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Astrocytes in Alzheimer's disease

Exploring the impact of amyloid-β pathology on neurotoxicity, metabolism and inflammation.

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Abstract

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Astrocytes play a central role in brain homeostasis, but are also tightly connected to the pathogenesis of Alzheimer's disease (AD). Yet, their exact role in amyloid-beta (Aβ) pathology and chronic neuroinflammation is unclear. The aim of this thesis was to elucidate the impact of astrocytes in AD progression. For this purpose, astrocytes in different culture set-ups were exposed to soluble Aβ aggregates. The astrocytes engulf and process, but fail to fully degrade the Aβ aggregates, which are instead stored as large intracellular deposits. In **Paper I**, we show that extracellular vesicles (EVs), secreted from the Aβ-containing cells induce synaptic loss, axonal swelling and vacuolization of primary neurons, which consequently leads to apoptosis.

Astrocytes play a central role in the brain's energy metabolism and we were therefore interested in how Aβ pathology affects their metabolism. In **Paper II**, we report that Aβ accumulation in astrocytes disrupts mitochondrial fission/fusion homeostasis, resulting in decreased mitochondrial respiration and altered glycolysis. Interestingly, the astrocytes switch to fatty acid β oxidation with the aid of peroxisomes to maintain stable energy production.

Another important task is to understand how astrocytes modify the ingested Aβ. In **Paper III**, we characterized the astrocytic Aβ inclusions by isolating them with magnetic beads. Our analysis showed that the astrocytes truncate and pack together the Aβ aggregates. Moreover, we found that astrocytes release specifically truncated forms of Aβ via different routes.

Astrocytes' involvement in lipid metabolism and inflammation has recently gained much interest, but many questions remain about the connection between these processes. In **Paper IV**, we show that Aβ pathology causes lipid droplet (LD) accumulation in astrocytes. Moreover, we could show that astrocytes frequently transfer LDs to neighboring cells, both through direct cell-to-cell contacts and via secretion. Astrocytes have previously been reported to express major histocompatibility complex II (MHCII) and have the capacity to perform as professional antigen presenting cells. Interestingly, our results demonstrate that LDs contain MHCII, identifying a link between LDs and inflammation in astrocytes.

Taken together, this thesis contributes with important knowledge of the role of astrocytes in AD pathology.

Keywords: Alzheimer's disease, astrocytes, amyloid-beta, extracellular vesicles, mitochondria, lipid droplets, lipid metabolism, inflammation

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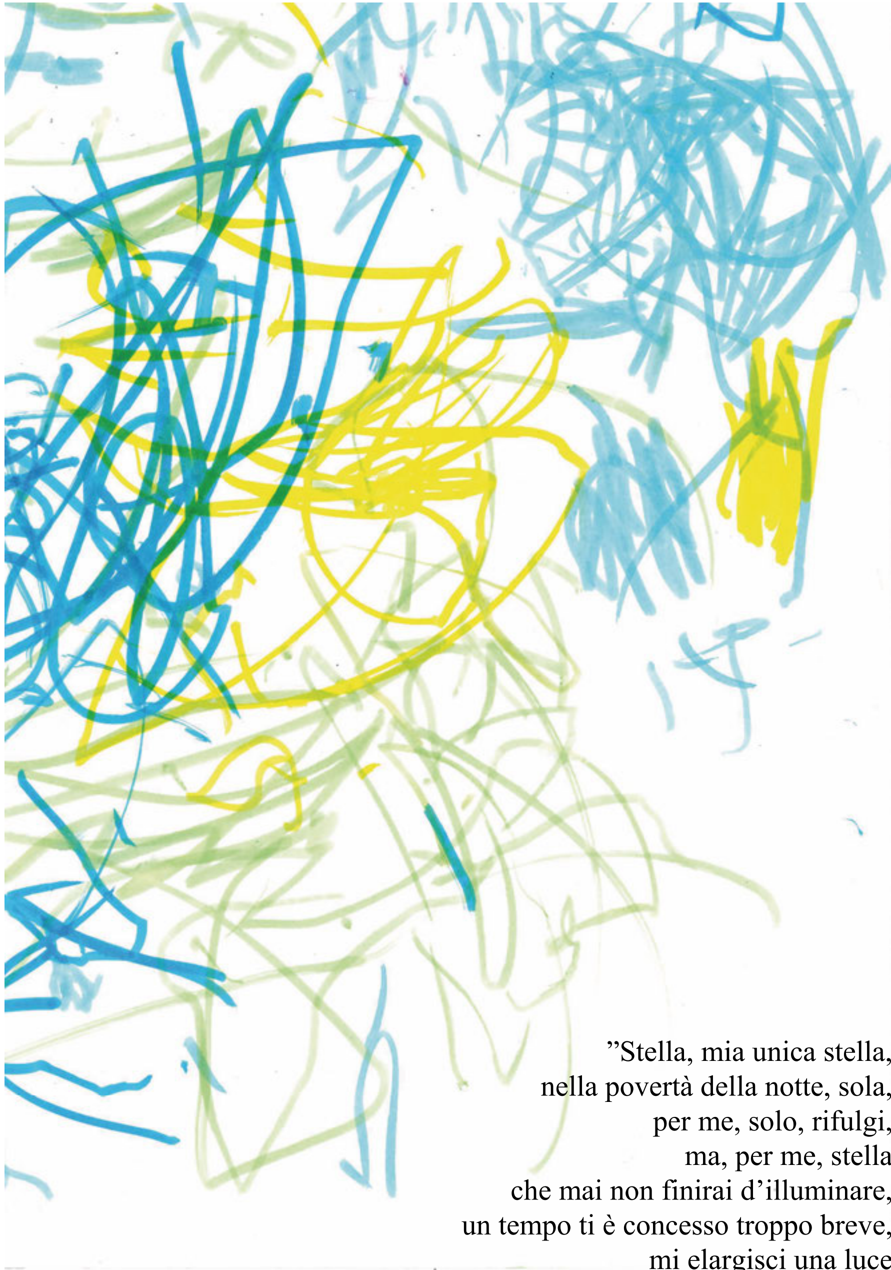
Till mamma och mormor



”Stella, mia unica stella,
nella povertà della notte, sola,
per me, solo, rifulgi,
ma, per me, stella
che mai non finirai d’illuminare,
un tempo ti è concesso troppo breve,
mi elargisci una luce
che la disperazione in me
non fa che acuire."

Giuseppe Ungaretti, Stella

”*Astrocytes, stars and their skeletons”*
Ada and Mia Beretta (2024)

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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.

I. **Beretta C**, Nikitidou E, Streubel-Gallasch L, Ingelsson M, Sehlin D, Erlandsson A. (2020) Extracellular vesicles from amyloid-β exposed cell cultures induce severe dysfunction in cortical neurons. *Scientific Reports,* 10(1):19656.

II. Zyśk M*, **Beretta C***, Naia L, Dakhel A, Påvénius L, Brismar H, Lindskog M, Ankarcrona M, Erlandsson A. (2023) Amyloid-β accumulation in human astrocytes induces mitochondrial disruption and changed energy metabolism. *Journal of Neuroinflammation,* 20(1):43. * Contributed equally

III. **Beretta C**, Svensson E, Dakhel A, Zysk M, Hanrieder J, Sehlin D, Michno W, Erlandsson A. (2024) Amyloid-β deposits in human astrocytes contain truncated and highly resistant proteoforms. *Molecular and Cellular Neuroscience,* 128:103916.

IV. **Beretta C**, Dakhel A, Rosqvist F, Uzoni S, Mothes T, Fletcher J, Risérus U, Sehlin D, Michno W, Erlandsson A. Astrocytes with Alzheimer's disease pathology provoke lipid droplet mediated cell-to-cell propagation of MHC II complexes. *Manuscript.*

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Additional papers

V. Konstantinidis E, Dakhel A, **Beretta C**, Erlandsson A. (2023) Long-term effects of amyloid-beta deposits in human iPSC-derived astrocytes. *Mol Cell Neurosci.* 125:103839
VI. Konstantinidis E, Portal B, Mothes T, **Beretta C**, Lindskog M, Erlandsson A. (2023) Intracellular deposits of amyloid-beta influence the ability of human iPSC-derived astrocytes to support neuronal function. *J Neuroinflammation*. 20(1):3.
VII. Rofo F, Ugur Yilmaz C, Metzendorf N, Gustavsson T, **Beretta C**, Erlandsson A, Sehlin D, Syvänen S, Nilsson P, Hultqvist G. (2021) Enhanced neprilysin-mediated degradation of hippocampal Aβ42 with a somatostatin peptide that enters the brain. *Theranostics.* 11(2):789-804.
VIII. Streubel-Gallasch L, Zyśk M, **Beretta C**, Erlandsson A. (2021) Traumatic brain injury in the presence of Aβ pathology affects neuronal survival, glial activation and autophagy. *Sci Rep.* 11(1):22982.

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Abbreviations

Aβ	amyloid β
AD	Alzheimer's disease
APC	antigen presenting cell
APOE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
BBB	blood-brain barrier
bFGF	basic fibroblast growth factor
CE	cholesterol esters
CNTF	ciliary neurotrophic factor
CoA	Co-enzyme A
CSF	cerebrospinal fluid
DRP-1	dynamin-related protein 1
ECAR	extracellular acidification rate
EEA1	early endosome antigen 1
EE	early endosome
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EV	extracellular vesicle
FAD	familial Alzheimer's disease
FADH	dihydroflavine-adenine dinucleotide
GFAP	glial fibrillary acidic protein
GTP	guanosine triphosphate
HRP	horseradish peroxidase
ICC	immunocytochemistry
IGF1	insulin-like growth factor
IHC	immunohistochemistry
Ii	invariant chain
iPSC	induced pluripotent stem cells
LC-MS	liquid chromatography mass spectrometry
LD	lipid droplet
LDH	lactate dehydrogenase
LE	late endosome
LOAD	late onset Alzheimer's disease
MHC	major histocompatibility complex

MIF	macrophage migration inhibitory factor
MLB	multilamellar bodies
MS	mass spectrometry
MVB	multivesicular body
NADH	nicotinamide adenine dinucleotide
NES	neuroepithelial-like stem cells
NFT	neurofibrillary tangles
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
PCA	principal component analysis
PLIN	perilipin
PSEN	presenilin
PVDF	polyvinylidene fluoride
SCR	spare respiratory capacity
SNARE	Soluble N-ethylmaleimide-Sensitive Factor Attachment Proteins receptor
TEM	transmission electron microscopy
TG	triacylglycerols
TNT	tunneling nanotubes
ToF-SIMS	Time of Flight secondary ion mass spectrometry
UPP	ubiquitin-proteosomal pathway
WB	western blot

Introduction

Alzheimer's disease

Alzheimer's disease (AD) is a growing public health concern and it is estimated that 139 million people will be affected by year 2050[1]. Alzheimer's disease was first described in 1906 by Alois Alzheimer[2]. It is a neurodegenerative disease, characterized by memory loss and cognitive impairment, due to synaptic dysfunction, neuronal cell death and brain atrophy[3]. Although it is such a common cause of dementia, the exact mechanisms behind the pathology are not well understood. Alzheimer's disease has very distinctive hallmarks: amyloid β (Aβ) plaques, neurofibrillary tangles (NFTs), and chronic neuroinflammation, represented by reactive astrocytes and activated microglia[4–6] (Figure 1).

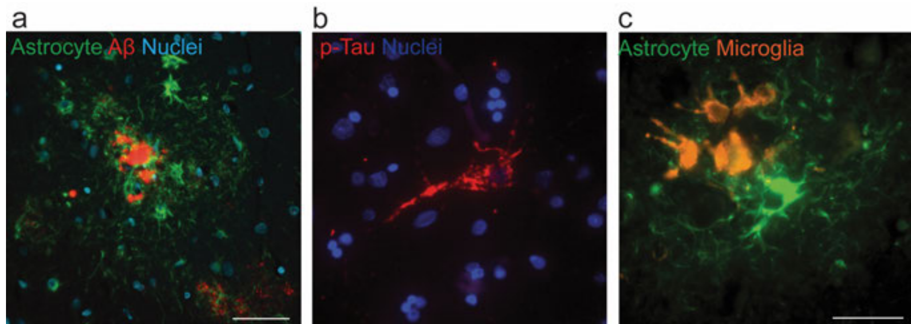

Figure 1. Amyloid plaques (a), neurofibrillary tangles (b), and chronic inflammation represented by astrocytes and microglia (c) are found in *post-mortem* cortical sections of AD patients. Scale bar (a, c): 20 µm. Image b was kindly provided by Tobias Mothes.

