

*Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Medicine 2022*

A stargazer's guide to neurodegeneration

*Astrocytes' role in the propagation of pathological
proteins*

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ACTA UNIVERSITATIS
UPSALIENSIS
2024

ISSN 1651-6206
ISBN 978-91-513-2045-8
urn:nbn:se:uu:diva-523852



UPPSALA
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Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbeckslaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Friday, 12 April 2024 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: PhD Maria Grazia Spillantini (Department of Clinical Neurosciences, University of Cambridge, Cambridge, United Kingdom).

Abstract

Mothes, T. 2024. A stargazer's guide to neurodegeneration. Astrocytes' role in the propagation of pathological proteins. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 2022. 76 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2045-8.

Alzheimer's disease (AD) and Parkinson's disease (PD) are characterized by brain accumulation of pathogenic protein aggregates. In the AD brain, amyloid- β (A β) and tau form plaques respective tangles, while in the PD brain α -synuclein (α -syn) form Lewy bodies and Lewy neurites. In addition, deposits of A β , tau and α -syn are frequently present in glial cells, including astrocytes. Historically, the focus was on neuronal dysfunction, leaving the involvement of glia largely understudied. The overall aim of this thesis was to investigate the role of glial cells in the disease progression, primarily focusing on astrocytes and the role they play in tau pathology.

Paper I focuses on the crosstalk between astrocytes and microglia in respects to degradation of α -syn and A β fibrils. Our results show that mono-cultured microglia are more effective than astrocytes at degrading exogenously added fibrils. However, when cultured together, microglia and astrocytes work synergistically, leading to an overall increase in the degradation.

In **Paper II**, we show that astrocytic tau inclusions are not benign, but in fact act as a reservoir for seeding competent tau species. The astrocytes engulf and process, but fail to fully degrade internalized material. Instead, seeding competent pathogenic tau spreads to nearby cells via secretion and tunneling nanotube mediated transfer. Furthermore, we show that tau and debris burdened astrocytes negatively affected the health of nearby neurons.

In **Paper III**, we investigated the cellular effects following astrocytic engulfment of human brain-derived tau. Our results show that astrocytes internalize and accumulate both AD and control tau fibrils. However, fibrils from AD brains were more neurotoxic and induced a stronger immune response in astrocytes, compared to fibrils derived from control brains.

In **Paper IV**, we studied the effects of *APOE*-genotype on astrocytic processing of tau by comparing astrocytes homozygous for *APOE* ϵ 2 and *APOE* ϵ 4. Our results showed that *APOE*2/2 astrocytes contained more and larger tau aggregates. Moreover, *APOE* 2/2 astrocytes excreted higher levels of pro-inflammatory cytokines, including IL-8, CCL2 and CXCL10 compared to *APOE* 4/4 astrocytes.

Paper V aimed to establish a cortical organoid model for studies of AD and PD. Exposure to α -syn especially led to internalisation by the organoid cells and active spreading throughout the tissue.

Our results demonstrate that astrocytes work closely with microglia to degrade internalised material. Furthermore, astrocytes actively contribute to neurodegeneration and disease propagation by affecting the health of neurons and by spreading seeding competent tau species.

Keywords: Alzheimer's disease, Parkinson's disease, tauopathies, astrocyte, microglia, neuron, tau, amyloid- β , α -synuclein, accumulation, degradation, inflammation, spreading, seeding.

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ISSN 1651-6206

ISBN 978-91-513-2045-8

URN urn:nbn:se:uu:diva-523852 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-523852>)

As excellent as our minds are, in the modern world we must know when
to discount them and turn our reasoning over to instruments

-Steven Pinker

Welcome dear stargazer to this guide to neurodegeneration! This work focuses on the glial cell components of neurodegenerative disease, and for us to fully appreciate the topic at hand there are several scientific principles we first need to discuss... Now don't *panic*! We shall do so now.

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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. Rostami J, **Mothes T**, Kolahdouzan M, Eriksson O, Moslem M, Bergström J, Ingelsson M, O’Callaghan P, Healy L, Falk A, Erlandsson A. Crosstalk between astrocytes and microglia results in increased degradation of α -synuclein and amyloid- β aggregates. *Journal of Neuroinflammation*, 2021; 18:124.
- II. **Mothes T**, Portal B, Konstantinidis E, Eltom K, Libard S, Streubel-Gallasch L, Ingelsson M, Rostami J, Lindskog M, Erlandsson A. Astrocytic uptake of neuronal corpses promotes cell-to-cell spreading of tau pathology. *Acta Neuropathologica Communications*, 2023; 11:97.
- III. Eltom K, **Mothes T**, Libard S, Ingelsson M, Erlandsson A. Astrocytic accumulation of tau fibrils derived from Alzheimer’s disease brains induces inflammation, cell-to-cell propagation and neuronal impairment. *Acta Neuropathologica Communications*, 2024; 12:34.
- IV. **Mothes T**, Konstantinidis E, Eltom K, Dakhel A, Rostami J, Erlandsson A. *APOE ϵ 2* drives tau processing and tau-mediated inflammation in human iPSC derived astrocytes. *Submitted for publication*.
- V. **Mothes T**, Dakhel A, Michno W, Erlandsson A. Spreading and morphology of α -synuclein, amyloid- β and tau deposits in cortical organoids. *Manuscript*.

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Additional Publications

- I. Konstantinidis E, Portal B, **Mothes T**, Beretta C, Lindskog M, Erlandsson A. Intracellular deposits of amyloid-beta influence the ability of human iPSC-derived astrocytes to support neuronal function. *Journal of Neuroinflammation*. 2023; 20(1):3.
- II. Behere A, **Mothes T**, Härskog E, Södergren M, Ingelsson M, Ekmark-Lewén S, Bergström J, Erlandsson A. Phosphorylation of α -synuclein triggers glial activation prior to intra-neuronal seeding in a mouse model of Parkinson's disease. *Under review*.

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Abbreviations

α -syn	α -synuclein
aa	Amino acid
ACM	Astrocyte conditioned medium
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
AQP4	Aquaporin 4
ATP	Adenosine triphosphate
A β	Amyloid- β
BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
CaMKII	Calmodulin dependent protein kinase II
CBD	Corticobasal degeneration
Cdk5	Cycline dependent kinase 5
CFP	Cyan fluorescent protein
CHI3L1	Chitinase-3-like protein 1
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
Cy3	Cyanine 3
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxynucleic acid
ECL	Electro-chemiluminescence
EGF2	Embryonic growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EV	Extracellular vesicle
FGF2	Fibroblast growth factor 2
FRET	Förster resonance energy transfer
FTD	Frontotemporal dementia
Fyn/Src	Proto-oncogene tyrosine-protein kinase
GFAP	Glial fibrillary acidic protein
Gpc-4/6	Glypican-4/6
Gsk3- α/β	Glycogen synthase kinase 3 α/β
H ₂ SO ₄	Sulphuric acid

HEK	Human embryonic kidney
HPC	Hematopoietic stem-cell
HRP	Horseradish peroxidase
iAPP	Islet amyloid polypeptide
Iba1	Ionized calcium-binding adaptor molecule 1
ICC	Immunocytochemistry
IDE-1	Definitive endoderm-1
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL-x	Interleukin-(1,2,3...)
INF- α/β	Interferon- α/β
IntDen	Integrated density
iPSC	Induced pluripotent stem-cell
LDH	Lactate dehydrogenase
MAO-B	Monoamine oxidase b
MAP	Microtubule associated protein
MAPK	Mitogen protein kinase
MBD	Microtubule binding domain
MCP-1	Monocyte chemoattractant protein 1
MCSF	Microphase colony-stimulating factor
mEPSC	Mini-excitatory postsynaptic current
MERGF10	Multiple epidermal growth factor-like domains protein 10
MERTK	Tyrosine-protein kinase Mer
MSA	Multiple system atrophy
MTBR	Microtubule binding repeat
NAC	Non-amyloid component
NFT	Neuro-fibrillary tangle
NMDA	N-methyl-D-aspartate
NPC	Neuronal progenitor cell
PD	Parkinson's disease
PHF	Paired helical filament
PiD	Pick's disease
PKA	Protein kinase A
PNS	Peripheral nervous system
PrP	Prion protein
PSP	Progressive supranuclear palsy
PTM	Post-translational modification
PVDF	Polyvinylidene
RNA	Ribonucleic acid
sEPSC	Spontaneous-excitatory postsynaptic current
SF	Straight filament
TDP-43	TAR DNA-binding protein 43
TEM	Transmission electron microscopy
TGF- β	Transforming growth factor β

TMB	3,3',5,5'-Tetramethylbenzidine
TNF- α	Tumour necrosis factor α
TNT	Tunnelling nano-tube
TREM2	Triggering receptor expressed on myeloid cells 2
TSP	Thrombospondin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UPS	Ubiquitin- Peroxisome system
VAMP2	Vesicle-associated membrane protein 2
WB	Western blot
WGA	Wheat germ agglutinin
YFP	Yellow fluorescent protein

Introduction

What are proteins?

To understand neurodegeneration, one must first grasp the core concepts regarding proteins. To start with the question stated above: A protein is, in principle, simply a chain of amino acids (aa) coupled together by peptide bonds (creating a polypeptide). The specific sequence of aa makes up a linear string and constitute the protein's primary structure. In reality, most proteins do not function as mere strings and need to be folded into an active state. The folding process often starts during translation by the ribosome and continues in specific cellular compartments like the endoplasmic reticulum (ER)¹, by aid of specialised peptides like chaperons². As a result, proteins gain their secondary and tertiary structures. Secondary structures include turns, α -helices and β -pleated sheets and refer to different local arrangements of the protein backbone. The tertiary structure, on the other hand, refers to the 3D-arrangement of the entire protein. To add yet another layer of complexity, proteins often form complexes with each other, in order to create functional units. This process is what makes our roughly 20.000 protein coding genes generate the cellular machines that make life possible. However, every now and then, mistakes are made, leading to misfolded proteins that cause dysfunction and disease.

Amyloid formation

Aggregation of misfolded proteins that escape the cellular quality-control mechanisms is common in the field of neurodegeneration. Amyloid is typically defined as a polypeptide that has formed unintended cross-linked β -sheet structures (**Fig. 1 a**)³. The amyloid then aggregates and forms insoluble fibrils that accumulate in the tissue over time, eventually leading to disease. Disorders caused by this mechanism are collectively referred to as amyloidosis and can occur in most tissues, including the brain. To date, more than 30 different amyloid proteins have been identified to cause various types of diseases⁴. To name a few, these include α -synuclein (α -syn), amyloid- β (A β), tau, TAR DNA-binding protein 43 (TDP-43), huntingtin, islet amyloid polypeptide (iAPP), transthyretin and prion protein (PrP). The process of amyloid formation is dependent on the intrinsic properties of the protein itself, and the combined effects of the cellular environment⁵. Theoretically, this could occur with any polypeptide given certain conditions⁶⁻⁸. Spontaneous misfolding of

protein monomers can for instance be a consequence of mutations that promote low energy state conformations. This means that the misfolded form of the protein is favoured simply due to the thermodynamics of the system⁹. Once a protein has become misfolded it can act as a nucleation point for naive monomers and initiate template driven polymerization (fibrillation) (**Fig. 1 b**)¹⁰. Amyloid is very stable and often resistant to normal cellular degradation pathways^{11,12}.

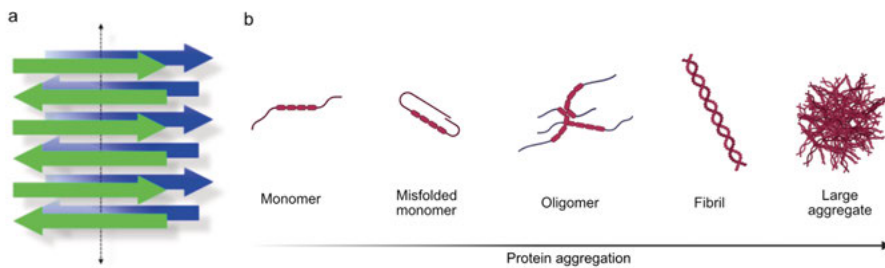


Figure 1. Schematic illustration of the aggregation stages of amyloid proteins. **(a)** Schematic of stacked anti-parallel β -sheet structures of amyloid¹³. **(b)** The native monomers misfolds and adopts a more energy conservative confirmation. The misfolded monomer can then act as a nucleation point and initiate fibrillation. This ultimately leads to larger protein species like oligomers, fibrils and eventually plaques or other characteristic deposits seen in amyloid disorders.

Neurodegeneration

Neurodegeneration refers to the biological process of progressive dysfunction and overall loss of neurons. Most neurodegenerative conditions are irreversible, in part because mature neurons do not regenerate on their own. Disorders include dementias, parkinsonian diseases and peripheral motor disorders and can be the cause of amyloid accumulation, head trauma or environmental toxins¹⁴. This pathological mechanism drives all neurodegenerative disorders of the peripheral and central nervous systems (PNS/CNS). However, which neurons that are affected and eventually die, and how this occur, depends on the disease. There are multiple subcategories of neurodegenerative disorders. Alzheimer's disease (AD) is the most common, affecting over 50 million people worldwide¹⁵. Tauopathies is a group that include roughly 20 separate diseases. Examples of these are progressive supranuclear palsy (PSP), frontotemporal dementia (FTD), Pick's disease (PiD) and corticobasal degeneration (CBD)¹⁶. Alpha-synucleinopathies is another category, including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA)¹⁷. These disorders are notoriously difficult to study for a couple of reasons. The brain is a highly complex organ consisting of many different cell types, working in concert during normal and pathological conditions. A lack

of models that accurately represent the brain in its entirety makes it a difficult organ to study. Furthermore, neurodegenerative diseases often develop over the span of decades. Studies in actual patients are therefore mostly limited to later stages of disease, when the cellular environment and potential mechanisms of early disease likely no longer apply. For these reasons, most currently available treatment options only work by limiting the symptoms and do not modify the disease directly.

Alzheimer's disease

In 1906, Alois Alzheimer described a peculiar neurological condition displayed by his patient Auguste Deter. She had troubles with memory, difficulties to express herself and displayed overall confusion in her daily life. *Post-mortem* analysis of her brain revealed the presence of what Alois would describe as senile plaques and fibrillary tangles. Today, AD is estimated to constitute 60-80% of the dementia cases and is defined by the three pathological hallmarks: extra-cellular A β plaques, intra-cellular neurofibrillary tangles (NFTs) consisting of hyper-phosphorylated tau (**Fig. 2 a**) and a state of chronic neuroinflammation¹⁸. Plaque pathology appears in patients about 10-30 years before onset of symptoms, and several years prior to signs of NFTs (**Fig. 2 b**). Historically, patients were diagnosed based on cognitive tests like the mini-mental state examination (MMSE). However, modern medicine have improved and includes more sophisticated diagnostics, like analysis of cerebrospinal fluid (CSF)/plasma biomarkers and amyloid positron emission tomography (PET)^{19,20}. There are two major types of AD: The sporadic variant, which give rise to late onset AD (LOAD) and the familial variant, resulting in early onset AD (EOAD). LOAD represents about 95% of all AD cases and is defined by a disease onset after age 65²¹. EOAD is instead caused by inherited mutant genes or chromosome abnormalities.

In the early 1990's, the amyloid cascade hypothesis was presented, suggesting A β to be the driving factor in AD²². In essence, the hypothesis states that A β precedes and instigates the aggregation of tau and induction of neuroinflammation. This reasoning was strengthened by the discovery of the EOAD causative mutations (all increasing A β levels or A β aggregation)²³. The amyloid cascade hypothesis has since then been central in AD research. However, there are still competing hypotheses as to how AD emerges and ultimately what would constitute a sound treatment strategy²⁴. Challengers to the hypothesis often state the fact that the A β plaque load does not correlate well with symptom severity. This argument became central after the 2021 FDA approval of Aducanumab (antibody targeting insoluble A β plaques), which managed to show efficacy in plaque removal but had conflicting results as to the degree of symptomatic relief²⁵. Current research suggests that most likely it is not the plaques themselves that drive pathology, but instead the more toxic soluble

species^{26–28}. The approval of Lecanumab (antibody targeting soluble A β protofibrils) in 2023 supports this notion as it, when compared with placebo, slowed the cognitive decline in AD patients after 18 months of treatment^{29,30}. These trials constitute the first direct evidence that removal of A β alleviates AD-related cognitive decline in humans. Nevertheless, other researchers have instead proposed that soluble tau species that precede NFT formation could be the instigator of A β aggregation that in turn initiates a feedback loop that accelerates the pathology³¹.

