Prion-like Proteins in Neurodegeneration

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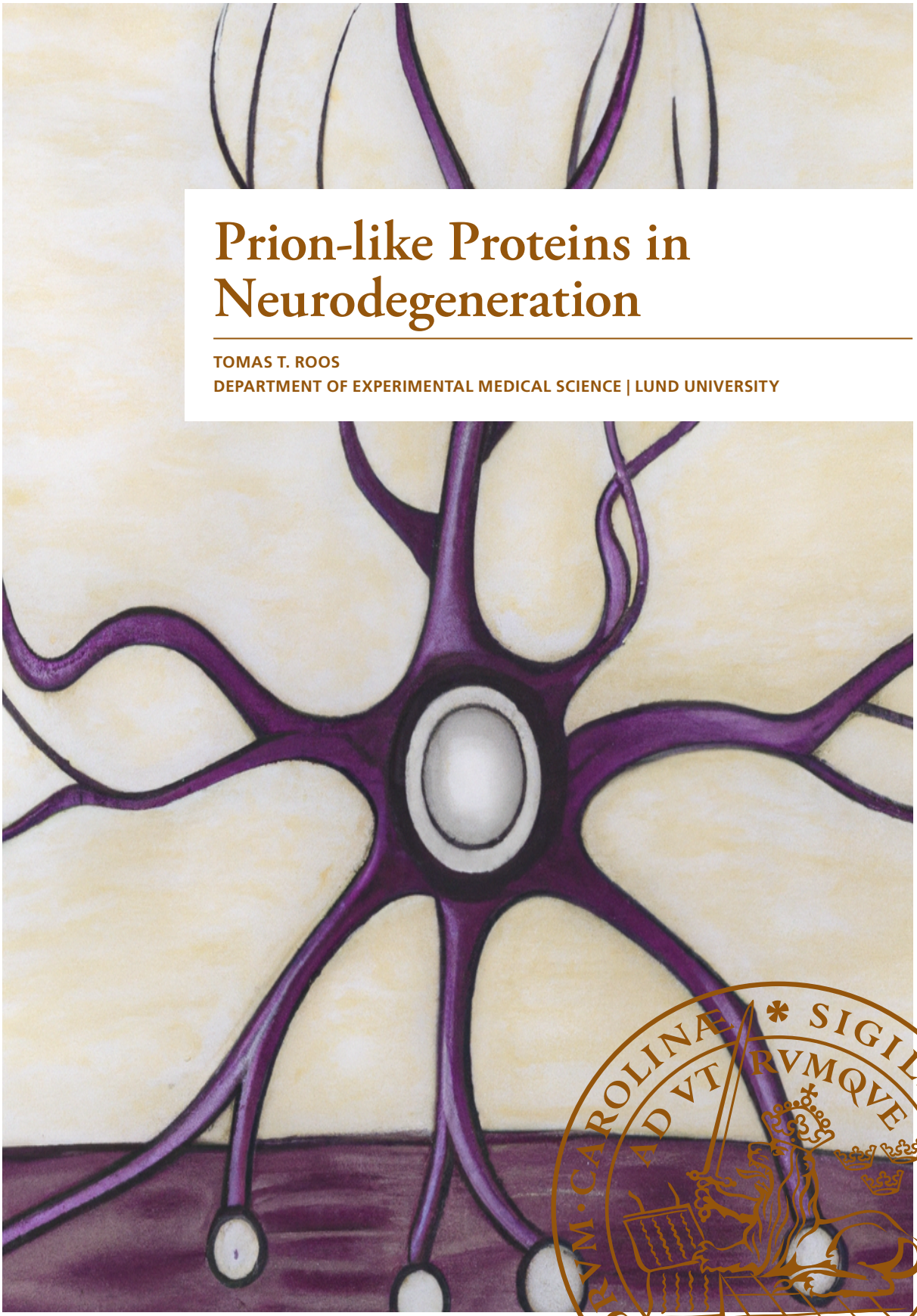

Prion-like Proteins in Neurodegeneration

TOMAS T. ROOS
DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY

Prion-like Proteins in Neurodegeneration

Tomas T. Roos

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the faculty of medicine at Lund University to be publicly defended 17th of February at 13:00 in Belfragesalen, Department of Experimental medical science, Sölvegatan 19, BMC I13, 221 84 Lund

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Title and subtitle: Prion-like Proteins in Neurodegeneration

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In paper I we developed a cellular model to quantify intercellular spread of alpha-synuclein. This system could be used to screen for compounds that inhibit intercellular spread of alpha-synuclein, a potential treatment against PD.

In paper II we show, for the first time, that prion-like Aβ can exist within a cell. Aβ plaques are extracellular so intracellular Aβ is easily overlooked. But here we argue that prion-like intracellular Aβ likely precedes plaques. Paper III is a continuation of paper II. We show that intracellular Aβ is capable of prion-like spread in an AD mouse model and that prion-like spread of Aβ cause extensive intracellular changes, partially preceding plaque induction. We also show that aggregated intracellular Aβ increases cellular production of Aβ, which may partly explain the massive Aβ increase in AD.

In paper IV we study the earliest plaques and earliest intracellular Aβ in a mouse model of AD and relate that to brain anatomy, inflammation and glia.

This research indicates that intracellular prion-like Aβ may play an essential role in the early pathogenesis of AD and could be a target for disease-modifying treatments.

Key words: Alzheimer's disease, Parkinson's disease, prion-like, amyloid-beta, intracelllular and alpha-synuclein

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Tomas T. Roos

LUND
UNIVERSITY

Coverphoto by DALL-E2, prompt: “A painting of a neuron in the style of Hilma af klint in gray and purple”.

Back cover: Primary neurons stained with MAP2 (purple) and Aβ (green).

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To my children

Table of Contents

Abstract

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most and second most common neurodegenerative disorders. There are no disease-modifying treatments for either disease.

Both diseases seem to be caused by proteins aggregating in the brain. In AD there are extracellular aggregates of amyloid-beta (Aβ plaques) and intracellular aggregates of tau (neurofibrillary tangles), and in PD intracellular aggregates of mainly alpha-synuclein (Lewy bodies). In this thesis we try to understand how these proteins aggregate and how the aggregates can spread throughout the brain. We use the paradigm of the prion-like hypothesis of aggregating proteins. If a protein misfolds to a prion-like state it can induce misfolding of proteins of the same type and thereby spread.

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Populärvetenskaplig sammanfattning

Alzheimers och Parkinsons sjukdom är de vanligaste och näst vanligaste neurodegenerativa sjukdomarna som globalt drabbar ca 30 respektive 6 miljoner människor. I och med vår åldrande befolkning kommer det antalet bara att öka. Alzheimers är en demenssjukdom som börjar med minnessvårigheter och slutar med en svårt dement patient där även grundläggande språk och motoriska funktioner är påverkade vilket gör patienten helt beroende av andra.

Parkinsons drabbar först motoriken och beror på dopaminbrist i hjärnans basala ganglier. Med tiden blir motorikstörningen mer och mer svårbehandlad och andra symtom tillkommer såsom sömnstörning, depression och kognitiv nedsättning. Det finns tämligen dålig symtomatisk behandling mot Alzheimers (främst acetylkolinesterashämmare) och tämligen bra symtomatisk behandling mot Parkinsons (Levodopa), men det finns ingen behandling som stoppar sjukdomsförloppet vid något av tillstånden. Dessa sjukdomar innebär alltså ett enormt lidande för de drabbade och deras närstående och en enorm kostnad för samhället.

Båda sjukdomarna verkar bero på att proteiner/peptider klumpar ihop sig i hjärnan. Vid Alzheimers ser man stora ansamlingar av amyloid-beta (Aβ) utanför nervcellerna, s.k. plack, samt ansamlingar av proteinet tau s.k. neurofibrillära trassel, inuti nervcellerna och vid Parkinsons ser man ansamlingar av alpha-synuclein, s.k. Lewy bodies, inuti nervcellerna. I denna avhandling försöker vi förstå hur dessa sjukliga proteinansamlingar startar och sprider sig genom hjärnan. Vi har fokus på den prion-liknande hypotesen vad gäller proteinansamlingarna. Enligt denna kan vissa proteiner, t.ex. Aβ, felvecka sig på ett sätt som gör att det kan sprida sin felveckning till andra protein av samma typ.

I det första arbetet utvecklade vi en modell för hur alpha-synuclein kan sprida sig mellan celler och hur det eventuellt skulle kunna stoppas. Utmaningen var att specifikt stoppa alpha-synucleins spridning mellan celler och inte andra protein.

I det andra arbetet visade vi för första gången att prion-liknande Aβ kunde finnas inuti celler. Detta är överraskande för många eftersom Aβ placken är utanför nervcellerna. Det pekar på en väldigt tidig start av Alzheimers sjukdomsprocess och att placken kanske är mindre viktiga. Den tredje studien var en utveckling av den andra. Vi visade att intracellulärt prion-liknande Aβ kunde ses i en musmodell av Alzheimers sjukdom och att ansamling av intracellulärt Aβ ökar dess produktion, en potentiell ond spiral alltså.

Slutligen, i den fjärde studien tittade vi på de tidigaste placken och den tidigaste ansamlingen av Aβ inuti celler och hur de relaterade till hjärncellernas kopplingar (synapser), inflammation och glia (celler i hjärnan som inte är nervceller).

Sammantaget, indikerar våra studier att Alzheimers sjukdom kanske startar med uppkomsten av prion-liknande Aβ inuti en cell vilket har konsekvenser för hur och när man skulle vilja behandla Alzheimers sjukdom.

List of Papers

Paper I

Reyes, J. F., **Olsson, T. T**., Lamberts, J. T., Devine, M. J., Kunath, T., & Brundin, P. (2015). A cell culture model for monitoring α-synuclein cell-to-cell transfer. *Neurobiology of disease*, *77*, 266–275.

Paper II

Olsson, T. T., Klementieva, O., & Gouras, G. K. (2018). Prion-like seeding and nucleation of intracellular amyloid-β. *Neurobiology of disease*, *113*, 1–10.

Paper III

Roos, T. T., Garcia, M. G., Martinsson, I., Mabrouk, R., Israelsson, B., Deierborg, T., Kobro-Flatmoen, A., Tanila, H., & Gouras, G. K. (2021). Neuronal spreading and plaque induction of intracellular Aβ and its disruption of Aβ homeostasis. *Acta neuropathologica*, *142*(4), 669–687.

Paper IV

Megg G. Garcia, **Tomas T. Roos**, Luis Quintino, Bodil Israelsson, Cecilia Lundberg, Sara Bachiller, Tomas Deierborg, Gunnar K. Gouras. Modeling pre-plaque amyloid-β and associated pathology in connected brain regions in 5xFAD Alzheimer's disease-transgenic mice. *Manuscript in preparation.*

List of papers outside the scope of this thesis

Petit, G. H., **Olsson, T. T.**, & Brundin, P. (2014). The future of cell therapies and brain repair: Parkinson's disease leads the way. *Neuropathology and applied neurobiology*, *40*(1), 60–70.

Gouras, G. K., **Olsson, T. T.**, & Hansson, O. (2015). β-Amyloid peptides and amyloid plaques in Alzheimer's disease. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*, *12*(1), 3–11.

Olsson, T. T., Svensson, M., Hållmarker, U., James, S., & Deierborg, T. (2020). Delayed Clinical Manifestation of Parkinson's Disease Among Physically Active: Do Participants in a Long-Distance Ski Race Have a Motor Reserve?. *Journal of Parkinson's disease*, *10*(1), 267–274.

Tomas Roos Startar Alzheimers sjukdom inuti nervcellerna? Neurologi i Sverige nr 1-22

Note: I changed my name from Tomas Olsson to Tomas Roos in 2020, after my marriage.

