Neuroinflammation and amyloid-β in early Alzheimer's disease. Insight into the earliest events using mouse models

Garcia, Megg Gonzales

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MEGG G. GARCIA-RYDE
DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY

Neuroinflammation and amyloid-β in early Alzheimer's disease

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Megg G. Garcia-Ryde

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Faculty opponent
Dr. Soyon Hong
UK Dementia Research Institute at UCL

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Abstract:

Alzheimer's disease (AD) is the leading cause of dementia and most common neurodegenerative disease worldwide, but there currently exists no effective treatment that can stop nor slow the progression of the disease. The current dogma in the field postulates that the appearance of extracellular amyloid-beta (Aβ) plaques, a histopathological hallmark of the disease, is the trigger for downstream, detrimental events, including neuronal loss, extensive neuroinflammation and cognitive decline. However, increasing evidence suggests that neuroinflammatory alterations and synaptic and neuronal dysfunction occur already before plaque deposition, which we have also noted in previous work done by our groups. In addition, we have found that Aβ aggregates intracellularly, especially within neurons, before plaque appearance, and this has the ability to impair synaptic function. Therefore, we wonder whether there is an interplay between the neuroinflammatory system, neuronal and synaptic alterations, and intracellular Aβ in the earliest stages of the disease. To address this, we utilize mouse-based models in vivo, primarily the 5xFAD transgenic mouse model, and in vitro neuronal culture models. In the scientific papers included in this thesis work, we explore aspects related to mechanisms and modulations related to early AD. This includes looking at the prion-like spread and properties of intracellular Aβ, identifying sex-specific effects of early-life stress on inflammatory systems as well as neurons and Aβ, and investigating the interaction between neuroinflammatory cells and early aggregated Aβ. Taken together, we have worked to elucidate the earliest events in the disease, including factors that can modulate pathogenesis and the underlying mechanisms. By fostering a greater understanding of AD, we attempt to aid efforts towards the development of an effective disease-modifying treatment.

Key words: Alzheimer's disease, amyloid-beta, neuroinflammation

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To the friends I made along the way

Table of Contents

Popular science summary

Alzheimer's disease is the most common cause of dementia, but there is no effective treatment to prevent nor slow the development of the disease. Amyloid-beta is a protein that aggregates into plaques and is a hallmark of the disease. This leads many scientists to believe that these plaques, which appear outside of cells, cause Alzheimer’s disease. Recent treatments that have or are seeking regulatory approval aim to decrease this extracellular amyloid-beta plaque load, but these treatments have only moderately succeeded in improving the brain function. Therefore, it is important to consider what keeps Alzheimer’s treatments from having a bigger impact on the disease, such as whether treatments can work on different aspects or at the earliest stages of the disease. An increasing number of studies have shown that numerous changes happen in the brain before plaque formation and that factors in the brain's immune system play a bigger role in driving the disease than previously believed. In our research, we observed and emphasized an initial accumulation of amyloid-beta inside of neurons before plaque appearance. This amyloid-beta can impact the function of neurons negatively, which at a large enough scale can impair brain function. Additionally, we and others have noticed that the immune cells and immune environment of the brain are altered in the pre-plaque phase of disease models. These observations have led to the present work as we asked whether there is a connection between amyloid-beta accumulation in neurons and inflammatory changes before plaques appear. Ultimately, an increased understanding of this and the earliest events of Alzheimer’s disease development may contribute to more effective future Alzheimer's treatments.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom är den vanligaste orsaken till demens, men trots detta finns det ingen effektiv behandling för att bota eller bromsa sjukdomsförloppet. Amyloid-beta är ett protein som aggregerar och samlar sig till plack vilket är ett av sjukdomens kännetecken. Detta har lett många forskare att tro att dessa plack, vilka dyker upp utanför celler, orsaker Alzheimers sjukdom. Därför försöker många nya behandlingar under utveckling att minska mängden extracellulära amyloid-betaplack, men dessa behandlingar har bara lyckats bättra hjärnfunktionen lite. Därför är det viktigt att tänka på vad som hindrar Alzheimers behandlingar från att ha större effekt på sjukdomen än vad de har, till exempel som att om behandlingarna skulle kunna göra något åt andra aspekter av sjukdomen eller vid ett tidigare skede av sjukdomen. Ett ökande antal studier har visat att många förändringar händer i hjärnan redan innan placken bildas och att faktorer i hjärnans immunsystem spelar en större roll i att driva sjukdomen än vad man tidigare trott. I vår forskning har vi sett och lagt vikt vid den inledande ansamlingen av amyloid-beta i neuronerna innan placken dyker upp. Denna amyloid-beta kan ha en negativ effekt på neuronernas funktion, vilket i stor nog skala kan ha en effekt på hjärnans funktion. Ytterligare har vi och andra lagt märke till att immunceller och hjärnans immunmiljö är förändrad i pre-plackstadiet i sjukdomsmodeller. Dessa observationer har lett oss till vårt nuvarande jobb då vi frågat oss om där är ett samband mellan amyloid-betaansamling i neuroner och förändringar i inflammation för placken dyker upp. Till sist skulle en ökad förståelse för detta och de tidigaste händelser i utvecklingen av Alzheimers sjukdom kunna bidra till effektivare behandlingar i framtiden.

List of papers

Note that all papers are published as Megg G. Garcia (maiden name).

Paper I

Tomas T. Roos, **Megg G. Garcia**, Isak Martinsson, Rana Mabrouk, Bodil Israelsson, Tomas Deierborg, Asgeir Kobro-Flatmoen, Heikki Tanila, Gunnar K. Gouras (2021) Neuronal spreading and plaque induction of intracellular Aβ and its disruption of Aβ homeostasis. *Acta Neuropathologica* 142:669–687. doi: 10.1007/s00401-021-02345-9

Paper II

Sara Bachiller, Isabel Hidalgo, **Megg G. Garcia**, Antonio Boza-Serrano, Agnes Paulus, Quentin Denis, Caroline Haikal, Oscar Manouchehrian, Oxana Klementieva, Jia-Yi Li, Kees-Jan Pronk, Gunnar K. Gouras, Tomas Deierborg (2022) Early-life stress elicits peripheral and brain immune activation differently in wild type and 5xFAD mice in a sex-specific manner. *Journal of Neuroinflammation* 19:151. doi: 10.1186/s12974-022-02515-w

Paper III

Megg G. Garcia, Agnes Paulus, Sandra Vázquez-Reyes, Oxana Klementieva, Gunnar K. Gouras, Sara Bachiller, Tomas Deierborg (2023) Maternal separation differentially modulates early pathology by sex in 5xFAD Alzheimer's disease-transgenic mice. *Brain, Behavior, & Immunity – Health* 32, 100663. doi: 10.1016/j.bbih.2023.100663

Paper IV

Megg G. Garcia, Emma Nyberg, Sabine Konings, Luis Quintino, Bodil Israelsson, Oxana Klementieva, Cecilia Lundberg, Sara Bachiller, Tomas T. Roos, Tomas Deierborg, Gunnar K. Gouras (2023) The medial mammillary nuclei are sites of early aggregated amyloid-beta and neuroinflammation in 5xFAD Alzheimer's disease-transgenic mice. Manuscript in preparation.

Co-authored papers not included in this thesis

- Isak Martinsson, Luis Quintino, **Megg G. Garcia**, Sabine C. Konings, Laura Torres-Garcia, Alexander Svanbergsson, Oliver Stange, Rebecca England, Tomas Deierborg, Jia-yi Li, Cecilia Lundberg, Gunnar K. Gouras. (2022) Aβ/Amyloid Precursor Protein-Induced Hyperexcitability and Dysregulation of Homeostatic Synaptic Plasticity in Neuron Models of Alzheimer's Disease. *Frontiers in Aging Neuroscience* 14:1-16. doi: 10.3389/fnagi.2022.946297
- Antonio Boza-serrano, Agathe Vrillon, Karolina Minta, Agnes Paulus, Lluís Camprubí-ferrer, **Megg Garcia**, Ulf Andreasson, Anna Antonell, Malin Wennström, Gunnar Gouras, Julien Dumurgier, Emmanuel Cognat, Laura Molina-porcel, Mircea Balasa, Javier Vitorica, Raquel Sánchez-valle, Claire Paquet, Jose Luis Venero, Kaj Blennow, Tomas Deierborg (2022) Galectin-3 is elevated in CSF and is associated with Aβ deposits and tau aggregates in brain tissue in Alzheimer's disease. *Acta Neuropathologica* 144:843-859. doi: 10.1007/s00401-022-02469-6
- Agnes Paulus, Anders Engdahl, Yiyi Yang, Antonio Boza Serrano, Sara Bachiller, Laura Torres-Garcia, Alexander Svanbergsson, **Megg Garcia**, Gunnar Keppler Gouras, Jia-Yi Li, Tomas Deierborg, Oxana Klementieva (2021) Amyloid Structural Changes Studied by Infrared Microspectroscopy in Bigenic Cellular Models of Alzheimer's Disease. *International Journal of Molecular Sciences* 22(7):3430.

Abstract

Alzheimer's disease (AD) is the leading cause of dementia and most common neurodegenerative disease worldwide, but there currently exists no effective treatment that can stop nor slow the progression of the disease. The current dogma in the field postulates that the appearance of extracellular amyloid-beta (Aβ) plaques, a histopathological hallmark of the disease, is the trigger for downstream, detrimental events, including neuronal loss, extensive neuroinflammation and cognitive decline. However, increasing evidence suggests that neuroinflammatory alterations and synaptic and neuronal dysfunction occur already before plaque deposition, which we have also noted in previous work done by our groups. In addition, we have found that Aβ aggregates intracellularly, especially within neurons, before plaque appearance and that this has the ability to impair synaptic function. Therefore, we wonder whether there is an interplay between the neuroinflammatory system, neuronal and synaptic alterations, and intracellular Aβ in the earliest stages of the disease. To address this, we utilize mouse-based models *in vivo*, primarily the 5xFAD transgenic mouse model, and *in vitro* neuronal culture models. In the scientific papers included in this thesis work, we explore aspects related to mechanisms and modulations related to early AD. This includes looking at the prion-like spread and properties of intracellular Aβ, identifying sex-specific effects of early-life stress on inflammatory systems as well as neurons and Aβ, and investigating the interaction between neuroinflammatory cells and early aggregated Aβ. Taken together, we have worked to elucidate the earliest events in the disease, including factors that can modulate pathogenesis and the underlying mechanisms. By fostering a greater understanding of AD, we attempt to aid efforts towards the development of an effective disease-modifying treatment.

Abbreviations

Includes only abbreviations used in more than one section.

AAV	Adeno-associated virus
Aβ	Amyloid-beta
AD	Alzheimer's disease
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BDA	Biotinylated dextran amine
CA	Cornu ammonis
CTF	C-terminal fragment
EOAD	Early-onset Alzheimer's disease
LOAD	Late-onset Alzheimer's disease
N2a	Neuro-2a (cell line)
NSAID	Nonsteroidal anti-inflammatory drug
P	Postnatal day
PSEN	Presenilin
sAPP	Soluble amyloid precursor protein
TBI	Traumatic brain injury
TREM2	Triggering receptor expressed on myeloid cells 2
WT	Wild type

Introduction

The *Space Invaders*-inspired cover image is meant to serve as an analogy for the area of Alzheimer's disease (AD) that is covered in this thesis. In the image, increasingly reactive microglia take the role of the offending aliens, and a pyramidal neuron stands in as the defending ship. The shots being exchanged between alien and ship can be likened to cytokines and other molecules that mediate communication between neurons and microglia. In the original game, there is a barrier that shields the ship from the attacks of the aliens, disintegrating with every shot from the aliens (or, in some cases, a misplaced shot from the ship). Here, synaptic boutons are the buffer between the microglia and neuron. The brain in the upper right corner stands in as the life counter, and the score is displayed with birthday candles as a nod to increasing age being the biggest risk factor for AD but also how long this PhD work took in years. Like how the difficulty increases in the original game – as the space invaders are picked off, the score increases – microglia also change with increasing age, functioning differently and potentially in less neuron-supportive ways.

Though the image depicts microglia as an antagonist to neurons, that is not quite the case in reality. Microglia support neuronal and brain function by protecting against foreign threats and maintaining their local environment, among other things. At times, it can seem like microglia are the "bad guys" in AD because they can create an inflammatory, neurotoxic environment by releasing cytokines and other signals and aberrantly prune synapses, ultimately affecting neuronal activity and function.

The remainder of this thesis will explore the topic of neuroinflammation and aggregated amyloid-beta in early AD – what is known, what we found, and what remains unknown. My hope is that, after going through this thesis, the reader revisits the cover image and understands how neuron-microglia interactions can be related to the game *Space Invaders* as well as what the analogy fails to capture.

What's in a name? – a brief historical perspective

In November of 1906, Alois Alzheimer, a Bavarian psychiatrist and neuropathologist, presented the peculiar case of Auguste D. at a meeting for psychiatrists (*37 Versammlung Südwestdeutscher Irrenarzte*) in Tübingen, Germany (Alzheimer, 1906). Auguste D. was a female psychiatric patient who was hospitalized in 1901 at the age of 51 for cognitive impairment and rapidly declining memory at the mental institute in Frankfurt (*Städtischen Anstalt für Irre und Epileptische in Frankfurt am Main*), where Alzheimer examined her (Maurer et al., 1997). In April 1906, Auguste D. passed away, and Alzheimer investigated her brain post-mortem. In addition to presenting his results at the 1906 conference, he published them in a paper in 1907, called "On an Unusual Illness of the Cerebral Cortex" ("*Über eine eigenartige Erkrankung der Hirnrinde*") (original German: Alzheimer, 1907; English translation: Stelzmann et al., 1995). There, Alzheimer described the presence of "minute miliary foci" deposited around the cortex and intraneuronal fibrils, which we now know were extracellular amyloid-beta (Aβ) plaques and neurofibrillary tau tangles, respectively. These observations taken together with Auguste D.'s relatively young age at hospitalization led Alzheimer to remark that the case at hand went beyond the diseases known at that time.

