Cellular responses to amyloid-beta protofibrils

Focus on astrocytes, extracellular vesicles and antibody treatment

ELISABETH NIKITIDOU
Abstract

Knowledge about the cellular mechanisms behind the initiation and propagation of Alzheimer’s disease (AD) is limited. Decades of research have focused on neuronal abnormalities in AD, but recently more attention has been given to the glial cells. Being the most numerous glial cell type in the brain, astrocytes are important for many functions, but their role in AD is poorly understood. The aim with this thesis was to clarify the involvement of astrocytes in AD by using a co-culture system of primary neurons and glia. The co-cultures were exposed to soluble amyloid-beta (Aβ) aggregates, i.e. protofibrils that are known to be particularly harmful.

In Paper I, the capacity of astrocytes to ingest and degrade Aβ protofibrils was investigated. We found that astrocytes effectively ingested Aβ, but were ineffective in degrading the material. The intracellular accumulation of Aβ in astrocytes resulted in lysosomal dysfunction, high intracellular load of partly N-terminally truncated Aβ and extracellular vesicle (EV) mediated neuronal cell death.

Cells can communicate by releasing cargo into EVs, but the role of EVs in the spreading of Aβ pathology is unclear. In Paper II, the protein content of EVs released specifically following Aβ protofibril exposure was analyzed. We found markedly increased levels of apolipoprotein E (apoE) in EVs from Aβ protofibril exposed co-cultures, suggesting a role for intercellular transfer of apoE in Aβ pathology.

Passive immunotherapy has been suggested as a promising therapeutic strategy for AD. In Paper III, we investigated if the Aβ protofibril-selective antibody mAb158 could affect Aβ clearance in the co-culture. The mAb158 treatment reduced Aβ accumulation in astrocytes and rescued neurons from Aβ-induced cell death.

In Paper IV, we explored the effect of EVs, isolated from Aβ protofibril exposed co-cultures on cultured neurons. In addition to increased cell death, we found that such EVs had a strong negative impact on the synapses, dendrites and mitochondria of the neurons.

Taken together, this thesis contributes with important knowledge about the role of astrocytes in Aβ pathology, the vesicle-mediated spreading of Aβ and the effects of anti-Aβ antibody treatment.

Keywords: Alzheimer’s disease, Amyloid-beta, Protofibrils, Astrocytes, Extracellular vesicles, Antibody, Neurons, Degradation
To my wonderful family
for your endless love and support
The good life is one inspired by love
and guided by knowledge.
Bertrand Russel
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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III  Söllvander S, Nikitidou E, Zyśk M, Söderberg L, Sehlin D, Lannfelt L, Erlandsson A. The Aβ protofibril selective antibody mAb158 prevents accumulation of Aβ in astrocytes and rescues neurons from Aβ-induced cell death. *Submitted*

IV  Nikitidou E, Ingelsson M, Erlandsson A. Extracellular vesicle-mediated amyloid-β toxicity in cortical neurons. *Manuscript*

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The front cover is designed in collaboration with my dear brother-in-law and talented neuroscientist Marco Ledri.
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Abbreviations

Aβ  Amyloid-beta
ABCA1  ATP-binding cassette transporter 1
AD  Alzheimer’s disease
ADL  Activities of daily living
AICD  APP intracellular domain
Aph-1  Anterior pharynx defective
apoE  Apolipoprotein E
APP  Amyloid precursor protein
BACE1  Beta-site APP cleaving enzyme 1
bFGF2  Basic fibroblast growth factor 2
cAMP  Cyclic adenosine monophosphate
CNPase  2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS  Central nervous system
CSF  Cerebrospinal fluid
CTF  Carboxyterminal fragment
DAPI  4',6-diamidino-2-phenylindole
DLB  Dementia with Lewy bodies
ECL  Enhanced chemiluminescence
EGF  Epidermal growth factor
ELISA  Enzyme-linked immunosorbent assay
EVs  Extracellular vesicles
FITC  Fluorescein isothiocyanate
FTD  Frontotemporal dementia
GFAP  Glial fibrillary acidic protein
HFIP  1,1,1,3,3,3,-Hexafluoro-2-propanol
HRP  Horseradish peroxidase
ICC  Immunocytochemistry
ILVs  Intraluminal vesicles
IP  Immunoprecipitation
LAMP1  Lysosomal-associated membrane protein 1
LAMP2  Lysosomal-associated membrane protein 2
LC-MS  Liquid chromatography-mass spectrometry
LDLR  Low density lipoprotein receptor
LRP  Low density lipoprotein receptor-related protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MMSE</td>
<td>Mini-mental state examination</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MVBs</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>MVs</td>
<td>Microvesicles</td>
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<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>NFTs</td>
<td>Neurofibrillary tangles</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Pen-2</td>
<td>Presenilin enhancer</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PiB</td>
<td>Pittsburgh compound B</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>PSEN2</td>
<td>Presenilin 2</td>
</tr>
<tr>
<td>p-tau</td>
<td>Hyperphosphorylated tau</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rab</td>
<td>Ras-related protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar-type ATPase</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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Dementia is the diagnostic term for a variety of symptoms, caused by many different diseases and injuries in the brain. Alzheimer's disease (AD) is the most common cause of dementia, which accounts for 60-80% of all dementia cases in the world. In AD, the brain tissue becomes atrophic due to an abnormal extent of neuronal cell death. This gradual process is ongoing for many years, before the first clinical symptoms arise. The symptoms vary depending on which brain region that is affected, but the parietal and temporal lobes are particularly diseased. The parietal lobe is a brain area that is important for our orientation skills, practical ability, and writing and counting skills, while the temporal lobe is important for learning, memory storage, language functions and emotional regulation. In AD, these and many other brain functions are impaired. At present, there is unfortunately no cure for the disease, but the symptoms can be relieved in some cases by medication. About 1-5% of all AD cases are caused by hereditary mutations in different genes, but the majority have an unknown cause. Research is conducted worldwide to investigate the underlying disease mechanisms in order to develop therapies for treating AD. A suggested explanation to why the neurons die in the AD brain is based on the storage of the deleterious protein, amyloid-beta (Aβ), between neurons in the form of plaques. In addition, another protein called tau is accumulated inside the neurons and forms tangles, which can induce their death. There is a theory that strongly supports that formation of Aβ occurs first and then drives the disease by the formation of tangles. Increasing evidence indicates that it is not the plaques that are deleterious to neurons, but rather the soluble precursors of the plaques, so-called Aβ oligomers and protofibrils.

Research has so far mainly focused on the neuronal impact in AD, but more attention has started to be given to the different glial cells of the brain. Astrocytes, the most common type of glial cells, have previously been considered to function primarily as support cells for the neurons. However, it has been found that astrocytes have several important functions, both in the healthy and the diseased brain. Their functions include regulating the brain’s pH, blood flow and chemical environment, nurturing neurons and contributing to the formation of the blood-brain barrier. Astrocytes also respond to brain damage by forming a so-called glial scar, as well as helping in the degradation of foreign particles and harmful proteins such as Aβ.

The aim of this thesis was to increase the knowledge about the role of astrocytes in the uptake and degradation of Aβ protofibrils, and to study whether
Astrocytes may contribute to the Aβ pathology by spreading Aβ and other proteins via vesicles. To our help, we cultured neurons, astrocytes and oligodendrocytes from neural stem cells isolated from embryonic mouse brains. The differentiated cells in the mixed co-culture were exposed to synthetic Aβ protofibrils to study the cellular responses.

In the first study, we found that astrocytes effectively engulf Aβ protofibrils, but that they are ineffective and slow in degrading the ingested material, which are instead stored in the cell’s garbage stations called lysosomes. We also noted that oligodendrocytes, which belong to the brain’s glial cells, accumulate Aβ protofibrils to a certain extent, while the neurons do not. No neuronal cell death occurred directly after the Aβ protofibril exposure. However, the high load of Aβ protofibrils in astrocytes resulted in the astrocytes releasing the partially degraded material into vesicles in the cell medium, which in turn induced neuronal cell death.

In the second study, we analyzed and compared the protein content of the vesicles that were secreted specifically after Aβ protofibril exposure with vesicles secreted from untreated cell cultures. The amount of released vesicles did not differ, but we found five proteins that showed markedly altered levels in vesicles from Aβ protofibril exposed cell cultures, compared to untreated cell cultures. The most prominent protein of them was apolipoprotein E, known to transport cholesterol between cells and to be strongly linked with AD.

In the third study, we investigated how the degradation of Aβ protofibrils in astrocytes is affected by treatment with the Aβ protofibril-selective antibody mAb158, which in humanized form (BAN2401) is currently in clinical trials for AD. We found that the Aβ accumulation in astrocytes ceased when the cell cultures were treated with mAb158 and the neurons were rescued from dying.

In the fourth study, we investigated how the neurons are affected by treatment with vesicles isolated from Aβ protofibril-exposed cell cultures. In addition to increased neuronal cell death, we found a significant negative impact on the neurons’ dendrites, synapses and mitochondria (the energy stations of the cell).

In summary, we have been able to demonstrate that astrocytes have an important role in the Aβ pathology and that vesicle-mediated spreading of Aβ may accelerate the disease. Our results also indicate that immunotherapy against Aβ can affect different pathological processes in both astrocytes and neurons.
Demens är den diagnostiska benämningen för en rad symptom som kan bero på många olika sjukdomar och skador i hjärnan. Den vanligaste orsaken till demens är Alzheimers sjukdom (AD), som utgör 60-80% av alla demenssjukdomar i världen. Vid AD börjar hjärnvävnaden gradvis förtvina, till följd av att nervcellerna dör i onormal omfattning. Denna process sker snygande och under en längre tid innan de första kliniska symptomen uppkommer. Symptomen varierar beroende på vilken del av hjärnan som drabbats, men framför allt påverkas parietal- och temporalloberna. Parietalloben är ett hjärnområde som är viktigt för vår orienteringsförmåga, praktiska förmåga samt skriv- och räkneförmåga, medan temporalloben är viktig för inlärning, minneslagring, språkfunktioner och emotionell reglering. Vid AD försämrar dessa och många andra hjärnfunktioner. Sjukdomen går dessvärre i nuläget inte att bota, men symptomen kan i vissa fall lindras med mediciner. Cirka 1-5% av alla AD fall orsakas av ärfliga mutationer i olika gener, men majoriteten har en okänd orsak. Forskning pågår världen över för att förstå de bakomliggande sjukdomsmekanismerna i hopp om att utveckla terapier för att kunna behandla sjukdomen. En huvudsaklig förklaring till att nervcellerna dör i AD hjärnan tros bero på att ett skadligt protein, amyloid-beta (Aβ), lagras mellan nervceller i form av plack. Dessutom ansamlas även små trådar, av proteinet tau, i s.k. tangles inuti nervcellerna, vilka också kan bidra till att de dör. Det finns en stark teori om att Aβ uppkommer först och därefter driver sjukdomsförloppet genom bildandet av tangles. Alltmer forskning tyder på att det inte är placken som är giftiga för nervcellerna, utan de lösliga förstadierna till placken, s.k. Aβ oligomerer och protofibriller.