Abbreviations

AD: Alzheimer's disease

PD: Parkinson's disease

NNT: Number needed to treat

Aβ: Amyloid-beta

APP: Amyloid precursor protein

sAPP: Soluble APP (alpha or beta-cleavage of APP produces sAPP and CTF)

CTF: C-terminal fragment (gamma cleavage of CTF produces Aβ and p3)

FAD: Familial Alzheimer's disease

PSEN: Presenilin (a component of gamma secretase)

CSF: Cerebrospinal fluid

ErC: Entorhinal cortex

HD: Huntington's disease

N2a: Neuro2a (a cell-line)

SDS: Sodium dodecyl sulfate (detergent that denatures polypeptides)

PAGE: Polyacrylamide gel electrophoresis

SDD: Semi denaturing detergent

BN: Blue native

FTIR: Fourier transform infrared spectroscopy

GFP: Green fluorescent protein

YFP: Yellow fluorescent protein

5xFAD: A transgenic mouse that expresses 5 different familial AD mutations: APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L (A>C), PSEN1 L286V

APP/PSEN1: A transgenic mouse that has one mutation in APP, APP KM670/671NL (Swedish) and one in presenilin, PSEN1 L166P

Introduction

This is a work about neurodegenerative disorders. Mainly Alzheimer's disease (AD) but also Parkinson's disease (PD). More than 30 million people suffer from AD globally, at least 100 000 just in Sweden, and with an aging population that number will only increase. AD starts insidiously with forgetfulness that can be ascribed to normal aging. But memory continues to deteriorate, executive ability and orientation also become affected. At the end even basic language and motor skills are compromised and the patient is completely dependent upon others. William Utermohlen was a painter who in 1995 was diagnosed with AD at the age of 61. He drew portraits of himself until he was no longer able to. He died of pneumonia in 2007 but his wife said: *"Bill died in 2000, when the disease meant he was no longer able to draw."* [50]. Utermohlen's case is not unusual. While AD is not directly fatal, you are your brain and AD destroys your brain. The spirit departs before the body shuffles off the mortal coil.

Figure 1: Utermohlen's self-portraits vividly and literally illustrate the progressive cognitive decline in AD. Images courtesy of Chris Boïcos Fine Arts.

This progressive neurodegeneration x 30 000 000 means an incomprehensible amount of suffering for patients and their relatives and a huge economic burden for society.

Current treatments against Alzheimer's disease

There are approved treatments against AD but they are strictly symptomatic. The first-line treatment is acetylcholinesterase inhibitors which counteracts the enzyme that degrades acetylcholine, thereby increasing the amount of that neurotransmitter in the brain. The cholinergic neurons of the basal forebrain degenerate early in AD so it makes sense that increasing acetylcholine might help. But, the effect on cognition with this medication is modest. One needs to treat an average of 7 (number needed to treat, NNT) AD patients for one to experience some cognitive benefit [25]. An NMDA receptor antagonist called Memantine is also used in AD. NMDA is a receptor for glutamate which is the main excitatory neurotransmitter in the brain. Blocking the NMDA receptor may then ameliorate excitotoxicity in AD. Memantine has a similar modest to moderate efficiency NNT as cholinesterase inhibitors [31]. These treatments help with the symptoms of AD but they do not modify the course of the disease.

In the course of writing this thesis a phase 3 trial of an antibody against amyloid-beta, Lecanemab, showed promising results by reducing plaques and ameliorating cognitive decline [8]. It has not been approved yet but very likely will be. From that phase 3 trial the cognitive benefit was in the same ballpark as acetylcholinesterase inhibitors. But the hope is that Lecanemab would actually be a disease-modifying therapy meaning that the pace of cognitive decline would slow. Time and further studies will tell.

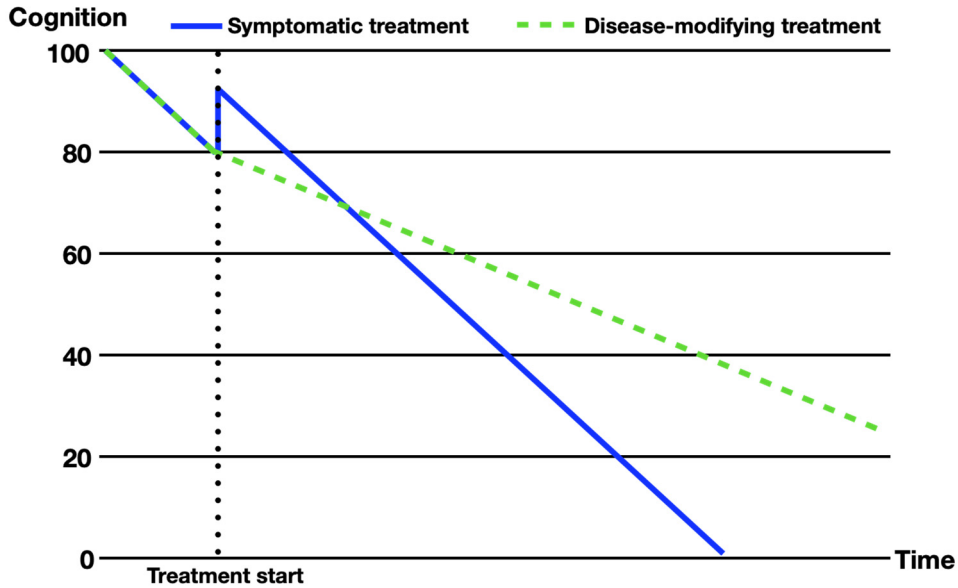


Figure 2: Cartoon model of symptomatic treatment (blue line) disease-modifying treatment (green dashed line). The former gives a one-time boost while the latter changes the trajectory of the disease leading to an increasing divergence between the green and blue line with time.

Parkinson's disease

PD affects about 6 million people globally, making it the second most common neurodegenerative disorder and its prevalence will also increase with the graying of the population. PD is mainly known as a motor disorder characterized by resting tremor, slow movement (bradykinesia), rigidity and postural instability. This is caused by degeneration of dopamine producing neurons in the substantia nigra which project to the striatum. PD also has many non-motors symptoms such as sleep disturbance, depression, reduced sense of smell, higher risk of dementia and autonomic dysfunction (PD can affect any function of the autonomic nervous system some examples are dysregulated blood pressure, constipation, urinary incontinence and dysregulation of body temperature). Like AD, PD is not directly fatal but it can severely affect quality of life and increase mortality from other causes. For example, PD may cause a fall causing a hip fracture causing immobility causing a pulmonary embolism leading to death. Unlike AD the gold standard symptomatic treatment against PD, levodopa, works very well, at least in the beginning. Levodopa can cross the blood brain barrier and once in the brain it is converted to dopamine. Early in PD the motor symptoms dominate and they are mainly caused by a dopamine deficiency in the striatum, levodopa fixes this. But

with disease progression dosing levodopa becomes more difficult as the substantia nigra loses its residual dopamine production and striato-nigral synapses deteriorate. Furthermore, levodopa induced dyskinesias (chorea and dystonia, that is sudden/erratic movements and abnormal muscle contractions) become a problem. This can be somewhat ameliorated with careful dosing of medications, or pumps that give a continuous dose of dopamine agonists. The non-motor symptoms are even harder to treat and treatment has to be tailored to the patient. Levodopa is an awesome drug but not a panacea, disease modifying treatments are sorely needed for PD as well.

Neuropathology of Alzheimer's and Parkinson's disease

Both PD and AD are neuropathologically characterized by protein aggregation. Extracellular plaques (plaques are insoluble fibrillar aggregates) of Amyloid-beta (Aβ) and intracellular tangles of microtubule associated protein tau in AD and intracellular Lewy body inclusions of alpha-synuclein in PD. In AD, the research has mainly been focused on Aβ. All dominantly inherited forms of AD are either due to the production of more Aβ or more aggregation-prone Aβ [54] and a gene variant that reduces Aβ production protects against AD [19]. The gene that produces Aβ is located on chromosome 21 and people with trisomy 21 (Down syndrome) invariably develop AD. Some but not all forms of dominantly inherited PD are caused by mutations in alpha-synuclein [23]. It should however be noted that inherited forms of both AD and PD are rare; most cases are caused by polygenic risk factors, environmental factors, unknown factors and most importantly advanced age. In AD Aβ is also the first biomarker to change, a decline of CSF Aβ42 can be detected decades before onset of dementia [6]. We thus have good evidence that Aβ is not only associated with AD but that it plays a causative role. In contrast, tau dysfunction, while important, seems to be downstream of Aβ.

This led to the formulation of the amyloid cascade hypothesis. That is that the deposition of amyloid in the brain is the trigger for tau dysfunction, synapse loss and finally neuron loss and dementia. There are problems with this hypothesis. The amount of amyloid plaques in the brain correlate only weakly to cognitive dysfunction, there are plenty of people with lots of plaques but no cognitive impairment [2]. Tau tangles on the other hand correlates both with severity of disease and the localization of tau tangles in the brain even correlates with the phenotype of the dementia [39]. Furthermore, many experimental therapies against Aβ have been developed. Many of them have successfully reduced Aβ levels and plaques. But they have all failed in their primary end-point, they have not ameliorated the cognitive decline of Alzheimer's dementia (the previously mentioned Lecanemab being a possible exception). Thus, we have a conundrum in

the field of AD. How do we square genetic and biomarker evidence pointing towards a clear causal role of Aβ with the seeming almost irrelevance of amyloid plaques? To begin to answer that question some background knowledge is necessary.

The production of Aβ

Strictly speaking Aβ is not a protein but a peptide, 37-43 amino acids long (Aβ can then be post-translationally truncated to various lengths). Aβ is cleaved from the much larger Amyloid Precursor Protein (APP) and from the name you understand which part of the protein scientists considered most important. APP is mainly processed in two different pathways: The amyloidogenic pathway that produces Aβ and the non-amyloidogenic that does not.

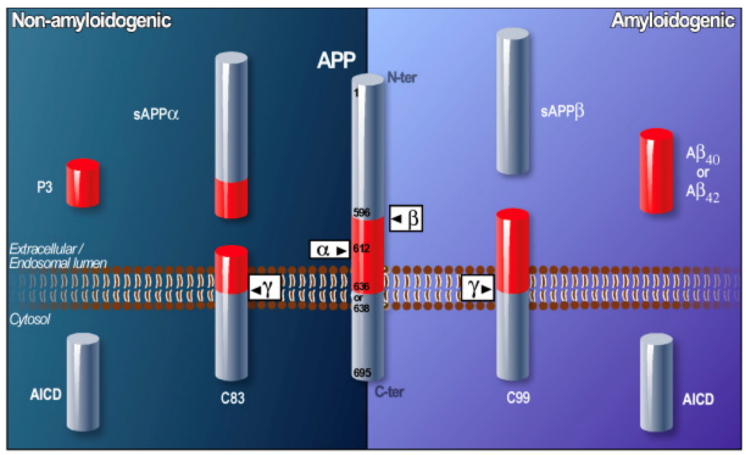


Figure 3: The two cleavage pathways of APP. Note that γ-clevage (gamma-cleavage) occurs within the phospholipid bilayer (picture from Bécot et al. 2020 [3] under creative commons license 4.0).

The non-amyloidogenic pathway mostly takes place at the cell surface where APP is cleaved in the Aβ region (between amino acid 16 and 17 of Aβ) by α-secretase which produces soluble (s)APPα and α-C-terminal fragment (CTF). α-CTF is then cleaved by γ-secretase to produce AICD (amyloid precursor protein intracellular domain) and the peptide p3. The amyloidogenic pathway mostly takes place in endosomes where APP is instead cleaved by β-secretase to produce sAPPβ and β-CTFs (β-CTFs is usually 99 amino acids long and can then also be called C99). Then, as in the non-amyloidogenic pathway β-CTF is also cleaved by γ-secretase here producing AICD and Aβ. This last step can happen both at the cell membrane or in endosomes. Thus, β-cleavage is the initial rate limiting step in the production of Aβ from APP. It should also be noted that α and β-cleavage compete with each

other [12]. Increasing α-cleavage should reduce β-cleavage and vice versa. APP is produced in many organ systems but only in brain is the amyloidogenic pathway favoured [12].

The γ-cleavage is not always at the same site of the CTF, leading to varying amino acid lengths of Aβ. The two most common are Aβ 1-40 and Aβ 1-42. It also seems that longer Aβ isoforms and intracellular Aβ are preferentially produced in acidic late endosomes/lysosomes [49]. Production of Aβ40 is much higher than Aβ42 but the latter aggregates easier and is thus thought to be pathologically more relevant. Indeed, if you quantify the Aβ of an AD brain, or even a non-AD brain, the vast majority will be Aβ42 [45] and the core of the amyloid plaque consists of Aβ42. Once formed Aβ can aggregate into soluble oligomers and insoluble fibrils. There are studies showing that Aβ (monomers and oligomers) may regulate synaptic function [51] but a definitive physiological role of Aβ has not been established.

