorylation of tau by cdk5 is one of the key events in neuronal cell death [Alvarez, Toro et al. 1999]. It is
therefore assumed, that tau is first phosphorylated by TPK II, which in turn facilitates phosphorylation by TPK I leading to the hyperphosphorylated tau forming NFTs [Arioka, Tsukamoto et al. 1993].

### 1.4. Amyloid plaques and amyloid beta

In the middle of the 1980ies, the main constituent protein of the amyloid plaques found and described by Alois Alzheimer was identified and called amyloid beta (Aβ) [Glenner and Wong 1984; Masters, Simms et al. 1985]. In 1991, the amyloidogenic hypothesis by Selkoe postulated that an aberrant processing of the so-called amyloid precursor protein (APP) must lead to the pathological structures called plaques consisting of Aβ [Selkoe 1991]. Finally, it was mainly genetic information that led to full identification of the origin of these plaques. The genetic locus of APP could be located on chromosome 21 [Kang, Lemaire et al. 1987]. The fact that individuals with Down Syndrome having three copies of this chromosome and generally exhibiting AD by 40 years of age [Hardy and Allsop 1991] supported the amyloidogenic hypothesis.

Further evidence supporting the hypothesis of cleavage dysfunction leading to plaques and AD came from the finding that transgenic mice expressing the so-called Swedish mutant form of the human APP gene where the two amino acids immediately precedent to the β-cleavage site are affected (Lys670→Asn, Met671→Leu) developed amyloid plaques and AD-like brain pathology [Hsiao, Chapman et al. 1996]. The plaques primarily found in the cortex were thought to be the cause of neuronal loss and brain atrophy.

In 2004 Schmitz et al. found that neuronal loss was not exclusively observed at sites of plaque deposition [Schmitz, Rutten et al. 2004]. In early human
trials, an experimental vaccine was found to clear the amyloid plaques, but it did not have any significant effect on dementia [Holmes, Boche et al. 2008]. As the Aβ deposits were ruled out as main toxic species, researchers suspected small non-plaque Aβ oligomers as the primary pathogenic form. These toxic oligomers bind to a surface receptor on neurons and change the structure of the synapse, thereby disrupting neuronal communication [Klein, Krafft et al. 2001].

The exact mechanism how Aβ promotes neuronal loss is of great interest to understand AD and find possible treatment options. Hence, several newer studies addressing this issue give possible explanations (see also Section 1.6). Since Aβ oligomers induce neuronal cell death, the involvement of an apoptotic cascade is probable. The non-receptor tyrosine kinase c-Abl is involved in many processes [Van Etten 1999]. Among others, c-Abl regulates p73, a member of the p53 family of tumour-suppressor proteins [Agami, Blandino et al. 1999; Ben-Yehoyada, Ben-Dor et al. 2003]. This pre-apoptotic system is involved in regulating neuronal cell death in response to Aβ fibrils. Aβ increases c-Abl activity in rat neuronal cells as well as its interaction with p73. Moreover, neuronal cell death in response to Aβ treatment can be prevented by inhibiting c-Abl activity or expression [Alvarez, Sandoval et al. 2004]. In addition, c-Abl influences cdk5 (see Section 1.6) linking the amyloidogenic hypothesis to the action of tau.
1.5. Formation of Aβ by APP processing

The Aβ fragment is processed from the 695 to 770 amino acids long type I membrane protein APP, containing a large N-terminal extracellular domain and a short intracellular C-terminus [Kang, Lemaire et al. 1987].

During the transit from the endoplasmic reticulum (ER) to the plasma membrane, APP is heavily modified [Weidemann, König et al. 1989; Griffith, Mathes et al. 1995]. Once the fully matured APP reaches the plasma membrane, it is quickly re-internalised by endocytosis and recycled through the trans-Golgi network (TGN) or finally degraded in the lysosome (Figure 1.1). Thus, in the steady state, most of the APP molecules are located at the ER and Golgi with only a small fraction localised at the plasma membrane [Thinakaran and Koo 2008].

Figure 1.1: APP trafficking. APP is shown as black bars. It is modified during traffic via the Golgi to the plasma membrane (1), where it is rapidly internalized (2) and recycled through the endosomal compartments to the TGN and the cell surface (3) or eventually degraded in the lysosome. From [Haass, Kaether et al. 2012].
Figure 1.2: Processing of APP. Schematic representation of non-amyloidogenic and amyloidogenic processing of APP by α-, β- and γ-secretases. The ectodomain of APP can be cleaved by α- or β-secretase, resulting in the release of soluble forms of APP (sAPPα and sAPPβ, respectively). The remaining membrane-bound fragments α-CTF and β-CTF are further cleaved by γ-secretase, releasing p3 or Aβ and AICD.

While trafficking to the plasma membrane and back, APP is cleaved sequentially at two of three possible cleavage sites (see Figure 1.2). First, either an α- or a β-secretase cleaves the ectodomain near the membrane
domain. A soluble fragment called sAPPα or sAPPβ, respectively, is released. The remaining C-terminal fragments (CTF) are further cleaved within the membrane at their γ-position by an enzyme complex called γ-secretase. This cleavage liberates the p3 (after α-cleavage) or the Aβ fragment (after β-cleavage) and an APP intracellular domain (AICD). Depending on the first cleavage, the pathway is called either non-amyloidogenic (after α-cleavage) or amyloidogenic (after β-cleavage), respectively, as the cleavage at APP’s α-site precludes the generation of the toxic Aβ [Haass, Kaether et al. 2012].

Cleavage of APP by the three secretases is dependent on the subcellular localization. α-cleavage preferentially occurs in the late endosomal compartments and at the plasma membrane [Sambamurti, Shioi et al. 1992; Sisodia 1992; De Strooper, Umans et al. 1993; Lammich, Kojro et al. 1999; Skovronsky, Moore et al. 2000]. In contrast, β-cleavage mainly takes place after endocytosis of APP in the early endosomes [Ehehalt, Keller et al. 2003; Kinoshita, Fukumoto et al. 2003]. The γ-cleavage finally is located predominantly at the plasma membrane or in early endosomes [Kaether, Schmitt et al. 2006]. There is evidence that γ-cleavage relies on the endocytic pathway, possibly for recycling of products built by the β-secretase [Koo and Squazzo 1994]. In addition, while β-cleavage and γ-cleavage both are supposed to takes place in lipid raft regions [Cordy, Hussain et al. 2003; Hur, Welander et al. 2008], α-secretases (e.g. ADAM10) are excluded from those rafts [Harris, Pereira et al. 2009]. This implies that a preference for one or the other APP cleavage pathway is dependent on APP localization within organelles and membrane domains as well as on membrane composition (see Section 1.7). This hypothesis is supported by the observation that cholesterol depletion hindering APP internalisation from the cell membrane favours sAPPα secretion [Kojro, Gimpl et al. 2001; Ehehalt, Keller et al. 2003].
In addition, it has been shown that posttranslational modifications of APP can influence APP cleavage preference with an increase in O-GlcNAcylation favouring the non-amyloidogenic pathway [Jacobsen and Iverfeldt 2011] and phosphorylation of the CTFs triggering γ-cleavage [Vingtdeux, Hamdane et al. 2005]. A comprehensive review on APP sorting mechanisms is given by [Small and Gandy 2006], while lipid rafts and their influence on APP cleavage are discussed by [Taylor and Hooper 2007].

Depending on the cleavage-pathway, APP is processed to either p3 or Aβ. Aβ exists in several forms depending on the exact residue of cleavage (see Section 1.5.3), the most important of which are Aβ40 and Aβ42. Aβ42 has been shown to be more prone to aggregation than Aβ40 [Schmechel, Zentgraf et al. 2003]. Therefore it is thought to be the building block of soluble toxic Aβ oligomers [Jarrett, Berger et al. 1993; Mann, Iwatsubo et al. 1996; Haass, Kaether et al. 2012]. In contrast, the soluble sAPPα fragment built by the non-amyloidogenic pathway has neuroprotective functions and seems to enhance memory functions [Furukawa, Sopher et al. 1996; Meziane, Dodart et al. 1998; Stein, Anders et al. 2004]. Thus, the non-amyloidogenic pathway not only mechanistically precludes the formation of Aβ but has per se a protective function on neurons.

While earlier studies using mice models indicated that α- and β-cleavage are coupled, with an increase in α-cleavage leading to a decrease in β-cleavage and vice versa [Postina, Schroeder et al. 2004], newer records indicate that sometimes a reduction in either of both cleavage pathways can also lead to a reduction in the other [Jorissen, Prox et al. 2010]. In agreement with those studies, in different cell lines, among them SH-SY5Y cells, no competition between constitutive α- and β- cleavage was observed [Kuhn, Wang et al. 2010].
Being possible targets for therapies against AD, the three types of secretases have been extensively studied. A comprehensive review of the α-, β- and γ-secretases can be found in [Lichtenthaler, Haass et al. 2011].

1.5.1. α-Secretases

In 1990, cleavage of APP within the Aβ sequence was reported for the first time [Esch, Keim et al. 1990]. Esch et al. found the cleavage site to be located between the amino acids lysine 16 and leucine 17 with the amino acid lysine 16 being removed by a yet unknown mechanism. Four years later, Roberts et al. identified the enzyme as a membrane bound metalloendopeptidase [Roberts, Ripellino et al. 1994]. Based on this findings several enzymes were proposed to act as α-secretases, among them members of the α disintegrin and metalloproteinase (ADAM) protein family such as ADAM9 [Koike, Tomioka et al. 1999], ADAM10 [Lammich, Kojro et al. 1999] and ADAM17, also called tumour necrosis factor-α converting enzyme (TACE) [Slack, Ma et al. 2001]. ADAM19 was proposed to have α-secretase activity by RNA interference experiments. However, this enzyme did not cleave APP in vitro leading to the hypothesis that ADAM19 might indirectly activate APP cleavage, possibly by activating other ADAMs [Tanabe, Hotoda et al. 2007]. Several studies led to the assumption that there is no unique α-cleaving enzyme, but rather the interplay of a group of ADAMs namely ADAM9, 10 and 17 and possibly other enzymes [Asai, Hattori et al. 2003; Allinson, Parkin et al. 2004]. In the absence of one of those enzymes another might thus compensate its enzymatic activity at least partially. Nevertheless, ADAM10 has been shown to possess the highest α-cleavage capability and, therefore, is proposed as the major α-secretase [Allinson, Parkin et al. 2004; Jorissen, Prox et al. 2010; Kuhn, Wang et al. 2010].
Table 1.3: Regulation of ADAM10. Data from [Endres and Fahrenholz 2010].

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA receptor</td>
<td>Promotes gene expression.</td>
</tr>
<tr>
<td>Sp1 (transcription factor)</td>
<td>Promotes gene expression.</td>
</tr>
<tr>
<td>USF (transcription factor)</td>
<td>Promotes gene expression.</td>
</tr>
<tr>
<td>Furin and other convertases</td>
<td>Prodomain cleavage, increases activity.</td>
</tr>
<tr>
<td>Prodomain of ADAM10</td>
<td>Transient inhibitor and internal chaperone, inhibits activity.</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Binding to ADAM10 inhibits maturation.</td>
</tr>
</tbody>
</table>

Enhancers of enzyme activity

<table>
<thead>
<tr>
<th>Enhancer</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Depletion of cholesterol enhances ADAM10 activity.</td>
</tr>
<tr>
<td>HMG-CoA reductase inhibitors (statins)</td>
<td>Depletion of cholesterol enhances ADAM10 activity.</td>
</tr>
<tr>
<td>Type-III secretory phospholipase A</td>
<td>Substrate availability at the cell surface enhances activity.</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td></td>
</tr>
<tr>
<td>Nardilsyn</td>
<td></td>
</tr>
<tr>
<td>Tetraspanin 12</td>
<td></td>
</tr>
<tr>
<td>G protein-coupled receptors (GPCR)</td>
<td></td>
</tr>
<tr>
<td>GPCR ligands</td>
<td></td>
</tr>
</tbody>
</table>

ADAM10 is a type I membrane protein mediating proteolysis of APP in the late compartments of the secretory pathway and at the plasma membrane [Sambamurti, Shioi et al. 1992; Sisodia 1992; De Strooper, Umans et al. 1993; Lammich, Kojro et al. 1999; Skovronsky, Moore et al. 2000]. Gene expression of ADAM10 is up-regulated by retinoic acid (RA) [Prinzen, Muller et al. 2005; Tippmann, Hundt et al. 2009]. This up-regulation can even be
reinforced by simultaneous addition of brain-derived neurotrophic factor (BDNF) [Holback, Adlerz et al. 2005]. Besides the up-regulation by retinoic acid, several other factors have been described to influence ADAM10 expression or activity, among them up-regulation by PI3-K which in turn is also influenced by RA [Holback, Adlerz et al. 2008], up-regulation by protein kinase C (PKC) [Lammich, Kojro et al. 1999] and cleavage by convertases (e.g. furin) leading to a fully maturated and functional enzyme [Hwang, Kim et al. 2006]. Interestingly, ADAM10 is itself cleaved by ADAM9, ADAM15 and the γ-secretase complex [Parkin and Harris 2009; Tousseyn, Thathiah et al. 2009]. The mechanisms of ADAM10 regulation are discussed in detail by Endres et al. [Endres and Fahrenholz 2010]. Some of the results are summarised in Table 1.3. Obviously, these regulations of ADAM10 might have important implications for the generation of either sAPPα or the Aβ peptide. Apart from APP, ADAM10 sheds over 30 different membrane proteins, among them Notch. A comprehensive overview of ADAM10’s substrates is given by Reiss and Saftig [Reiss and Saftig 2009].

1.5.2. β-Secretase / BACE1

In 1999, the β-site APP-cleaving enzyme 1 (BACE1) was identified as the first β-secretase. It is a membrane-bound aspartic protease [Sinha, Anderson et al. 1999; Vassar, Bennett et al. 1999]. Up to date, BACE1 still is the only known enzyme featuring relevant β-secretase activity towards APP. Although a homologous aspartic protease called BACE2 was identified by database search, several studies suggest it to function as alternative α-secretase, since cleavage at the β-site is only of subordinate significance (reviewed by [Vassar 2004]). A singular role of BACE1 in β-cleavage is supported by the fact that its knock-out completely abolishes Aβ production [Cai, Wang et al. 2001; Luo,
Bolon et al. 2001] and no compensatory genes, e.g. BACE2, are up-regulated [Luo, Bolon et al. 2003]. Moreover, BACE1 deficient mice do not develop major behavioural or morphological deficits [Luo, Bolon et al. 2001; Roberds, Anderson et al. 2001]. Thus, BACE1 is an interesting target in AD therapy (see Section 1.11).

BACE1 is ubiquitously expressed with especially high levels in the brain and pancreas. It localizes to the Golgi, the TGN and the endosomes [Vassar, Bennett et al. 1999]. At the cell surface BACE1 co-localizes with APP. BACE1 and APP are rapidly internalized and remain co-localized in the endosomes, where the enzymatic activity of the β-secretase appears to be highest due to a favourable pH milieu [Kinoshita, Fukumoto et al. 2003; Lichtenthaler, Haass et al. 2011].

Figure 1.3: The γ-secretase complex. The four subunits nicastrin, Aph-1, Pen-2 as well as the catalytic subunit presenilin form the active γ-secretase. From [Schellenberg and Montine 2012].
1.5.3. The $\gamma$-secretase complex

The $\gamma$-secretase consists of a complex of four proteins: the catalytic subunit presenilin 1 or 2 (PS1 or PS2) [Xia, Zhang et al. 1997; De Strooper, Saftig et al. 1998], nicastrin (NCT) [Yu, Nishimura et al. 2000], anterior pharynx defective (APH-1) and presenilin enhancer (PEN)-2 [Francis, McGrath et al. 2002] (Figure 1.3). Those four subunits are necessary as well as sufficient for $\gamma$-secretase activity [Edbauer, Winkler et al. 2003]. Presenilin is an aspartyl protease cleaving within lipid bilayers [Wolfe, Xia et al. 1999]. Mutations in the presenilin gene are the major genetic causes for inheritable forms of AD (see Section 1.9.1). While PEN-2 seems to facilitate presenilin endoproteolysis into its active heterodimeric state and stabilizes it within the $\gamma$-secretase complex [Prokop, Shirotani et al. 2004] little is known about the biological functions of APH-1 and NCT.