Syftet med den här avhandlingen var att öka kunskapen om astrocyters roll i upptaget och nedbrytningen av Aβ protofibriller samt studera om astrocyter...
bidrar till Aβ patologin genom att sprida Aβ och andra proteiner i vesiklar. Till vår hjälp odlade vi nervceller, astrocyter och oligodendrocyter från neurala stamceller som isolerats från embryonala mushjärnor. De färdigutvecklade cellerna i den gemensamma cellkulturen behandlades med syntetiska Aβ protofibriller för att studera det cellulära svaret.

I den första studien fann vi att astrocyter effektivt tar upp Aβ protofibriller, men att de är ineffektiva och långsamma på att bryta ner materialet, som istället lagras i cellens sopstationer som kallas lysosomer. Vi noterade även att oligodendrocyter, vilka också tillhör hjärnans gliaceller, lagrar Aβ protofibriller i viss utsträckning, medan nervcellerna inte gör det. Ingen nervcelldöd infann sig direkt efter exponeringen av Aβ protofibriller. Den höga belastningen av Aβ protofibriller i astrocyter resulterade dock i att astrocyterna släppte ut det delvis nedbrutna materialet i vesiklar i cellmediet, vilka i sin tur inducerade nervcelldöd.

I den andra studien analyserade och jämförde vi proteininnehållet från vesiklar som utsändes specifikt efter Aβ protofibrill-behandling med vesiklar som utsändes från obehandlade cellkulturer. Mängden vesiklar skiljde sig inte åt, men vi fann fem proteiner som visade markant förändrade nivåer i vesiklar från Aβ protofibrill-behandlade cellkulturer jämfört med obehandlade cellkulturer. Det mest framträdande proteinet av dem var apolipoprotein E, känd för att transportera kolesterol mellan celler och som är starkt kopplad till AD.

I den tredje studien undersökte vi hur nedbrytningen av Aβ protofibriller i astrocyter påverkas genom behandling med den Aβ protofibrill-selektiva antikroppen mAb158, som i humaniserad form (BAN2401) just nu befinner sig i klinisk prövning för behandling av AD. Vi fann att Aβ ackumuleringen i astrocyter upphörde när cellkulturerna behandlades med mAb158 och nervcellerna räddades från död.

I den fjärde studien undersökte vi hur nervcellerna påverkas av behandling med vesiklar isolerade från Aβ protofibrill-behandlade cellkulturer. Utöver ökad nervcellsdöd fann vi kraftig negativ påverkan på nervcellernas utskott, synapser och mitokondrier (cellens energistationer).

Sammanfattningsvis har vi kunnat påvisa att astrocyter har en viktig roll i Aβ patologin och att vesikel-medierad spridning av Aβ skulle kunna påskynda sjukdomsförloppet. Våra resultat tyder också på att immunterapi mot Aβ kan påverka olika patologiska processer i både astrocyter och nervceller.
Introduction

Alzheimer’s disease

It is estimated that more than 46.8 million people worldwide are affected by dementia and, according to the World Alzheimer Report 2016, the number of cases are expected to reach 131.5 million by 2050 [1]. Dementia has therefore become an important health and socioeconomic issue in our society. The most common cause of dementia is Alzheimer’s disease (AD), which constitutes 60-80% of all dementia cases [2]. Other forms of dementia include vascular dementia, dementia with Lewy bodies (DLB) and frontotemporal dementia (FTD). Alzheimer’s disease is an irreversible, progressive neurodegenerative disorder that was first described by the German psychiatrist and neuropathologist Alois Alzheimer in 1906. He observed and clinically investigated a 55-year old patient named Auguste Deter, who was suffering from severe memory loss, disorientation and other cognitive impairments. Following her death, Alois Alzheimer examined the autopsied brain and discovered neuro-pathological lesions in the form of dense amyloid plaques around neurons and neurofibrillary tangles (NFTs) inside the neurons (Figure 1) [3]. The extracellular amyloid plaques and intraneuronal NFTs were later shown to consist of amyloid-beta (Aβ) fibrils and hyperphosphorylated tau (p-tau), respectively [4]. They represent the two major neuropathological hallmarks of AD.

Figure 1. The neuropathological hallmarks of AD consist of extracellular amyloid plaques (green, A) and intracellular NFTs (red, B). Pictures kindly provided by Paul O’Callaghan.
Moreover, AD is characterized by vascular damage from extensive plaque deposition, neuroinflammation, gradual loss of neurons and degeneration of axons and dendrites that lead to enlarged ventricles and brain atrophy [5]. In most cases, the NFTs initially involve the medial temporal lobe structures (e.g. hippocampus and entorhinal cortex) and then extend to the temporal, parietal and frontal lobes as the disease progresses [6]. Consequently, this will give rise to various behavioral, psychological and cognitive symptoms affecting learning, memory, language and other higher executive functions in AD patients [7, 8]. The severity of symptoms will gradually worsen as the disease progresses and affected individuals will also suffer from impaired movements and end up completely dependent on their caregivers in the late stage of the disease [9]. Due to heterogeneity in the rate of progression among AD patients, it may be difficult for clinicians to give an accurate prognosis after a clinical diagnosis of AD [10] and the life expectancy can vary between 3-20 years [11], with the definite cause of death usually being pneumonia or ischemic heart disease [12]. Based on the age of onset, AD is divided into familial or sporadic AD, which helps us better understand the development and severity of the disease.

**Familial Alzheimer’s disease**

Early-onset or familial AD generally affects individuals under the age of 65 years and constitutes 1-5% of all AD cases [13]. It is associated with a more rapid rate of disease progression because of inheritance factors. In familial AD, the cause is a mutation with an autosomal dominant inheritance pattern. The first gene to be identified in familial AD was the *amyloid precursor protein* (*APP*) gene on chromosome 21, discovered in 1987 by Kang and colleagues [14]. The APP is an integral, transmembrane glycoprotein (cell-surface receptor) with a large extracellular domain and a small cytosolic domain [15]. Alternative splicing of the mammalian *APP* gene produces different isoforms; whereas the APP\(_{695}\) isoform is primarily expressed in the brain the APP\(_{751}\) and APP\(_{770}\) isoforms are mainly expressed in the periphery [16, 17]. The physiological role of APP is only partly understood, but it has been suggested to be important for neuronal function, including neuronal migration, neurite outgrowth, synapse formation and synaptic plasticity [18, 19]. Point mutations (insertion, deletion or substitution to a single nucleotide base) in the *APP* gene lead to an improper post-translational proteolytic processing of APP, resulting in enzymatic cleavage of the APP fragment to form A\(\beta\) by \(\beta\)- and \(\gamma\)-secretases [20]. More than 16 disease-causing mutations located at or in the vicinity of the \(\beta\)- and \(\gamma\)-secretase cleavage sites or within the APP sequence have been discovered in various families around the world [21, 22]. Among them is the Swedish mutation, discovered in 1992 and caused by two base pair transversions at the \(\beta\)-secretase cleavage site, which results in an increased
total Aβ production [23]. The London mutation is instead caused by mutations in the γ-secretase cleavage sites, which leads to an increased production of the 42-amino acid Aβ sequence compared to the 40-amino acid Aβ sequence (Aβ42/Aβ40 ratio) [24]. Increased formation of soluble Aβ protofibrils due to a mutation located within the Aβ sequence was found in the Arctic mutation in 2001 [25]. Interestingly, it was found a few years ago that a group of individuals on Iceland with a missense mutation (amino acid substitution) in the APP gene, referred to as the Icelandic mutation, are protected against developing AD [26]. An important parallel to familial AD was the discovery that Down’s syndrome trisomy 21 individuals, who have an extra copy of chromosome 21 on which the APP gene is located, most often develop amyloid pathology and AD-like symptoms before the age of 35 years [27, 28].

Other mutations located outside the APP gene locus are missense mutations in the Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2) genes located on chromosomes 14 and 1, respectively. These genes encode for the catalytic subunits of γ-secretase and lead to an increased Aβ42/Aβ40 ratio [29-32]. Although mutations only represent a small percentage of all AD cases, they have enabled generation of suitable animal models for AD and increased the understanding of the disease pathogenesis.

Sporadic Alzheimer’s disease

The majority of AD patients (>95%) have a late-onset, sporadic disease variant that affects individuals over the age of 65 years [13]. The cause of this form of the disease is unclear, but several factors have been reported to increase the risk of developing sporadic AD and it may likely be multifactorial, resulting from a combination of different factors [33]. Aside from the clear risk factor of age, many epidemiological studies have found lifestyle-related associations linked to the cardiovascular system, including hypertension, hypercholesterolemia, atherosclerosis, coronary heart disease, smoking, obesity and diabetes [34-39]. In addition, patients with a history of stroke, traumatic brain injury and chronic neuroinflammation are at higher risk of developing AD, probably due to a decreased clearing capacity and a greater Aβ-related burden [40-42]. Other studies have found that people with a higher educational level may be protected against developing AD because of a higher cognitive reserve, but it is unclear whether such a factor affects the disease process or not [43-45]. Nevertheless, time spent in engaging in mental, physical and leisure activities has been associated with a reduced risk of dementia in cognitively healthy individuals over 65 years [46].
Apolipoprotein E

The strongest genetic risk factor for sporadic AD is the association with the apolipoprotein E (apoE) genotype [47, 48]. The apoE gene is located on chromosome 19 and encodes a 299-amino acid protein with a native molecular mass of ~34 kDa [49]. In peripheral tissues, apoE is primarily produced by the liver and macrophages, while in the central nervous system (CNS) it is primarily produced by astrocytes and to some extent by microglia, oligodendrocytes and ependymal layer cells [50-53]. Under certain conditions, such as excitotoxic injury, neurons are also able to generate apoE [54, 55]. The physiological function of apoE mainly relates to packaging and transporting cholesterol and other lipids, as well as binding to cell surface apoE receptors [56]. Upon production in the CNS, apoE is secreted by astrocytes [51, 57] where cholesterol and other lipids are transferred to apoE predominantly via ATP-binding cassette transporter 1 (ABCA1), a brain lipid regulator protein expressed on astrocytes, microglia and neurons [58, 59]. The transport to cells is mediated by the interaction of apoE with abundant cell surface receptors, including the low density lipoprotein receptor (LDLR), low density lipoprotein receptor-related protein 1 and 2 (LRP1 and LRP2) and very low density lipoprotein (VLDL), followed by endocytosis [60].

