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Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE

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Samira Parhizkar

"*Don't let the muggles get you down.*"

- Ron Weasley

Publications of the thesis

The FTD-like syndrome causing TREM2 T66M mutation impairs microglia function, brain perfusion, and glucose metabolism

Kleinberger G, Brendel M, Mracsko E, Wefers B, Groeneweg L, Xiang X, Focke C, Deussing M, Suarez-Calvet M, Mazaheri F, **Parhizkar S**, Pettkus N, Wurst W, Feederle R, Bartenstein P, Mueggler T, Arzberger T, Knuesel I, Rominger A, and Haass C.

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Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE

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Contents

Abbreviations

TSPO	18-kDa translocator protein
ADAM	A disintegrin and metalloproteinases
ABCA1	Adenosine triphosphate-binding cassette transporter A1
AD	Alzheimer's disease
APP	Amyloid precursor protein
Aβ	Amyloid-β
ALS	Amyotrophic lateral sclerosis
APO	Apolipoprotein
APOE	Apolipoprotein E
AICD	APP intracellular domain
ADAD	Autosomal dominant AD
BBB	Blood brain barrier
PrP^C	Cellular prion protein
CAA	Cerebral amyloid angiopathy
CSF	Cerebrospinal fluid
CSF1R	Colony stimulating factor 1 receptor
CTF	C-terminal fragment
DAM	Disease-associated microglia
DAP12	DNAX-activating protein of 12 kDa
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FAD	Familial AD
FDG	Fluorodeoxyglucose
FTD	Frontotemporal dementia
GCV	Ganciclovir
GWAS	Genome wide association studies
GFAP	Glial fibrillary acidic protein
HSTK	Herpes simplex virus thymidine kinase
HDL	High-density lipoproteins
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motifs
ISF	Interstitial fluid
IBA1	Ionized calcium binding adaptor molecule 1
LOAD	Late onset AD
LDL	Low-density lipoproteins
LDLR	Low-density lipoproteins receptor
LRP	Lipoprotein receptor-related protein
MRI	Magnetic resonance imaging
MGnD	Microglia of neurodegenerative phenotype
NFT	Neurofibrillary tangles
NF	Nuclear factor

PI3K	Phosphoinositide 3-kinase
PET	Positron emission tomography
PS1	Presenilin 1
PS2	Presenilin 2
PrP^{SC}	PrP-scrapie
sAPPα	Secreted APPα
sTREM2	Soluble TREM2
SYK	Spleen tyrosine kinase
TREML	TREM-like
TREM2	Triggering receptor expressed on myeloid cells-2
WT	Wild-type

Summary

Alzheimer's disease (AD) is the most common cause of dementia and presents a growing challenge to global health. AD is pathologically characterised by extracellular amyloid plaques (Aβ) and intraneuronal neurofibrillary tangles. Another key pathological feature of AD is chronic neuroinflammation suggesting that microglia, the major immune cell type in the brain, play an important role in AD progression. These pathological features have been known for over 115 years, yet the underlying mechanisms that connect these pathologies and result in AD are still unknown. Extensive evidence from recent genome wide association studies suggest that the innate immunomodulatory receptor, the triggering receptor expressed on myeloid cells-2 (*TREM2*[1]), plays a crucial role in sustaining the microglial response to disease pathogenesis. *TREM2* is second only to apolipoprotein E (*APOE*[2]), the strongest genetic risk factor of AD, in terms of the magnitude of its effects as a late-onset AD risk factor.

Studies using AD mouse models show that Trem2 is selectively expressed in microglia that cluster around Aβ plaques and is required for a variety of important microglial functions related to phagocytosis of cellular debris and Aβ fibrils. Although loss of Trem2 expression in AD mouse models was reported to decrease Aβ-associated microglia, contradictory effects on Aβ pathology were published. Therefore, to study the effects of TREM2 on premature Aβ plaque formation and deposition, I experimentally induced cerebral amyloidosis by stereotaxically injecting brain extract from aged Aβ plaque-bearing APPPS1 mice into the hippocampi of APPPS1/Trem2$^{+/+}$, APPPS1/Trem2$^{-/-}$, and APPPS1/Trem2$^{p.T66M}$ mice, at an age when no plaques had yet formed. I used APPPS1/Trem2$^{p.T66M}$ mice as an additional loss of Trem2 function control as I, together with Gernot Kleinberger, published that the frontotemporal dementia-like disorder-associated p.T66M mutation reduces soluble Trem2 levels in vivo as a result of impaired Trem2 maturation and shedding (Kleinberger *et al.*, 2017). Using this experimental paradigm, I found that Aβ seeding was significantly increased in the loss of Trem2 function APPPS1 mice compared to controls. I also confirmed that Trem2-deficiency impaired the capacity of microglia to remove Aβ seeds, most likely due to reduced IBA1- and CD68-positive microglia around plaques. Similar increase in plaque deposition and reduction in microglial activity was observed in Trem2-deficient APPPS1 mice during aging using longitudinal positron emission tomography. Surprisingly, while the rate of amyloidogenesis in Trem2-deficient APPPS1 mice consistently contradicted APPPS1/Trem2$^{+/+}$ controls throughout aging, the plaque deposition in both models levelled out at 12 months. These data indicate that additional factors may influence Aβ accumulation, including its aggregation and clearance during aging. Moreover, the seeded plaque morphology in loss of Trem2 function APPPS1 mice appeared diffuse with less compact cores despite the increase in total Aβ burden. This was contradictory to what I expected considering that the reduction in microglial clearance of Aβ plaques may instead result in increased cored aggregates due to decreased phagocytosis of the plaque halo.

[1] TREM2 – Human ortholog for gene and protein. Italicized for gene. Also used to describe the protein generally. Trem2 – Mouse ortholog for both gene and protein.

[2] APOE – Human and mouse ortholog. Italicized for gene. Also used to describe the protein generally. ApoE – human and mouse ortholog to describe the protein.