In the same year that Alzheimer published that article, Oskar Fischer, a Czech psychiatrist and neuropathologist, published an article describing his observations in post-mortem brains from senile dementia[*] cases wherein he described, for the first time, neuritic plaques (Fischer, 1907). It is important to note that neither Alzheimer nor Fischer were the first to describe plaques: Georges Marinesco, a Romanian neurologist, and Paul Blocq, a French pathologist, noted plaques in 1892 in the brain of epilepsy patients (Blocq and Marinescu, 1892), and Emil Redlich, an Austrian neurologist, described plaques, even using that term, in two cases of senile dementia (Redlich, 1898). What differentiated Fischer's observations of plaques from those of his predecessors was that Fischer noted neuronal elements within the plaques (Fischer, 1907, 1910, 1912). Figure 1 shows his observations that he illustrated himself of club-like, distended neurites associated with plaques (Fischer, 1907; Goedert, 2009).

[*]Senile dementia refers to dementia that was thought to be due to old age (65 years and older). This term is considered outdated as it is derived from the idea that dementia is a normal part of aging and the word "senile" often carries a negative connotation in modern usage.

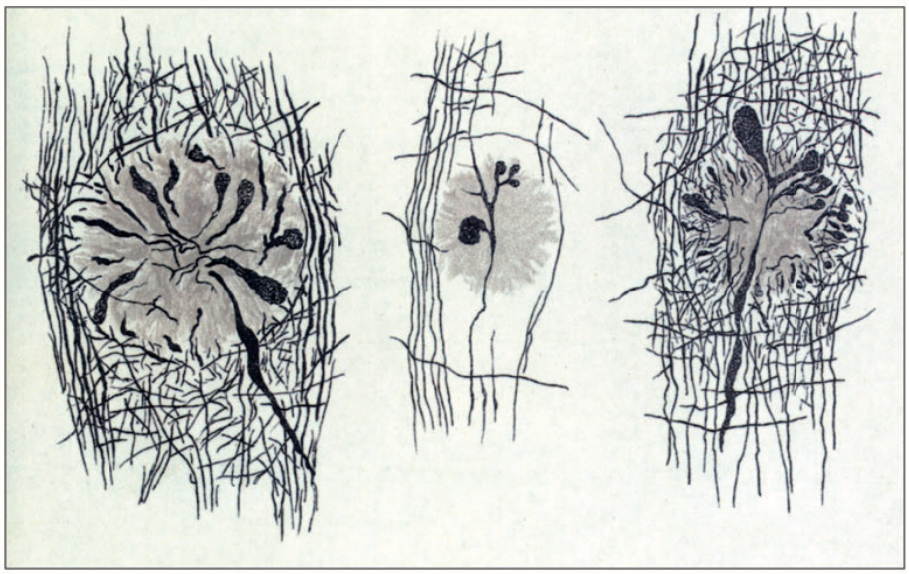

Figure 1. Original illustrations from Oskar Fischer's 1907 paper showing neuritic plaques in senile dementia. Illustrations were compiled into the figure shown here in Goedert (2009). Reproduced under a CC BY-NC 2.0 UK license.

Alzheimer was not alone in trying to understand what was going on in the brains of dementia patients, so how did the disease come to be called "Alzheimer's disease"? One of the biggest contributors to this is likely Emil Kraepelin, a German psychiatrist who was already prominent at the time and mentor to Alzheimer. In 1910, Kraepelin published a section in his textbook on Alzheimer's findings and called the disease Alzheimer's disease (*Alzheimersche Krankheit*) (Kraepelin, 1910). Still, Alzheimer's disease as it was known then was differentiated from senile dementia as the occurrence of symptoms and pathology occurred before the age of 65 (Berchtold and Cotman, 1998).

We know now that Alzheimer was describing a case of early-onset AD and that Fischer, as well as others, described pathology in late-onset AD. However, in its original usage, Alzheimer's disease exclusively referred to early-onset cases, so how did the term come to encapsulate both early- and late-onset forms of the disease? The answer is likely the culmination of many events. Some factors proposed by Michel Goedert (2009) on why the name "Alzheimer's disease" stuck – without a nod to Fischer – include the codification of Alzheimer's disease in textbooks, the link between Fischer's discoveries to the diagnosis of presbyophrenic

dementia[†], which was a concept that fell out of favor, and Fischer's unfortunate circumstances as a victim of the Nazi German regime at the time. Nevertheless, it was realized that both early- and late-onset cases shared symptoms and histopathology. In a twist of time, modern-day usage of the term Alzheimer's disease, more often than not, refers to the late-onset variant of the disease, which constitutes the largest proportion of cases.

Since it was given its moniker over 100 years ago, the concept of *Alzheimer's disease* has broadened, and great strides have been made in our understanding of the disease by many researchers both then and now. Though always advancing technology sheds light on aspects of the disease that would have been impossible before, many of the astute observations of past researchers like Fischer still hold and continue to be elaborated on in modern research.

Alzheimer's disease as we know now

Worldwide, it is estimated that 50 million people have AD, a number that is projected to increase to 150 million people in 2050 (Nichols et al., 2022; Prince et al., 2015). Taking into consideration preclinical[‡] and prodromal[§] AD cases, in addition to diagnosed cases, it is estimated that around 416 million people fall within the AD continuum (Gustavsson et al., 2023). Looking only at Sweden and clinical cases, an estimated 100 000 people are currently affected by AD, though this number is also expected to increase in the future (Hjärnfonden, 2021). In regard to sex, women make up disproportionately more AD cases compared to men, though the complete reason behind that remains unclear (Beam et al., 2018; Ferretti et al., 2018).

The vast majority of AD cases are idiopathic[**], meaning the cause of the disease is unknown. Similar to past categorizations of dementia (presenile and senile dementia), AD can be divided into early- (EOAD) and late-onset AD (LOAD) based on the age at which clinical symptoms appear with the cutoff most commonly being either 60 or 65 years of age. LOAD is overwhelmingly more common than EOAD as EOAD accounts for between 1-10% of all AD cases (Reitz et al., 2020; Zhu et al., 2015).

[†] Presbyophrenic dementia was considered a subset of dementia characterized primarily by confabulation (false memories) and memory impairment. See Berrios (1986) for a historical review.

[‡] Defined as positive for Aβ and tau biomarkers but without symptoms.

[§] Defined as positive for Aβ and tau biomarkers mild cognitive impairment

[**] Idiopathic AD is commonly referred to as sporadic AD.

Risk factors

Insight from genetic studies

There is a misconception that most, if not all, EOAD cases are due to autosomal dominant mutations, meaning that one copy of a mutant gene is enough to cause AD. However, of EOAD cases, only around 10% are estimated to be due to autosomal dominant mutations, meaning they cause ≤ 1% of all AD cases (Wingo et al., 2012). These autosomal dominant mutations occur in genes related to Aβ production, namely the genes for amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*), which are represented in the upper left region of Figure 2 and will be discussed more later. The remaining ~90% of EOAD cases are idiopathic, though genetic risk factors that contribute to LOAD risk likely also contribute to EOAD risk (Cruchaga et al., 2018; Sirkis et al., 2022). Interestingly, even LOAD is estimated to have a relatively high degree of heritability (Bergem et al., 1997; Gatz et al., 2006; Ridge et al., 2016).

Since the late 2000s, an increasing number of genome-wide association studies have given us insight into not only genetic variants that increase AD risk but also the biological pathways that are involved in the development of the disease. Figure 2 plots out genes that have variants that may impact one's risk of AD; genetic variants that confer a higher risk of AD are less common in the population. Functionally, AD risk genes tend to involved in Aβ or tau processing, lipid metabolism, the endosome-lysosome system, or neuroinflammatory function (Baker et al., 2023; Bellenguez et al., 2022).

Of all the genes that can modulate AD risk, apolipoprotein E (*APOE*) is the most notable. *APOE* has three major allele forms: ε2, ε3, and ε4. Each person has two copies of the *APOE* gene and can inherit any combination of alleles. Worldwide, ε3 is the most common allele followed by ε4 then ε2, and this holds generally true between different populations though the exact proportions of each allele vary within each (Corbo and Scacchi, 1999; Farrer et al., 1997; Kern et al., 2015). *APOE* ε4 is the strongest genetic risk factor for AD and lowers the age on onset of LOAD (Corder et al., 1993; Poirier et al., 1993; Sando et al., 2008; Tsai et al., 1994). Conversely, *APOE* ε2 seems to protect against AD (Corder et al., 1994; Serrano-Pozo et al., 2015).

Besides mutations in the sequence, genes with a causative role in AD, namely APP and PSEN1, can also cause AD due to the presence of an extra genetic copy within an individual. Notably, individuals with Down syndrome, who have a partial or whole extra third copy of chromosome 21, have a high chance of developing EOAD due to having an extra copy of *APP*, which is located on chromosome 21. Extra *APP* copies have also been found in individuals without Down syndrome due to a duplication of *APP* within the chromosome; these rare duplications have been noted

as causing familial EOAD in the affected individuals (Blom et al., 2008; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006).

Beyond variants that increase the risk of AD, there are also genetic variants that seem protective, i.e., decrease the risk of AD. *APOE* ε2, which was previously mentioned, is one example. Additionally, there are variants of *APOE* ε3 that may be protective, called the Christchurch (Arboleda-Velasquez et al., 2019) and Jacksonville variants (Liu et al., 2021; Medway et al., 2014). Another is the Icelandic mutation in *APP*, which will be discussed more later. More recently, a protective mutation in *RELN*, which encodes the protein reelin, was reported in a Colombian man with a familial-AD *PSEN1* mutation, similarly to how the *APOE* ε3 Christchurch variant was discovered (Lopera et al., 2023). Still, reliable knowledge of protective genetic variants in AD remains limited compared to risk-conferring genetic variants and more work remains to be done in this area to determine the functional role of protective variants (Andrews et al., 2019).

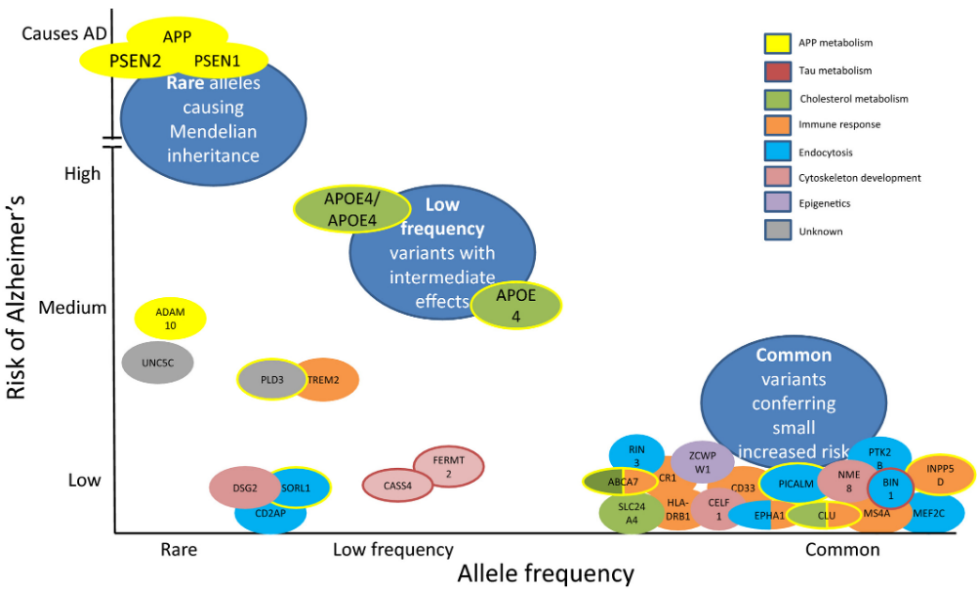

Figure 2. Genes with AD risk variants graphed according to their frequency in the population (x-axis) and the degree of risk they confer of developing AD (y-axis). Genes are color coded according the primary functional pathway(s) they are associated with (key in upper right). Genes with a yellow or red outline indicate that the gene may influence APP or tau metabolism, respectively. Reproduced from Lane et al. (2017) © EAN with permission. As figure is a derivative work, permission obtained for reproduction of the relevant original works in Karch and Goate (2015) © Society of Biological Psychiatry and Guerreiro et al. (2013) © Elsevier.

Other factors

The biggest risk factor for AD is increasing age; starting from 65 years of age, the risk of developing AD doubles every five years (Ziegler-Graham et al., 2008). Among those aged 65 years and older, women have nearly double the remaining lifetime risk of developing AD compared to men (Seshadri et al., 1997). Moreover, race and ethnicity also seem to play a role as, in the U.S., African Americans have the highest risk of AD compared to other races/ethnic groups (Lim et al., 2022).

Modifiable risk factors are of particular interest as they have the potential to be leveraged therapeutically to modulate the risk of developing AD. Head injury is generally associated with an increased risk of dementia (Schneider et al., 2021). Studies looking specifically at heady injury AD risk have also shown increased risk of the disease after experiencing a prior head injury (Fleminger et al., 2003; Plassman et al., 2000). With traumatic brain injury (TBI) [††], APP, Aβ, and tau are upregulated, linking TBI with AD on a molecular level (Edwards et al., 2017; Tsitsopoulos and Marklund, 2013).

Unlike TBI, there are a handful non-genetic risk factors with mostly unclear mechanistic connections to AD that do show a connection to AD risk. Higher education is generally associated with a lower risk of AD; conversely, lower education is associated with an increased risk of AD (Maccora et al., 2020; Xu et al., 2016). Developing type 2 diabetes mellitus (Gudala et al., 2013) or late-life depression (Diniz et al., 2013) also seem to increase AD risk. Additionally, there are risk factors that seem or are highly suggestive to be risk factors for AD, which are reviewed in Bellou et al. (2017).