The apoE gene shows a genetic polymorphism in humans and exists in three major isoforms; ε2, ε3 and ε4. Despite a single amino acid substitution among the apoE isoforms, this will result in differences in several functional properties, including their ability to bind lipids, receptors and Aβ [61, 62]. The apoE ε3 allele (~78%) is the most frequent in the general population, followed by ε4 (~14%) and ε2 (~8%) [63, 64]. Individuals with one or two copies of the apoE ε4 allele have a significantly higher risk of developing AD and with a lower age of onset, while the apoE ε2 allele seems to be protective [47, 65, 66]. There is evidence that apoE co-deposits with Aβ in amyloid plaques of AD patients, particularly amongst apoE ε4 carriers [67] and astrocytic apoE has been shown to be important for the uptake and degradation of Aβ [68]. However, it is up to date not entirely clear why apoE ε4 carriers have an increased risk of developing AD.

APP processing

Since APP is a protein, it will be degraded and recycled. Processing of the APP occurs by proteolytic cleavage with the enzymes α-, β- and γ-secretase in either the non-amyloidogenic or the amyloidogenic pathway (Figure 2). In the non-amyloidogenic pathway, APP is first cleaved by α-secretase, a member of the ADAM family of proteases, within the Aβ domain (at the Lys16-Leu17 bond). This cleavage prevents the formation of Aβ [69, 70] and results in extracellular release of a large soluble N-terminal ectodomain
known as sAPPα, whereas the carboxyterminal fragment (CTF) APP-CTF83, remains bound to the plasma membrane. It has been shown that sAPPα has a neuroprotective role, preventing neurons from excitotoxicity and promoting neurite outgrowth, synaptogenesis and cell adhesion [71, 72]. The plasma membrane-bound fragment APP-CTF83 is then further cleaved by γ-secretase, which is a tetrameric protease complex consisting of the four subunits presenilin, nicastrin, anterior pharynx defective (Aph-1) and presenilin enhancer (Pen-2) [73]. This cleavage will result in the release of a small extracellular P3 fragment (with unknown physiological function) that is rapidly degraded, as well as the generation of an amino-terminal APP intracellular domain (AICD) [74].

In the amyloidogenic pathway, APP is instead first cleaved by the membrane-bound aspartyl protease β-secretase (also known as beta-site amyloid precursor protein cleaving enzyme 1, BACE1), causing the extracellular release of a large N-terminal ectodomain called sAPPβ, while leaving the C-terminal fragment APP-CTF99 bound to the plasma membrane [73]. The function of sAPPβ is poorly understood, but it has been suggested to be important for synaptic pruning during CNS development [75]. The APP-CTF99 is then further cleaved by γ-secretase, resulting in the production and extracellular release of Aβ, as well as the generation of intracellular AICD that is released in the cytosol.

Figure 2. APP processing. The APP transmembrane protein can be processed in two different pathways. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase with a subsequent cleavage by γ-secretase. In the amyloidogenic pathway, APP is instead cleaved by β-secretase with a subsequent cleavage by γ-secretase, resulting in formation of the Aβ peptide.
Aβ aggregation

Various Aβ peptides with different chain lengths are produced, depending on the γ-secretase cleavage site. The majority of Aβ produced following APP processing consists of the 4 kDa Aβ40 monomer along with a minor portion of the Aβ42 monomer, but additional shorter or longer Aβ variants have been observed [76, 77]. Although the Aβ42 has only two additional amino acids, its hydrophobic structure makes it more prone to aggregate and more toxic to neurons than Aβ40 [78]. In addition, Aβ42 has been found to be the predominant component of the amyloid plaques [79, 80]. Depending on conformational changes, monomeric Aβ can either enter an off-pathway, preventing Aβ to aggregate into fibrils by forming soluble end-stage oligomers, or an on-pathway with gradually increased fibrillization. The Aβ monomers will then adopt a β-hairpin structure and start to assemble and aggregate into small soluble oligomers, including Aβ dimers, trimers, tetramers and protofibrils (Figure 3) [81-83]. Protofibrils are described as elongated, curvilinear >70 kDa intermediates of up to 200 nm in length and with a general diameter of 4-10 nm [25, 84-87]. As the end-stage of the Aβ aggregation process, insoluble Aβ fibrils are deposited as plaques. Interestingly, it has been shown that small soluble Aβ aggregates have the ability to seed high-molecular weight Aβ aggregates into insoluble Aβ fibrils and thus induce the deposition of Aβ, suggesting that the seeding propagation of Aβ may contribute to the progression of AD [88, 89].

Figure 3. The Aβ aggregation pathway. The Aβ monomers aggregate and form soluble oligomers that act as building blocks for protofibrils. Elongation of protofibrils generates insoluble Aβ fibrils, the main component of amyloid plaques. Plaque image kindly provided by Paul O’Callaghan.
Aβ toxicity

The cause of neurodegeneration in AD has been a central question for many years, but it is well known that Aβ plays an important role. However, which Aβ species that cause the neurological damage, directly or indirectly, has been debated. For many years, it was considered that insoluble fibrillar Aβ found in the plaques were the main neurotoxic species, but recently the research has shifted more towards the soluble forms of Aβ as the main causative agents. One of the strongest arguments supporting this theory is the lack of correlation between the amount of Aβ plaques in human AD brains and the severity of AD-like symptoms [86, 90, 91], whereas other studies have suggested that the concentration of soluble Aβ oligomers correlates with the pathological picture [84, 86, 92-95]. However, it should not be excluded that fibrillar Aβ may have a partial role in AD [96]. Oligomeric Aβ have been found to bind with high affinity to synapses and impair neurotransmission by rapidly inhibiting long-term potentiation (LTP) in rodent hippocampal slice preparations [97, 98]. Moreover, injection of various Aβ oligomers directly into the rodent brain leads to cognitive impairments [99, 100]. In addition to synaptic dysfunction, Aβ oligomers also cause mitochondrial dysfunction and oxidative stress [101].

The amyloid cascade hypothesis

In 1992, Hardy and Higgins proposed a theory on the causative mechanisms of progression in AD, known as the amyloid cascade hypothesis, wherein they postulate that Aβ is the causative agent that initiates a sequence of events that ultimately lead to dementia [102]. The main support of the amyloid cascade hypothesis is based on the discovery of the genetic factors described above (related to APP, PSEN1, PSEN2 and apoE), which all promote and increase Aβ deposition in AD before the onset of neuroinflammation, NFT pathology, neuronal loss and dementia [103]. Moreover, mutations in the tau gene on chromosome 17 are known to cause FTD, but without the appearance of amyloid plaques, thus indicating that tau pathology itself can cause neuronal loss but that tau pathology is a downstream process of Aβ pathology [104]. Since most AD cases occur in a sporadic fashion, Hardy and Higgins propose that other external factors, such as head trauma, may trigger Aβ deposition and in this way lead to downstream pathological processes [102]. Since the amyloid cascade hypothesis was first published, the focus of research has shifted from amyloid plaques to the soluble oligomeric Aβ forms as the main neurotoxic species in AD, since they predict the disease progression more accurately than the Aβ plaque burden [81, 83, 86, 105, 106].
Diagnosis and treatment of Alzheimer’s disease

Standardized diagnostic criteria for AD were first published in 1984 [107]. In order to give a valid clinical diagnosis of AD, it is necessary to rule out other conditions that can also cause dementia. The typical dementia of AD is characterized by a reduction of multiple cognitive functions, such as prominent episodic memory impairment, deficits in spatiotemporal cognition and executive functions, aphasia and apraxia that combined will adversely affect the patient’s activities of daily living (ADL) [108]. To assess the cognitive status of the patient, the Mini-Mental State Examination (MMSE) is often used, which tests various neuropsychological parameters, including orientation, memory, language, construction and abstract reasoning [109]. Additional diagnostic criteria for AD involve physical and neurological examination, blood- and serological tests to rule out other causes of the symptoms and analysis of Aβ42, total-tau and p-tau in cerebrospinal fluid (CSF) [110]. The decreased CSF levels of Aβ42 are suggested to be related to the increased aggregation of Aβ peptides in the AD brain, whereas the increased tau levels are suggested to reflect the extracellular leakage of the microtubule-associated tau protein following neuronal cell death of the AD brain. Detection of fibrillar Aβ from amyloid plaques can further be visualized by positron emission tomography (PET) imaging with the radioligand Pittsburgh Compound B (PiB) [111].

At present, there is no curative treatment for AD, but pharmacological treatment is often used in an attempt to alleviate the symptoms. Acetylcholinesterase inhibitors are most often used in early-to-moderate AD to increase acetylcholine levels in the brain and thus improve memory. In moderate-to-severe AD, N-methyl-D-aspartate (NMDA) receptor antagonists can be used to reduce excitotoxicity [112, 113].

Aβ immunotherapy in Alzheimer’s disease

Antibody-based immunotherapy against Aβ has been proposed as a therapeutic strategy for AD and several clinical trials have been performed, using both active and passive immunization. In active immunization trials, synthetic intact Aβ42 or conjugated Aβ42 fragments have been used to activate the immune system to produce endogenous antibodies against Aβ [114, 115]. Unfortunately, all active immunization trials to date have failed, presumably due to reduced responsiveness of the immune system to produce the right kind of antibodies in older individuals as well as severe side effects, such as meningoencephalitis [116, 117].

Passive immunization with direct administration of anti-Aβ antibodies has instead become the more common strategy to target the soluble Aβ aggregates. Two large phase III clinical trials using the monoclonal antibodies Bapineuzumab (targeting the N-terminal region of Aβ) from Pfizer/Janssen and
Solanezumab (targeting the central region of Aβ) from Eli Lilly both failed to improve cognition or functional ability in mild-to-moderate AD. In addition, the extent of adverse effects, such as cerebral microbleeds, were rather high [118, 119]. Antibodies targeting soluble oligomeric forms of Aβ aggregates are currently being investigated in clinical trials. The BAN2401 monoclonal antibody, targeting Aβ protofibrils was developed by BioArctic AB and is currently in a phase 2b clinical trial conducted by Eisai Pharmaceutical [120]. The murine version of BAN2401, called mAb158, has been shown to reduce brain levels of Aβ protofibrils and prevent Aβ deposition in mouse models of AD. Another monoclonal antibody targeting soluble aggregated forms of Aβ is Aducanumab by Biogen, which was recently reported to slow down cognitive decline in a phase I study and a large phase III clinical study is currently underway for the treatment of AD [121]. However, the underlying mechanisms of how Aβ antibodies affect the Aβ deposition, mediate their therapeutic effects and the role of different cell types for its action remain to be elucidated.