![Figure 1.4: Sequential processing of APP by $\gamma$-secretase.](image)

Two product lines are discussed leading either to the production of $A\beta_{40}$ (top) or $A\beta_{42}$ (bottom). Each line starts with cleavage at the $\varepsilon$-site, followed by the $\zeta$-site and one of the $\gamma$-sites. From [Haass, Kaether et al. 2012].
It is supposed that the $\gamma$-secretase cleaves sequentially within the transmembrane domain at so-called $\varepsilon$- [Sastre, Steiner et al. 2001; Weidemann, Eggert et al. 2002], $\zeta$- [Zhao, Mao et al. 2004] and $\gamma$-sites. In addition, the final $\gamma$-cleavage is also not precise giving rise to several $A\beta$ peptides differing in length from 37 to 43 amino acids, with $A\beta40$ and $A\beta42$ being the most common forms. It is supposed that those two peptides arise due to two product lines in a stepwise cleavage mechanism (Figure 1.4) [Zhao, Mao et al. 2004; Qi-Takahara, Morishima-Kawashima et al. 2005; Zhao, Cui et al. 2005]. Because the cleavage site lies within the lipid bilayer, it is thought that the $\gamma$-secretase does not recognize a special sequence, but rather an $\alpha$-helical structure common in transmembrane regions. Such a model would place the respective cleavage sites for $A\beta40$ and $A\beta42$ on opposite sides of the helix, giving a hint why several mutations may favour either of the two peptides [Wolfe, Xia et al. 1999]. Recently, it has been proposed that APP might form homodimers further influencing $\gamma$-cleavage by limiting access of the secretase complex to certain residues [Munter, Voigt et al. 2007].

Besides APP, the $\gamma$-secretase also cleaves Notch and other membrane proteins by a yet poorly understood mechanism [Lichtenthaler, Haass et al. 2011].

### 1.6. Tau and A$\beta$ are likely to interact in AD

Nowadays, a combined action of both tau and A$\beta$ is thought to be responsible for the brain pathology found in AD patients. Therefore, the updated amyloidogenic hypothesis also includes the neurotoxic action of tau, where an excess of A$\beta$ triggers the hyperphosphorylation of tau leading to increased
tangle production. Indeed, Aβ treatment of neuronal rat cells led to neuronal cell death and up-regulation of TPK II [Alvarez, Toro et al. 1999], which is thought to trigger phosphorylation of tau by TPK I, necessary for the formation of NFT [Arioka, Tsukamoto et al. 1993]. In addition, inhibition of the catalytic TPK II subunit cdk5 prevented this neuronal cell death in a concentration dependent manner [Alvarez, Toro et al. 1999]. A new study gives evidence on the mechanism of this interaction: Aβ increases c-Abl total protein and activity (see Section 1.4) as well as triggering phosphorylation of c-Abl at tyrosine 412, which is linked to enhanced c-Abl activity [Cancino, Perez de Arce et al. 2011]. C-Abl in turn participates in the Aβ induced phosphorylation of tau through activation of cdk5. Activation is achieved by phosphorylation at tyrosine 15 [Cancino, Perez de Arce et al. 2011].

In addition, while neurons expressing tau protein are degenerated in the presence of Aβ, tau depleted neurons are not [Rapoport, Dawson et al. 2002]. Therefore, tau protein seems to be involved in Aβ induced neurotoxicity in the central nervous system (CNS). Microtubule stabilization by taxon restores Aβ induced neurotoxicity in tau depleted cells [Rapoport, Dawson et al. 2002]. More dynamic microtubule formations might thus confer resistance to Aβ triggered neurodegeneration.

While Aβ toxicity leads to loss of synapses and spines and impairs long-term potentiation, tau aggregation interferes with axonal transport and mitochondrial respiration [Ittner and Gotz 2011]. These effects finally lead to massive neuronal loss, triggering chronic inflammation of whole brain regions [Sastre, Richardson et al. 2011].
1.7. **Fatty acids and membrane composition in AD progression**

The fatty acid composition heavily influences physicochemical properties of membranes, such as fluidity, thickness and permeability. Unsaturated fatty acids pack less dense than saturated fatty acids due to their kinked conformation and, therefore, provide higher fluidity and smaller thickness to membranes [King, Stavens et al. 1977; Alberts, Johnson et al. 2002]. Studies of the topology of the C-terminal part of APP led to the conclusion that the α-cleavage site loops back to the plasma membrane (Figure 1.5) [Beel, Mobley et al. 2008]. Such a conformation situates the α-cleavage site of APP only few amino acids away from the membrane. Thus, membrane thickening could block the access of cleaving enzymes to the cleavage site. Therefore, APP or its cleaving enzymes BACE1 and ADAM10 could indirectly be influenced by the fatty acid composition of membranes.

Cholesterol was the first membrane constituent shown to affect APP cleavage stimulating the non-amyloidogenic pathway [Kojro, Gimpl et al. 2001; Ehehalt, Keller et al. 2003]. Ehehalt showed that lipid rafts are involved in regulating β-cleavage of APP. Co-localization of APP and BACE1 in lipid rafts at the plasma membrane led to an increase in Aβ production [Ehehalt, Keller et al. 2003]. Moreover, it has been shown that lipid rafts influence the trafficking of APP cleaving enzymes and thus the preferential APP cleavage-site as reviewed in [Bali, Halima et al. 2010]. Beside cholesterol, other lipids came in the focus of interest. In a cohort study, increased uptake of docosahexaenoic acid (DHA), the most abundant poly-unsaturated fatty acid (PUFA) present in the human brain, was shown to reduce the risk for AD [Engelhart, Geerlings et al. 2002]. Similarly Mediterranean diet or diet rich in fish, containing high amounts of DHA or other PUFAs, appeared to have a neuroprotective effect.
in humans [Morris, Evans et al. 2003] and a rat model for AD [Hashimoto, Hossain et al. 2002]. However, several other studies investigating the effects of DHA or other PUFAs on AD failed to demonstrate a benefit. Animal studies in transgenic mice [Arendash, Jensen et al. 2007] and studies in AD patients [Schaefer, Bongard et al. 2006] could not support the theory that DHA would protect from cognitive decline. Therefore, the impact of dietary intake of PUFAs on disease progression is still heavily discussed, but no conclusion has been drawn so far [Cunnane, Plourde et al. 2009; Huang 2010; Jicha and Markesbery 2010].

Figure 1.5: Sequence and topology of C-terminal APP. The estimated topology of the 99 C-terminal amino acids of APP. Cleavage sites of α- and γ-secretase as well as sites of known disease-linked mutations are indicated. Schematic from [Beel, Mobley et al. 2008].
Another class of fatty acids prone to influence the APP cleavage process are unsaturated fatty acids with a *trans* configuration [Morris, Evans et al. 2003]. The amount of *trans*-fatty acids in western diets was shown to increase [Emken 1984]. Dietary *trans*-fatty acids are incorporated in membranes and may alter membrane properties, affecting enzyme activities [Larque, Garcia-Ruiz et al. 2003]. The non-natural elaidic acid (EA) present in hardened oils was found to be the most abundant fatty acid in atheromatous plaques of patients undergoing surgery due to atherosclerotic stenosis of the abdominal aorta, iliac or femoral arteries [Stachowska, Dolegowska et al. 2004].

Yang et al. showed that fatty acids containing 3 or less double bounds, such as *trans*-vaccenic acid (VA), EA or stearic acid (SA), do not change membrane fluidity and sAPPα secretion [Yang, Sheng et al. 2011]. On the other hand, fatty acids with more than 3 double bonds, e.g. DHA, clearly increased membrane fluidity and concomitantly also increased sAPPα secretion in SH-SY5Y cells [Yang, Sheng et al. 2011].

In order to elucidate the membrane’s influence on APP cleavage, a previous study in our group analysed the cleavage of a short synthetic peptide at the α-cleavage site. The amino acid sequence of the peptide was similar to the sequence of APP around its α-cleavage site and transmembrane domain. This peptide was incorporated into liposomes of differing lipid composition. Trypsin, cleaving at the α-site, was used as model secretase. The cleavage kinetics were measured and a correlation between kinetics and lipid composition was established [Marenchino, Williamson et al. 2008]. The outcome of this study showed clear correlation between fatty acid composition and cleavage kinetics at α-site and provided support for the theory of membrane composition driven α-cleavage of APP.
1.8. Altered cortisol levels – cause or result of AD?

The steroid hormone cortisol is produced by the adrenal gland in response to acute stress. Its release is controlled by the hypothalamic-pituitary-adrenal (HPA) axis: The hypothalamus secretes the corticotropin-releasing hormone which induces the adrenocorticotropic hormone in the pituitary gland. Adrenocorticotropic hormone in turn is transported via the blood to the adrenal gland stimulating cortisol release. Since AD is characterized by a degeneration of brain structure, the HPA axis might be disrupted and an influence on cortisol levels is conceivable. Nevertheless, cortisol might in turn also influence AD by various means and it is not yet clear whether these elevated levels are a cause or a result of AD. Moreover, the hippocampus, one of the most vulnerable structures to AD, is rich in glucocorticoid receptors. It therefore is influenced by cortisol, but in turn might also result in a reduced negative feedback loop on the HPA-axis leading to increasing cortisol production. This cycle of regulation between hippocampus and HPA-axis is generally referred to as the glucocorticoid cascade hypothesis for hippocampal aging [McEwen 1992; Landfield and Eldridge 1994; Sapolsky 1994]. Indeed, AD patients generally display elevated serum cortisol levels [Laske, Stransky et al. 2009], whereas adrenalectomy in rats lowering corticoid levels has been shown to retard brain aging [Landfield, Baskin et al. 1981].

Moreover, cortisol has also been shown to be toxic to neurons, especially in the hippocampus where high concentrations of glucocorticoid receptors occur. Its effects on the hippocampus are dose dependent. While chronic intermediate doses are neuroprotective in rats, chronic low or high doses potentiate Aβ toxicity [Abraham, Harkany et al. 2000]. This differing effect is based on the different glucocorticoid receptors. Mineralocorticoid receptors showing high affinity for cortisol are activated by basal cortisol concentrations
leading to a long-term potentiation of the hippocampus. Lower affinity glucocorticoid receptors on the other hand need high levels for activation. In addition, prolonged elevation of cortisol levels has been associated with pathology in most body systems leading to diseases such as diabetes, hypertension, hypercholesterolemia, arterial diseases, impairment of growth and tissue repair (reviewed by [Sapolsky 1994]). Under stress conditions, cortisol leads to an energy crisis in brain tissue by redistribution of the available energy and its concentration significantly correlates with hippocampal atrophy [de Leon, McRae et al. 1988]. This energy crisis has been proposed to be at least in part responsible for neuronal cell death in AD, as reviewed by [Sapolsky 1994].

Another interesting feature of cortisol is its ability to influence immune reactions. Activation of microglia cells, which function as brain macrophages, leads to a reduction of neuronal cell survival in mice, where functional loss proceeds cell death [Chao, Hu et al. 1992]. Activation of microglia cells seems thus to be associated with neurodegenerative diseases such as AD [Rogers, Luber-Narod et al. 1988; Chao, Hu et al. 1992; Gonzalez-Scarano and Baltuch 1999]. Activated microglia cells release nitric oxide and tumour necrosis factor-α (TNF-α), both having a dual role by protecting individuals against pathogens but meanwhile destroying innate cells, e.g. neurons [Merrill and Zimmerman 1991; Chao, Hu et al. 1992; Merrill, Ignarro et al. 1993]. Cortisol is able to repress this release [Frei, Siepl et al. 1987; Chao, Hu et al. 1992; Drew and Chavis 2000]. Hence, neurons might be protected by hindering production of those cytotoxic molecules. This influence on the immune system as well as its simple access through the blood-brain barrier makes cortisol and its synthetic forms an interesting choice for the treatment of a variety of diseases.
Csernanski and co-workers found that increased plasma cortisol levels were associated with more rapid disease progression in patients with AD [Csernansky, Dong et al. 2006]. In addition, a German research group found elevated cortisol concentrations in cerebrospinal fluid (CSF) in AD patients, but not in subjects with mild cognitive impairment (MCI) [Popp, Schaper et al. 2009]. Higher cortisol levels were associated with better memory performance in healthy elderly, while higher cortisol levels were correlated with poorer memory performance in patients with MCI. No correlation between elevated cortisol levels and memory dysfunction was found in the AD group [Souza-Talarico, Chaves et al. 2010]. Animal studies with transgenic mice bearing several mutations increasing production of Aβ and hyperphosphorylated tau suggested cortisol to be rather a cause than a consequence for neuropathology in AD: cortisol administration to these mice further increased the Aβ load [Green, Billings et al. 2006].

Cortisol levels not only increase in AD but also with age [Yen and Laughlin 1998] and might contribute to cognitive deficiencies related to normal human aging [Lupien, de Leon et al. 1998]. Aged humans with high basal cortisol levels and an increase in cortisol concentrations show a hippocampal volume reduced by up to 14% as well as deficits in hippocampal related memory tasks [Lupien, de Leon et al. 1998]. A positive slope of cortisol change is therefore associated with cognitive impairment [Lupien, Lecours et al. 1994] and appears to be related to the clinical progression of AD but independent of length of survival [Weiner, Vobach et al. 1997].

It has been proposed that cortisol might act via glutamate by influencing calcium signalling pathways, although the exact mechanism of cortisol action in any of the presented fields is not yet clear [Landfield and Eldridge 1994]. Energy depletion triggers depolarization of neurons which in turn release glutamate. Glutamate leads to elevated calcium levels in the cytosol, thus
triggering many events, e.g. activation of proteases and phosphorylation of tau (reviewed by [Sapolsky 1994]). In this way, elevated cortisol levels might have a direct influence on AD progression by increasing tau hyperphosphorylation and thus favour the formation of NFTs.

1.9. Genetic risk factors for Alzheimer’s disease

Two types of AD are distinguished: inherited AD and sporadic AD. Inherited AD usually starts at a remarkably young age of <60 years. Hence, it is often called early-onset AD. It is genetically inherited in an autosomal dominant pattern and children of an affected parent have a 50% risk for developing early-onset AD. The far more common sporadic form of AD is not inherited. It starts in late age and is therefore termed late-onset AD. Only rarely cases of inherited AD also have a late onset.

1.9.1. Genes involved in inherited AD

Already very early in AD research (between 1987 and 1995) three autosomal dominant genes were identified to be involved in early-onset AD: APP, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Table 1.4). Individuals with mutations in one of these genes inevitably develop AD. Mutations in APP, PSEN1 and PSEN2 are proposed to account for 30 to 50% of all cases of early-onset AD. Although those mutations are rare causes of AD in general (approximately 0.5%), discovery of the responsive genes led to a broader understanding of the mechanisms involved in AD (reviewed by [Schellenberg and Montine 2012]).
Table 1.4: Autosomal dominant AD genes. From [Schellenberg and Montine 2012].

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Inheritance</th>
<th>Location</th>
<th>Onset range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid precursor protein</td>
<td>APP</td>
<td>Autosomal dominant</td>
<td>21q21.3</td>
<td>38–69</td>
</tr>
<tr>
<td>Presenilin 1</td>
<td>PSEN1</td>
<td>Autosomal dominant</td>
<td>14q24.2</td>
<td>25–65</td>
</tr>
<tr>
<td>Presenilin 2</td>
<td>PSEN2</td>
<td>Autosomal dominant, reduced penetrance</td>
<td>1q42.13</td>
<td>41–88</td>
</tr>
</tbody>
</table>

Based on the work of Glenner and Wong [Glenner and Wong 1984], the APP sequence was resolved in 1987 and the genetic locus could be located on chromosome 21 [Kang, Lemaire et al. 1987], explaining why people with Down Syndrome having three copies of this chromosome generally exhibit early-onset AD [Hardy and Allsop 1991]. To date, 24 mutations in APP are known to cause AD [Cruts 2007]. Those mutations are mostly located at either of three sites: immediately before the beginning of the Aβ sequence, in the Aβ sequence itself or after the C-terminal end of it (Figure 1.6). One of the most famous mutations at the beginning of the Aβ sequence is the Swedish mutation: A double amino acid substitution at position 670/671 [Mullan, Crawford et al. 1992] increases the amount of Aβ peptide produced by up to six fold in vitro [Citron, Oltersdorf et al. 1992] or up to twofold in vivo [Rabe, Reichwald et al. 2011] due to an increase in β-cleavage of APP. Alterations at the C-terminal end of Aβ affect γ-cleavage. They favour building of the more amyloidogenic Aβ42 instead of shorter forms of Aβ [Herl, Thomas et al. 2009]. While those two types of mutations change overall Aβ levels, mutations inside the gene either affect Aβ levels by influencing α-cleavage [Haass, Hung et al. 1994] or they affect aggregation and neurotoxicity of the peptide by changing its sequence [Murakami, Irie et al. 2002; Lam, Teplow et al. 2008].
Figure 1.6: Mutations in APP leading to early-onset AD. The amino acid sequence of APP and the respective sites of mutations are indicated. The most famous mutations are the Swedish mutation (K670L/M671L double mutation), the arctic mutation (E693G) and the Flemish mutation (A692G). From [Schellenberg and Montine 2012].