It is still unclear how these hallmarks relate to each other. Familial AD (FAD) is caused by mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) [7,8]. All these genes are involved in Aβ production, implicating that Aβ has an initiating role in the pathology[9]. The prevalence of Aβ related genetic causes has led the field to focus on a linear pathological model, where Aβ starts a cascade that leads to the formation of NFTs, increased inflammation, and finally neuronal loss. According to the amyloid cascade hypothesis, the pathology in AD is a direct consequence of misfolding and aggregation of Aβ[10,11]. However, recent studies have shown

that Aβ and tau pathology work in synergy, leading to enhanced seeding properties for both proteins[9,12,13]. Moreover, both Aβ and Tau interact with apolipoprotein E (ApoE)[14], which is linked to sporadic late onset AD (LOAD)[15].

ApoE is an important cholesterol and lipid transporter in both the peripheral and central nervous system[15–17]. The *APOE* gene has three alleles: ε2, ε3, and ε4. The presence of the ε4 allele increases the risk of AD, while the ε2 allele seems to have a protective effect[15,18]. The different *APOE* isoforms have diverse effects on lipid transport, neuronal health, inflammation, and Aβ burden[15,16,19–22].

Amyloid β

Aβ is the main component of plaques found in the brain of AD patients[23,24], but it is also found in the healthy brain where it is important for neuronal health[25]. It is a cleavage product of APP[26], which is a large protein found in the cell membrane that has different functions, e.g. in synaptic plasticity and neurotransmission[27,28]. APP cleavage can lead to two different pathways: the non-amyloidogenic or amyloidogenic pathway, depending on which enzymes cleave it first. In the non-amyloidogenic pathway, α-secretase and subsequently γ-secretase cleave APP, producing soluble APPα (sAPPα) and P3. In the amyloidogenic pathway, APP is first cleaved by β-secretase and then γ-secretase, resulting in the release of Aβ (Figure 2)[28–30].

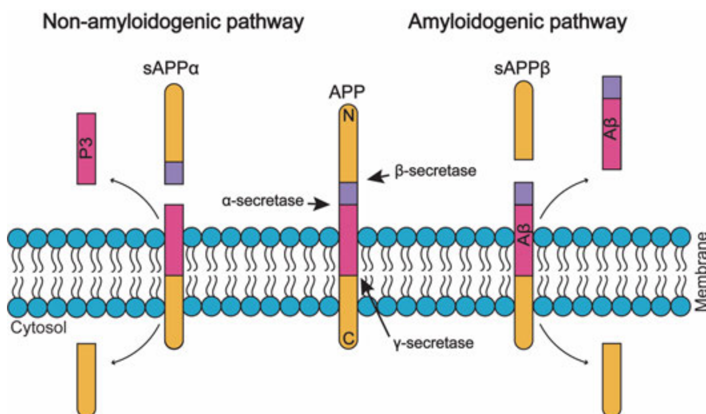

Figure 2. APP is a transmembrane protein that can be processed via two different pathways: the non-amyloidogenic pathway, and the amyloidogenic pathway. Aβ is released by the amyloidogenic pathway, in which APP is cleaved by β- and γ-secretase.

The Aβ peptide can be between 37 and 49 amino acids in length. The most commonly found variants in AD are Aβ1-40 and Aβ1-42, where the latter is more prone to aggregate[31]. Aβ monomers that start to aggregate at first result

in soluble low-molecular weight oligomers, that then aggregate further, forming larger oligomers, protofibrils and fibrils[31] (Figure 3). The fibrils are insoluble and the main constituent of plaques. As the plaques are one of the hallmarks of AD, it was long believed that they were the main cause of neurodegeneration[10]. However, the plaque burden does not directly correlate with the severity of the disease[31–33]. In fact, it has been shown that the levels of soluble Aβ correlate better with synaptic disruption[34,35] and the severity of neurodegeneration[36,37]. In the AD research field, the focus has therefore shifted to the smaller and soluble aggregates as the main toxic culprit.

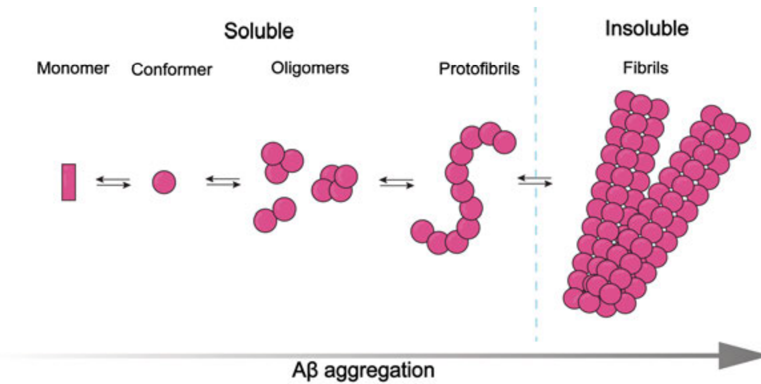

Figure 3. Aβ monomers form soluble aggregates of various size that finally deposit as insoluble fibrils.

Cellular effects of Aβ

The cellular effects of Aβ, leading to toxicity, are complex and not fully understood. Aβ aggregates have been shown to interact with membranes, receptors and different organelles[31].

Neurons are the cell type that is believed to be most vulnerable to Aβ toxicity[38]. In fact, extracellular Aβ aggregates can bind to ion channels and cause synaptic dysfunction and neuronal death[39–41]. Accumulation of Aβ, in close proximity to lipid rafts on membranes, has been shown to seed further aggregation and affect signal transduction and cell signaling[32]. Oligomeric Aβ may also create pores in the membranes disrupting membrane integrity[32]. Many organelles can interact with Aβ oligomers and may also be sites of Aβ generation and aggregation, e.g. endoplasmic reticulum (ER), lysosomes, endosomes, autophagosomes and mitochondria[32]. Moreover, Aβ accumulation leads to ER stress, mitochondrial damage, including disruption of the electron transport chain, alterations in proteolysis and finally apoptosis[31,32,42]. In addition, aggregated Aβ promotes the production of free radicals, causing elevated oxidative stress, resulting in DNA damage[31].

In addition to neurons, Aβ affects the glial cells in the brain. By binding to calcium permeable receptors on astrocytes, Aβ oligomers cause the release of glutamate, leading to synaptic loss[43]. Moreover, extracellular aggregated Aβ causes microglia and astrocyte activation and chronic inflammation which results in excessive phagocytosis and synaptic pruning. This also leads to an increased release of inflammatory mediators, e.g. cytokines, which can promote neurotoxicity[31].

Astrocytes

Astrocytes, named after their star-like morphology, are the most abundant glial cell in the brain[44]. As the focus has been on neurons, astrocytes have until recently been considered to have a passive role in brain function. However, studies have now shown that astrocytes are extremely complex and heterogeneous cells[45,46], with functions that are vital for brain homeostasis (Figure 4). Astrocytic end-feet interact with the endothelial blood vessels in the brain, contributing to the blood-brain barrier (BBB)[47], and regulating the blood flow[48] (Figure 4b). Moreover, astrocytes are very important for synapse formation and signaling[49,50], and they ensure that the neurons are healthy and have optimal conditions to function by giving them metabolic support[51](Figure 4b). They are fundamental for the removal and recycling of neurotransmitters, e.g. glutamate[52]. Furthermore, astrocytes seem to contribute to the glymphatic system, which has been hypothesized to promote waste clearance through cerebral spinal fluid (CSF) movement[53].

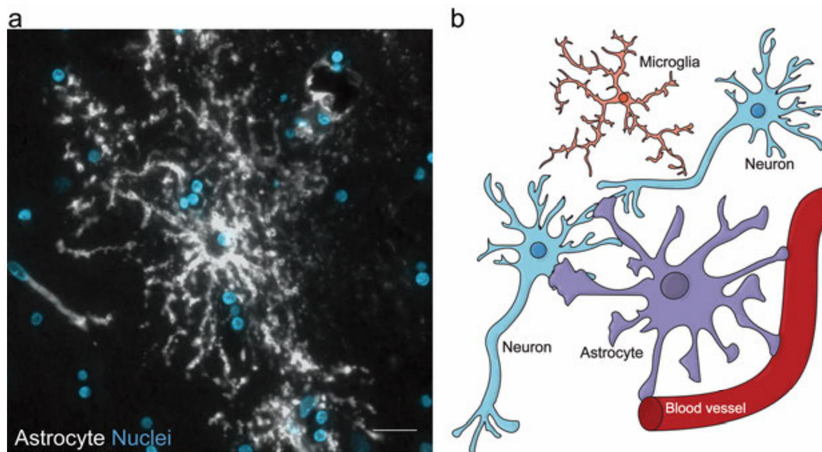

Figure 4. Immunofluorescent staining of an astrocyte in the human brain cortex (a). Astrocytes are in close contact with neurons, microglia and blood vessels in the brain (b). Scale bar (a): 20 µm.

Already in the mid-19th century, it was recognized that astrocytes are involved in neuropathology[54]. However, what happens to astrocytes during pathology and what role they play in AD progression are still not completely understood. Reactive astrogliosis is a process in which the astrocytes become hypertrophic and upregulate proteins, such as glial fibrillary acidic protein (GFAP), S100β, vimentin and nestin. Moreover, they start to proliferate and form glial scars. During this process, the astrocytes become more phagocytic, presumably to protect neurons from dead cells and toxic proteins[51]. Astrocytes also play a central role in neuroinflammation, and interact closely with microglia[55,56] (Figure 4b). This happens through direct cell contact, as well as production and secretion of inflammatory molecules from both cell types[55,57]. In AD, astrocytes have been shown to lose their homeostatic functions, preventing them from effective cross-talk with microglia[58,59]. Both reactive astrocyte and microglia are commonly found in proximity to amyloid plaques[60,61] and have been shown to engulf and degrade Aβ through ApoE-binding receptors[62,63]. These changes in the astrocytic homeostatic functions, do not only affect microglia, but neurons as well[64]. In fact, Aβ exposed astrocytes have been shown to be unable to metabolically support neurons[65]. Similarly, in AD mouse models disrupted astrocyte-neuron cross-talk has been shown to cause neuronal circuit instability[66].

Cellular degradation pathway

There are different degradation pathways for proteins and organelles in the cell: the ubiquitin-proteasomal pathway (UPP), the endo-lysosomal pathway, and the autophagy-lysosome pathway[67].

The UPP is important for degradation and removal of short lived or damaged proteins in the cytosol[68]. The proteasome is composed of a core particle (20S) that has multiple proteolytic sites and a regulatory particle (19S) that has ubiquitin receptors and regulates the access of substrates to the core[69–72] (Figure 5).

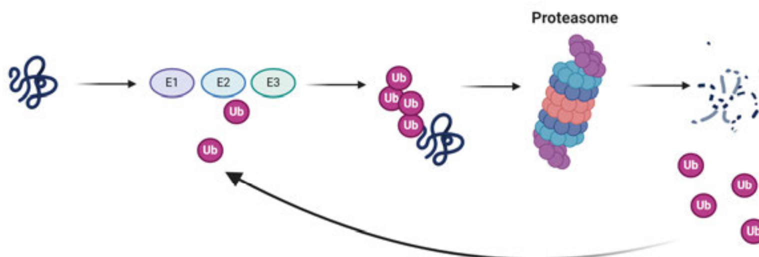

Figure 5. Proteins entering the UPP system are first ubiquitinated by different enzymes. The protein is then recognized by the proteasome, deubiquitinated and subsequently degraded. Created with BioRender.com

The endo-lysosomal pathway is important for the degradation of exogenous proteins and debris. Exogenous cargo is first engulfed through endocytosis. This process can happen in many different ways but the best studied is clathrin-coated endocytosis, which starts with protein-protein interactions in clathrin-coated pits[73]. The proteins are then internalized and the clathrin coating is removed by the early endosomes (EE). The EEs start to fuse with each other with the help of Rab5, early endosome antigen 1 (EEA1) and Soluble N-ethylmaleimide-Sensitive Factor Attachment Proteins receptor (SNARE) proteins[73,74]. The EEs then move towards the center of the cell and mature into late endosomes (LE) by lowering the pH with the acquisition of acid hydrolases and the transition from Rab5 to Rab7[73–75]. The late endosomes fuse with lysosomes and form endo-lysosomes, where the degradation happens[76] (Figure 6).