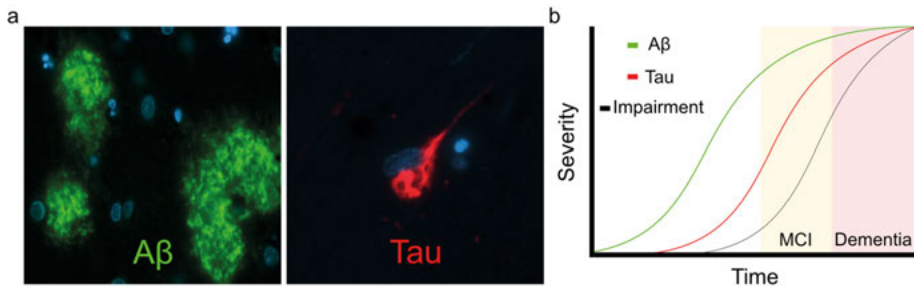


Figure 2. Immunostaining of human AD brain. (a) Example images of amyloid plaques consisting of aggregated A β and NFTs, consisting of hyper-phosphorylated tau. (b) Schematic illustration of the time-line for AD pathology. A β plaques appear first, years prior to NFTs. Cognitive impairment starts later and correlates better with NFT load than A β load. Mild cognitive impairment (MCI) is the first stage of cognitive symptoms, eventually advancing into dementia in AD patients.

Amyloid- β

A β is a 36-43 aa long peptide, produced through enzymatic cleavage of the membrane bound amyloid precursor protein (APP). The physiological function of A β is unknown, but it has been suggested to play a part in various processes, from protecting against oxidative stress and microbes to facilitating neuronal growth³². The enzymes α -, β - and γ -secretase cleave APP at different sites. Depending on which secretases that are involved, APP is processed through either the non-amyloidogenic or the amyloidogenic pathway. The non-amyloidogenic pathway releases two soluble peptide (the short P3 and sAPP α) extracellularly, leaving the remaining C83 peptide bound to the membrane. This pathway is a result of α - and γ -secretase cleavage and is believed to promote neuronal survival³³. In contrast, β - and γ -secretase cleavage result in the amyloidogenic pathway, which releases the soluble sAPP β and the highly aggregation prone A β peptide³³. Out of all the different A β species, A β 42 and A β 40 are believed to be the most important in pathology³⁴. A β 42 is more prone to aggregate and is the main constituent in amyloid plaques^{35,36}. A β 40 also aggregates, but are only found in certain types of plaques, even though the AD brain concentration is often several fold higher than A β 42^{36,37}. The Swedish (increases A β production) and London (increases the A β 42/40 ratio) mutations both directly alter the APP sequence and result in overall

increased levels of A β ^{38,39}. Conversely, presenilin (*PSEN1/2*) mutations indirectly increase A β pathology by altering the secretase enzymatic activity⁴⁰.

Risk factors

Many risk factors have been reported for AD: Age, level of education, diabetes, diet/obesity and brain injuries are some examples. Age is by far the greatest risk factor. At a young age the risk for developing AD is virtually zero, only to gradually increase as we get older. The prevalence is roughly 20% at the age 75, increasing to over 35% at 85⁴¹. Number of years in school is also a risk factor, where higher levels of education reduces the risk of dementia. Supposedly, this is because of an increase in cognitive resilience by formal education during ones early years⁴². Additionally, traumatic brain injuries (TBI) greatly increase the risk of AD, where a history of moderate brain trauma doubles the risk⁴³.

Apolipoprotein E

In addition to environmental risk factors, as stated above, there is a plethora of genetic risk factors. Variance in *APOE* is the greatest genetic risk factor for developing AD. Apolipoprotein E (ApoE) is a 299 aa long protein, important for shuttling fatty acids/cholesterol and is expressed in all tissues, including the brain. Humans have three ApoE isoforms; ApoE ϵ 2, ApoE ϵ 3 and ApoE ϵ 4 with an allele frequency of 8.4%, 77.9% and 13.7% respectively⁴⁴. Although isoforms only differ by two aa (ApoE ϵ 2 [Cys112, Cys158]; ApoE ϵ 3 [Cys112, Arg158]; ApoE ϵ 4 [Arg112, Arg158]), this significantly alters their ability to interact with lipids⁴⁵. Since *APOE* ϵ 3 is the most common variant, it is considered the base line for relative AD risk. Individuals homozygous for the *APOE* ϵ 4 allele have an estimated 8-16 fold increased risk of developing AD (appears ~40% of AD cases)⁴⁶. Conversely, *APOE* ϵ 2 appears half as frequently in the AD population as expected (~4% of AD cases) and therefore considered protective⁴⁶. Exactly how ApoE contributes to disease is not clear. However, ApoE ϵ 4 has been shown to affect plaque formation, and co-localizes well with amyloid-PET imaging^{47,48}. More recently, ApoE ϵ 2 has instead been reported to increase phosphorylation of tau in mice, a finding that appears to correlate well in PSP patients⁴⁹. This suggests that ApoE ϵ 2 may be “protective” for AD but increase the risk for other diseases⁵⁰. Moreover, ApoE from the periphery also affect AD risk, where lower levels of plasma ApoE increases the overall risk of dementia, irrespective of genotype⁵¹.

Tau

Tau was first described in 1975 as a microtubule-associated protein (MAP) important for tubulin stabilization/polymerization in the axons of neurons (**Fig. 3 a**)⁵². It was not until a decade later that the protein was identified as

the main constituent of NFTs^{53,54}. Human tau protein is expressed as six isoforms (in the CNS), ranging from 352 to 441 aa. The isoforms are produced during translation via alternative splicing of exon 2, 3 and 10 resulting in proteins containing 0-2 N-terminal inserts and 3-4 microtubule-binding repeats (MTBR) (**Fig. 3 b**)⁵⁵. In adult humans all isoforms maintain a relatively even expression⁵⁶⁻⁵⁸. Axonal tau has two main functional domains, the microtubule-binding domain (MBD) and the projection domain. MBD directly interacts with tubulin heterodimers promoting their polymerization, while the projection domain determines microtubule spacing by interaction with other cell components⁵⁹. Non-axonal tau is less investigated and includes nuclear, mitochondrial, dendritic and extra-cellular tau⁶⁰⁻⁶². The purpose of nuclear tau is still unclear, but it has been suggested to be involved in DNA and RNA processing^{63,64}. In contrast, mitochondrial tau has mainly been linked to toxic effects in pathology and it is unclear if it has any physiological function^{62,65,66}. Extracellular tau is believed to primarily be a toxic agent. However, some studies suggest that it could play a role in transcellular signalling⁶⁷.

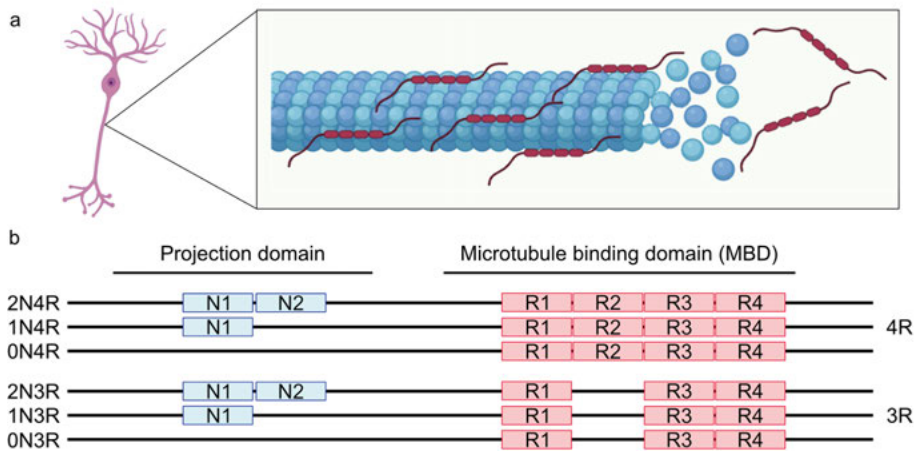


Figure 3. (a) Schematic of axonal tau. The MBD section bind to tubulin heterodimers and allow for microtubule polymerization. (b) Schematic illustration of the six isoforms of human tau present in the CNS, spanning from the N-terminus (left) to the C-terminus (right). N1/N2 refers to the N-terminal inserts, which are transcribed from exon 2 and 3 respectively. R1-R4 refers to the MTBR. The isoforms are therefore called 2N4R-, 1N4R-, 0N4R-, 2N3R-, 1N3R- and 0N3R-tau.

Tau phosphorylation

Tau is a highly modified protein, with post-translational modifications (PTMs), including phosphorylation, isomerization, glycation, nitration, O-GlcNAcylation, acetylation, oxidation, polyamination, SUMOylation and ubiquitinylation^{68,69}. Phosphorylation is the most common PTM and is be-

lieved to be the most important for physiological function as well as pathology^{54,70}. Tau is rich in serine and threonine, which constitute most of the phosphorylation sites of the protein⁷⁰. Many kinases have been shown to phosphorylate tau at different locations, where some appear more important to pathology than others. Glycogen synthase kinase 3- α/β (Gsk3- α/β), cyclin dependent kinase 5 (Cdk5), mitogen-activated protein kinase (MAPK), protein kinase A (PKA) and calmodulin dependent protein kinase II (CaMKII) are some of the most well described⁷¹. Gsk3- β is a serine/threonine kinase, known to be upregulated in AD and to directly phosphorylate tau in hippocampal neurons as a response to A β fibril exposure⁷²⁻⁷⁴. Controlled phosphorylation of tau is highly important for regulation of normal physiological function. Phosphorylation inside the MBD decreases the affinity to microtubule, dissociating the network⁷⁵. Therefore, a balance between kinase and phosphatase activity is critical for neuronal plasticity. Interestingly, the level of hyper-phosphorylated tau greatly increases in the brain of animals during hibernation and is believed to be protective during that process^{76,77}. Remarkably, phospho-tau quickly reverts back to normal levels once the animals wake up⁷⁶.

Some phosphorylation sites are strongly associated with pathology, in particular those within the MBD. pS202/pT205/(pS208) phosphorylation (detected by the AT8 antibody) is a defining feature in AD and other tauopathies⁷⁸. The pS396-pS404 (detected by the PHF1 antibody), in combination with pS202/pT205/pS208 give rise to disease specific paired helical filaments (PHFs)⁷⁹. Recently, tau phosphorylation has gained a lot of interest as a biomarker for AD and other tauopathies, most notably, pT181, pT217 and pT231⁸⁰⁻⁸². pT181 was the first to be discovered in the early 2000, where it showed promise as a CSF and later also as a plasma biomarker^{80,83-85}. More recently, pT217 was suggested to have the best predictive value as an AD plasma biomarker^{86,87}, while pT231 appears earliest in the pathological process^{82,88}. While *ex vivo* labelling using antibodies targeting these phospho-sites label NFTs in AD brain, they do not represent tau pathology when used as a biomarker. Instead, these phospho-tau species in CSF and plasma are more indicative of amyloid pathology during the preclinical stages of disease (pre-NFT formation)⁸⁹. Biomarkers indicative of tangles, such as MTBR-tau(p243) appear much later in the pathological process^{90,91}.

Tau aggregation

Tau forms two major fibril types, PHFs and straight filaments (SF). PHFs represent roughly 80% of the tau fibrils found in the human AD brain. In cross section, cryo-electron microscopy data have revealed that PHFs and SFs consist of two misfolded monomers (with different connecting points) constructing a dense core and a fuzzy outer layer⁹². These filaments are what constitute the NFTs and other larger deposits that can be seen in late stage tauopathies.

Several things contribute to tau aggregation; PTMs, mutations, mechanical trauma and polyanions are some examples.

As mentioned above, an increase in tau phosphorylation decreases its affinity towards tubulin. This ultimately results in a higher concentration of free tau monomers available for aggregation. Unlike most proteins, free tau is innately disordered and has no real tertiary structure⁵⁵. This has multiple effects on protein fibrillation. A lack of defined tertiary structure eliminates the need of any partial unfolding of native protein prior to aggregation. Any unfolding of protein structures requires energy that can be viewed as a bottleneck to amyloid formation and the polymerization of fibrils. Conversely, natively flexible proteins (like tau) are also less likely to conform to a given structure necessary for fibrillation. Pseudo-phosphorylation of Ser202/Thr205 and Ser396-Ser404 results in the so-called paperclip fold, giving tau a more rigid structure⁹³. The fold generates a pathology-associated tau species, detectable with the conformational dependent antibody MC-1 and Alz50^{93,94}. This shows that certain phosphorylation or equivalent PTMs can conform monomers to an aggregation prone state. Another important factor to consider is how an overall phosphorylation affects the proteins electrical charge. Native tau is polar, with a positively charged core. Excessive phosphorylation alters the charge (phosphates are negatively charged), negating the electrical repellent effect of native tau.

A few tauopathies are indeed caused by tau mutations. Most notably, the P301S, P301L and P301T point mutations generate tau species with greatly increased aggregation tendencies that eventually lead to development of FTD⁹⁷⁻¹⁰³. A152T-tau reduces microtubule affinity, theoretically increasing the risk of all tauopathies^{104,105}. While certain intronic mutations have been shown to promote the isoform shifts to 4R-tau expression (indicative of 4R-tauopathies like FTD, PSP and CBD)^{106,107}. Notably, no tau mutations have been linked to AD. Head injuries also result in hyper-phosphorylation and aggregation of tau, where even repeated sub-concussive head trauma is sufficient to develop pathology¹⁰⁸. Moreover, artificial ways to alleviate the positive charge of native tau is to incubate with polyanions, like arachidonic acid, heparin or heparan sulfate¹⁰⁹. These have been proven very useful for the creation of synthetic tau aggregates for research purposes¹¹⁰.

Tauopathies

Tauopathy is an umbrella term for neurodegenerative diseases categorized by abnormal deposits of hyper-phosphorylated tau^{4,5}. As mentioned above, PSP, FTD, PiD and CBD are a few examples, each with distinct features. Tauopathies are often classified as being either 3R- or 4R-dominant (referring to the predominant type of tau)¹¹¹⁻¹¹³. Interestingly, imbalance in the ratio of 3R/4R

is a strong sign of neurodegeneration^{114,115}. Mutations have been shown to skew the ratio mostly in favour of 4R¹¹⁶, possibly accounting for the 4R predominance seen in tauopathies. Although tau is primarily expressed inside neurons, glial tau inclusions are abundant in primary tauopathies and even the defining feature of some¹¹⁷⁻¹²⁰. PSP, CBD and PiD for instance, all have a distinct astrocytic tau phenotype. “Tufted” astrocytes is a hallmark of PSP, which is the accumulation of 4R-tau aggregates inside astrocytes mainly found in the neocortex and striatum¹¹⁹. Interestingly, tufted astrocytes stains poorly for glial fibrillary acidic protein (GFAP), a marker for reactive astrocytes¹¹⁹. CBD is also a 4R-dominant tauopathy defined by astrocytic plaques^{121,122}, while PiD is a 3R-tauopathy with both astrocyte and oligodendrocyte components as well as the characteristic neuronal Pick bodies¹²³. In contrast, AD is sometimes considered a secondary tauopathy, due to the presence of NFTs. The 3R:4R ratio is initially even in AD, but over time transitions to become more 3R-dominant¹²⁴⁻¹²⁶.