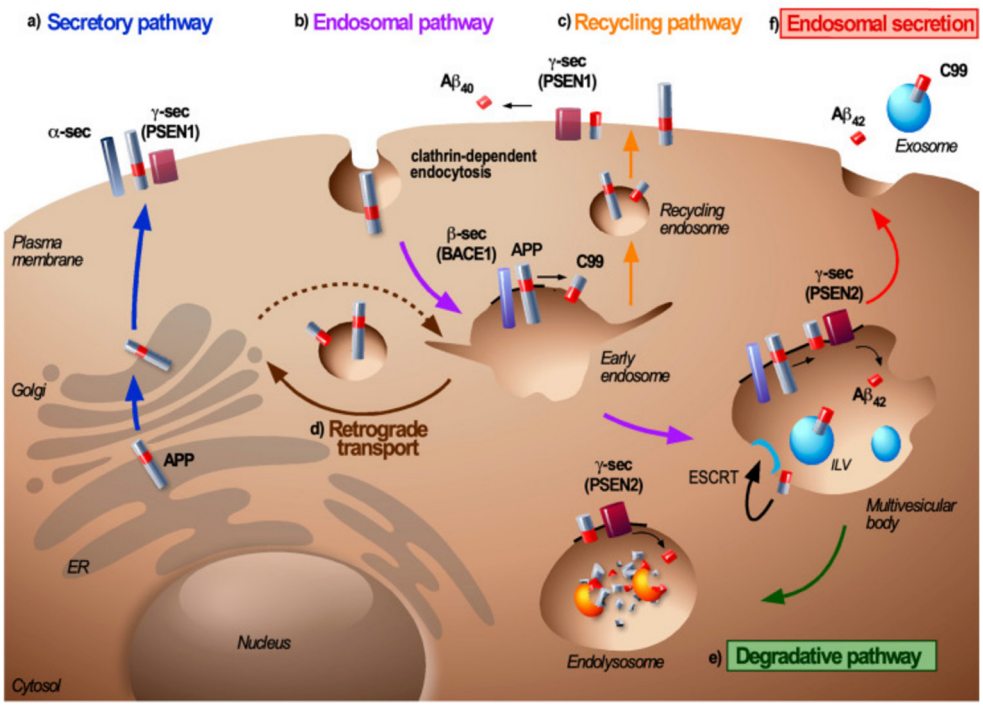


Figure 4: The subcellular localizations of Aβ production. Note that β-cleavage and Aβ production mostly happens in endo/lysosomes while α-cleavage is at the cell membrane. Picture adapted from Becot et al. 2020 [3] adapted under Creative Commons license 4.0.

Anatomy of Alzheimer's Disease

AD does not affect the entire brain equally. The hippocampus and medial temporal lobes are severely affected while, for example, the cerebellum is spared. The entorhinal cortex (ErC) is located in the medial temporal lobe and is the main connection between the hippocampus and neocortex. The hippocampus is essential for forming new memories. Arguably the most famous patient case in neuroscience, H.M. pertains to this. In the 1950s he underwent a double hippocampoectomy to treat his seizures. This did reduce seizure frequency but it also caused an almost complete inability to form new episodic memories. The hippocampus is vital for forming new memories. Furthermore, the hippocampus-entorhinal cortex system is also very important for our orientation and spatial awareness. The 2014 Nobel prize in medicine and physiology was awarded for this discovery; the so-called place cells of the hippocampus and grid cells of the ErC. The attentive reader will recall that memory impairment and disorientation are among the earliest symptoms of AD.

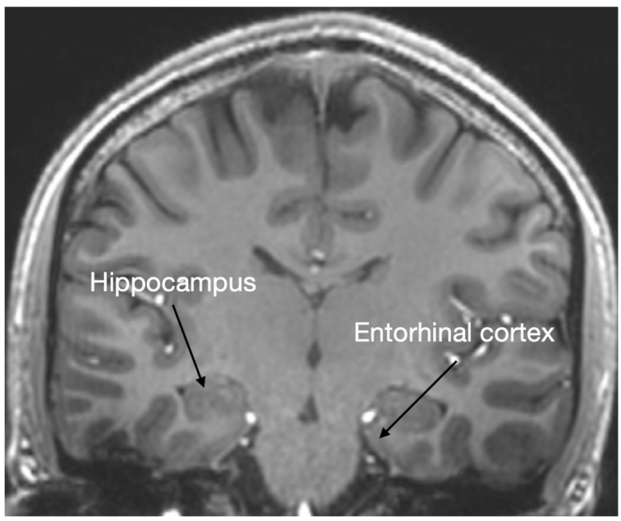


Figure 5: Coronal MRI section of the author's brain showing the hippocampi and entorhinal cortices within the medial temporal lobes (at the level of anterior pons).

The ErC is also the first cortical area to develop tau tangles. Notably though, ErC layer II, does not develop plaques [55]. The first plaques instead appear in the basal neocortex [5] and the first detectable changes with Aβ PET are in the default mode network [41]. After Aβ and tau have started aggregating reduced glucose metabolism is first noted with FDG PET in the medial temporal lobes and with disease progression this reduced metabolism is seen throughout the brain. Finally, brain atrophy also becomes evident, again starting in the medial temporal lobe [42].

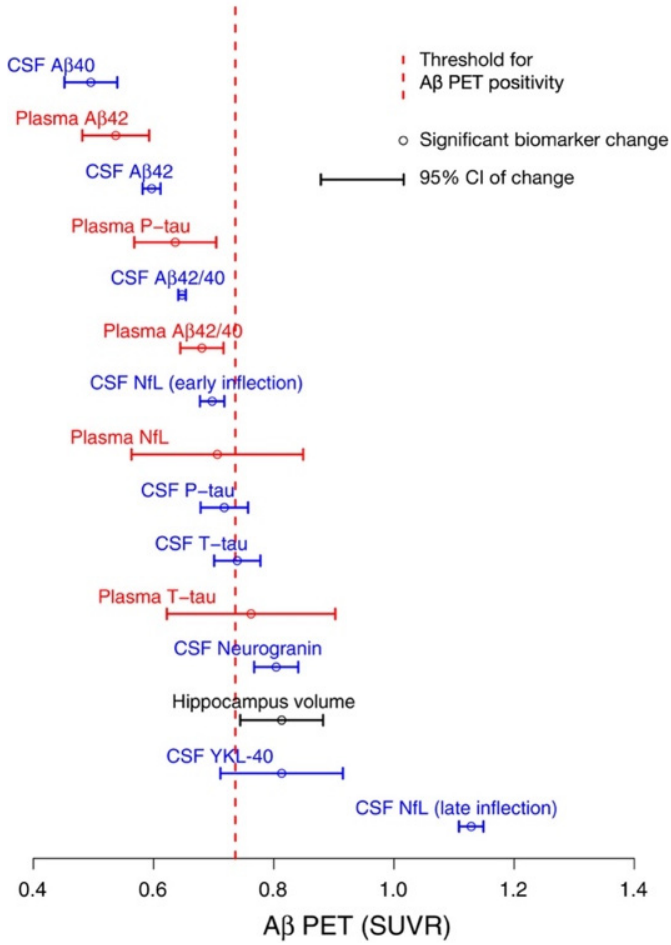


Figure 6: Change points for various AD biomarkers related to Aβ PET (plaque levels). Note that CSF Aβ 40 and 42 are the first biomarkers to change, followed by tau and after plaques hippocampal atrophy also becomes apparent (from Palmqvist et al. 2019 [40] adapted under Creative Commons license 4.0).

The Failure of the Amyloid Cascade Hypothesis

In 1992 Hardy and Higgins formulated the amyloid cascade hypothesis: "*Our hypothesis is that deposition of amyloid β protein (AβP), the main component of the plaques, is the causative agent of Alzheimer's pathology and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition*" [14]. On this basis the field developed transgenic animal models of AD, mainly mice. These animals expressed human APP together with known AD

causing mutation both in APP such as the Swedish mutation [13] and in the enzymes that cleave APP (Presenilin) [22]. These animal models often have several, in humans, AD-causing mutations. Something you would never find in any one human and indeed, these mice develop a lot of plaques and they develop them early. The 5xFAD mouse, that we use in paper III and IV, sports 5 different AD causing mutations and develop its first plaques around 2 months, just around the time they are fully grown. The transgenic AD mice do get cognitively impaired and suffer some neuronal loss but they do not get nearly as impaired as a human does nor do they suffer the massive brain atrophy seen in humans. Furthermore, the second neuropathological hallmark of AD, intracellular tau tangles is not found in AD-model animals, unless a tau mutation is also introduced. Thus, at least in rodents Aβ overproduction and plaques do not really cause AD although it does replicate many features of AD. This fact together with the previously mentioned lack of correlation between plaques and cognition in humans poses a problem for the amyloid cascade hypothesis.

Then, on top of that, we have now had more than two decades of various anti-Aβ therapies that have been successes in mice but failures in humans. These therapies have not only targeted plaques, antibodies against soluble Aβ oligomers and therapies inhibiting β-secretase have also been tried and found wanting. As mentioned in the *current treatments against Alzheimer's disease* there is an antibody directed mainly against soluble oligomeric/protofibrillar Aβ that showed promise in phase III trials but at the time of writing is not yet approved. It should also be mentioned that experimental therapies against tau have also failed [34]. Huntington's disease (HD) is also interesting. It is arguably a simpler disease than AD. Its sole cause is a mutation (or to be precise excessive CAG repeats) in a single gene that codes for the huntingtin protein. The mutation is dominant with 100 % penetration. If you have enough CAG repeats you will, if you live long enough, develop HD. If any disorder could be ameliorated by targeting the aggregating protein it should be HD. But these therapies too, have failed [24]. No one would argue that aggregation of huntingtin is not the causative agent in HD, yet this anti-sense oligonucleotide treatment that successfully lowered the amount of huntingtin still failed to alter disease progression. In a way that is evidence that the failure of anti-Aβ lowering therapies is not that informative in assessing the etiological role of Aβ in AD. Perhaps, we just need to understand when and what form of Aβ should be targeted. Nevertheless, it is clear that amyloid plaques by themselves do not drive cognitive deterioration in AD.

Aβ, More Than Plaques

Plaques are huge. Looking at a brain, riddled with plaques it seems hard to believe that they do not affect cognition much.

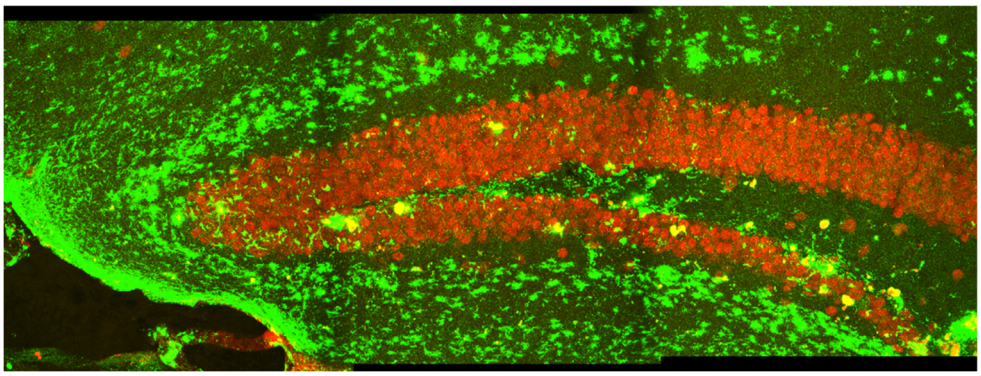


Figure 7: Dentate Gyrus of hippocampus in a transgenic mouse, injected with mouse AD brain extract to accelerate plaque pathology. Neuronal cell bodies in red and amyloid plaques in green.

But we must follow the data and look elsewhere to explain the cognitive deterioration in AD. Most Aβ quickly leaves the cell, either the β-CTF is cleaved at the plasma membrane or is secreted via the endosomal route (see Figure 3). But some is cleaved in endosomes and remain in the cell. In fact, aggregation of intraneuronal Aβ can be seen in humans before the onset of plaques and tangles, this finding was published in 2000 by my supervisor Gunnar Gouras [11]. In animal models intraneuronal Aβ also appears before the onset of plaques [36, 37]. Specifically, it is in layer II of the ErC in the medial temporal lobe that much intraneuronal Aβ is observed [55]. The ErC layer II is one of the first areas to accumulate phosphorylated tau and the first area to develop tau capable of seeding (more on that later) [21]. As previously mentioned, the ErC is also one of the first areas that degenerates in AD. The Aβ found inside cells is soluble [38, 55], in contrast to the Aβ of plaques which is insoluble. And evidence is accumulating that soluble oligomers of Aβ are much more toxic than insoluble fibrils [7]. Inside neurons Aβ accumulates before any other biomarker changes in AD [15, 33]. Inside the endosomes of cells there is a high concentration of Aβ and a low pH, ideal for aggregation [17]. Inside neurons we have soluble oligomers of Aβ that are likely more pathologically relevant than fibrils. Therefore, intraneuronal Aβ may be more pathologically relevant than plaques. In the studies of this thesis, we connect intracellular Aβ with another exciting hypothesis for the pathogenesis of AD.