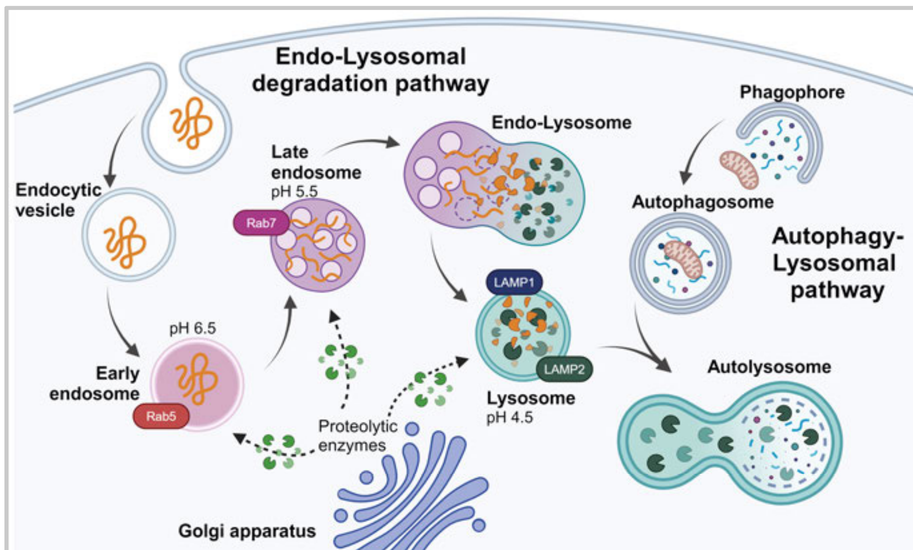

Figure 6. Extracellular proteins are internalized and end up in early endosomes, which mature to late endosomes. Late endosomes fuse with lysosomes and the proteins are degraded. In autophagy, autophagosomes sequester cytosolic proteins. Lysosomes and autophagosomes fuse and the cargo is degraded. Created with BioRender.com

Autophagy is responsible for the degradation of cytoplasmic components and for organelle turnover, and can be divided in microautophagy, chaperone-mediated autophagy and macroautophagy. Macroautophagy is the most well-studied in mammalian cells[77]. It involves the formation of phagophores and autophagosomes that sequester part of the cytosol. The autophagosomes then fuse with lysosomes that degrade the inner membrane, as well as the cargo[78] (Figure 6). In microautophagy, the lysosomes engulf cytosolic material directly, while in chaperon-mediated autophagy the targets are moved through the lysosomal membrane with the help of chaperones[78].

Neuroinflammation

Inflammation is a protective mechanism against infections or endogenous damage[79]. Under pathological conditions, several cell types produce and secrete inflammatory mediators, i.e. cytokines and chemokines[79,80]. Cytokines are small molecules that can have pro- or anti-inflammatory effects on other cells; while chemokines have specific chemoattractant proprerties[81]. This way of communication of the immune system is important for the recruitment, maturation and activation of different immune cells[82,83].

In the brain, microglia and astrocytes are the major modulators of neuroinflammation. Microglia are the brain's immune surveillance system and have macrophage-like functions[84]. In the healthy brain, microglia patrol their environment and they are essential for synaptic pruning and removal of dead cells[85]. However, their phenotypes and functions are drastically changed in AD, where they become more pro-inflammatory, leading to neuronal damage[86–89].

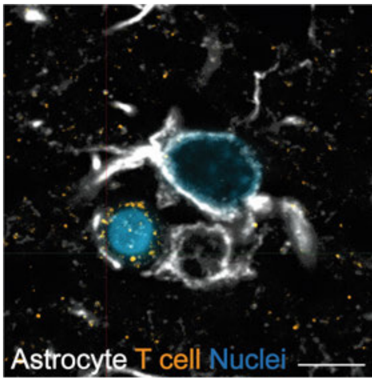

Figure 7. Astrocytes in close contact with T cell in AD brain. Scale bar: 5 µm.

Recent studies have demonstrated that astrocytes are intrinsically involved in neuroinflammation[90–92]. Not only is there a tightly regulated cross-talk between astrocytes and microglia, that modulates their activation[55,90,93,94], but astrocytes have also been shown to interact and mediate inflammatory responses from the periphery[95,96]. Notably, astrocytes have the capacity to be antigen presenting cells (APCs) and have been found in close contact with infiltrating T cells in the human brain (Figure 7)[56,97–99]. T cells have been observed in the brain and CSF in AD patients[100,101]. Recent studies have shown that microglia and astrocytes have an active role in recruitment and activation of T cells in AD models[102–104]. However, the exact role of different T cells in the brain is still unclear.

T cell activation

T cells are part of the adaptive immune system and rely on APCs for activation either via major histocompatibility complex class I or class II (MHCI or MHCII)[105]. MHCI activates CD8 positive T cells by presentation of endogenous peptides, while MHCII activates CD4 positive T cells by presentation of exogenous peptides[105,106].

MHCII is synthesized in the ER and has an invariant chain (Ii) where the antigen will bind later on. Ii-MHCII is trafficked through the Golgi apparatus to the plasma membrane, where it is internalized. Following endocytosis, Ii-MHCII enters the endo-lysosomal pathway, first through EEs, then LEs, and finally it is transported to the endo-lysosomal compartments. Here, Ii-MHCII is processed and Ii is cleaved off, leaving MHCII free to bind to the antigen[107]. Antigen bound MHCII is trafficked back to the plasma membrane, where it can interact with and activate T cells. The cleavage of the invariant chain results in the formation of CD74, which moves to the cell membrane, to serve as a receptor for macrophage migration inhibitory factor (MIF)[108] (Figure 8).

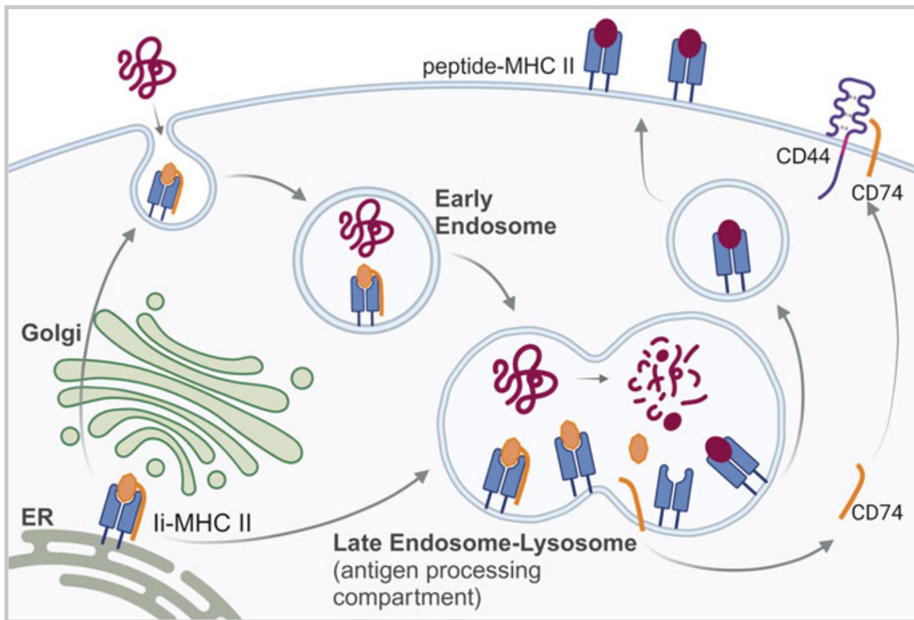

Figure 8. Ii-MHCII is trafficked to the plasma membrane, where it is endocytosed by the cell and thereby end up in the endo-lysosomal pathway. In the late endosome-lysosome, Ii-MHCII is cleaved and MHCII can bind the antigen and be transported back to the plasma membrane. Created with BioRender.com

Cell-to-cell communication pathways

Cells have many ways to communicate with each other. They can interact by direct contact, e.g. via tunnelling nanotubes (TNTs), or by secretion of signalling molecules, for example within extracellular vesicle (EVs)[109].

Tunnelling nanotubes are thin protrusions between two cells, containing actin filaments. They can range between 50 nm and 1 µm in diameter, and can

differ drastically in length[110,111]. Tunnelling nanotubes have been shown to be involved in cellular transfer of organelles and toxic protein aggregates[112–116]. Studies have shown that in the brain, neural cells commonly use TNTs for mitochondrial transfer[117,118]. Moreover, TNTs are a common communication pathway for immune cells[119].

Extracellular vesicles are an important way of communication and cargo exchange between cells over long distances. They can be divided into two types: exosomes and microvesicles. Exosomes are between 50 and 150 nm in diameter, originate from endosomes, and are released from multivesicular bodies (MVB); while microvesicles are larger (50 nm to 1 µm in diameter) and bud directly from the plasma membrane[120]. Extracellular vesicles can have varied cargo containing mRNA, lipids , and a wide range of proteins, giving them the ability to perform different functions, e.g. activating immune cells, initiating programmed cell-death, and spreading diseases, just to name a few[121]. It has been shown that exosomes from AD brains contain neurotoxic Aβ oligomers[122]. Because of their involvement in disease, EVs are being evaluated to be used as biomarkers and therapeutic targets[123].

Energy Metabolism

The brain consumes up to 20 % of the body´s overall glucose-derived energy[124]. In fact, neurons have a very fast energy turnover, but have to rely on astrocytes for metabolites, as they cannot store glucose as glycogen and lipids[124].

Energy metabolism is divided into three main pathways: glycolysis, which takes place in the cytosol, oxidative phosphorylation (OXPHOS), which takes place in the mitochondria, and fatty acid β oxidation, which takes place in mitochondria and peroxisomes (Figure 9).

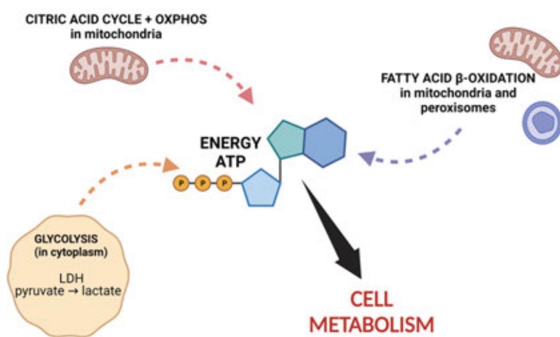

Figure 9. The three main pathways for energy production: glycolysis, OXPHOS, and fatty acid beta oxidation. Created with Biorender.com

The first step of the energy production is glycolysis, where glucose is broken down to two pyruvates, generating adenosine triphosphate (ATP)[125]. In mammalian cells, pyruvate can also be converted to lactate by lactate dehydrogenase (LDH). In the brain, astrocytes produce and

release lactate for neurons to use in their energy production[126]. This process is called the astrocyte-neuron lactate shuttle and is important for memory formation and learning, because of its neuroprotective function[127–130].

A more effective pathway for ATP production is the citric acid cycle, followed by OXPHOS. The citric acid cycle oxidizes Acetyl Coenzyme A (CoA) and produces after many intermediate steps, two CO_2, three nicotinamide adenine dinucleotide (NADH), one dihydroflavine adenine dinucleotide ($FADH_2$), and one guanosine triphosphate (GTP)[131]. NADH and $FADH_2$ are needed for OXPHOS, and by a series of enzymatic reactions throughout the complexes in the electron transport chain, ATP is generated[132]. Astrocytes have the capacity to sense metabolic changes in their surroundings and adapt their energy production to maintain homeostasis[133]. Although astrocytes also use OXPHOS for energy production, it is more dispensable as they can upregulate glycolysis when OXPHOS is inhibited[134,135]. Moreover, astrocytic OXPHOS has been shown to decrease in neurodegenerative diseases[136,137].

During starvation, cells can use fatty acids as an energy source. Fatty acids from autophagic degradation of organelles or from lipids stored in lipid droplets (LDs) are then imported into mitochondria[138] or peroxisomes[139]. There, the fatty acids are shortened to chain-shortened acyl-CoA, acetyl CoA and propionyl-CoA following multiple steps, in the process called fatty acid β oxidation that takes place in both organelles[140]. Recent studies have shown that astrocytes can use fatty acid β oxidation to prevent lipotoxicity[19,20,141]. Moreover, decreasing astrocytic fatty acid β oxidation causes increased inflammation[142].

Lipid metabolism

The brain is only second to adipose tissue in lipid compositions, with lipids being 50% of the brains dry weight[143,144]. It is mainly composed of long chain polyunsaturated fatty acids. Some fatty acids, including essential fatty acids can pass the BBB and be taken up by neurons and astrocytes[143,144]. Cholesterol, on the other hand, cannot cross the healthy BBB and therefore needs to be synthesized *de novo* locally in the brain[145]. The lipid production and storage mechanisms differ from one cell type to another. There is a tightly regulated lipid cross-talk between neurons and astrocytes, as neurons are dependent on other cells to store and produce lipids[143,145]. The *APOE4* genotype has been shown to affect and disrupt lipid homeostasis not only in astrocytes, but in all glial cells in the brain[20,87,146,147].

Lipid droplets

Lipid droplets are organelles formed by a core of neutral lipids surrounded by a monolayer of phospholipids[148] (Figure 10). Lipid droplets are present in many different cell types and are very dynamic, changing their size and numbers depending on nutrient availability[149,150]. Lipid droplet biogenesis occurs in the ER and starts with the synthesis of neutral lipids, mainly triacylglycerols and cholesterol esters[151]. As the concentration of neutral lipids increases, an oil lens is formed in the leaflets of the ER bilayer, eventually budding off as a LD [149,152]. Different and tightly regulated proteins are essential for the biogenesis of LDs from the ER[148].