Two recent studies independently reported that TREM2 binds to APOE and APOE mRNA expression is upregulated in plaque-surrounding microglia similarly to TREM2, suggesting that the TREM2-APOE interaction may regulate the microglial phenotypic changes in AD. As APOE is known to strongly localise with Aβ dense core plaques in AD as well as affect Aβ aggregation and clearance, I hypothesized that plaque-associated microglia may be critical to mitigating amyloid metabolism by inadvertently promoting Aβ aggregation in attempt to clear Aβ instead. To address this hypothesis, I performed three different sets of experiments. Firstly, I performed immunohistochemical analyses on exogenously seeded, that is, Aβ brain extract injected, as well as aged APPPS1 mice, with and without functional Trem2. By performing costainings for ApoE, Aβ, IBA1-expressing microglia and GFAP-expressing astrocytes, I observed a dramatic decrease in plaque-associated ApoE levels upon loss of Trem2 function in both seeded and aged mice. I found that compared to loss of Trem2 function mice, the IBA1-positive microglia around plaques in APPPS1/Trem2$^{+/+}$ showed a strong colocalisation with ApoE, whereas no Trem2-dependent changes in ApoE/GFAP colocalisation was detected. This was unexpected considering ApoE is reported as mainly being produced by astrocytes and only to a lesser extent in microglia. Secondly, in a collaborative effort, we analysed microglia-enriched and microglia-depleted fractions in APPPS1 mice, the latter fraction containing mostly astrocytes as well as oligodendrocytes and neurons to a lesser extent, to compare relative ApoE protein induction in these fractions using mass spectrometry. Indeed, the microglia-enriched fraction produced more ApoE in APPPS1/Trem2$^{+/+}$ compared to APPPS1/Trem2$^{-/-}$ mice. Microglia-depleted fractions in these mice did not show any Trem2-dependent effects, suggesting that while ApoE may mainly be synthesized by astrocytes under healthy physiological conditions, microglia may contribute to the overall ApoE production in the brain during inflammation. Thirdly, to confirm these findings, I compared plaque-associated ApoE levels and its glial origin in microglia-depleted APPPS1 mice to age-matched controls. ApoE was significantly reduced upon microglia-depletion, indicating that the ApoE content in plaques may largely derive from disease-associated microglia. Lastly, to test whether these preclinical findings could be translated to humans, I investigated microglia clustering around Aβ plaques and its ApoE content in AD patients with different *TREM2* coding variants. AD patients with *TREM2* variants presented impaired microglial association to plaques as well as reduced ApoE levels compared to AD cases with the common *TREM2* variant, regardless of their *APOE* genotype.

Taken together, my studies provide the first evidence that loss of Trem2 function not only increases early plaque formation due to impaired microglial phagocytosis, but also alters the kinetics of amyloidogenesis during aging. In addition, I found that ApoE protein levels are strongly induced in plaque-associated microglia under disease conditions in a Trem2-dependent manner. While the ApoE contribution of astrocytes cannot be completely excluded, microglia-depletion in mice and loss-of-function *TREM2* coding variants in AD patients drastically reduce ApoE in plaques, which in turn may affect Aβ metabolism. These results suggest that the TREM2-APOE interaction may initially serve as protective means to modulate Aβ plaque clearance but in parallel may exacerbate Aβ pathology. Therefore, any therapeutic strategy attempting to target microglial function to modulate Aβ pathogenesis must consider the effects of TREM2 on APOE.

Zusammenfassung

Die Alzheimer-Krankheit (AD) ist die häufigste Ursache für Demenz und stellt eine wachsende Herausforderung für die globale Gesundheit dar. AD ist pathologisch durch extrazelluläre Amyloid-Plaques (Aβ) und intraneuronale neurofibrilläre Bündel gekennzeichnet. Ein weiteres pathologisches Merkmal von AD ist die chronische Neuroinflammation, was darauf hindeutet, dass Mikroglia, der wichtigste Typ von Immunzellen im Gehirn, eine bedeutende Rolle bei der AD-Progression spielen. Obwohl diese pathologischen Merkmale seit über 115 Jahren bekannt sind, sind die zugrunde liegenden Mechanismen, die zu diesen Pathologien und zu AD führen, weiterhin unbekannt. Umfangreiche Beweise aus kürzlich durchgeführten genomweiten Assoziationsstudien legen nahe, dass der angeborene immunomodulatorische Rezeptor, der auf myeloischen Zellen-2 (TREM2) exprimiert wird, eine entscheidende Rolle bei der Aufrechterhaltung der Mikroglia-Reaktion und der Pathogenese der Erkrankung spielt. Darüber hinaus steht TREM2an zweiter Stelle hinter Apolipoprotein E (APOE), dem stärksten genetischen Risikofaktor von AD, was die Stärke seiner Auswirkungen als spät einsetzender AD-Risikofaktor angeht.

Studien mit AD-Mausmodellen zeigen, dass Trem2 selektiv in Mikroglia exprimiert wird, die sich um Aβ-Plaques herum ansammeln, und für eine Vielzahl wichtiger Mikrogliafunktionen benötigt wird, die mit der Phagozytose von Zelltrümmern und Aβ-Fibrillen zusammenhängen. Obwohl berichtet wurde, dass der Verlust der Trem2-Expression in AD-Mausmodellen die Aβ-assoziierten Mikroglia verringert, wurden widersprüchliche Auswirkungen auf die Aβ-Pathologie veröffentlicht. Um die Auswirkungen von TREM2 auf die Bildung und Ablagerung von neu entstandenen Aβ-Plaques zu untersuchen, induzierte ich daher experimentell die cerebrale Amyloidose durch stereotaktisches Injizieren von Hirnhomogenat aus gealterten APPPS1-Mäusen in die Hippocampi von APPPS1/Trem2$^{+/+}$ sowie, APPPS1/Trem2$^{-/-}$ und APPPS1/Trem2$^{p.T66M}$ Mäusen in einem Alter, in dem sich noch keine Plaques gebildet haben. Ich verwendete APPPS1/Trem2$^{p.T66M}$ Mäuse als zusätzliche Kontrolle zum Verlust der Trem2-Funktion, da ich zusammen mit Gernot Kleinberger veröffentlichte, die mit der frontotemporalen Demenz-ähnlichen Störung assoziierte p.T66M-Mutation in vivo lösliche Trem2-Spiegel reduziert, als Folge einer beeinträchtigten Trem2-Reifung und -Spaltung (Kleinberger et al. 2017). Darüber hinaus fand ich heraus, dass ‚Aβ-Seeding' in den APPPS1/Trem2$^{-/-}$ und APPPS1/Trem2$^{p.T66M}$-Mäusen im Vergleich zu den APPPS1/Trem2$^{+/+}$ Kontrollen signifikant erhöht war. Mit diesem Modell bestätigte ich auch, dass der Trem2-Mangel die Fähigkeit von Mikroglia zur Entfernung von ‚Aβ-Seeds' beeinträchtigt, höchstwahrscheinlich durch reduzierte IBA1- und CD68-positive Mikroglia um Plaques. Ein ähnlicher Anstieg der Plaqueablagerung und der Verringerung der Mikroglia-Aktivität wurde während des Alterns bei Trem2-defizienten APPPS1-Mäusen unter Verwendung der longitudinalen Positronenemissionstomographie beobachtet. Während die Geschwindigkeit der Amyloidogenese bei Trem2-defizienten APPPS1-Mäusen während der gesamten Alterungsdauer entgegengesetzt zu den APPPS1/Trem2$^{+/+}$ Kontrollen verlief, stagnierte die Plaqueablagerung bei beiden Mausmodellen überraschenderweise nach 12 Monaten. Diese Daten weisen darauf hin, dass zusätzliche Faktoren die Aβ-Akkumulation und -Beseitigung beeinflussen können, und zwar der Stoffwechsel während des Alterns. Darüber hinaus schien

die induzierte Plaque-Morphologie beim APPPS1/Trem2$^{-/-}$ und APPPS1/Trem2$^{p.T66M}$ Mäuse trotz der Zunahme der gesamten Aβ-Belastung diffus mit weniger kompakten Kernen zu sein. Dies stand im Gegensatz zu dem was ich erwartete, da eine Verminderung des Abbaus von Aβ-Plaques durch Mikroglia ebenso zu vermehrten Kernaggregaten aufgrund einer erhöhten Aβ-Verfügbarkeit sowie einer verringerten Phagozytose des Plaque-Halos führen kann.