Inflammation in Alzheimer’s disease

The neuroinflammatory aspects of AD have been widely studied, but it still remains unclear whether inflammation is the cause, a contributor or merely a consequence of the disease. According to the amyloid cascade hypothesis, the immune system is activated following Aβ accumulation. Regardless, it is well known that there is an ongoing neuroinflammation in the AD brain, manifested by activation of microglia, astrocytes, T-cells and the complement system. Moreover, there is a release of proinflammatory cytokines and chemokines, e.g. IL-1β, IL-6 and TNF-α, production of immunoglobulins and upregulation of microglial cell surface markers, e.g. major histocompatibility (MHC) complex II, CD36, CD14, CD11c and Fc receptors [122-126]. Free radicals such as reactive oxygen species (ROS) are also generated in the AD brain [127-134]. Overproduction and release of ROS and other cytotoxic factors lead to oxidative stress, mitochondrial dysfunction, cell damage and neurotoxicity in AD [135]. During neuroinflammation, some microglia have an immunosuppressive role by producing anti-inflammatory cytokines and neurotrophic growth factors [136]. Microglia and astrocytes are, as the brain’s resident immune cells, important for maintaining homeostasis by removing misfolded proteins such as Aβ [137]. Their activation in the early stages of AD suggests an initial protective role in preclinical AD, but during the course of the disease, microglia and astrocytes may be less efficient in their task and may instead switch to a hypersensitive proinflammatory state [138]. The interplay between microglia and astrocytes may also be important in the disease. It was recently reported that activated microglia induce a specific subpopulation of astrocytes, termed A1 astrocytes, by secreting IL-1α, TNF-α and C1q. Activation prevented A1 astrocytes to promote neuronal survival, outgrowth,
synaptogenesis and phagocytosis. The presence of A1 astrocytes in several neurodegenerative post-mortem brains, including AD, suggests that astrocytes may also contribute to neuronal cell death [139].

Astrocytes

Astrocytes, named after their star-like appearance, were long considered as mere support cells for neurons, but the knowledge has advanced and it is now well known that they are specialized glial cells with many important functions in the CNS. Their role includes buffering of ions [140], pH [141] and neurotransmitters [142, 143], regulation of cerebral blood flow [144], water transport by aquaporin channels [145], metabolic support for neurons [146], modification of synapse signaling [147], contribution to the glymphatic clearance pathway [148] and the blood-brain barrier [149]. The population of glial cells in relation to neurons in the human brain has been debated. Quantitative data from brain tissue analyses have shown conflicting results throughout the years, due to the samples analyzed (various distribution of cells in different parts of the brain) and histological counting methods. It was long proposed that the glia/neuron ratio was 10:1, but recent studies suggest about a 1:1 ratio [150]. Although the number of astrocytes varies uniformly in different brain areas of mammalian species, the proportion of astrocytes is estimated to be 20-40% of the total mammalian brain cell number, making it the most abundant glial cell type in the brain [151]. Based on morphology and anatomic location in the brain, astrocytes are divided into fibrous or protoplasmic astrocytes. Fibrous astrocytes connect to other cells by their long fiber-like processes and are present in the white matter, but the majority of astrocytes are protoplasmic, displaying short branches in the grey matter [152]. Unlike neurons, astrocytes are electrically non-excitable neural cells that instead possess excitability based on movements of Ca$^{2+}$ ions between intracellular compartments and Ca$^{2+}$ fluxes across the plasma membrane [153]. Astrocytes respond to almost all neurotransmitters, neuromodulators and hormones by cellular changes in cytosolic Ca$^{2+}$ or cyclic adenosine monophosphate (cAMP) [154].

Astrocytes in Alzheimer’s disease

Astrocytes respond to lesions and pathogenic insults through a process referred to as reactive astrogliosis. During reactive astrogliosis, astrocytes become hypertrophic, start to proliferate and upregulate their intermediate filaments (glial fibrillary acidic protein (GFAP), nestin and vimentin) [155]. In AD, reactive astrocytes are frequently found around amyloid plaques in both transgenic AD mice [156-158] and in human post-mortem brains [159, 160]. Being the most numerous glial cell type in the brain, astrocytes may have a
great impact on the degradation and clearance of Aβ and the role of astrocytes in the maintenance of brain homeostasis may be severely affected in AD. It has been suggested that in the early stages of AD, astrocytes have a more neuroprotective role and the ability to phagocytose Aβ aggregates for degradation. However, with increased brain atrophy in the later stages of AD, astrocytes have been implicated to contribute to the neuroinflammation and Aβ pathology by failure of Aβ clearance [161]. In addition, Aβ has also been found to affect astrocytes by inducing Ca²⁺ oscillations, which may contribute to the neurotoxicity [162, 163].

**Aβ clearance**

There are various mechanisms to clear Aβ from the brain, including degradation by the ubiquitin-proteasome pathway [164], clearance by the blood-brain barrier interstitial fluid bulk flow [165], CSF absorption [166], and endocytic pathways by phagocytosis and pinocytosis [167]. Phagocytosis is primarily conducted by microglia in the brain, but also astrocytes, neurons and oligodendrocytes possess phagocytic abilities. In fact, it has previously been reported that astrocytes can engulf whole dead cells, Aβ aggregates, synapses and α-synuclein [168-172]. However, astrocytes have been found to store, rather than degrade, ingested whole dead cells [173]. The reason behind the slow digestion was found to be poor lysosomal acidification, due to high levels of Rab27a in astrocytes. The Rab27a is known to reduce the acidity of lysosomes by Nox2 recruitment, in order to preserve antigens for presentation, as astrocytes express MHC class II.

There are different uptake mechanisms of Aβ by the cells. Receptor-mediated phagocytic internalization occurs by interaction of specific receptors on the phagocytic cell with ligands on the surface of the Aβ peptide, which then triggers actin filaments of the phagocyte to zipper up around the particle and engulf it [174]. Pinocytosis, on the other hand, occurs in all cell types and involves the invagination of particles into endocytic vesicles upon stimulation by growth factors or other signals and can be divided into four subgroups based on the uptake mechanisms; macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin/caveolae-independent endocytosis (Figure 4) [175]. In microglia, soluble Aβ have been shown to be internalized mainly through pinocytosis and fibrillar Aβ through receptor-mediated phagocytosis [176]. The uptake mechanisms of Aβ by astrocytes is poorly understood, but both macropinocytosis and receptor-mediated phagocytosis have been suggested as engulfment mechanisms [177, 178].
Figure 4. Endocytic cellular pathways. Extracellular material can be taken up by endocytosis through various mechanisms, including receptor-mediated phagocytosis and micropinocytosis. After internalization, the material is transported to early endosomes, where it can either be recycled back to the plasma membrane or be degraded by the endosomal-lysosomal pathway. In this pathway, the pH will subsequently drop causing activation of degrading enzymes. Important proteins of the endosomal-lysosomal pathway are Rab5, Rab7, LAMP-1 and LAMP-2. Exosomes are derived from MVBs, whereas MVs bud directly from the plasma membrane.

Following internalization, the cargo first arrives to the early endosomes, the main sorting stations in the endocytic pathway, characterized by the presence of the Ras-related proteins Rab4 and Rab5 [179]. The majority of cargo, including most receptor proteins, is returned to the plasma membrane via recycling endosomes. Cargo destined for degradation, such as Aβ, will follow the endosomal-lysosomal pathway. In the early endosomes, formation of intraluminal vesicles (ILVs) begins, and in the late endosomes (also known as multivesicular bodies, MVBs) the proteins are sorted between the endosomal membrane and ILVs, characterized by the replacement of Rab4 and Rab5.
the late endosomal markers Rab7 and Rab9. During the maturation process from early endosomes to lysosomes the vacuolar-type ATPase (V-ATPase) proton pump will subsequently drop the pH from around 6.2 in early endosomes to 5.5 and 5.0 in late endosomes and lysosomes, respectively, by delivering and increasing the concentration of acid hydrolases (degrading enzymes) [180, 181]. Except for the Rab proteins, the lysosomal-associated membrane proteins LAMP-1 and LAMP-2 are also involved in the maturation process of early endosomes to mature lysosomes, the end-stage where the waste material is degraded by proteolytic activity with lysosomal enzymes [182]. Studies have shown the endosomal-lysosomal pathway to be heavily affected in the AD brain, before Aβ and tau pathology begin, by dysregulation of pH and cellular indigestion, which may lead to impaired clearance of Aβ [183, 184].

Extracellular vesicles

Intercellular communication is essential for all multicellular organisms. Extracellular vesicles (EVs) is one form of intercellular communication, delivering various proteins, lipids, nucleic acids and sugar molecules between cells. The EVs can be isolated from a wide range of fluids, including cell culture medium, blood, CSF, saliva, breast milk and urine [185-187] by different techniques, including differential ultracentrifugation, density gradient centrifugation, size-exclusion chromatography, filtration and polymer-based precipitation [188]. Differential ultracentrifugation is commonly used and consists of several steps. First, a low-speed centrifugation is applied to remove cells and cell debris, then by a higher speed spin to remove any remaining cell remnants, followed by high-speed ultracentrifugation. Characterization of EVs is based on size, properties and cellular origin of the vesicles. Microvesicles or ectosomes (MVs) are produced by direct budding from the plasma membrane and range in size from 100-1000 nm. Exosomes are small membranous vesicles (20-100 nm) that are formed when late endosomes/MVBs fuse with the plasma membrane by involvement of Rab11, Rab27 and Rab35 (Figure 4) [189-192]. The EVs are released from various cells in the brain, including microglia, astrocytes and neurons, and the uptake of released EVs is suggested to occur mainly by phagocytosis, alternatively by macropinocytosis or membrane fusion [193-196]. Typical EV protein markers are the tetraspanins CD9, CD63 and CD81; the lipid rafts flotillin-1, flotillin-2 and caveolin-1, and the intravesicular markers alix, TSG101 and syndecan. Although several EV isolation protocols have been developed, there is currently a challenge within the research community to fully characterize different EV populations in practice, due to limitations in the purity of the isolated EVs, the risk of contamination of other EVs or cell remnants, and the lack of explicit protein markers for the different EVs. However, smaller EVs, such as exosomes, are isolated at higher
speed of centrifugation and longer ultracentrifugation step than larger EVs, such as MVs.