Alpha-synucleinopathies

Besides PD, Parkinson’s disease with dementia (PDD), dementia with lewy bodies (DLB) and multiple system atrophy (MSA) are the primary conditions classified as alpha-synucleinopathies. They are all characterised by the accumulation of α -syn in distinct brain regions. PD is the most common disorder, followed by DLB. PD and DLB share the same pathological hallmark; Lewy bodies (LBs) and Lewy neurites (LNs) (LB and LN are intra-neuronal α -syn aggregates)¹²⁷. After AD, DLB is the second most common type of dementia (roughly 15% of the cases), where damage to dopamine and cholinergic neurons lead to cognitive decline (dementia) as well as parkinsonian motor symptoms¹²⁸.

Parkinson’s disease

PD is primarily a condition defined by motor dysfunction, where patients are mostly cognitively unaffected in the earlier stages of disease. However, in some cases PD progresses into PD-MCI and eventually into PDD. PDD is a more advanced disease stage than PD, where cognitive impairment has become the predominant symptom^{129,130}. There are currently an estimated 7 million PD patients worldwide constituting roughly 1% of the population above 60 years of age¹³¹. Motor symptoms include tremors, bradykinesia and rigidity arising due to a loss of dopaminergic neurons in substantia nigra pars compacta (SNpc)¹³². SNpc is part of the basal ganglia in the mesencephalon and is involved in the modulation of voluntary movements¹³³. Why this region is specifically affected is still unknown. Additional non-motor symptoms like sleep disturbance, depression and loss of smell are also frequent¹³⁴. Several mutations have been linked to the development of PD. Alterations of leucine-

rich repeat kinase 2 (*LRRK2*), phosphatase and tensin homologue-induced kinase 1 (*PINK-1*) and the α -syn gene *SNCA* all increase the risk of developing PD^{135–137}.

Alpha-synuclein

Brain α -syn is mostly present in neuronal pre-synaptic compartments of the neocortex, hippocampus, cerebellum and striatum¹³⁸. α -syn also appears in peripheral organs like bone-marrow, kidney and liver^{139–141}. It is encoded by the *SNCA* gene, resulting in a 140 aa long protein with three functional domains: (1) the N-terminal domain (aa 1-60) interacts with membranes to anchor the protein in place while performing its function. (2) the non-amyloid component (NAC) region (aa 61-95), which becomes the fibrillary core during aggregation and (3) the C-terminal domain (aa 96-140) where many post-translational modifications (PTMs) occur. Under physiological conditions, α -syn is believed to play a role in synaptic recycling and release of neurotransmitters^{142–144}. By binding to vesicle-associated membrane protein 2 (VAMP2), α -syn stabilizes SNARE complexes necessary for vesicle fusion and subsequent transmitter release^{145–148}.

Prion propagation

For a long time, the prevailing theory was that infectious agents required DNA or RNA components in order to self-replicate and spread. Stanley Prusiner challenged this idea in 1982 with the discovery of the prions, a proteinaceous infectious particle¹⁴⁹. Prions are remarkable pathogens, solely made out of misfolded protein, which altogether lack the ability to replicate. Instead, they initiate misfolding in native proteins by acting as a nucleation point for template driven amplification (as mentioned in the section on amyloid). Contaminated food sources (containing prions) can lead to the internalization and eventual neuroinvasion of prion “strains”, resulting in very rapid forms of neurodegeneration and ultimately death¹⁵⁰. There are only a handful of so called “true” prions, each being a variant of the endogenous PrP¹⁵¹. Some examples of diseases include, Bovine spongiform encephalopathy in cattle (mad cow disease), as well as Creutzfeldt-Jakob disease and Kuru in humans¹⁵².

Prion-like proteins

Unlike true prions, “prion-like” proteins are not contagious but otherwise share many commonalities¹⁵³. Let us take tau in AD as an example: Tau is referred to as a prion-like protein, in that misfolded aggregates serve as a template and promote the misfolding of native tau^{95,96}. Prion-like propagation has for a long time been seen as the main mode of pathology propagation in humans¹⁵⁴. The hypothesis states that prion-like protein inside one neurons can

traverse the synaptic gap by various mechanisms and thereby “infect” their downstream neighbours (**Fig. 4 a**). This assumption fits well with the Braak staging of AD, indicating that the pathology starts in one specific region and subsequently spreads to other neuronally connected areas^{155–157}. The earliest NFT are found in the entorhinal cortex of the temporal lobe and appears shortly thereafter in the hippocampus^{158,159}. The hippocampus is a highly connected brain region from where tau can spread throughout the rest of the brain (**Fig. 4 b**). Various tau species derived from brain tissue of individuals with different tauopathies have been demonstrated to seed endogenous pathology both in cells cultures, transgenic and non-transgenic animal models^{160–163}. Interestingly, material from different tauopathies possess a greatly varied potency for seeding pathology¹⁶⁴. Similarly, α -syn pathology initiates in the SNpc and traverses through the brain in a prion-like manner (**Fig. 4 b**). Some evidence even suggest that it may be contagious, which would warrant a re-classification of α -syn as a true prion¹⁶⁵.

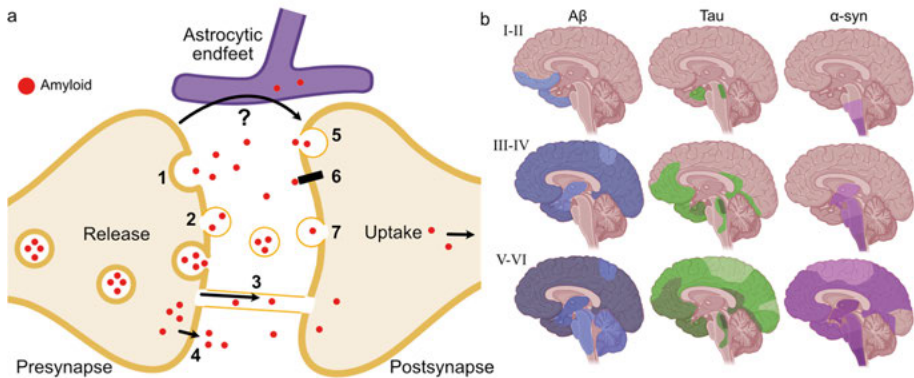


Figure 4. (a) Schematic of prion-like transmission over the tripartite synapse. Intra-neuronal amyloid is first released via; (1) exosomal release, (2) ectosomal release, (3) TNT mediated transfer, (4) release of free amyloid into the synaptic cleft. The amyloid is then taken up by the postsynaptic neuron via (5) endocytosis/micropinocytosis, (6) receptor mediated uptake, (7) membrane fusion. (b) Distribution of A β , tau and α -syn pathology (AD and PD patients) for Braak stages I-II, III-IV and V-VI. Image adapted from paper by Colin et al, and Goedert, M^{154,166}.

Neurons

Neurons are specialised to transmit information to interconnected regions throughout the body via electro-chemical signalling. Constantly active Na⁺/K⁺-pumps transport sodium and potassium ions against their osmotic gradients to maintain a polarized cell membrane¹⁶⁷. These pumps account for an estimated 50% of the brains energy consumption and make the neurons the

most energy intensive units in our bodies¹⁶⁸. Neurotransmitter-receptor interaction (chemical signalling) leads to a rapid depolarization of the cellular membrane (electric signalling). This electrical signal travels through the axon and initiates the release of other neurotransmitters from the synapse. Synapses consist of three parts (referred to as the tripartite synapse), where the first two are neuronal compartments (pre- and postsynapses). The third element of the synapse is the astrocytic compartment (astrocytic endfeet)¹⁶⁹. The astrocytic component is important for normal physiological function of neurons. Cross-talk between neurons and astrocytes influence basic cellular mechanisms, like calcium signalling and lipid/cholesterol metabolism¹⁷⁰⁻¹⁷². Moreover, glutamatergic neurons require astrocytic endfeet for recycling excess glutamate to avoid overstimulation and excitotoxicity¹⁷³.

How to kill a neuron?

Neurons are progressively damaged during neurodegeneration, which ultimately lead to disease-associated symptoms. Dysfunction precedes neuronal loss and can be caused by loss of synapses and reduction in plasticity or long-term potentiation (LTPs)¹⁷⁴. In proteinopathies, many mechanisms have been proposed for how amyloid damages cells. Misfolded protein aggregates for instance induce ER stress which in the long-term initiates apoptotic pathways in neurons^{175,176}. Amyloid structures have also been suggested function similarly to some bacterial toxins by forming of membrane-pores that disrupt cellular membrane (hugely important for neurons as discussed)^{177,178}. Moreover, damage can be caused more indirectly by activation of certain cellular pathways, in particular inflammation. Induction of certain microglial states have been shown damage neurons through several different mechanisms¹⁷⁹⁻¹⁸¹, which in turn have been reported to induce neurotoxicity in astrocytes¹⁸². Additionally, infiltration of CD8⁺ T-cell have been shown to cause damage to neurons both *in vivo* and *in vitro*^{180,183,184}. Together, these reports demonstrate that the mechanisms for neurodegeneration are complex, and most likely disease specific, making this an important avenue of enquiry.

Microglia

The human brain has a neuron to glial ratio of about 1:1 with some regional variation¹⁸⁵. Microglia are considered the brains resident macrophages and originate from the hematopoietic stem cells in the yolk sac. These cells infiltrate and occupy the CNS during neuronal development¹⁸⁶, setting them apart as the only non-neuronal derived glial cell type. Microglia are crucial for the brain's ability to fight potential threats and has been shown to engulf pathogens, protein aggregates, cellular debris and create neuronal scars. They are highly malleable by their environment and possess a remarkable ability for

swift activation when necessary. Even resting microglia have been shown to actively survey the brain parenchyma in search of threats¹⁸⁷. Once a potential threat is discovered they enter a reactive state and move to intercept the potentially harmful substance^{188,189}. They have been shown to mediate synaptic pruning that aids in neuronal plasticity both during normal development and as a response to injury^{190,191}. However, inflammation is not strictly positive, since immunosuppression following TBI has shown neuroprotective effects¹⁹². Moreover, microglia have been suggested to contribute to the gradual damaging of motor neurons causing the clinical symptoms in amyotrophic lateral sclerosis (ALS)¹⁹³. Additionally, cell culture and animal experiments suggest that they directly propagate α -syn and tau pathology^{194,195}. Taken together, microglial functions in neurodegeneration is complex and appears to be both neuroprotective and neurotoxic, perhaps at the same time.

Astrocytes

Etymologically the word “astrocyte” comes from the greek “astron”, meaning star, and “kytos”, meaning body. They are involved in regulating the blood brain barrier (BBB), synaptic support, neuronal development, neurotransmitter regulation and cholesterol production¹⁹⁶⁻²⁰¹. All these features make them crucial for maintaining brain homeostasis. The tripartite synapse highlights the astrocytes’ importance for a proper synaptic function¹⁶⁹. Additionally, astrocytic excretion of thrombospondins (TSPs), glypican-4/6 (Gpc4/6) and transforming growth factor beta (TGF- β) initiates synaptic generation, necessary for neuronal plasticity and memory formation²⁰²⁻²⁰⁵. They are also highly involved in synaptic pruning via the multiple epidermal growth factor-like domains protein 10 (MEGF10) and tyrosine-protein kinase Mer (MERTK) phagocytic pathways²⁰⁶. Furthermore, astrocytes regulate the water levels via aquaporin-4 (AQP4) expressed on their end feet²⁰⁷. Tau monomers are at least in part cleared from the brain in an AQP4 dependent manner, protecting against tau aggregation²⁰⁸. Astrocytes are also important for the production and maintenance of many substances necessary for normal neuronal function. Brain ApoE is one of these, which is predominantly produced by astrocytes and is required for lipid and steroid transportation²⁰⁹. Interestingly, the source of ApoE have different effects on neuronal activity. In a recent study, astrocytic ApoE4 was shown to have the most prominent effect of all isoforms and acutely increase the activity of neurons, whilst this was not the case for neuronal ApoE4²¹⁰.

Astrocytes in AD

Surprisingly, neurons are hyperactive in the early stages of AD, where one would expect the opposite²¹¹. This is supposedly due to a compensatory mechanism to synaptic dysfunction, however, exactly how this process is regulated remains unknown. Dysfunction of pre-synaptic calcium stores has been suggested as one mechanism that lead to an increase in N-methyl-D-aspartate (NMDA) receptor activation²¹². During the 1990's, the concept of a glial Ca^{2+} -“wave” started to emerge as a new non-neuronal signalling system in the CNS²¹³. Glutamate was shown both *in vitro*, *ex vivo* and *in vivo*²¹⁴⁻²¹⁶ to trigger these Ca^{2+} -waves, which then travel throughout the astrocytic network²¹⁷. Prostaglandin, as well as spontaneous oscillations in astrocytic Ca^{2+} levels, can initiate glutamate release and thereby directly affect NMDA activation in neurons²¹⁸⁻²²⁰. Combined, this supports the fact that astrocytes can influence neuronal activity both directly and indirectly.

Astrocytes' role in pathology appears even more complex than that of microglia. It might even be ill advised to think of astrocytes merely as a single cell type, considering their complex and heterogeneous nature²²¹. There is increasing evidence that astrocytes directly contribute to the propagation of pathology in neurodegenerative diseases by distributing amyloidogenic seeds. For example, tunnelling nanotubes (TNTs) are formed by astrocytes to share α -syn aggregates with nearby cells *in vitro*²²². Tau is also known to be excreted in its free form, suggesting that any cell that conceivably could contain tau without degrading it could serve as a vector for transmission²²³. Cellular stress can induce astrocytic excretion of extracellular vesicles (EVs) containing various proteins²²⁴. EVs as a mode for intercellular spreading of amyloid protein is known to happen from the pre- to the post-synapse. However, some studies indicate release of glial EVs as a potential major spreading mechanism for several proteinopathies²²⁵. Considering that astrocytes are highly interconnected and directly interact with all cell types of the brain, this constitute a possible transmission pathway with potential far-reaching effects.

Astrocytes in neuroinflammation

Historically, astrocytes have been somewhat overlooked when it comes to immunological function. Like microglia, astrocytes enter a reactive state by certain stimuli, a condition called reactive astrogliosis²²⁶. This entails a change in their morphology and expression of certain proteins, classically categorized by an upregulation of GFAP (**Fig. 5**), vimentin, nestin and S100- β ²²⁷. Reactive astrocytes are known for their involvement in glial scar formation. As a response to injury the astrocytes divide and occupy the area around the lesion to create a scar²²⁸. This process was believed to mainly induce damage to surrounding cells and inhibit neuronal regeneration. However, fairly recently this

process was shown to also have a regenerative effect on neurons post injury^{229,230}. Moreover, microglia activate astrocytes to become phagocytic and even clear debris from the brain parenchyma together with microglia in a well-orchestrated manner^{182,231,232}. This highlights the importance of microglia-astrocyte cross-talk during inflammation. In fact, these cell types are often interacting through direct contact during this process (**Fig. 5**). Additionally, experiments show that astrocytes are able to act as antigen presenting cells (APCs) post α -syn exposure²³³. Reports indicate that α -syn exposure does not trigger astrocytic release of pro-inflammatory cytokines²³⁴, although they are indeed capable of producing both cytokines and chemokines. Cytokines like interleukin-1/6/10 (IL-1/6/10), interferon- α/β (IFN- α/β), tumour necrosis factor- α (TNF- α) and the chemokines IL-8, monocyte chemoattractant protein 1 (MCP-1) are all produced by astrocytes^{235,236}. The appearance of astrocytic biomarkers sparked greater investigation into their role in inflammation²³⁷. For example, plasma GFAP and chitinase-3-like protein 1 (CHI3L1/YKL40), two astrocytic markers with an inverse relation to A β levels, have shown promise as early biomarker for AD²³⁸⁻²⁴⁰. Importantly, plasma GFAP correlates with clinical severity in TBI, making it an assessable and practically useful biomarker²³⁹. Furthermore, parallel to biomarkers some astrocytic PET tracers have been reported, such as the [11C]-L-deprenyl-D2 tracer for monoamine oxidase b (MAO-B) (yet another astrocyte marker)²⁴¹.