Unlike the genetic risk factors discussed previously, non-genetic risk factors for AD tend to be less disease-specific and affect dementia risk in general (Mentis et al., 2021). Nevertheless, consideration of non-genetic risk factors, particularly modifiable ones, are of interest for efforts to reduce one's overall AD risk. Overall, consideration and continued study of both genetic and non-genetic risk factors is important, especially since most AD cases are idiopathic and likely have a multifactorial etiology.

Histopathology

Unchanged from what Alzheimer and others described more than 100 years ago, AD is histopathologically characterized post-mortem by extracellular Aβ plaques and intraneuronal neurofibrillary tau tangles such as those in Figure 3. Indeed, a quick Google search of the exact phrase "Alzheimer's disease is characterized by" is followed by 884 000 results with the phrase being completed with some variation

[††] Traumatic brain injury is a kind of head injury. Colloquially, the terms are sometimes used interchangeably.

of wording to express extracellular amyloid-beta plaques and neurofibrillary tau tangles due to the importance of these two hallmarks. More so, identification of plaques and tangles post-mortem is used to definitively diagnose AD, though other changes in the tissue can also be seen (Hyman and Trojanowski, 1997).

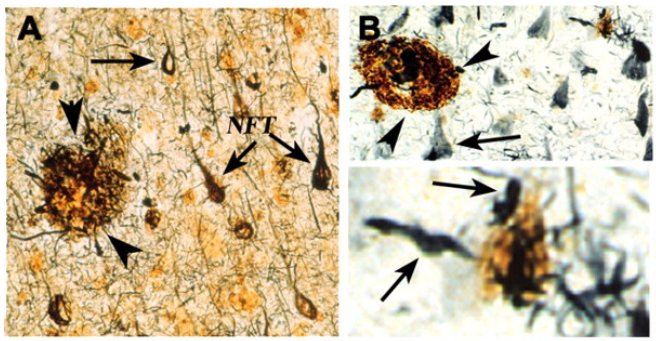

Figure 3. Histopathological hallmarks in AD. Description of the figure from the original article (Nixon, 2007): "(A) The two hallmark features of Alzheimer disease, β-amyloid plaques (arrowheads) and neurofibrillary tangles (arrows) in AD brain are revealed by the Bielschowsky silver stain. (B) Antibodies against paired-helical-filament (PHF) tau (arrows) and β-amyloid (arrowheads) label PHF-containing neurites associated with amyloid deposits." Reproduced with permission from Journal of Cell Science.

Current therapies

Though we know more now than when the disease first got its name, there still exists no treatment that can prevent nor effectively modify the progression of the disease. There are three types of drugs approved to treat AD: acetylcholinesterase (AChE) inhibitors, an N-methyl-D-aspartate (NMDA) receptor antagonist, and anti-Aβ immunotherapies. With the recent approval of an anti-Aβ immunotherapy, all three types of AD treatment are available in the U.S. In Europe, as of writing this thesis, no anti-Aβ immunotherapies have been approved yet, so the only treatments for AD in the European marker are AChE inhibitors and an NMDA receptor antagonist.

AChE inhibitors were the first type of drug to be approved to treat AD with the approval of tacrine in 1993 (Crismon, 1994). Used to treat mild to moderate cases of AD, these drugs work by inhibiting the breakdown of acetylcholine, levels of which are lower in AD brain. Following this came the NMDA receptor antagonist memantine, which was approved to treat cases of moderate to severe AD in the early 2000s.

The most recent drugs approved to treat AD are anti-Aβ immunotherapies. As of writing this thesis, there are two antibody treatments on the market: aducanumab (brand name Aduhelm®), released in 2021, and lecanemab (brand name Leqembi®), released in 2023. Both drugs had received accelerated approval by the

U.S. Food and Drug Administration as both showed the ability to reduce Aβ plaque load (Cavazzoni, 2021; Office of the Commissioner, 2023a). However, only lecanemab has gone on to receive traditional approval as it had a moderate effect on cognitive decline (Office of the Commissioner, 2023b; van Dyck et al., 2023). Though aducanumab has been shown to reduce Aβ plaque load, the cognitive benefit was not significant (Sevigny et al., 2016).

Amyloid-beta

The primary constituent of extracellular plaques in AD is $A\beta_{42}$. First isolated and identified from plaques in the 1980s, Aβ is a relatively small peptide that is commonly either 40 ($A\beta_{40}$) or 42 ($A\beta_{42}$) amino acids long (Asami-Odaka et al., 1995; Dovey et al., 1993; Glenner and Wong, 1984). Aβ is produced from the sequential cleavage of amyloid precursor protein (APP), which is covered here.

APP processing

APP is a transmembrane protein that is primarily processed via one of two pathways, the non-amyloidogenic pathway or the amyloidogenic pathway, illustrated in Figure 4.

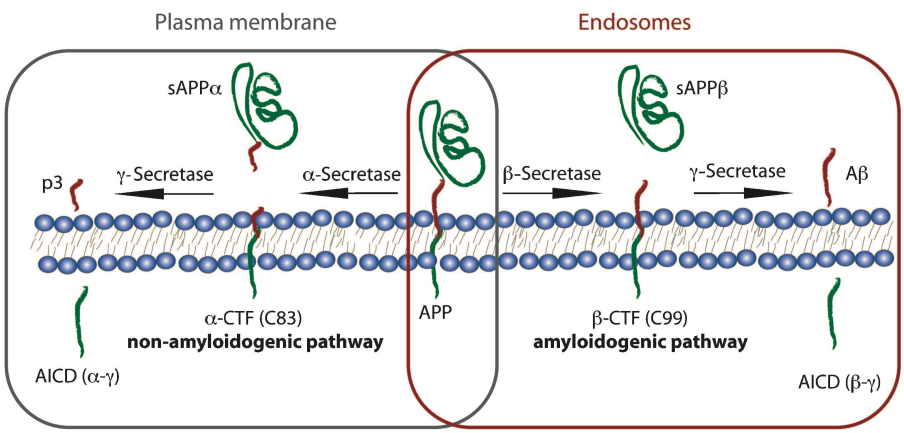

Figure 4. APP processing pathways. To the left is the non-amyloidgenic pathway that occurs primarily at the cell surface. To the right is the amyloidgenic pathway that preferentially takes place in endosomes. Original figure from Rajendran and Annaert (2012). Reproduced with permission from John Wiley and Sons.

The non-amyloidogenic pathway takes place primarily at the cell surface. There, APP is first cleaved by α-secretase into soluble APP α (sAPPα), which is released

into the extracellular space, and α C-terminal fragment (α-CTF, also known as C83), which remains in the membrane. This is followed by cleavage of α-CTF into p3 and APP intracellular domain (AICD) by γ-secretase.

In the amyloidogenic pathway, APP is first cleaved by β-secretase to produce soluble APP β (sAPPβ) and β C-terminal fragment (β-CTF, also known as C99). From there, β-CTF is cleaved by γ-secretase into Aβ and AICD. The most common lengths of Aβ produced are 40 and 42 amino acids long, but other lengths of Aβ can be produced due to different cleavage patterns or truncation (Kummer and Heneka, 2014). Moreover, as Figure 5 shows, the amyloidogenic pathway preferentially takes place in the endosome-lysosome system, and Aβ production has also been noted in the endoplasmic reticulum and trans-Golgi network (Hartmann et al., 1997).

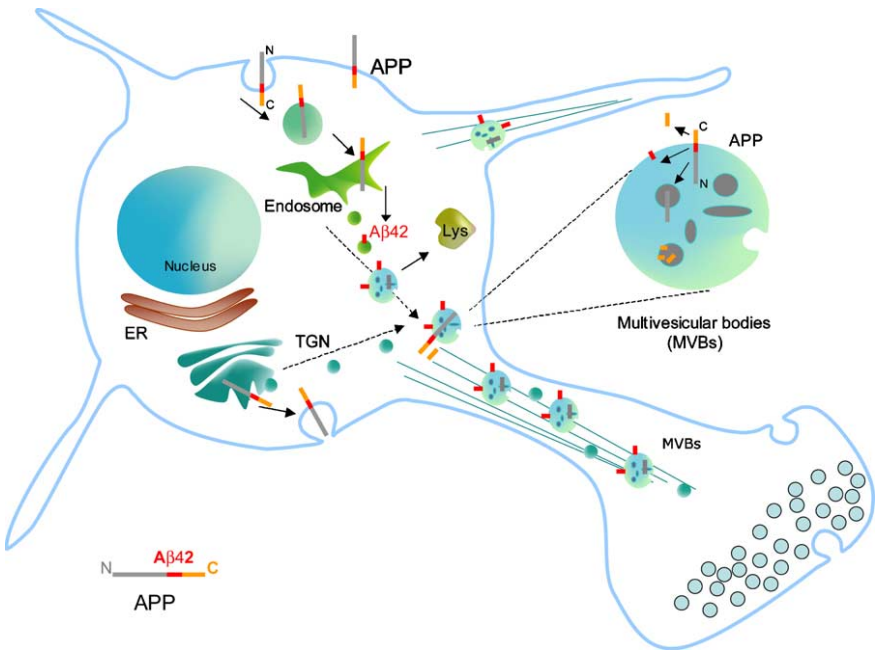

Figure 5. Description from the original article (Gouras et al., 2005): "Schematic diagram of APP and Aβ trafficking within a neuron. APP is trafficked from the ER, where some Aβ may be generated, to the Golgi apparatus and then the plasma membrane (PM) where additional Aβ appears to be generated. Significant Aβ is produced in the trans-Golgi network (TGN). An important site of Aβ generation is in the endocytic pathway after APP internalization from the PM. Although APP localizes especially to the TGN, both APP and Aβ localize to vesicles within neuronal processes. In Alzheimer's disease Aβ42 accumulates within multivesicular bodiess of vulnerable neurons, especially within distal neuronal processes and pre- and post-synaptic compartments." ER = endoplasmic reticulum. MVBs = multivesicular bodies. Reproduced with permission from Elsevier.

Familial Alzheimer's disease

A number of families around the world have genetic mutations that lead to EOAD. These mutations lie within the genes for amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) and affect APP processing or Aβ aggregation propensity. Mutations in *PSEN1* are the most common, followed by *APP* and *PSEN2*. PSEN1 and PSEN2 are part of the γ-secretase complex, which cleaves the CTF left after α- or β-cleavage. How *PSEN* mutations cause AD remains mostly unclear, but evidence suggests that *PSEN* familial AD mutations are loss-of-function (De Strooper, 2007). The resulting impaired function of PSEN1 seems to result in a different proportion of Aβ forms which has pathological consequences (Weggen and Beher, 2012; Petit et al., 2022).

Compared to *PSEN* mutations, familial AD mutations in *APP* are relatively easier to understand. As shown in Figure 6, these mutations can be located both within and outside of the Aβ sequence. Table 1 lists the mutations presented in Figure 6 and states their primary effect on Aβ.

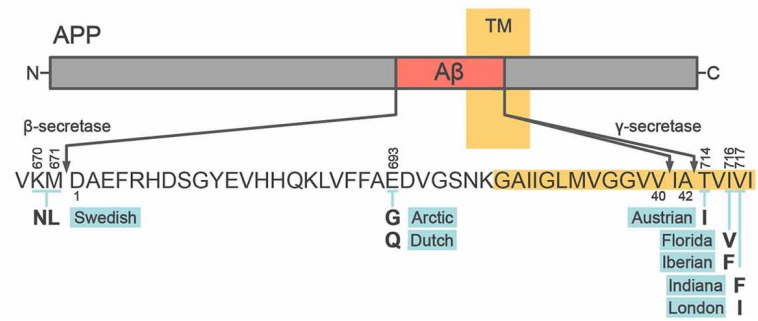

Figure 6. Aβ mapped to APP. Aβ amino acid sequence with cleavage sites and familial AD mutations indicated. Adapted from Figure 1B in Yokoyama et al. (2022). Reproduced here under a CC BY 4.0 license.

Table 1. Select familial AD mutations in APP in order of their location and their effect on Aβ. Data was obtained from the Alzforum mutations database (Alzforum, n.d.).

Mutation name	Amino acid changes	Effect
Swedish	K670N/M671L	Overall increased Aβ levels
Arctic	E693G	More readily aggregates into protofibrils
Dutch	E693Q	More readily aggregates into fibrils
Austrian	T714I	Increased Aβ42/Aβ40 ratio
Florida	I716V	Increased Aβ42/Aβ40 ratio
Iberian	I716F	Increased Aβ42/Aβ40 ratio
Indiana	V717F	Increased Aβ42/Aβ40 ratio, promotes longer Aβ
London	V717I	Increased Aβ42/Aβ40 ratio

Unlike the mutation in Table 1, the Icelandic mutation (A673T) is particularly interesting as it was the first mutation in *APP* that was found to be protective and reduce the risk of developing AD (Jonsson et al., 2012). Mechanistically, this mutation seems to impact cleavage of APP by β-secretase and attenuate the amyloidogenic pathway. As a result, less Aβ is generated.

Taken together, the effects produced by the mutations that cause familial AD support the notion of Aβ being critical for the pathogenesis of AD. Though Aβ in AD is most notably seen in plaques, accumulation of intracellular Aβ precedes plaque appearance and may have its own, early role to play in AD.

Intracellular amyloid-beta and plaque origin

Before discussing what intracellular Aβ is, it may be insightful to look briefly into why intracellular Aβ is much less focused on in the field compared to plaques. A seemingly obvious reason is that plaques can become very large and are a hallmark of AD. A lesser known but potential factor is that, as plaque deposition increases, intracellular Aβ decreases, making intracellular Aβ difficult to study in areas saturated with plaques (Gouras et al., 2000; Mori et al., 2002). Practically, Aβ, especially $A\beta_{42}$, can be difficult to work with – even more so when trying to analyze intracellular Aβ (Christensen et al., 2009; Gouras et al., 2012). In these situations, this quote by Carl Sagan is especially relevant: "Absence of evidence is not evidence of absence."