The prion-like hypothesis

Prions are proteins that when misfolded can induce other proteins to also misfold and then aggregate. That is prions can propagate their misfolded state via templated seeding. This makes it possible for the misfolded prion state to spread within the organism and in rare cases even between organisms. The most famous prion disorder is variant Creutzfeldt-Jakob disease (mad cow's disease) which could spread via meat containing misfolded prions. These prion diseases are incurable and devastating but are, fortunately, also very rare. However, more and more studies indicate that the proteins and peptides that aggregate in many common neurodegenerative disorders may have prion-like properties; alpha-synuclein in PD and Lewy body dementia, tau in AD and other tauopathies, TDP43 in ALS and frontotemporal dementia and the focus of this thesis Aβ in AD [18]. In 2000 it was discovered that plaque pathology could be accelerated in a transgenic AD mouse by intracerebrally injecting AD brain homogenate [20]. This has been replicated many times, including in paper III of this thesis [46].

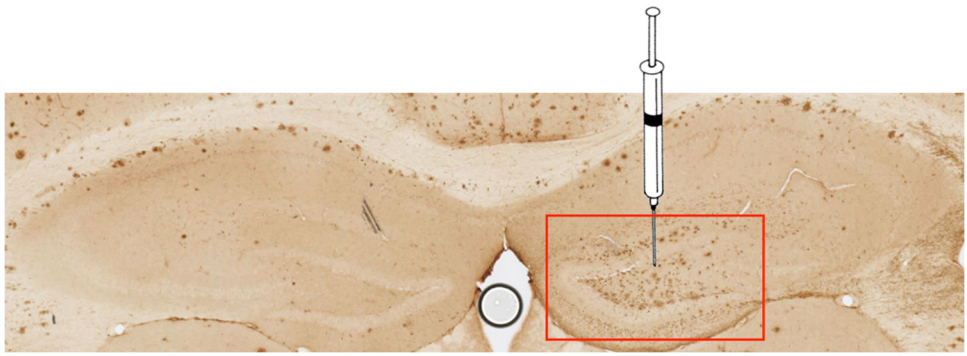


Figure 8: Mouse hippocampus that has been injected with a small amount of mouse AD brain homogenate and then, months later, sacrificed. Note the plaques, in brown in the injected hippocampus.

It is the Aβ of the injected brain that accelerates plaque formation. Injection of non-AD brains does not accelerate plaque formation, nor is plaque formation accelerated if the extract is mixed with antibodies against Aβ and finally, the host must also express human Aβ, a wild-type (WT) mouse will not develop plaques [32]. Brain-derived Aβ is a very potent seed, as little as a femtogram of brain-derived Aβ can seed plaques [9], while synthetic Aβ requires much higher concentrations and pre-treatment [52] and CSF-derived Aβ does not seed at all [9]. Outside of rare iatrogenic circumstances prion-like seeds of Aβ are not introduced into humans [18]. Thus, if the prion-like hypothesis is true then a very important question is: *Where is the first prion-like seed of Aβ formed?* Answering that question has been

the focus of this thesis. Considering the preceding section, the reader may not be surprised that we believe that it may be inside neurons.

Aims

Paper I: It had recently been shown that alpha-synuclein could perhaps transfer between neurons in the brains of embryonal stem cell recipients [30]. We therefore wanted to develop an *in vitro* system to quantify and inhibit the intercellular transfer of alpha-synuclein.

Paper II: We believed that Aβ had prion-like properties and that it can be found intracellularly. It should then be possible to induce prion-like Aβ in an APP-producing cell-line.

Paper III: Here we wanted to expand on the results from paper II and show intracellular prion-like activity of Aβ *in vivo.* We also wanted to explore how APP processing was changed by accumulation of intracellular Aβ.

Paper IV: We wanted to study the earliest Aβ aggregation and inflammation. We focused on two anatomical areas and their connection: The mamillary body which develops early Aβ aggregates and the subiculum which develops early intraneuronal Aβ.

Methodology

Mouse models

We research Alzheimer's disease, a human disease. But we do not use any human samples. Instead, we use different models that mimic certain aspects of AD. One very useful model is mice that have been genetically modified to express human Aβ, along with mutations that enhance its aggregation. The mouse model used in paper III and IV is called 5xFAD [36]. This mouse expresses human APP/Aβ along with five disease causing mutations in the APP/Aβ protein itself as well as in the proteins that cleave APP. This is an extreme situation that would never occur in any one human. However, the aggressivity of this model makes disease development fast and thus easy to work with. We were mainly interested of the extra-intracellular dynamics of Aβ and its anatomical spread so for us the 5xFAD mouse was ideal.

The Neuro 2a cell-line

In paper I, II and III we use the Neuro 2a (N2a) cell-line. In contrast to non-stem cells from multicellular organisms, these cell-line cells can divide indefinitely and they can be cloned. N2a cells were originally derived from a spontaneous neuroblastoma (a type of nerve cell tumor and as you may know tumor cells can divide forever) from a mouse. This means that the cells have some neuronal characteristics and with certain treatments they can be differentiated to develop even more neuronal characteristics (we do not do this). But they are not neurons. However, as a simple model to study protein transfer and Aβ production and aggregation the N2a cells work well as they can be maintained indefinitely *in vitro,* they are robust and as they are clonal, variability is low compared to primary neurons or induced neurons.

Details on the culture and cloning of N2a cells can be read in the methodology section of paper I, II and III.

Primary mouse neurons

These cells come from dissociated cortical-hippocampal neurons from mouse embryos at day 16. These neurons then mature in a dish and can be maintained for up to a month, or even longer. These are *bona fide* neurons; they express all neuronal markers and develop active synapses. Albeit, the cells are from mouse, not man. Since, Alzheimer's disease is a neurodegenerative disorder, that is primarily affecting neurons findings in cell-lines should be replicated in primary neurons. Something we do in figure 5 and figure 7 of paper III.

Western blot

Is a method to detect one specific protein out of a mixture, for example brain or cell lysate. First proteins are separated based on size via gel electrophoresis, all proteins will move through the gel due to their electric charge but the smaller the protein the faster it will move through the pores of the gel. After migration the proteins of the gel are transferred to a membrane (much easier to work with) and on that membrane specific proteins can be detected with antibodies. The most common type of western blot is SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). SDS is a powerful anionic surfactant (i.e. a detergent, it is a common ingredient of shampoos and cleaning products) which makes it good at lysing cells and denaturing proteins. With the application of heat, SDS and a reducing agent (to break disulfide bonds) proteins are denatured down to their primary structure. SDS then covers the protein in negative charge, thus giving all proteins the same charge, which means that with SDS-PAGE proteins are separated solely based on size. We use SDS-PAGE in paper I, II and III.

In paper II we were interested in protein aggregates. SDS PAGE completely breaks up these aggregates. We therefore used BN-PAGE (blue native polyacrylamide gel electrophoresis). Here we used small amounts of the detergent triton-X to lyse cells in a way which does not break up the protein complexes, that is we blot the proteins in a more native confirmation. The dye Coomassie Blue-G250 coats the protein complexes with negative charge so that the aggregates can be separated by size as in SDS PAGE.

In paper II we developed a technique we call SDD-PAGE (semi denaturing detergent PAGE). To test stability of protein aggregates we used a lower amount of SDS than in regular SDS PAGE, no heat and no reducing agent. The exact protocol can be found in the methods section of paper II.

Dot blot

Is like a non-denaturing western blot but without the gel electrophoresis to separate proteins. You simply apply the protein solution directly onto a nitrocellulose membrane that you then dry, wash, block and then stain that with an antibody. This means you do not get any information about the size of the protein/protein aggregate. But with conformation specific antibodies we are not interested in that, indeed we do not know the size of what we are looking for. We therefore used dot blot with conformation specific antibodies in paper II and III.

Immunocytochemistry and immunohistochemistry

Are used to tag proteins with fluorescent proteins in cell culture (immunocytochemistry) or in tissue (immunohistochemistry). This does not give any information about the size of the protein but it tells you where the proteins are located in relation to other proteins, cellular and/or anatomical structures. We use these techniques in all papers.

Microscopy

To see the immunostained brains and cells we use fluorescent microscopy. We have used epifluorescence microscopy where the entire sample is illuminated at the same time so you are seeing/detecting light that is both in and out of focus. This makes it sensitive and can be good for quantifying the fluorescent signal; we use epifluorescence microscopy for this purpose in paper III. It is also a relatively cheap and simple to use microscope.

If you want higher resolution, for example to more precisely and accurately determine relative co-localization of two fluorophores you can use laser scanning confocal microscopy. Unsurprisingly, this method uses lasers as a source of excitation light, the light of which is significantly narrower in its wavelength than epifluorescence, i.e., more precise. Furthermore, con*focal* microscopy only illuminates one focal plane at a time, out of focus light is blocked. This gives you greater resolution and makes it possible to determine that something (say Aβ or alpha-synuclein) is indeed inside a cell, not just on it or below it. We have used laser scanning confocal microscopy in all our papers.

Fourier transform infrared spectroscopy (FTIR)

Is a method where you measure what wavelengths of infrared light are absorbed by your sample. Then, through complex mathematics (Fourier transform) this yields information about the 3D structure of a protein, mainly the secondary structure. In paper II we used this method on N2a cell preparations. A caveat is that FTIR is mainly validated in pure protein preparations. As we used it on cells, we cannot be certain whether the changes in FTIR are due to structural changes of Aβ or if it was from secondary changes within the cells due to intracellular Aβ. Second author, Oxana Klementieva performed the FTIR in paper II.

Statistics

"The first principle is that you must not fool yourself — and you are the easiest person to fool." Richard Feynman

During my PhD studies I have come to realize that statistics is really hard and really, really important. As Feynman pointed out we want to avoid fooling ourselves that we have found something that is not there and statistics should help with this. However, we are also easy to fool, because usually scientists are not disinterested in our experimental outcomes. We do want particular results as that may confirm our hypotheses and further our careers. We are very focused on p-values and we all want a star (*), a p-value < 0.05. The p-value assesses how consistent your data is with the null hypothesis i.e., no difference between two groups. The p-value tells you the probability that you would find a difference at least as extreme as the observed one if the null hypothesis was true. Most often we use t-test or the related ANOVA (which is used when you have more than 2 groups). If you defined your question and the intended test before you saw the data and your data is normally distributed this works fine (ideal question is something like comparing the heights of two different large populations). But in pre-clinical science we do lots of exploratory research, thus to a degree hypotheses are generated while the data is collected. What particular statistical test to use and to what degree you should adjust for multiple hypotheses is therefore a bit of an open question; for a deeper dive into this read The garden of Forking paths by Gelman and Loken 2013 [10]. Furthermore, the number of replicates (n) is usually too low to ascertain with confidence whether your data is normally distributed or not. The solution to this is of course replication. When you replicate an experiment, you can pre-determine the statistical test to use and you have an idea of the expected effect size so you can choose an appropriate n. Unfortunately, there is not enough prestige in replication

studies so they are not performed as much as they ought too, hence we have a replication crisis.

Summary of Key Results

A cell culture model for monitoring α-synuclein cell-to-cell transfer (Paper I)

According to the prion-like hypothesis of neurodegenerative disease misfolded proteins spread their misfolded state via templated seeding. Thus, for a prion-like protein to spread its misfolded state throughout the brain it must be able to spread cell to cell. In this paper we studied the intercellular transfer of alpha-synuclein and how that spread could be arrested.

We used N2a cells (an immortalized cell-line from a mouse neuroblastoma) that had been transfected to express alpha-synuclein tagged with the fluorophore mCherry or GFP at the C-terminal end, as well as N2a cells expressing the fluorophores alone.