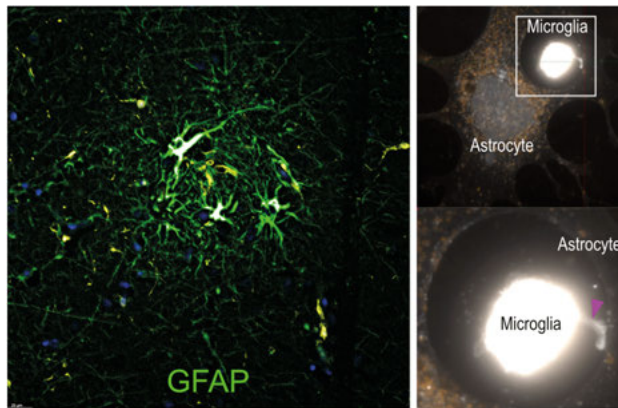


Figure 5. Image of a GFAP positive astrocyte cluster in human AD brain, and iPSC derived astrocytes and microglia interacting *in vitro* (membrane contact marked by purple arrow).

Protein processing

Degradation

Active degradation of debris, excess proteins and invading pathogens are all-important for maintaining homeostasis in healthy tissues. The two major cellular degradation pathways are the ubiquitin proteasome system (UPS) and the autophagy-lysosomal pathway (ALP). The UPS consists of a series of protein complexes that primarily degrade cytosolic proteins and is heavily involved during apoptosis. For a protein to be degraded, it first needs to be “tagged” by enzymatically attaching a chain of ubiquitin. Ubiquitin tags are recognized by the 26S-proteasome complex, which then degrades the tagged proteins²⁴². The ALP instead degrades larger components, such as phagocytosed proteins, pathogens or damaged organelles. Phagocytic cells (like microglia and astrocytes) internalise foreign debris/pathogens inside phagosomes²⁴³. Phagosomes then go through several stages of maturation, first fusing with early endosomes (lowers pH), followed by the recruitment of proteolytic enzymes like lysosomal-associated membrane protein 1/2 (LAMP-1/LAMP-2). This progression results in the formation of late endosomes and subsequently lysosomes²⁴⁴. Under normal circumstances, astrocytes uses both the UPS and the ALP to degrade internalised materials^{245–247}. However, these systems are not necessarily always effective. Dysfunction within the ALP system gives rise to an entire category of disorders, called lysosomal storage diseases (LSDs). Symptoms of LSDs often resemble those of neurodegenerative conditions, highlighting link between degradation and degeneration²⁴⁸.

Cell-to-cell transmission

Intercellular transfer of trophic factors, organelles and proteins is another important process in tissues under normal physiological conditions. Transmission of material occurs either through direct cellular contact or by excretion via extracellular vesicles (EVs). Direct contact includes several mechanisms, such as membrane fusion and formation of tunnelling nanotubes (TNTs)²⁴⁹. TNTs are thin, actin rich projections between cells²⁵⁰. Exposure to lipopolysaccharides (LPS), H₂O₂ and TNF- α has been shown to promote the formation of TNTs, suggesting an increased need during cellular stress^{251–253}. Furthermore, TNTs have been suggested as a major transmission pathway of prions in the brain²⁵⁴.

EV is a collective term used to describe exosomes and ectosomes. Exosomes is a heterogeneous group of vesicles created intracellularly and released into the extracellular space²⁵⁵. Ectosomes are much smaller and are directly created by pinching off the cellular membrane²⁵⁶. All cells use these mechanisms for cell-to-cell transportation of for instance RNA and proteins²⁵⁷. CSF derived

EVs from PD patients have been shown to induce secondary pathology, indicating their importance in PD pathogenesis²⁵⁸. Similarly, neuronal exosomes containing tau have been shown to be released following membrane depolarization, showing that neuronal activity can directly propagate pathogenic tau²⁵⁹.

Aims

The overall aim of this thesis was to investigate the role of glial cells in the progression of neurodegenerative diseases, primarily focusing on astrocytes and the role they play in tau pathology.

Study specific aims:

- I. Investigate how the interaction between astrocytes and microglia affect uptake, degradation, and transmission of α -syn and A β .
- II. Study how tau and neural cell debris affect astrocytes' ability to support neighbouring neurons. An additional aim was to assess whether astrocytic tau inclusions are benign or may serve as a reservoir of pathogenic seeds.
- III. Analyse astrocytes' capacity to process and spread human AD brain derived tau fibrils.
- IV. Investigate the effects of *APOE*-genotype on astrocytes' inflammatory response, as well as their capacity to process and spread pathological tau species.
- V. Establish an organoid model for studying penetration and pathology progression of prion-like proteins.

Methods

Cell culture model

The brain is a highly interconnected organ consisting of multiple different cell types working together to perform various functions. This makes neurological disorders difficult to study. Therefore, the use of appropriate models is crucial when researching this topic. In this thesis, we extensively used human induced pluripotent stem cells (iPSCs) originally derived from patient fibroblasts. This system allowed us to generate multiple cell types (astrocytes, neurons and microglia), simply by varying the differentiation protocols (**Fig. 6**). In **Paper I**, we differentiated astrocytes and microglia from the same iPSC line (Cntrl 9 II) to generate immunologically compatible cells^{260,261}. This was important for the purpose of co-culturing to avoid adverse immune reactions. Neural induction of iPSCs was performed to create viable neural progenitor cells (NPCs), from which astrocytes could be differentiated (**Fig. 6 a**). The NPC-intermediate stage are known as long-term neural epithelial-like stem (ltNES) cells, which were differentiated into astrocytes over a 28-day period. This was achieved by the addition of basic fibroblast growth factor (bFGF), activating A, heregulin β and insulin-like growth factor 1 (IGF-1). The same iPSC line was used in **Paper II** and **Paper III**, but with slight modifications to the astrocyte differentiation protocol. In addition to the factors mentioned above, we also included ciliary neurotrophic factor (CNTF) during the second half of the differentiation (extra push to the maturation). The aim of **Paper IV** was to investigate how astrocytic *APOE*-genotype might affect processing of tau aggregates. For this we used two different isogenic iPSC lines (EBiSC BIONi037-A-2 and EBiSC BIONi037-A-4), generated using the CRISPR/Cas9 system. Since both lines originate from the same person, they share the same genetic profile with the exception for *APOE*. The BIONi037-A-2 line is homozygous for *APOE* ϵ 2 while BIONi037-A-4 is homozygous for *APOE* ϵ 4. Astrocytes from these lines were generated in parallel and cultured in the same way as described above (with CNTF). Neurons could be differentiated by the addition of the ubiquitous neurotrophic supplement B27 to the same NES cells used for astrocyte differentiation²⁶². In **Paper II** we varied the differentiation times between 28-66 days depending on the experiments, while in **Paper III** and **Paper IV** we kept to the shorter differentiation times. While astrocytes originate from neuronal precursors during embryogenesis, microglia do not. Due to their cellular origin they cannot be generated from NES cells. To generate

microglia in **Paper I**, we first differentiated iPSCs into hematopoietic stem cells (HPCs) over a period of 14 days (**Fig. 6 b**). Microglia were then generated by addition of interleukin-34 (IL-34), transforming growth factor- β /definitive endoderm-1 (TGF- β /IDE-1), microphage colony-stimulating factor (MCSF), CXCL1 and CD200. In **Paper V** we utilised human cortical organoids (generated from Cntrl 9 II line). Organoid maturation theoretically mimics the more complex microenvironment of the human brain through a more embryo-like differentiation. First, spheroids were generated by centrifuging iPSC single cell suspension in Aggrewell plates (**Fig. 6 c**). Organoids were then matured, initially by the addition of small molecules like SB431542, XAV939 and dorsomorphine hydrochloride and later by the addition of fibroblast growth factor 2 (FGF2), embryonic growth factor (EGF), NT-3 and brain derived neurotrophic factor (BDNF) for minimum of 10 weeks.

In specific experiments, we also utilised a custom close-culture chamber. This chamber allows cells to be cultured in close proximity (1 mm separation) without cell-to-cell contact. We used this in **Paper I** to assess the transmission potential between astrocytes-to-microglia and vice versa. In **Paper IV** we instead used this model to investigate how the presence of astrocytic *APOE ϵ 3/3* affects tau processing in *APOE ϵ 2/2* and *APOE ϵ 4/4* astrocytes.

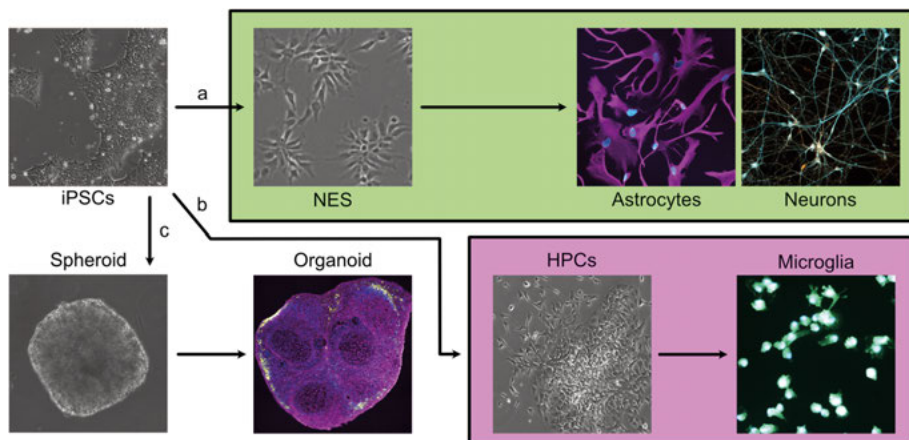


Figure 6. Schematic illustration of the cell systems, including intermediate cell types and distinct differentiation pathways. (a) Neural induction; iPSCs were differentiated into neuronal precursors, the NES cell stage. The NES cells were then used to produce both astrocytes and neurons by addition of different culture medium and trophic factors. (b) Hematopoietic differentiation; HPCs were generated from iPSCs colonies and further matured into microglia. (c) Spheroid generation; Single cell suspension of iPSC was centrifuged in Aggrewells to create spheroids that can be further differentiated into cerebral organoids.

Amyloid fibrils

In vitro (synthetic) formed fibrils are a great tool for studying the effects of a particular protein. We used *in vitro* fibrils of α -syn, A β and tau throughout this thesis (**Fig. 7**). In **Paper I** we exposed microglia and astrocytes to aggregates of both α -syn, and A β , while for **Paper II** and **Paper IV**, we performed similar experiments but with tau fibrils¹¹⁰. All fibrils were created using recombinant monomers of the respective wild type proteins. Aggregation is a highly concentration dependent process and monomers are therefore dissolved in buffer solutions at a high concentration. Additionally, we speed up the process by placing the solution on high shake at 37°C, since both physical agitation and temperature are determining factors. In the case of tau we also included heparin to promote fibrillation. In order to compare the infiltration capacity of α -syn, A β and tau, we included all of them in **Paper V**. Even though *in vitro* fibrils are useful, they differ in many ways, compared to fibrils formed *in vivo* (brain derived). Fibrillation is an important step in pathogenesis and fibrils derived from patient brains may behave very differently. For that reason, we extracted tau fibrils from human brain samples in **Paper III**. Fibrils could be extracted by homogenizing the brains with sodium lauryl sarcosinate (sarkosyl), followed by multiple ultra-centrifugation steps^{92,263}.

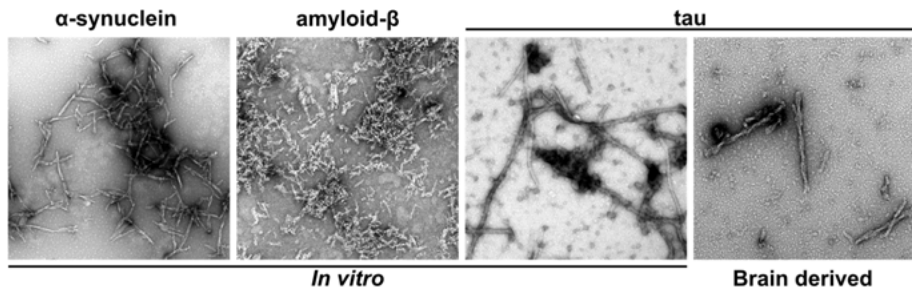


Figure 7. Transmission electron microscopy images of *in vitro* fibrils of α -synuclein, amyloid- β , tau as well as brain derived tau fibrils prior to sonication.

Exposure to fibrils

Throughout this thesis, we incubated cells/organoids with sonicated fibrils for a fixed number of days. Sonication was required to reduce the size of the fibrils to allow for internalization by cells. Several different time points were used for the different papers, indicated as “xd+yd”. Cells were washed with medium/PBS after incubation (number of days indicated as “xd”) to remove free protein from the medium and subsequently cultured in aggregate free medium (number of days indicated as “yd”). Example: 3d+12d; i.e 3 days incubation with fibrils, followed by 12 days of fibril free incubation.

Protein analysis

Immuno-stainings

Immunocytochemistry (ICC) and immunohistochemistry (IHC) are common methods used to visualize specific antigens in cells and tissues, respectively. They are in principle the same, utilizing the specificity of primary antibodies that bind to unique epitopes, corresponding to specific proteins of interest. Chromogens and/or fluorophores coupled to the antibodies allow for the visualisation by microscopy. We primarily used fluorescence as detection in this thesis. Different fluorophores can be coupled either to the primary antibody (direct), or to a secondary antibody, that in turn bind to the primary (indirect). Since more than one secondary antibody can bind to a single primary antibody, the indirect variant allows for signal amplification, making it more suitable for detecting weaker signals. Indirect ICC also allows for more flexibility since each primary antibody can be detected using secondary antibodies coupled to different fluorophores. This thesis exclusively used indirect ICC. Fluorescent dyes (labelling specific targets) can also be used, either on their own or in combination with antibodies. Tetramethylindo(di)-carbocyanines are synthetic dyes that include cyanine 3 (Cy3) which is extensively used in **Paper I-II, IV-V** to visualise protein aggregates. In **Paper III** we instead utilize the Amytracker range to directly detect β -sheet structures. Additionally, we used 4',6-diamidino-2-phenylindole (DAPI) to label DNA and molecules from the BioTracker range or wheat germ agglutinin (WGA) to label cellular membranes.

Western blot

Western blot (WB) is a method frequently used as a semi-quantitative analysis of protein content. The technique is based on separating proteins by molecular weight through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The polyacrylamide gel functions as a mesh through which proteins can migrate. First, SDS denatures and applies a negative charge to proteins in a sample. Once a current is applied, these proteins are “pulled” through the gel towards the positive pole. Small proteins can more easily traverse through the gel and end up towards the bottom, while larger ones experience more resistance, and thus remain closer to the top. The proteins can then be transferred to a polymer membrane, most commonly nitrocellulose or polyvinylidene difluoride (PVDF). PVDF membranes have greater protein binding capacity compared with nitrocellulose and is therefore better suited for detecting proteins of lower concentrations. Similar to ICC, specific protein on the membrane can be stained using antibodies and visualised by either fluorescence or electrochemiluminescence (ECL). We used WB extensively in **Paper II-IV** for detecting intracellular tau aggregates.

Sandwich ELISA

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based technique useful for precise quantification of protein levels in a sample. In this thesis, we exclusively used “sandwich” ELISA; which means that the antigen of interest is “sandwiched” between a capture antibody and a detection antibody. The capture antibody coated onto a plate is pinning the antigen to the surface, allowing unwanted antigens in the sample to be washed away. The detection antibody targets the same antigen and is frequently coupled to horseradish peroxidase (HRP), allowing for subsequent detection. Unless the aim is to detect multimers, the capture and detection antibodies need to bind different epitopes of the same protein since each epitope only allow a single antibody to bind simultaneously. Addition of the chromogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB) initiates a chemical reaction, where the TMB is progressively oxidized by the HRP, resulting in a blue water-soluble solution. Subsequent acidification (by addition of H₂SO₄) halts the reaction and yields a yellow solution with a peak absorbance of 450 nm. The absorbance can then be measured by spectrophotometry, where values can be compared with a designated protein standard of known concentration. In **Paper I**, we measured α -synuclein and amyloid- β concentrations in the cell medium, while **Paper II-IV** utilized the same principle to detect tau.