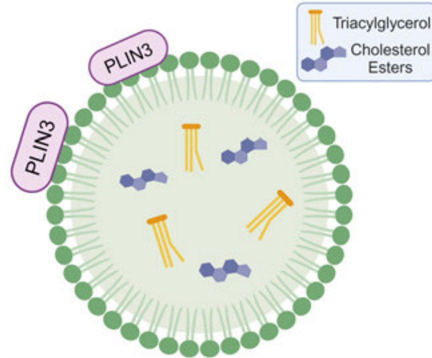

Figure 10. Lipid droplets contain triacylglycerols and cholesterol esters, surrounded by a phospholipid monolayer. PLIN3 coats LDs in astrocytes. Created with Biorender.com

A family of proteins, called PAT proteins or perilipins (PLINs), have been found to be the main coating proteins of LDs[153]. There are 5 known PLINs, PLIN1-5. All the PLINs have different functions and are to some extent cell type specific[149,153–156]. In the brain, it has been shown that PLIN3 coats astrocytic LDs and to a lesser extent microglial LDs, while PLIN2 is found in neurons[157].

For a long time, the only function of LDs was believed to be lipid storage. However, novel data show that they are involved in many cellular mechanisms. Lipid droplets can interact with mitochondria, peroxisomes, lysosomes and Golgi[158–160], suggesting a complex lipid cross-talk between cellular organelles. Interestingly, recent studies indicate that LDs play a role in immune responses, including regulation of immune cells[161,162]. In fact, studies in dendritic cells have shown that LD accumulation interferes with MHCI mediated cross presentation, and that depletion of LDs decreases the cross-presentation[163,164]. In addition, dendritic cells with LD accumulation were found to be better at activating other immune cells[165]. T cell reprogramming needs a lot of energy and therefore activated T cells switch to use fatty acids[166]. As a result, lipid metabolism plays a central role in T cell mediated immune responses[167,168].

In the brain, LDs seem to be involved in both pathology and normal aging[157]. In fact, both microglia and astrocytes accumulate LDs in the aging brain, suggesting a role for LDs in neuroinflammation[87,146,169]. Moreover, astrocytes store fatty acids released from stressed neurons in LDs, to be used for

energy production through fatty acid β oxidation[19]. Several studies have reported that *APOE4* affects lipid metabolism and LD formation in glial cells, suggesting that disruption of LD homeostasis is central in AD development[20,146,170–172]. Furthermore, astrocytic LD accumulation has been linked to increased reactivity, decreased neuronal support, and elevated activation of microglia[142]. Interestingly, decreasing the LD load in glial cells has shown promising results in ameliorating inflammation and tau pathology in AD mouse models[173].

Aims

The overall aim of this thesis was to investigate the role of astrocytes in Alzheimer's disease progression, focusing on cell-to-cell spreading, energy homeostasis, lipid metabolism, and inflammatory responses.

Specific aims:

I. To isolate EVs from Aβ exposed co-cultures and study their effect on neurons.

II. To investigate how Aβ exposure influences mitochondria and energy metabolism in astrocytes.

III. To study in which way Aβ in astrocytic inclusions has been modified and processed by the cell.

IV. To investigate the effects of Aβ pathology on lipid metabolism in astrocytes and uncover the link between lipid droplets and inflammation.

Methods

Cell culture systems

Cell culture systems are of central importance for this thesis. They have enabled detailed studies of different AD mechanisms in astrocytes and neurons.

Murine cell culture systems. In **Paper I**, we used cortical neural cell cultures obtained from C57/BL6 mice. Cerebral cortices were dissected from embryonal day 14 (E14) mouse embryos. Neural stem cells were first expanded as neurospheres in DMEM/F12 cell culture medium, supplemented with B27, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF). The cells were then dissociated and plated on coated dishes. The growth factors were removed to start differentiation, resulting in mixed co-cultures composed by 75% astrocytes, 20% neurons, and 5% oligodendrocytes (Figure 11a-c).

To obtain pure neuronal cultures (Figure 11d), the cells were plated directly following dissection and cultured in neurobasal cell medium, supplemented with B27 and L-glutamine.

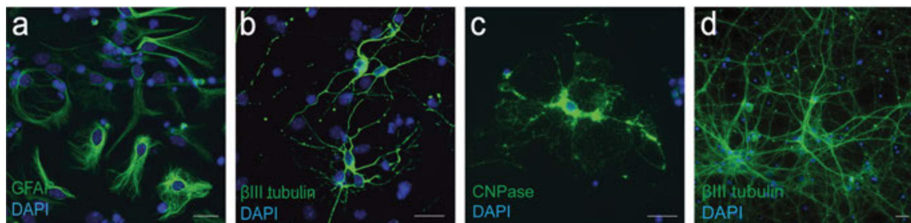

Figure 11. Characterization of murine cell culture systems. The murine co-cultures were stained for the astrocytic marker GFAP (a), neuronal marker βIII tubulin (b), and oligodendrocytic marker CNPase (c), allowing us to quantify the percentage of each cell type. Immunocytochemistry of pure neuronal cultures showed βIII tubulin neurons (d), creating neuronal networks. Scale bar: 20 μm.

Human astrocytes cultures. Human astrocytes have been shown to be significantly more complex than their murine counterparts[174,175]. To set up a culture system more translatable to the human brain, we used astrocytes derived from human induced pluripotent stem cells (iPSC) in **Paper II-IV**. The iPSC cells were first differentiated to neuroepithelial-like stem (NES) cells [176]. To differentiate the cells to mature astrocytes, we cultured the NES cells in Advanced DMEM/F12 medium supplemented with insulin-like growth factor 1 (IGF1),

basic fibroblast growth factor (bFGF), activin A, heregulin b, for 28 days with the addition of ciliary neurotrophic factor (CNTF) from day 15 in the differentiation[177]. The astrocytes were characterized by immunocytochemistry, targeting different astrocytic markers such as GLAST-1, GFAP, AQP4, S100β and vimentin (Figure 12)

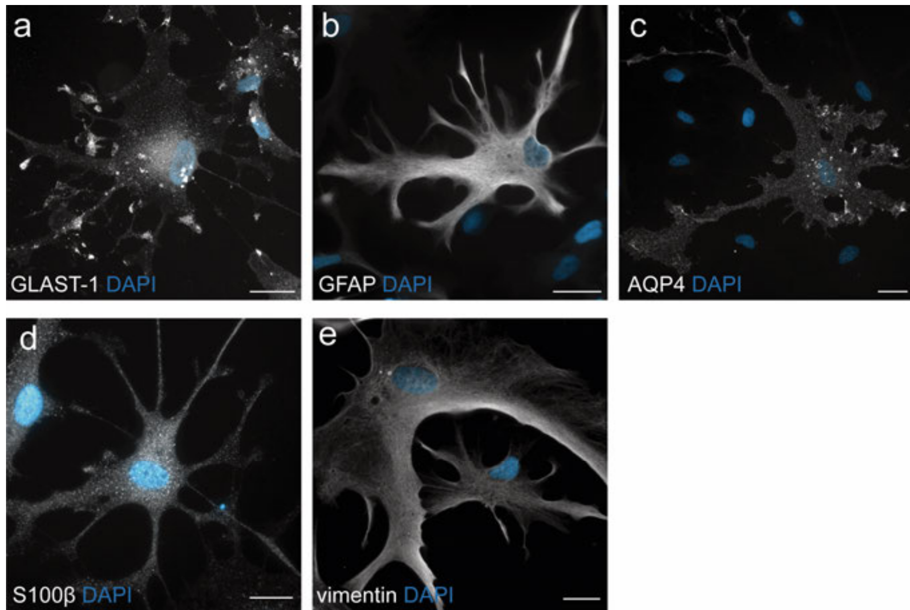

Figure 12. Characterization of human iPSC-derived astrocytes. Immunocytochemistry was used to detect the astrocytic markers GLAST-1 (a), GFAP (b), AQP4 (c), S100β (d), and vimentin (e). Scale bar: 20 µm.

Immunofluorescent stainings

Immunocytochemistry. In order to be able to visualize the proteins of interest and investigate how the morphology of the cells were affected by Aβ pathology, we used immunocytochemistry (ICC). In this technique, primary antibodies are used to target the proteins of interest and subsequently fluorophore-labelled secondary antibodies are used to detect the primary antibodies, and increase the signal. In this thesis, ICC was used to detect organelles, cellular markers, toxic proteins, markers for cellular stress and inflammatory responses. Different types of fluorescent dyes were also used to detect actin filaments, membranes, cholesterol, mitochondria, nuclei, and neutral lipids.

Immunohistochemistry. Cortical sections of paraffin-embedded brain tissue from AD patients and age-matched controls were analysed in **Paper IV** using immunohistochemistry (IHC). We followed a similar approach as for

ICC, using primary antibodies, after removing the paraffin, to detect the proteins of interest, followed by fluorophore labelled secondary antibodies to target the primaries.

Microscopy techniques

Microscopy techniques were central for this thesis, to visualize cell morphology, organelles, as well as cellular changes during pathology.

Fluorescent microscopy was performed after ICC and IHC to visualize, analyze and quantify the proteins of interest. With fluorescent microscopy, different fluorescent wavelengths can be detected and, therefore, many different proteins can be visualized at the same time, by using different fluorescently labeled secondary antibodies. Using this technique, we investigated Aβ uptake and degradation, organelle organization, and cell-to-cell communication throughout this thesis. Fluorescent microscopy was also used in **Paper IV** to visualize astrocytic markers in combination with lipid droplets, T cells, microglia, and MHCII in human brain sections.

Time lapse microscopy made it possible to follow dynamic processes in and between the cells in this thesis. The cells were placed in an incubator with optimal culture conditions and Nikon Biostation Live Cell Recorder captured images at different intervals. We were able to visualize uptake of extracellular vesicles and Aβ, mitochondrial motility, and organelle transfer between the cells.

Transmission electron microscopy (TEM) was performed to study cell and organelle morphology and pathological processes in detail. TEM creates very high resolution images as a result of an ion beam that passes through the sample section. In addition, immunoTEM was used in **Paper IV** to detect different proteins on the surface of isolated lipid droplets.

Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) is an antibody based method to detect protein levels. Sandwich ELISA was used in **Paper I**, **III** and **IV**. In short, for this method a plate is coated with a primary antibody that will bind to the protein of interest, later a secondary antibody is added that will also target the same protein. The secondary antibody is biotinylated or conjugated to horseradish peroxidase (HRP) creating a detectable signal following development. In **Paper I** and **IV**, this method was used to confirm Aβ protofibril concentration, while in **Paper III** it was used to investigate how intracellular Aβ was truncated.

Synthetic Aβ_{42} aggregation

Aβ_{42} protofibrils were used in **Paper I** and **IV**. The protofibrils (called soluble aggregates in **Paper IV**) were produced following well established, published protocols[178–180]. Synthetic Aβ_{42} monomers were resuspended in 10 mM NaOH and PBS and left to aggregate at 37°C for 30 minutes. The mAb158 based protofibril-specific ELISA was used to confirm the concentration of the protofibrils. In addition, the protofibrils were characterized using TEM (Figure 13a).

In **Paper II** and **III**, Aβ_{42} monomers were aggregated to fibrils at 37°C over 4 days (Figure 13b). The fibrils were then sonicated to break them up into smaller pieces, which was confirmed by TEM (Figure 13c).

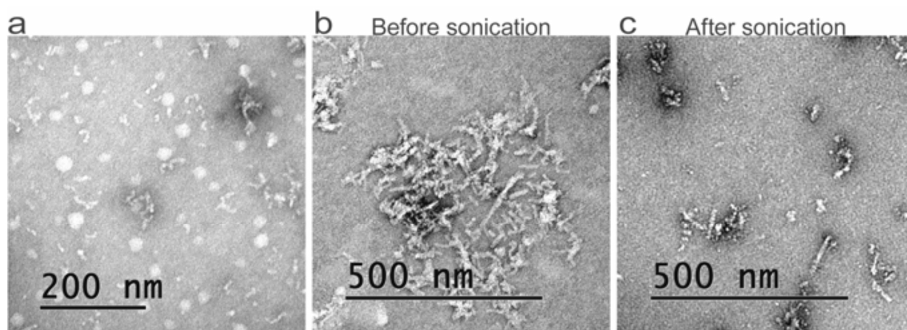

Figure 13. TEM images of Aβ protofibrils (a), Aβ fibrils before and after sonication (b and c, respectively).