Over 180 mutations in \textit{PSEN1} and 13 in \textit{PSEN2} are known to cause AD [Cruts 2007]. While all mutations in \textit{PSEN1} immediately lead to early-onset AD, those in \textit{PSEN2} have a lower penetrance. Thus, people carrying a mutation on \textit{PSEN2} do sometimes develop late-onset instead of early-onset AD. The two presenilin proteins PS1 and PS2 are closely related and either of them can be part of the $\gamma$-secretase complex by building the catalytic subunit (see Section 1.5.3). Some mutant forms of PS1 (L392V, M146L and L286V) were shown to favour the building of amyloidogenic A$\beta$42 [Citron, Eckman et al. 1998].
Table 1.5: Genes implicated in late-onset AD risk. Odds ratio (OR) on 95% confidence interval (CI), data for SORL1 from [Reitz, Cheng et al. 2011], for all other genes from [Naj, Jun et al. 2011]. Adapted from [Schellenberg and Montine 2012].

<table>
<thead>
<tr>
<th>Gene</th>
<th>ORs (CI)</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE</td>
<td>3.84 (3.56–4.14)</td>
<td>Lipid metabolism and transport, Aβ trafficking, synaptic function, immune regulation, intracellular signalling.</td>
</tr>
<tr>
<td>CLU</td>
<td>0.89 (0.85–0.93)</td>
<td>Lipid transport, eventually: extracellular chaperone influencing Aβ aggregation and clearance.</td>
</tr>
<tr>
<td>CR1</td>
<td>1.16 (1.11–1.22)</td>
<td>Cell surface receptor, which is part of the complement system, eventually: participates in clearance of Aβ.</td>
</tr>
<tr>
<td>PICALM</td>
<td>0.87 (0.84–0.91)</td>
<td>Clathrin-mediated endocytosis.</td>
</tr>
<tr>
<td>BIN1</td>
<td>1.17 (1.13–1.22)</td>
<td>Not much known, member of a family of proteins involved in clathrin-mediated endocytosis.</td>
</tr>
<tr>
<td>CD33</td>
<td>0.91 (0.88–0.93)</td>
<td>Cell surface immune receptor, the role in the brain is unknown.</td>
</tr>
<tr>
<td>ABCA7</td>
<td>1.15 (1.09–1.21)</td>
<td>Member of the ABC superfamily of transporters, lipid metabolism, eventually: efflux of phospholipids and cholesterol.</td>
</tr>
<tr>
<td>EPHA1</td>
<td>0.90 (0.86–0.93)</td>
<td>Not much known, member of the ephrin family of receptor protein-tyrosine kinases involved in cell adhesion, migration and axon guidance, synapse formation and regulation of apoptosis in neuronal progenitor cells.</td>
</tr>
<tr>
<td>CD2AP</td>
<td>1.11 (1.07–1.15)</td>
<td>Scaffolding protein involved in cytoskeletal organization and synapse formation.</td>
</tr>
<tr>
<td>MS4A4A/ MS4A6A/ MS4A4E</td>
<td>0.89 (0.87–0.92)</td>
<td>Not much known, eventually: immune-related function (high homology to CD20).</td>
</tr>
<tr>
<td>SORL1</td>
<td>1.08 (1.03–1.13)</td>
<td>Intracellular APP trafficking, lipoprotein receptor for ApoE.</td>
</tr>
</tbody>
</table>
1.9.2. **Risk factors for sporadic AD**

For a long time, it was not possible to find risk factors for sporadic AD which could be reproduced. The only exceptions were the ε4 allele of APOE [Corder, Saunders et al. 1993; Strittmatter, Saunders et al. 1993] and the SORL1 gene [Scherzer, Offe et al. 2004; Andersen, Reiche et al. 2005; Rogaeva, Meng et al. 2007]. Only in the last three years new techniques using genome-wide association studies allowed reproducibly identifying an additional nine genes able to augment the risk for AD. Although those genes increase susceptibility for late-onset AD, none of them *per se* is a sufficient cause for AD. All susceptibility genes known so far are listed in Table 1.5 (reviewed in [Schellenberg and Montine 2012]). By far the most important of these still is the ε4 allele of APOE.

![Figure 1.7: Possible mechanisms of ApoE in influencing AD. From [Kim, Basak et al. 2009].](image-url)
The 299 amino acids long apolipoprotein E (ApoE) is involved in receptor-mediated endocytosis of lipoprotein particles and, thus, is essential for the catabolism of cholesterol and triglycerides [Pitas, Boyles et al. 1987; Fagan, Holtzman et al. 1999]. Newer studies show an influence on lipid transport, Aβ trafficking, synaptic functions, immune regulation and intracellular signalling (reviewed in [Kim, Basak et al. 2009]). Nevertheless, the main influence of ApoE on AD progression is thought to be due to on Aβ aggregation and Aβ clearance (Figure 1.7).

Several single-nucleotide polymorphisms of APOE are known, with the most common ones leading to the ε2, ε3 and ε4 alleles (Figure 1.8). While the ε4 allele has been linked to an increase in AD susceptibility [Corder, Saunders et al. 1993], ε2 lowers the risk for developing AD [Corder, Saunders et al. 1994; Farrer, Cupples et al. 1997]. Those effects are additive, increasing the risk for people having two copies of the ε4 allele compared to those, having only one [Corder, Saunders et al. 1993]. Likewise, two alleles of ε2 offer more protection than just one [Farrer, Cupples et al. 1997]. The risk for an individual homozygous for ε4 to develop AD by the age of 75 is estimated to be around 33%, while it increases to 52% for men and 68% for women, respectively, by the age of 85 [Schellenberg and Montine 2012].

ApoE ε4 leads to increased cholesterol concentration. As cholesterol depletion favours sAPPα secretion [Kojro, Gimpl et al. 2001; Ehehalt, Keller et al. 2003], its increase in contrast is suspected to significantly influence plaque deposition and APP metabolism, as reviewed in [Poirier 2000]. Interestingly, APOE influences onset age in people carrying mutations in either PSEN1 or PSEN2.
Due to the known interaction of ApoE with low-density lipoprotein receptors, a possible link of those receptors to AD was suspected. A first study showing that loss of the receptor SorL1 (also known as LR11 or SorLA) being linked to AD [Scherzer, Offe et al. 2004] was confirmed by others: SorL1 has been shown to be an important factor in APP trafficking. While cells expressing normal levels of SorL1 direct APP to the Golgi, decreased levels lead to APP trafficking to late endosomal compartments favouring β-cleavage (Figure 1.9) [Andersen, Reiche et al. 2005; Rogaeva, Meng et al. 2007].
1.10. Diagnosis of AD

In order to evaluate severity of AD and treatment efficacy, specific and stable biomarkers are necessary. Such markers allow evaluation of treatment success already in a small group of patients, without the need for an elongated treatment period. Up to date, several markers in the CSF are used. Tau in CSF of AD patients is elevated compared to patients with other types of dementia, such as frontotemporal dementia or vascular dementia, as well as compared to Parkinson’s disease [Sjogren, Davidsson et al. 2001; Sunderland, Linker et al. 2003; Hampel, Buerger et al. 2004]. Some studies state an inverse correlation of CSF tau with education, suggesting education being a possible protective factor [Sunderland, Linker et al. 2003]. It might be
speculated that total tau protein reflects the amount of NFTs. However, correlation with the commonly used mini-mental state examination score range from not significant to a weak but significant correlation [Sunderland, Linker et al. 2003; Haense, Buerger et al. 2008].

A more reliable marker might be hyperphosphorylated tau, which has been shown to be necessary for NFT formation. Several of those hyperphosphorylated tau isoforms are commonly used as biomarkers: tau phosphorylated at threonine 181 (P-tau$_{181}$P) [Vanmechelen, Vanderstichele et al. 2000; Sjogren, Davidsson et al. 2001; Hampel, Buerger et al. 2004; Buerger, Alafuzoff et al. 2007], at serine 199 (P-tau$_{199}$P) [Hampel, Buerger et al. 2004] or at threonine 231 (P-tau$_{231}$P) [Kohnken, Buerger et al. 2000; Hampel, Buerger et al. 2004; Buerger, Ewers et al. 2006]. P-tau$_{199}$P as well as P-tau$_{231}$P is phosphorylated by TPK I [Ishiguro, Omori et al. 1992] and known to be involved in NFT formation [Ishiguro, Takamatsu et al. 1992]. Levels of all three phosphorylated tau subtypes are augmented in CSF of AD patients [Hampel, Buerger et al. 2004]. Notably, the site of phosphorylation might nevertheless have a great influence on tau aggregation.

P-tau$_{181}$P is elevated in CSF of AD patients compared to patients with other types of dementia [Sjogren, Davidsson et al. 2001; Hampel, Buerger et al. 2004]. Nevertheless, no correlation to neurofibrillary pathology could be found [Buerger, Alafuzoff et al. 2007]. In contrast, P-tau$_{231}$P measured in CSF correlate with levels of P-tau$_{231}$P measured post mortem in the diseased neocortical brain and with NFTs and other neuropathological measures for AD. Interestingly, this correlation is not seen in the hippocampus [Buerger, Ewers et al. 2006]. This lack of correlation in hippocampus is not yet explained, but might be due to its relatively small size leading to only a small effect on total P-tau levels in CSF. Taken together, alternative
phosphorylation of tau proteins might lead to big differences in neurofibrillary pathology.

Another commonly used AD marker is Aβ42. Interestingly, total CSF Aβ42 levels are significantly lower in AD patients than in control individuals [Sunderland, Linker et al. 2003]. Compared to tau, Aβ42 shows higher inter-case variability [Blennow, Zetterberg et al. 2007]. Since AD is a disease with long clinical course, biomarkers should provide long-lasting stability. Both tau and Aβ42 show stable concentrations in CSF for more than 6 month and therefore seem to be suitable for clinical use [Blennow, Zetterberg et al. 2007].

In summary, it has to be stated that none of the so far used biomarkers is optimal for AD diagnosis. A combination of the biomarkers can increase the accuracy of diagnosis but much more work is needed to find a good measure for AD severity, which is an inevitable prerequisite for an early and adapted treatment and drug development.

Table 1.6: Approved drugs against AD in Switzerland [Documed 2012].

<table>
<thead>
<tr>
<th>Drug</th>
<th>Trade name (Company)</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donepezil</td>
<td>Aricept (Pfizer)</td>
<td>AChEI</td>
</tr>
<tr>
<td></td>
<td>Generics</td>
<td></td>
</tr>
<tr>
<td>Rivastigmine</td>
<td>Exelon (Novartis)</td>
<td>AChEI</td>
</tr>
<tr>
<td>Galantamine</td>
<td>Reminyl (Janssen-Cilag)</td>
<td>AChEI</td>
</tr>
<tr>
<td>Memantine</td>
<td>Axura (Merz)</td>
<td>NMDA receptor antagonist</td>
</tr>
<tr>
<td></td>
<td>Ebixa (Lundbeck)</td>
<td></td>
</tr>
</tbody>
</table>
1.11. Treatments for Alzheimer’s Disease

AD is reaching epidemic proportions (see Section 1.2) and therefore effective treatments are greatly needed. Table 1.6 summarises the currently approved AD treatments in Switzerland. Current drugs for AD target cholinergic and glutamatergic neurotransmission (e.g. AChEI and NMDA receptor antagonists), thus alleviating symptoms. Nevertheless their neuroprotective activity is still debated [Wenk, Parsons et al. 2006]. None of the currently approved drugs significantly slows down disease progression, although much effort is directed towards identifying disease-modifying therapies, with several compounds in different phases of clinical development [Mangialasche, Solomon et al. 2010]. Several lines of clinical trials for new drugs are ongoing.

New treatment strategies target Aβ to either prevent its aggregation or to raise an immune response. Bapineuzumab is one example of an antibody targeting soluble oligomers of Aβ [Kerchner and Boxer 2010]. Other therapies aim at blocking BACE1 thereby enhancing the non-amyloidogenic α-cleavage. Since a singular role of BACE1 in β-cleavage leading to Aβ formation is supposed [Cai, Wang et al. 2001; Luo, Bolon et al. 2001] and BACE1 deficient mice do not develop major behavioural or morphological deficits [Luo, Bolon et al. 2001; Roberds, Anderson et al. 2001] BACE1 is one of the most interesting targets in AD therapy. However, development of such agents has turned out to be extremely challenging. The first orally available non-peptidic BACE1 inhibitor, LY2811376 [(S)-4-(2,4-difluoro-5-pyrimidin-5-yl-phenyl)-4-methyl-5,6-dihydro-4H-[1,3]thiazin-2-ylamine], led to significant Aβ reduction in mice [May, Dean et al. 2011]. Nevertheless, toxicological studies revealed retinal pathology in long term examinations making it necessary to stop further development of the drug [May, Dean et al. 2011].
CHAPTER 1: INTRODUCTION

Figure 1.10: Drugs in clinical trials. Drugs being investigated for Alzheimer's disease therapy, reported according to the most advanced phase of study and main therapeutic properties (including data from studies in vitro and from animal models). BBS1: anti-β-site antibodies. BDNF: brain-derived neurotrophic factor. EGCg: epigallocatechin-3-gallate. IVIg: intravenous immunoglobulin. LG1: leuco-methylthioninium chloride. NGF: nerve growth factor. NGXsc: NGX series compounds. PUFAs: polyunsaturated fatty acids. GSM: γ-secretase modulator. Randomised, controlled trials in Alzheimer's disease not ongoing. †Drugs approved for the treatment of Alzheimer's disease. From [Mangialasche, Solomon et al. 2010].
Aside, therapies augmenting $\alpha$-cleavage by up-regulation of ADAM10 through treatment with BDNF (see Section 1.5.1) or inhibiting c-Abl necessary for $A\beta$ triggered neuronal cell death are a new promising options for AD therapy (see Section 1.4). Drugs in various clinical phases are summarized in Figure 1.10.

1.12. **Hypothesis and aim of this thesis**

Motivated by previous findings that lipid composition of liposomes influence the kinetics of APP cleavage at its $\alpha$-site [Marenchino, Williamson et al. 2008], our aim was to go one step further and investigate whether supplied nutritive compounds would show similar effects in an *in vitro* cell system. Such a cellular model allows neglecting systemic responses and is ideal to determine the influences of fatty acids or other membrane-modifying molecules by measuring the effect of these molecules on APP processing with a simple and quantitative method. ELISA measurement was considered the method of choice due to the very low sAPP concentrations to be determined. For quantification, levels of sAPP$\alpha$ and sAPP$\beta$ were measured. The sAPP$\alpha$ and sAPP$\beta$ levels were then directly compared using the ratio of sAPP$\alpha$ to sAPP$\beta$. Calculating the ratio allowed to eliminate possible differences in cell number. With this model at hands, we wanted to test the effect of plasma membrane composition by varying the amount of selected fatty acids incorporated into the plasma membrane as well as the influence of serum from different species or cortisol on APP cleavage at the $\alpha$- and $\beta$-site.
We hypothesised that

1. the amount of APP $\alpha$- or $\beta$-cleavage is dependent on membrane composition, which can be influenced through nutrient supplementation in a cell culture model.

2. supplementation of fatty acids would alter APP cleavage.

We wanted to measure the effect of natural (VA) and unnatural (EA) trans-fatty acids, since they strongly influence physico-chemical properties of membranes. Moreover, Morris et al. described a positive association of the risk of incident AD with increased trans-fatty acid uptake [Morris, Evans et al. 2003]. We further wanted to investigate the effect of DHA, since its positive effect on disease progression has been intensively discussed and reviewed [Huang 2010]. And finally, we included the most common fatty acid, SA, as a control in our studies.

3. using serum as medium supplement from different species origin would influence APP cleavage.

Sera from different origin contain varying amounts and composition of fatty acids and growth factors. Serum choice thus was expected to lead to different APP cleavage.

4. cortisol would reduce the $sAPP\alpha/sAPP\beta$ ratio when supplemented to SH-SY5Y cells.

Although it is not yet clear, whether cortisol is a cause or result of AD, cortisol levels are elevated in AD patients and an effect on neuronal cell death is seen (see Section 1.8).
2. Materials and Methods

2.1. Cell Culture

All cell culture experiments were performed with SH-SY5Y cells purchased from ECACC (Salisbury, UK; ECACC No. 94030304). Purchased cells were at passage number 17. The cells were propagated in 25 cm² or 75 cm² cell culture flasks (TPP). The culture was routinely tested for mycoplasma contaminations using a direct DAPI staining method [Uphoff, Gignac et al. 1992]. Two growth conditions were used for cell propagation: low and high cell density culture conditions are described in the following sections. All experiments were performed with cells of passage number lower than 50.