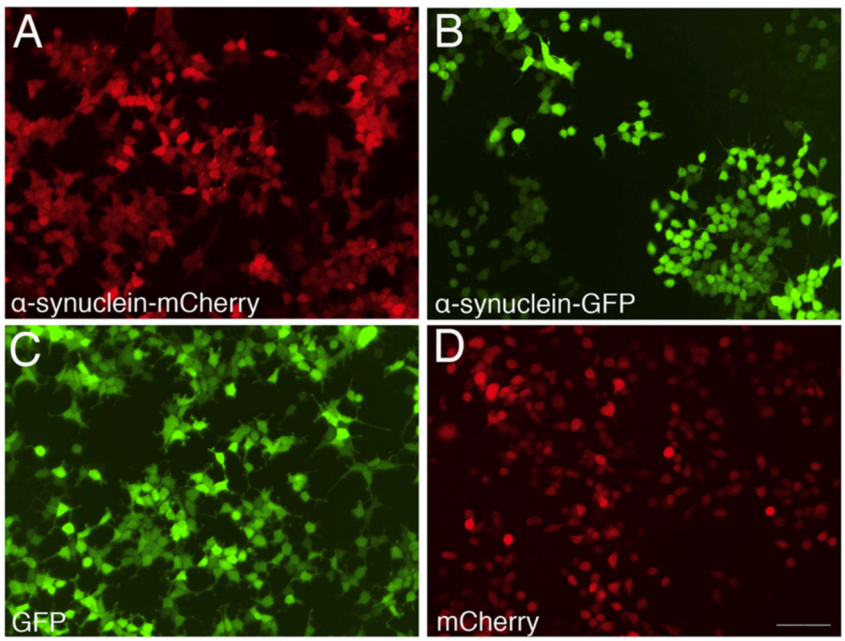
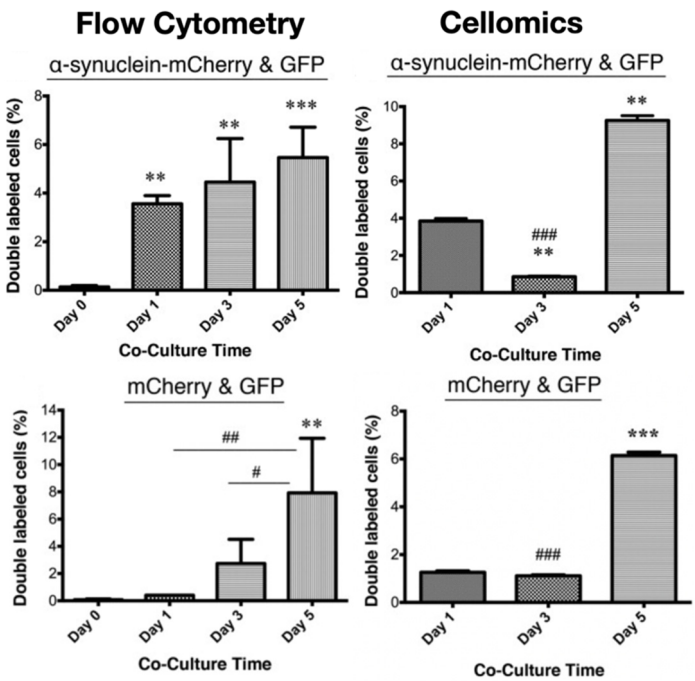


Figure 1: N2a cells expressing fluorophores alone or alpha-synuclein tagged with fluorophores.

By co-culturing these cells, we could asses transfer of alpha-synuclein by quantifying the number of cells that contained both fluorophores. We found the combination alpha-synuclein mCherry (donor cell) co-cultured with GFP expressing cells (recipient cell) to be the most robust. We quantified the number of double labeled cells with flow cytometry and automated microscopy (we used the Cellomics system).



Adapted combination of figure 2 and 4: We observed an increasing number of double-labeled cells with time in co-culture with both flow cytometry and Cellomics. Double labeled cells were not unique to alpha-synyuclein but were also observed when N2a cells expressing fluorophores alone were co-cultured.

The number of double-labeled cells was similar in co-cultures of mCherry-alpha synuclein + GFP and mCherry alone + GFP. Indicating that much of the transfer was likely unspecific, any extracellular protein, sufficiently overexpressed, would likely transfer cell-cell.

The intercellular transfer of alpha-synuclein (and in the case of dynasore treatment, fluorophores as well) could be inhibited. We could do this via pharmacological inhibition of dynamin (a GTPase important in endocytosis) using dynasore, by siRNA knockdown of dynamin or by heparin treatment (this blocks interaction with cell-surface heparan-sulfate proteoglycans).

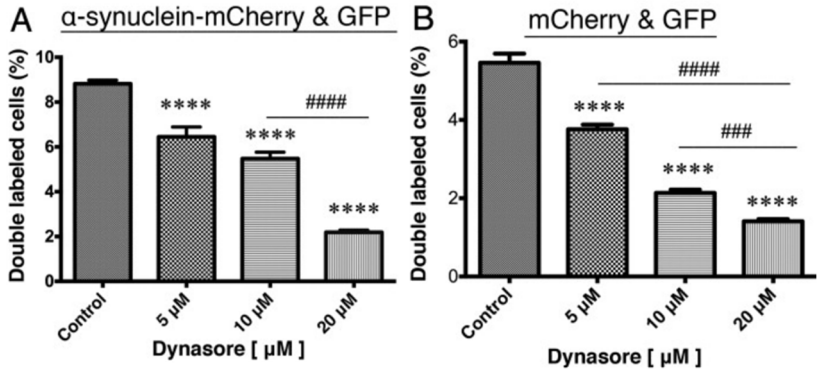


Figure 5: Dynasore reduces the number of double labeled cells after co-culture.

The initial goal of this work was to develop an assay suitable for screening chemical compounds that could reduce alpha-synuclein cell-cell transfer. That was also the reason that we used the Cellomics system. Treatment with dynasore showed that our assay had a relatively large dynamic range 8.81 % vs 2.19 % with low variability (small standard deviations), indicating suitability for screening [56].

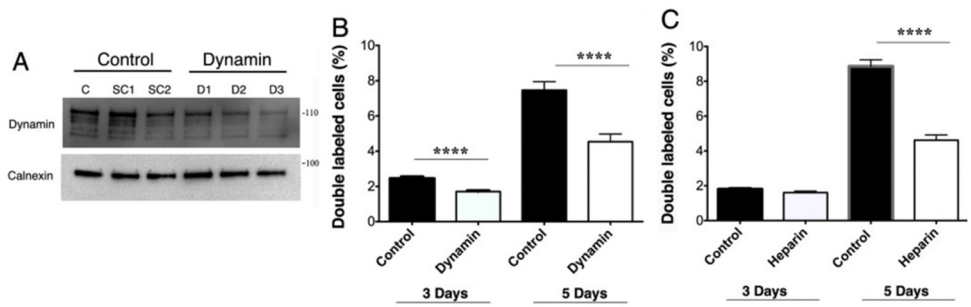
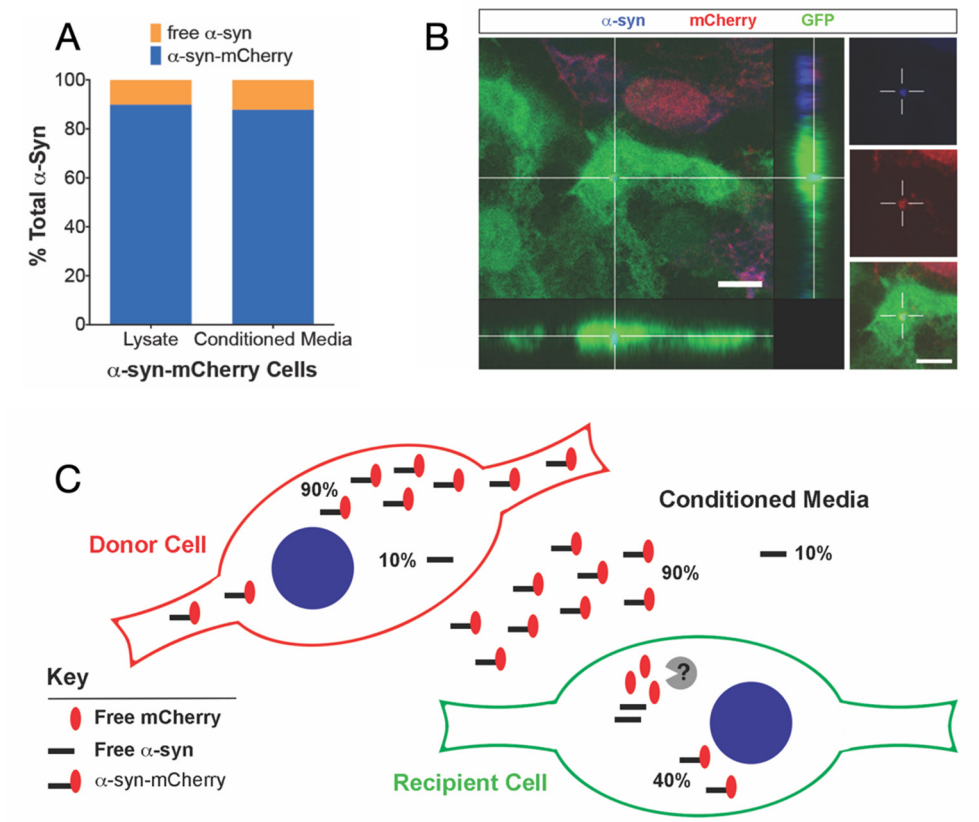


Figure 6: SiRNA knockdown of dynamin as well as heparin treatment reduced the number of double-labeled cells after 5 days of co-culture of alpha-synuclein mCherry cells and GFP cells.

One of the more interesting results was hidden away in the supplementary figures. To ascertain that alpha-synuclein stayed tagged with mCherry we measured the amount of alpha-synuclein in conditioned media and in cell lysate of mCherry-alpha synuclein cells with western blot. Using an alpha-synuclein antibody we found 90 % of alpha-synuclein to be tagged to mCherry and 10 % free, in both lysate and media. However, using a mCherry antibody we only found full length mCherry tagged alpha-synuclein proteins, so in contrast to alpha-synuclein we never found any free mCherry. Furthermore, we performed immunostains against alpha-syncuclein on GFP cells that had been co-cultured with mCherry-alpha synuclein cells and found that only 40 % of transferred alpha-synuclein was co-localized with

mCherry. Since 90 % of alpha-synuclein was still tagged in donor cells and in conditioned media we surmised that the mCherry could be cleaved from the alpha-synuclein and degraded within the recipient cell.



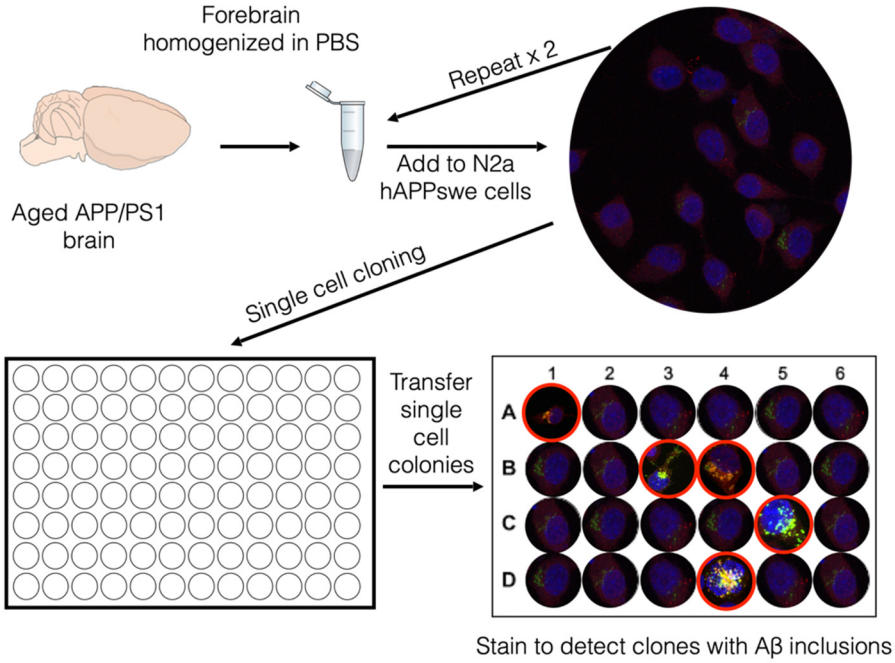
Adapted supplementary figure 3, cleavage of fluorophores from alpha-synuclein: A: In lysate and conditioned media from donor cells 90 % of alpha-synuclein is tagged with mCherry. **B:** In recipient cells only 40 % of alpha-synuclein co-localize with mCherry. **C:** Schematic of what may happen after intercellular transfer of alpha-synuclein.