Isolation of extracellular vesicles and lipid droplets

To be able to study the content of extracellular vesicles released by astrocytes in more detail, we isolated the EVs from conditioned media through different centrifugation steps. In the first step, we removed cell debris and dead cells, and in the next step we used ultracentrifugation to isolate the EVs (Figure 14a), including both larger microvesicles and smaller exosomes. The EVs were then resuspended in medium or lysis buffer depending on the experimental setup.

In **Paper IV**, we isolated LDs from astrocytes, by detaching the astrocytes, centrifuging them and washing the cell pellet thoroughly with PBS. We then homogenized the astrocytes and used a sucrose gradient and high speed centrifugation to isolate the lipid droplets (Figure 14b).

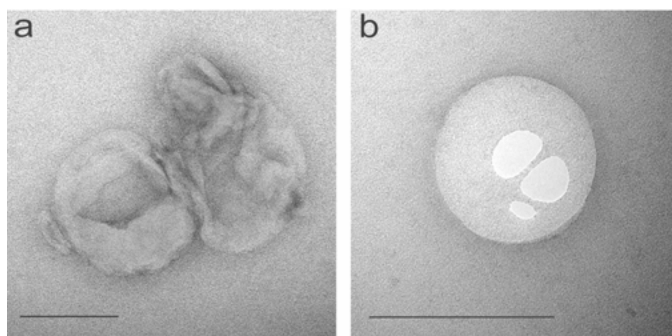

Figure 14. TEM images of isolated extracellular vesicles (a) and lipid droplets (b). Scale bar: 200 nm.

Western blot

Western blot (WB) is a technique commonly used to analyze protein levels in different samples. WB was used to detect proteins in lysed cell and EV samples. The proteins were first separated by gel electrophoresis, followed by protein transfer to polyvinylidene fluoride (PVDF) membranes. The membrane was then blocked and incubated with primary antibodies against the proteins of interest. Secondary antibodies linked to HRP were used to detect the primaries by chemiluminescence. In **Paper I**, we used WB to analyze if the levels of the synaptic protein synaptophysin were changed in the neurons under different pathological conditions. In **Paper II**, different mitochondrial and peroxisomal markers linked to energy metabolism were analyzed with WB. In **Paper III,** Aβ levels in cell lysates were measured with WB, and in **Paper IV**, we used WB to analyze the presence of the LD marker PLIN3 in EVs.

Techniques for energy metabolism analysis

In **Paper II** we focused on the effects of astrocytic Aβ pathology on energy metabolism and we therefore used different methods to analyze metabolic changes.

ATP assay is a colorimetric assay, which was used to study the ATP levels in the cells.

Seahorse assay was performed to get a more detailed view of the energy metabolism in real time. With this method it is possible to determine both oxygen consumption rate (OCR), which is the mitochondrial respiration or OXPHOS, and extracellular acidification rate (ECAR), which measures glycolysis. By using different inhibitors of these two energy pathways, Seahorse analysis can measure total ATP production and baseline respiration rates.

Mass spectrometry

Mass spectrometry (MS) is used to quantify and analyze e.g. proteins or lipids. While previously described techniques analyze specific target proteins, MS can be used to identify the overall content of a sample. Moreover, MS is useful for characterization of post-translational modifications and truncations of specific proteins.

In MS, the sample is first ionized with an ion beam, causing the sample to fragment. The ions are then sorted by their mass-to-charge ratio, creating a spectrum.

In **Paper III**, we performed liquid chromatography mass spectrometry (LC-MS) analysis on the Aβ deposits, isolated from astrocytes using magnetic beads to study what happens to the protein inside the astrocytes. We also analyzed conditioned media and EVs with LC-MS.

Time of flight Secondary ion mass spectrometry (ToF-SIMS) uses a primary ion beam to cause sputtering and emission of a secondary ion beam from cell surfaces. Depending on which primary ion beam that is used, different chemical species are detected[181]. In **Paper IV**, we used ToF-SIMS to analyze the presence of different lipids in astrocytes and investigate how they are affected by Aβ exposure.

Fatty acid profiling

In **Paper IV**, we performed fatty acid profiling by gas chromatography to investigate how different fatty acids in the astrocytes are affected by Aβ exposure. Total lipids were extracted from the cells and separated in four different fractions using solid-phase extraction[182], prior to analysis.

Data Analysis and Statistics

For WB, the intensity of the protein band of interest was analysed using ImageJ. The signal was normalized to housekeeping proteins in **Paper I** and **II**, and to total protein in **Paper IV**.

For ICC, different ImageJ macros were used to analyse: Aβ accumulation, and mitochondrial numbers and length in **Paper II**, expression of the lysosomal marker LAMP, and the overlap between Aβ and LAMP in **Paper III**, and the number, area and average size of LDs in **Paper IV**.

For statistical analysis, we first performed normality tests, followed by t-test or one-way ANOVA with multiple comparison test, or the non-parametric counterparts. In **Paper II**, Wilcoxon rank test was performed to analyse mitochondrial motility.

For the big data set created by SIMS analysis in **Paper IV**, principal component analysis (PCA) was used.

Results and discussion

Aβ exposed astrocytes secrete neurotoxic extracellular vesicles.

We have previously shown that astrocytes engulf Aβ, but are unable to degrade it, leading to the release of N-truncated Aβ in EVs[183]. In **Paper I**, we aimed to investigate how the spread of partially degraded Aβ in EVs affects neurons. For this purpose, murine neural co-cultures, consisting mainly of astrocytes, were exposed to Aβ protofibrils for 24 h after which they were cultured in Aβ free medium for another 12 days. Extracellular vesicles were then isolated from the conditioned medium. Pure neuronal cultures were exposed to normal medium, Aβ protofibrils, or EVs from either control or Aβ exposed co-cultures. The effect of the different treatments were analysed after 2 or 4 days. Quantification of the relative number of cells positive for the apoptotic marker TUNEL, revealed a significant increase in apoptotic neurons in cultures exposed to Aβ protofibril EVs, compared to the other conditions. Apoptosis was induced already 2 days after exposure, pointing to a faster neurotoxicity in Aβ protofibril EVs compared to Aβ protofibrils alone (Figure 15a-b). Possible reasons for this could be that the N-truncations or other modifications caused by the astrocytes make the Aβ more toxic. Another explanation could be that the neuronal uptake of EVs is faster and more effective, compared to the uptake of free-floating Aβ protofibrils. Using ICC and TEM, we studied different processes that could give insight about neuronal health after the addition of EVs. First, we analysed the mitochondria by transfecting the neurons with CellLight Mitochondria-GFP and quantifying the number of cells with healthy respective unhealthy mitochondrial networks (Figure 15c). There was a significant decrease in healthy mitochondrial networks in the neuronal cultures exposed to Aβ protofibril EVs compared to those that were exposed to control EVs. Analysis with TEM revealed similar results, demonstrating that mitochondria in Aβ protofibril EV exposed neurons showed signs of dysfunctional fission and fusion.

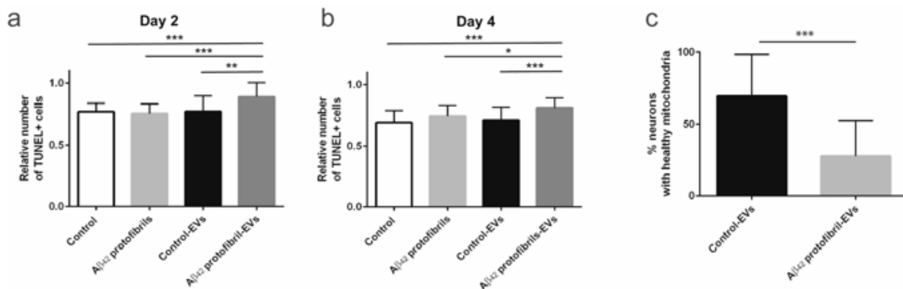

Figure 15. Neuronal cell death increased after addition of EVs from Aβ exposed co-cultures at both 2 day and 4 days after exposure (a-b). A decrease in number of neurons with healthy mitochondrial networks was observed in neurons exposed to Aβ protofibril EVs compared to control EVs (c).

Next, we sought to study if there was any synaptic changes in the EV exposed neurons. Hence, we used the synaptic vesicle marker synaptophysin. Indeed, we found that the synaptic organization was impaired in neurons exposed to Aβ protofibril EVs, compared to control EVs (Figure 16a). TEM was performed to study the morphology changes further. The soma of neurons exposed to Aβ protofibril EVs had an increased number of vacuoles, compared to control EVs (Figure 16b).

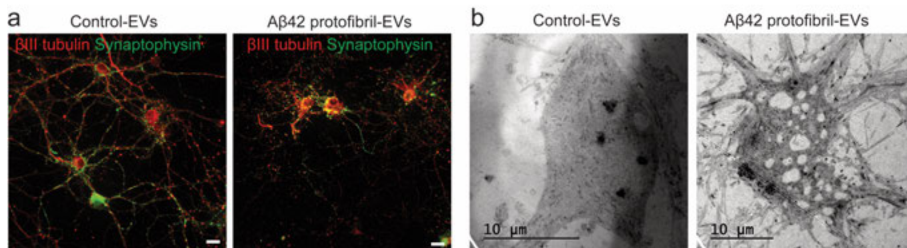

Figure 16. Synaptic organization was studied by ICC. Neurons displayed healthy and elongated synaptic networks after addition of control EVs, while they were clearly disrupted after addition of Aβ protofibril EVs (a). Morphology studies with TEM showed that neurons exposed to control EVs had a compact and healthy soma, while the soma of Aβ protofibril EV exposed neurons was filled with vacuoles (b). Scale bar: (a) = 20 µm.

TEM analysis also demonstrated the presence of multilamellar bodies (MLB) in neurons after addition of control EVs or Aβ protofibril EVs (Figure 17a-b). Multilamellar bodies are organelles found in a variety of cells under both normal and pathological conditions. They are important for lipid storage and secretion[184]. Multilamellar bodies accumulations are commonly found in lysosomal storage disorders and contain cholesterol, sphingolipids and apolipoproteins[184–186]. To further study the presence of MLBs, we performed ICC using the cholesterol binding dye filipin III and the lysosomal marker LAMP1. We could then identify LAMP1 positive cholesterol deposits (Figure 17c).

Taken together our results show that astrocytes spread neurotoxic EVs, which affect neurons to a great extent.

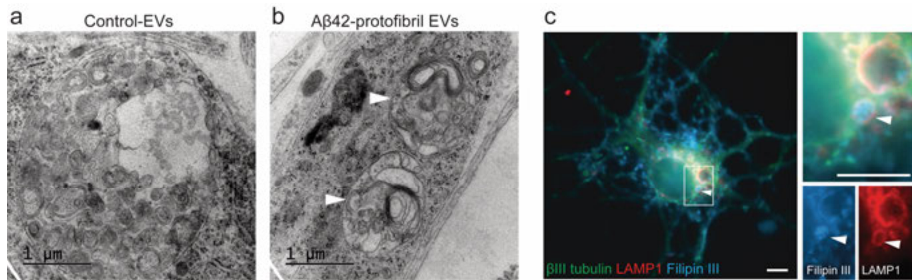

Figure 17. Multilamellar bodies observed with TEM in neurons exposed to control EVs (a) or Aβ protofibril EVs (b). Stainings for cholesterol (blue) and lysosomal marker (red) showed the presence of cholesterol deposits in LAMP1 positive vesicles (c). Scale bar: (c) = 5 µm.

Aβ accumulating astrocytes present disrupted energy metabolism.

Neurons have a very high energy turnover and rely on astrocytes for energy metabolites[187]. Hence, changes in astrocytic energy metabolism would have detrimental effects on neuronal function and signaling. In **Paper II**, we sought to investigate if Aβ exposure would affect the different energy pathways in astrocytes. For this purpose, human iPSC-derived astrocytes were exposed to sonicated Aβ fibrils for 7 days after which they were further cultured for 0, 6 or 12 days in Aβ free medium. First, we studied the morphology of the mitochondria with ICC and TEM. Quantifications of mitochondria showed a decrease in their numbers at 7d+6d, followed by an increase at 7d+12d (Figure 18a). TEM confirmed that while mitochondria in control astrocytes showed healthy morphology, mitochondria in Aβ exposed astrocytes were very long and unusually branched at 7d+6d (Figure 18b). In contrast, abnormally short mitochondria were observed in Aβ exposed astrocytes at 7d+12d (Figure 18c). These results indicate that Aβ pathology in astrocytes affects fission/fusion homeostasis of the mitochondria.

WB analysis of dynamin related protein 1 (DRP-1) and its phosphorylated forms confirmed a disruption in mitochondrial fission and fusion mechanisms.