2.1.1. Low cell density culture condition

Cells were maintained in foetal calf serum (FCS) supplemented medium (FSM, see Table 2.1) and split 1:20 every seven to ten days, before they reached confluence. At least one medium exchange was necessary after five to seven days. In some experiments, the FCS was substituted with human (HSM, Sigma, lot 039K8716) or horse (ESM, Sigma, lot 078K0375) serum. If not stated otherwise, sera were inactivated in a 56 °C water bath for one hour.

2.1.2. High cell density culture condition

Experiments with fatty acid complementation were performed in medium without serum to avoid undefined amounts of fatty acids contained in the serum. Therefore, cells were grown alternately in FSM and in StartV (Biochrom) only supplemented with penicillin/streptomycin. The cells were
Table 2.1: Cell culture medium composition.

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced DMEM:F12</td>
<td>Gibco</td>
<td>500 ml</td>
</tr>
<tr>
<td>Foetal calf serum (lot: A01123-415)</td>
<td>Omnilab</td>
<td>50 ml</td>
</tr>
<tr>
<td>Sodium hydrogencarbonate 7.5%</td>
<td>Sigma</td>
<td>15 ml</td>
</tr>
<tr>
<td>L-Glutamine 200 mM</td>
<td>Sigma</td>
<td>10 ml</td>
</tr>
<tr>
<td>Penicillin (5000 units/ml)/streptomycin (5000 µg/ml)</td>
<td>Gibco</td>
<td>5ml</td>
</tr>
</tbody>
</table>

split 1:5 once a week and the medium was exchanged two and five days after splitting. At each splitting, the medium was switched from FSM to StartV and vice versa.

2.2. Cell counting

Cells in 25 cm² flasks were counted with either of two methods: whole cell counting or nuclei release. If whole cells were counted, they were washed once with Earl's balanced salt solution (EBSS, Gibco). In order to detach the cells, 0.5 ml trypsin was added and cells incubated for 3 minutes at 37 °C. After addition of 4.5 ml culture medium, the cells were gently passed once through a sterile 20G needle. An aliquot of the cell suspension was diluted (usually 1:10) with EBSS and the cells were counted in a Neubauer hemocytometer.

For nuclei release, cells from a 25 cm² flask were washed in saline (0.9% NaCl (w/v)) and incubated in 2 ml hypotonic solution (10 mM HEPES, 1.5 mM magnesium chloride hexahydrate, pH 7.4). After 5 minutes 200 µl lysis solution (3% (v/v) glacial acetic acid, 5% (w/v) ethylhexadecyl-dimethylammonium bromide, Fluka) was added and the suspension shaken
for another 5 minutes at room temperature. The suspension was diluted to a volume of 10 ml with a 1:1 mixture of saline and formaldehyde (37% (w/v) in water). The resulting nuclei suspension was diluted (usually 1:10) with saline and the nuclei were counted in a Neubauer hemocytometer.

2.3. Sample preparation for ELISA and Western blot

SH-SY5Y cells were grown in 25 cm$^2$ cell culture flasks. To quantify APP cleavage products in the medium with ELISA, 4-5 ml supernatant were carefully collected and floating cells were sedimented by centrifugation (5 minutes, 2000 g). The cellfree medium was stored at -20 °C for later ELISA analysis. The cell pellet from centrifugation was pooled together with the adherent cells and washed twice with Dulbecco's PBS (DPBS, Gibco). Under conditions where the cells grew strongly adherently, the washing was carried out directly in the culture flask. If the cells only weakly adhered to growth support, the cells were detached by pipetting with DPBS and recovered by centrifugation (2 minutes, 2000 g). Cells were lysed and proteins solubilised with 1 ml RIPA buffer (50 mM Tris base pH 8.0, 150 mM sodium chloride, 1% (v/v) NP-40, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.5% (w/v) sodium deoxycholate) containing protease inhibitor complete mini® (Roche) for 30 minutes on ice. The lysate was forced ten times through a 21G needle. Insoluble proteins were removed from solution by centrifugation (2 minutes, 10'000 g). The protein concentration of the whole cell extract was determined using the Biorad Dc assay. The protein extract was diluted 1:2 (in addition to the assay inherent dilution of 1:10). These whole cell extracts were frozen at -20 °C and used for further Western blot analysis.
2.4. ELISA

ELISA measurements of sAPPα and sAPPβ were performed using two corresponding ELISA kits (IBL, Japan) according to the manufacturer’s instructions: sAPP-alpha ELISA, sAPP-beta ELISA, sAPPalpha high sensitive ELISA and sAPPbeta high sensitive ELISA. In brief, 200 µl EIA buffer (IBL, Japan) was mixed with 800 µl supernatant from cell cultures. For high sensitive ELISA, 200 µl sample supernatant was mixed with 800 µl EIA buffer. Lyophilised standard protein (kit component) was dissolved in pure water and diluted to a concentration of 100 ng/ml, as described above for the supernatants, and further diluted to get four to five calibration solutions between 0.39 and 50 ng/ml. Samples or standard solutions (100 µl) were dispensed in precoated wells from a 96-well ELISA plate. Triplicate measurements were recorded for each sample. The plate was incubated for 16 to 20 hours at 4 °C on a rocking platform. The analyte was discarded and remaining liquid removed by snapping the whole plate on a paper tissue. The wells were plunged into freshly prepared washing buffer and the liquid was removed as described above. This washing procedure was repeated eight times in total, the washing buffer being renewed after four washing steps. The labelled antibody was diluted 1:30 with the appropriate antibody dilution buffer (IBL). Labelled antibody solution (100 µl) was dispensed to each well right after the last washing step to avoid drying of the well. The plate was incubated for 30 minutes at 4 °C on a rocking platform and subsequently washed ten times as described above with a change of the washing buffer after the fifth washing step. Chromogen tetramethylbenzidine (TMB, 100 µl) was added to each well and the plate was incubated in the dark without shaking at room temperature. The reaction was stopped between 30 and 60 minutes with
100 µl stop solution (IBL) and the absorbance at 450 nm recorded on a Synergy HT plate reader (BioTek). All chemicals were kit components.

**2.5. Statistical analysis of data**

For ELISA measurements, experiments were usually performed in three flasks per condition prepared from one cell culture batch, but grown separately for three days before starting the experiment. We assumed independency of each flask. In addition, for each flask ELISA was performed in triplicate measurements. Several experiments were repeated with cells of different passage numbers.

Cell culture samples were measured in ELISA, if they matched the following criteria:

- The cells were not completely detached at harvesting.
- The standard curves for sAPPα and sAPPβ had a slope $x$ between 0.014 and 0.081 for sAPPα and between 0.01 and 0.063 for sAPPβ, respectively, and a $R^2$ of at least 0.96

\[ A = x \times \text{concentration} \left( \frac{ng}{ml} \right) + y \]

where $A$ is absorbance at 450 nm.

Statistical analysis of ELISA measurements was conducted using R-software. Significance of differences was analysed with ANOVA. As the measured sAPPα and sAPPβ values differed greatly from one experiment to another, the date of experiment was introduced as block parameter. To test variability of one condition’s effect during several experiments, analysis of datasets with more than one date of experiment was calculated using an interaction model.
(interaction of condition and date). Therefore, data originating from more than one experiment were analysed with a two-way ANOVA (date and condition), data from single experiments with a one-way ANOVA (condition only). For datasets with more than two comparators, post-hoc Bonferroni corrected p-values were calculated with R-software.

Effects of cell numbers were eliminated calculating the ratio between sAPPα and sAPPβ. Differences were considered significant for p<0.05.

Table 2.2: Composition of SDS-PAGE gels.

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4% Stacking gel, pH 6.8</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris base</td>
<td>Promega</td>
<td>130 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>Sigma</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide 37.5:1 (2.6% C)</td>
<td>Biorad</td>
<td>4% (w/v)</td>
</tr>
<tr>
<td>APS</td>
<td>Sigma</td>
<td>0.05% (w/v)</td>
</tr>
<tr>
<td>TEMED</td>
<td>Fluka</td>
<td>0.1% (v/v)</td>
</tr>
<tr>
<td><strong>12% Separating gel, pH 8.8</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris base</td>
<td>Promega</td>
<td>380 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>Sigma</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide 37.5:1 (2.6% C)</td>
<td>Biorad</td>
<td>12% (w/v)</td>
</tr>
<tr>
<td>APS</td>
<td>Sigma</td>
<td>0.05% (w/v)</td>
</tr>
<tr>
<td>TEMED</td>
<td>Fluka</td>
<td>0.05% (v/v)</td>
</tr>
</tbody>
</table>
Table 2.3: Composition of PAGE-buffer.

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>Promega</td>
<td>25 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>Sigma</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma</td>
<td>192 mM</td>
</tr>
</tbody>
</table>

2.6. SDS-PAGE and immunoblotting

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% acrylamide separating and 4% stacking gels [Gallagher 2006]. The compositions are given in Table 2.2. Protein samples were incubated at 60 °C for 10 minutes with sample buffer (5x stock solution: 0.313 M Tris, 10% (w/v) SDS, 35% (v/v) glycerol, 17% β-mercaptoethanol, 0.1% (w/v) bromophenol blue, pH 6.8).

A sample volume corresponding to 10 µg total protein was loaded on a SDS-PAGE gel. As molecular size marker prestained dual color protein marker (Biorad) was loaded. The gels were run with PAGE-buffer (see Table 2.3) for 90 minutes at 120 V in a Mini-PROTEAN 3 Electrophoresis Module (Biorad). The device was cooled with ice. The separated protein bands were blotted onto a nitrocellulose membrane (Amersham) using a wet-blot protocol (PAGE-buffer with 20% methanol, 200 mA current per gel, 90 minutes).

Table 2.4: Composition of T/TBS.

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base (pH adjusted to 7.4 with HCl)</td>
<td>Promega</td>
<td>20 mM</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Fluka</td>
<td>500 mM</td>
</tr>
<tr>
<td>Tween20</td>
<td>Sigma</td>
<td>0.1% (v/v)</td>
</tr>
</tbody>
</table>
CHAPTER 2: MATERIALS AND METHODS

The membranes were blocked for one hour in Tris-buffered saline with 0.1% Tween20 (T/TBS, see Table 2.4) containing 5% (w/v) low fat milk powder (grocery store, Coop) on a rocking platform. Antibodies were diluted in T/TBS containing 1% milk powder according to Table 2.5. The membranes were incubated with the diluted antibodies at 4 °C overnight, then washed three times for 15 minutes with T/TBS with 1% milk powder and incubated at 4 °C for 4 hours with the corresponding secondary antibody. The membranes were washed three times for 15 minutes with T/TBS and incubated with either Immunostar substrate (Biorad) for the alkaline phosphatase (ALP) conjugates or with ECL (gift of Dr. A. Müller) for the horseradish peroxidase (HRP) conjugates. The membrane was exposed to a film and developed in an AGFA Curix photo developing device. Exposure times varied from 1 to 60 minutes, depending on signal strength.

Table 2.5: Antibodies used in Western Blot analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Product Code</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit anti-APP (C-terminus)</td>
<td>Abcam</td>
<td>ab32136</td>
<td>1:10'000</td>
</tr>
<tr>
<td>rabbit anti-cyclophilin</td>
<td>Abcam</td>
<td>ab41684</td>
<td>1:20'000</td>
</tr>
<tr>
<td>mouse anti-ADAM9</td>
<td>Abcam</td>
<td>ab57934</td>
<td>1:5'000</td>
</tr>
<tr>
<td>rabbit anti-ADAM10</td>
<td>Abcam</td>
<td>ab1997</td>
<td>1:3'333</td>
</tr>
<tr>
<td>mouse anti-ADAM17</td>
<td>Abcam</td>
<td>ab57484</td>
<td>1:10'000</td>
</tr>
<tr>
<td>rabbit anti-BACE1</td>
<td>Abcam</td>
<td>ab2077</td>
<td>1:4’000</td>
</tr>
<tr>
<td>mouse anti-neuron specific βIII tubulin</td>
<td>Abcam</td>
<td>ab7751</td>
<td>1:5’000</td>
</tr>
<tr>
<td>goat anti-rabbit IgG coupled to ALP</td>
<td>Pierce</td>
<td>31340</td>
<td>1:25’000</td>
</tr>
<tr>
<td>goat-anti rabbit IgG coupled to HRP</td>
<td>Sigma</td>
<td>A0545</td>
<td>1:50’000</td>
</tr>
<tr>
<td>goat anti-mouse IgG coupled to ALP</td>
<td>Thermo</td>
<td>31320</td>
<td>1:15’000</td>
</tr>
</tbody>
</table>
2.7. Fatty acid pattern quantification of cell membranes by HPLC

2.7.1. Supplementing growth medium with fatty acids

StartV medium was supplemented with one of the following fatty acids: all-cis-docosa- 4,7,10,13,16,19-hexa-enoic acid (DHA), (E)-octadec-11-enoic acid (trans-vaccenic acid, VA), (E)-octadec-9-enoic acid (elaidic acid, EA) or octadecanoic acid (stearic acid, SA). The structures of the fatty acids are shown in Figure 2.1.

![Fatty acid structures](image)

**Figure 2.1: Fatty acids used in medium supplementation experiments.** SA: stearic acid, natural saturated fatty acid, 18:0; VA: trans-vaccenic acid, natural trans-fatty acid, 18:1 trans-11; EA: elaidic acid, non-natural trans-fatty acid, 18:1 trans-9; DHA: docosahexaenoic acid, natural poly-unsaturated cis-fatty acid, 22:6(n-3).
DHA was purchased from TCI while SA, VA and EA were purchased from Sigma. Upon arrival, fatty acids were dissolved in chloroform to obtain a final concentration of 100 mM and stored under argon at -20 °C to avoid oxidation. When culture medium was supplemented with fatty acids, the stock solution was diluted 1:10 with ethanol in order to get manageable volumes. The pH was increased by adding 20 µl of a 5 M sodium hydroxide solution to 180 µl of the diluted fatty acid solution. The solvents were evaporated under nitrogen and the dried fatty acid salts immediately dissolved in 1.5 ml boiling water. This aqueous solution was immediately added to 15 ml cell culture medium containing 1 mg/ml essentially fatty acid free human serum albumin (Sigma). The pH was adjusted with up to 0.5 ml diluted hydrochloric acid to pH 7.4. The medium was filtered through a 0.2 μm sterile filter and immediately used. To obtain a concentration of 30 μM fatty acid, the prepared medium was diluted 1:4 with cell culture medium during the cell splitting or medium replacement.

2.7.2. Fatty acid extraction from cell membranes

Cells were grown at high densities as specified in Section 2.1.2. The cells were detached by pipetting and harvested to a 15 ml falcon flask. The medium was removed by centrifugation (2000 g, 5 minutes, 4 °C) and the cell pellet was washed twice with phosphate buffered saline (PBS, Gibco). Membrane lipids were extracted from the cell pellet with 3 ml of a 2:1 (v/v) chloroform-methanol solution for 15 minutes in an ultrasound bath at room temperature. The extraction was repeated with 2 ml 2:1 (v/v) chloroform-methanol and the organic phases were pooled and stored in a glass vial at -20 °C. From the organic phase, 0.5 ml (corresponding to 1-100 µg total lipids) was dried in a nitrogen stream. The lipids were hydrolysed in 1 ml of 1 M potassium hydroxide in 95% ethanol at 70 °C for one hour. The solution was
neutralised with 0.2 ml 6 N hydrochloric acid. The free fatty acids were extracted twice with 0.5 ml hexane and stored under argon at -20 °C.

2.7.3. HPLC analysis of fatty acid extracts

The solvent from 250 µl of free fatty acid extract or from 20 µl diluted fatty acids in ethanol (see Section 2.7.1) was evaporated in a nitrogen stream. The derivatisation solution was prepared freshly: 40 µl bromomethylmethoxycoumarin (1 mg/ml in dry acetonitrile, TCI) were mixed with 2 µl 18-crown-6 (2.6 mg/ml in dry acetonitrile, Sigma) and 2 µl of dry acetonitrile. One spatula tip dry sodium sulfate (Fluka) was added and 100 µl of this derivatisation solution was incubated with a spatula tip of dry potassium carbonate (Fluka) and the dried fatty acids at 60 °C for 15 minutes. The reaction mixture was cooled on ice and filtered through a 0.22 µm nylon filter.