With those aspects considered, the existence of intracellular Aβ should not come as a surprise as, mentioned earlier, amyloidogenic processing of APP preferentially occurs in the endosome-lysosome system. Indeed, we have noted that Aβ accumulates in multivesicular bodies, which is illustrated in Figure 5, and that accumulation may be a driver of early synaptic impairment in AD (Takahashi et al., 2002). Moreover, evidence from Down syndrome and patients with mild cognitive impairment support the accumulation of Aβ intracellularly before plaque appearance (Gouras et al., 2000; Mori et al., 2002). Even more so, aggregated intracellular Aβ may serve as the starting point for plaques (D'Andrea et al., 2001; Friedrich et al., 2010; Gouras et al., 2005; Lee et al., 2022; Pensalfini et al., 2014).

Aggregation and prion-like spread

Plaques are composed of Aβ in an aggregated state. These Aβ aggregates can be classified into different groups depending on the number of peptides bound together and the overall structure, starting from monomers and followed by oligomers, proto-fibrils, and fibrils. One significant reason for consideration of Aβ aggregate species is that Aβ oligomers and not fibrils nor monomers are considered to be the most damaging (Cline et al., 2018).

As different species of Aβ have different properties, detection of the aggregation state is something to be considered. Traditionally, this was done in tissue using Congo red or thioflavin S (Kelényi, 1967). These dyes are understood as binding to β-sheet structures and indicate that with a visual change: Congo red shows birefringence under polarized light, and thioflavin S fluoresces green. Plaques that are positive for one of those dyes – or newer analogues – are considered dense-core and are the plaques typically thought of in AD. Conversely, diffuse plaques lack such signal. Interestingly, the Arctic mutation in *APP* promotes the development of diffuse plaques (Kalimo et al., 2013). The distinction between dense-core and diffuse plaques is important as they exhibit different pathological phenomena, such as neuroinflammation, which will be discussed later.

The last point of consideration for this section on Aβ is how plaques and aggregated Aβ progressively appears in more brain regions in AD patients. Where Braak stages track neurofibrillary tau tangle progression throughout the brain (Braak and Braak, 1991; Braak et al., 2006), Thal phases does so for amyloid plaques (Thal et al., 2002). From looking at the Thal phases as well as the Braak stages, it can be seen that there is an order to where the AD hallmark aggregates appear, so what is mediating that spread? That some Aβ aggregates possess a prion-like nature is one idea that is proposed (Watts and Prusiner, 2018), which is explored in Paper I of this thesis.

Neuroinflammation

As ~99% of AD cases are not directly caused by a familial AD mutation, mechanisms other than altered Aβ levels or structure must come into play. In recent years, the role of neuroinflammation in driving AD has been increasingly highlighted due to the finding of an AD risk-increasing variant of triggering receptor expressed on myeloid cells 2 (*TREM2*), which can be noted in Figure 2, below *APOE4/4* (Guerreiro et al., 2013b; Jonsson et al., 2013). Still, the implications of neuroinflammation in AD is not new; immunoglobulins and complement factors were found in plaques decades ago (Eikelenboom and Stam, 1982). As the resident immune cell of the brain, microglia were suspected to be the prime actors of the complement in AD brain (Eikelenboom and Veerhuis, 1996). Moreover, the association between nonsteroidal anti-inflammatory drug (NSAID) use and lower AD risk also implicated neuroinflammation as playing a role in AD pathology (in ’t Veld et al., 2001; Rich et al., 1995).

Microglia – the brain's resident immune cell

Microglia are the innate immune cell resident to the brain. During development, microglia begin in the yolk sac and then migrate to what will become the brain (Alliot et al., 1999). These cells exist in a continuum of states from highly ramified cells in a steady-state like mode to very round and ameboid cells with a high phagocytic capacity (Paolicelli et al., 2022). Though the ramified microglia are often thought of as "resting," they are doing everything but that; the ramifications allow the microglia to sample their environment, surveilling in case they need to step in (Bernier et al., 2019; Nimmerjahn et al., 2005). During development, microglia play an important role as cullers of excess synapses and neurons, which is important for proper network formation (Schafer and Stevens, 2015).

In AD, the role of microglia is perhaps even more complicated. There are arguments that microglia are "bad" as dramatically reducing microglial numbers has seemingly beneficial effects in mouse models (Olmos-Alonso et al., 2016; Sosna et al., 2018). On the other hand, *TREM2* variants that are associated with increased AD risk appears to be loss-of-function mutations (Cheng-Hathaway et al., 2018; Prokop et al., 2019). Additionally, microglia with AD-related *TREM2* mutations seem less able to interact with plaques and attenuate the plaque microenvironment (Wang et al., 2016). Therefore, it will be interesting following the development of a TREM2-based treatment that aims to enhance microglial function (Paul et al., 2021), especially given how clinical trials for the use of NSAIDs to treat AD have not panned out as expected (Meyer et al., 2019; The Alzheimer's Disease Anti-inflammatory Prevention Trial Research Group, 2013).

Nevertheless, in AD brain, microglia can often be found associated with plaques, such as in Figure 7, and likely play a role in plaque development and toxicity (Haga et al., 1989; McGeer et al., 1987; Stalder et al., 1999). In addition to phagocytosing and digesting Aβ, other functions of microglia at plaques may be to act as a barrier, keeping more toxic forms of Aβ contained, and compacting plaques (Bolmont et al., 2008; Condello et al., 2015; Huang et al., 2021; Zhao et al., 2017). In support of this, studies of microglia with impaired TREM2 function show that those microglia have a diminished capacity to interact with plaques, leading to reduced plaque compaction and a more toxic plaque environment (Wang et al., 2016; Yuan et al., 2016). However, not all plaques are created equal, and diffuse plaques tend to show markedly reduced microglial reactivity compared to dense-core plaques (Mackenzie et al., 1995; Ohgami et al., 1991). One perspective on this is that diffuse plaques are a precursor to dense core plaques and that microglia in a less-reactive state process the Aβ to facilitate plaque development (Sheng et al., 1997). Alternatively, diffuse plaques may lack the substrates needed to activate microglia (D'Andrea et al., 2004; Jung et al., 2015). Still, it remains unclear what the role of microglia are during the earliest stages of AD and, even more basic, how plaques develop, which is what we investigated in this thesis work.

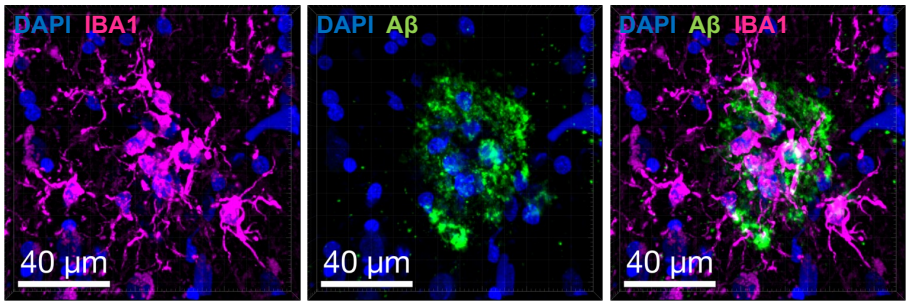

Figure 7. Microglia (magenta, IBA1) are intertwined with a plaque (green, Aβ) in cortex from an AD patient. DAPI (blue) shows cell nuclei. Image is same as in Fig. 3a in Boza-Serrano et al. (2022). Original image was aquired by me and is adapted for presentation here.

Astrocytes

Compared to microglia, astrocytes, also known as astroglia, are more abundant in the brain and are well-known as support cells. These large, star-shaped glia help form and maintain the blood brain barrier (Abbott et al., 2006) and are crucial for neuronal function (Durkee and Araque, 2019). While microglia are the immune specialists in the brain, astrocytes can also exert neuroinflammatory roles and enter a reactive state in response to injury or threat (Han et al., 2021; Sofroniew and Vinters, 2010). In addition, astrocytes can step up and perform some microglial functions when necessary (Konishi et al., 2020). In AD, astrocytic activation biomarkers seem elevated in patients and may be useful in future diagnostic efforts (Bellaver et al., 2021).

As mentioned previously, *APOE* ε4 is the strongest genetic risk factor for AD, and astrocytes are the primary producers of APOE in the brain, which can be seen in Figure 8 (Boyles et al., 1985; Pitas et al., 1987). However, under pathological conditions like AD, microglia can upregulate APOE (Mathys et al., 2019) which may play a role in the disease (Kang et al., 2018). Interestingly, when microglia lack functional TREM2, there is a reduction in APOE associated with plaques (Parhizkar et al., 2019). For example, if the microglia in the mice in Figure 8 lacked functional TREM2, there would likely be much less APOE associated the Aβ plaque than seen here. Furthermore, microglia and astrocytes seem to produce different species of APOE (Lanfranco et al., 2021), so APOE expression by each cell type likely plays different functions. Though not completely clear, there seems to be a pathologically relevant interplay between microglia, astrocytes, and APOE (Parhizkar and Holtzman, 2022; Wang et al., 2021) This may especially be the case at synapses, where microglia, astrocytes, and APOE all play a role in synaptic function (Lane-Donovan and Herz, 2017; Schafer et al., 2013).

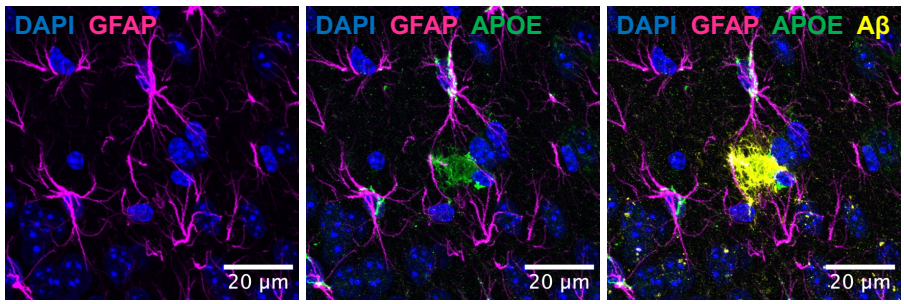

Figure 8. Astrocytes (magenta, GFAP) surrounding a plaque (yellow, Aβ) in the hippocampus of a 5xFAD mouse. Note the APOE (green) associated with the astrocytes but also with the plaque. DAPI (blue) stains cell nuclei.

Peripheral immune system

The role of the peripheral immune system in AD is a significant development from when the brain was thought to be immunologically privileged – mainly meaning, that the brain was isolated from the peripheral immune system on account of the blood brain barrier (Carson et al., 2006). We know now that the reality is not so straightforward. To some degree, the brain can be considered as having immune privilege as the means of peripheral immune cell entry is limited under physiological conditions (Engelhardt and Coisne, 2011; Wilson et al., 2010). In AD, both the peripheral and brain immune system are considered affected, and the altered interaction between the two may promote pathology (Bettcher et al., 2021). In Paper II, we touch on the potential for the peripheral immune system to impact brain function.

Selective vulnerability

In addition to being the most common cause of dementia, AD is also the most common neurodegenerative disease. Though there are many diseases that fall under the neurodegenerative disease umbrella, one aspect that makes each unique is the brain regions affected and, as a result, the cognitive and behavioral effects. For example, in Parkinson's disease, the loss of dopaminergic neurons in the substantia nigra leads to the characteristic motor symptoms of that disease. In AD, the hippocampus is often the poster child for AD-related brain regions as the hippocampus is well-known for being involved in learning and memory, but more regions are affected.

Braak staging and Thal phases spatio-temporally outline the development of AD histopathology in the brain and show that cortical regions and subcortical nuclei are

among the earliest regions affected (Braak et al., 2006; Thal et al., 2002; Braak and Braak, 1991). Some regions identified histopathologically are also a part of the default mode network, which consists of brain regions that are active during a resting or default state (Greicius et al., 2003; Harrison et al., 2008; Raichle et al., 2001). In AD, this network is impaired and seems to be altered early in the disease (Greicius et al., 2004; Mevel et al., 2011; Palmqvist et al., 2017; Scherr et al., 2021; Wang et al., 2013).

The entorhinal cortex, a part of the medial temporal lobe, which in turn is part of the default mode network, is of particular interest in AD (Stranahan and Mattson, 2010). This region, particularly the lateral entorhinal cortex, is considered to be the first region where changes in AD can be detected (Ball, 1978; Khan et al., 2014; Llamas-Rodríguez et al., 2022). This even extends to intracellular Aβ as reelin-positive neurons in layer II of the lateral entorhinal cortex were shown to be among the earliest to develop intracellular Aβ (Kobro-Flatmoen et al., 2016). Still, it remains mostly unknown what underlies such selective vulnerability. One idea is that selectively vulnerable regions are very active areas, which makes them susceptible to activity-modulated Aβ production. (Small and Swanson, 2018). Ultimately, this remains an active field of investigation and is important to consider when doing *in vivo* work.

Aims

The overall aim of this project is to determine what, if any, alterations are occurring in the interplay between neurons and synapses with microglia and neuroinflammation in association with early AD mechanisms, disease modulating risk factors, and intracellular Aβ. To that end, the following four papers included in this thesis take up more specific aspects and aims.