Kürzlich berichteten zwei unabhängige Studien, dass TREM2 an APOE bindet und die APOE-mRNA-Expression in Plaque-umgebender Mikroglia, ähnlich wie TREM2 hochreguliert, wird, was darauf hindeutet, dass die TREM2-APOE-Wechselwirkung die mikroglialen phänotypischen Veränderungen in AD regulieren könnte. Da bekannt ist, dass APOE mit dichten Aβ-Core-Plaques in AD stark lokalisiert ist und die Aβ-Aggregation und -Beseitigung beeinflusst, stellte ich die Hypothese auf, dass Plaque-assoziierte Mikroglia kritisch sein können, um den Amyloid-Metabolismus zu mildern, indem bei dem Versuch Aβ zu beseitigen, stattdessen versehentlich die Aβ-Aggregation begünstigt wird. Um dieser Hypothese nachzugehen, führte ich drei verschiedene Experimente durch. Zunächst führte ich immunhistochemische Analysen an exogen induzierten und gealterten APPPS1-Mäusen, mit und ohne funktionellem Trem2, durch. Durch die Durchführung von Parallelfärbungen für ApoE, Aβ, IBA1-exprimierende Mikroglia und GFAP-exprimierende Astrozyten, konnte ich eine dramatische Abnahme der Plaque-assoziierten ApoE-Spiegel nach Verlust der Trem2-Funktion sowohl in injizierten als auch in gealterten Mäusen feststellen. Außerdem stellte ich fest, dass die IBA1-positiven Mikroglia um Plaques in APPPS1 im Vergleich zu APPPS1/Trem2$^{-/-}$ und APPPS1/Trem2$^{p.T66M}$-Mäusen eine starke Kolokalisation mit ApoE zeigten, während keine Trem2-abhängige Veränderung der ApoE/GFAP-Kolokalisation festgestellt wurde. Dies war unerwartet, da zuvor berichtet wurde, dass ApoE hauptsächlich von Astrozyten produziert wird und nur in geringerem Maße in Mikroglia. Zweitens haben wir in gemeinschaftlichen Bemühungen mikroglia-angereicherte und mikroglia-depletierte Fraktionen in APPPS1-Mäusen analysiert, wobei die letztere Fraktion hauptsächlich Astrozyten sowie Oligodendrozyten und, in einem geringeren Ausmaß, Neuronen enthielt, um die relative Induktion von ApoE-Protein in diesen Fraktionen mittels Massenspektrometrie zu vergleichen. In der Tat erzeugte die mit Mikrogliazellen angereicherte Fraktion mehr ApoE in APPPS1/Trem2$^{+/+}$ im Vergleich zu APPPS1/Trem2$^{-/-}$ Mäusen. Darüber hinaus zeigten die an Mikrogliazellen depletierten Fraktionen in diesen Mäusen keine Trem2-abhängigen Effekte, was darauf hindeutet, dass ApoE unter gesunden physiologischen Bedingungen zwar hauptsächlich durch Astrozyten synthetisiert werden kann, Mikroglia jedoch während einer Entzündung zur gesamten ApoE-Produktion im Gehirn beitragen können. Drittens, zur Bestätigung dieser Befunde, verglich ich die Plaque-assoziierten ApoE-Spiegel und ihren Glia-Ursprung in Mikroglia depletierten APPPS1 Mäusen mit den altersüblichen APPPS1-Kontrollen. Bei Mikroglia-Depletion fehlte ApoE nicht nur weitgehend in den Plaques, sondern zeigte auch eine signifikante Reduktion der IBA1/ApoE-Kolokalisationsfärbung. Es wurden keine Veränderungen der GFAP/ApoE-Kolokalisationen im Vergleich zu den Kontrollen beobachtet, was darauf hindeutet, dass der ApoE-Gehalt in Plaques weitgehend von krankheitsassoziierten Mikroglia herführen kann. Um zu testen, ob diese präklinischen Ergebnisse auf den Menschen übertragen werden können, untersuchte ich Mikroglia, die sich um Aβ-Plaques herum ansammeln, und deren ApoE-Gehalt bei AD-Patienten mit

verschiedenen TREM2-Codierungsvarianten. AD-Patienten mit TREM2-Varianten zeigten im Vergleich zu AD-Fällen mit der üblichen TREM2-Variante unabhängig von ihrem APOE-Genotyp eine beeinträchtigte Mikrogliazellen-Assoziation mit Plaques sowie verringerte ApoE-Spiegel.

Zusammenfassend liefern meine Studien den ersten Beweis dafür, dass der Verlust der Trem2-Funktion nicht nur die frühe Plaquebildung aufgrund einer gestörten mikroglialen Phagozytose erhöht, sondern auch die Kinetik der Amyloidogenese während des Alterns verändert. Darüber hinaus fand ich heraus, dass ApoE-Proteinspiegel in Plaque-assoziierten Mikroglia unter Krankheitsbedingungen auf eine Trem2-abhängige Weise induziert werden. Während der ApoE-Beitrag von Astrozyten nicht vollständig ausgeschlossen werden kann, reduzieren Mikroglia-Depletion bei Mäusen und TREM2-Codierungsvarianten bei AD-Patienten ApoE in Plaques drastisch, was wiederum den Aβ-Metabolismus beeinflussen kann. Diese Ergebnisse legen nahe, dass die TREM2-APOE-Wechselwirkung anfänglich als Schutzmittel zur Modulation der Aβ-Plaque-Beseitigung dienen kann, parallel dazu jedoch die Aβ-Pathologie verschlimmern kann. Daher muss bei jeder therapeutischen Strategie, die versucht, die Mikrogliafunktion zur Modulation der Aβ-Pathogenese zu beeinflussen, die Auswirkungen von TREM2 auf APOE berücksichtigt werden.

I. Introduction

1 Alzheimer's disease

Alzheimer's disease (AD) is a terminal, age-related neurodegenerative disease, which affects over 20 million people worldwide and is known to be the most common cause of dementia (www.alz.org/alzheimers-dementia). Given the accelerated aging of the population, the number of individuals with AD is predicted to increase dramatically and create a worldwide public health crisis (www.alz.org/alzheimers-dementia/facts-figures). AD is clinically distinguished by loss of memory and progressive decline in cognitive function. Current medications for AD only provide symptomatic relief by delaying cognitive decline, however there are no disease-modifying therapies available to intervene the course of AD dementia or delay its onset.