From the filtered fatty acid sample, 5 µl were directly injected into a HPLC column or spiked with 10% (v/v) of derivatised diluted fatty acids. The fatty acids were separated on a Waters ODS column (C18, 250 mm x 4 mm) using a gradient elution profile (see Figure 2.2) with a flow rate of 1 ml/min. The column was kept at 30 °C. The peaks were detected using a fluorescence detector (Ex: 325 nm, Em: 398 nm). All reagents and solvents were of HPLC grade. The chromatograms of spiked samples were used to identify peaks from corresponding fatty acids. The area under the curve was normalised to the peak at 10.5 minutes and the normalised value used as measure for the fatty acid amount. For a representative chromatogram, see Figure 3.19. Samples from cells supplemented with fatty acids were compared to a control sample from cells grown in FSM without fatty acid complementation.
Figure 2.2: HPLC elution profile. Fatty acids were eluted with acetonitrile and water as eluents. Acetonitrile was gradually increased from 70% to 95.5% over ten minutes and then kept constant for another 15 minutes.

2.7.4. Cortisol and retinoic acid treatment of cells

Cortisol was bought as 50 µM solution (Sigma, H6909). The final concentration of 50 nM was achieved by pipetting 1/1000 volume cortisol solution to the already prepared cell culture medium.

Retinoic acid (RA, Fluka, order number: 95152) was dissolved in ethanol. Cells were seeded in a 25 cm² cell culture flask at a density of 2000 cells/cm² and grown for 3 days. RA was added to a final concentration of 10 µM RA and 1‰ ethanol. The cells were differentiated for five days in RA with medium changes twice weekly. Thereafter, they were incubated for another five days without medium change to accumulate sAPPα and sAPPβ for ELISA measurements.
3. Results

3.1. Characterisation of SH-SY5Y cells

The human neuroblastoma cell line SH-SY5Y is frequently used to investigate the processing of APP \textit{in vitro}. In this work, we studied the effect of various conditions on $\alpha$- and $\beta$-cleavage. Therefore, we first characterised the cells regarding their expression of APP as well as the main secretases ADAM10 and BACE1. Figure 3.1 shows the corresponding immunoblot analysis of whole cell extracts confirming the expression of detectable levels of APP, ADAM10 and BACE1. The neuronal phenotype was verified by staining for the neuron specific $\beta$III tubulin, a common neuronal marker, with an expected band at 50 kDa. Staining against APP resulted in a broad double band at 90-120 kDa.

![Figure 3.1: Expression of $\beta$III tubulin, APP, ADAM10 and BACE1 in SH-SY5Y cells. Immunoblot analysis of a whole cell extract from SH-SY5Y cells grown in FSM. Lane 1: anti-$\beta$III tubulin; lane 2: anti-APP; lane 3: anti-ADAM10 and lane 4: anti-BACE1.](image-url)
The bands of the APP cleaving enzyme ADAM10 showed an apparent molecular weight around 75 kDa. Analysis of BACE1 led to several bands between 40 and 70 kDa. Multiple band patterns for both ADAM10 and BACE1 showed that these proteins were present in immature and mature forms, as indicated on a reference blot of the supplier of the antibody.

3.2. ELISA pilot experiments

3.2.1. Buffer conditions

ELISA conditions first needed to be optimised for the quantification of sAPP$\alpha$ and sAPP$\beta$ in cell culture flasks. Preparing standard curves in EIA buffer instead of medium led to an increased slope and hence to a higher sensitivity at lower concentrations (Figure 3.2). Therefore, we used EIA diluted samples to prepare ELISA measurements. As concentrations of sAPP$\alpha$ and sAPP$\beta$ were very low, we refrained from the proposed dilution of 1:10 as indicated in the manufacturers manual, but used a 4:1 dilution (i.e. 800 µl sample with 200 µl of EIA buffer) for ELISA and a 1:4 dilution (i.e. 200 µl sample with 800 µl of EIA buffer) for highly sensitive ELISA. Only three peptide concentrations were used in these optimisation experiments to save ELISA antibodies.

3.2.2. Influence of protein load on ELISA

We determined the effect of protein load on ELISA by measuring standard peptide (sAPP$\beta$) in EIA diluted medium (4:1 dilution) adding 0%, 0.5% or 4% bovine serum albumin (BSA, Fluka). The absorbance at 450 nm was not influenced by any of the tested protein concentrations (Figure 3.3).
CHAPTER 3: RESULTS

Figure 3.2: Medium effect on ELISA calibration. A calibration for sAPPα (diamonds) and sAPPβ (circles) was prepared in EIA buffer (closed symbols, solid lines) as well as in pure medium without serum (open symbols, dashed lines).

Figure 3.3: Effect of protein load on ELISA measurements. Standard curves of sAPPβ were prepared in EIA diluted medium (4:1 dilution) supplemented with 0% BSA (open diamonds), 0.5% BSA (triangles, dashed line) or 4% BSA (closed diamonds, solid line).
Figure 3.4: Influence of pH on ELISA measurements. Absorbance at 450 nm of sAPPβ standard peptide was measured in samples adjusted with HCl (solid lines, closed symbols) or HEPES (dashed lines, open symbols). Samples of pure EIA at pH 7.4 were included as controls (circles). The absorbance of samples at pH 7.4 (squares) or at pH 9.0 (triangles) was measured.

3.2.3. Influence of pH on ELISA

The pH of ELISA samples changed overnight resulting in a colour change of the medium’s phenol red from red to orange. Preliminary experiments showed that the pH of the measured sample does influence ELISA measurements. Samples were buffered with EIA (20% v/v for standard ELISA and 80% v/v for highly sensitive ELISA). EIA buffers around pH 7.4, but samples from cell culture supernatant eventually had a pH of about 9 when collected. We tested the effect of medium’s pH adjustment by HCl (Titrisol, Merck) or HEPES (Sigma) on the absorbance of sAPPβ ELISA measurements (Figure 3.4). Adjusting the pH with HEPES led to a stronger deviation of the absorbance from the control value compared to HCl. There was practically no influence on the calibration slope between medium at pH 9.0 and medium adjusted to pH
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7.0 for HCl, whereas HEPES showed a pH dependent decrease of the calibration slope. Therefore, we chose to adjust ELISA samples with HCl to a pH of around 7.0 before ELISA measurements to avoid differences due to pH or HEPES concentration.

3.3. Effects of sera from different species origin on sAPPα and sAPPβ levels in SH-SY5Y cells

Sera from different species origin vary in their composition of e.g. fatty acids, hormones and growth factors. In order to find out whether any serum component affects APP processing, SH-SY5Y cultures were grown in DMEM:F12 medium with different heat inactivated serum supplementations: foetal calf serum supplemented medium (FSM), human serum supplemented medium (HSM) or equine serum supplemented medium (ESM). The sAPPα and sAPPβ concentration of cell culture supernatants was measured in ELISA and the different conditions were compared (Figure 3.5). The ratio between sAPPα and sAPPβ was calculated and is shown in Figure 3.6. The sAPPα concentration as well as the sAPPα/sAPPβ ratio was significantly different in all three conditions. The sAPPα values were highest in HSM and lowest in FSM in all experiments. Differences in sAPPβ concentration between all conditions were much smaller, but nevertheless significant for FSM vs. HSM, but not for FSM vs. ESM. Post-hoc Bonferroni-corrected p-values are given in Table 3.1. We observed a significant interaction of the experiment dates and conditions on all variables but the sAPPα/sAPPβ ratio. However, the results remained consistent, e.g. sAPPα concentration were always highest in HSM and lowest in FSM.
Figure 3.5: Influence of serum on sAPPα (A) and sAPPβ (B) levels. Circles: FSM; diamonds: HSM; triangles: ESM. All sera were heat-inactivated. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 3 experiments are shown individually.
As SH-SY5Y cells were routinely grown in FSM, we wanted to test whether changing growth medium immediately prior to ELISA experiment would lead to a “memory effect”. We therefore propagated SH-SY5Y cells in FSM and switched to HSM at the beginning of the test experiment. We compared this “switched” samples to samples of cells grown in HSM or FSM only. The experiment’s design is shown in Figure 3.7.

Table 3.1: Bonferroni-corrected p-values of serum comparisons (two-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>FSM vs HSM</th>
<th>FSM vs ESM</th>
<th>HSM vs ESM</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>&lt; 10⁻³</td>
<td>0.111</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
</tr>
<tr>
<td>sAPPα/sAPPβ</td>
<td>&lt; 10⁻³</td>
<td>0.013</td>
<td>&lt; 10⁻³</td>
<td>0.053</td>
</tr>
</tbody>
</table>
Figure 3.7: Experimental design to test the influence of a serum switch on sAPP levels.

Figure 3.8: Influence of serum switch on sAPP levels and the sAPP\(\alpha\)/sAPP\(\beta\) ratio. Open circles: FSM; closed diamonds: Switch (from FSM to HSM); open diamonds: HSM. All sera were heat-inactivated. Concentrations and sAPP\(\alpha\)/sAPP\(\beta\) ratios are represented as mean values ± standard deviations from three flasks per condition.
Table 3.2: Bonferroni-corrected p-values for serum-switch experiment (one-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>FSM vs Switch</th>
<th>HSM vs Switch</th>
<th>FSM vs HSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPP(\alpha)</td>
<td>&lt; (10^{-3})</td>
<td>1.000</td>
<td>&lt; (10^{-3})</td>
</tr>
<tr>
<td>sAPP(\beta)</td>
<td>&lt; (10^{-3})</td>
<td>0.069</td>
<td>&lt; (10^{-3})</td>
</tr>
<tr>
<td>sAPP(\alpha)/sAPP(\beta) ratio</td>
<td>&lt; (10^{-3})</td>
<td>0.520</td>
<td>&lt; (10^{-3})</td>
</tr>
</tbody>
</table>

Concentrations of sAPP\(\alpha\) and sAPP\(\beta\) were measured and the corresponding ratio was calculated (Figure 3.8). Cells grown in FSM only showed a significantly lower sAPP\(\alpha\) and higher sAPP\(\beta\) concentration than cells grown in HSM or cells switched from FSM to HSM. Switching to HSM resulted in the same values as growth in HSM only. Bonferroni-corrected p-values of the comparison of sAPP\(\alpha\), sAPP\(\beta\) and the sAPP\(\alpha\)/sAPP\(\beta\) ratio between FSM, HSM and adaptation to HSM within three days (Switch) are given in Table 3.2.

Figure 3.9: Existing sAPP levels in serum supplemented cell culture medium. The concentration of sAPP\(\alpha\) and sAPP\(\beta\) was measured by ELISA in samples of sterile medium containing 10% FCS (grey bar) or human serum (black bar). Results from two batches are shown as mean values ± standard deviations.
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We analysed the already existing sAPPα and sAPPβ content of sterile medium containing 10% FCS or human serum (Figure 3.9). Concentrations of sAPPα and sAPPβ remained far below 1 ng/ml. The contribution of already existing sAPPα and sAPPβ was therefore neglected for FSM as well as for HSM.

The differences in sAPPα or sAPPβ levels between different serum addition did not result from altered expression levels of cleaving secretases (e.g. ADAM10 or BACE1), as changes in concentrations were not reflected by changes the enzyme levels (shown in Section 3.9).

Table 3.3: Preliminary experiments of fatty acid supplementation on APP cleavage. Data from [Legnani 2009].

<table>
<thead>
<tr>
<th>fatty acid</th>
<th>sAPPα/sAPPβ-ratio</th>
<th>p-value (t-test statistics)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fatty acid supplied</td>
<td>control</td>
</tr>
<tr>
<td></td>
<td>Mean value ± st. dev.</td>
<td>Mean value ± st. dev.</td>
</tr>
<tr>
<td>LA</td>
<td>1.68 ± 0.17</td>
<td>1.65 ± 0.17</td>
</tr>
<tr>
<td>LA</td>
<td>1.27 ± 0.19</td>
<td>1.34 ± 0.10</td>
</tr>
<tr>
<td>VA*</td>
<td>2.49 ± 0.20</td>
<td>2.13 ± 0.09</td>
</tr>
<tr>
<td>VA*</td>
<td>2.05 ± 0.11</td>
<td>1.88 ± 0.18</td>
</tr>
<tr>
<td>VA</td>
<td>1.88 ± 0.14</td>
<td>1.87 ± 0.21</td>
</tr>
<tr>
<td>VA</td>
<td>2.06 ± 0.19</td>
<td>2.01 ± 0.18</td>
</tr>
<tr>
<td>EA*</td>
<td>3.13 ± 0.18</td>
<td>2.42 ± 0.31</td>
</tr>
<tr>
<td>EA*</td>
<td>3.30 ± 0.49</td>
<td>2.30 ± 0.28</td>
</tr>
<tr>
<td>EA*</td>
<td>1.74 ± 0.21</td>
<td>1.42 ± 0.19</td>
</tr>
<tr>
<td>EA†</td>
<td>1.66 ± 0.19</td>
<td>1.89 ± 0.16</td>
</tr>
</tbody>
</table>
3.4. Influence of supplemented fatty acids on APP processing in SH-SY5Y cells

Comparing human and bovine serum, several factors differ significantly in their concentration, among them fatty acids [Lagarde, Sicard et al. 1984], growth factors and hormones. One such constituent or a group of them could have caused the differences in sAPP\textsubscript{α} and/or sAPP\textsubscript{β} levels described in Section 3.3. We tested the hypothesis that changes of membrane composition elicited by fatty acid supplementation affect \(\alpha\)-cleavage of APP by measuring the influence of supplemented fatty acids on the sAPP\textsubscript{α} and sAPP\textsubscript{β} levels and calculating the corresponding ratio. Preliminary ELISA experiments have been performed by G. Legnani in her master thesis [Legnani 2009]. In order to exclude effects from serum components we used serum-free medium for these experiments. The use of StartV medium and the high density culture protocol (see Section 2.1.2) allowed excluding effects from fatty acids present in FCS or human serum. The omission of serum complementation however had the drawback that the SH-SY5Y cells, although further dividing, did not increase in size and mainly grew as suspension culture.

The cleared supernatant was diluted to 80\% (v/v) with EIA buffer and sAPP\textsubscript{α} and sAPP\textsubscript{β} concentration was measured by ELISA. The sAPP\textsubscript{α}/sAPP\textsubscript{β} ratios as well as p-values (t-test statistic) of independent experiments with linoleic acid (LA), \textit{trans}-vaccenic acid (VA) and elaidic acid (EA) supplementation compared to the corresponding control (unsupplemented StartV medium) are shown in Table 3.3. Providing LA did not significantly change the sAPP\textsubscript{α}/sAPP\textsubscript{β} ratio compared to control. In some experiments with VA and EA (marked with * in Table 3.3) the ratio was significantly higher for fatty acid supplemented cells. In other experiments however, the ratio did not change
significantly (VA) or the ratio was even higher for control than for the fatty acid supplemented samples (EA, marked with † in Table 3.3).

These preliminary results were verified by a series of experiments. We studied the effects of the before tested trans-fatty acids EA and VA as well as the PUFA docosahexaenonic acid (DHA) and the saturated stearic acid (SA). The reasons for the choice of these fatty acids are discussed in Section 1.12.

In one single experiment, we repeated the measurement of VA supplementation testing its effect on sAPP levels and the sAPPα/sAPPβ ratio. Supplementing VA, we observed a small, weakly significant sAPPα lowering effect (Figure 3.10). Nevertheless, no significant effect on sAPPβ or the ratio was observed (Table 3.4).

![Figure 3.10: Influence of VA on sAPP levels (A) and the sAPPα/sAPPβ ratio (B). Open circles: StartV; closed diamonds: StartV supplemented with VA. Concentrations are represented as mean values ± standard deviations from three flasks per condition in one single experiment.](image-url)
Table 3.4: p-values for the effect of VA (one-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>VA vs StartV</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPP(_{\alpha})</td>
<td>0.048</td>
</tr>
<tr>
<td>sAPP(_{\beta})</td>
<td>0.766</td>
</tr>
<tr>
<td>sAPP(<em>{\alpha})/sAPP(</em>{\beta}) ratio</td>
<td>0.250</td>
</tr>
</tbody>
</table>

Since we observed changing effects of EA supplementation on the sAPP\(_{\alpha}\)/sAPP\(_{\beta}\) ratio in the preliminary experiments, we verified these results with three additional ELISA measurements. EA was added to serum-free medium and the effect on sAPP\(_{\alpha}\) and sAPP\(_{\beta}\) concentration was measured in ELISA (Figure 3.11). The sAPP\(_{\alpha}\)/sAPP\(_{\beta}\) ratio was calculated (Figure 3.12) and the results were statistically analysed (Table 3.5). We observed statistical interaction on the sAPP\(_{\alpha}\) concentration: differences between experiments were greater than differences between supplementation and control. No statistical influence of EA supplementation on any of the measured or calculated parameters was observed.