Extracellular vesicles in Alzheimer’s disease

Extracellular vesicles have been implicated to contribute to the pathogenesis of AD and other neurodegenerative disorders by transmitting protein aggregates, and they have also been proposed to take part in cell-to-cell transfer of prions [197, 198]. Specifically, Aβ, APP and its cleaving products have been found to be sorted in MVBs and released by exosomes into the extracellular space in transgenic AD animal models, suggesting that exosomes promote aggregation of neurotoxic Aβ in amyloid plaques [199-201]. The EV proteins alix and flotillin-1 have also been found to be enriched in amyloid plaques in the human AD brain and in a transgenic AD mouse model [202, 203]. Moreover, there is a significant correlation between the amount of microglial MVs and atrophy of the hippocampus in AD patients [204]. However, it should be mentioned that exosomes have also been suggested to have neuroprotective roles, e.g. by acting on extracellular Aβ to promote uptake and clearance by microglia [205]. Since the EV content mirrors the intracellular environment, it could contribute with important information about ongoing pathological processes and may be a useful source for biomarkers, reflecting the progression of various diseases, including AD.
Aim of the thesis

The overall aim of this thesis was to increase the knowledge of astrocytes in Aβ pathology, focusing on Aβ clearance and Aβ-induced toxicity by the secretion of EVs. All the included studies have been performed using a co-culture system of primary neurons, astrocytes and oligodendrocytes.

Specific aims

I To clarify the role of astrocytes in Aβ pathology by studying uptake, degradation and toxicity of Aβ$_{42}$ protofibrils.

II To analyze the protein content of EVs, specifically released following Aβ$_{42}$ protofibril exposure.

III To investigate if Aβ degradation in astrocytes can be enhanced by treatment with the Aβ protofibril-selective antibody mAb158.

IV To examine the mechanisms behind the neurotoxicity, mediated by EVs released following Aβ$_{42}$ protofibril exposure.
Experimental procedures

Model systems for Alzheimer’s disease

Epidemiological and population-based studies on AD patients are important for evaluating data, in order to find common denominators and risk factors of the disease. However, experimental studies on humans are limited due to various ethical issues. The existence of both in vitro and in vivo models in AD research has been beneficial and enabled researchers to study various aspects of the disease. Many transgenic animal models of AD have been developed to study the disease in vivo thanks to the genetic discoveries linked to AD. Also, new ways of studying AD pathology have been made possible by breeding rodents carrying more than one AD mutation. One such example are mice expressing the APP gene with both the human Arctic and Swedish mutations (tg-ArcSwe). The tg-ArcSwe mice develop an early (4-8 months) and prominent Aβ pathology, with elevated soluble Aβ aggregates such as Aβ protofibrils, and amyloid plaques [206-208]. Animal models are useful for studying the disease phenotype and for the development of new drugs and treatments, as they allow behavioral and functional analyses. On the other hand, cell culture models are a more direct way to study cellular and molecular processes of individual cells, which could increase our understanding of some aspects of the disease. Moreover, cell culture models are easily reproduced and require reduced number of experimental animals. However, one limitation is that cell culture models do not reflect the complexity of the brain.

Neural cell culture model

Throughout this thesis, we have used an in vitro cell culture model, consisting of primary astrocytes, neurons and oligodendrocytes. In order to obtain these, cerebral cortices from C57/BL6 mice of embryonal day 14 (E14) were dissected and the dissociated cells were cultured in serum-free medium. Neural stem cells were expanded as neurospheres in DMEM/F12-GlutaMAX cell culture medium supplemented with B27 and growth factors (epidermal growth factor, EGF and basic fibroblast growth factor 2, bFGF2) and passaged every second or third day. The neurospheres were dissociated and plated as a monolayer on coated coverslips or cell culture dishes in medium with growth factors, in order to let them recover from the passage. After 24 h incubation, the
neural stem cells were differentiated in mitogen-free medium for 7 days, with medium changes performed every second or third day. The differentiated neural cultures were quantified after immunocytochemistry (ICC) and consisted of ~70% astrocytes, ~25% neurons and ~5% oligodendrocytes (Figure 5) [209]. Microglia originate from the hematopoietic stem cell line and were therefore not present in our cultures. This enabled us to fully study the capacity of astrocytes as scavenger cells without the presence of professional phagocytes.

Figure 5. Mixed neural cell cultures from embryonal cortical stem cells. Differentiation of the neural stem cells leads to development into a co-culture of astrocytes, neurons and oligodendrocytes. GFAP, βIII-tubulin, CNPase (all green), DAPI blue. Scale bar: 20 µm.

After the differentiation period, cultured cells were exposed to synthetic Aβ42 protofibrils or left untreated, and were followed over time. In Paper I and Paper III, cells were exposed to Aβ42 protofibrils for 24 h prior to fixation or cell lysis, or were washed thoroughly in culture medium without Aβ42 protofibrils and incubated for an additional 6 or 12 days prior to fixation or cell lysis. In order to study the effect of mAb158 in Paper III, Aβ42 protofibrils were pre-incubated for 5 min with the hybridoma-produced murine mAb158 (IgG2a) antibody or the recombinantly produced mAb158, RmAb158 (IgG2c)
antibody, with or without the N297D mutation prior to addition to the cultures. To analyze the release and protein content of EVs in Paper II, cells were constantly exposed to Aβ42 protofibrils for 2 or 5 days prior to medium collection and ultracentrifugation in order to maximize the EV content. The time points in Paper II were based on the finding in Paper I that formation of enlarged endosomes start to appear after 2-3 days. In Paper I and Paper IV, primary cortical E14 neurons were cultured 12 days in vitro (12DIV) in neurobasal medium. The neuronal cultures were treated with EVs isolated 6 and 12 days after Aβ42 protofibril exposure of neuroglial co-cultures (Aβ42 protofibril-EVs) or EVs from untreated neuroglial co-cultures (Control-EVs). The viability and cellular effects of the neurons in response to EV treatment were investigated.

Synthetic Aβ42 protofibrils

Throughout this thesis, we have used synthetic Aβ to study the cellular responses. Since the quality of Aβ and its propensity to aggregate varies between different manufacturers and lots, we consistently used the same lot of Aβ in all experiments throughout each study. Due to the hydrophobicity of Aβ, it has a propensity to stick to glass and plastic surfaces in tubes, plates, pipette tips and chromatography columns. However, this may be avoided by addition of small amounts of detergents. Fluorescent HiLyte™ Fluor 555-labeled Aβ42 (Aβ42-555) peptides or unlabeled Aβ42 peptides were used as starting material to prepare Aβ42 protofibrils. In many laboratories, organic compounds such as 1,1,1,3,3,3,-Hexafluoro-2-propanol (HFIP) are used to dissolve and monomerize Aβ. However, we and other research groups routinely use sodium hydroxide (NaOH) to dissolve the lyophilized Aβ peptide, since increase in the pH of Aβ (pH ~10.5-11) followed by neutralization with PBS avoids the Aβ to pass its isoelectric point. This will lead to formation of peptide solutions with higher yields of low-molecular weight Aβ and lower levels of pre-existing aggregates [210-212]. After dissolution, both Aβ42-555 and unlabeled Aβ42 peptides were centrifuged for 5 min at 17 900 x g to remove any insoluble aggregates. All solutions and material used to prepare Aβ42 protofibrils were sterile and final aliquots were stored at -70°C. The expected concentration of Aβ was routinely analyzed and confirmed in our laboratory, using the protofibril specific ELISA, based on mAb158 [206]. We defined Aβ protofibrils as curvilinear structures with a size larger than 75 kDa, based on cryo-TEM studies [206] and HPLC-SEC analysis using a Superdex 75 column (>95% purity of Aβ42 protofibrils), respectively. The HiLyte™ Fluor 555-label did not disturb the protofibril conformation of Aβ42 protofibrils.
Immunofluorescence and other staining techniques

Immunofluorescence staining techniques are based on the direct or indirect binding of antibodies, chemically conjugated with a fluorophore to a specific antigen of interest, thus enabling visualization of the specimen under a fluorescence microscope. In direct immunofluorescence, the primary antibody is labeled with a fluorophore. However, in all the papers we have used indirect immunofluorescence, in which we have selected primary antibodies that bind to the antigen of interest and then added fluorescent secondary antibodies to detect the primary antibody. All cells used for ICC were cultured on coated coverslips and were fixed in 4% paraformaldehyde (PFA). A list of all primary antibodies used in the papers are shown in Table I. Specific markers were used in all papers to stain the three cell types present in the mixed co-culture. Astrocytes were stained with the intermediate filament protein GFAP, neurons with the microtubule-associated protein βIII-tubulin and oligodendrocytes with the myelin-associated enzyme protein 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase). It is important to keep in mind that GFAP does not stain the whole cell, but only approximately 15% of the astrocyte, leading to underestimation of actual astrocyte size [213]. Various Aβ antibodies (mAb1C3, 6E10, mAb158 and a polyclonal Aβ42 antibody) were used in the papers. Although they target different epitopes on Aβ, they all stained Aβ equally well. Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI), which binds strongly to A-T rich regions in the DNA, was used to visualize the cell nuclei.

Table 1. List of all primary antibodies used for ICC in the different papers of this thesis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised in</th>
<th>Dilution</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
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<td>DakoCytomation</td>
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<td>Invitrogen</td>
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35
To study degradation of Aβ by the endosomal-lysosomal pathway, we performed additional stainings in **Paper I** against the lysosomal-associated membrane proteins LAMP-1 and LAMP-2, as well as the early and late endosomal proteins Rab5 and Rab7, respectively. To investigate if the lysosomes in the Aβ42 protofibril accumulating astrocytes were acidic or not, we performed experiments using LysoTracker® Red DND-99, which is a dye that labels acidic organelles in live cells. In **Paper II**, we used antibodies against apoE to study the expression of this protein in the three different cell types and in combination with the EV marker CD9 and trans-golgi subcellular marker GM-160. The treatment effect of mAb158 was evaluated by analysis of the intensity and area of Aβ inclusions in **Paper III**. Phalloidin is a phallotoxin produced by the death cap mushroom *Amanita phalloides* and binds actin filaments of cells. Fluorescein isothiocyanate (FITC) conjugated phalloidin was used in **Paper III** in combination with Aβ42 antibodies to visualize the cell contours in the presence of Aβ. In order to visualize and analyze the amount of apoptotic cells following EV treatment of cortical neuronal cultures in **Paper I** and **Paper IV**, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used. This assay detects DNA fragmentation by labeling 3′-hydroxyl termini generated during apoptosis. However, it is important to be aware of that fragmented DNA may also be visualized by necrotic cells. Therefore, it should not be excluded that TUNEL assay may also stain for necrosis [214]. Neurons were transfected with CellLight® Mitochondria-GFP for 24 h in **Paper IV** to visualize mitochondria in live cells.

### Microscopy techniques

Studies of cellular expression, cellular responses, EV release, co-localization, Aβ42 accumulation and clearance were possible by the use of different microscopy techniques. Following ICC, images of cells were captured using conventional fluorescence microscopy. For detailed analysis on co-localization of Aβ42 protofibrils and mAb158 in astrocytes, confocal microscopy was used in **Paper I** and **Paper III**. This technique allows scanning across the layers of the specimen to make up a z-stack that can be used to create a three-dimensional image.