Microscopy

Several different microscopy techniques were used in this thesis, each with a designated microscope (**Table 1**). “Wide-field” microscopy encompasses a broad range of techniques and refers to when the whole sample is illuminated to maximize the number of photons that reach the detector. We used fluorescence as a detection method. Fluorophores are excited by certain wave-lengths of light and result in red-shifted emission. The light emitted is registered once it reaches the microscopes detector. By using emission filters that only allow light within a specified range, one can separate the signal depending on the fluorophore of origin (different for each target). This was the most utilized microscopy technique and used to a great extent in all five papers on fixed cells and tissue. The same principle was also used in the live-cell imaging techniques in **Paper I-IV**. Live cell imaging functions much the same from a microscopy perspective. The core difference is that the cells remain alive inside an incubator chamber while images are captured. Confocal microscopy utilizes a pinhole to greatly increase the contrast of an image. This is achieved by constricting the pinhole and thereby removing the out-of-focus light. In **Paper IV-V** we also used a pseudo-confocal technique (THUNDER) which lies somewhere in between. In terms of hardware it is functionally a widefield

microscope (i.e. no pinhole) but with specific software additions that computationally removes out-of-focus light. Transmission electron microscopy (TEM) uses electrons as an illumination source instead of photons. Electrons can have a wavelength 100,000 times shorter than photons (within the visible spectrum), giving this method a much greater resolution compared with conventional microscopy. We utilized this technique to visualise *in vitro/in vivo* fibrils in **Paper II-IV**.

Table 1. List of all microscopy techniques that were used in the thesis, including which microscope was used for each technique and in which paper.

Microscopy technique	Microscope	Used in
Wide-field	Observer Z1 Zeiss	Paper I-IV
Live-cell imaging (wide-field)	Nikon Biostation IM CellR	Paper I-III
Pseudo-confocal (THUNDER)	Leica DMi8	Paper IV-V
Confocal	LSM 700	Paper 1-V
Transmission electron microscopy (TEM)	Tecnai G2	Paper II-IV

Image analysis

ImageJ software analysis was crucial for this thesis with multiple custom macros designed to answer specific scientific questions. In all papers, we quantified the level of intra-cellular Cy3-protein aggregates (fluorescently labelled) using custom macros. In **Paper I**, we also quantified α -synuclein and amyloid- β levels in astrocytes and microglia for both mono- and co-cultures. In short, macros were designed to measure the intra-cellular signal upon staining for a cellular marker (often vimentin, Iba1 or BioTracker490) to establish the outline of the cells. Next, this outline (ROI) was overlaid onto the channel corresponding to protein aggregates, allowing for intracellular measurements of the integrated density (IntDen). IntDen is a measurement that combines intensity (pixel value) and size of an object. This means that an object twice as bright but half the size of another, obtain the same IntDen value. We utilized the same principle in **Paper II-IV**, where we instead quantified the levels of Cy3-labelled tau inside astrocytes. A more complicated macro was needed for analysis in **Paper V**, although the core principle was the same. Here we used a string function to separate images into multiple measurements to estimate the average penetration of labelled protein aggregates into the organoid tissue over time.

Cytokine assays

Cytokines are a group of molecules produced by activated immune cells that play various roles in both the innate and adaptive immune response. In **Paper I** we used a comprehensive cytokine array to detect cytokines released by astrocytes and microglia, both in mono- and co-cultures. The technique is similar to that of WB, where a membrane has multiple dots pre-coated with anti-cytokine antibodies. Cytokines in the cell medium are then captured during incubation with the membrane. HRP-conjugated detection antibodies can be used in the same way as for WB and be visualised by ECL. We used a similar array in **Paper III-IV**, to study the astrocyte cytokine release following tau exposure. Additionally, we designed a custom Multiplex ELISA from Meso Scale for more accurate readings. The custom U-Plex Meso Scale Discovery electro-chemiluminescence (MSD-ECL) analysis was designed to measure astrocyte medium concentrations of IL-1 β , IL-6, IL-8, IL-10, IL-12/IL-23p40, IL-17A, CXCL11 (I-TAC), CXCL10 (IP-10), CCL2 (MCP-1) and TNF- α . This technique is in practice an ultra-sensitive ELISA. It utilizes and uses ECL as a signal amplifier and standards of known concentration for each cytokine to become quantitative.

Tau seeding assay

The tau RD P301S FRET Biosensor is a biological assay initially developed for evaluating seeding efficiency of prions¹⁶². We utilized this model in **Paper II-IV** to show that astrocytes continuously excrete tau species that are capable of propagating pathology. The assay is based on human embryonic kidney 293T (HEK293T) cells, transfected with two separate lentivirus constructs (RD-P301S-CFP and RD-P301S-YFP). This results in HEK293T cells over-expressing P301S-tau (mutation result in highly aggregation prone tau species) conjugated to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). Exogenous tau seeds act as nucleation points that allow the aggregation prone endogenous tau to aggregate (**Fig. 8**). CFP and YFP have peak excitation at 405 and 513 nm respectively. This is so far enough apart that the 405 excitation primarily result in CFP emission (under normal circumstances). Endogenous aggregation can be detected when CFP and YFP molecules comes within range for Förster resonance energy transfer (FRET) to occur. FRET refers to the process of energy directly transferred (enough to initiate excitation) between light sensitive molecules (like fluorophores). Once CFP is excited, some energy is transferred to YFP (via FRET), resulting in YFP emission. We can thereby detect aggregated endogenous tau by measuring YFP emission resulting from CFP excitation. Since fluorophores have to be within 1-10 nm for FRET to occur, this detection method yields some of the highest resolution possible in a biological system.

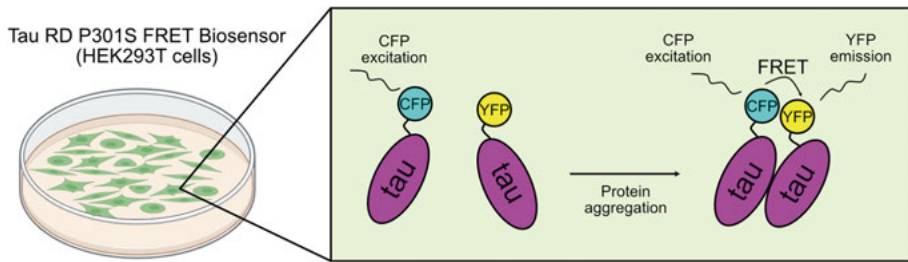


Figure 8. Schematic illustration of the tau seeding assay. HEK293T cells overexpressing the endogenous tau constructs (tau-CFP and tau-YFP). Aggregation is detected by YFP emission, resulting from CFP excitation, made possible due to FRET (CFP to YFP energy transfer when within 1-10 nm).

Neuronal functionality and viability

To evaluate astrocyte cytotoxicity we exposed neuronal cultures to astrocyte conditioned medium (ACM). In **Paper II**, we evaluated the effect of tau burdened astrocytes on neurons both by addition of ACM and by co-culturing. In this study we investigated the neuronal death by terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL). TUNEL is a fluorescent dye that binds double-strand DNA breaks, staining late phase apoptotic nuclei. In addition, we used single cell patch-clamp to directly measure the mini-/spontaneous- excitatory postsynaptic currents (m-/s-EPSCs) of individual neurons to estimate their electro-physiological functionality. In **Paper III** we analysed the pattern of the pre-synaptic marker synaptophysin to investigate the impact of tau burdened astrocytes on neurotoxicity. We also utilized a more direct measurement of toxicity by analysing the cellular adenosine triphosphate (ATP) levels inside the neurons after ACM exposure. In **Paper IV** we analysed the neuronal ATP levels as well as lactate dehydrogenase (LDH) activity following ACM exposure. LDH activity acts as a proxy for cellular respiration (produced during cellular stress) and works well in combination with ATP to estimate general cellular dysfunction.

Statistics

In this thesis, we utilised several statistical tests depending on the dataset in question. Many factors, like data variation and missing values affect the statistical analysis. Before considering the statistical analysis we best address the following core concepts: (1) distribution and (2) n-number.

- (1) Gaussian or non-Gaussian (normal/ distribution is important to consider. This determine whether parametric or non-parametric testing of

the dataset is advised. We always tested the distribution of the datasets prior to further analysis to determine the appropriate test.

- (2) The n-number is important in a mathematical context but does not necessarily make sense in a biological setting. Throughout this thesis, we typically do not use the term “n-number” (**Paper III** being the exception) but instead explain how the experiments are conducted. In short, our biological “n-number” is values from individual wells of a culture plate.

The choice of test was determined based on number of groups and number of parameters used in the comparison (**Table 2**). Note that time was not considered a statistical parameter in this thesis since the effects measured by time are separate cultures (started at the same time and cultured for varying time).

Table 2. List of statistical tests used for analysis in this thesis and which type of dataset they were used for.

Statistical test	Gaussian distribution?	Number of groups	Number of parameters
Standard t-test	Yes	2	-
Mann-Whitney U test	No	2	-
One-Way ANOVA	Yes	>2	1
Kruskal-Wallis test	No	>2	1
Two-Way ANOVA	Yes	>2	2

Results and discussion

Paper I

The aim of **Paper I** was to determine how microglia and astrocytes act in synergy when exposed to α -syn or A β fibrils. More specifically, we sought to investigate how the uptake and degradation of each fibril type is affected by the presence of respective glial cell. For this purpose, we exposed mono-cultures of astrocytes or microglia to Cy3-labelled fibrils and compared the results to a parallel co-culture. Under all conditions, astrocytes and microglia engulfed both α -syn and A β fibrils (**Fig. 9**). To compare microglia and astrocytes with respects to their phagocytic ability we used constant exposure (varied the incubation time; 24h, 4d and 7d) without any washing steps. In contrast, incubation (24h and 4d) followed by washing steps and subsequent culturing in fibril free medium (24h+3d and 24h+6d) allowed us to compare degradation and excretion of α -syn and A β fibrils over time.

Astrocytes and microglia showed a roughly equivalent α -syn uptake at 24h, and a similar trend with respects to degradation (**Fig. 10 a, c**). The measurements of cultures with constant exposure indicated an overall gradual increase in the levels of intracellular Cy3- α -syn (**Fig. 10 a, c**). This was most evident in astrocytes, indicating that they accumulate α -syn over time. ELISA analysis of astrocyte and microglia cell medium supported this notion, as similar amount of α -syn aggregates remained in the medium at 24h, 4d and 7d post exposure. These results show that both cell types are equivalent when it comes to uptake of α -syn aggregates. In contrast, A β exposed astrocytes and microglia cultures differed substantially. ELISA measurements of respective cell medium indicated a similar uptake after 24h. However, analysis of medium from following time points suggested that microglia are much more effective in removing A β fibrils from the medium, compared to astrocytes. Image analysis of astrocytes revealed similar level of intracellular A β at all time points, indicating a slower uptake (**Fig. 10 b**). Moreover, washed astrocyte cultures (4d+3d) showed very little A β degradation (**Fig. 10 b**). A β signal inside microglia on the other hand, reduced substantially over time, both in the unwashed (uptake) and the washed (degradation) cultures (**Fig. 10 d**). Combined with the ELISA measurements this must be due to degradation since the A β levels in medium remain low post wash. Taken together, these data suggest that both astrocytes and microglia can engulf and degrade α -syn and A β fibrils.

However, microglia are far superior to astrocytes in this regard when it comes to A β aggregate.

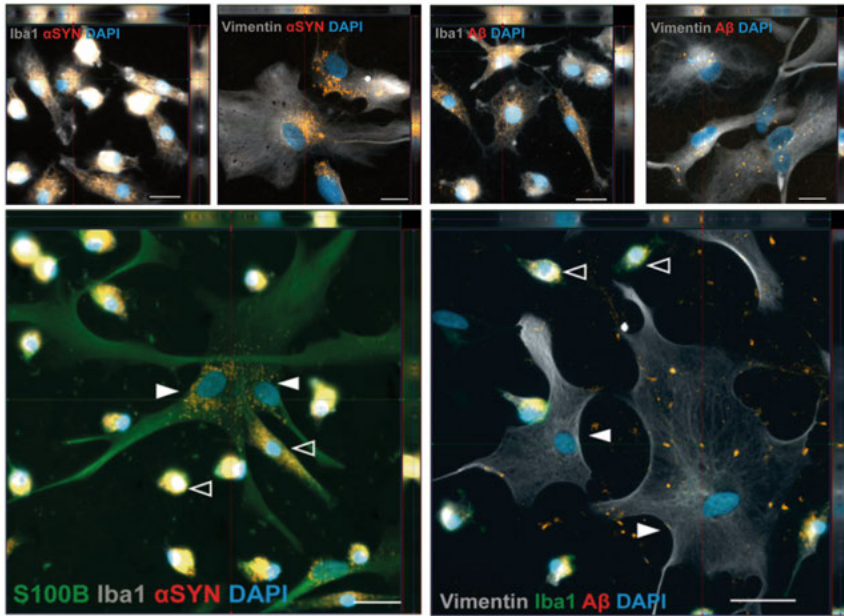


Figure 9. Intracellular cy3-labelled α -syn and A β fibrils in mono and co-cultures of astrocytes and microglia. White arrows indicate astrocytes and black arrows indicate microglia in the co-culture setup.

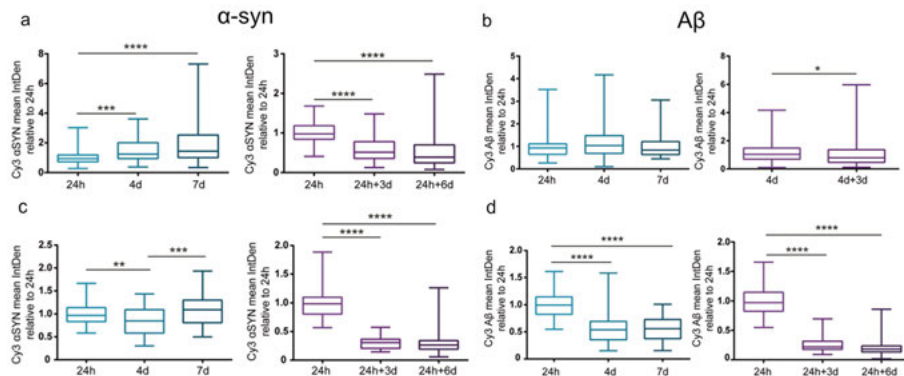


Figure 10. Image analysis data from α -syn and A β exposed astrocytes and microglia mono-cultures. Quantification of (a) astrocyte α -syn, (b) astrocyte A β , (c) microglia α -syn and (d) microglia A β uptake and degradation (intracellular Cy3 signal).

Once we had established a base line for uptake and degradation for both cell types, we compared the mono-cultures to astrocyte/microglial co-cultures. As a whole, the levels of total Cy3 signal decreased more with time in the co-cultures, for both α -syn and A β fibrils. To gain more information we distinguished between aggregates inside astrocytes and microglia by using separate markers to determine the cellular outline (vimentin as a marker for astrocytes and Iba1 for microglia). Plotting data for each individual cell type in a co-culture setting allowed us to assess any synergistic effects. Interestingly, the α -syn levels inside microglia appear much the same in a co-culture setup as they did for mono-cultures, where the signal decreased over time (**Fig. 11**). However, corresponding astrocytes also displayed a reduction in α -syn 7d relative 24h (unlike their mono-cultures) (**Fig. 11**). Similarly, A β levels decreased over time in astrocytes co-cultured with microglia (**Fig. 11**). Taken together, these results suggest that astrocytes have an increased degradation capacity when in close proximity to microglia. Another interpretation could be that astrocytes instead transport aggregates to microglia for degradation. To test this, we utilized the close-culture chamber to determine which cell type had a higher tendency to secrete aggregates. Our results showed that astrocytes secrete much more of internalized protein aggregates than microglia, meaning that at least a part of the Cy3 signal reduction seen in astrocytes is due to aggregates being transferred over to microglia for degradation.