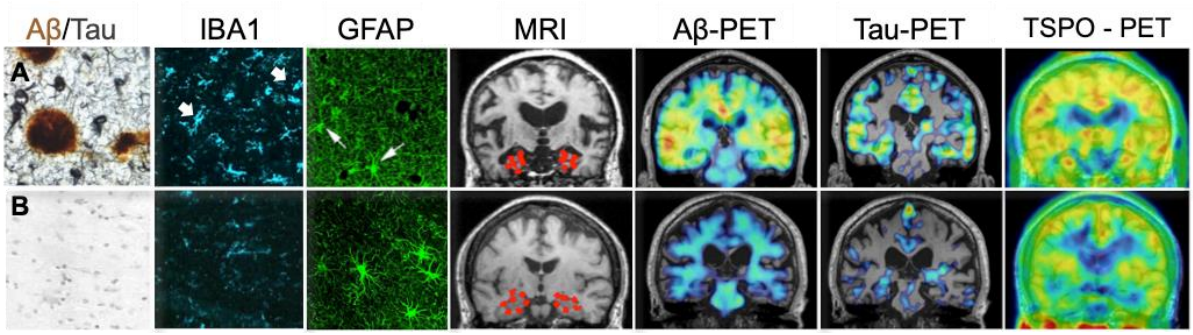

Figure 1.1 Multifaceted pathology of AD. Amyloid-β (Aβ) plaques and tau aggregates are often found throughout the (A) AD brain, particularly in the temporal cortex upon post-mortem histological examination, compared to (B) healthy elderly brain. Ionized calcium binding adaptor molecule 1 (IBA1)-positive microgliosis and glial fibrillary acidic protein (GFAP)-stained astrogliosis are often observed in AD. Arrows indicate areas of increased gliosis. Cortical and hippocampal brain areas are most commonly affected and begin to shrink with increasing severity of neurodegeneration as seen by enlarged ventricles in AD compared to healthy control. Red dotted area in the magnetic resonance imaging (MRI) scan shows significant hippocampal atrophy in AD patient compared to control. In vivo positron emission tomography (PET) images show increased Aβ and tau signal in AD compared to healthy cases. Similarly, increased 18-kDa translocator protein (TSPO) signal is observed due to increased gliosis and inflammation in the AD brain. (Adapted from (Higuchi *et al.*, 2016)).

Post-mortem AD brains present key histopathological hallmarks accompanied by widespread brain atrophy (Figure 1.1) (Braak and Del Tredici, 2011). These include aggregation and accumulation of extracellular Aβ plaques and intracellular tau neurofibrillary tangles (NFTs) (Figure 1.1). AD was initially considered a cell-autonomous neurodegenerative disorder, although Aβ and tau pathology is often accompanied by marked gliosis in AD patients and respective mouse models. In fact, Alois Alzheimer already illustrated the abnormal morphology of glial cells in the initial publication describing the disease (Alzheimer, 1907). Since then, extensive post-mortem immunohistochemical examinations illustrate abnormal protein aggregates that are often associated with activation of microglia and astrocytes (Ransohoff, 2016). Although AD is often accurately diagnosed only after death, recent biomarker and imaging studies indicate that AD pathology begins decades before the onset of clinical symptoms (Gonneaud *et al.*, 2017, Insel *et al.*, 2017, Suarez-Calvet *et al.*, 2016a,

Suarez-Calvet *et al.*, 2016b). Imaging modalities such as MRI provide detailed three-dimensional structural information of the brain by using tissue characterisation with soft tissue contrast, while PET specifies functional and metabolic changes in the brain by detecting gamma rays emitted indirectly by a radioactive tracer (Zhang *et al.*, 2017). Thus, a combination of PET/MRI has significantly improved our understanding of different stages of AD by measuring changes in Aβ and tau deposits as well as gliosis over time (Figure 1.1). Extensive neuroimaging studies involving a large cohort of AD patients at different disease stages imply that degrees of hippocampal atrophy, ventricular and whole-brain volumes can predict the disease progression (Fox *et al.*, 2001, Gispert *et al.*, 2016). To date, the cause of AD is not well understood. However, accumulating genetic and functional evidence strongly indicates an active role of brain innate immunity in AD pathogenesis and progression (Pimenova *et al.*, 2018).

1.1 Early onset AD

Aging is the greatest risk factor for sporadic AD (Guerreiro and Bras, 2015). Besides aging, environmental, epigenetic, and genetic factors contribute to the risk of developing AD. The majority of AD cases are sporadic with familial (FAD) cases accounting for less than 1% (Campion *et al.*, 1999). This small percentage of autosomal dominant AD (ADAD) cases manifest with early dementia onset, typically between the age of 30 and 60 years old due to mutations in presenilin 1 (PS1), presenilin 2 (PS2) and amyloid precursor protein (APP). While presenilins are mainly known as the catalytic subunit of the γ-secretase complex, APP has several important physiological functions in neuronal plasticity, synaptic transmission, cognition and neuroprotection during brain development as well as in the mature and ageing brain (Muller *et al.*, 2017, Richter *et al.*, 2018, Rice *et al.*, 2019, Haass and Willem, 2019). In the healthy brain, APP functions as a cell surface receptor-like protein or ligand and may mediate its effects from the cell surface or via its secreted proteolytic fragments, particularly secreted APPα (sAPPα) (Figure 1.2). However, APP mutations located within or immediately flanking the Aβ region of APPβ affect Aβ production or aggregation, for example by altering the proteolytic activity of β- and γ-secretases (Figure 1.2) (Citron *et al.*, 1992). Mutations in components of the γ-secretase complex PS1 or PS2 also favour the amyloidogenic pathway by reducing the γ-secretase complex activity to increase $A\beta_{42/40}$ ratio or by generating longer amyloidogenic peptides such as $A\beta_{42}$ and $A\beta_{43}$ (Saito *et al.*, 2011, Kretner *et al.*, 2016). Moreover, certain mutations in APP or PS do not necessarily affect Aβ production but may instead affect its hydrophobic properties and therefore its aggregation propensity and clearance (Tsubuki *et al.*, 2003). For example, the London APP mutation (V717I) increases the $A\beta_{40/42}$ ratio by modifying the γ-secretase cleavage (Goate *et al.*, 1991), while the Swedish mutation (K670N/M671L) enhances the β-secretase cleavage, thereby promoting Aβ production (Hardy and Selkoe, 2002). Additionally, the Icelandic APP mutation (A673T) based N-terminal to the β-secretase cleavage site not only reduces Aβ production, but also protects against AD (Jonsson *et al.*, 2012). These findings suggest that Aβ plays an important role in the initiation and development of AD pathology.