In order to dynamically study mechanisms of Aβ protofibril clearance over time in the co-cultures, we used time-lapse microscopy in **Paper I** and **Paper III**. For this purpose, cells were cultured in time-lapse culture dishes with a glass bottom and experiments were performed at 37°C in humidified 5% CO2 in air using a Nikon Biostation Live Cell Recorder. Images were captured every 10 minutes up to 5 days and were combined to a sequential movie.

EV-secreting cells and EVs in **Paper I**, **Paper II** and **Paper IV** were visualized by transmission electron microscopy (TEM). This technique can be utilized to study very small cellular structures down to nanometer range. It uses
a beam of electrons to pass through an ultra-thin specimen and only electrons that pass through the specimen will convert into light and create an image. Compared to a regular light microscope, TEM creates images with very high resolution and magnification, due to the lower wavelength of electrons than light. The EV-secreting cells were fixed in 2.5% glutaraldehyde and following ultracentrifugation, EVs were resuspended in PBS before preparation of TEM.

Differential ultracentrifugation

One of the most common methods used to isolate EVs from a solution is by differential ultracentrifugation. It requires centrifugation in multiple steps, first at low speed to eliminate cell debris, then at higher spin speed to remove larger vesicles and debris and finally at high speed in the 100 000 x g range. Therefore, ultracentrifugation must always be performed in high vacuum to reduce friction of rotor and air. Differential ultracentrifugation was used in Paper I, Paper II and Paper IV to isolate EVs from the cell culture media of mixed co-cultures (Figure 6). The ultracentrifugation protocol was the same in all three papers, with an initial low-spin speed of 300 x g for 5 min, followed by 2000 x g for 10 min and finally 135 000 x g for 1.5 h at 4°C. Although the EV pellets are not visible by eye after ultracentrifugation, using a swinging bucket rotor (SW28) will make the EVs sediment to the bottom center during ultracentrifugation. The EVs were resuspended in PBS, lysis buffer with protease inhibitors or neurobasal medium depending on the experimental set-up. Analysis of EVs was performed by TEM, mass spectrometry (MS) analysis, western blot analysis and immunoprecipitation (IP).

In Paper I and Paper IV, we aimed to investigate the viability and the cellular effects of primary cortical neurons following treatment with EVs isolated from Aβ42 protofibril exposed co-cultures. For this purpose, neuroglial co-cultures were either exposed to Aβ42 protofibrils for 24 h or left untreated, followed by thorough washes in medium to remove any residual Aβ. Cells were kept in the Aβ-free medium and the cell culture medium was collected after 6 and 12 days, pooled, centrifuged in two steps, ultracentrifuged, resuspended in neurobasal medium and added to cortical neurons. In Paper II, we focused more on analyzing the protein content of EVs released by the co-cultures specifically in response to Aβ42 protofibril exposure. Co-cultures were constantly exposed to Aβ42 protofibrils or left untreated in order to maximize the protein release. In order to discriminate between exosomes and larger MVs in Paper II, we performed experiments in which the samples (following the two initial centrifugation steps) were first ultracentrifuged for 30 min at 10 000 x g at 4°C (to pellet MVs). The supernatant was then ultracentrifuged a second time for 1.5 h at 135 000 x g at 4°C (to pellet exosomes). The cellular effects of EV treatment on primary cortical neurons were investigated in closer detail in Paper IV.
Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is an immunological technique used for identifying and quantifying the concentration of specific antigens in a sample by using antibodies. Its high sensitivity allows detection of antigens in very low ranges, depending on the interaction between antigen and antibody. A commonly used type of ELISA in laboratories is the sandwich ELISA. In the first step of a sandwich ELISA, a capture antibody is immobilized on a microplate surface. The surface is then blocked to prevent non-specific binding with other proteins of the sample, which are added in the next step. Only antigens that are recognized by the antibody will be captured, while the remaining non-bound proteins are washed away. A biotinylated secondary antibody, linked with a detection enzyme e.g. horseradish peroxidase (HRP), is then added. The addition of a substrate e.g. Enhanced K-blue® substrate will lead to a detectable signal in the presence of antigen due to a measurable color change.
In our studies, we have used sandwich ELISAs to specifically measure total 
Aβ (Aβ_{1-x} and Aβ_{x-42} ELISAs) in **Paper I** and **Paper III**, and Aβ protofibrils 
(mAb158 ELISA) in **Paper I**. It is important to consider the aggregation state 
of Aβ when measuring synthetic or biological Aβ. The hydrophobic C-terminus 
of Aβ is often hidden in the core of Aβ aggregates, leading to underestimation 
of actual Aβ levels in C-terminus dependent ELISAs. To circumvent 
this issue, samples containing Aβ can be boiled in sodium dodecyl sulfate 
(SDS) to break up the Aβ aggregates into monomeric forms. In this way, the 
C-terminus of Aβ is accessible to allow detection of total Aβ. Prior to ELISA, 
all samples (except lysate pellets) in the Aβ_{1-x} and Aβ_{x-42} ELISAs were denatured 
by boiling in SDS and then further diluted to decrease the SDS concentration, 
in order to avoid interference from SDS. No SDS treatment or boiling was performed 
in samples analyzed with the mAb158 ELISA. As capture antibodies in the Aβ_{1-x} and Aβ_{x-42} ELISAs 
we used the Aβ N-terminal specific mAb82E1 and polyclonal Aβ_{42} antibody, respectively. The biotinylated 
maAb4G8, specific for the mid-region of Aβ, was used as detection antibody. 
In the mAb158 ELISA, the monoclonal mAb158 antibody was used as both 
capture antibodies in the Aβ_{1-x} and Aβ_{x-42} ELISAs we used the Aβ N-terminal specific 
maAb82E1 and polyclonal Aβ_{42} antibody, respectively. The biotinylated 
maAb4G8, specific for the mid-region of Aβ, was used as detection antibody. 
In the mAb158 ELISA, the monoclonal mAb158 antibody was used as both 
capture and detection antibody. The mAb158 ELISA is designed to specifically 
measure larger Aβ protofibrils and does therefore not detect Aβ monomers or low molecular weight Aβ oligomers. However, more than one 
epitope has to be present to be detected in the mAb158 ELISA.

**Mass spectrometry analysis**

Mass spectrometry is a common tool for protein identification and quantification. The mass spectrometer consists of an ion source, a mass analyzer and anion detection system. To enable analysis of proteins by MS, the proteins are first ionized, then separated according to their (or their fragments) mass to charge ratio (m/z) and finally detected by the system. This allows detection of a large scale of proteins with a relatively high specificity and sensitivity [215]. By coupling a liquid chromatography to the mass spectrometer (LC-MS) and separate the molecules in an additional dimension (retention time), one can further enhance MS sensitivity. In **Paper II**, we performed a type of LC-MS analysis called shotgun MS based label free (LF) quantification analysis, to study the expression and relative levels of proteins in EVs. Following differential ultracentrifugation, the lysed proteins in the EV samples were first digested to peptides by trypsin/Lys-C and then run through the LC-MS. The peptide sequences were identified using a search engine against UniProt/Swiss-Pro human database (in combination with a decoy database), containing more than 20 000 human protein sequences. The protein identification was then inferred from the peptide sequences. A lower limit of the false discovery rate for proteins had to be selected (p<0.05) in order for them to be considered present in the samples.
Western blot analysis

Western blot analysis is a molecular biology technique used for immunodetection of proteins by visualizing the size of your protein of interest. For quantitative data of protein concentration, ELISA is preferred as it is more reliable than western blot analysis. However, an advantage over ELISA is that western blot analysis allows one to detect the size of the proteins, which makes it possible to be more confident about the specificity of the antibodies. In our studies, the proteins were first denatured by heating in SDS (LDS sample buffer) and dithiothreitol (reducing agent) to give them a negative charge that was proportional to their molecular weight. After sample loading onto a SDS-polyacrylamide (SDS-PAGE) gel, an electric field was applied across the gel to enable migration of the negatively charged proteins towards the positive electrode, thus separating the proteins according to their molecular weight. All proteins on the gel were transferred to a polyvinylidene fluoride (PVDF) membrane or nitrocellulose membrane (used for Aβ detection) by applying an electrical field, making the proteins migrate towards the positive electrode. The membrane was then blocked to prevent non-specific binding with other proteins of the samples and the primary antibody specific against the protein of interest was added to the membrane. Following overnight incubation, a HRP-conjugated secondary antibody was added and the protein of interest was visualized by enhanced chemiluminescence (ECL). Development by ECL is an indirect method for detecting proteins bound on a membrane. It utilizes a combination of a chemiluminiscent substrate and a strong oxidizing agent to form excited intermediates. When these excited intermediates decay to a lower energy level, they release a strong blue emission at 450 nm wavelength. By applying ECL, it produces intense, prolonged light emission and low background noise. It is also possible to use secondary antibodies directly conjugated to fluorescent dyes for visualization and quantitative analysis of proteins. By using fluorophores with different excitation and emission spectra, differentiation of proteins can be performed on the same membrane.

In Paper II, we performed western blot analysis to study the amount of released EVs with various antibodies (flotillin-1, TSG101 and CD9). Moreover, antibody against apoE was used to visualize and quantitatively analyze apoE levels from the EV lysates compared to flotillin-1 levels. In order to evaluate the clearance of Aβ42 in Paper III, we performed western blot analysis on medium samples from untreated and Aβ42 protofibril exposed co-cultures with or without preincubation of mAb158. Western blot analysis on neuronal cell lysates was performed following EV treatment with synaptophysin antibodies in Paper IV to study the intracellular expression of the synaptic marker synaptophysin.
Immunoprecipitation

Immunoprecipitation is a technique used for isolating and concentrating a specific protein of interest by precipitating the protein antigen out of a solution, e.g. by a magnetic particle concentrator. First, antibodies are added in the solution to allow specific binding to the antigen. In order to precipitate the antigen-antibody complex, a superparamagnetic bead is added to bind to the antibody, thus enabling precipitation by the magnetic particle concentrator. In Paper II, we immunoprecipitated apoE from lysed and intact EVs to investigate the localization of apoE. Dynabeads™ with recombinant Protein G coupled to the surface were used as magnetic beads. After extensive washes in washing buffer, premixed LDS sample buffer and reducing agent were added together with elution buffer to the samples for further investigation by western blot analysis.
Results and comments

Being the most numerous glial cell type in the brain, astrocytes have many important functions in the brain, but their role in AD is poorly understood. Insufficient lysosomal degradation has been suggested to be the main cause of sporadic AD [181, 216]. The ineffective degradation may lead to spreading of AD pathology, due to Aβ secretion from the phagocytic cells [217]. Many studies have been focusing on the role of microglia in AD, but the importance of astrocytes has not been thoroughly investigated. Our research group has recently shown that astrocytes effectively engulf whole dead cells, but store, rather than degrade the ingested material [173]. In this thesis, we were interested in investigating the role of astrocytes in Aβ pathology and clearance. Since soluble Aβ aggregates, including protofibrils, have been suggested to be the most neurotoxic Aβ species, we focused on examining the cellular responses of Aβ42 protofibrils.