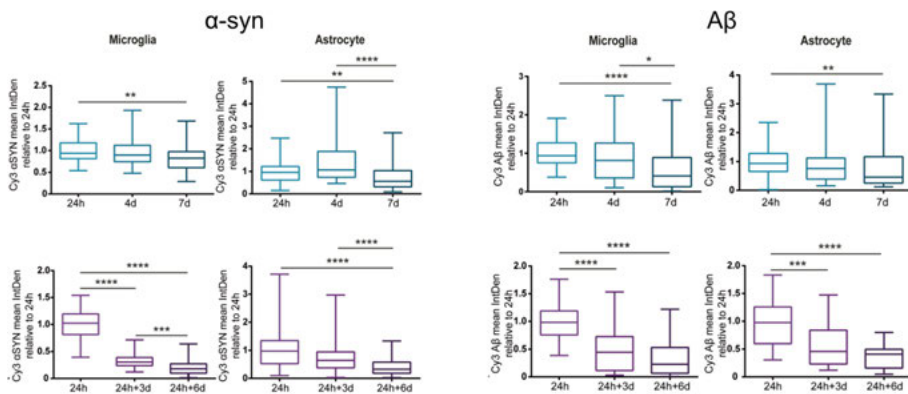


Figure 11. Image analysis data from α -syn and A β exposed co-cultures. Quantification of α -syn/A β in astrocytes and microglia (intracellular Cy3 signal).

Paper II

Here we aimed to investigate how tau fibrils affect astrocytes and neighbouring neurons. We previously showed that astrocytes accumulate α -syn and A β fibrils and wanted to extend our investigations to include tau and neuronal cellular debris. For this purpose, we exposed astrocyte cultures to Cy3-tau fibrils for three days before washing and further culturing for up to 28 days.

Moreover, we added astrocytes to culture dishes with apoptotic neurons and allowed the astrocytes to engulf the debris. Image analyses indicated that astrocytes effectively engulf large amounts of tau during the three days of incubation. This was supported by analysis of the medium (~7% of the starting concentration remained in 3d cell medium, as measured by ELISA). Astrocytes are known to clear cellular debris in response to injury. Our experiments showed that astrocytes exposed to apoptotic neurons engulf entire nuclei (**Fig. 12 a**), highlighting a large capacity to internalize extracellular debris. There was no difference in total number of condensed nuclei (dead neurons) comparing 3d and 7d, suggesting that no degradation occurred during this time (**Fig. 12 b**). Moreover, we could assess uptake by quantifying the average amount of condensed nuclei inside each astrocyte. At 3d, roughly half of the astrocytes had internalized at least 1 dead cell and about 10% had ingested 5 or more (**Fig. 12 c**). This shifted by 7d, when about 75% of the astrocytes contained at least one dead neuron and 20% contained 5+ (**Fig. 12 c**). To determine long-term effects in astrocytes exposed to tau fibrils, we analysed cell morphology directly after washing (3d) and after additional 14 and 28 days (3+14d, 3+28d) (**Fig. 12 d**). Initially, tau exposure did not seem to affect the astrocytes much, which appeared stable at d14. However, we noticed a significant decline in the total number of cells by d28 (**Fig. 12 e**), which is likely due to a latent toxic effect posed by the tau fibrils. In addition, the astrocytes experienced a gradual shift in morphology during this time. Comparing the overall trend from 3d to 28d, astrocytes became both larger in size (increased area) and less ramified (fewer branching points) when exposed to tau (**Fig. 12 f-g**). As a whole, these results show that astrocytes engulf large amounts of both protein aggregates and neuronal debris, which accumulate inside the cell.

In paper I, we established that astrocytes excrete large amounts of internalized α -syn and A β fibrils. Thus, we wanted to investigate if the same was true for tau. For this we utilized live-cell imaging, to observe astrocyte-astrocyte interactions and transfer events of Cy3-tau. TNTs were frequently found in both control and tau exposed cultures. Several transfer events of tau could be observed, with aggregates transmitted from one astrocyte to another (**Fig. 13**). In order to quantify the transfer efficiency we exposed one set of astrocytes (donors) to Cy3-tau and labelled another set of astrocytes with a membrane marker (acceptors). By combining these cultures and quantifying the tau content for each population (donor/acceptor), we could assess the transfer efficiency of internalized tau. Interestingly, there was an equivalent Cy3-tau signal in the donor and acceptor population after three days in co-culture, suggesting a highly effective transfer mechanism (**Fig. 13**).

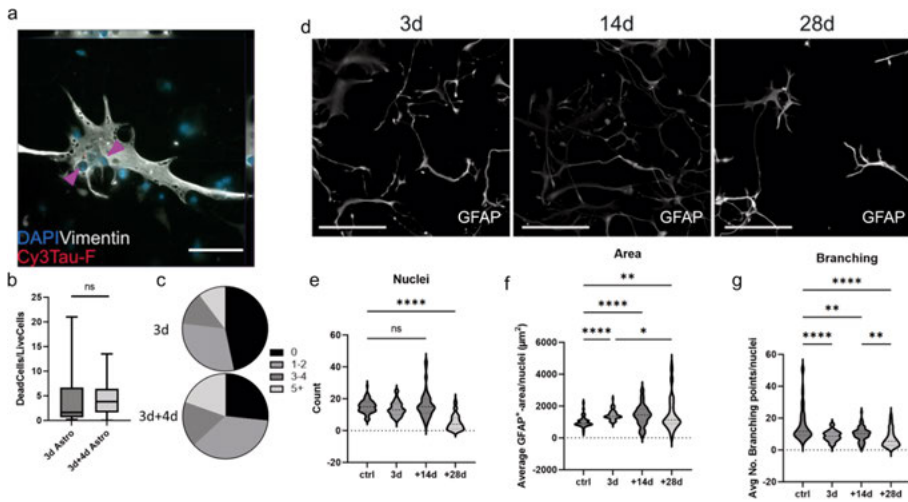


Figure 12. Astrocytes accumulate apoptotic nuclei and tau aggregates over time. **(a)** Example of an astrocyte with internalised condensed nuclei (marked by purple arrows). **(b)** Quantification of the total number and **(c)** intracellular (average/astrocyte) of condensed nuclei. **(d)** Astrocytes after long-term exposed to tau (unlabelled). **(e)** Total number of astrocytes over time, **(f)** average area and **(g)** number of branch points per cell.

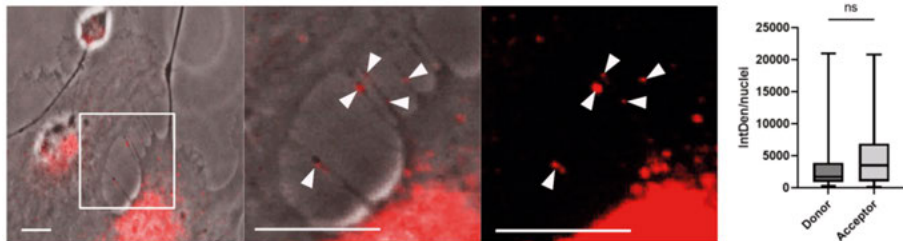


Figure 13. Astrocytes effectively distribute internalised tau aggregates between each other via TNTs. Cy3-Tau fibrils within TNTs (white arrows). Quantification of Cy3-tau in donor respective acceptor astrocytes after 3d of co-culture.

Additionally, we have investigated how debris and tau burdened astrocytes affect surrounding neurons. To do this, we exposed astrocytes to either tau fibrils or neuronal corpses prior to co-culturing with healthy neurons. Counting the number of TUNEL positive nuclei within each culture and comparing it to control co-cultures allowed us to assess the damage caused by the tau/debris burdened astrocytes (**Fig. 14 a**). Our results suggest that tau and debris burdened astrocytes induce apoptosis in neighbouring neurons (**Fig. 14 b**). However, there was no difference between tau to neuronal debris. We also performed patch-clamp experiments, which display a similar trend. Neurons in co-culture with tau and debris burdened astrocytes had a reduced frequency

of sEPSCs, which is an indicative of declined cellular function. Interestingly, we have noticed that tau internalised by astrocytes stain poorly using commercial tau antibodies. We hypothesized that this could be due to cellular modifications that affect the binding of the antibodies. This led us to investigate whether the astrocyte-mediated modifications of tau affect its seeding efficiency. To test this, we exposed RD tau P301S FRET cells to ACM from control and tau exposed astrocytes (**Fig. 14 c**). Control medium gave no signal, while the positive control (starting medium) proved that the starting tau fibrils were indeed capable of seeding pathology in this model (**Fig. 14 d**). Interestingly, the level of YFP signal of the positive control was equivalent to that of ACM from exposed cells (**Fig. 14 d**). Since the tau concentrations in the positive control were much higher than in the ACM (**Fig. 14 e**), we reasoned that this effect is most likely due to modifications of tau excreted by astrocytes. Combined, these data show that astrocytes have a great capacity of accumulating debris, which affect their overall function. Astrocytes can become neurotoxic and can serve as a reservoir for distributing toxic tau species, capable of seeding further pathology.

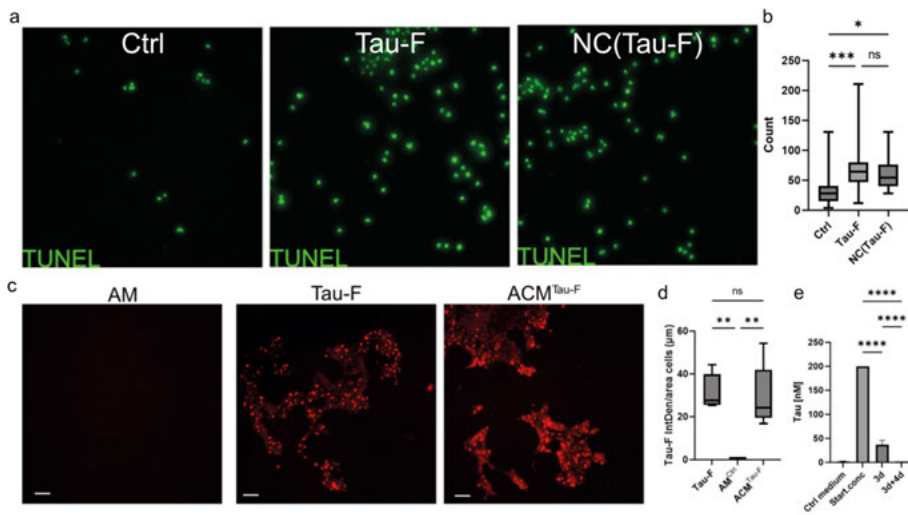


Figure 14. Tau burdened astrocytes damage surrounding neurons and excrete highly seeding competent tau species. **(a)** Neurons in co-culture with control, tau, and debris-burdened astrocytes, stained for TUNEL. **(b)** Count of the average number of TUNEL positive nuclei. **(c)** YFP-images of RD tau P301S FRET cells (negative control, positive control and ACM exposed cells). **(d)** Quantification of the YFP signal. **(e)** ELISA measurements of tau concentration in astrocyte medium over time.

Paper III

Building on the premises of Paper II, we sought to investigate how astrocytes process tau aggregates derived from human brain. Brain derived fibrils differ substantially from those created *in vitro*. Moreover, there is a large variation in fibril types between different diseases, highlighting their importance for pathology. For this reason it is conceivable that the astrocytic response to brain derived fibrils are different from those of *in vitro* fibrils. First, we extracted tau fibrils from human AD brain (Braak stage V-VI) and aged matched control brain, from both parietal and temporal lobes (**Fig. 15 a**). EM of the respective samples revealed fibrils with an AD characteristic spiral structure, indicative of PHFs, in both parietal and temporal samples (**Fig. 15 b**). Notably, we did also observe PHFs in the control brains, but to a lesser extent. Importantly, WB analysis revealed that AD samples contained far more tau and phospho-tau compared with control brain samples (**Fig. 15 c-d**). Additionally, isoform analysis revealed that the 3R-tau was the dominant isoform (**Fig. 15 e-f**), mostly present in AD samples. This finding fits well with late Braak stage AD cases, where 3R-tau is the dominant form^{113,124,126}. In short, the analysis of the samples support that the extracted tau preparations are representative for AD tau.

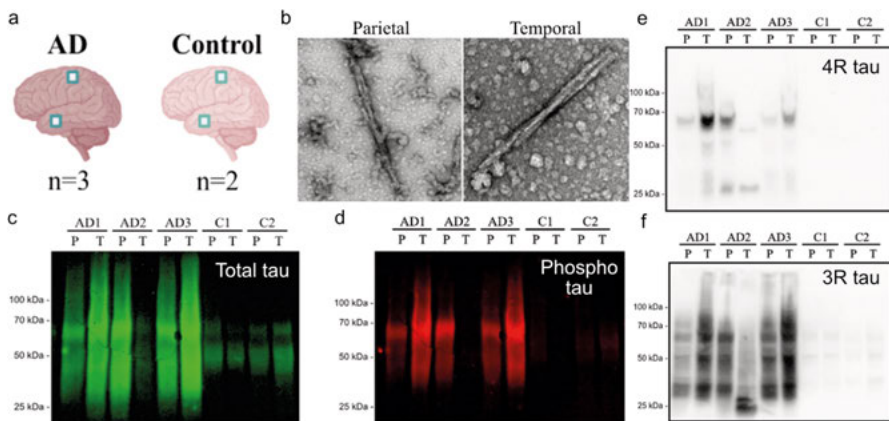


Figure 15. Analysis of AD and control brain derived tau fibrils. **(a)** Fibrils were extracted from both parietal and temporal lobes. **(b)** EM image of parietal and temporal samples, confirming the presence of PHFs. **(c)** Total tau and **(d)** phospho-tau (pS202, pT205) levels. Isoform analysis, showing **(e)** 4R-tau and **(f)** 3R-tau levels.

In Paper II, we established that astrocytes accumulate tau aggregates and are very effective in transferring internalised tau between each other. To test whether this also applied to brain derived tau we used a fluorescent Amytracker dye, which label the β -sheet structures in the PHFs. Measurements of intracellular Amytracker signal showed that astrocytes accumulate fibrils from both brain regions and do not appear to degrade the tau during the time

of the experiment (**Fig. 16 a**). We also observed TNT-mediated transfer of brain derived tau using live-cell imaging (**Fig. 16 b**).

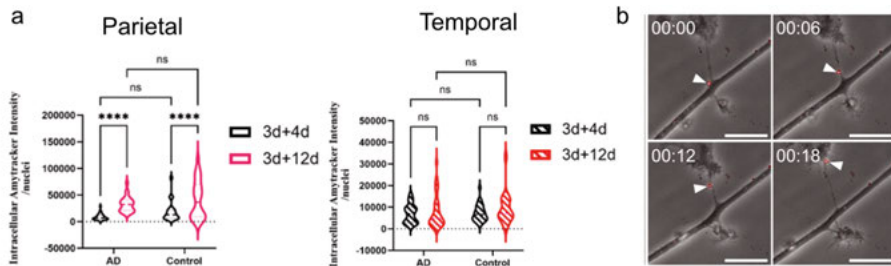


Figure 16. Astrocytes accumulate and transfer brain derived tau between each other. **(a)** Intracellular Amytracker signal in astrocytes exposed to parietal and temporal, AD/control brain derived fibrils. **(b)** TNT mediated transfer of brain-derived tau aggregates (labelled with Amytracker).