Paper I

- To determine the competence of intracellular Aβ as a prion-like seed *in vivo*
- To understand the dynamics between intra- and extracellular pools of Aβ and how aggregated intracellular Aβ may affect that

Paper II

- To investigate the inflammatory mechanisms that may underlie the effects of early-life stress and its link to neurological diseases and disorders

Paper III

- To examine whether early-life stress can modulate pre-plaque AD-related phenomena

Paper IV

- To define the interaction between microglia and presynaptic early aggregated Aβ

Methodological consideration

There are often multiple ways to accomplish a task. It often seems that identifying the "wrong" solution is much easier than determining the "right" solution. Adding to that, lab protocols usually remain unchanged for years, following the adage "if it ain't broke, don't fix it." The amounts and concentrations that are listed in a protocol can feel arbitrary as they are usually determined empirically, and what works in one lab may not work in another. Regardless, every scientist should understand the general logic behind a protocol. Practically speaking, this understanding is extremely valuable when, for example, a tried-and-true protocol yields no or inconsistent results, so troubleshooting is required (this happens often). Additionally, applications for funding or ethical permission may require justification of the materials and methods used for the question being asked.

In this section, key materials and methods will be briefly discussed. Full details of the materials and methods used can be found in their respective paper(s).

Ethical permission

In Sweden, virtually all research done using animals requires an application to the Swedish Board of Agriculture (*Jordbruksverket*) and approval from a regional board for research animal ethics (*regional djurförsöksetisk nämnd*) (Jordbruksverket, 2022). Researchers in Sweden conducting animal-based experiments must also follow the European Union (EU) directive for conducting research on animals though Swedish rules are considered stricter in comparison. In Sweden as well as the EU, consideration of the 3 R's is required when conducting research on animals: replacement, reduction, and refinement (EU Parliament and Council, 2010; Jordbruksverket, 2019). Replacement refers to whether an alternative to the use of live animals is available and adequate to address the research question at hand. Examples of such alternatives are cell cultures or computer modelling. Reduction poses the question whether fewer animals can be used in answering the research question. Lastly, refinement is the optimization of the experimental procedure to minimize suffering and promote the wellbeing of the research animal(s).

As this PhD project required the use of laboratory animals, ethical permission was obtained. From our perspective, the use of laboratory animals was necessitated by

the fact that cell cultures, including 3D cultures, do not yet capture the complexity of the brain in terms of regionality, connectivity, and cellular diversity, among other reasons. Even the use of primary neuronal cultures, which requires an ethical permit, is not yet entirely replaceable by, at best, human cells reprogrammed into neurons when studying synapses and due to some practical aspects, but advances in methodology make this argument increasingly less convincing.

Briefly, the relevant ethical permits approved by the Malmö/Lund Ethics Committee on Animal Testing in Sweden used in this PhD work will be described. In Paper I, two ethical permits were applicable: Ethical permit 5.8. 18-05983/2019 allowed for the sacrifice of mouse embryos and neonates for primary cell cultures. Ethical permit 5.8 18-12561/2020 allowed for the intracerebral injections of cell and tissue homogenate into mice. In papers II and III, ethical permit 5.8. 18-01107/2018 was needed to conduct the maternal separation protocol and behavioral tests. Note that all ethical permits also require information on animal breeding and sacrifice.

Mouse models

All papers included in this thesis utilize 5xFAD AD-transgenic mice on a B6SJL hybrid background from The Jackson Laboratory and their non-carrier, WT littermates as controls (The Jackson Laboratory, 2023). 5xFAD mice are also available on the more common C57BL/6J background, but according to the breeding company, the phenotype in 5xFAD mice with a congenic C57BL/6 background is less robust compared to in 5xFAD with a B6SJL hybrid background.

The name 5xFAD comes from the fact that they have 5 familial AD mutations. These mutations are located in transgenes encoding mutant human *APP* and *PSEN1*: there are three mutations in *APP* (the Swedish double mutation [K670N/M671L], the Florida mutation [I716V], and the London mutation [V717I]) and two mutations in *PSEN1* (M146L and L286V). This is summarized in Figure 9. The transgenes are expressed under a neuron-specific mouse *Thy1* promotor leading transgenic protein expression to increase over time similarly to the expression of Thy1 (Feng et al., 2000). As the equivalent endogenous genes are not knocked-out, 5xFAD mice also express mouse APP and PSEN1. The transgenic mice used are hemizygous as these mice are viable and can be used for breeding.

The primary reason for using 5xFAD mice in this PhD project was practicality. These mice develop Aβ plaques at 10 weeks of age and intraneuronal Aβ even earlier (Oakley et al., 2006). However, our observations in Paper IV and others suggest that plaques can be detected earlier beginning at 4 to 6 weeks of age in the mammillary nuclei (Gail Canter et al., 2019). Given the time-limited contract and requirement to publish a first-author, peer-reviewed paper as a PhD student, the use of a more aggressive mouse model makes sense.

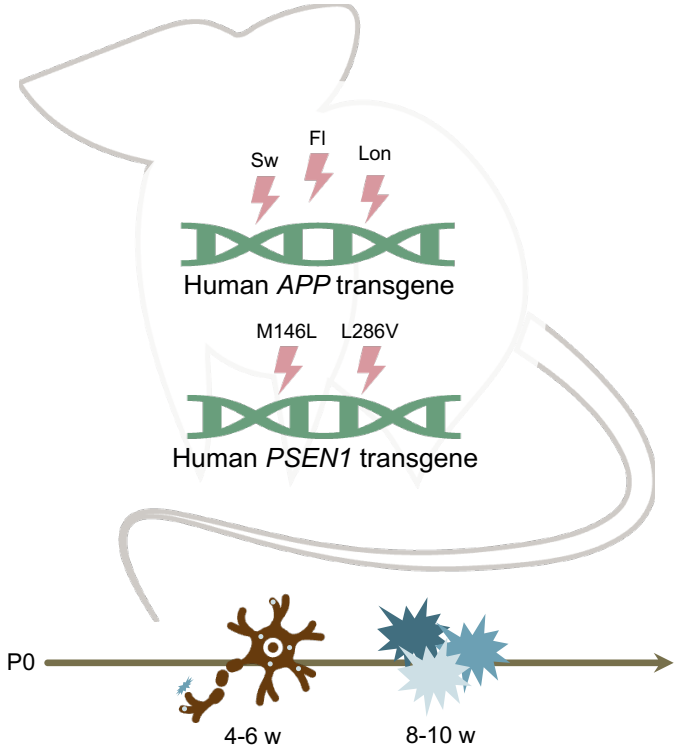

Figure 9. Illustrated description the of 5xFAD mose model. These mice express two transgenes: a mutant human *APP* with three familial AD mutations and a mutant human *PSEN1* with two familial AD mutations. The timeline below the mouse indicates aggregated Aβ progression. Four to six weeks after birth (P0), these mice show prominent intraneuronal Aβ and some early plaques. By eight to ten weeks of age, the amount of plaques in the brain becomes notable and continues to increase with age. Sw = Swedish, Fl = Florida, Lon = London.

We are aware that knock-in mouse models present a more ideal alternative because they express proteins of interest at a more physiological level. For the work done here, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice would have been the knock-in alternative as these mice develop cortical plaques at two months of age (Saito et al., 2014). Still, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice have the Arctic mutations (*G*), in addition to the Swedish (*NL*) and Beyreuther/Iberian (*F*) mutations, which promotes Aβ aggregation but produces primarily diffuse plaques rather than dense-core plaques and, thus, a different neuroinflammatory response (Kalimo et al., 2013). On that note, regardless of the mouse model, they all have their differences, which comes into play. For a comparison of different AD mouse models, see Yokoyama et al. (2022).

Intracerebral injection

Using the mouse models, we can devise experiments that allow us to ask region-specific questions. Intracerebral injections are one technique that allow us to target specific brain regions of interest and downstream regions. To achieve this, stereotactic frames are a key tool as they allow the precise targeting of brain regions based off coordinates in three planes: anterior-posterior, usually relative to bregma, the point where the anterior cortical suture and sagittal suture intersect on the skull; medial-lateral, wherein positive or negative values can indicate right or left relative to the midline; and dorsal-ventral starting from the dura. The desired coordinates can be obtained from species-specific stereotaxic atlases, such as *The Mouse Brain in Stereotaxic Coordinates* that was used to in our work (Franklin and Paxinos, 1997), but optimization of the coordinates is often required due to differences in strain, sex. For the work done here, coordinates were primarily obtained from previous publications and work.

In Paper I, brain homogenate or cell lysate containing seed-competent Aβ was injected into the hippocampus, particularly Cornu ammonis (CA1) stratum lacunosum moleculare, of 5xFAD mice. Mice injected with brain homogenate were sacrificed at different times post-injection in order to observe where and how the Aβ induction progressed. Additionally, an adeno-associated virus (AAV) with the code for the fluorophore mCherry was injected into the lateral entorhinal cortex layer II in order visualize the downstream axon terminals.

In Paper IV, biotinylated dextran amine (BDA) 10,000 molecular weight, an anterograde tracer, was used to show the axon terminals of dorsal subiculum neurons burdened with intraneuronal Aβ in young 5xFAD mice. Note that BDA-10,000 can be transported retrogradely but is preferentially transported anterogradely (Brandt and Apkarian, 1992; Veenman et al., 1992); lower molecular weight BDA, such as BDA-3000, are preferentially transported retrogradely and are better suited for retrograde tracing (Fritzsch, 1993). There are two main ways to inject BDA: pressure injection or iontophoretic injection. Both methods work for anterograde tracing with BDA-10,000, though iontophoretically injecting BDA can help with producing smaller injection sites compared to pressure injecting (Reiner et al., 2000). Here, we used pressure injection.

In both Papers I and IV, intracerebral injection was used to help visualize downstream axon terminals. There are pros and cons for using a virus or a tracer. For a visual comparison, the Allen Brain Atlas systematically injected a recombinant AAV followed by BDA-10,000 into various regions in the mouse brain and uploaded the processed samples into an online resource (Allen Brain Atlas, 2023; Allen Mouse Brain Connectivity Atlas, 2014). Here, the decision whether to use virus or BDA came down to practicality, for the most part. The use of an AAV with mCherry in Paper I was a choice made by collaborators (Rana Mabrouk and Professor Heikki Tanila) who conducted the experiment.

In Paper IV, the first tracing experiments were done using a lentiviral vector encoding EGFP under a synapsin promotor, generated by local collaborators (Luis Quintino and Professor Cecilia Lundberg). One major advantage of using viral vectors is that fluorophore expression can be made cell-specific by using the appropriate promotor. In our case, the synapsin promotor restricted EGFP expression to neurons. Still, though the injections were precise, they were not accurate as EGFP was not detected in the target region (dorsal subiculum). We attributed this to the coordinates being inaccurate and needing optimization as the 5xFAD mice that we were using were only 4 weeks old. Moreover, there came an additional issue: EGFP fluorescence intensity drops at low pH (Haupts et al., 1998). This was problematic as optimal detection of intraneuronal Aβ uses an antigen retrieval step with relatively concentrated formic acid, which will be discussed more later (Christensen et al., 2009). The use of a different fluorophore that is more tolerant to low pH could remedy this issue (see Shinoda et al., 2018 for a table listing fluorophores and their pK_a). However, because of time constraints and other practical issues, we decided to move forward with BDA-10,000 and using slightly older mice (around 6 to 8 weeks of age at time of injection) to improve coordinate accuracy.

A last point to consider is that the insertion of a needle when doing experiments like these is that increased $A\beta_{42}$ expression can be noticed in the needle track (Gouras et al., 2010). The implications of this may not be so relevant if analyses will be done on downstream regions. However, this also raises the possibility of studying the needle track area as a model of TBI.

Maternal separation

Early-life stress in mice can be induced by different means. Here, maternal separation was used in Papers II and III, and the sequelae were studied. The protocol used involved separating pups from their dams starting from postnatal day 2 to postnatal day 14 for 3 hours per day, illustrated in Figure 10. In Paper II, mice were sacrificed at postnatal day 15 and at 4 months of age, and in Paper III, mice were sacrificed at 6 weeks of age.

Aside from maternal separation, there exists other paradigms for inducing early-life stress (for a review early-life stress, see Cattane et al., 2022). These other paradigms differ not only in the way early-life stress is achieved but also when the protocol is administered. As early-life stress is not a strictly defined term, the life stages that are included spans from gestation up to adolescence. As many biological changes occur during development, stress that is induced during different early life stages will likely have a differential effect based on the exact timing. In addition, sex also requires consideration as there are sex-specific sequalae, which we noted in Papers II and III. Ultimately, paradigms of early-life stress help us understand the resulting

molecular and cellular alterations, among other aspects, that may promote the development of disorders and diseases.

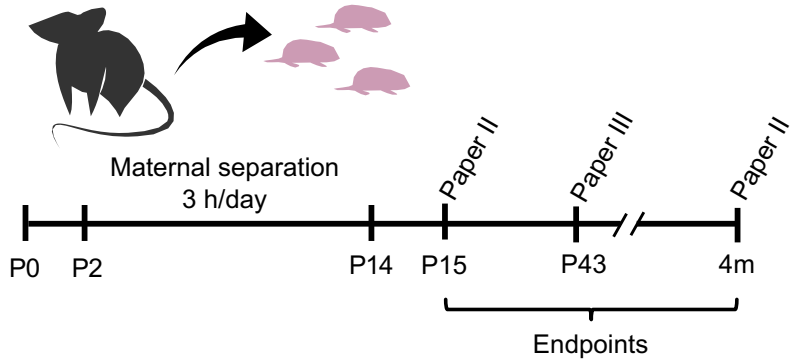

Figure 10. Modified schematic from Paper Ⅲ showing the timeline for maternal separation and endpoints analyzed according to their respective paper.

Behavioral tests

Alterations in cognitive function can be seen as a culmination of molecular and cellular alterations towards some tipping point. Viewed another way, there can be molecular and cellular alterations without cognitive impairment. For example, Aβ plaques can be found in clinically normal aged individuals (Mormino and Papp, 2018). When it comes to animal models, behavioral tests are valuable for evaluating the cognitive effects of a treatment or manipulation. There are many types of behavioral tests that aim to evaluate a specific cognitive aspect. Here, we used behavioral tests to determine whether maternal separation affected cognition.