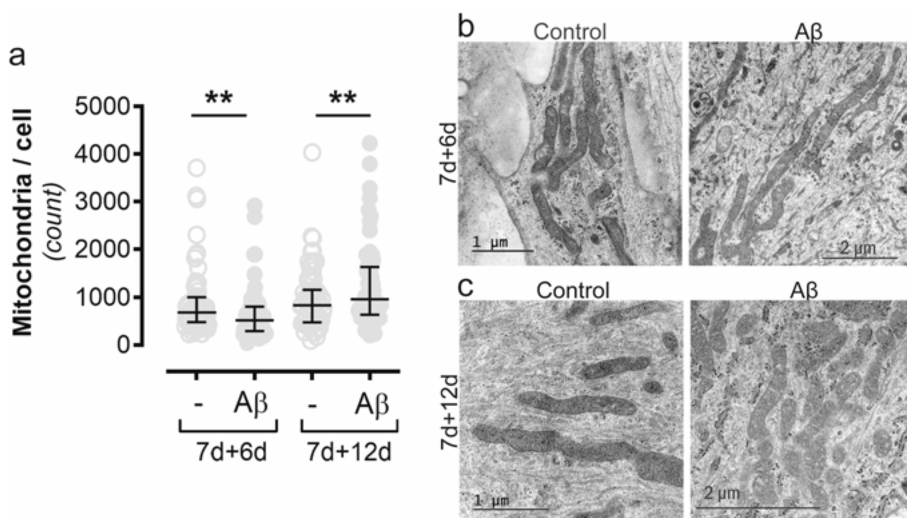

Figure 18. Labelled mitochondria were visualized with ICC and quantified. The results showed a decrease in the number of mitochondria in Aβ exposed astrocytes at 7d+6d, while there was an increase at 7d+12d (a). TEM analysis demonstrated that control astrocytes had healthy mitochondria, while mitochondria in Aβ exposed astrocytes were elongated and branched at 7d+6d (b). At 7d+12d the mitochondria in the control astrocytes still had healthy morphology, while the mitochondria in Aβ accumulating astrocytes were very short (c).

As the mitochondria appeared to be severely affected by Aβ pathology, it was of great interest to study mitochondrial functionality. Hence, we performed Seahorse assay to assess if it was altered. Measuring OCR showed no significant changes in OXPHOS at 7d and 7d+6d. However, Aβ exposed astrocytes at 7d+12d had significantly decreased maximal mitochondrial respiration and spare respiratory capacity (SRC), compared to control astrocytes (Figure 19a-b), suggesting impaired ability to increase OXPHOS to meet the energy needs. However, ATP levels were stable throughout the experiment (Figure 19c), implying that ATP is also generated with a different energy pathway in Aβ exposed cells. We therefore measured glycolysis by Seahorse ECAR analysis. Glycolysis and glycolytic capacity were both increased in Aβ accumulating astrocytes at 7d and 7d+6d, whilst unchanged at 7d+12d (Figure 19d-e), suggesting that glycolysis compensated the ATP production in the early time points. As glycolysis did not explain how ATP levels were unchanged at 7d+12d in Aβ exposed astrocytes, we studied another energy pathway: fatty acid β oxidation.

When the cultures were exposed to the LD inhibitor Triacsin C, ATP levels were decreased in Aβ accumulating astrocytes (Figure 19f), suggesting that LDs play a central role in their energy production. Interestingly, when we inhibited the shuttling of fatty acids inside the mitochondria, there were no changes in ATP, indicating that the ATP was produced somewhere else as

well. Another organelle involved in fatty acid β oxidation is the peroxisome. Therefore, we next analyzed two different peroxisomal markers: PMP70, situated on the membrane, and catalase, an enzyme responsible for the breakdown of hydrogen peroxidase. The levels of both markers increased in astrocytes already 7d after Aβ exposure, suggesting a switch to fatty acid β oxidation for energy production and an involvement of peroxisomes in energy production in Aβ accumulating astrocytes.

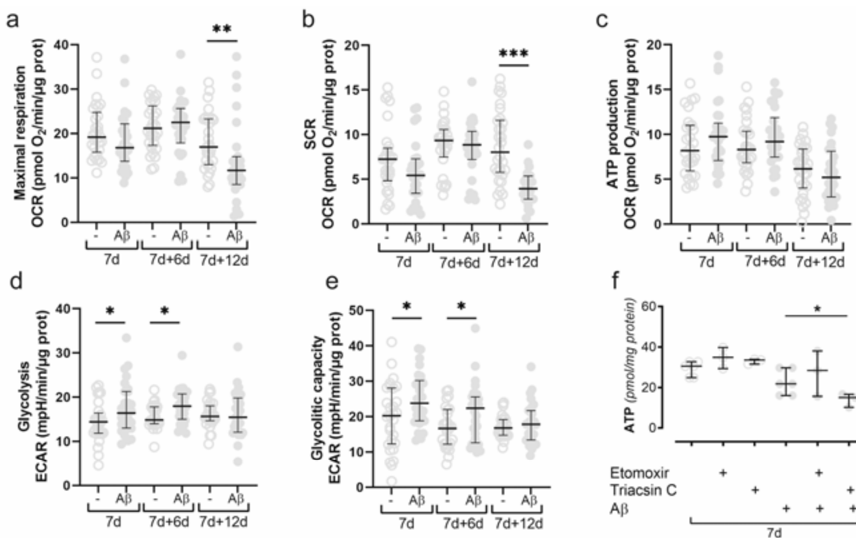

Figure 19. Seahorse OCR analysis showed a significant decrease in maximal mitochondrial respiration and SRC in Aβ exposed astrocytes at 7d+12d (a-b). ATP levels were stable in both groups throughout the experiment (c). Seahorse ECAR analysis demonstrated that glycolysis and glycolytic capacity were both increased in Aβ accumulating astrocytes at 7d and 7d+6d (d-e). Inhibitor experiments showed a decrease in ATP levels when astrocytes were exposed to Aβ and the LD inhibitor Triacsin C (f).

Astrocytes modify Aβ to create highly resistant proteoforms.

Astrocytes are known to turn more phagocytic when they become reactive[188]. It has previously been shown that astrocytes engulf Aβ[51,60,62,63] but are unable to fully degrade it, resulting in the release of N-truncated Aβ[183]. In **Paper III**, we aimed to investigate what happens to the Aβ that is accumulated inside astrocytes. For this purpose, iPSC-derived astrocytes were incubated with sonicated Aβ fibrils and magnetic beads for 4 days, after which they were cultured in Aβ and beads free medium for 0, 7 or 14 days. The magnetic beads were then used to isolate Aβ inclusions from the cell lysates, followed by treatment with formic acid to detach Aβ from the beads (Figure 20).

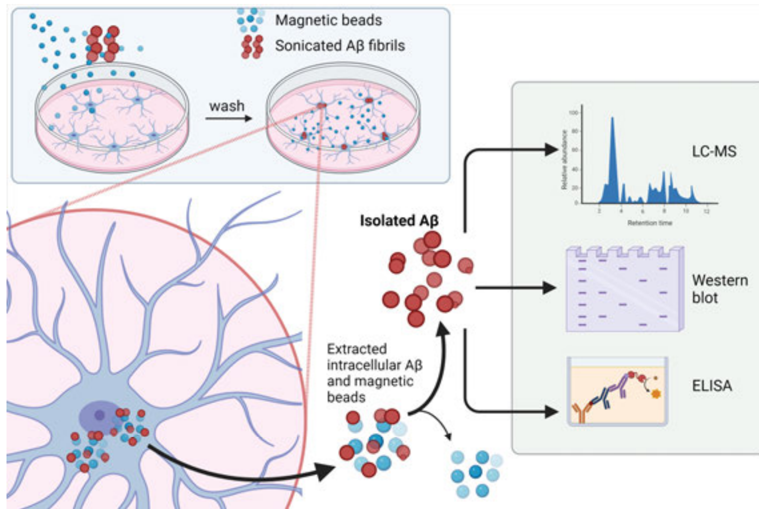

Figure 20. Graphical representation of experimental setup. Astrocytes were exposed to sonicated Aβ fibrils and magnetic beads. The intracellular deposits were isolated and analysed. Created with BioRender.com

Importantly, the astrocytes engulfed Aβ and magnetic beads simultaneously and stored them together, which was crucial for successful isolation. Over time the Aβ and beads were packed together and co-localized with LAMP1 positive lysosomes (Figure 21a). ELISA analysis of isolated inclusions showed that there was an increased N-truncation of Aβ over time. LC-MS confirmed that the intracellular Aβ was N-truncated at different positions, and this was also observed in EVs (Figure 21b). Interestingly, LC-MS of conditioned medium contained Aβ truncated at the C-terminus (Figure 21b). Taken together these results suggest that astrocytes truncate Aβ intracellularly and store N-truncated Aβ. Furthermore, our data indicates that there are different secretion pathways depending on the type of truncated Aβ released.

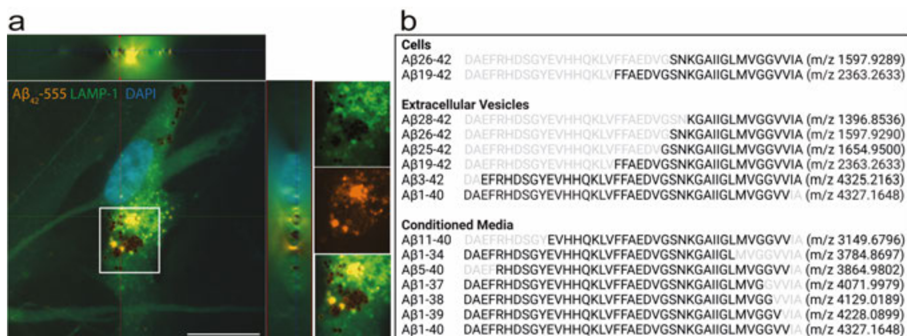

Figure 21. Magnetic beads and Aβ clearly co-localized with the lysosomal marker LAMP1 (a). LC-MS analysis showed different patterns of Aβ truncation in intracellular inclusions, conditioned media, and EVs (b). Scale bar: 20 µm.

Western blot analysis, using Aβ specific antibodies demonstrated the presence of large proteoforms at 55 kDa in the astrocytic inclusions. These proteoforms were resistant to harsh denaturing steps, such as formic acid and SDS, and increased over time in culture (Figure 22a). TEM analysis of sonicated Aβ isolates confirmed the presence of large protein structures in the astrocytic inclusions that were more resistant to sonication than the original Aβ fibrils (Figure 22b-c). In conclusion, we showed that astrocytes truncate Aβ in different ways and create very stable proteoforms.

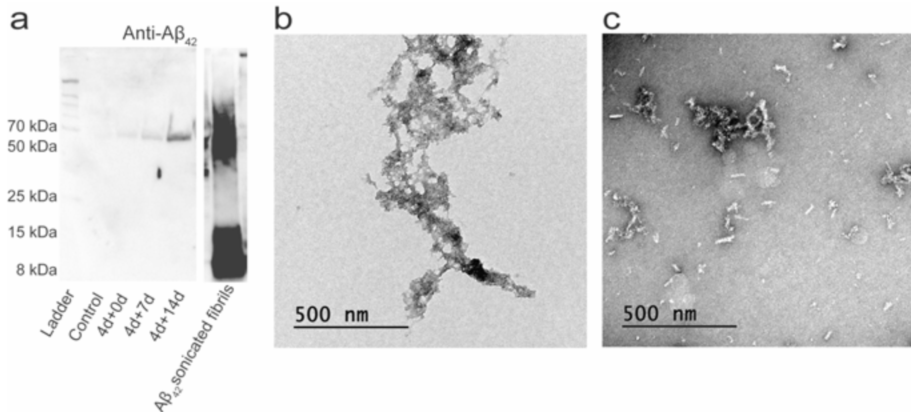

Figure 22. WB analysis showed the presence of large proteoforms resistant to denaturing steps (a). Large fibrillary structures were observed with TEM analysis of sonicated Aβ isolates (b) that were very different from the sonicated Aβ fibrils that were added to the astrocytes in the first place (c).

Aβ accumulation alters LD metabolism in astrocytes.

One of the many processes regulated and influenced by astrocytes is lipid metabolism. The brain is a very lipid dense organ and astrocytes have a crucial role in the maintenance of the brain's lipid homeostasis[143,189]. In **Paper IV**, we sought to study how exposure to Aβ soluble aggregates influences lipid metabolism in astrocytes. For this purpose, iPSC-derived astrocytes were exposed to Aβ soluble aggregates for 7 days after which the cultures were washed and cultured for additional 0 or 6 days. Our first goal was to identify a reliable lipid droplet marker that we could use in this study. It has previously been shown that LDs in astrocytes are coated with PLIN3[157]. We replicated these results in both brain tissue samples and *in vitro*, and confirmed that PLIN3 clearly coated astrocytic LDs (Figure 23a). PLIN3 quantification in iPSC-derived astrocytes showed a significant increase in the number of LDs in Aβ exposed cultures at 7d+12d compared to control cultures (Figure 23b), suggesting that Aβ disrupts the LD metabolism. To further investigate the effects of Aβ pathology on the overall lipid metabolism we performed ToF-

SIMS, which demonstrated no differences between groups. However, fatty acid profiling showed that Aβ exposure altered the expression of several specific fatty acids involved in cytokine production, neuroprotective functions, and inflammation. Interestingly, these differences were mainly found in the cholesterol ester (CE) fraction and the triacylglycerol (TG) fractions, which are the main components of LDs.