Cellular uptake and accumulation of Aβ42 protofibrils

In order to investigate the uptake and accumulation of Aβ42 protofibrils by the major cell types in the brain, we performed experiments in Paper I, using mixed co-cultures of primary cortical astrocytes (70%), neurons (25%) and oligodendrocytes (5%). The cell cultures were exposed to Aβ42-555 protofibrils for 24 h, fixed and stained with antibodies against the three cell types to study the amount of intracellular Aβ. Astrocytes, neurons and oligodendrocytes were stained with GFAP, βIII-tubulin and CNPase, respectively. We found that astrocytes contained large deposits of Aβ42-555, while neurons had almost no detectable levels of intracellular Aβ. Accumulation of Aβ42-555 was also noted in the oligodendrocytes (Figure 7). Interestingly, the Aβ42-555 inclusions frequently co-localized with DAPI-stained condensed nuclei. The intracellular deposits of Aβ42-555 in astrocytes was further confirmed by confocal microscopy. To verify that the 555-label was still bound to Aβ after engulfment, we exposed parallel co-cultures with unlabeled Aβ and stained them with four different Aβ antibodies (mAb1C3, 6E10, mAb158 and a polyclonal Aβ42 antibody). The immunostainings revealed that all antibodies displayed the same staining pattern as the labeled Aβ42-555 protofibrils, indicating that the 555-label was still bound to Aβ in the detected intracellular Aβ deposits. To follow the engulfment of Aβ42 protofibrils over time, we performed time-
lapse experiments, in which we recorded the cell culture during the 24 h Aβ42-555 protofibril exposure. Our results showed that already after 30 min, astrocytes had accumulated Aβ42-555 and the intensity of the staining increased during the 24 h time period.

Immunostainings of brain sections from 14-month-old tg-ArcSwe mice in Paper I demonstrated that reactive astrocytes were tightly localized around the amyloid plaques and Aβ co-localized with astrocytes, confirming that our findings in the cell cultures reflected cellular processes of Aβ pathology in vivo.

Incomplete clearance of Aβ42 protofibrils by astrocytes

To follow the degradation of ingested Aβ42 protofibrils, the co-cultures in Paper I were washed after the 24 h incubation with Aβ42-555 protofibrils and were cultured for additional 6 or 12 days prior to fixation and immunostaining. Our results demonstrated that astrocytes effectively engulfed Aβ42 protofibrils, but that the ingested material was only partially degraded within 12 days. The oligodendrocytes contained large or medium-sized Aβ42-555 inclusions that did not significantly change over time. Intensity- and area measurements of the intracellular Aβ42-555 confirmed that astrocytes degraded the Aβ42-555 protofibrils to some degree, although very slowly. In contrast to our results with Aβ42-555 protofibrils, cell cultures exposed to synthetic Aβ40-555 monomers showed no accumulation of Aβ in astrocytes.

To investigate the intracellular localization of ingested Aβ42 protofibrils in Paper I, immunostainings with the lysosomal marker LAMP-1 were performed. Only a limited co-localization between Aβ42 and LAMP-1 was found directly after exposure (24 h), but the co-localization increased over time. At 12 days after Aβ42 protofibril removal, most of the intracellular Aβ deposits...
co-localized with LAMP-1, indicating that Aβ42 protofibrils were transported to lysosomal compartments within the astrocytes. However, to clarify if the astrocytic lysosomes were acidic enough to degrade the ingested Aβ42 protofibrils, additional experiments using the LysoTracker® dye, that labels acidic organelles in live cells, were performed. Interestingly, we found that although the lysosomes were LAMP-1 positive, the Aβ42-containing lysosomes did not stain with the LysoTracker® dye, indicating that Aβ42 protofibrils stored in astrocytes were located in immature lysosomes. We aimed to find out if the Aβ42 stored in the immature lysosomes were partially degraded. For this purpose, we analyzed cell culture lysates using three different sets of sandwich ELISAs (the N-terminal dependent Aβ1-35, the protofibril specific mAb158 ELISA and the N-terminal independent Aβx-42 ELISA). The ELISA results showed that a high proportion of the intracellularly stored Aβ was N-terminally truncated, indicating that ingested Aβ42 protofibrils were partially degraded by the astrocytes and processed in a way that could alter their properties and toxicity.

Formation of enlarged endosomes in astrocytes following Aβ42 protofibril exposure

Time-lapse recording of cell cultures after Aβ42 protofibril exposure in Paper I revealed an interesting finding. The accumulation of Aβ induced formation of enlarged, dynamic vacuoles in the astrocytes after 2-3 days (Figure 8). The vacuoles were built up successively over time and increased in size (up to ~50 µm in diameter) by fusing with adjacent vacuoles. The giant vacuoles eventually shrank or collapsed, but concurrently, formation of new vacuoles appeared in the astrocytes, without causing astrocytic death.

Figure 8. Formation of enlarged endosomes in astrocytes following Aβ42 protofibril exposure. Time-lapse recording taken 5 days after Aβ42 protofibril exposure showed formation of large dynamic vacuoles (white asterisks) that fused inside the astrocytes. The vacuoles were identified to be enlarged early endosomes. Scale bar: 10 µm.
Immunostainings with the specific endosomal markers Rab5 (early endosomal marker) and Rab7 (late endosomal marker) identified the giant vacuoles to be enlarged early endosomes. Thus, these data indicate that the accumulation of Aβ induced severe endosomal-lysosomal defects in the phagocytic astrocytes.

Secondary neuronal toxicity following Aβ42 protofibril exposure

To investigate how Aβ42 protofibril exposure affected the viability of astrocytes, neurons and oligodendrocytes in Paper I, the number of each cell type was manually quantified directly after 24 h exposure, as well as 6 and 12 days after Aβ42 protofibril removal. The total number of living cells was compared to the cell number in parallel, unexposed cell cultures. A significant increase in astrocytes and oligodendrocytes was observed after 12 days, indicating that Aβ42 protofibril exposure triggered proliferation of glial cells. However, the number of neurons significantly decreased in the Aβ42 protofibril exposed cultures (Figure 9). This was noted after 12 days and not directly after Aβ42 protofibril removal, indicating that the neuronal cell death was due to a secondary mechanism, most likely connected to the incomplete astrocytic clearance of Aβ42 protofibrils.

Figure 9. Secondary neuronal toxicity following Aβ42 protofibril exposure. The number of astrocytes increased 12 days (24 h + 12 days) after Aβ42 protofibril exposure (A). Neuronal cell death was observed 12 days (24 h + 12 days) after Aβ42 protofibril exposure and not directly after exposure (B). Data is presented as relative number of each cell type.
Spreading mechanisms of Aβ pathology by the secretion of extracellular vesicles

Failure of proper Aβ42 protofibril clearance and the formation of enlarged endosomes in astrocytes following Aβ42 protofibril exposure made us hypothesize that astrocytes may try to get rid of the ingested material by secreting it into EVs. To identify if this was a possible mechanism for the secondary neuronal toxicity, we isolated EVs from the cell culture medium by differential ultracentrifugation in Paper I and added these to cortical neuronal cultures. Neurons were fixed after 48 h and the number of apoptotic TUNEL-labeled neurons was quantified. Our results revealed that Aβ42 protofibril-EVs significantly induced neuronal cell death compared to Control-EVs, demonstrating that Aβ42 protofibril accumulation induced secretion of EVs with neurotoxic content. There was no difference in the amount of released EVs between exposed and unexposed co-cultures, indicating that the toxicity was due to their content. Interestingly, Aβ1-40 and Aβ3-42 ELISA analysis of the EV content demonstrated that EVs contained N-terminally truncated Aβ42, suggesting spreading of Aβ pathology by the secretion of EVs.

The presence of Aβ in EVs gave us a hint that additional proteins might be altered in response to Aβ42 protofibril exposure, which may be of interest from a biomarker point of view. To elucidate the protein content of the EVs in response to Aβ42 protofibril exposure, we performed LC-MS analysis of the lysed EVs in Paper II. In order to detect early changes of protein levels at the time of formation of enlarged endosomes and maximize the protein content, co-cultures were constantly exposed to Aβ42 protofibrils for 2 (day 0-2) or 5 (day 2-5) days prior to ultracentrifugation. The LC-MS analysis of the EV content revealed a total of 807 unique proteins, of which five displayed altered levels in Aβ42 protofibril exposed cultures. These proteins were apoE, CNPase, clathrin heavy chain 1, 60S ribosomal protein L4 and cytoplasmic dynein 1 heavy chain 1, all known to be involved in central cellular processes but not known to be directly linked to each other. In particular, we noted that apoE stood out among these proteins and it was the only protein that was increased at both time points. We selected to further study apoE due to this fact and because of its strong link to AD. Western blot analysis revealed that the EV release did not change markedly in response to Aβ42 protofibril exposure, but a threefold increase in apoE levels was found in Aβ42 protofibril-EVs compared to Control-EVs, both at day 0-2 and day 2-5 (Figure 10A). The apoE was mainly situated inside the EVs, particularly in the exosome fraction (Figure 10B), indicating an endosomal origin. Immunoreactivity of apoE was seen mainly in astrocytes and neurons following Aβ42 protofibrils, suggesting a release of EVs mainly from the secretory astrocytes. The role of apoE in EVs in the spreading of Aβ pathology remains to be further investigated.
Figure 10. Increased release of apoE in EVs isolated from Aβ42 protofibril exposed co-cultures. At both day 0-2 and day 2-5, western blot analysis of lysed EVs from Aβ42 protofibril exposed co-cultures (+) demonstrated a threefold increase of apoE, compared to control cultures (-). The EV markers flotillin-1, TSG101 and CD9 showed that the amount of EVs did not change markedly in response to exposure (A). Apolipoprotein E was primarily situated in the exosome fraction, indicating an endosomal origin (B).