In this study we developed a system to quantify intercellular transfer of alpha-synuclein and showed that transfer could be inhibited. However, it is uncertain how specific our system was as fluorophores alone also transferred. Furthermore, considering that the mCherry was often cleaved from alpha-synuclein we likely missed a lot of alpha-synuclein transfer. It is also uncertain how the fluorophore (which is about twice as large as the alpha-synuclein molecule itself) affected transfer and aggregation of alpha-synuclein.

Prion-like seeding and nucleation of intracellular amyloid-β (Paper II)

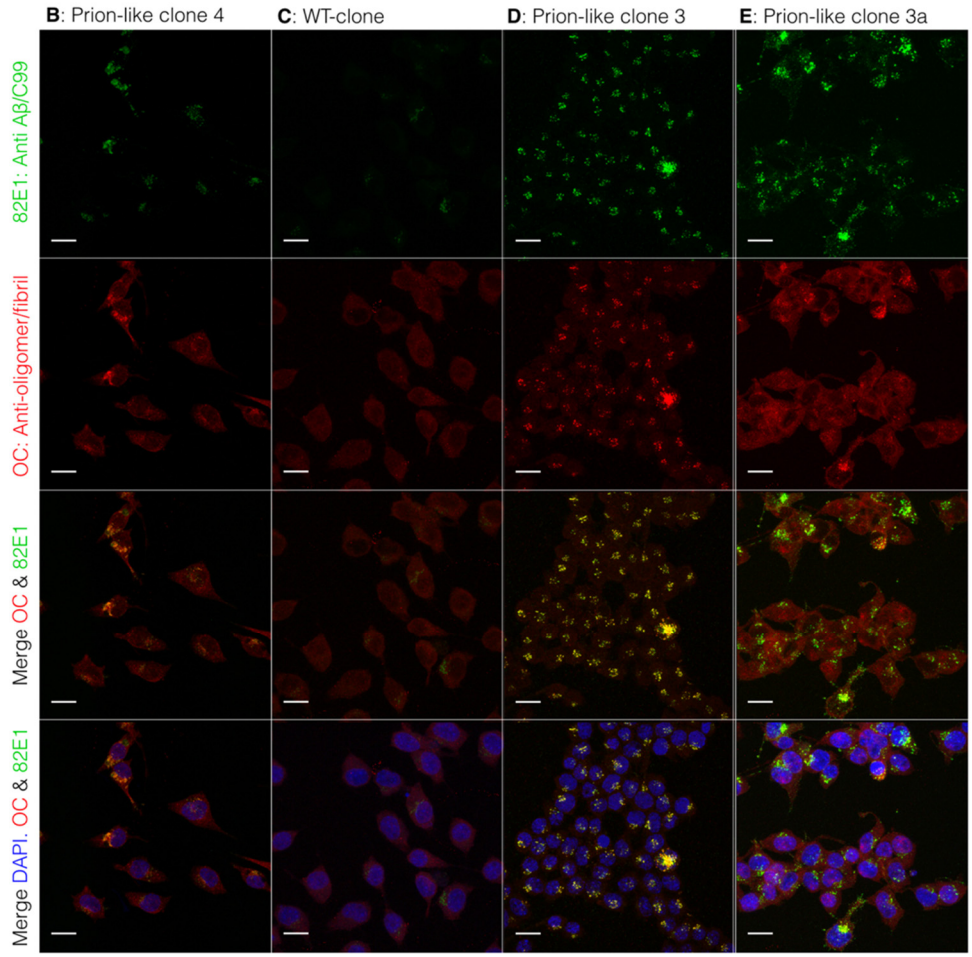
The inspiration for this paper was from a talk by Marc Diamond at AD/PD 2015 where he presented a lot of the data from Sanders et al. 14 [48]. In that paper they had HEK-293 cells that expressed the most aggregation-prone region of human tau (4 repeat domain) tagged with the fluorophore YFP (as in paper I here too the fluorophore is larger than the protein/peptide of interest). They treated these cells with fibrils of tau and by doing that they induced stable aggregates of tau in the HEK-293 cell. Presumably, they had achieved prion-like conversion of the cellular tau.

Since we hypothesized that prion-like Aβ could exist intracellularly we wanted to show that prion-like Aβ could be found within cells. Prior work had reported that synthetic/recombinant fibrillar Aβ has low to no seeding capability [52] (this is in contrast to tau where tau fibrils readily induces seeding) while brain Aβ is quite potent [9]. We therefore treated N2a cells expressing human APP with the "Swedish" AD-causing mutation (henceforth referred to as SWE cells) [13] with homogenized brain from AD transgenic mice. The idea was to induce prion-like conversion of Aβ in a subset of the N2a cells. Now Aβ, in contrast to tau is not a protein but a peptide produced by sequential cleavage of APP. Thus, it is not as straightforward to tag Aβ with a fluorophore. One could transfect a cell to produce only Aβ with a tagged fluorophore but that would not be physiologically produced Aβ. Furthermore, Aβ is a small peptide, YFP is 6 times larger. Presumably, fusion with such a relatively large protein would affect aggregation. We also saw in paper I that fluorophore tags can be cleaved from the protein of interest. We therefore eschewed fluorophores and used immunostaining to identify Aβ aggregates.



Part of figure 1: The workflow of inducing intracellular prion-like conversion in SWE cells.

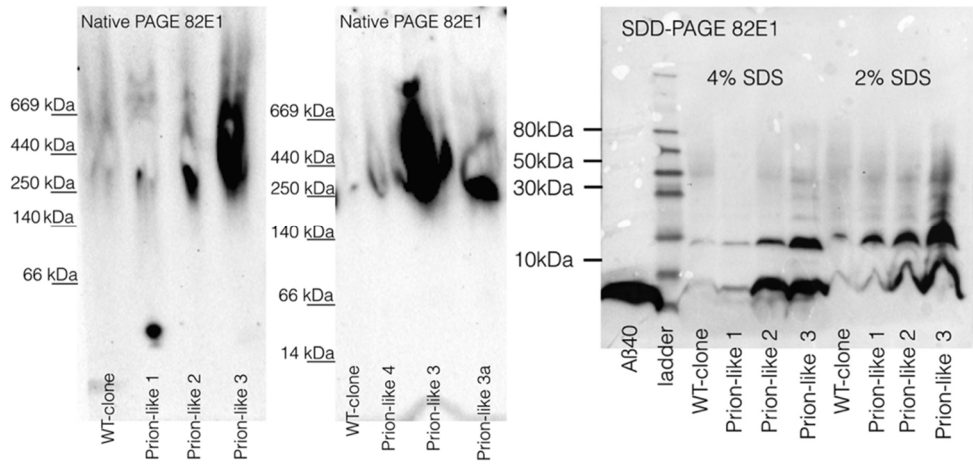
The brain homogenate treatment and subsequent single-cell cloning (illustrated above) produced several clones with aggregates positive for 82E1 (antibody against the N-terminus of Aβ and C99) and OC (confirmation specific antibody against oligomers and fibrils), henceforth referred to as prion-like clones. These prion-like clones retained their aggregates over multiple replicative generations (cell passages) and time in the freezer. We also treated SWE cells with lysate from prion-like clone 3 instead of brain as in the above figure and thereby produced another prion-like clonal cell-line, prion-like clone 3a.



Part of figure 1, B and D: Here we see two different primary prion-like clones (more were produced but they are not pictured) with many puncta/aggregates positive for both 82E1 and OC. **C:** In the WT-clone, that had been treated with wild-type (WT) mouse brain we do not see these aggregates. **E:** Prion-like clone 3a was produced by treating SWE cells with lysate from prion-like clone 3 and this also induced aggregates.

The co-localization of antibodies 82E1 and OC indicated the presence of fibrillar and/or oligomeric Aβ in the prion-like clones. To study this further we performed blue native (BN) PAGE, semi denaturing detergent (SDD)-PAGE, proteinase K digestion and dot blots with confirmation-specific antibodies on lysates of the prion-like clones. This data indicated that the Aβ of the prion-like clones existed as 250-670 kDa soluble aggregates (BN-PAGE data), that showed some resistance to denaturation with SDS (SDD-PAGE data), like with the immunostainings we saw an increase in OC signal on dot blot in the prion-like clones but not with another

confirmation specific antibody A11 and finally the Aβ aggregates were not resistant to proteinase K. Thus, there were likely large soluble fibrils within the cells but no insoluble fibrils.



Part of Figure 2: BN-PAGE showing large soluble oligomers positive for 82E1 and SDD-PAGE showing greater resistance to SDS in the prion-like clones.

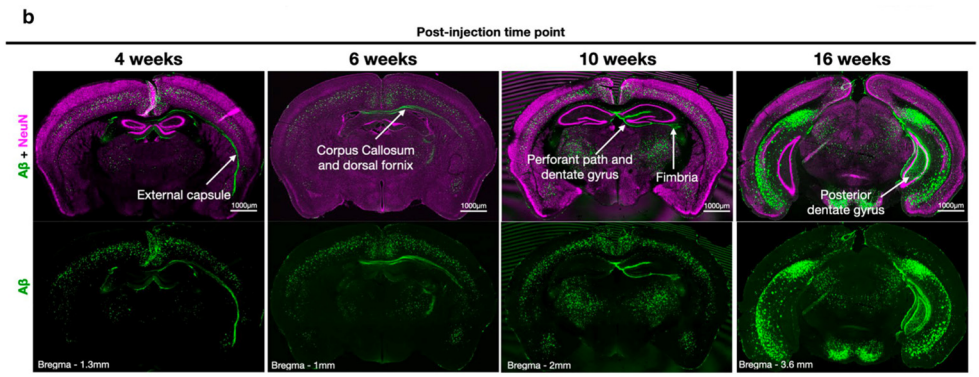
We also performed Fourier Transform Infrared Spectroscopy (FTIR). In FTIR you analyse the absorbance of infrared light, which gives you information about the secondary structure of proteins and peptides. The prion-like clones had different absorbance spectra compared to WT-clones, specifically more unordered structure and more anti-parallel β-sheet structure. Both of these changes in FTIR are, like the biochemical evidence, consistent with oligomeric but not fibrillar structures.

We also noted that the processing of APP was changed in the prion-like clones. There was more APP and seemingly more APP going into the amyloidogenic pathway and less into the non-amyloidogenic pathway (see Figure 3 of the introduction).

In paper II we showed, for the first time that prion-like Aβ could form inside cells. We also showed that amyloidogenic processing of APP was exacerbated by intracellular accumulation of Aβ. Finally, there are many studies indicating that soluble Aβ may be more pathologically relevant than fibrillar. It has a greater seeding capacity [9, 26] and correlates better with cognitive decline [53]. Paper II adds to this literature. Notably, another group published a similar cellular assay for prion-like Aβ a year later (without citing us…). This was however with cells producing fluorescently tagged Aβ as opposed to our APP-producing cell-line without fluorescent tags [1].

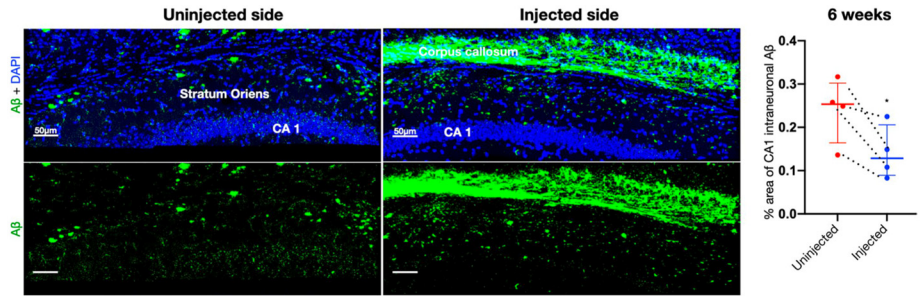
Neuronal spreading and plaque induction of intracellular Aβ and its disruption of Aβ homeostasis (Paper III)

This is to a large degree a continuation of paper II. We look at *in vivo* effects of prion-like Aβ with a focus on intracellular Aβ and extend the cell-culture findings from paper II. As seen in figure 8 of the introduction, plaque pathology can be accelerated via intracerebral injection of AD brain homogenate in an AD transgenic mouse. In paper III we inject lysate of the prion-like clones from paper II and thereby induce a modest number of seeded plaques in the hippocampi of 5xFAD mice. We then wanted to look at the anatomy of seeded Aβ, both plaques and cellular effects. To this end we injected 5xFAD mice with brain homogenate from APPPSEN1 transgenic mice and analyzed the injected brains at different time-points.