The effect of DHA on APP cleavage was tested in five independent experiments. After DHA supplementation, measured concentrations strongly varied between experiment dates, but the effect on sAPP\(_{\alpha}\) and sAPP\(_{\beta}\) levels remained unchanged: both sAPP\(_{\alpha}\) and sAPP\(_{\beta}\) concentrations were significantly increased (Figure 3.13). No significant interaction between condition and date was observed (Table 3.6.). The effect on sAPP\(_{\alpha}\) was equal to the effect on sAPP\(_{\beta}\) resulting in no net effect on the sAPP\(_{\alpha}\)/sAPP\(_{\beta}\) ratio (Figure 3.14).
Figure 3.11: Influence of EA on sAPPα (A) and sAPPβ (B) levels. Open circles: StartV (control); closed diamonds: StartV supplemented with EA. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 3 experiments are shown individually.
Figure 3.12: Calculated sAPPα/sAPPβ ratio for the effect of EA. Open circles: StartV (control); closed diamonds: StartV supplemented with EA. Ratios are represented as mean values ± standard deviations from three flasks per experiment. Data from 3 experiments are shown individually.

Table 3.5: p-values for the effect of EA (two-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>EA vs StartV</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>0.186</td>
<td>0.001</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.193</td>
<td>0.401</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>0.680</td>
<td>0.113</td>
</tr>
</tbody>
</table>
Figure 3.13: Influence of DHA on sAPPα (A) and sAPPβ (B) levels. Open circles: StartV (control); closed diamonds: StartV supplemented with DHA. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 5 experiments are shown individually.
Figure 3.14: Calculated sAPPα/sAPPβ ratio for the effect of DHA. Open circles: StartV (control); closed diamonds: StartV supplemented with DHA. Ratios are represented as mean values ± standard deviations from three flasks per experiment. Data from 5 experiments are shown individually.

As last fatty acid SA, the most abundant saturated fatty acid in mammalian cell membranes, was investigated. Addition of SA did not lead to consistent results. While a clearly decreased sAPPα level upon SA addition was seen in one experiment, this influence was absent in the second experiment (Figure 3.15), indicating a significant interaction of experiment date and condition. No effect on sAPPβ and the sAPPα/sAPPβ ratio was observed (Figure 3.16). Statistical analysis is summarized in Table 3.7.

Table 3.6: p-values for the effect of DHA (two-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>DHA vs StartV</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>0.019</td>
<td>0.230</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.011</td>
<td>0.402</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>0.382</td>
<td>0.733</td>
</tr>
</tbody>
</table>
Figure 3.15: Influence of SA on sAPPα (A) and sAPPβ (B) levels. Open circles: StartV (control); closed diamonds: StartV supplemented with SA. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 2 experiments are shown individually.
We verified the effect of DHA and VA in cells that were cultivated in human serum supplemented medium (Figure 3.17 and Figure 3.18). The effect of DHA or VA supplementation was identical. We observed a significant increase of sAPPα and sAPPβ for both DHA and VA compared to HSM. However, the increase of sAPPα was almost identical to the increase of sAPPβ, resulting in an invariant sAPPα/sAPPβ ratio. For the statistical analysis, Bonferroni’s correction for multiple comparisons was used (Table 3.8).

Table 3.7: p-values for the effect of SA (two-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>SA vs StartV</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>&lt; 10^{-3}</td>
<td>0.001</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.634</td>
<td>0.455</td>
</tr>
<tr>
<td>sAPPα/sAPPβ</td>
<td>0.554</td>
<td>0.629</td>
</tr>
</tbody>
</table>
Figure 3.17: Influence of DHA and VA on sAPPα (A) and sAPPβ (B) levels in HSM. Circles: HSM (control); diamonds: HSM + DHA; triangles: HSM + VA. Untreated human serum was used in these experiments. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 2 experiments are shown individually.
Figure 3.18: Calculated sAPPα/sAPPβ ratios for the effects of DHA and VA in HSM. Circles: HSM (control); diamonds: HSM + DHA; triangles: HSM + VA. Untreated human serum was used in these experiments. Ratios are represented as mean values ± standard deviations from three flasks per experiment. Data from 2 experiments are shown individually.

In order to verify the incorporation of fatty acids into the cell membranes, we prepared lipid extracts from cell membranes and analysed the fatty acid contents with HPLC analysis. A representative chromatogram confirming DHA-incorporation into the cell membrane is shown in Figure 3.19.

Table 3.8: Bonferroni-corrected p-values for the effect of DHA and VA in HSM (two-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>DHA in HSM vs HSM</th>
<th>VA in HSM vs HSM</th>
<th>DHA in HSM vs VA in HSM</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>&lt; $10^{-3}$</td>
<td>&lt; $10^{-3}$</td>
<td>0.561</td>
<td>0.448</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>&lt; $10^{-3}$</td>
<td>&lt; $10^{-3}$</td>
<td>0.180</td>
<td>0.014</td>
</tr>
<tr>
<td>sAPPα/sAPPβ</td>
<td>0.091</td>
<td>0.090</td>
<td>1.000</td>
<td>0.871</td>
</tr>
</tbody>
</table>
Peaks of interest are indicated with an arrow. Peak areas were normalised to an internal standard. Additional peaks were identified as oleic acid, linoleic acid and conjugated linoleic acid. The relative amount of the provided fatty acids in cells grown in supplemented medium compared to cells grown in pure StartV confirmed an increased incorporation of the supplemented fatty acids (Table 3.9). While the amount of incorporated DHA increased more than eight-fold, VA, EA and SA were less well absorbed. Nevertheless, supplementation led to an increase of roughly two-fold for EA and SA and 1.5-fold for VA. Supplemented fatty acids were therefore incorporated in the respective cells.

Figure 3.19: Fatty acid content determination of a membrane extract from DHA samples. Free fatty acids of a membrane extract of samples from DHA supplied cells were quantified by HPLC measurement. Peaks of interest are indicated with an arrow.
Table 3.9: Fatty acid analysis of supplemented cells.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Amount relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not supplemented control</td>
<td>100%</td>
</tr>
<tr>
<td>VA</td>
<td>143%</td>
</tr>
<tr>
<td>EA</td>
<td>186%</td>
</tr>
<tr>
<td>DHA</td>
<td>886%</td>
</tr>
<tr>
<td>SA</td>
<td>231%</td>
</tr>
</tbody>
</table>

3.5. Effect of serum inactivation on APP processing

Besides fatty acids, we also tested other parameters in order to specify the factor in serum responsible for the observed differences in the sAPPα/sAPPβ ratio between different sera (see Section 3.3). We hypothesised that, if the responsible factor in serum was a biomolecule, it possibly could be temperature sensitive, especially in case of a protein or peptide. Heat treatment of serum is commonly used to inactivate serum components such as complement [Bradbury, Aparicio et al. 1984]. Therefore, we tested whether heat inactivation of fetal calf serum or human serum for 1 hour at 56 °C influences APP cleavage. Levels of sAPPα were greatly affected by heat inactivation (Figure 3.20), whereas levels of sAPPβ and cell growth (data not shown) were not. For FSM, the sAPPα levels of samples with untreated serum were significantly increased compared to the samples with inactivated serum, but slightly decreased for HSM. The sAPPβ concentration for inactivated FSM and HSM did not differ significantly from the corresponding untreated sera.
Figure 3.20: Influence of serum inactivation in FSM and HSM on sAPPα (A) and sAPPβ (B) levels. Closed circles: FSM; open circles: inactivated FSM; closed triangles: HSM; open triangles: inactivated HSM. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 2 experiments for FSM and from 1 experiment for HSM are shown individually.
The sAPP\alpha/sAPP\beta ratio reflected the differences in sAPP\alpha (Figure 3.21): inactivated serum samples had a significantly lower ratio than untreated serum samples for FSM. There was no significant difference between HSM samples. P-values of the statistical analysis are summarised in Table 3.10. Changes in sAPP levels were not due to changes in the enzyme expression (see Section 3.9). Using untreated FSM instead of inactivated FSM led to a decrease in APP, but not of the enzymes ADAM10 and BACE1. Untreated HSM showed slightly higher levels of ADAM10 as well as BACE1 expression compared to inactivated HSM.

Figure 3.21: Calculated sAPP\alpha/sAPP\beta ratios for the effect of inactivation in FSM and HSM. Closed circles: FSM; open circles: inactivated FSM; closed triangles: HSM; open triangles: inactivated HSM. Ratios are represented as mean values ± standard deviations from three flasks per experiment. Data from 2 experiments for FSM and from 1 experiment for HSM are shown individually.
Table 3.10: p-values for the effect of serum inactivation in FSM (A, two-way ANOVA) and HSM (B, one-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>FSM vs FSM (inactivated)</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>&lt; 10^{-3}</td>
<td>&lt; 10^{-3}</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.783</td>
<td>0.226</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>&lt; 10^{-3}</td>
<td>&lt; 10^{-3}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HSM vs HSM (inactivated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>0.021</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.068</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>0.51</td>
</tr>
</tbody>
</table>

3.6. Effect of cortisol on α- and β-cleavage

As shown in Section 1.8, AD patients generally display elevated cortisol levels. It is still debated, whether cortisol is the cause of neuronal degeneration or rather a consequence of inflammation in AD brains. Furthermore, the cortisol concentration greatly differs between sera of different species [Laske, Stransky et al. 2009], which could explain the differences that we have seen for sAPP levels in cells grown in sera of different species origin. In addition, heat inactivation of serum was shown to increase the free fraction of cortisol by denaturing the binding sites of carrier proteins of cortisol [Lewis, Bagley et al. 2005]. Therefore, we measured the effect of cortisol on sAPPα and sAPPβ concentration and the respective ratio in ELISA (Figure 3.22). Effects of cortisol addition in both FSM and FSM with inactivated serum were not reproducible: in one experiment levels of sAPPα decreased upon cortisol addition while in a second one they increased significantly. However, the values for sAPPβ remained remarkably constant within one set of experiments resulting in a changing effect on the sAPPα/sAPPβ ratio (Figure 3.23).
Figure 3.22: Effect of cortisol addition on sAPPα and sAPPβ levels. Open circles: inactivated FSM; closed circles: inactivated FSM + 50 nM cortisol; open triangles: FSM; closed triangles: FSM + 50 nM cortisol; closed squares: FSM + 100 nM cortisol. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 3 experiments for inactivated FSM, from 2 experiments for FSM + 50 nM cortisol and from 1 experiment for FSM + 100 nM cortisol are shown individually.
Figure 3.23: Calculated sAPPα/sAPPβ ratios for the effect of cortisol. Open circles: inactivated FSM; closed circles: inactivated FSM + 50 nM cortisol; open triangles: FSM; closed triangles: FSM + 50 nM cortisol; closed squares: FSM + 100 nM cortisol. Ratios are represented as mean values ± standard deviations from three flasks per experiment. Data from 3 experiments for inactivated FSM, from 2 experiments for FSM + 50 nM cortisol and from 1 experiment for FSM + 100 nM cortisol are shown individually.

These findings were confirmed in the statistical analysis, which showed a significant interaction between date and condition for the sAPPα levels but not for sAPPβ (Table 3.11). Thus, the high statistical significance has to be interpreted in the way, as cortisol has a strong but not directed effect on the sAPPα/sAPPβ ratio. It has to be noted though, that only the sAPPα levels were affected by cortisol addition. However, increasing the added cortisol concentration to 100 nM did not produce any effect in one single experiment.
Table 3.11: p-values for the effect of cortisol addition. Addition of 50 nM cortisol to inactivated FSM (A, two-way ANOVA), to FSM (B, two-way ANOVA) and of 100 nM cortisol to FSM (C, one-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>FSM (inactivated) vs FSM (inactivated) + 50nM cortisol</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sAPPα</td>
<td>&lt; 10^-3</td>
<td>&lt; 10^-3</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.046</td>
<td>0.103</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>&lt; 10^-3</td>
<td>&lt; 10^-3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FSM vs FSM + 50nM cortisol</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sAPPα</td>
<td>&lt; 10^-3</td>
<td>&lt; 10^-3</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.338</td>
<td>0.110</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>&lt; 10^-3</td>
<td>&lt; 10^-3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FSM vs FSM + 100nM cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>sAPPα</td>
<td>0.837</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.265</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>0.509</td>
</tr>
</tbody>
</table>

The actual increase of cortisol in the medium was verified by ELISA measurement of freshly prepared medium with and without cortisol addition (Figure 3.24). The cortisol content in HSM was elevated compared to FSM. Although no clear influence of cortisol on APP-cleavage was observed, we tested whether addition of inhibitors of its receptors might show any effect. Neither of the corticoid receptor inhibitors eplerenone nor mifepristone led to an effect when comparing cells with cortisol supplementation, cells with inhibitor treatment and cells with both cortisol and inhibitor addition (data not shown). Cortisol treatment led to a decrease in protein levels of APP, ADAM10 and BACE1 (see Section 3.9).
Figure 3.24: Cortisol content of media. The cortisol content was measured by ELISA in freshly prepared FSM, HSM and FSM supplemented with 50 nM cortisol. Values are given as mean values ± standard deviations from three ELISA triplicate measurements.

Figure 3.25: Effect of serum dialysis on sAPP levels and the sAPPα/sAPPβ ratio. Closed circles: FSM; open circles: inactivated FSM; closed diamonds: dialysed FSM sample; open diamonds: combined sample. Concentrations and ratios are represented as mean values ± standard deviations from three flasks per condition.
Table 3.12: Bonferroni-corrected p-values of the dialysis experiment (one-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>FSM vs FSM (inactivated)</th>
<th>FSM vs dialysed</th>
<th>FSM vs combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>1.000</td>
<td>0.106</td>
<td>&lt; 10⁻³</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>0.003</td>
<td>1.000</td>
<td>0.005</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>dialysed vs combined</th>
<th>FSM (inactivated) vs dialysed</th>
<th>FSM (inactivated) vs combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPPα</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
<td>&lt; 10⁻³</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>0.317</td>
<td>0.058</td>
<td>&lt; 10⁻³</td>
</tr>
<tr>
<td>sAPPα/sAPPβ ratio</td>
<td>0.001</td>
<td>&lt; 10⁻³</td>
<td>1.000</td>
</tr>
</tbody>
</table>

3.7. sAPPα and sAPPβ concentrations after incubation of SH-SY5Y cells with dialysed serum

In order to get an idea about the molecular size of the factor leading to the differences between untreated and inactivated foetal calf serum, untreated FCS was dialysed using dialysis tubes with an exclusion size of 12 kDa. The high molecular weight (MW) fraction was used as medium additive (“dialysed” sample). The low MW fraction (“dialysate”) was diluted with an equal volume of inactivated FCS and also used as medium additive (called combined sample). Corresponding untreated FSM and inactivated FSM samples were used as controls. The ELISA result from one experiment is shown in Figure 3.25 and the corresponding statistical summary in Table 3.12.

As seen before (Section 3.5), the sAPPα/sAPPβ ratio was significantly higher in untreated than inactivated samples. The combined sample containing small serum components and inactivated serum did not increase the ratio, although
sAPPα concentrations were significantly increased. On the other hand, the dialysed sample significantly increased the ratio in the same way as untreated FSM. This result shows that the α-cleavage increasing factor must be in the dialysed sample and, thus, has a molecular size of at least 12 kDa.

### 3.8. APP α- and β-cleavage after cell differentiation with retinoic acid

SH-SY5Y cells can be differentiated to a more neuron-like phenotype using retinoic acid (RA) [Pahlman, Hoehner et al. 1995], a method also used in recent publications [Yang, Sheng et al. 2011]. Treating cells with 10 µM RA resulted in a clear morphologic change: the cells elongated and formed outgrowths (Figure 3.26). Differentiated cells grew slowly and exclusively adherently while undifferentiated cells detached easily and also grew in suspension.