We have previously shown that astrocytes transfer different organelles and toxic protein aggregates through TNTs[55,113–115]. Moreover, astrocytes are very secretory cells[183,190,191]. However, it remains unclear if LDs are transferred between astrocytes via TNTs or by secretion. In this study, we show that LDs are indeed present in TNTs connecting neighboring astrocytes (Figure 23c). Moreover, WB analysis confirmed the presence of the PLIN3 in EVs, suggesting another route of LD transfer (Figure 23d). Taken together our data indicates that Aβ significantly disrupts LD metabolism and that astrocytes are able to transfer LDs via different cell-to-cell communication pathways.

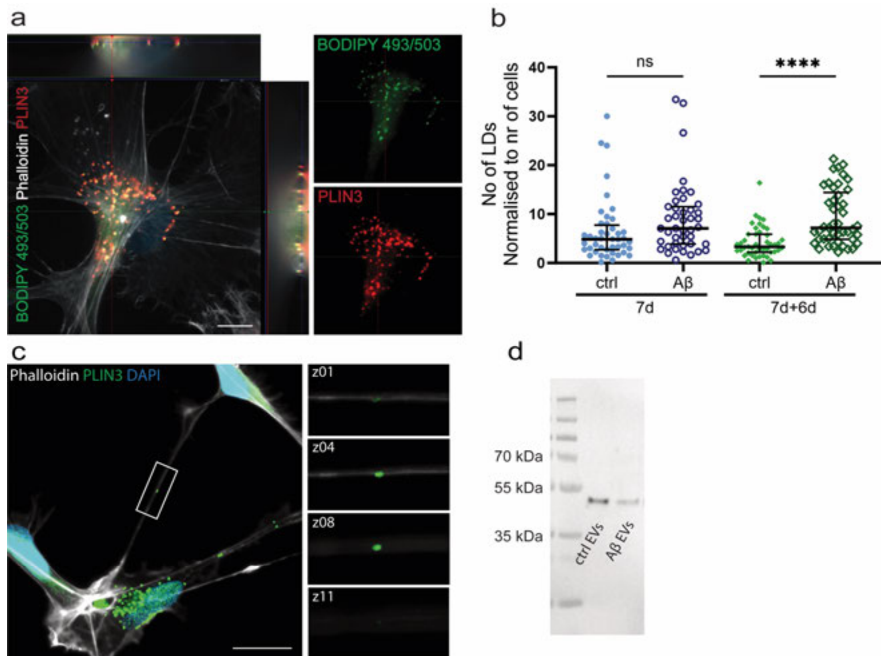

Figure 23. ICC of PLIN3 and BODIPY 493/503 showed a clear co-localization of the two markers in astrocytes (a). Quantification of the PLIN3 signal revealed an increased number of LDs at 7d+6d after Aβ exposure (b). LDs were observed to be situated in TNTs between astrocytes (c). WB analysis confirmed the presence of PLIN3 in EVs (d). Scale bar: 20 µm.

Astrocytic lipid droplets: a link between lipid metabolism and neuroinflammation.

Previous studies have shown that astrocytes are antigen presenting cells in the brain[56]. In peripheral immune cells, LDs have long been used as a marker for ongoing inflammation[165], and have also been shown to affect cross-presentation in dendritic cells and T cell stimulation[163–165]. In **Paper IV**, we sought to investigate if there is a link between antigen presentation and LDs in astrocytes. MHCII stainings revealed two different patterns in iPSC-derived astrocytes: either the astrocytes were completely MHCII positive and found in close proximity to lipid laden cells (Figure 24a), or the MHCII was found inside the LDs (Figure 24b). This finding was very intriguing as MHCII positive LDs have not been described previously in the literature. We next sought to investigate if MHCII positive LDs were located within the lysosomal degradation system. However, there was no co-localization with lysosomal or endosomal markers. This data suggests that the MHCII positive LDs are not within the traditional antigen presentation pathway, but may instead be packed together for different reason.

Moreover, IHC of cortical brain section of AD patients confirmed the presence of astrocytes positive for both PLIN3 and MHCII (Figure 24c). We next sought to investigate if astrocytes and CD4 positive T cells interact in the brain. Indeed, T cells were found to be in close contact with MHCII positive astrocytes. When we stained for PLIN3, we observed that T cells interacting with astrocytes had LD accumulation (Figure 24d), suggesting a lipid interplay with astrocytes. In conclusion, our data suggests that LDs play a role in antigen presentation in astrocytes.

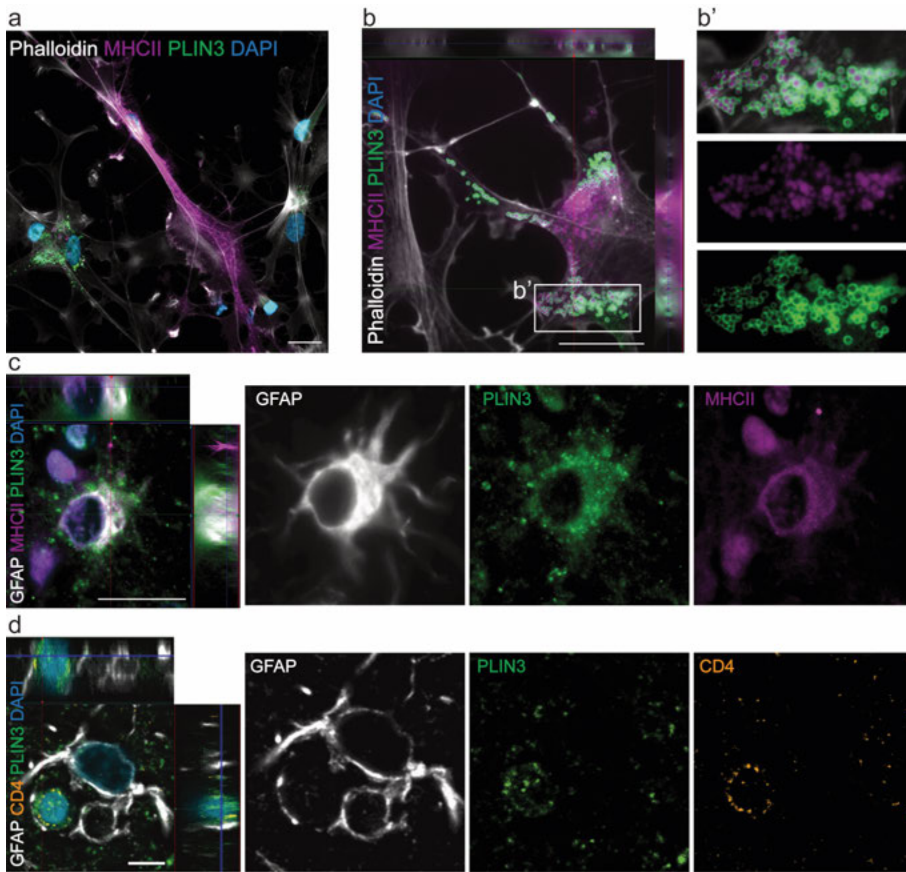

Figure 24. ICC showed two different MHCII patterns in iPSC-derived astrocytes: astrocytes that were MHCII positive (a) and astrocytes that clearly contained MHCII within LDs (b). IHC of cortical brain sections of AD patients confirmed the presence of LDs in MHCII positive astrocytes (c) and that T cells are in close contact with astrocytes (d). Scale bar (a-c): 20 µm; (d): 5 µm.

Future perspectives

Throughout this thesis, we have described several detrimental effects of Aβ pathology in astrocytes, indicating how they may be involved in disease propagation. However, as it often happens, when you answer one question, many more arise. In this section, I will discuss possible follow up investigations that will further increase our understanding of astrocytes' involvement in AD.

Aβ accumulation in astrocytes leads to severe cellular stress, affecting their lipid metabolism, their ability to support neurons and their release of neurotoxic EVs[65,115,136,183,190,191]. Hence, there is a great need to understand why astrocytes do not effectively degrade ingested Aβ aggregates and how they modify the stored Aβ. We have shown that astrocytes form large, very compact Aβ inclusions after being exposed to sonicated Aβ fibrils. Interestingly, the intracellular Aβ and the secreted Aβ contained different truncation patterns, suggesting that there are preferred secretion pathways depending on the Aβ proteoforms. In future experiments, it would be compelling to study the toxicity of specific Aβ species by exposing neurons to different astrocytic Aβ isolates, and compare the cellular effects. It would also be interesting to investigate if the various astrocyte modified Aβ species have different seeding capacities by using an *in vitro* seeding assay.

Lipid droplets are often seen as markers for cellular stress. Moreover, astrocytes are known to be effective at storing fatty acids in LDs to reduce the risk of lipotoxicity in neurons[19]. We have demonstrated that Aβ exposure increases the number of LDs in astrocytes, but if this is a pathological or a rescuing effect is still unclear. By inhibiting LD formation or promoting accumulation, it would be possible to assess if LDs have positive or negative effects on cellular stress in Aβ exposed astrocytes or surrounding neurons. Lipidomics and proteomics analysis of isolated LDs would also provide important insight on how Aβ exposure affects their content.

The link between LDs and antigen presentation would be very interesting to study further. We have demonstrated that MHCII has two different patterns in human astrocytes: either the astrocytes express MHCII throughout the cell, in different cellular compartments and membranes, or it is expressed in high concentrations within LDs. This finding was very intriguing, as MHCII in LDs has not been reported previously. LDs have been suggested to be involved in MHCI mediated cross-presentation[163,164], but the mechanisms behind it remain

unclear. The role of MHCII positive LDs remain to be identified, but hypothetically it could constitute a mechanism of MHCII shuttling between APCs or a way to pack MHCII with fatty acids for T cells to use as an energy source during reprogramming (Figure 25). An *in vitro* model containing both astrocytes and T cells would make it possible for us to study LD involvement in antigen presentation and T cell activation. For example, a microfluidity chamber could be used to study the involvement of astrocytic LDs in T cell recruitment, when oleic acid or an LD inhibitor is added to alter the cellular LD load. This models would clarify if T cell migrate to a higher extent towards astrocytes that have a high or low lipid load. Furthermore, super resolution microscopy could be used to study interactions between LD markers and inflammation markers in detail, both in cell cultures and in human brain tissue.

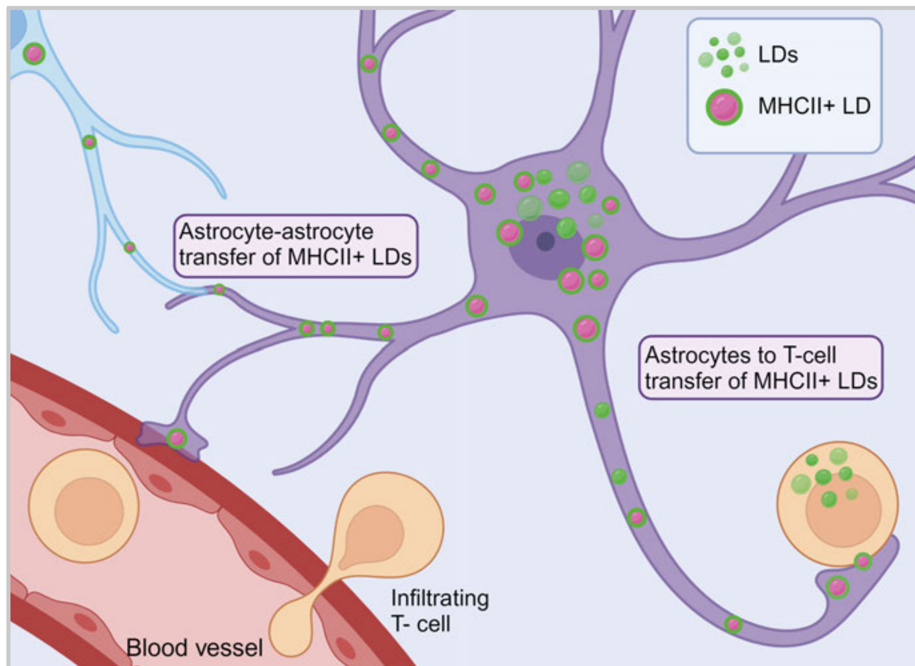

Figure 25. MHCII positive LDs' role in antigen presentation is still unclear. They may act as shuttling vehicles for cell-to-cell transfer between antigen presenting cells, or be involved in antigen presentation, by supplying T cells with fatty acid for energy production. Created with BioRender.com

Riassunto di divulgazione scientifica

Il morbo di Alzheimer é la forma piú comune di demenza senile, ed è caratterizzato da decadimento cognitivo e perdita graduale della memoria. Decenni di ricerca non hanno ancora chiarito cosa causi questa malattia, ma hanno trovato che i tratti distintivi sono le placche di β amiloide, i grovigli di tau e l'infiammazione cronica. La β amiloide è una proteina che, in condizioni patologiche, forma strutture sempre piú grandi fino a diventare placche. Normalmente, la proteina tau è essenziale per il funzionamento dei neuroni. Tau, peró, nel morbo di Alzheimer, viene modificata e perció si assembla nei grovigli di tau. Questi cambiamenti patologici causano gravi danni alle cellule del cervello. L'ipotesi piú comune sul come inizi la patologia é che l'aggregazione di β amiloide sia la causa principale di tossicitá nel morbo di Alzheimer, e che poi questo induca di conseguenza la formazione dei grovigli di tau. Nuovi studi hanno però dimostrato che le due proteine hanno meccanismi patologici sinergici.