Different species of tau may vary in terms of astrocyte-mediated toxicity. To test this we utilized the same seeding assay as previously described. We noticed a positive signal in all groups that was consistent over time, supporting the notion that astrocytes continuously excrete seeding competent tau species. Additionally, exposing neurons to ACM from tau exposed astrocytes allowed us to assess neurotoxicity. Our result show that neurons exposed to ACM from astrocytes containing AD fibrils reduced the number of synaptophysin positive puncta (**Fig. 17 a**). These data highlight an AD dependent toxic effect, since synaptophysin levels in neurons exposed to control ACM were comparable to those of unexposed neurons (**Fig. 17 a**). Furthermore, ATP concentration followed the same trend, with a significant decrease observed for both fibril types. However, ACM from astrocytes with AD fibrils resulted in the lowest ATP concentrations (**Fig. 17 b**), which was in line with the synaptophysin result. Moreover, astrocytes exposed to AD fibrils secreted higher levels of the cytokines IL-8 and CCL2 (**Fig. 17 c**). Together, our data show that fibrils derived from AD brain induce more of a toxic effect in surrounding neurons, and a stronger inflammatory response in astrocytes.

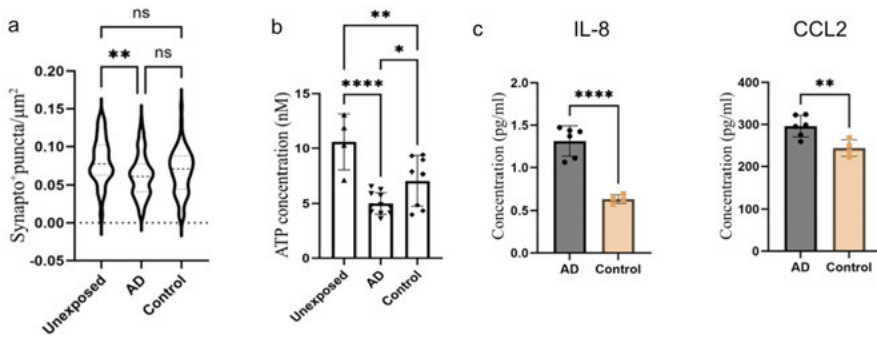


Figure 17. AD fibrils promote astrocyte neurotoxicity and induce a stronger inflammatory profile than control fibrils. **(a)** Quantification of synaptophysin positive puncta and **(b)** ATP concentration in neurons following ACM exposure. **(c)** Concentration of cytokines IL-8 and CCL2 in astrocyte medium following AD or control fibril exposure.

Paper IV

Variation in *APOE* is the greatest genetic risk factor for developing AD. Here we wanted to investigate how the *APOE* genotype influences astrocytes' ability to process recombinant tau fibrils. More specifically, we were interested in how it affects the astrocytes' ability to store/degrade tau aggregates, their inflammatory response as well as their potential neurotoxic effects. For this we utilized isogenic astrocyte lines, homozygous for *APOEε2* (*APOE 2/2*) and *APOEε4* (*APOE 4/4*) and exposed them to tau fibrils for up to 15 days. Our first observations revealed a striking difference between *APOE 2/2* and *APOE 4/4* cells. Aggregates were larger and more distinct in the *APOE 2/2* astrocytes, compared to the *APOE 4/4* astrocytes (**Fig. 18 a-b**). Quantification of the Cy3 signal confirmed an overall decrease over time for both genotypes. However, the Cy3 signal always remained higher in *APOE 2/2* astrocytes through the experiments (**Fig. 18 b**). Analysis of the ACM by ELISA showed that equivalent levels of tau are secreted from the two cell types, suggesting that this effect would be due to a greater degradation capacity of *APOE 4/4* astrocytes. To verify this result, we also performed WB analysis on cell lysates. The WB revealed no differences when comparing tau levels in the soluble fraction (**Fig. 18 c-d**). However, WB of the insoluble fraction revealed two tau positive bands in *APOE 2/2* astrocytes, while the second band was virtually non-existent in *APOE 4/4* astrocytes (**Fig. 18 e-f**). Together, this suggests that tau is stored and processed differently between the genotypes, where *APOEε2* favoured the accumulation of tau. This fits well with previous observations of *APOEε2* being a risk factor for developing certain primary tauopathies, like PSP⁴⁹. Thus, *APOEε2* seems to be mainly promoting tau pathology,

suggesting that perhaps *APOEε4* drives AD pathology by primarily influencing Aβ pathology.

Chronic inflammation is a hallmark of many neurodegenerative diseases. Astrocytes are also known to be involved in the inflammatory response but has historically been overlooked in that regard. Since ApoE affects cells in many ways, we wanted to investigate whether it had any effect on the astrocytes' ability to induce inflammation. To assess the astrocytic inflammatory response we analysed the cytokine concentrations in ACM from control and tau exposed *APOE 2/2* and *APOE 4/4* cells. Cytokine analysis revealed that IL-8, CCL2 and CXCL10 were released at high concentrations in the ACM. Interestingly, *APOE 2/2* astrocytes released significantly elevated levels of all three cytokines, compared *APOE 4/4* astrocytes (**Fig. 19**). However, there was little difference in cytokine concentrations between control and tau exposed cultures for both genotypes. We noted the same phenomenon in **Paper I**, where cytokine release appeared unaffected by exposure to *in vitro* α-syn and Aβ fibrils. With respect to inflammation, our data show that astrocytes release only certain subsets of cytokines and are largely unaffected by the accumulation of tau. However, some cytokines (IL-8, CCL2 and CXCL10) are released in high concentrations and strongly favoured by *APOEε2*, suggesting that astrocytes' role in inflammation may be more impactful in diseases where *AP- OEε2* is more prevalent.

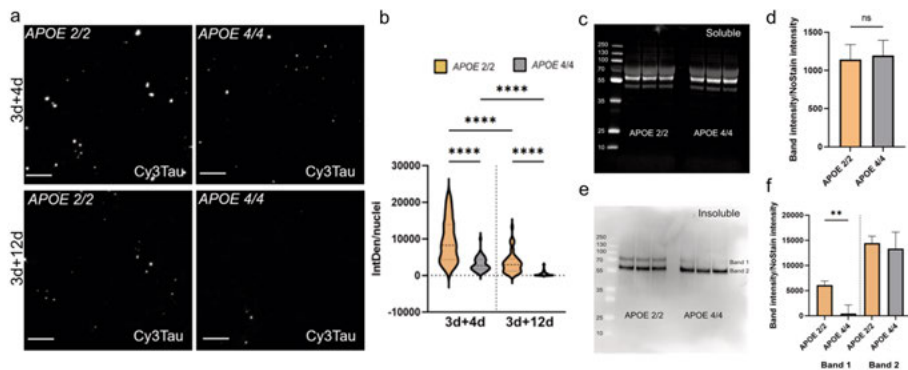


Figure 18. Astrocytic *APOEε2* and *APOEε4* genotype greatly affect the processing of internalized tau fibrils. (a) Images of Cy3-tau exposed *APOE 2/2* and *APOE 4/4* astrocytes (only the Cy3-channel) at 3d+4d and 3d+12d. (b) Quantification of signal in a. (c) WB of the soluble fraction of astrocyte lysates (stained for tau) (d) Intensity of bands in c. (e) WB of the insoluble fraction of astrocyte lysates. (f) Intensity of bands in e.

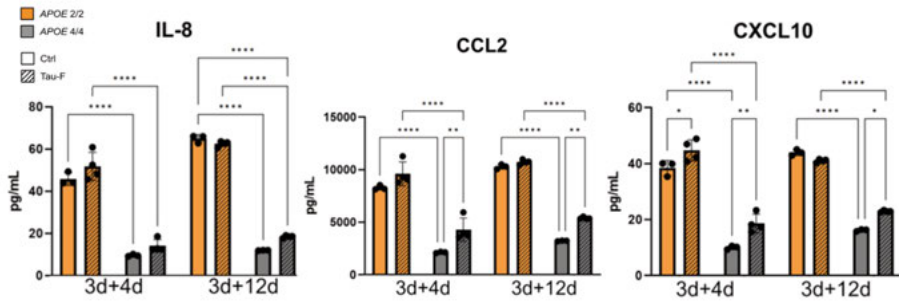


Figure 19. Cytokine concentrations (IL-8, CCL2 and CXCL10) in ACM from control and tau exposed *APOE 2/2* and *APOE 4/4* astrocytes over time.

Paper V

In this paper, we wanted to establish an organoid culture system to study pathological processes of neurodegenerative diseases. The first step was to investigate how the protein aggregates (α -syn, $A\beta$ and tau) traverse the organoid tissue. To do this we exposed individual organoids to fluorescent aggregates (direct) or to astrocytes containing fluorescent protein inclusions (astrocyte-mediated) (**Fig. 20 a**). Microscopy of the organoid tissue after 1-4 weeks of incubation revealed large differences between the different protein aggregates. Direct exposure to α -syn resulted in very distinct inclusions inside the cells of the organoid (**Fig. 20 b**), while astrocyte-mediated tau exposure revealed what appeared as cellular projections, invading the organoid tissue (**Fig. 20 b**). Analysis of cross-sections of the whole organoids allowed us to determine the average penetration of the fluorescent protein aggregates. One week of incubation with α -syn resulted in little penetration, where most of the Cy3 signal was located by the outer region of the organoid tissue (**Fig. 21**). Remarkably, this shifted significantly over time. Organoids incubated for four weeks had a much more even distribution of Cy3- α -syn (**Fig. 21**). This could easily be visualised by plotting the data as a percentage of the total Cy3 signal. The penetration was especially evident with α -syn. Interestingly, we noticed what appeared to be an active transport between connected regions of the organoid. Four weeks of α -syn exposure revealed a Cy3 positive central channel, suggesting something other than general diffusion through the tissue. This fits well considering that we observed α -syn internalised within the cells of the organoid (**Fig. 20 b**). Astrocyte mediated α -syn did penetrate the organoid as well, but much slower than the free aggregates. Similar trends of penetration were observed for direct and astrocyte-mediated, $A\beta$ and tau. As a whole, this model show that protein aggregates traverse organoid tissue both as free aggregates and as astrocytic inclusions. α -syn was especially affected by the mode of transport, where free aggregates much more easily traversed the tissue.

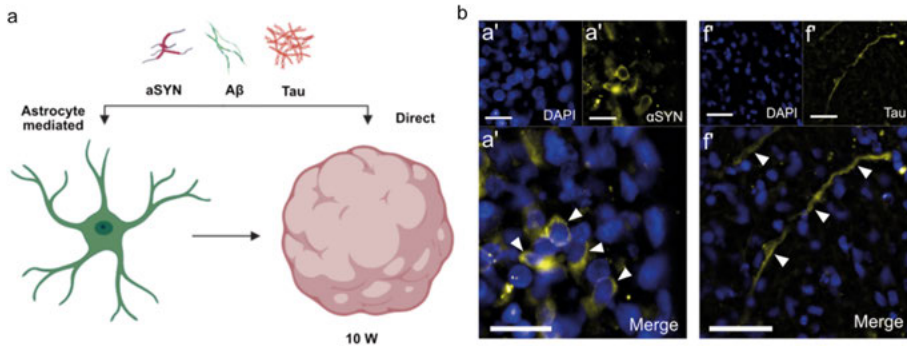


Figure 20. (a) Schematic outline of the experimental setup. Organoids exposed to aggregates (direct), or astrocytes with protein inclusions (astrocyte-mediated). (b) Example of cellular inclusions in organoids following direct α -syn and astrocyte-mediated tau exposure.

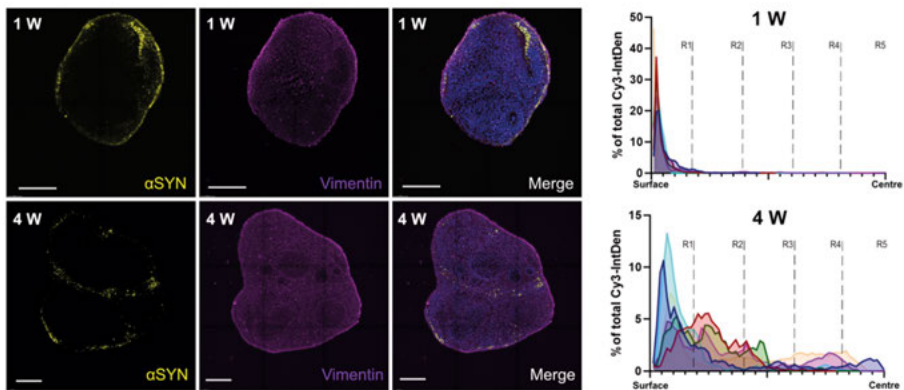


Figure 21. Distribution of α -syn aggregates one and four weeks following direct exposure.

Concluding remarks and future perspectives

This thesis focuses on the glial cell component of neurodegenerative disease. Taken together, the results indicate that astrocytes are highly involved in processing and spreading of pathological proteins to both neighbouring neurons and other astrocytes. Moreover, astrocytes work together with microglia to degrade protein aggregates in a synergistic manner. They also play an active role in the production of pro-inflammatory cytokines and directly affect the health of neurons. Nevertheless, many questions remain to fully understand the role of astrocytes in disease progression; some of these are discussed here.

Throughout this thesis, we show that astrocytes engulf and accumulate protein aggregates and neuronal debris, but mostly fail to degrade the internalised material (**Fig. 22 a**). However, astrocytes in co-culture with microglia work in synergy to more effectively degrade protein aggregates than each cell type individually (**Fig. 22 b-c**). This highlights the importance of multiple cell types with respects to disease, and that effects on one cell type likely affect other cell types downstream. To understand how the microglia-astrocyte interaction influence processes like uptake, degradation and seeding would be highly valuable to the field in general. Considering our findings of glial cross-talk, it would be interesting to investigate how the astrocytes and microglia interact with other immune cells, such as invading T-cells. Peripheral immune cells are known to infiltrate the brain parenchyma in AD and other conditions, but the astrocytes' role in that process is understudied. We show that astrocytes produce and excrete certain types of cytokine, such as IL-8 and CCL2 (**Fig. 22 d**). Both of these are known to promote infiltration and mobility of immune cells (**Fig. 22 e**). Moreover, since *APOEε2* seems to promote the release of these cytokines it would be informative to investigate whether the number of T-cells differ in patient brains, dependent on their *APOE*-genotype. Granted, this may be difficult to do in practice due to the low prevalence of patients homozygous for *APOEε2*. Alternatively, *APOEε2* and *APOEε4* homozygous organoids could be used for studying microglia and T-cell invasion *in vitro*. An iPSC system would allow us to culture immunologically compatible T-cells to be used for this purpose. In this regard, organoid cell cultures has an enormous potential. Simpler *in vitro* experiments using migration assays would also be of great value. Culturing astrocytes and T-cells in connected chambers would provide valuable information on how this affects the motility of infiltrating T-cells. Moreover, this setup would also make it easier

to apply methods like flow cytometry to investigate cellular activation of those T-cells. The same experiments could naturally also be conducted with microglia instead of T-cells.

Amyloid species can vary greatly between each other, even if they consist of the same protein. This was apparent when we compared different fibril types of tau in this thesis. Tau fibrils derived from brain tissue had for instance the characteristic PHF appearance, while the synthetic tau did not. Fibrils made *in vitro* also appeared to be degraded more easily by astrocytes (most clearly displayed in Paper IV), compared to brain derived fibrils. It is conceivable that this is because certain protein folds are more resistant to cellular degradation. Furthermore, synthetic tau seemed to gain seeding efficiency when processed and excreted from astrocytes relative the native aggregate, in a way that brain derived tau did not (**Fig. 22 g**). This makes it so synthetic tau fibrils appear more malleable by astrocytes. This could potentially be because brain derived tau is more stable (possibly due to prior glial processing in the brain) and thus less easily influenced by astrocytic modification. Further investigation of how astrocytes modify the internalised protein aggregates would be most interesting. A potential way to perform such a study would be to extract tau from astrocytes using magnetic beads that are ingested together with the tau fibrils. Cryo-EM and mass spectrometry analysis of the extracted fibrils would be very informative with respect to astrocytic modifications. This would shine some light on whether astrocytes promote pathology by directly altering the fibrils themselves. Such a study could also include brain derived α -syn and A β , or tau derived from different tauopathies. Moreover, techniques like single cell RNA sequencing that can sift through large amounts of data regarding many different pathways would be very useful to compare astrocytic response to different types of fibrils.