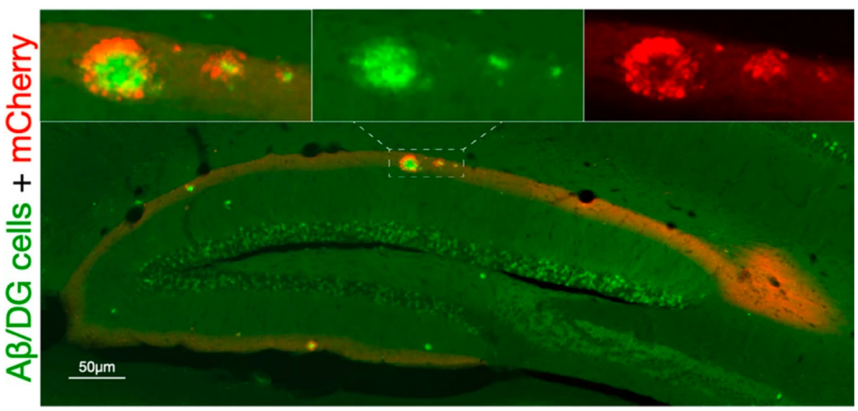
Part of figure 2: 5xFAD mice unilaterally injected with brain homogenate at 7 weeks and then sacrificed 4, 6, 10 and 16 weeks later. 4 and 6 weeks post-injection seeded Aβ is in white matter tracts or adjacent to white matter tracts. At 10 and 16 weeks seeded plaques have spread into the hippocampus, particularly in the perforant pathway and dentate gyrus.

At the 4 weeks timepoint the induced Aβ stayed mostly in white matter tracts such as the external capsule and corpus callosum, after 6 weeks most was still in white matter tracts but we also observed spread into adjacent areas, especially in the stratum oriens. In the stratum oriens adjacent to the plaques we also noted a decline in the number of NeuN (an antibody against neuronal cell bodies) positive cells, indicating damage to interneurons.



Part of figure 4: 6 weeks post-injection lots of Aβ can be seen in the corpus callosum and adjacent to that plaque-like structures can be seen in the stratum oriens. We also note a significant decline of intraneuronal Aβ in the CA1 pyramidal neurons.

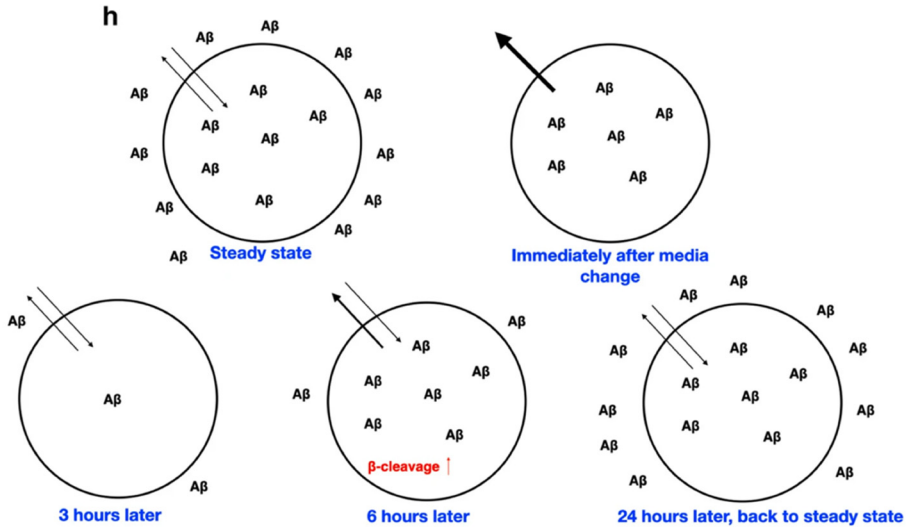
We then wanted to look at how intracellular Aβ was affected by the introduction of prion-like Aβ. The stratum oriens was the first site with significant plaque induction and it is adjacent to the pyramidal neurons of CA1 and is the site of their basal dendrites. We observed a significant decline of intraneuronal Aβ adjacent to plaques compared to the uninjected side 6 weeks post-injection (pictured above). At the 10 and 16 week timepoints we see many induced plaques around the dentate gyrus that correspond well to the localization of the axon terminals of the ErC layer II and III neurons, this is called the perforant pathway. We therefore looked at intracellular Aβ in ErC layer II before plaque induction (6 weeks) and after plaque induction (16 weeks). At 6 weeks we found a significant increase of Aβ in layer II ErC neurons followed by a significant decrease at 16 weeks. Thus, it seemed that ErC layer II neurons first took up the injected Aβ. Later, when plaques had formed at the axon terminals the Aβ was then lost, presumably to the plaques; similar to what was observed at 6 weeks in the CA1 neurons.



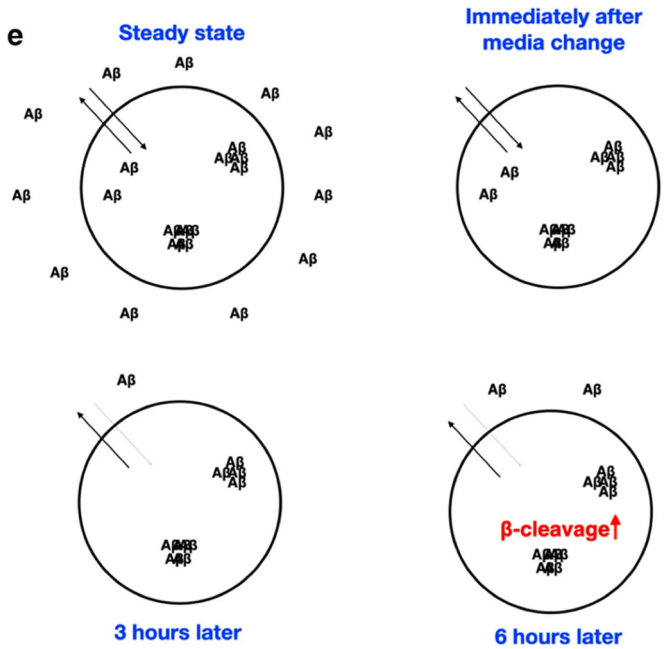
Part of figure 7: Mouse hippocampus where the ErC neurons produces the fluorescent protein mCherry. We can therefore see the perforant path in red as well as dystrophic neurites from the ErC surrounding a plaque in the perforant path (upper pictures).

Another possibility is that Aβ was routed away from the cell body towards processes. With NeuN we can only see neuronal cell bodies, it is very hard to immunostain specific neuronal processes (better to use viral vectors to induce neurons to express fluorescent proteins as in the figure above). We therefore treated primary neurons with ultracentrifugated brain lysate, where we saw redistribution of Aβ away from cell bodies towards processes.

Finally, we had already seen in paper II that accumulation of intracellular Aβ affected APP processing. Now we also saw possible equilibrium effects *in vivo* where extracellular plaques may have affected intraneuronal Aβ. Thus, we studied the extra-intracellular equilibrium of Aβ in N2a SWE cells and in prion-like clone N2a cells. First, we cultured N2a SWE cells, we then replaced the media with conditioned Aβ-free media. 3 hours after this media change, we saw a dramatic decrease of extracellular Aβ that also led a loss of intracellular Aβ, a simple equilibrium. 6 hours after the media change, extracellular Aβ was still low while the intracellular pool had recovered, interestingly we also saw an increase of β-CTF, indicating an increase of amyloidogenic APP processing: a dynamic equilibrium. 24 hours after the media change both the intra and extracellular pools had recovered and β-CTF was normal. We then repeated this experiment with prion-like cells. At the 3-hour timepoint, there was now no decline of intracellular Aβ, breakdown of the simple equilibrium. However, after 6 hours we still saw an increase of β-CTF, indicating that it was low extracellular Aβ that triggered that rather than low intracellular Aβ. This opens up the possibility of a vicious spiral of more and more β-cleavage and Aβ production in AD. Intracellular aggregated Aβ increases β-cleavage, this aggregated Aβ cannot be secreted which lowers extracellular Aβ which also increases β-cleavage and further increases intracellular Aβ. There is a massive increase of Aβ42 in AD. A healthy 50-year-old has about 100µg of brain Aβ42 while a 70-year-old AD patient has around 5mg [45]. Reaching that amount would require a linear deposition rate of 28ng/hour Aβ42 over 20 years. Even if all CSF Aβ42 were deposited into plaques during those 20 years that would still only account for half of the Aβ42 increase. It is thus likely that there are also mechanisms that increase Aβ42 production in AD. The disrupted equilibrium from aggregated intracellular Aβ42 provides such a mechanism.



Part of figure 5: A model of the dynamic equilibrium of extra and intracellular Aβ in normal N2a APPSWE cells.

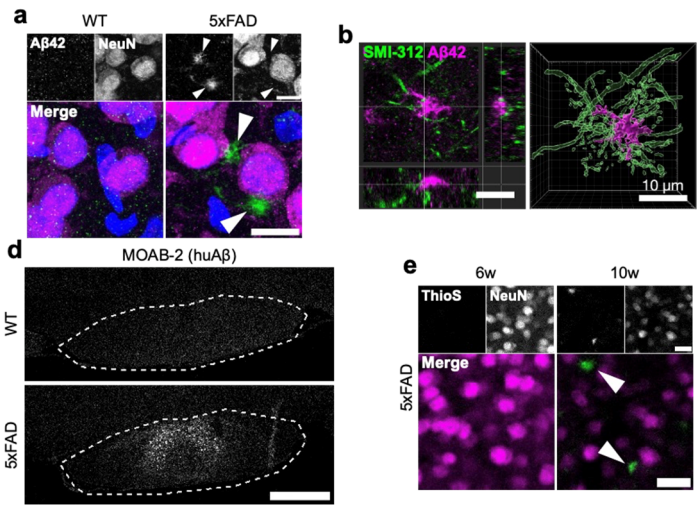


Part of figure 6: In the prion-like clones the simple equilibrium between extra and intracellular Aβ is disrupted, intracellular Aβ is not secreted. However, β-clevage is still up-regulated. This could lead to even more Aβ production.

In this study we showed that the introduction of prion-like Aβ into the brain of a 5xFAD mouse had extensive effects on intracellular Aβ. These intracellular Aβ changes paralleled those seen in human AD brains with an increase followed by a decrease of intraneuronal Aβ in ErC layer II. We also provide a possible mechanism for upregulation of Aβ production due to aggregated intracellular Aβ.

Modelling pre-plaque amyloid-β and associated pathology in connected brain regions in 5xFAD Alzheimer's disease-transgenic mice (Paper IV)

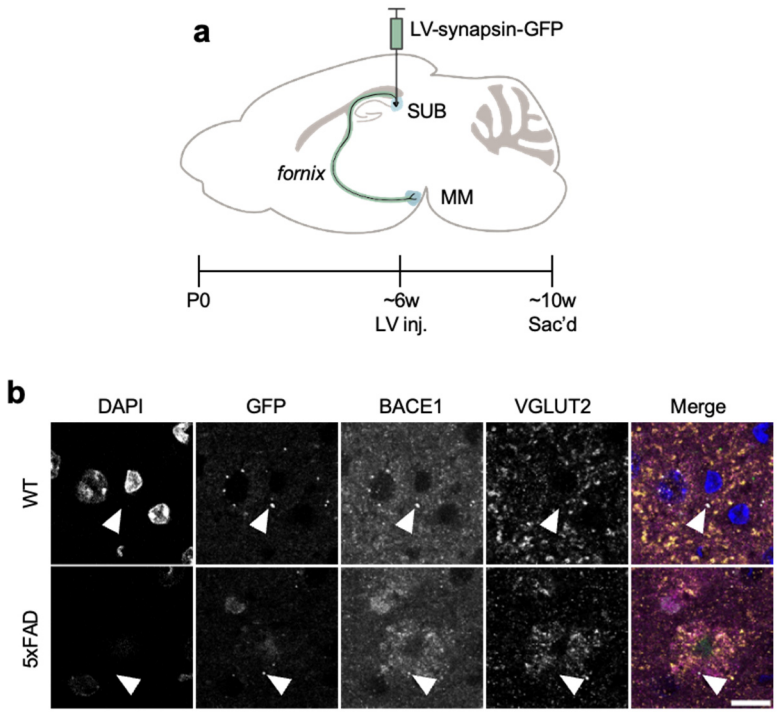
In this paper we wanted to look at the earliest Aβ aggregation. Furthermore, microglia and neuroinflammation play an important role in AD [28]; microglia phagocytose Aβ [43] and (inappropriately) prune synapses in AD [16]. We therefore had a special focus on microglia and their reaction to the earliest Aβ both inside and outside of neurons. We again used the 5xFAD mouse model of AD but studied them around 1.5 months of age when the first plaques appear. We noted that some of the first aggregates of Aβ appeared in the medial mammillary nuclei (mMn) at 6 weeks. They resembled early plaques but were thioflavin negative. While at 10 weeks thioflavin-positive aggregates were present in 5xFAD mice but not in WT mice.