Il cervello è composto da tanti tipi di cellule, tra i quali i neuroni e gli astrociti. I neuroni sono cellule molto dinamiche che attraverso complesse reti di comunicazione creano i nostri pensieri, memorie, e che ci permettono di imparare cose nuove. Peró sono anche cellule molto sensibili a cambiamenti nel cervello, infatti sono le cellule che muoiono in maggior grado nel morbo di Alzheimer.

Gli astrociti sono importanti per il mantenimento della salute del cervello. Recenti studi hanno dimostrato che gli astrociti aiutano i neuroni a produrre energia, e a formare le reti neuronali. In condizioni patologiche queste funzioni vengono, però, alterate e gli astrociti diventano infiammatori e fagocitici ("mangiando" il materiale intorno a loro). Nel morbo di Alzheimer é stato provato che gli astrociti fagocitano β amiloide ma non riescono a degradarla. L'obiettivo di questa tesi è di elucidare il ruolo degli astrociti nella patologia di β amiloide e nel morbo di Alzheimer.

Il nostro gruppo ha precedentemente dimostrato che gli astrociti, dopo aver internalizzato β amiloide, rilasciano la proteina parzialmente degradata in vescicole extracellulari. Le vescicole extracellulari sono un modo che viene usato dalle cellule per comunicare tra di loro, anche a grandi distanze. Nel **primo articolo**, abbiamo dimostrato che l'aggiunta di vescicole extracellulari isolate da astrociti esposti a β amiloide, "uccideva" piú neuroni e causava seri danni alla loro morfologia e ai loro compartimenti intracellulari. Per esempio,

i mitocondri, che sono essenziali per le cellule per la produzione di energia, erano gravemente danneggiati dall'aggiunta di vescicole extracellulari da astrociti esposti a β amiloide. Inoltre, queste vescicole extracellulari disturbavano la capacità dei neuroni di formare reti di comunicazione. Questi cambiamenti dannosi sono stati osservati solo quando i neuroni erano stati esposti a vescicole extracellulari isolate da astrociti esposti a β amiloide, e non quando erano stati esposti a vescicole extracellulari da colture "normali" o a β amiloide direttamente. Questo indica che gli astrociti hanno un ruolo nella diffusione della patologia del morbo di Alzheimer, e rilasciano vescicole tossiche quando sono stati esposti a β amiloide.

Il cervello è un organo che usa grandi quantitá di energia per il suo funzionamento ottimale. Gli astrociti sono importanti perché possono assorbire il glucosio direttamente dai vasi sanguigni, per poi usarlo per la produzione di energia, o lo conservano per quando ce ne sará bisogno. Nel **secondo articolo,** ci siamo concentrati sugli effetti che l'accumulazione di β amiloide ha sul metabolismo energetico degli astrociti. La β amiloide aveva grandi ripercussioni sui mitocondri, avendo cosí effetti negativi sulle loro capacitá di produzione di energia. Peró i livelli di energia complessivi erano stabili. Quindi abbiamo analizzato un'altra via di produzione energetica: la glicolisi, che usa il glucosio. I livelli di glicolisi erano aumentati negli astrociti esposti a β amiloide, indicando un tentativo di compensazione per la diminuita produzione energetica nei mitocondri. Ma col tempo erano diminuiti a livelli normali, il che suggerisce che gli astrociti esposti a β amiloide non riuscivano a incrementare la glicolisi per lungo tempo. Un altro percorso di produzione di energia cellulare è l'ossidazione degli acidi grassi, che di solito si trovano nelle gocce lipidiche. Infatti, abbiamo dimostrato che gli astrociti usano l'ossidazione di acidi grassi e le gocce lipidiche per la produzione energetica quando sono stati esposti a β amiloide.

Poiché é stato osservato che gli astrociti fagocitano β amiloide, ma che non riescono a degradarla, nel **terzo articolo** abbiamo voluto vedere come gli astrociti modificano la β amiloide intracellulare. Usando perle magnetiche abbiamo potuto isolare la β amiloide intracellulare e ci è stato possibile analizzarla. Gli astrociti troncano β amiloide e la conservano internamente nei compartimenti di degradazione, creando forme di β amiloide molto stabili.

Il cervello è un organo principalmente composto da lipidi, ed é noto che gli astrociti hanno un ruolo centrale nella produzione e conservazione di lipidi. Nel **quarto articolo**, abbiamo visto un aumento significativo nel numero di gocce lipidiche negli astrociti esposti a β amiloide, e che c'era un trasporto attivo di gocce lipidiche sia via contatto diretto che via rilascio delle cellule nel medium. Questi risultati suggeriscono che la β amiloide influenzi la conservazione di lipidi e causi un'accumulazione di gocce lipidiche negli astrociti. Oltretutto, abbiamo anche visto una connessione tra gocce lipidiche e infiammazione. Infatti gli astrociti sono capaci di interagire e attivare cellule del sistema immunitario attraverso una proteina chiamata MHCII. In questo

articolo, abbiamo osservato che alcuni astrociti contenevano MHCII dentro le gocce lipidiche. Questi risultati indicano che le gocce lipidiche negli astrociti possono avere un ruolo attivo nelle funzioni infiammatorie.

In conclusione, i risultati in questa tesi dimostrano che gli astrociti fagocitano e modificano β amiloide, inducendo cosí il rilascio di vescicole molto tossiche per i neuroni. Questo indica che gli astrociti hanno un ruolo nella diffusione della patologia. In piú, abbiamo mostrato che la β amiloide causa grandi danni e alterazioni sia nella produzione di energia che nel metabolismo dei lipidi negli astrociti. Poiché gli astrociti sono di centrale importanza per entrambi i meccanismi nel cervello, questi cambiamenti avrebbero degli effetti deleteri sulle cellule che si trovano intorno, per esempio lasciando i neuroni ancora piú vulnerabili.

Popular science summary

Alzheimer's disease (AD) is the most common cause of dementia among the elderly. The disease is characterized by cognitive impairment and gradual memory loss. However, after decades of research the causes still remain unclear. The common pathological hallmarks, observed in the AD brain, are amyloid β plaques, tau tangles and chronic inflammation. Amyloid β is a protein that, under pathological conditions, starts to aggregate into bigger and bigger structures until it forms plaques. The protein tau is normally important for neuronal health. However, in AD, tau becomes modified and assembles, forming tau tangles. These changes cause severe cellular damage in the brain. The most common hypothesis of how the pathology begins states that the main toxic culprit in AD is amyloid β aggregation. It is believed that amyloid β pathology then leads to tau tangles and consequently to neuronal loss. However, recent studies have shown that both proteins have synergistic pathological mechanisms.

The brain contains many different cell types: neurons and astrocytes are the most common. Neurons are very dynamic cells that create our thoughts, memories, and let us learn new things through complex communication networks. However, they are also very sensitive to changes in the brain; they are, in fact, the cells that die to the highest degree in AD.

Astrocytes are important for the maintenance of the brain's health. Recent studies have shown that astrocytes help neurons with energy production and the formation of neuronal networks. In pathological conditions, these functions are altered and the astrocytes become inflammatory and phagocytic ("eating up" material around them). The aim of this thesis was to elucidate astrocytes' role in AD, focusing on amyloid β pathology.

Our group has previously demonstrated that astrocytes engulf amyloid β but are not able to degrade it efficiently. Instead, the astrocytes release partially degraded amyloid β in extracellular vesicles. Extracellular vesicles are used by cells to communicate with each other, even over long distances. In **Paper I**, we demonstrated that extracellular vesicles from astrocytes exposed to amyloid β cause severe damage to neurons and eventually kill them. For example, mitochondria, which are essential for the cells' energy production, were severely affected. Moreover, amyloid β extracellular vesicles disrupted the neurons' capacity to form neuronal networks. These detrimental changes were only seen when the neurons were exposed to extracellular vesicles from

amyloid β accumulating astrocytes, not when the neurons were exposed to extracellular vesicles from control cultures or amyloid β directly. This suggests that astrocytes have a role in the pathological spreading in AD and release neurotoxic extracellular vesicles after amyloid β exposure.

The brain is an organ that uses great quantities of energy for its optimal function. Astrocytes are important because they can take up glucose directly from the blood vessels, and utilize it for energy production or store it to be used when needed. In **Paper II**, we focused on the effects of amyloid β accumulation on astrocytic energy production. We found that amyloid β exposure had great repercussions on the mitochondria, affecting their ability to produce energy. Interestingly, the overall energy levels were still stable. We therefore analysed glycolysis, which is a different energy production pathway that uses glucose. The levels of glycolysis were increased in amyloid β exposed groups, indicating an attempt by the astrocytes to compensate for the diminished energy production in the mitochondria. However, overtime glycolysis levels were decreased to normal levels again, suggesting that amyloid β exposed astrocytes were not able to increase glycolysis for a long time in order to sustain stable energy levels. This drew our attention to another pathway of cellular energy production: the oxidation of fatty acids, usually found in lipid storage compartments, called lipid droplets. In fact, we demonstrated that astrocytes switch to oxidation of fatty acids and use lipid droplets for energy production when exposed to amyloid β.

It has been demonstrated that astrocytes engulf and store amyloid β and in **Paper III**, we sought to understand how the astrocytes might change the intracellular amyloid β. By using magnetic beads, we were able to isolate the amyloid β from the astrocytes and analyse it. From the analysis of intracellular amyloid β we could gather that astrocytes truncate and pack it in degradation compartments, creating very stable forms of the protein.

The brain is an organ mainly composed of lipids, and it is well known that astrocytes have a central role in the production and storage of lipids. In **Paper IV**, we detected a significant increase in the number of lipid droplets in astrocytes exposed to amyloid β. We also observed active transportation of lipid droplets between astrocytes both by direct contact and by release into the medium. These results suggest that amyloid β affects astrocytic fat storage and causes lipid droplets accumulation. Moreover, we demonstrated a connection between lipid droplets and inflammation. Astrocytes can interact and activate immune cells from the rest of the body through a protein called MHCII. We observed that some astrocytes contained MHCII within the lipid droplets. Taken together, these results indicate that lipid droplets in astrocytes have an active role in their inflammatory functions.

In conclusion, the results of this thesis demonstrate that astrocytes engulf and modify amyloid β, leading to the release of vesicles that are toxic for neurons. Hence, astrocytes seem to play a central role in disease spreading. Moreover, amyloid β pathology causes severe damage and changes to both energy

production and lipid metabolism in astrocytes. As astrocytes are central for both mechanisms in the healthy brain, these changes would have detrimental effects on the surrounding cells, leaving for example neurons even more vulnerable.

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It feels surreal to be writing this… I am finally *DONE*!!! I hope you enjoyed reading this thesis. It has been so fun and I feel so lucky to have been part for the MolGer family for so long! But now it's time to wrap it up and get to the really important things.

When I was looking for a lab to do my master thesis, I knew I wanted to do it in Uppsala, I wanted to study Alzheimer's disease, and if possible I wanted to work with glia cells. I couldn't find any groups doing research on Alzheimer's on then university website. Then, after a lot of frustration, I remembered that a few years prior I had a lecture on AD in a neurobiology course. Surely the lecturer must have worked with AD research. After a bit of detective work, I found him. That lecturer was **Dag**! So thank you **Dag,** for leading me to MolGer! You have been a great co-supervisor, helping out with protofibril production and the troubleshooting that it always entails. You have always provided great insights and helpful comments to my projects, and helped me take them a step further! I have always felt that I can come to you with any questions or problems!

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