To investigate the cellular effects of Aβ42 protofibril-EVs on neurons in closer detail, we performed immunostaining and TUNEL assays of neurons, after 2 and 4 days of EV incubation in Paper IV. Consistent with the results from Paper I, we found significantly less viable neurons and an increased number of apoptotic cells. Moreover, addition of Aβ42 protofibril-EVs resulted in loss of neuronal synapses and dendrites in the living neurons (Figure 11A-B). However, western blot analysis with specific antibodies against the synaptic marker synaptophysin revealed no altered intracellular levels of the protein in presence of Aβ42 protofibril-EVs, compared to Control-EVs. This result suggests that there is a reorganization of the existing synaptophysin rather than a change in protein levels. Furthermore, TEM analysis and the CellLight® Mitochondria-GFP dye, that labels mitochondria in live cells, demonstrated severe mitochondrial impairment in neurons after treatment with Aβ42 protofibril-EVs, as seen by their disrupted mitochondrial network and mitochondrial swelling (Figure 11C-D). These results indicate a possible role of EVs in the progression of Aβ-induced pathology.
Figure 11. Neuronal effects after Aβ₄₂ protofibril-EV treatment. Cortical neurons displayed long branches of dendrites and synapses after treatment with Control-EVs (A), whereas Aβ₄₂ protofibril-EVs had detrimental effects on the neurons (B). In addition, mitochondria from neurons treated with Control-EVs exhibited healthy mitochondrial (C), but Aβ₄₂ protofibril-EVs induced mitochondrial impairment and swelling (red asterisks) (D). Synaptophysin (red), βIII-tubulin (green), DAPI (white). Scale bars: A-B: 20 µm, C-D: 2 µm.

Antibody-mediated effects on astrocytic Aβ₄₂ protofibril clearance

The humanized version of the murine Aβ protofibril selective antibody mAb158, called BAN2401, is currently evaluated in phase 2b clinical trials for AD. Although the mAb158 antibody has previously been shown to reduce the levels of pathogenic Aβ and prevent Aβ deposition in tg-ArcSwe mice, the cellular mechanisms of mAb158 and other therapeutic Aβ antibodies remain elusive. In Paper III, we aimed to investigate if mAb158 (IgG₂a) could affect the Aβ₄₂ protofibril clearance by astrocytes in the mixed co-culture. Co-cultures were exposed to Aβ₄₂-555 protofibrils for 24 h in the presence or absence of mAb158 prior to fixation, or cultured for an additional 6 or 12 days after removal of Aβ₄₂-555 protofibrils and mAb158 prior to fixation. Our ICC data demonstrated that preincubation of Aβ₄₂-555 protofibrils with mAb158 almost abolished the accumulation of astrocytic Aβ₄₂-555 protofibrils, already at 24 h
A reduced effect on astrocytic Aβ accumulation was seen when mAb158 was added 1 h prior to Aβ42-555 protofibril exposure. However, the effect of mAb158 was lost when added 3 days after the Aβ42-555 protofibril exposure, indicating that the preformed extracellular antigen-antibody complex is crucial for the effect to occur. Time-lapse and confocal microscopy confirmed that Aβ42-555 protofibrils and mAb158 co-localized inside the astrocytes and that the intracellular accumulation of Aβ was much weaker at 24 h in presence of mAb158, compared to Aβ42-555 protofibrils only. Similar to mAb158, co-incubation with the mAb1C3 antibody, binding pan-Aβ, also lowered the Aβ42-555 accumulation in astrocytes. To investigate further if the effect was specific for Aβ antibodies, co-cultures were exposed to Aβ42 protofibrils together with the irrelevant antibody Ly-128 (IgG1) or the irrelevant antibody MOPC-173 (IgG2a). The Ly-128 did not reduce the intracellular Aβ and MOPC-173 had a significantly lower effect on the Aβ accumulation, as compared to mAb158, indicating that the effect was mainly specific for Aβ antibodies.

The mAb158 antibody prevents accumulation of Aβ in astrocytes. Accumulation of Aβ42-555 protofibrils was notable in astrocytes at 24 h (A). However, antibody treatment almost abolished the accumulation of astrocytic Aβ42-555 protofibrils (B). Scale bar: 20 µm.

The intracellular concentration of Aβ42 was significantly reduced after mAb158 treatment in Paper III, as demonstrated by ELISA analysis. Western blot analysis showed an increased clearance of Aβ42 from the cell culture medium in the presence of mAb158, indicating that Aβ was not secreted as much by the cells after mAb158 treatment. Importantly, in Paper I and Paper IV, we found that Aβ42 protofibril exposure resulted in increased neuronal cell death by the secretion of Aβ-containing EVs, but mAb158 treatment in Paper III rescued the neurons from Aβ-induced toxicity. However, the effect of
mAb158 was not observed when the antibody was added 3 days after Aβ42 protofibril removal.

We sought to elucidate the mechanism mediating the Aβ clearance by astrocytes. To investigate if the mAb158 effect on Aβ protofibril accumulation is Fcγ receptor dependent or Fcγ receptor independent, we used the recombinantly produced mAb158 antibody, N297D (IgG2c), which has a mutation on the glycosylation site, leading to loss of receptor-binding and reduced C1q binding. We found that mAb158 with the N297D mutation (asparagine replaced by aspartic acid) showed almost as good clearing effect of Aβ in the astrocytes, as the mAb158 without the mutation. Moreover, studies were performed to investigate if the antigen-binding sites of the mAb158 antibody, called F(ab')2, could mediate Aβ clearance in the absence of an Fc region. The divalent F(ab')2 fragment of an antibody is generated by pepsin digestion to remove the Fc region while leaving intact some of the hinge region, thus preventing the fragment to bind to any of the Fcγ receptors. The F(ab')2 fragment of mAb158 also significantly reduced the accumulation of Aβ in astrocytes, suggesting that the effect of mAb158 on Aβ42 protofibril clearance is mainly a Fcγ receptor independent mechanism.

In order to investigate the influence of the proteosomal and endosomal-lysosomal pathway on mAb158-mediated Aβ clearance in astrocytes, we preincubated co-cultures with the proteosomal inhibitor mg-132 or the lysosomal inhibitor bafilomycin for 30 min prior to Aβ42 protofibril exposure in the presence or absence of mAb158. The inhibitors were also present during the 24 h exposure. Neither mg-132 nor bafilomycin had any effect on the mAb158-mediated reduction of Aβ deposits in astrocytes, demonstrating that inhibition of the proteosomal or lysosomal pathway did not have an impact on the reduced Aβ accumulation. Taken together, our results indicate that the majority of the antigen-antibody complexes may be degraded by another, not yet described mechanism.

Future perspectives

Based on the results of this thesis, one major issue that still remains to be clarified is why the neurons die following Aβ42 protofibril-EV treatment. The EVs may contain other cargo aside from N-terminally truncated Aβ that may induce toxicity. Immunocytochemistry against Aβ42 should be performed in order to assess if EVs transport the Aβ into the neurons. Furthermore, it remains unclear if apoE from EVs promote or counteract the Aβ pathology. Nevertheless, it would be of interest to evaluate the biomarker potential of apoE in EVs isolated from human AD CSF samples, including apoE ε4 carriers, in order to reflect the in vivo role of apoE in EVs. Whether neurons in culture could be rescued from EV-induced cell death by inhibition of the cellular uptake, e.g. by the endocytic inhibitor Dynasore is a relevant question.
In addition, this may provide useful insights into the mechanistic action of EVs, i.e. if EVs are required to be endocytosed in order to cause cellular damage or if the toxic action occurs extracellularly. The positive effect of mAb158 on the reduction of Aβ accumulation in astrocytes was evident, but the exact mechanism mediating the clearance effect remains unclear. It is likely that mAb158 not only affects the Aβ accumulation, but may also limit the spreading of Aβ pathology by EVs. Although our experiments showed that mAb158 still had an effect on the Aβ clearance, regardless of the inhibition of either the proteosomal or endosomal-lysosomal pathway, full blocking is not guaranteed by the inhibitors. Experiments using different parts of the mAb158 antibody still resulted in increased Aβ clearance. In addition, Aβ was cleared rapidly from both the cell culture medium and intracellularly by mAb158 treatment, but only when the antigen and antibody were present at the same time to allow formation of antigen-antibody complexes. Time-lapse recording showed low background signals without increased amount of Aβ complexes floating in the medium. It would therefore be interesting to examine if there are other ways to activate astrocytes to clear Aβ. One hypothesis that may be worth investigating is if the antigen-antibody complexes are able to stimulate the astrocytes to respond by releasing certain molecules that may help clear Aβ already in the extracellular space, thus limiting the uptake by the astrocytes.
Concluding remarks

The work presented in this thesis has increased our understanding of the cellular responses to Aβ42 protofibrils, the spreading of Aβ pathology by the secretion of EVs and the mechanisms of antibody treatment in a mixed cell culture model exposed to Aβ42 protofibrils.

In Paper I, we showed that astrocytes effectively engulf Aβ42 protofibrils, but store, rather than degrade the ingested material. The incomplete degradation of Aβ42 protofibrils resulted in formation of enlarged endosomes in astrocytes and induced secondary neuronal cell death by the release of N-terminally truncated Aβ in EVs. Our results demonstrate that astrocytes are highly involved in Aβ pathology, by accumulating and spreading toxic Aβ species.

In Paper II, we found that the release of EVs did not vary in response to Aβ42 protofibril exposure, but the protein content in EVs changed markedly. Specifically, a threefold increase of apoE was confirmed in Aβ42 protofibril-EVs, compared to Control-EVs. This increase of apoE in EVs may be a “defense” mechanism of the cells to fibrillize soluble Aβ to less toxic fibrils, but this needs to be further addressed.

In Paper III, we demonstrated that mAb158 reduced the astrocytic Aβ protofibril accumulation and rescued neurons from Aβ-induced cell death. Antigen binding was crucial for the effect to occur and increased clearance of Aβ was confirmed from both the extracellular and intracellular space. In the AD brain, this type of passive immunization targeting Aβ pathology might enhance Aβ clearance and thus preserve the functionality of astrocytes.

In Paper IV, we showed that spreading of Aβ-induced pathology by the secretion of EVs has detrimental effects on neurons. The Aβ42 protofibril-EVs induced neuronal cell death, destroyed synapses and dendrites and caused mitochondrial impairment. These findings may be involved in the neuroinflammatory process and exacerbation of AD pathology.

Taken together, the work presented in this thesis demonstrates a chain of cellular events that occur in response to Aβ42 protofibril exposure, eventually leading to neuronal cell death. Aβ protofibril-selective antibodies may constitute a promising therapeutic strategy against AD. By preventing vesicle-mediated spreading of Aβ, progression of neurodegeneration may be limited.
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The day before I moved to Uppsala to start my PhD, I read two interesting quotes that left an impression on me. The first quote was the following: *It is not because things are difficult that we do not dare, it is because we do not dare that they are difficult.* The second quote was: *You cannot cross the ocean unless you have the courage to leave the shore.* In retrospect, I am very happy I left the shore because, despite a few headwinds at times, it took me on an amazing and unforgettable PhD journey. A journey that has now reached its final destination. This journey would not have been possible or the same without a few people. I would like to take the opportunity to acknowledge them here.

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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)