In Paper II, we utilized the open field test, novel object recognition test, and the forced swim test. The open field test was used to measure anxiety-like behavior. To evaluate recognition memory, we utilized the novel object recognition test. The forced swim test was used to measure depressive-like behavior.

In Paper III, we used the Y maze, elevated plus maze, and the tail suspension test. The Y maze tested spatial working memory. The elevated plus maze was used to evaluate anxiety-like behavior. The tail suspension test was used to examine depressive-like behavior.

Ultimately, treatments for AD should improve cognitive function or prevent cognitive decline, so the inclusion of behavioral tests when testing treatments at the preclinical phase can be a valuable complement.

Tissue collection

Though part of many experiments, tissue collection may not seem like the most glamorous part of the materials and methods section. However, failure to adapt tissue collection parameters to the research question can lead to larger consequences downstream, the worst possibly being significantly lost time and wasted resources.

In all the work done here, how the tissue would be processed was considered before sacrificing the mice based on the planned downstream analyses and research questions. For tissue to be used only for immunohistochemistry, mice were perfused with paraformaldehyde to fix and preserve the tissue more evenly before collection. From there, downstream analysis involved immunolabeling followed by microscopy and image analysis (see Figure 11 for an overview).

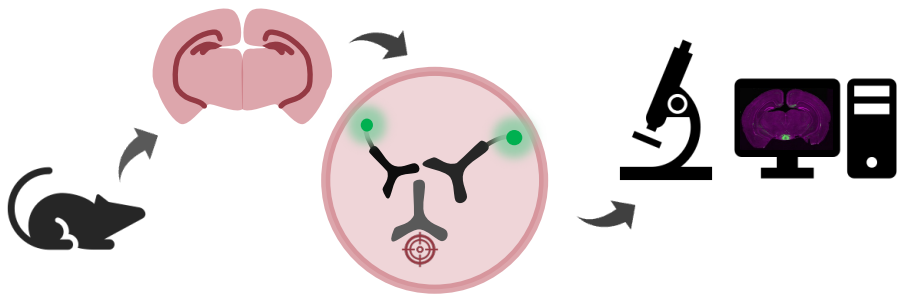

Figure 11. General workflow for fixed tissue. Brain tissue is collected from mice and sectioned. These sections are then used for immunolabeling, which is visualized using a microscope. The images are then analyzed at a computer.

When downstream analysis included using molecular biology techniques, like in Papers II and III, perfusion with paraformaldehyde was not done but rather saline to clear out the blood. Instead, the brains were split into their two hemispheres; one hemisphere was snap frozen for use with molecular biology techniques, and the other hemisphere was incubated in paraformaldehyde for immunohistochemistry. There a few advantages for splitting the brain like this: fewer mice and materials are needed and results from different methods can be correlated within the same mouse. However, using only one hemisphere means less tissue available, which can make analysis more difficult. Moreover, in Paper II, spleens were also collected but processed almost immediately for flow cytometry. Lastly, in Paper I, the collection of embryos for primary neurons might be considered tissue processing collection in a way but will be discussed in a later section.

Cell culture

Cell cultures are particularly useful for studying molecular mechanisms and subcellular compartmentalization. They can be manipulated relatively easily and are fairly easy to obtain and use. Paper I utilized both cell lines and primary neurons to study Aβ dynamics.

To study Aβ intra- and extracellular pool dynamics, neuro-2a (N2a) cells were used. These cells were derived from a mouse neuroblastoma and are used to model neurons in culture (Augusti-Tocco and Sato, 1969). Compared to primary neurons, N2a cells are easier to use, more readily available, and more robust. However, they are not neurons *per se* and do not form synapses like neurons would. In addition, as a cell line, N2a cells risk genetic drift and significant variation in responses with increasing passage number (Ben-David et al., 2018; Gutbier et al., 2018). Still, the availability of N2a lines that express WT APP and human APP with the Swedish mutation made this model suitable for the questions being asked (Thinakaran et al., 1996).

Primary neuronal cultures, as used here, originate from embryonic mouse brain though they can also be obtained from neonatal mice (Kaar et al., 2017). Unlike N2a cells, primary neurons can better capture the morphology of a neuron and the connectivity between neurons. Another advantage is that more region-specific neurons can be obtained by careful dissection of the brain before the cell dissociation step. However, primary neuronal cultures tend to be more finicky and difficult to obtain a than cell line. Nevertheless, research questions involving synapses and neuronal compartmentalization like in Paper I are better answered using primary neuron cultures rather than N2a cells or similar cell lines.

Molecular biology techniques

In Papers I, II, and III, levels of messenger ribonucleic acid (mRNA) and/or protein were measured from cell or brain homogenate. For mRNA, we utilized quantitative real-time PCR analysis with brain homogenate in Papers II and III. In Papers I, II, and III, protein was analyzed using dot blot, Western blot, a sensitive multiplex immunoassay, or some combination of the methods. The different methods used to measure proteins was dependent on the level of sensitivity needed, i.e., how much protein was available in the samples of a given experiment that would fall within the measurable range of a technique.

Compared to mRNA, protein measurements are often more insightful as proteins are the primary participants in cellular function. Protein can also be easier to work with as RNA is more sensitive to degradation than protein and requires thorough

cleaning and preparation to ensure adequate sample quality for analysis. Still, mRNA and proteins levels do not always correspond, though these differences can be indicative of a cellular state, for example, if both were measured in a given experiment (Liu et al., 2016; Perl et al., 2017).

Immunolabeling

Immunolabeling was used extensively in all the papers included here. In all papers, mouse brain tissue was immunolabeled. Additionally, in Papers I and II, cells were also immunolabeled.

As the name suggests, antibodies are the key component of this method to detect desired targets within intact tissue or cells. Beyond identifying the presence of a protein, antibody can bind specifically depending on conformation, post-translational modifications, and more. Thus, antibodies are powerful tools that can address a variety of research questions depending on the specific target of the antibody and the method for detecting the antibody or antibodies after binding.

Antibodies can be detected either directly or indirectly. Direct detection refers to the use of a primary antibody with the label directly conjugated to it, such as a fluorophore or enzyme. There were two experiments wherein direct antibody detection was used: to define peripheral immune cell populations using flow cytometry in Paper II and to characterize microglial and neuronal cell populations in the mammillary bodies with spatial profiling in Paper IV (see Figure 12).

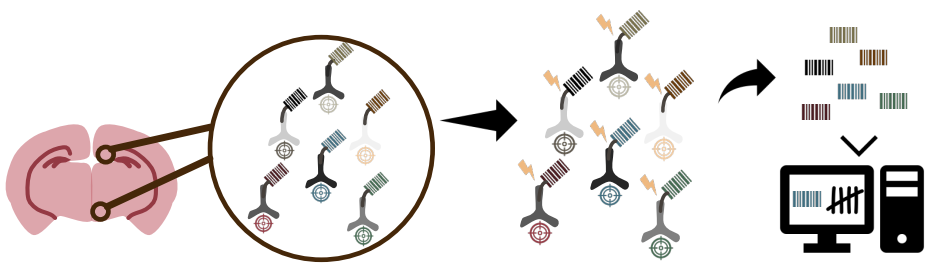

Figure 12. Direct detection in spatial profiling (Paper Ⅳ). A panel of antibodies directly conjugated to a type of barcode were incubated on a slide with mouse brain sections. Using a directed light, the barcodes were cleaved in the regions (circled on the brain to the left) and cells of interest, allowing for a degree of spatial resolution. The barcodes were then carefully collected for each region and counted to yield a quantitative measure of expression that could be analyzed.

Indirect detection requires the use of a secondary reagent to detect the primary antibody. For the majority of the immunolabeling experiments we conducted, we

utilized indirect detection via species-specific secondary antibodies conjugated to a label, usually a fluorophore, to detect the primary antibody like in Figure 13. This provides several advantages over direct detection immunolabeling: Signal from the label can be more easily detected for proteins with less-than-high expression due to the amplifying nature of indirect detection. Additionally, being able to use a given secondary antibody with several primary antibodies reduces costs. Likewise, being able to utilize different labels for different experiments with a given primary antibody has a similar benefit.

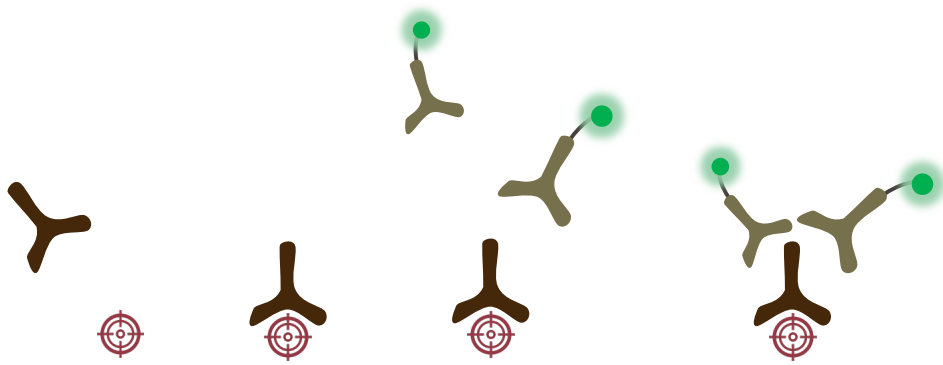

Figure 13. The process of indirect immunolabeling starting from left to right. A primary antibody binds to a target. Several secondary antibodies conjugated to a fluorophore bind to the primary antibody, allowing for detection with an amplified signal.

However, running an immunolabeling experiment using indirect detection may also require more optimization and steps in a protocol and may be more limiting compared to directly labeled primary antibody. This includes considering which primary antibodies to include in an experiment based on host species and conducting negative control tests.

Moreover, there are other aspects to consider when immunolabeling, for example, primary antibody concentration, cross-reactivity, antigen retrieval, and microscopes and their equipped hardware. Case in point, pretreatment with formic acid is often preferred to detect aggregated intracellular Aβ and enhances detection of extracellular Aβ in plaques, which we do in Papers I, III, and IV (Christensen et al., 2009; Kitamoto et al., 1987). Still, this is not the only option for enhancing Aβ signal (Kai et al., 2012).

Microscopy and image analysis

Microscopy and, in turn, image analysis are reliant on sample quality, which begins long before the sample reaches the microscope. “Garbage in, garbage out” is a key tenant when doing microscopy and image analysis. Even the best microscope cannot make a poor-quality sample better, and trying to make a poor-quality sample and/or microscopy image better at the image analysis phase risks crossing over into data falsification territory.

However, even with a good quality sample, microscopy is a skill of its own, and there are many things to consider before and at the microscope. First and foremost, what is the research question being asked? For example, a question regarding a whole brain region may require differently sized field of view and microscope objective compared to the requirements for a question looking at subcellular components. Is confocal quality necessary or is the epifluorescence microscope good enough? Though obtaining the best quality images via confocal microscope would be ideal, this comes at the cost of time and file size.

There are other points to keep in mind that may seem inconsequential but can have a large impact under certain circumstances as they can reduce image quality or cause aberrations. For example, microscope objectives tend to be configured for a specific thickness of coverslip, which is typically 0.17 mm or a #1.5 coverslip. (There are microscope objectives that can be adjusted to account for different coverslip thicknesses but seem less common than the fixed kind.) Moreover, mismatching refractive indices can have a similar impact, though this is more notable when imaging at higher numerical aperture. For example, it may be that the mounting medium does not have the same refractive index as the immersion medium between the coverslip and the objective.

Similarly, the workflow when doing image analysis also requires consideration of many factors. However, for much of the quantitative image analysis done in the work here, deciding how to threshold an image was the biggest point of consideration. Is manual or algorithm-based thresholding better? Do the images need some kind of pre-processing to work with the threshold better? (For a perspective on thresholding, see the section “Considerations during image segmentation” on the Principles of Scientific Imaging webpage at imagej.net.) Nevertheless, all images should be treated the same, so optimization of the image analysis pipeline can take considerable time but is often a necessary step to ensure accurate results.

Statistics

In our humble efforts to understand nature, statistics helps us make sense of the data that we acquire. Though statistics is often described last in the materials and methods section of journal articles, statistical consideration ideally occurs when the experiment is being planned. Deciding the statistical tests later when the data is already collected risks biasing the analysis and, in worst case, producing false positive results. Nevertheless, the reality of exploratory work or even planned experimental studies that evolve with time is that statistical tests may be determined once the is data acquired, though not in a "choose the test that gives the best p-value" kind of way. An example of this is in Paper I, wherein datasets first underwent normality[‡‡] testing to decide whether to move forward with a parametric or non-parametric statistical test.

Core to statistical hypothesis testing is the null hypothesis and the alternative hypothesis (note that there can be multiple alternative hypotheses). Statistical hypotheses are not the same as the experimental or research hypotheses; statistical hypotheses are specific, mathematical statements that can be rejected based on statistical test results. The statistical hypotheses are not often stated within a research article, but they can be inferred based on the statistical tests used, which should always be stated. Understanding null and alternative hypotheses is important for understanding p-values.

The use of p-values is widespread in scientific literature, and low p-values, usually $p \leq 0.05$, are emphasized as they are used to show that a significant difference or effect exists. The actual definition of a p-value is the probability of observing a given or more extreme result assuming that the null hypothesis is true. A more concrete way of viewing this is taking a p-value, for example $p = 0.01$, and reading it as "there is a 1% chance that we would observe the given result or a result even more extreme if the null hypothesis was true, i.e., there is, in reality, no difference. Because the significance threshold was set to $p \leq 0.05$, the null hypothesis is rejected. It is important to note that the significance threshold can be set to any value between 0 and 1, but 0.05 is conventional. (This author's preference is to avoid the use of asterisks to denote significance levels in graphs, which this author did in Paper III, so that the reader can more easily critically examine the results.) Even more ideal would be to include confidence intervals and estimated effect size to give an expanded view of whether a statistically significant effect is biologically relevant or not (Nakagawa and Cuthill, 2007).