Adapted figure 1 Aβ aggregates are apparent at 6 weeks in the medial mamillary nuclei. a: Wispy plaque-like aggregates are observed already at 6 weeks in the 5xFAD mouse. **b:** These aggregates are intertwined with axons (SMI-312 is a pan-axonal marker). **d:** Overview of Aβ signal in the mMn at 6 weeks. **e:** No thioflavin signal at 6 weeks but it is apparent at 10 weeks.

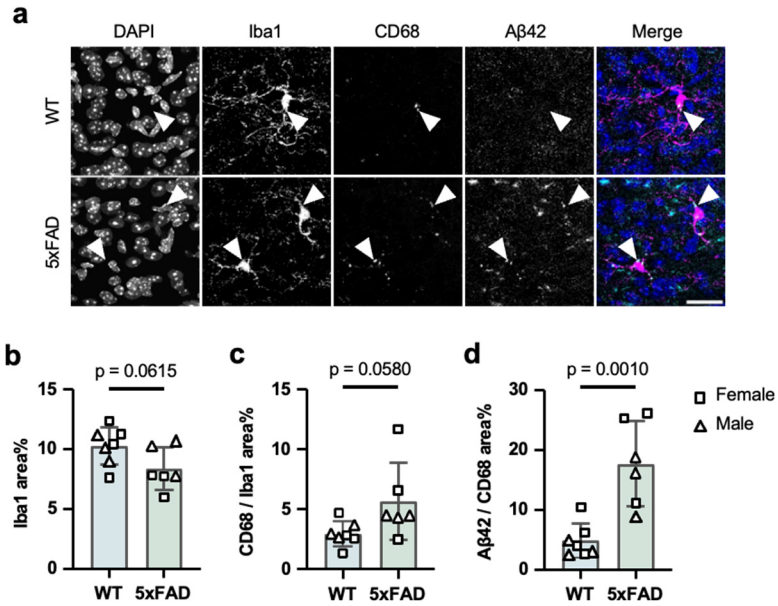
We hypothesized that these early aggregates had an intracellular origin. Namely from the pyramidal neurons of the subiculum. The subicular neurons are among the first to accumulate intraneuronal Aβ in the 5xFAD mouse [36] and they have a direct synaptic connection to the mMn via the fornix (part of the Papez circuit). At 6 weeks we therefore injected the subiculum with a lentiviral vector to induce GFP-expression in the subiculum and could thereby identify subicular synaptic terminals in the mMn by their expression of GFP at 10 weeks. In the 5xFAD mouse virtually

all GFP-signal co-localized with BACE-1, a marker of dystrophic neurites that accumulate around plaques which was not the case in the WT mice. This indicates that the extracellular Aβ aggregates in the mMn perhaps partly originated from the subiculum. We also observe co-localization of GFP with CTFs (another marker of dystrophic neurites) and that it is adjacent or even co-localizes with Aβ (data not shown).



Part of figure 2: GFP from the subiculum co-localizes with dystrophic neurites (BACE-1).

We then looked at microglia and noted no increased number of microglia in the mMn at 6 weeks. However, at 10 weeks we saw Iba1 and galectin3-positive microglia around thioflavin-positive plaques in agreement with earlier studies [4]. We did, however, observe a morphological change in the microglia towards a more reactive, ameboid, morphology. We quantify this by looking at total area of Iba1 (a marker of microglia) signal and since the number of microglia is not decreased a lower area indicates activated microglia and so does a higher CD68/Iba ratio (CD68 is a protein mainly expressed in the lysosomes of monocytes such as microglia). Both of these quantifications trended towards more activated microglia.



Part of figure 3 a: Qualitatively we observe a more amoeboid morphology of the 5xFAD microglia at 6 weeks. **b and c:** Quantitatively we observe a trend towards more reactive microglia.

To summarize, even before the appearance of dense-core thioflavin-positive plaques there are plaque-like structures in the 5xFAD mouse. At this time-point we also see subtle alterations in glia, mainly microglia. In the context of intracellular Aβ we show here that the plaque-like structures of the mMn may originate intracellularly from the subiculum which is consistent with the findings in paper III, particularly figure 7 and figure 4 there.

Discussion and Future Perspectives

There are three warring sects vying to explain the pathogenesis of Alzheimer's disease. The βaptists, who believe in the primacy of the Aβ peptide. The tauists, who think tau is more important and the heretics who believe in something other than Aβ or tau e.g., an infectious origin. As described in the introduction the βaptists have historically reigned supreme based on neuropathological, genetic and biomarker evidence, but there are cracks in their edifice. Most notably the clinical failure of Aβ-targeting therapies. Thus, attention is increasingly directed towards tau and "heretical" ideas such as neuroinflammation. Yet, this thesis is old-fashioned, it could fairly be called to belong in the βaptist camp. To explain the failures of Aβ-directed immunotherapies and remain a βaptist the immunotherapies can either target the wrong form of Aβ and/or be given too late in the disease. The hypothesis of prion-like intracellular Aβ fits both of these conjectures. Both we (in paper III) and Frithschi et al 2014 [9]. Have shown that a very small part of brain Aβ confers a lot of the seeding activity so it seems eminently possible to remove the vast majority of Aβ and still leave the most toxic fraction. Since this fraction is so small it may also be hard to detect. Furthermore, if the most pathologically relevant Aβ is intracellular it may be harder to reach via immunotherapy as it would be sequestered from both antibodies and immune cells. We showed in paper III that intracellular aggregated Aβ is not readily secreted, even via equilibrium mechanisms. Gouras et al. 2000 [11] showed that intracellular Aβ preceded plaques (and plaques precede dementia). We also show, in paper III, that intracellular changes of Aβ in the ErC layer II precedes plaque formation in the hippocampus. We also show that accumulation of intracellular Aβ could explain the earliest biomarker changes in AD (the decline of CSF Aβ42). This would mean that accumulation of intracellular Aβ is one of the earliest steps in the pathogenesis of AD.

If AD is to be targeted decades before symptoms, before even mild cognitive impairment then we need specific and scalable biomarkers for it, such as blood-based biomarkers for Aβ and phosphorylated tau [29]. If intracellular Aβ is important then we need therapies that eliminate this small but potentially pathologically essential pool of Aβ. My hypothesis/scenario for the pathogenesis of AD: A prion-like seed of misfolded Aβ is formed within a neuron, likely in ErC layer II. To some degree this is a stochastic event but it is made more likely by age-

dependent decline of proteostasis, mitochondrial function, inflammation. The intracellular environment is also auspicious for forming prion-like Aβ as within small endosomes the Aβ concentration is high and pH is low. As we showed in paper III this accumulation of intracellular Aβ increases β-cleavage of APP and may also decrease secretion of Aβ as aggregated Aβ is not secreted from cells, decreased extracellular Aβ may also by itself increase β-cleavage of APP. This could cause both the decline of CSF Aβ (mainly 42) and the massive increase of brain Aβ42 that one sees in AD. The prion-like Aβ is then transported via neuronal processes and can initiate plaque formation by dystrophic neurites (as seen in figure 7 of paper III) and also spread between cells.

However, this is to a large degree speculation. Almost certainly someone working on some "heretical" hypothesis of AD pathogenesis could also link their experimental results to biomarkers and neuropathological findings. We have performed pre-clinical experimental studies in a cell-line of mouse neuroblastoma, mouse neurons in a dish and in one particularly aggressive mouse model of AD. The results have of course been replicated within our models, but they also need to be replicated and expanded upon in other models. We are right now working on replicating some of the results from paper III in the TM2 mouse model of AD; this model is an APP knock-in (so no overexpression of APP) with the Swedish and Iberian mutation [47]. If the results are replicated in another and likely more physiologically relevant mouse model, then, I would like to explore how the intracellular Aβ can be targeted for elimination, especially in early AD.

Age is an essential component in the pathogenesis of AD. Even with dominant AD-causing mutations no one below age 30 develops AD. To some degree this argues against a pure stochastic genesis of prion-like Aβ. Compare this with cancers, the mutations of which are also stochastic but made more likely by age or environmental insults. However, some unlucky children do get tumors (admittedly, children get a different spectrum of tumors than adults) yet a child or young adult never gets AD. Is it impossible to form prion-like Aβ without some aging? Or perhaps the incubation time from the formation of prion-like Aβ to AD is very long; i.e., the very unfortunate person that develops AD in their thirties perhaps formed the prion-like Aβ in their childhood. There are suspected cases of iatrogenic cerebral amyloid angiopathy via prion-like mechanisms through dura mater transplantation, human growth hormone injections and other neurosurgical procedures and the "incubation" time there seems to be several decades [27, 44]. Regardless of the special cases of young onset AD, neurodegenerative disorders rarely afflict those below 65 and aging is by far the most important environmental risk factor. What are the components of age that give rise to AD and other neurodegenerative disorders? Failing proteostasis must play a role; these are diseases of protein aggregation, but why does someone get Lewy bodies and PD, while someone else gets plaques, tangles and AD? The brain is an energy intense organ and the early victims of both

AD and PD (ErC and substantia nigra respectively) are particularly energy demanding neurons with extensive processes. Declining mitochondrial function must be important in neurodegeneration. Inflammation is also very relevant, GWAS studies have shown that microglial regulators are significant risk factors for AD [35]. Perhaps targeting ageing is a better idea than targeting Aβ to prevent AD, it would certainly have other positive side effects as well.

We started this section with a tongue in cheek description of Alzheimer's research as a religious war (the amyloid-cascade hypothesis was formulated in 1992, so at time of writing, a 30-years' war). But although, the focus on the research here has been βaptist I believe that neurodegeneration and AD, like aging, is multi-factorial. I believe that we will have treatments (note the plural) against AD in the coming decades and I hope that this work has contributed towards that.

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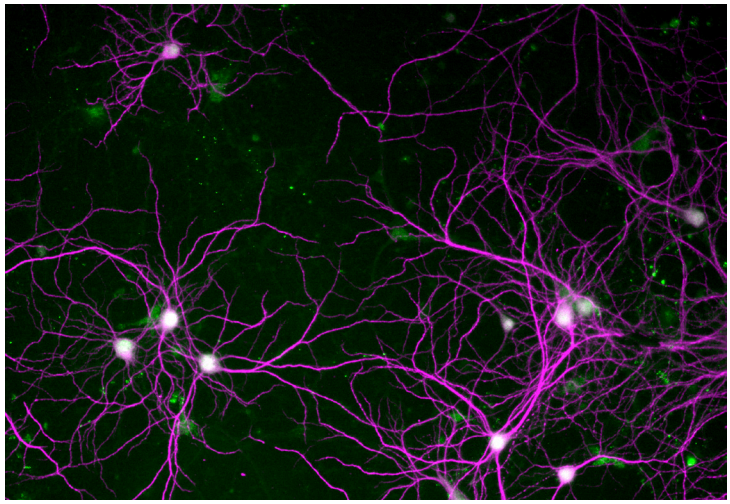

About the author

I started my PhD in 2014. During this time I have also worked as a physician and neurologist, gotten married, had my first child and have another child due in April. I have been interested in neuroscience since reading Oliver Sacks and V.S. Ramachandran as a young teenager. I love the brain and want to protect it, so I hope to continue research into neurodegeneration and clinical neurology. Though, I will try to broaden my research horizons into the aging brain and its failures of proteostasis, energy homeostasis and dysregulated inflammation.

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