![Figure 3.26: Cell differentiation with RA. (A) SH-SY5Y cells were grown for eight days in FSM (control) or (B) differentiated for 5 days by addition of 10 µM RA after three days.](image)
Figure 3.27: Effect of retinoic acid on sAPPα (A) and sAPPβ (B) levels. Closed circles: FSM; closed diamonds: FSM + RA; closed triangles: FSM + RA + 50 nM cortisol. Concentrations are represented as mean values ± standard deviations from three flasks per experiment. Data from 2 experiments (FSM, FSM + RA) or from 1 experiment (FSM + RA + 50 nM cortisol) are shown individually.
Since RA induced major morphological changes, the effect on the APP cleavage products was investigated by ELISA. Differentiation with RA led to a significant decrease in sAPPα levels compared to control (Figure 3.27). This effect was reversed upon cortisol addition. The same effect was observed for sAPPβ. The sAPPα/sAPPβ ratio did not significantly change between non-differentiated cells and RA differentiated cells (Figure 3.28). There was no significant effect of cortisol in RA differentiated cells on sAPP levels compared to non-differentiated cells (Table 3.13).
### Table 3.13: Bonferroni-corrected p-values for the effect of RA (two-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>FSM vs FSM + RA</th>
<th>FSM vs FSM + RA + cortisol</th>
<th>FSM + RA vs FSM + RA + cortisol</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAPP(_\alpha)</td>
<td>&lt; 10(^{-3})</td>
<td>0.44</td>
<td>&lt; 10(^{-3})</td>
<td>0.685</td>
</tr>
<tr>
<td>sAPP(_\beta)</td>
<td>&lt; 10(^{-3})</td>
<td>0.060</td>
<td>0.002</td>
<td>&lt; 10(^{-3})</td>
</tr>
<tr>
<td>sAPP(<em>\alpha)/sAPP(</em>\beta) ratio</td>
<td>0.250</td>
<td>0.330</td>
<td>1.000</td>
<td>0.004</td>
</tr>
</tbody>
</table>

### Figure 3.29: Western blot analysis of APP. Upper part: whole cell extracts from cells grown in inactivated or untreated FSM or HSM, untreated ESM, StartV medium, or from cells treated with either 50 nM cortisol or 10 µM RA in inactivated FSM were separated by SDS-PAGE followed by immunoblot analysis using an anti-APP antibody. Lower part: anti-cyclophilin staining was used as loading control.
3.9. APP, ADAM10 and BACE1 levels in SH-SY5Y cells under various culture conditions

Differences in sAPPα concentration found under different growth conditions may result from changes in APP cleavage enzyme expression. Hence, we examined the protein expression of APP, ADAM10 (the major α-secretase) and BACE1 (the only known β-secretase). Immunoblot analysis of whole cell extracts showed the protein bands at their expected size: APP appeared as double band at 90-120 kDa (Figure 3.29), ADAM10 at around 75 kDa (Figure 3.30) and BACE1 as band pattern between 40 and 70 kDa (Figure 3.31).

Figure 3.30: Western blot analysis of ADAM10. Upper part: whole cell extracts from cells grown in inactivated or untreated FSM or HSM, untreated ESM, StartV medium, or from cells treated with either cortisol or RA in inactivated FSM were separated by SDS-PAGE followed by immunoblot analysis using an anti-ADAM antibody. Lower part: anti-cyclophilin staining was used as loading control.
**Figure 3.31: Western blot analysis of BACE1.** Upper part: whole cell extracts from cells grown in inactivated or untreated FSM or HSM, untreated ESM, StartV medium, or from cells treated with either cortisol or RA in inactivated FSM were separated by SDS-PAGE followed by immunoblot analysis using an anti-BACE1 antibody. Lower part: anti-cyclophilin staining was used as loading control.

Other bands represented immature forms, cleavage products or arose from unspecific binding of staining antibodies. The bands were scanned and quantified using ImageJ software. Band densities were normalised to the corresponding cyclophilin band and correlated to the corresponding control. The results are shown as intensity percentage of the control sample (Table 3.14). Most interestingly, cortisol and RA treatment led to lowered ADAM10 and BACE1 expression compared to control, whereas serum inactivation did not influence the enzyme abundance. StartV showed decreased expression levels for all proteins, indicating starvation. Human serum interfered with the secondary antibodies and led to very strong signals. Hence, HSM samples were difficult to interpret and, therefore, the blot was not comparable to FSM.
control. Nevertheless, no major difference between untreated and inactivated serum could be stated for HSM. ESM displayed an interesting feature: while APP showed increased expression, ADAM10 expression remained constant and BACE1 expression decreased by around 40% as compared to inactivated FSM. This is in agreement with the finding that ESM led to a higher sAPP\(\alpha\)/sAPP\(\beta\) ratio compared to FSM (see Section 3.3).

### Table 3.14: Western blot band quantification.

Relative protein amount as percentage of control (inactivated FSM).

<table>
<thead>
<tr>
<th></th>
<th>APP</th>
<th>ADAM10</th>
<th>BACE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (inactivated FSM)</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>untreated FSM</td>
<td>67%</td>
<td>108%</td>
<td>102%</td>
</tr>
<tr>
<td>inactivated HSM</td>
<td>391%</td>
<td>200%</td>
<td>103%</td>
</tr>
<tr>
<td>untreated HSM</td>
<td>442%</td>
<td>213%</td>
<td>127%</td>
</tr>
<tr>
<td>inactivated ESM</td>
<td>162%</td>
<td>109%</td>
<td>57%</td>
</tr>
<tr>
<td>StartV</td>
<td>80%</td>
<td>48%</td>
<td>59%</td>
</tr>
<tr>
<td>inactivated FSM + cortisol</td>
<td>87%</td>
<td>36%</td>
<td>48%</td>
</tr>
<tr>
<td>inactivated FSM + RA</td>
<td>23%</td>
<td>43%</td>
<td>64%</td>
</tr>
</tbody>
</table>
4. Discussion

It is plausible that fatty acids could impair APP cleavage via changing physico-chemical membrane properties. A study performed in our lab earlier gave a first hint for this hypothesis: in a liposomal cleavage assay a change in the lipid composition altered APP $\alpha$-cleavage kinetics [Marenchino, Williamson et al. 2008]. In this thesis, we studied the effect of several saturated and unsaturated fatty acids on the cleavage of APP by the non-amyloidogenic and the amyloidogenic pathway, respectively. We could confirm an influence of cell membrane composition on $\alpha$-cleavage in a SH-SY5Y cell culture model. Cells grown in StartV supplemented with DHA and VA both showed a significant increase in $\alpha$-cleavage, while those supplemented with SA showed a significant decrease. It is interesting to compare the significance of the change in $\alpha$-cleavage with the amount of fatty acids incorporated into the membranes. DHA showed the highest incorporation of fatty acids compared to the control, namely 886%. VA had an incorporation of only 143%. Nevertheless, the increase in $\alpha$-cleavage was significant for both conditions, with a p-value of 0.019 for DHA and 0.040 for VA. The higher significance in cells supplemented with DHA might therefore rely on a higher increase of the fatty acid content of the membrane. SA incorporation of 231% was only minor compared to DHA, but nevertheless led to the most significant result with a p-value $<10^{-3}$. No effect of EA supplementation on $\alpha$-cleavage could be stated. The comparably low incorporation of 186% could, however, be the explanation for this finding, since all other fatty acid supplementation led to changes in at least sAPP$\alpha$. We suggest that EA supplementation interferes with normal lipid metabolism and leads to pleiotropic effects on cell growth and metabolism. This would be
in line with reports of Furstova et al. showing that incorporation of EA induces cell death in NES2Y β-cells [Furstova, Kopska et al. 2008].

It has to be noted that in the previous study we used an artificial model of liposomes with trypsin as a model protease mimicking α-secretase activity [Marenchino, Williamson et al. 2008]. Liposomes are a simple model for cell membranes, lacking important features such as lipid rafts or protein patches. Although trypsin cleaves in the same place as α-secretase, there are several differences. First, while ADAM10 is a type I transmembrane protein, trypsin is a soluble enzyme. Thus, it might not be affected equally by the membrane composition as a membrane associated enzyme and in addition it “attacks” its substrate from a different angle, which might change the influence of the lipid surrounding on cleavage site accessibility. Second, in contrast to ADAM10, trypsin is not specialised to cleave at a unique cleavage site but rather at certain amino acid sequences. It is a serine protease cleaving proteins after the amino acids lysine or arginine. Third, while ADAMs have been localised to various tissues [Seals and Courtneidge 2003], trypsin activity is limited to the intestine with its very singular environmental conditions. It is therefore plausible that small effects on cleavage kinetics seen in liposomes are indeed due to a change in the lipid surrounding of APP. Those effects on cleavage kinetics might be influenced by other factors when using a cell cultural model or even an in vivo assay, which in addition contains systemic interactions. Nevertheless, in agreement with results of Marenchino et al. we could confirm an influence of the fatty acid composition of the cell membranes on α-cleavage in the SH-SY5Y cells.

Our results are in contrast to a report of Yang et al. stating that supplementing SH-SY5Y cells with fatty acids containing 3 or less double bounds namely VA, EA or SA has a negligible influence on α-cleavage [Yang, Sheng et al. 2011]. Nevertheless we could confirm their findings that those containing more
double bonds, namely DHA, lead to an increase in sAPPα secretion [Yang, Sheng et al. 2011]. Those differences could be due to the fact that Yang et al. used cells differentiated with RA. When we differentiated cells with RA, the sAPPα/sAPPβ ratio did not change compared to non-differentiated cells, but both sAPPα and sAPPβ levels decreased. Our differentiated cells did not respond to further cortisol addition. It is possible that differentiation of SH-SY5Y cells interferes with APP cleavage and that differentiated cells therefore react differently upon changes in growth conditions compared to non-differentiated SH-SY5Y cells.

Several clinical studies investigated the effect of dietary intake of trans-fatty acids on AD development. While no significant relationship between trans-fatty acid consumption and AD was observed in the Rotterdam study [Engelhart, Geerlings et al. 2002], other reports showed that individuals with a high dietary intake of trans-fatty acids have an elevated relative risk of developing AD [Morris, Evans et al. 2003]. It has to be speculated whether the reduced risk of developing all-cause dementia by 47% in the Framingham Heart study [Schaefer, Bongard et al. 2006] is really due to an increase in the sAPPα/sAPPβ ratio or rather due to a yet unknown effect, which might rely on systemic interactions. One hint might be given the above mentioned in vitro study of Grimm showing the mechanism by which DHA reduces Aβ production. BACE1 activity remained relatively unchanged upon DHA addition, whereas γ-secretase activity was markedly reduced and sAPPα secretion increased significantly [Grimm, Kuchenbecker et al. 2011]. The decrease in β- and γ-secretase activity could be confirmed in vivo by isolating cells from mice fed with DHA rich diet [Grimm, Kuchenbecker et al. 2011].

In addition to the α-cleavage studied in the liposomal assays in our lab before [Marenchino, Williamson et al. 2008], here we examined both the α- and the
β-cleavage. In the set-up used in this thesis, DHA led to an increase in β-cleavage, while sAPPβ levels remained constant upon supplementation with VA, EA or SA. For all fatty acids, the sAPPα/sAPPβ-ratio remained unchanged.

Table 4.1: Summary of effects on sAPP levels and sAPPα/sAPPβ ratio.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Comparator</th>
<th>Effect on sAPPα</th>
<th>Effect on sAPPβ</th>
<th>Effect on sAPPα/sAPPβ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSM</td>
<td>FSM</td>
<td>✅</td>
<td>✗</td>
<td>✅</td>
</tr>
<tr>
<td>HSM</td>
<td>ESM</td>
<td>✅</td>
<td>✗</td>
<td>✅</td>
</tr>
<tr>
<td>ESM</td>
<td>FSM</td>
<td>✅</td>
<td>✗</td>
<td>✅</td>
</tr>
<tr>
<td>Inactivated FSM</td>
<td>FSM</td>
<td>✗</td>
<td>✅</td>
<td>✗</td>
</tr>
<tr>
<td>Inactivated HSM</td>
<td>HSM</td>
<td>✅</td>
<td>✗</td>
<td>✗</td>
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Although changes in expression levels were found between growth conditions (see Section 3.9), these changes were not translated to altered sAPP levels. For ESM samples, APP expression was elevated, ADAM10 expression remained stable and BACE decreased, but we did not observe decreased sAPPβ but elevated sAPPα concentrations. The changes in sAPP levels were therefore due to either complex combinations of enzyme expression levels or independent of enzyme expression.

Considering the results on α-cleavage and β-cleavage respectively (Table 4.1), it is interesting to compare whether the two cleavage pathways are coupled or not, where coupled means that an increase of the cleavage-rate in one site leads to a decrease in the other. Newer studies show that α- and β-cleavage are not necessarily coupled. Instead part of the protein might remain uncleaved. Although in some in vivo experiments the cleavage pathways are coupled [Postina, Schroeder et al. 2004], in others, no competition between constitutive α- and β-cleavage was observed [Jorissen, Prox et al. 2010]. In vitro, no competition was observed in different cell lines, among them SH-SY5Y cells [Kuhn, Wang et al. 2010]. Interestingly, in some conditions tested here we observed an increase in one of both APP cleavage-pathways accompanied by a decrease in the other, while in others both showed the same tendency. In detail, we observed a parallel evolution of sAPPα and sAPPβ for DHA in serum supplemented StartV medium, while for EA divergent effects on sAPPα and sAPPβ were observed and VA as well as SA only had a significant effect on sAPPα but not on sAPPβ. In summary, our findings provide evidence for α- and β-cleavage being influenced separately. Grimm et al. claim that fatty acids act on both the amyloidogenic and the non-amyloidogenic pathway via pleiotropic mechanisms. While trans-fatty acids resulted in up-regulation of Aβ combined with a decrease in α-cleavage [Grimm, Rothhaar et al. 2011], the opposite was true for DHA [Grimm,
Kuchenbecker et al. 2011]. This might indicate that a competition is either not pronounced but under extreme conditions or dependent on the cell type used. Such extreme conditions might be found when certain genes are overexpressed e.g. in SH-SY5Y cells stably transfected with APP that are often used in AD research e.g. in the study of Grimm et al. [Grimm, Rothhaar et al. 2011]. Alternatively, inhibition of one cleavage-pathway might not lead to an increase in the other due to APP being translocated in a compartment not optimal for the corresponding cleavage. As discussed previously, APP cleavage by the respective secretases depends on the cellular localization. Our results indicate that the cell membrane composition plays an important role in this decision. While all tested fatty acids apart from the not evaluable EA had a clear effect on \( \alpha \)-cleavage, only DHA had also a significant influence on \( \beta \)-cleavage. This is in line with the previously discussed reports showing that \( \alpha \)-cleavage takes place at the plasma membrane while \( \beta \)-cleavage preferentially occurs after endocytosis of APP in the early endosomes (see Section 1.5).

Comparing the results of Grimm et al. with our own it has to be noted that Grimm et al. added DHA in ethanol [Grimm, Kuchenbecker et al. 2011], while in our experiments ethanol was evaporated prior to addition to the cell culture medium. It is known that ethanol can activate signal transduction pathways in SH-SY5Y cells [Klinker, Lichtenberg-Kraag et al. 1996], which could influence experimental outcome.

It is an ongoing discussion, which marker for AD should be looked at. To our knowledge, we are the first ones to consider the cleavage rate of APP into either sAPP\( \alpha \) or sAPP\( \beta \) measured by the corresponding ratio. Therefore, we examined both sAPP\( \alpha \) and sAPP\( \beta \) throughout all experiments. This gives evidence on the amount of APP cleaved by both the amyloidogenic and the non-amyloidogenic pathway when SH-SY5Y cells are supplemented with sera
from different species origin, fatty acids or cortisol. This is especially important, since the different APP cleavage products have antagonistic effects \textit{in vitro} [Jarrett, Berger et al. 1993; Furukawa, Sopher et al. 1996] as well as \textit{in vivo} [Mann, Iwatsubo et al. 1996; Meziane, Dodart et al. 1998; Stein, Anders et al. 2004]. A significant change in Aβ concentration might therefore be compensated by a corresponding change in sAPPα concentration. In addition, no competition between constitutive α- and β- cleavage in SH-SY5Y cells was observed [Kuhn, Wang et al. 2010]. Therefore, it is not sufficient to focus on the expression level of Aβ only, neither on another single product of either the amyloidogenic or the non-amyloidogenic pathway. Rather the whole APP processing has to be considered. With our model, we thus aim to have a deeper insight into the overall effect of biomolecules, giving further evidence for the preponderance of either neuroprotective functions of sAPPα or neurotoxic effects due to Aβ generation estimated by the amount of sAPPβ measured. When interpreting our recent data showing no effect of any of the fatty acids on the sAPPα/sAPPβ ratio, it is interesting to have a closer look on the mechanism leading to these results (see Table 4.1). Whereas the effect on the sAPPα/sAPPβ ratio is evident if the effects on sAPPα and sAPPβ diverge, it is interesting to look in detail at the ratio of those conditions where both APP cleavage-pathways show the same alteration. In our experiments, the corresponding increase or decrease in sAPPα after DHA or SA supplementation, respectively, was outweighed by an identical although for SA not significant change in sAPPβ, leading to no overall significant change of the ratio. The increase in α-cleavage after VA supplementation was not pronounced enough to result in a significantly elevated sAPPα/sAPPβ ratio. Interestingly, the same experiment performed in HSM led to a significant increase in both sAPPα and sAPPβ upon DHA as well as VA supplementation. Nevertheless, the ratio did not significantly change for any
of the two fatty acids. The different behaviour of VA in StartV and HSM can be explained by starving conditions in StartV medium. VA was shown to be metabolised in human cells to conjugated linoleic acid [Banni, Angioni et al. 2001]. It is likely that in the starved StartV medium, VA was directly used to build up lipids whereas in the serum complemented medium HSM, VA was first metabolised to conjugated linoleic acid and, therefore, had a different effect on membrane properties.