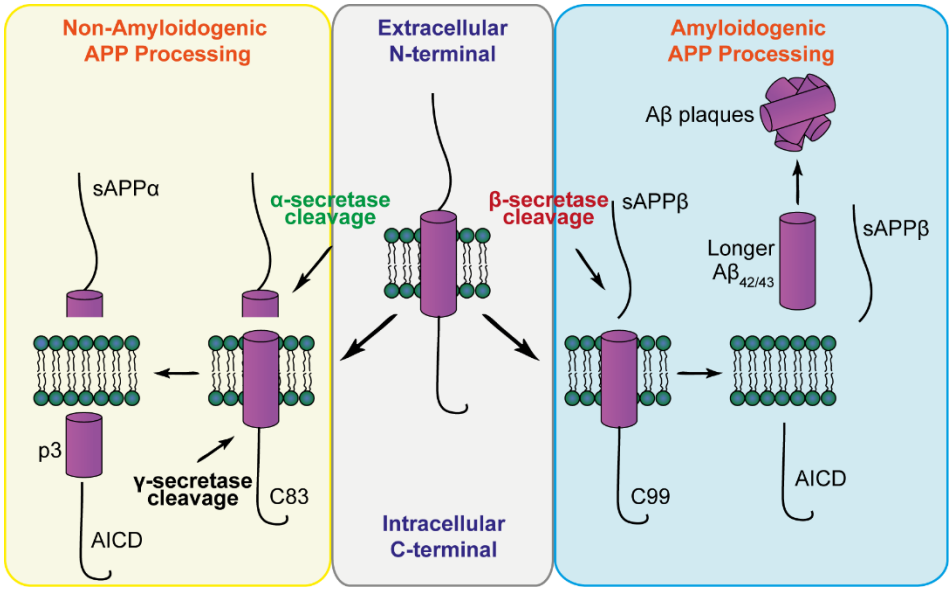

Figure 1.2 Proteolytic processing of APP. APP is a type 1 transmembrane protein with the carboxyl terminus within the cytosol and amino terminus within the extracellular space. In the non-amyloidogenic pathway, APP is processed consecutively by α- and γ-secretases to yield sAPPα, p3, and the APP intracellular domain (AICD). However in the amyloidogenic pathway, APP is first cleaved by membrane-bound endoprotease β-secretase to release a large fragment called sAPPβ. The remaining membrane bound C99 fragment is sequentially cleaved by γ-secretase to yield Aβ peptides. The longer Aβ peptides, for example $A\beta_{42}$ and $A\beta_{43}$, are the more hydrophobic and aggregation-prone species that are commonly found in Aβ core plaques. Amyloid plaque cores composed of fibrillar Aβ in a β-sheet conformation are surrounded by a halo of diffuse Aβ.

1.2 Late onset AD

Genome wide association studies (GWAS), a methodology that compares allele frequencies of polymorphisms between cases and controls, have identified many genetic risk factors implicated in increasing susceptibility for late onset AD (LOAD). The majority of AD cases clinically manifest later than FAD, that is, after the age of 60 but is pathologically identical to FAD. In the non-AD brain, the Aβ peptide is cleared from the brain via several means: (1) enzymatic degradation (Carson and Turner, 2002), (2) secretion across the blood brain barrier (BBB) (Cirrito *et al.*, 2005, Ma *et al.*, 2018), (3) glymphatic clearance system (Jessen *et al.*, 2015), or (4) microglia phagocytosis (Lee and Landreth, 2010). However, LOAD cases often display deficits in Aβ clearance and Aβ production (Figure 1.3) (Mawuenyega *et al.*, 2010, Selkoe, 2001). For example, apolipoprotein E (*APOE*) – the strongest genetic risk factor for LOAD - modulates Aβ clearance and aggregation isoform-dependently (Castellano *et al.*, 2011, Holtzman *et al.*, 2000). The *APOE* gene resides on chromosome 19 with three common alleles present in the human population: ε2 (Cysteine 112, Cysteine 158), ε3 (Cysteine 112, Arginine 158) and ε4 (Arginine 112, Arginine 158). The ε4 allele is associated with an increased risk, whereas ε2 is relatively protective compared to the ε3 allele. A single copy of ε4 increases AD risk by ~3-fold, while two copies increases the risk by ~12-fold, thereby affecting the age of onset and disease severity significantly (Corder *et al.*, 1993, Saunders *et al.*, 1993). More than 50% of LOAD patients carry at least one copy of the ε4 allele. Homozygous ε4/ε4 individuals include 15% of LOAD cases, however only 2% of the healthy population are ε4/ε4 carriers (Farrer *et al.*, 1997). Although carrying the ε4 haplotype does not

necessarily guarantee that an individual will develop LOAD, the ε4 isoform appears to affect brain function associated with both altered neurological and metabolic functions decades prior to any clinical symptoms becoming obvious (Filippini *et al.*, 2009, Michaelson, 2014) (further discussed in section 4.0).

Over 50% of GWAS-identified risk factors are involved in microglial and innate immune cell function, some of which include *ABCA7*, *ABI3*, *CD33*, *CLU*, *CR1*, *MS4A4A*, and *PLCγ2* (Harold *et al.*, 2009, Lambert *et al.*, 2013, Naj *et al.*, 2011, Sims *et al.*, 2017, Hollingworth *et al.*, 2011). Additionally, the triggering receptor expressed on myeloid cells type 2 (TREM2) significantly impacts the risk of LOAD with similar odds ratio to the single *APOE* ε4 allele (Guerreiro *et al.*, 2013a). Innate immunity is an indispensible component in AD pathogenesis as chronic glial activation is a prominent feature accompanying pathological protein accumulation in the AD brain. Moreover, immune cells such as microglia and astroglia that react to disease-associated protein aggregates may not simply be bystanders, but may instead contribute to AD pathogenesis (Wyss-Coray and Rogers, 2012). For example, long-lasting inflammation may cause neuronal damage and death via several mechanisms, such as: (1) release of toxic substances including reactive oxygen species and nitric oxide, (2) enhanced expression of "eat-me" signals including phophatidylserine and calreticulin which induce phagoptosis, (3) activation of complement system to induce cell lysis, and (4) activation of inflammasomes (Shi and Holtzman, 2018). Thus, Aβ build-up due to impaired innate immune cell functions may serve as a driving force for disease progression by influencing neurodegeneration.

1.3 Amyloid cascade hypothesis

The amyloid cascade hypothesis is strongly supported by genetic evidence not only from FAD cases, but also because multiple copies of APP cause ADAD with cerebral amyloid angiopathy (CAA) (Rovelet-Lecrux *et al.*, 2005) or Down's syndrome (Delabar *et al.*, 1987) with increased accumulation of Aβ peptides. The hypothesis proposes that excessive Aβ peptide accumulation, particularly of the longer, more aggregation-prone Aβ species, is the causative agent in AD pathology that sets of a series of downstream events that ultimately leads to dementia and neurodegeneration (Hardy and Selkoe, 2002, Selkoe, 1991, Hardy and Higgins, 1992) (Figure 1.3). For example, the build-up of extracellular Aβ aggregates may result in increased oxidative and endoplasmic reticulum (ER) stress as well as disrupted ionic homeostasis, which may create an imbalance of phosphatases and kinases that stimulate the hyperphosphorylation of tau, enhance vascular and neuronal damage, and induce widespread neuroinflammation observed as the disease progresses (Hardy and Selkoe, 2002, Haass and Selkoe, 2007). However, the hypothesis has also been criticized as not all observations necessarily fit. For instance, early symptoms of dementia and neuronal loss have been more closely linked to amount of NFT deposits rather than Aβ plaques (Arriagada *et al.*, 1992, Musiek and Holtzman, 2015). Additionally, Aβ plaques appear to be neurotoxic particularly in the presence of NFTs (Rapoport *et al.*, 2002). These seemingly contradicting findings may be explained by the prerequisite of Aβ oligomers or deposits to be initially present to consequently trigger tau-mediated toxicity (Haass and Selkoe, 2007). Nonetheless, while rare variants in the microtubule associated tau-encoding gene have been linked to tauopathies, such as