[‡‡] Normality refers to whether the frequency of outcome values follows a normal or Gaussian distribution, a.k.a. a bell curve.

Summary of key results

Detailed results can be found in their respective paper, which are included in the printed version of the dissertation book (see Appendix).

Paper I – Prion-like spread of intracellular Aβ and Aβ dynamics

Here, we showed that a purely intracellular source of Aβ from prion-like N2a cell lysates can induce plaque pathology in an AD-transgenic mouse model though less potently than injecting brain homogenate from an aged AD-transgenic mouse. Follow-up experiments using the brain homogenate as the Aβ seeding source showed spatial-temporal patterns of plaque induction in regions downstream of the injection site. Interestingly, we noted small wisp-like Aβ aggregates near the site of injection in CA1 stratum oriens. The appearance of these aggregates coincided with a drop in NeuN-immunoreactivity in CA1 stratum oriens neurons in their vicinity. In addition, we observed that intracellular Aβ levels were lower in the injected side CA1 pyramidal neurons, which have their basal dendrites in stratum oriens where the wisp-like Aβ was located. Meanwhile, we observed increased intracellular Aβ in layer II lateral entorhinal cortex neurons which had seeded induced Aβ near in their terminal fields in the dentate gyrus. We then injected an AAV encoding a fluorophore into the lateral entorhinal cortex and observed that the terminal fields were sites of early plaque formation.

Looking *in vitro*, we treated primary neurons with aged AD-transgenic mouse brain ultracentrifugate as the Aβ seed source and noticed a relocation of intracellular Aβ from the soma to the neurites, which is consistent with what we saw *in vivo*. Lastly, we explored the connection between the extra- and intracellular pools of Aβ. There, we also utilized the prion-like N2a cell line that maintains aggregated intracellular Aβ. to disturb the extra-/intracellular Aβ dynamic and study the consequences.

Key takeaways

- An intracellular source of aggregated Aβ can seed plaques *in vivo*.

- Seeding using brain homogenate leads to Aβ plaque induction in downstream, connected brain regions (Figure 14).

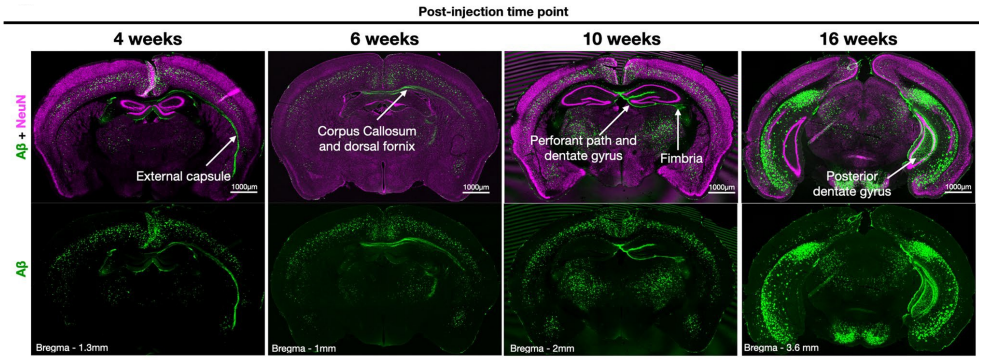

Figure 14. Mice were sacrified a different time points (4, 6, 10, and 16 weeks) post-injection of the seeding material and induction of plaques was noted on the side ipsilateral to the injection compared to the contralateral uninjected side. Corresponds to Fig. 1b in the original article.

- Intracellular pools of Aβ are coordinated with extracellular levels of Aβ such that low levels of extracellular Aβ promote increased β-cleavage and efflux of non-aggregated intracellular, thus replenishing the extracellular pool (Figure 15 and 16).

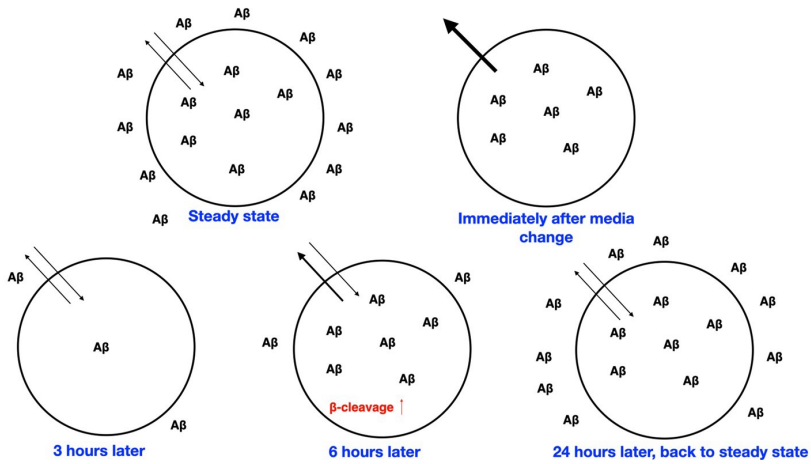

Figure 15. Schematic of Aβ intra- and extracellular dynamics with a media change experiment to deplete the extracellular pool of Aβ. Intracellular Aβ efflux is followed by increased β-cleavage,replentishing intracellular Aβ pools and, eventually, extracellular Aβ levels. Figure corresponds to Fig. 5h in the original article.

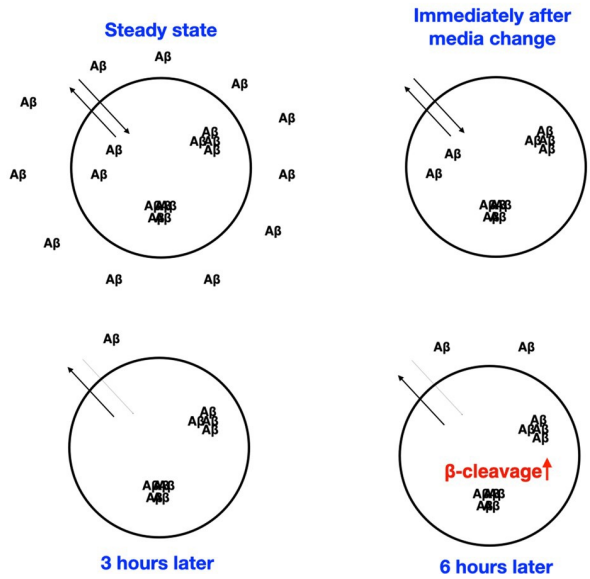

Figure 16. Aggregated intracellular Aβ is resistant to efflux and disrupts the Aβ intra-/extracellular equilibrium. Even with low extracellular Aβ levels, the aggregated intracellular Aβ remains within the cell. However, like in Figure 13, β-cleavage is upregulated with low extracellular Aβ levels. This suggests that the extracellular pool of Aβ determines β-secretase acitivty as a means of eventually replenish that pool. However, intracellular aggregation is exacerbated with increased intracellular production of Aβ, implying that aggregated intracellular Aβ can become a compounded problem with removal of extracellular pools of Aβ. This may have implications for anti-Aβ immunotherapies seeking to reduce extracellular Aβ plaque load. Figure corresponds to Fig. 6e in the original article.

Paper II – Immediate and long-term effects of early-life stress on inflammatory systems and brain function

In Paper II, we observed that exposure to early-life stress has the potential to modulate inflammatory systems in a sex-dependent manner. In addition, immediate effects may foreshadow long-term effects depending on the genotype. To model early-life stress, we used a protocol for maternal separation in WT and 5xFAD AD-transgenic mice. We noted an immediate effect of maternal separation on microglia: microglial area was increased in the hippocampus of male WT mice, and microglial morphology was more reactive in prefrontal cortex of female WT mice compared to their control counterparts. In the prefrontal cortex of adult mice, maternally separated WT male mice had increased microglial coverage. The last aspect of inflammation that we looked at was peripheral immune cell populations in the spleens of adult mice: we found that different peripheral immune cell populations were affected by maternal separation in a sex- and genotype-specific manner.

While analyzing brain function, we found that maternal separation was associated with impaired recognition memory in female and male WT mice and female 5xFAD mice. Depressive-like behavior was also affected as it was increased in maternally separated male WT mice and female 5xFAD mice. At a molecular level, we found that levels of *Arc* and *Bdnf* expression were decreased in maternally separated male mice: 5xFAD male mice with *Arc* and WT and 5xFAD mice with *BDNF*. With the genetic background of the 5xFAD mice, we could also study whether early-life stress affects Aβ levels in adulthood. There, we noted that female mice had increased plaque load and proto-fibrils in the prefrontal cortex associated with maternal separation.

Key takeaways

- Maternal separation can lead to immediate effects on microglial status in a sex- and genotype-specific manner (Figure 17).

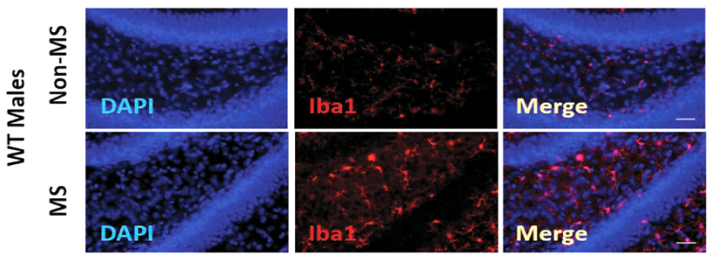

Figure 17. Microglial coverage (Iba1) is increased in maternally separated (MS) male WT mice. Figure corresponds to part of Fig. 1c in the original article.

- Early-life stress can alter peripheral immune cell populations even into adulthood of mice.

- By adulthood, early-life stress may impact recognition memory and modulate depressive-like behavior in a sex- and genotype-specific manner.

Paper III – Alterations in Aβ and neuroinflammation due to early-life stress in young AD-transgenic mice

Like in Paper II, we utilize maternal separation as a model of early-life stress but focus on effects during the adolescence only in an AD-vulnerable mouse model. We found that early-life stress can modulate intracellular Aβ levels as well as overall Aβ levels in specific brain regions. Likewise, microglial state was affected by early-life stress but primarily in the basolateral amygdala. Looking more at the neuroinflammatory environment, we noted some changes in cytokine expression in hippocampal extract of only male mice due to maternal separation. Moreover, we found a broad effect of maternal separation on neuronal marker gene expression in both male and female mice. Lastly, we noted no significant alterations in spatial working memory nor anxiety-like and depressive-like behavior.

Key takeaways

- Early-life stress promote increased Aβ levels, including increased intraneuronal Aβ levels, in a sex-specific manner (Figure 18).

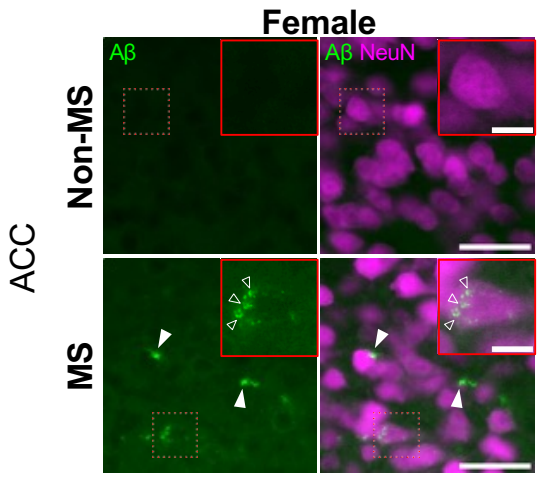

Figure 18. The anterior cingulate cortex (ACC) of maternally separated (MS) 6-week-old female 5xFAD mice had increased overall and intraneuronal Aβ-immunoreactivity compared to their coutnerparts. Figure corresponds to Fig. 1B in the original article.

- Synapse and neuronal gene marker expression seems broadly affected by early-life stress but does not yet manifest as behavioral alteration by the adolescent stage.

- The microglia in the basolateral amygdala may be particularly sensitive to the effects of maternal separation even into the adolescent stage (Figure 19).

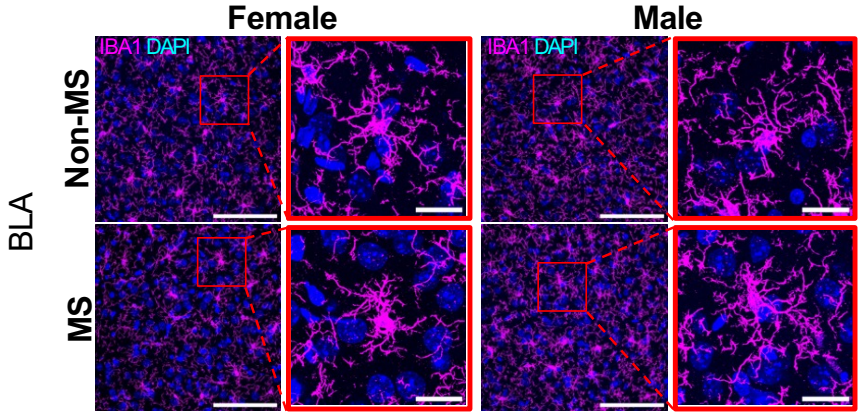

Figure 19. Measures of microglial coverage and morphology were different in maternally separated (MS) male and female mice in the basolateral amygdala (BLA). Figure corresponds to part of Fig. 2B in the original article.

Paper IV – Neuroinflammatory interactions with early aggregated Aβ in the medial mammillary nuclei of young AD-transgenic mice

In Paper IV, we observed that the medial mammillary nuclei of 5xFAD mice are early sites of aggregated Aβ and started to probe for neuroinflammatory alterations at that point. Previous literature and our own preliminary data suggest that the Aβ in the medial mammillary nuclei originate from the terminal fields of dorsal subicular neurons. This connection gives us the potential to probe how neuroinflammatory systems interface with plaque development of presynaptic-associated Aβ to gain a better understanding of early neuroinflammatory alterations in AD.