Considering the reports showing competition for APP as a substrate in vivo in transgenic mice overexpressing some of the relevant factors such as APP [Postina, Schroeder et al. 2004] but not in knock-out mice [Jorissen, Prox et al. 2010] or in vitro in normal cell lines [Jorissen, Prox et al. 2010; Kuhn, Wang et al. 2010], experiments using transfected cell lines might lead to a different or even adulterated outcome. This might explain the discrepancy between different results from diverse in vivo and in vitro studies and the ones observed in this thesis.

An in vivo study reported an increase in Aβ production in transgenic mice when DHA was administered together with a peptamen diet, but a reduced Aβ production in co-administration with a low-fat diet [Amtul, Keet et al. 2011]. These findings point at potential interaction of the effect of DHA with other nutrient’s effect on APP processing. Therefore, differences in the medium composition between StartV, HSM and FSM could be the reason for the different effect of DHA supplementation seen in our experiments and the study of Grimm et al. [Grimm, Kuchenbecker et al. 2011]. In conclusion, there still exist a lot of differing observations on the effect of DHA and other fatty acids, depending on the experimental set-up used (transgenic mice, knock-out mice or the cell lines used as well as the growth conditions). Thus, for the in vitro assays it would be helpful to agree on one or two cell models for mechanistic studies. SH-SY5Y cells having been extensively studied would
Chapter 4: Discussion

make an ideal candidate cell line for such a standard model. However, the major challenge is to establish such a standard model in combination with a defined medium for standardised cell growth. The importance for a standardised cell culture set-up was demonstrated by our experiments. We have seen significant effect of serum choice and inactivation of serum on APP cleavage. We hypothesised that the different effects seen using different sera could be due to different cortisol concentration, since serum cortisol levels vary between species [Laske, Stransky et al. 2009] and heat inactivation of serum has been shown to increase the free fraction of cortisol denaturing carrier proteins of cortisol [Lewis, Bagley et al. 2005]. Increased cortisol levels are still discussed to be either a cause of disease progression or a consequence of chronic inflammation in the diseased brain. In our experiments, we found that supplementation of cortisol both in- and decreased sAPP\(_{\alpha}\) levels in SH-SY5Y cells, depending on cell batch and experiment. As the sAPP\(_{\beta}\) levels changed also from experiment to experiment but did not differ between cortisol addition samples and control, we excluded a general effect on protein metabolism or enzyme activity. Our results suggest that cortisol enhances effects already present in a positive feedback manner. This would be in agreement with earlier studies in AD patients. We therefore hypothesised that the previously described increased cortisol concentrations are rather a consequence of the inflammatory response in diseased brains. It should be mentioned, however, that \textit{in vivo} concentrations of 500 nM were needed to repress induction of nitric oxide production and TNF-\(\alpha\) release from activated microglia cells [Drew and Chavis 2000]. These reports depend on systemic interactions of cortisol, explaining different results in cell culture models. Nevertheless, concentration of 50 – 100 nM cortisol used in our experiments might have been too low.
We excluded the tested fatty acids and cortisol as cleavage modifying factors responsible for the differences seen using different sera. Further analysis showed that heat inactivation of serum changed the sAPPα/sAPPβ ratio for FSM but not HSM (shown in Section 3.5). Interestingly, heat inactivation of fetal calf serum only affected α-cleavage while β-cleavage remained unchanged leading to a net reduction of the sAPPα/sAPPβ ratio. This strengthened the hypothesis that at least fetal calf serum contains an APP cleavage influencing factor, which is heat sensitive. Dialysing FCS we could show that at least one influencing factor must be larger than 12 kDa. The fraction containing only smaller molecules did not increase the sAPPα/sAPPβ ratio even when combined with heat inactivated serum. However, using the high molecular weight fraction resulted in the same ratio as untreated serum. The factor responsible for the differences between untreated and heat-inactivated serum is therefore very likely to be a protein. We cannot exclude the possibility of a soluble enzyme present in serum that is able to cleave APP. ADAM33 was shown to be a soluble α-secretase cleaving APP [Zou, Zhang et al. 2005]. Its cleavage efficiency however was low and ADAM33 was shown to be primarily expressed in lung fibroblasts and bronchial smooth muscle cells. Nevertheless, other yet unknown soluble enzymes, possibly from the ADAM family, could be present in the serum. The effect of serum can therefore be due to either a direct cleavage of APP or to an effect on the already known secretases.
5. Conclusions and outlook

We studied the effect of serum origin, serum inactivation, fatty acids and cortisol on APP cleavage. Serum from different species origin significantly affected APP cleavage. Furthermore serum inactivation led to a significant decrease in sAPPα levels in FSM but an increase in HSM. Serum therefore contains factors able to affect APP cleavage in cell culture models. This has to be considered when performing mechanistic studies in cell culture, but also opens a new track for the research of molecules able to reduce Aβ load. When cells were incubated with fatty acids, levels of both sAPPα and sAPPβ were affected, although the sAPPα/sAPPβ ratio remained unchanged. These results strengthen the hypothesis of membrane composition affecting APP cleavage. We could not provide supporting data of cortisol treatment affecting α- or β-cleavage of APP. Our results rather suggest that previously reported effects of cortisol are due to its influence on inflammatory response in diseased brains. We could show that foetal calf serum contains a molecule of at least 12 kDa that was able to increase the sAPPα/sAPPβ ratio. We expect this molecule to be a protein due to its heat sensitivity. The effect of this factor was not due to increased expression of neither APP, the most important α-secretase ADAM10, nor BACE1. The next steps to further characterise this factor would go in direction of differential protein analysis between dialysed and full serum. A powerful proteomic approach could narrow down the number of possible candidates. Individual testing of these candidates then might help to identify the factor and open up new questions to investigate. However, it cannot be excluded that several factors could be necessary and only the combined supplementation of these factors would produce an effect on APP cleavage.
6. **Appendix: Tight junction projects**

In this appendix separate projects about tight junctions are presented. We terminated these projects because purification as well as synthesis of claudin-1 was not successful.

**Background**

Tight junctions are molecular barriers hindering paracellular diffusion at epithelia and endothelia. The tightness of these barriers is mainly determined by proteins of the claudin family, which consists of more than 20 transmembrane proteins, with claudin-1 being the most common and providing the tightest junctions. All claudins have a common domain structure: a short cytoplasmic N-terminus, 4 transmembrane domains, two extracellular "loop" domains and a longer cytoplasmic C-terminus. At so-called "kissing points" of tight junctions, the loop domains of claudins on one cell are thought to interact with proteins (probably also claudins) on the adjacent cell providing a tight fence that even can hinder the diffusion of water molecules.

The tightest junctions are found in the endothelium forming the blood-brain barrier, which is, along with all its transport proteins such as P-glycoprotein, one of the main obstacles for drugs targeting brain structures. Therefore, a deeper understanding of the exact mechanism of tight junctions is of great pharmaceutical interest. Strategies to loosen the tight junctions temporarily might allow blood-brain passage of otherwise non-permeating drugs. This is of particular interest in life-threatening diseases such as brain cancer.
Figure 6.1: Scheme of a tight junction assay. Claudin loops are attached on a Biacore chip and on liposomes via biotin-streptavidin interactions. The association of liposomes and chip-anchored loops is measured. Small molecules are tested to break the association.

The goal of this project was to set up an *in vitro* system to study the loop-loop interactions of claudins and to use this system to screen peptide or chemical libraries for molecules that disrupt claudin loop-loop interactions. Figure 6.1 shows how such an in vitro assay could be set up.

**Claudin-1 purification from MDCK cell culture**

First we tried to purify claudin-1 from MDCK cells. MDCK cells (passage numbers up to 234) were grown to confluence and maintained in culture for 11 days. The cells were harvested, washed and the cell pellets frozen in liquid nitrogen for later use. Cell pellets were homogenised with 50 strokes in a Brown homogenizing system. Different buffers for protein solubilisation (IP buffers) were used for preparing membrane fractions:
Only OG was able to solubilise claudin-1. After solubilisation of claudin-1 with OG containing buffers, claudin-1 was immunoprecipitated with anti-claudin-1 antibodies and protein G beads. Since OG was interfering with the antibody-antigen interaction, claudin-1 could not be purified. Dialysis of samples after solubilisation to reduce the amount of OG was not successful. Although higher amounts of SDS did also improve solubilisation, purification of claudin-1 from those samples was not possible, because higher SDS concentration impaired antibody-antigen interactions. In order to enrich claudin-1 we prepared membrane fractions of whole cell extracts from MDCK cells which were subjected to sucrose density centrifugation (continuous gradient from 10% to 40% sucrose). SDS-PAGE analysis of fractions showed that claudin-1 accumulated in raft fractions, assigned by the presence of caveolin.

**Solid phase peptide synthesis of the first loop of claudin-1**

In a next step, the first loop domain of claudin-1 was synthesized using solid phase peptide synthesis (SPPS). The first loop domain of claudin-1 is 53 amino acids long, which is too long for an efficient one-batch synthesis. A single cysteine almost in the middle of the peptide (position 26) however can be used to conduct a native chemical ligation of the two subdomains claudin-1-loop1-C-terminus (25 amino acids, hydrophobic peptide) and claudin-1-loop1-N-terminus (28 amino acids). The method of native chemical ligation is
described in [Dawson, Muir et al. 1994]. In addition, using special resins allowed direct introduction of a biotinylated PEG-linker.

The main mass found in mass spectrometry (MS) after a successful synthesis of claudin-1-loop1-C-terminus was 3455.8 m/z as expected. However the peptide contained several impurities. Using a previously described high performance liquid chromatography (HPLC)-method [Marenchino, Williamson et al. 2008] did not allow separating the impurities from claudin-1-loop1-C-terminus. Adjustments in gradient or detection did not improve the resolution power of the method. As we weren’t successful purifying claudin-1-loop1-C-terminus (see Figure 6.2), we refrained from the synthesis of further claudin-1 fragments.

Figure 6.2: HPLC Chromatogram of SPPS product. The product of solid phase peptide synthesis was run over a C8 reverse phase HPLC column. This figure shows one representative chromatogram. MS analysis of pooled fractions (bars) revealed the indicated masses. The desired mass of 3455 m/z was found in the fourth fraction together with several impurities.
Cloning of loop-domain of claudin1

Finally, we tried to purify recombinant claudin-1 loops after expression in *E. coli*. Therefore, we cloned the loop into the pTYB vector able to express peptides in *E. coli* cells. The IMPACT kit (NEB) allowed expressing a desired protein fused with an intein-tag that could be purified over a chitin column. During the purification step, the intein-tag was cleaved off.

Correct cloning of the loop peptide was verified by sequencing. Nevertheless, transformation in *E. coli* did not yield a coomassie-detectable peptide expression. Repetitive cloning of the loop as well as adding an alanine before the intein-tag to avoid cleavage inhibiting effects of an arginine did not further improve peptide expression.

Pilot experiments to test streptavidin binding on biotinylated liposomes

In order to avoid dimerization of liposomes while adding biotinylated claudin loops using streptavidin-biotin interactions, pilot Zetasizer experiments were set up. Liposomes (POPC:POPE:Chol:Biotin-DOPE, 50:40:10:0.1) were prepared using a standard freeze-thaw protocol followed by extrusion through a 200nm pore membrane. Dimerization of liposomes was measured as function of biotin addition. The most homogenous size distribution at around 200 nm was found when equimolar amounts of streptavidin and additional biotin (excluding the covalently bound biotin on DOPE) were used. Adding excess biotin reduced dimerization: dimers only appeared after 22 hours measurement time.
Pilot experiments for a cellular tight junctions-disruption assay

A cellular assay based on the very tight monolayer of MDCK cells is a very interesting tool to confirm molecules found to promote dissociation of claudin loops. In order to quantify tight junction permeability, we measured trans-epithelial electrical resistance (TEER) or the diffusion of a Dextran-AlexaFluro-594-dye in a spectrophotometer. The Dextran-AlexaFluro-594-dye resulted in a good linear response when the cell layer was treated with ethylene glycol tetraacetic acid (EGTA, Ca$^{2+}$-shift experiments). TEER-measurements in contrast provided less reliable and far less quantitative results, as no linear range of response was detected.

Methods used in the tight junction projects

*Cell culture.* MDCK cells, strain II, were a kind gift of Michel Paccaud (Geneva, Switzerland). They were grown up to passage 234 as previously described [Hofmann 2007].

*Antibodies.* For Westernblot analysis and immunoprecipitation of claudin-1, monoclonal mouse anti-claudin-1 was used (catalogue number: 37-4900, Zymed). Raft fractions were identified in Westernblot analysis using a polyclonal anti-caveolin antibody (catalogue number: C13630, BD Biosciences).

*Sucrose density gradient centrifugation.* Samples in TBS buffer were mixed with an 80% sucrose solution to obtain a final concentration of 60% sucrose. In a centrifugation tube a step-gradient with 80%, 60% (including the sample), 40%, 20% and 5% sucrose was carefully prepared. The tube was centrifuged
at 39'000 rpm (200'000 g) for 19 hours in a SW41Ti rotor in a Beckmann ultracentrifuge at 4°C. After centrifugation, fractions of 1 ml were carefully taken from the top. The density of the fractions was calculated from the refractive index measured with a refractometer (RFM90, Bellingham and Stanley, UK).

**Solid phase peptide synthesis of claudin-1-loop-1-C-terminus.** The Biotin-PEG NovaTag® resin was washed in dimethylformamide and deprotected with piperidine. The first amino acid (Fmoc-arginine with pmc-protected side chain) was activated with HATU and coupled to the resin. A positive Kaiser’s test confirmed the completeness of the reaction. If the test was negative, further rounds of arginine-coupling reactions were added. The further amino acids were coupled in a Synthesizer with automated fast-moc coupling chemistry (Applied Biosystems Peptide Synthesizer Model 431). After coupling of the last amino acid, the peptide was cleaved from the resin with a mix of trifluoroacetic acid (94%), pure water (2.5%), ethanediol (2.5%) and triisopropylsilane (1%). The peptide was precipitated with ice-cold ethylester. The solvents were evaporated and the peptide dissolved in pure water and desalted over a PD size exclusion column. The peptide was lyophilized for longer storage.

**Cloning of pSM2.** The DNA sequence of the first loop domain of claudin-1 was amplified in PCR reaction using Pfu DNA-polymerase and two primers (3’-CAGTGGAGGATTTTACTCC-5’ and 3’-ACGGGTTGCTTGCAATG-5’) on the complete human cDNA of claudin-1 (GenBank accession No. BC012471) obtained from the German Resource Center for Genome Research (Heidelberg, Germany). The resulting DNA sequences were purified by agarose gel electrophoresis, visualised with GelGreen to avoid damaging wavelengths when extracting the purified DNA-bands from the gel. The purified DNA was digested with Ndel and SapI to obtain sticky ends for
ligation with an identically digested pTYP1 vector (Invitrogen Kit). The correct sequence was verified by sequencing (Microsynth). The correct ligation product (pSM2) was used to transform ER2566 *E. coli* cells.

*Expression of claudin-1 loop 1 in *E. coli*.* *E. coli* ER2566 cells were transformed with pSM2 using a standard heat-shock transformation protocol as provided by the IMPACT kit’s manufacturer (NEB). The cells were grown in LB medium with ampicillin as transformation marker. At an OD of 0.2 the cells were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown over night to produce the fusion protein of claudin-1-loop-1 with an intein tag. The intein tag was cleaved during the affinity chromatography (Kit components) using dithiothreitol (DTT). The eluates were analysed with SDS gel electrophoresis and coomassie blue stain.

*Measurement of trans-epithelial electrical resistance (TEER).* MDCK cells were grown on a cell culture insert for 6-well plates (pore size: 4 µm, Corning). After 5 days growth, the cells were subjected to specific treatments (like calcium-depletion by EGTA). The cell insert was placed in a EndOhm-12 resistance measurement chamber and the electrical resistance was measured with an EvomX device (World Precision Instruments, USA).

**Conclusion**

Due to its localisation to lipid rafts, the tetraspanin-like membrane protein claudin-1 is very difficult to purify. Furthermore, the sequence of claudin loop domains was not suitable to be synthetized by SPPS. We prepared liposomes ready to be loaded with claudin-1 loop peptides and were able to set up a cellular assay for tight junction permeability. However, we could not introduce
the claudin loops to the assays and, therefore, stopped to pursue the tight junction projects.
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