frontotemporal dementia (FTD) (Hutton *et al.*, 1998, Neumann *et al.*, 2009), AD onset is primarily influenced by genetic variations affecting Aβ production (Hardy and Allsop, 1991, Hardy and Higgins, 1992). On the other hand, the presence of senile Aβ plaques in the absence of cognitive abnormalities suggests that Aβ may not necessarily initiate the downstream events but may instead be associated with aging (Bennett *et al.*, 2006, Morris *et al.*, 2010). Although, why would Aβ in AD patients act differently compared to healthy elderly cases? Several immune cell receptors expressed particularly on microglia in the brain are responsible for engulfing and degrading Aβ plaques. Many of these microglial receptors, including *TREM2*, have been recently linked to increased LOAD risk (discussed in section 1.2), suggesting that the difference in AD patients and healthy aging cases may be the failure of microglia to appropriately respond to the pathogenic Aβ. Therefore, the inability to remove the initial threat in time becomes increasingly destructive leading to overwhelming Aβ production as well as loss of microglial function (discussed in section 2). In parallel, the accumulating Aβ may trigger an inflammatory response leading to increased free reactive oxygen species and a cytokine profile that promotes neuroinflammation, synaptic degradation and neurodegeneration (Wyss-Coray and Rogers, 2012) (further discussed in section 2.0). Thus, the microglia-Aβ interaction may significantly impact AD onset and severity as a consequence of an imbalance between Aβ-peptide production and clearance.

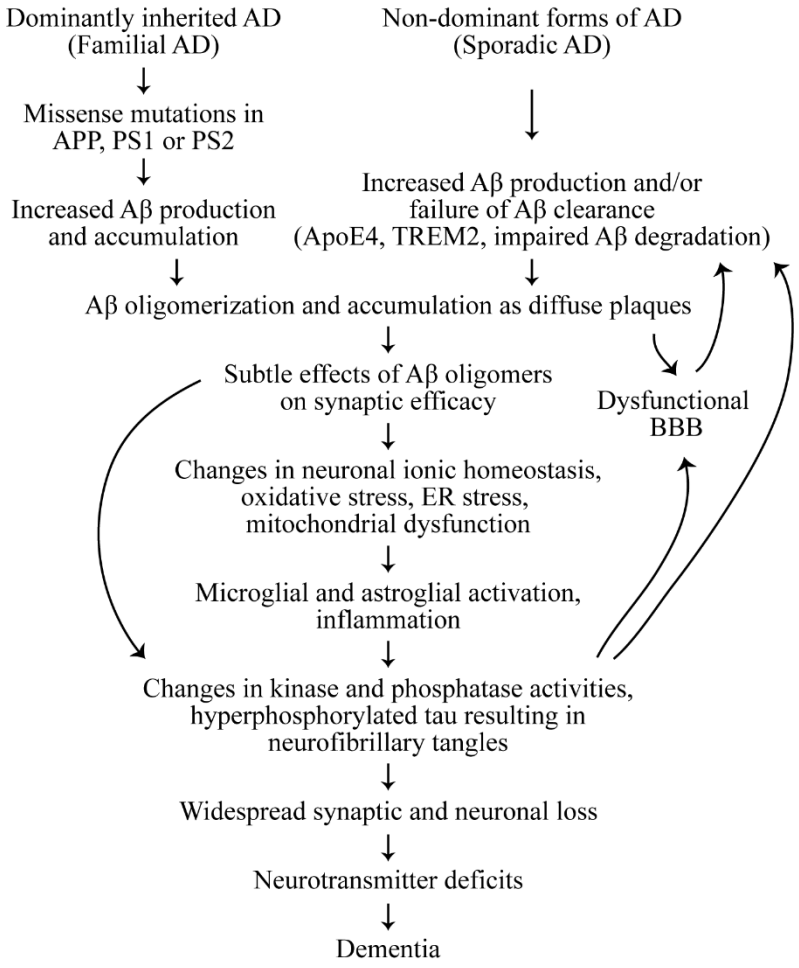

Figure 1.3 Schematic representation of the amyloid cascade hypothesis.

1.4 Mouse models of amyloid pathology

As there are no efficient naturally occurring animal models for AD, the discovery of APP and PS mutations in AD gave rise to indispensable opportunities to study amyloidosis in transgenic mouse models harbouring these mutations (Götz *et al.*, 2004, Sasaguri *et al.*, 2017, LaFerla and Green, 2012). Using "humanised" mice carrying human APP and PS variants can be used to not only investigate Aβ pathology and associated disease phenotypes, but also the affinity of a drug designed to bind to the relevant human protein (Sasaguri *et al.*, 2017, Webster *et al.*, 2014). Ideally, animal models of AD should entirely recapitulate the complete human pathological scenario, that is, synaptic dysfunction and loss, progressive cognitive decline, amyloid plaques and NFT deposition, inflammation, neuronal death as well as brain atrophy (Sasaguri *et al.*, 2017). However, current models recapitulate only certain aspects of the disease. For example, even though many APP and PS1 mouse models exhibit extracellular Aβ deposits, reminiscent of plaques in the human AD brain, to date none of the amyloidogenic transgenic mouse models show aggregated tau deposits or neuronal loss in the brain.

Several Aβ-depositing mouse models have been extensively studied, some of which include PDAPP (Games *et al.*, 1995), Tg2576 (Hsiao *et al.*, 1996), APP23 (Sturchler-Pierrat *et al.*, 1997), J20 (Mucke *et al.*, 2000), TgCRND8 (Chishti *et al.*, 2001), APP/PS1dE9 (Jankowsky *et al.*, 2004) and APP/PS1 (Radde *et al.*, 2006) among many others. Most amyloid mouse models currently available overexpress one or more of the FAD mutations such as the London or Swedish mutations. As a result, these mice develop substantial Aβ deposits between 6-11 months of age, eventually displaying progressive plaque-associated gliosis and dystrophic neurites with age (Sasaguri *et al.*, 2017). Certain APP transgenic mouse models, such as the Swedish mutation-overexpressing mice, show apparent behavioural deficits at a young age compared to wild-type (WT) mice. Even though PS1 mutations cause the majority of FAD cases (Karch *et al.*, 2014), overexpression of mutant PS1 or a knock-in of the human gene mutation alone fails to induce Aβ pathology in mice. This may most likely be due to insufficient production of pathogenically longer Aβ species (De Strooper *et al.*, 1995). Alternatively, the difference in three amino acids in murine $A\beta_{1\text{-}42}$ sequence from human Aβ reduces the amyloidogenic potential of murine Aβ to self-aggregate as well as its degree of neurotoxicity (Chui *et al.*, 1999, Guo *et al.*, 1999, Schmitz *et al.*, 2004, Xu *et al.*, 2015). Thus, Aβ pathology in transgenic mouse models is only induced with the overexpression of both mutated human APP and PS, such as Tg2576 and PS1M146L (Holcomb *et al.*, 1998), APPKM670/671NL and PSA246E (Borchelt *et al.*, 1997), APP KM670/671NL and PS1 deltaE9/S290 (Jankowsky *et al.*, 2004), APP KM670/671NL and PS1 L166P (Radde *et al.*, 2006), and others.