Another topic that would be interesting to pursue is linked to the various transfer mechanisms discussed in the different papers. Astrocytes are highly effective in transferring material between each other through the release of EVs and the formation of TNTs (**Fig. 22 c, f**). It will require more research to investigate whether these processes are beneficial or detrimental. The astrocytes' distribution of toxic protein species may in fact be a protective mechanism, by essentially sharing the burden of heavily affected areas. However, amyloid resistant to degradation will eventually accumulate to critical levels, simply becoming too much for the cells to handle. Inhibiting TNT formation (for instance by siRNA²⁶⁴), and/or the EV release²⁶⁵, would be useful in determining the relevance of amyloid transfer between astrocytes and other cells. The use of such inhibitors would also allow us to estimate which transfer method is more important and if it differs over time. Plenty of research papers have pointed out differences in cellular processes between 2D mono-cultures and more complex 3D cultures, consisting of multiple cell types. Thus, organ-

oids is the logical next step in order to investigate determine whether astrocytic inclusions can promote pathology in a more physiological setting. Another avenue of inquiry would be to compare astrocytes with and without the presence of microglia within the organoid.

In conclusion, astrocytes are highly interconnected, sharing material with each other and other cell types for better or worse, meaning that astrocytes likely act in both a protective and a destructive way during pathogenesis. It is an important distinction to make in order to enable the development of astrocyte directed medications in the future.

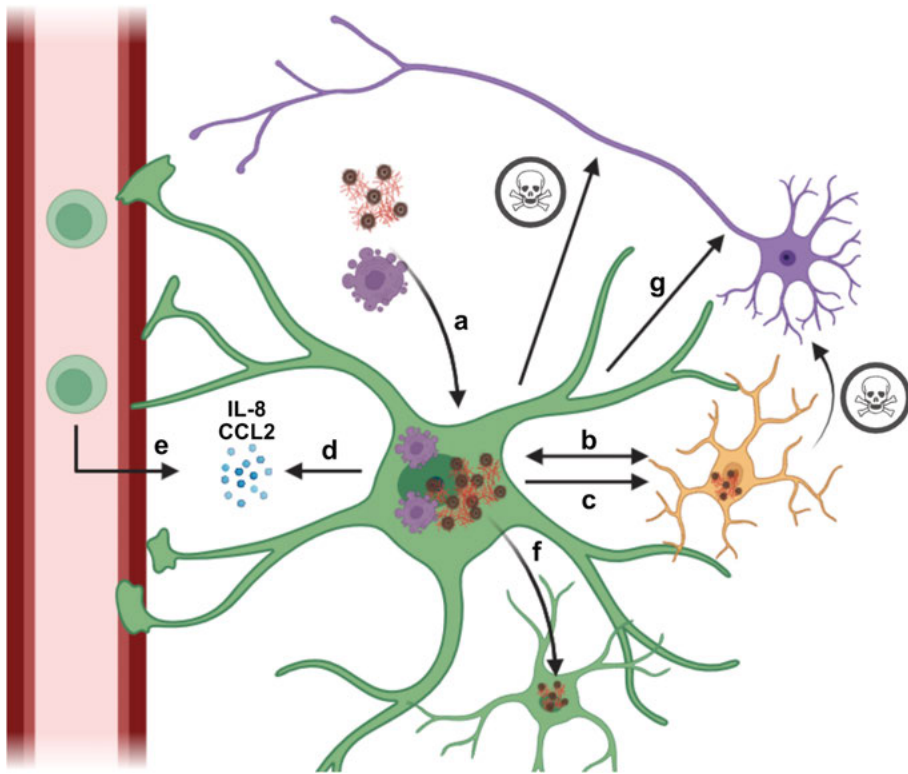


Figure 22. Possible mechanisms by which astrocytes contributes to neurodegeneration. **(a)** Astrocytes engulf both dead neurons (Paper II) and protein aggregates (Paper I-V) that accumulate intracellularly over time. **(b)** Astrocyte and microglial crosstalk boosts both cells' degradation capacity (Paper I). **(c)** Internalised protein aggregates are transported from astrocytes to microglia (Paper I). **(d)** Astrocytes excrete a subset of cytokines, (Paper I, III-IV), possibly leading to **(e)** subsequent infiltration of peripheral immune cells. **(f)** Astrocytes effectively distribute internalised protein aggregates between each (and to microglia) by excretion of EVs and via TNTs (Paper II-IV). **(g)** Release of highly seeding prone tau species show a non-neuronal progression mechanism within the astrocyte network (Paper II-IV). Furthermore, compromised astrocytes negatively affect the neuronal health (Paper II-IV).

Populärvetenskaplig sammanfattning

Neurodegenerativa sjukdomar är ett samlingsbegrepp för flera olika tillstånd som karakteriseras av en gradvis förlust av nervceller. Över tid leder döden av hjärnans celler till en försämrad hjärnfunktion. Vilka symptom som uppstår beror på vilka områden i hjärnan som påverkas. I nuläget uppskattas över 50 miljoner människor leva med Alzheimers sjukdom och ytterligare 7 miljoner med Parkinsons sjukdom. Alzheimers sjukdom är den vanligaste orsaken till demens och orsakar stort lidande för både den sjuka individen och dess närmaste anhöriga. Demensen beror på att nervcellerna i hjärnans minnescentrum (hippocampus) förtvinar. Anledningen till varför detta händer är inte helt känt men det finns många hypoteser som baseras på årtionden av forskning. Den bästa förklaringen är att vissa proteiner veckas fel och formar en typ av giftiga aggregat som kallas amyloid. Amyloid ansamlas i hjärnan över årtionden och skadar succesivt närliggande celler. I Alzheimer-hjärnan hittar man huvudsak två olika typer av aggregat. De första är plack, som består av proteinet beta-amyloid. Den andra typen av aggregat kallas för ”tangles” och består av proteinet tau. Felveckade varianter av beta-amyloid och tau har en förmåga att orsakar felveckning av det normala proteinet. Detta leder till en kedjereaktion som sprider sig igenom hjärnan. I synnerhet tau sprids på ett förutsägbart sätt, där aggregeringen börjar i hjärnstammen och sedan sprids via nervcellsbanor till hippocampus, och därefter vidare till resten utav hjärnan. I Parkinson hjärnan är det istället proteinet alfa-synuclein som utgör aggregat som kallas Lewykroppar.

Utöver nervceller består hjärnan också av olika stödjeceller, så kallade ”glia”celler. Det finns flera olika typer av gliaceller som är specialiserade för olika funktioner. De två vanligaste är astrocyter och mikroglia. Astrocyter är involverade i näst intill alla funktioner i hjärnan, såsom uppbyggnad av blod-hjärn barriären samt allmänt stöd för nervceller. Mikroglia är istället något mer specialiserade som hjärnans immunförsvar. Historiskt har forskningen fokuserat på nervcellerna men fler studier har visat att även gliacellerna är högst involverade under sjukdomsförloppen. Idag är de flesta behandlingarna riktade mot att dämpa symptomen och gör ingenting för att bota själva sjukdomen. Studier med fokus på gliaceller utgör nya spännande områden som förhoppningsvis kan leda till bättre behandlingsmöjligheter i framtiden.

Min avhandling är fokuserad på hur hjärnans stödjeceller bidrar till sjukdomsförloppen av neurodegenerativa sjukdomar. I projekt **I** fokuserade vi på hur astrocyter och mikroglia hjälper varandra för att bryta ner beta-amyloid och alfa-synuklein. Våra resultat tyder på att mikroglia är generellt bättre på att bryta ner proteinaggregat än astrocyter. Vi visar också att båda celltyperna hjälps åt för att mer effektivt göra sig av med amyloid-ansamlingar. I projekt **II-IV** intresserade vi oss istället för hur astrocyter behandlar tau-proteinet. I dessa studier visar vi att astrocyter ansamlar stora mängder av tau som påverkar deras förmåga att hjälpa nervceller. Genom att använda en ”seeding assay” kunde vi visa att astrocyter konstant utsöndrar tau aggregat som i sin tur kan orsaka felveckning av ”friska” proteiner och därmed sprida sjukdomen vidare. Astrocyter behandlade med tau verkade också negativt påverka funktionen av närliggande nervceller. I projekt **IV**, studerade vi den största genetiska riskfaktorn för utveckling av Alzheimers sjukdom. Vi använde genetiskt modifierade celler för att påvisa att olika varianter av genen *APOE* drastiskt påverkar astrocyters förmåga att hantera tau proteinet. I projekt **V** utvecklade vi ett organoidsystem för att studera spridning av amyloid i en mer hjärnlik miljö. Jämfört med enskilda celler i kultur har organoider fördelen att de beter sig mer som cellerna i hjärnan. Vid odlingen av organoider återskapar vi hjärnans naturliga utvecklings process och får då en bättre modell för sjukdomarna. Med denna modell kunde vi visa att både tau, beta-amyloid och alfa-synuklein kan färdas genom vävnaden. I synnerhet alfa-synuklein verkar aktivt spridas via organoidens celler.

Sammanlagt visar denna avhandling att astrocyter och mikroglia arbetar mer effektivt tillsammans. Astrocyter är dåliga på att rensa bort ”skräp” och sprider aktivt amyloid vidare till andra astrocyter och till nervceller. Trots att vi observerat flera potentiellt viktiga funktioner av gliaceller som kan påverka sjukdomsförloppet återstår fortfarande många frågor. Till exempel är det viktigt att förstå exakt vilka mekanismer som ligger bakom spridning och nedbrytning av amyloid-proteiner, samt hur man eventuellt skulle kunna påverka dem. Framtida studier med ett sådant fokus är av stort intresse för utvecklingen av nya läkemedel.

Acknowledgements

First, I'd like to acknowledge the financial support that made the work in this thesis possible. A great many thanks to the Vetenskapsrådet, Alzheimerfonden, Parkinsonfonden, Hjärnfonden, Åhlén-stiftelsen, Stohnes Stiftelse, Gamla Tjänarinnor, Torsten Söderbergs Stiftelse, Olle Engkvist Stiftelse, Edla Johanssons Stiftelse, Sävstaholms Stiftelse and Stiftelsen Forska Utan Djur for funding the research.

This next bit goes out to my supervisors! **Linn**, you were my most hands-on tutor early in my PhD journey. That time without a doubt had an enormous impact on my scientific thinking and lab skills, which will continue to define my career. And you **Martin**! I've always admired you as an incredibly intelligent and kind person. It has truly been a privilege to learn from you and I wish you all the best with your life in Canada. This goes to the both of you... I only wished that we'd had more time to work together but alas that's how it goes. Perhaps we will at some future date. Now where do I even begin with you **Anna**? You have taught me so much throughout the years and I can't imagine a better supervisor. It was such a momentous moment in my life when you decided to take a chance and hire me as a PhD student. You've been so incredibly supportive even when I felt I didn't deserve it and I can truly say that you've definitely change me for the better! Thank you so much for that. These were truly some of the best years of my life, in great part thanks to you. I'd also take this opportunity to thank **Fredrik** for introducing us, and serving as my first real introduction to science.

To the remaining tribe elders. **Stina** and **Dag**, a true PI dream-team! Even though you haven't been involved in my research directly you have for sure made an impression on me. My guess without knowledge, but you've taught me a bunch throughout the years. Never doubt that! And **Dag**, I'll keep swinging by lab for some snus and pipe smoking to be sure! According to von Clausewitz, a well-disciplined force prevails. Many thanks to **Ximena** for keeping the troops in line. Good general practice is something you've taught me a great deal of, which will serve me well in the future for sure. **Sahar**! I know I'm not the only one that noticed your absence when you left the lab. Was always hilarious having you around. **Vilmantas** and **Jocke** same goes for you two! Always enjoyable to have a chat about anything and nothing. To the

youngest PI, **Wojciech**. I must thank you firstly for teaching me how to culture organoids. I'm confident that your legacy will be legendary!

To the current and former members of the Astro-group. **Jinar**, you are a true role model for what to strive for as a scientist. I remember being so impressed during your defence, and felt very motivated by it. On top of personifying a great scientist, you are also one of the nicest people I've ever met. You'll be a grand MD I'm sure! **Evangelos** and **Chiara**, I wish you luck in your future endeavours. Both of yah are destined for greatness! The kiddos won't stand a chance. **Abdul**! You're the future of the astro-group and I'm confident you'll make us all very proud. Also happy, I managed turn your heart green. Forza Bajen! It has truly been a pleasure to work with you **Khalid**. You've have been a fantastic addition to both the group as a whole and this thesis in particular. Super proud of your work and have really appreciated our scientific and philosophical discussions. I hope that we can stay in touch and discuss our favourite books!

Now to our younglings... **Amelia**, **Elin** and **Sona** you've been such a nice addition to the group, both scientifically and socially. I've no doubt that you'll completely smash it when your time comes! Hope I can join the parties! They say people come and go, and nowhere is that more true than in science. To all former members of MolGer! **Marlena**, **Agnieszka**, **Anish**, **Tobbe G**, **Silvio**, **Rebecca**, **Emma**, **Maria**, **Ulrika** and **Mengfei** it has been a pleasure! Thank you all for contributing to a great atmosphere and a fantastic place to work. By the way **Anish**! I don't think tau-infused beer is such a good idea... I'll make something else! **Benjamin** and **Mia**, it has been such a pleasure to work together with you. Thank you so much for a fantastic addition to my thesis!

I'd also like to thank all members at **BioVis**, which provided invaluable support in the makings of this thesis. Thanks to **Karin** and **Monica** for assisting us with electron microscopy. Head of the unit, **Pacho** for helping us with Flow cytometry and to **Jeremy** for all the aid with image analysis and in the creation of macros. All of these features were instrumental for the thesis as a whole.

N. Machiavelli said that a leader's wisdom is measured by his choice of ministers. I may not strive for world conquest, but nonetheless have chosen my friends carefully. Old and new, scientists or no, this section is for you. **Gillian** and **Eva**, we came into lab around the same time. What a ride it's been! We've been through it all a few times over and really happy to call you friends. May there be many more nights of sports and games in the future! It was only chance that brought us together **Sara**... and I'm so happy it did! You've become one of my closest friends in a very short time and can't believe my luck that we now live in the same town! The whole situation is the very definition of "livet på en pinne"! **Tiffany** the same goes for you! Even though it wasn't that long ago we first met it feels like I've known you for years. I'm especially

thankful for introducing me to **Dave**! Always a great time watching sports and going for runs. Hope for much more to come! **Viktor** you also became a very close friend in a very short time. You're definitely a friend I can count on and I'm super happy to have you in my life.

To some of my oldest friends **Alice** and **Marcus**. We have in a sense grown up together, so you know me more intimately than most. Though, we don't meet that often, it's always something I look forward to! My mate, **Ernie**! I still remember the very instance we fell in love. Across the table at Värmlands nation, you asked me the simplest of questions... "Är du Bajare?", and the rest is history. Didn't know it at the time but just made a friend for life!

The rest of my old University Posse. My time at Uni wouldn't have been the same without **Jerkan**, **Floris**, **Hanki**, **Lois**, **Julle**, **Heddahedis** and **Ida** (and of course **Alex** by proxy).

To my boys, boys, boys! **Johan**, **Philip**, **Andreas**, **Gustav**, **Sebbe** and **Kalle** for allowing me into their inner circle. Mates! For all the dancing, poker, sports and nights out... Let's keep it up!

Also, for obvious reasons I would like to thank **Kennedy** and every other member of **HIF**, past and present, and every one of the original **Dalarö** crew.

To my partner **Sofia**. There are too many things for me to put into text here so I shall not even try. However, I'll say this! Really appreciated you taking up some cooking responsibilities during the writing phase! Nevertheless, you don't have to worry! I'll be back in the kitchen shortly.

Sist till min närmsta familj. **Mamma**, **Pappa** och lille-bror **Jonathan**. Ni var aldrig speciellt involverade i min utbildning, något som jag verkligen uppskattat. Jag fick alltid valet att gå min egen väg och hitta det som intresserade mig. Tack för all stöd under åren och hoppas ni gillar populärvetenskapliga sammanfattningen.

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