Key takeaways

- The aggregated Aβ in the medial mammillary nuclei likely originates from the axon terminals of subicular neurons.

- The medial mammillary nuclei develop early, plaque-like aggregated Aβ alongside the punctate intracellular Aβ in the subiculum of 5xFAD mice (Figure 20).

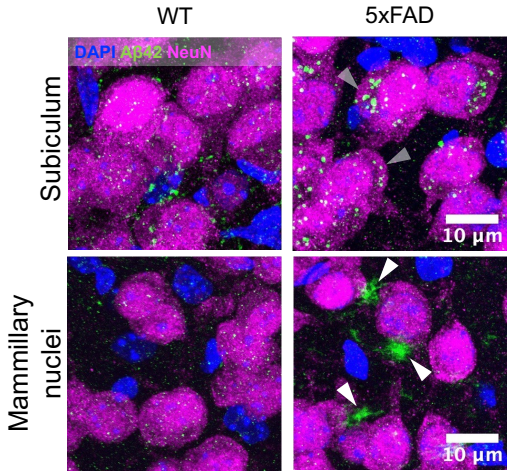

Figure 20. Early aggregated Aβ in 6-week-old pre-plaque 5xFAD mice in the subiculum and medial mammillary nuclei. Arrowheads highlight the morphological difference between aggregated Aβ in the subiculum compared to the mammillary nuclei.

- Microglia seem to physically interact with the aggregated Aβ in the mammillary nuclei of young 5xFAD mice (Figure 21).

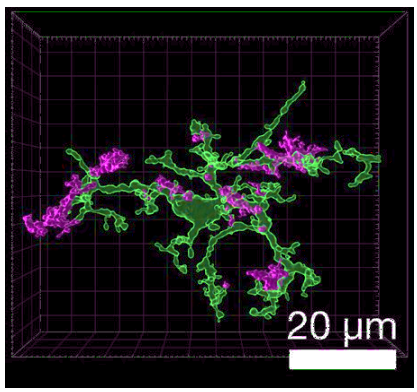

Figure 21. 3D surface model of a microglial cell (green) in contact with aggregated Aβ (magenta) in the mammillary nuclei of a 5xFAD mouse.

Discussion

In the grand scheme of things – putting the papers in context

Our overarching goal for this work was to understand mechanisms and modulations related to early AD. We looked from different angles, including prion-like spread of intracellular Aβ, sex-specific early-life stress effects on inflammatory systems as well as neurons and Aβ, and neuroinflammatory cell interactions with early aggregated presynaptic-associated Aβ.

Paper I built upon the work done by the first author previously. In the first part of Paper I, the source of aggregated intracellular Aβ came from an isolated clonal line that had persistent aggregated intracellular Aβ, referred to as prion-like cells (Olsson et al., 2018). Paper I then went further by exploring whether that aggregated intracellular Aβ source would persist *in vivo* by inducing Aβ plaques, which had not been done in the 2018 paper. Additionally, Paper I also utilized the prion-like cells when testing the dynamics of extra- and intracellular pools of Aβ. Because the aggregated intracellular Aβ in the prion-like cells does not efflux, this helped show that low extracellular but not low intracellular Aβ levels induce β-cleavage and, consequently, intracellular Aβ generation. One implication of this is that, if an anti-Aβ immunotherapy removes extracellular Aβ, then more intracellular Aβ will be generated to replenish the extracellular pool. Interestingly, a previous study by Oddo et al. (2006) explored this by injecting an anti-Aβ antibody *in vivo*. They found that extracellular pools of Aβ, i.e., plaques, were cleared first by the antibody with intracellular Aβ depleting after. After 15 days post-injection, intracellular Aβ levels recovered to control levels with plaques reappearing later, consistent with intracellular Aβ generation to replenish extracellular Aβ levels. It would be interesting to build on this study and Paper I with constant anti-Aβ treatment, which would be used with Aβ immunotherapies in humans. This could continually drive the extra-/intracellular dynamic so that the continual production of intracellular Aβ may increase the possibility of the Aβ aggregating. All in all, consideration needs to be given with therapeutics that aim to remove extracellular Aβ.

Papers II and III highlight the importance of considering sex (and gender) in AD research. Papers II and III do so explicitly Though not considered as a factor during statistical analysis in Paper I, both mice of both sexes were used for the experiments. This was similar for Paper IV, but plans for future experiments and analyses include

looking for sex-specific effects when possible. As can be inferred from the disproportionate demographic of women with AD compared to men, sex is an important aspect to consider in AD research. Adding to that, sex and gender[§§] can have modifying effects on other AD risk factors, but the mechanisms underlying this are still poorly understood. In epidemiological studies of AD, the same lack of understanding exists, and sex and gender require further exploration (Mielke et al., 2014; Nebel et al., 2018). More still is the lack of differentiating between sex and gender and looking more specifically into subgroups within each.

Paper IV builds off of previous work in the lab on neuroinflammatory alterations in 5xFAD mice of similar age (Boza-Serrano et al., 2018). However, here, additional consideration is given to neuroinflammatory alterations that interact with early aggregated Aβ and synapses. This interplay is one that is not well known, but there have been studies pointing towards potential mechanisms, including complement-mediated synaptic pruning (Hong et al., 2016) and neuron-based inflammatory signaling (Welikovitch et al., 2020). Furthermore, we have the opportunity to study some of the earliest appearing Aβ aggregates in this model (Gail Canter et al., 2019) in a relatively well-mapped region, the mammillary bodies (Shibata, 1989; Umaba et al., 2021). If our educated guess holds – that the source of the Aβ in the medial mammillary nuclei is the terminal fields of subicular projection neurons– then we would also have the possibility to study potential differences in neuroinflammation with early aggregated Aβ at the axon terminals versus in the somatodendritic compartment.

In sum, based on our work and others, we believe that Aβ has a crucial role in AD pathogenesis and that the pathogenesis of AD can be exacerbated by neuroinflammation and even factors that promote peripheral inflammation. These arguments for the role of Aβ in AD are sometimes conflated with the amyloid cascade hypothesis, which proposes that extracellular plaque appearance is the cause of downstream effects, including neurofibrillary tau tangles and cognitive impairment (Hardy and Allsop, 1991; Hardy and Higgins, 1992). However, the two are not one and the same, and reevaluating the amyloid cascade hypothesis may be a beneficial for the field and development of therapeutics. Adding to that point is evidence that there is a poor correlation between plaques and cognitive decline (Terry et al., 1991) and that plaques can be present in the brains of cognitively normal aged individuals (Nelson et al., 2012). Therefore, the overlooked phenomena of intracellular Aβ aggregation, which precedes plaque appearance, and its detrimental effects on synapses and neuronal function may help explain these gaps.

[§§] Sex is broadly defined as the biological and physiological characteristics that determine male or female categorization. Gender can be defined as the socially constructed role that an individual adopts based on a complex combination of factors, such as culture and biology. Sex and gender classifications are often aligned in an individual but can differ.

Limitations

The age of the mice used here is a potential point of contention. Aging is the biggest risk factor for AD, none of the mice that we used were older than 5 months. When equating mouse age to human life stages, 5-month-old mice are only "mature adults" and not "old," which in humans would be when the risk of AD starts to increase (Hagan, 2017). Nevertheless, the overall research interest here was the earliest events in AD. Additionally, it may be informative to see whether changes that occur earlier on persist later. In Paper II, we looked at the effect of maternal separation already one day after the end of the protocol. We also looked at mice months post-protocol, allowing us some idea of whether earlier alterations laid some groundwork to the effects seen later.

Sample size, especially with experimental models, is usually brought up as they often fall short of numbers that are considered "ideal" for statistical analysis. This is a fair concern as statistical power is a function of sample size and effect size, so experiments with small sample sizes may fail to capture phenomena with a smaller effect size. In Papers II and III, a balance had to be reached between the number of groups and sample size per group with practicality. In addition, sex-specific effects due to stress are documented in the literature, so pooling sexes together to achieve a higher *n* would not have made sense given that and our research question. An alternative strategy that we took was to leverage paired design. In Paper I, because the injections were done unilaterally, the uninjected side could be used as the control for the injected size to reduce variability and improve statistical signal to noise.

As the goal of using mouse models of disease is to apply that knowledge to humans, translatability is always a concern. Ideally, we would be able to study AD in human brains in a non-invasive manner and with the same cellular and molecular resolution of *in vivo* and *in vitro* models. Practically though, we rely on models to answer research questions that can be difficult, if not impossible, to do in humans. The use of knowledge gained from animal models to humans holds some validity as many biological molecules and processes are conserved across species. Table 2 lists the $A\beta_{42}$ amino acid sequence in different animals to illustrate how the sequence is relatively conserved across species.

Table 2. The sequence for Aβ42 is relatively conserved between species. Select animals and their Aβ (1-42) amino acid sequence. Amino acids in red denote a difference relative to the human sequence. Sequences were obtained from UniProt (The UniProt Consortium, 2023).

Species	Aβ (1-42) amino acid sequence
Human (Homo sapiens)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Mouse (Mus musculus)	DAEFGHDSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Cat (Felis silvestris catus)	DAEFRHESGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Dog (Canis lupus familiaris)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Guinea pig (Cavia porcellus)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Chimpanzee (Pan troglodytes)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

Future perspectives

Looking beyond Alzheimer's disease for clues

Some pathological phenomena observed in AD can be seen in other animals, which may aid our understanding of the disease in humans. For example, senior cats, which are cats age 7 and older, are at risk of developing feline dementia, which has histopathological similarities with AD (Head and Gunn-Moore, 2017), and cats are not the only animals that experience this with aging (Youssef et al., 2016). In additional, as brought up earlier, there is a strong degree of conservation in the sequence of Aβ between species. Moreover, observations from less conventional animal models may provide insight into factors that modulate Aβ or tau AD-like pathology as well as promote healthy aging. Consider these examples: In hibernating animals, tau becomes hyperphosphorylated in neurons, and this hyperphosphorylation is reversed after coming out of hibernation (Arendt et al., 2015). Naked mole rats do not appear to develop plaques despite their relatively high expression of $A\beta_{42}$ and long lifespan (Edrey et al., 2013). Bowhead whales are the longest-lived mammals with an estimated lifespan of over 200 years and seem resistant to age-related diseases (Keane et al., 2015).

Relative to how much we know about Aβ in disease conditions, relatively little is known about its "normal," i.e., physiological, functions. While it is easy to dismiss Aβ as just being a toxic byproduct because of its role in AD, the interplay between Aβ, activity, and synaptic function, among other things, suggests that the peptide has a role to play under non-pathological conditions (Brothers et al., 2018; Galanis et al., 2021; Martinsson et al., 2022). Similarly, the physiological role of APP remains unclear but also likely plays a role in synaptic function (Martinsson et al., 2019). Moreover, because protein aggregation is a big part of the pathology of AD as well as other neurodegenerative diseases, the topic of functional amyloids is also worth considering (Otzen and Riek, 2019). In sum, the understanding of "normal" functions can help guide treatment strategies but also optimization as further

disruption of physiological function could decrease the effectiveness of a treatment (Bishop and Robinson, 2004).

The next experiments

It feels like there are a near infinite number of questions that can be asked as follow-up to the work presented here. Some of these unanswered questions were raised by the reviewers during peer review but never got addressed then because they involved impractical – for one reason or another – experiments. Others are questions that we would have liked to explore and include in our paper but never managed to do so – again, for one reason or another. Regardless, after being involved in the papers included in this thesis, there remain some areas that this author would like to explore further (if time and money allowed).

Of particular interest is the exploration of selective vulnerability and the role of neuroinflammatory system plays in driving the pathology in those vulnerable regions. For example, interneurons are a vulnerable cell population in AD, and their loss has far-reaching, network-level consequences. Uncovering whether there are region-specific markers that are involved in the interactions between cell types, namely microglia and astrocytes with neurons, particularly at synapses, could have therapeutic implications, assuming such markers exist. In addition, studying this in human tissue would be extremely insightful and desired, but mouse models would allow for manipulations, such as intracerebral injections, and study of the sequalae. One such manipulation could be modulating activity in a specific region as microglial and astrocytic activity and the production of Aβ both have an activity-dependent component.

A common question asked at a PhD defense is what experiments the PhD student would do if they had a large amount of money to do so. In that scenario, some kind of -omics experiment could be on the table, though consideration needs to be given to the large amount of data that -omics produces and the time and effort needed to work through it. Still, as a continuation to this PhD work, it would be interesting to analyze the molecular signatures of different cell populations, such as interneurons, microglia, and astrocytes, in AD vulnerable brain regions using the newer knock-in mouse models. Like with the other work in this thesis, the focus would be on the earliest stages of intraneuronal Aβ accumulation and appearance of extracellular, wispy aggregated pre-plaque Aβ. Overall, with the development and increasing availability of knock-in mouse models of AD, more experiments remain to be done in those models as they may give more translatable insight into the earliest molecular and cellular events in AD.

Concluding remarks

The overall aim of this PhD work was to uncover any neuroinflammatory and Aβ alterations, mainly related to intracellular Aβ and Aβ aggregation, in early AD. Factors that may modulate pathogenesis and their mechanisms were also investigated experimentally. Ultimately, an increased understanding of how AD develops and progresses in early stages may guide the development of effective AD treatment strategies that can, one day, slow or prevent the disease.

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Appendix (Papers I – IV)

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Neuroinflammation and amyloid-β in early Alzheimer's disease

This California native was a shared PhD student between the Experimental Neuroinflammation Laboratory and Experimental Dementia Research Unit in the Biomedical Center at Lund University. The PhD project written here was a unique opportunity bringing together the expertise of her two research groups in order to understand a research area that synergizes the specialties of both: the interplay between neurons and synapses with microglia and neuroinflammation in early Alzheimer's disease.

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