Overexpressing several transgenic insertions may destroy the endogenous gene loci (Saito *et al.*, 2016) and overproducing APP fragments unrelated to Aβ may interact abnormally with cellular proteins leading to artefacts (Mitani *et al.*, 2012, Saito *et al.*, 2016). To overcome the drawbacks of APP overexpression paradigm, APP knock-in mice were generated to overproduce pathogenic $A\beta_{42}$ without changing APP expression (Saito *et al.*, 2014). For this, the authors inserted humanised Aβ region containing two or three APP mutations (Swedish KM670/671NL and Beyreuther/Iberian I716F mutations, respectively) into the endogenous murine APP gene resulting in increased Aβ plaque deposition and high $A\beta_{40/42}$ ratio (Saito *et al.*, 2014). The Beyreuther/Iberian mutation increases the $A\beta_{40/42}$ ratio by 30-fold

(Lichtenthaler *et al.*, 1999), which was later identified as an aggressive form of FAD in Iberia (Guerreiro *et al.*, 2010). Although the APP knock-in model may offer an improved mouse model for FAD, the mice do not develop fibrillar core Aβ plaques until much later in age, with almost no vascular pathology compared to the APP and PS1 double transgenic mice. Thus, the APP overexpression and knock-in mice each independently present distinct advantages and disadvantages depending on the aim of the experiment.

1.4.1 The APPPS1 mouse model

APPPS1 mice (line 21) carry human transgenes for the Swedish APP mutation (K670N/M671L) and PS1 (L166P) mutation. Both genes are expressed under thymidine 1 (Thy.1) promoter to ensure high levels of neuron-specific transgene expression in the postnatal brain (Radde *et al.*, 2006). The human APP transgene is expressed approximately 3-fold higher than the endogenous murine APP, and mainly expressed in neocortical, hippocampal, and brain stem neurons. Consequently, APP overexpression results in roughly 3-fold increased Aβ generation compared to WT mice due to enhanced β-secretase cleavage (Radde *et al.*, 2006). The aggressive PS1 L166P mutation, which causes onset of AD as early as 24 years of age (Moehlmann *et al.*, 2002, Bentahir *et al.*, 2006), favours the production of human $A\beta_{42}$ over $A\beta_{40}$, all of which increase with age (Li *et al.*, 2016).

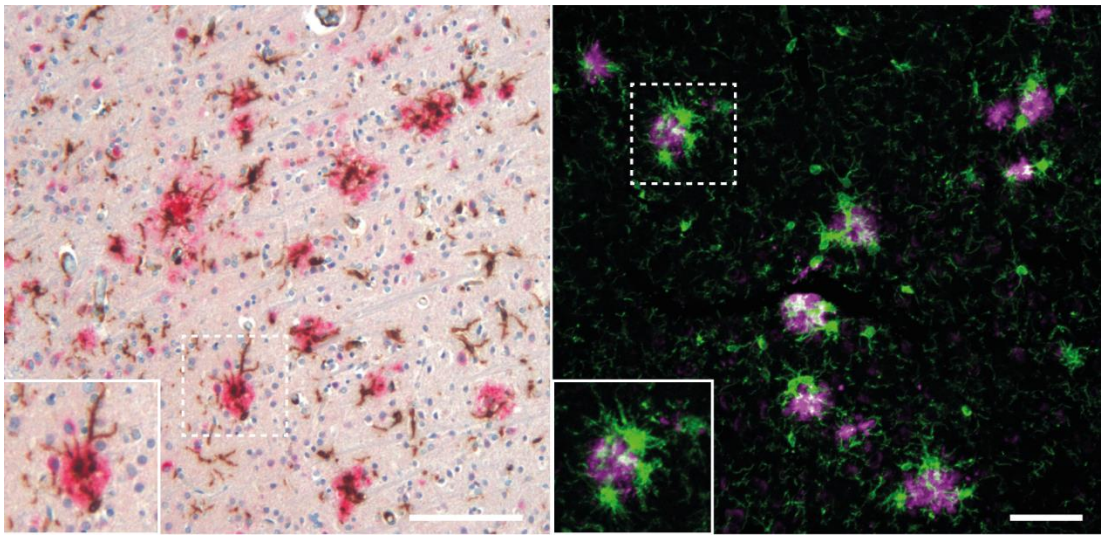

Figure 1.4 Microglia clustering around Aβ plaques. Left: Aβ plaques (red) and microglia (brown) stained temporal cortex of an AD case. Scale bar - 100μm. Right: Aβ plaques (magenta) and microglia (green) stained in APPPS1 mouse cortex. Scale bar - 20μm. Insets show a higher magnification of the area indicated in white dotted boxes. (Experiments performed and imaged by S. Parhizkar).

Amyloid plaque deposition starts in the neocortex at six weeks of age (Radde *et al.*, 2006). Despite their small size, nearly 100% of the plaques are congophillic in nature. Deposits in the hippocampus appear at around three months of age in males and four months in females. Congophillic plaques are also observed in the striatum, thalamus and brain stem after five months of age. By eight months, these mice exhibit substantial plaque load with the majority of plaques consisting of a dense core surrounded by a diffuse halo, resembling plaques observed in the human AD brain (Figure 1.4). Tau-positive neuritic processes are present at later stages in proximity to dense cored plaques, however fibrillar tau inclusions fail to

accumulate (Radde *et al.*, 2006). Moreover, microgliosis and astrogliosis are often observed in plaque-bearing regions similar to the AD brain (Figure 1.4). Additionally, cognitive impairments manifest around six to eight months of age, often presented by deficits in spatial learning and memorizing a maze task (Radde *et al.*, 2006, Serneels *et al.*, 2009). Neuron loss is largely absent in this mouse model, with only modest loss found in the granule cell layer of the dentate gyrus after 15 months of age (Rupp *et al.*, 2011).

Besides investigating Aβ pathology in the brain, the Aβ levels in cerebrospinal fluid (CSF) have also been extensively studied (Bacioglu *et al.*, 2016, Maia *et al.*, 2013). $A\beta_{42}$ and $A\beta_{40}$ levels in CSF decrease with age by approximately 80% and 45%, respectively. This reduction in Aβ levels strongly correlates with increased Aβ aggregates depositing in the brain. Additionally, the total tau concentrations increase at six months, reaching a 5-fold increase by 18 months of age (Maia *et al.*, 2013). Altogether, these findings in the APPPS1 mouse model closely corroborate the amyloid pathology observed in AD patients (Figure 1.4), and therefore serve as a suitable system to study amyloidosis-related pathomechanisms allowing relatively rapid experimental readouts.

1.5 The prion paradigm – Propagation of proteopathic seeds

Numerous studies on the role of protein aggregation in neurodegenerative diseases have speculated that the essential cause of neuronal dysfunction and death may result from cumulative protein misfolding and aggregation (Goedert *et al.*, 2017, Walker and Jucker, 2015). Although neurodegenerative diseases such amyotrophic lateral sclerosis (ALS), Parkinson's disease, FTD and AD present different pathological and clinical characteristics, the diseases share a central pathogenic mechanism: the seeded aggregation of disease-specific proteins (Prusiner, 2012, Jucker and Walker, 2013, Guo and Lee, 2014). The prion paradigm proposes that assemblies of misfolded protein act as seeds for the template misfolding of other uncorrupted molecules (Figure 1.5). The seeds may be the agents by which the aggregates proliferate and spread elsewhere in the brain, once again initiating seeding and thereby sustaining the disease process (Prusiner, 1984, Gajdusek, 1994). The hypothesis arose from decades of research on a group of proteinaceous infectious diseases, known collectively as transmissible spongiform encephalopathies (Collins *et al.*, 2004). Prusiner and colleagues showed that prions are infectious agents consisting of abnormally folded prion protein (PrP) (Prusiner, 1982). Initially, prion diseases were thought to be present in humans only, e.g. Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, kuru and fatal insominas. However, during the years several rare human spongiform encephalopathies were found to be transmissible to nonhuman primates (Gajdusek, 1977), for example scrapie in sheep, chronic wasting disease of deer and elk, bovine spongiform encephalopathy, and others (Caughey *et al.*, 2009, Prusiner, 1998). Prion diseases are best known for their infectivity, however they can also be genetically heritable or sporadic in origin (DeArmond and Prusiner, 1995), similar to AD. Under normal conditions, the cellular prion protein (PrP^{C}) assumes a nonpathogenic α-helical conformation with little β-sheet. The disease manifests when PrP^{C} molecules misfold into a shape abnormally enriched in β-sheet – similar to Aβ peptide in AD, and propagate through the nervous system after self-assembly. This corrupted form of PrP is called PrP-scrapie (PrP^{Sc}). An important characteristic of misfolded prion protein is its enhanced potential

to form amyloid, that is, abnormal quantities of fibrillar protein (DeArmond and Prusiner, 1995). Thus, similarities in biophysical properties of amyloidogenic proteins suggest that diseases characterised by abnormal protein deposition share certain etiological mechanisms (Gajdusek, 1994, Lansbury, 1997, Prusiner, 1998, Trojanowski and Lee, 1998, Goedert *et al.*, 2017).

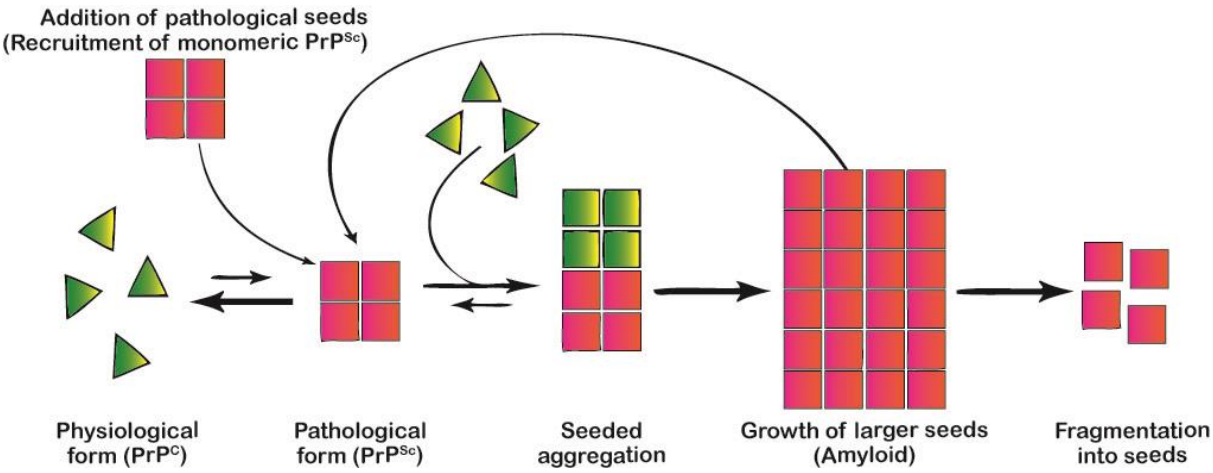

Figure 1.5 Prion-like template and seeding. A small portion of pathological seeds act as a template to induce misfolding and aggregation of the soluble protein, similar to the way amyloid fibril formation is accelerated upon addition of pre-formed pathological seeds in vitro (Harper *et al.*, 1997, Hasegawa *et al.*, 1999). The initial lag phase of the primary nucleation step follows a secondary nucleation step, during which existing fibrils are broken down into smaller fragments that become the building blocks for further seeding of soluble peptide monomers. Therefore, the second step not only depends on the rate at which the fibrils are created but also the availability of the soluble peptide monomers (Jarrett and Lansbury, 1993, Knowles *et al.*, 2009). Thus, new preformed seeds can facilitate the formation of larger multimeric amyloid fibrils. (Adapted from (Goedert, 2015)).

1.5.1 Amyloid seeding

AD is not a classic prion disease, but shares several similarities. Aβ peptide multimerisation is long known to be an early and central process in the pathogenic cascade in AD (Selkoe, 2003, Hardy and Selkoe, 2002). However, little is known about the mechanisms that govern the initiation of Aβ aggregation and deposition in vivo. In the late 80's, several groups performed inoculation experiments to test the hypothesis that AD, like spongiform encephalopathies, was transmissible (Goudsmit *et al.*, 1980, Baker *et al.*, 1993). Baker et al showed that intracerebral injections of AD brain homogenate into marmoset monkeys led to increased senile plaque load, however only after a long incubation period of approximately six to seven years. Moreover, the inductive agent as well as the mechanism of action remained unknown (Baker *et al.*, 1993). As the first APP transgenic mouse models became available (Games *et al.*, 1995, Hsiao *et al.*, 1996, Sturchler-Pierrat *et al.*, 1997), Aβ seeding experiments were carried out to determine whether Aβ deposits could be generated de novo in a prion-like manner. Brain extracts from autopsy-derived AD patients and controls were stereotaxically injected into the hippocampal formation and overlying neocortex in young, predepositing APP-transgenic mice (Meyer-Luehmann *et al.*, 2006, Walker *et al.*, 2002, Kane *et al.*, 2000). Three to five months post-injection, fibrillar Aβ deposits increased significantly at a point when the transgenic model would not yet have begun to develop endogenous Aβ plaques. Since then, the seeding paradigm

has been widely used in many different APP transgenic mice such as, Tg2576, APP23, APPPS1 and 5xFAD as an inoculation model to induce Aβ aggregation and cerebral amyloidosis (Watts *et al.*, 2014, Langer *et al.*, 2011, Meyer-Luehmann *et al.*, 2006, Kane *et al.*, 2000, Morales *et al.*, 2012, Rasmussen *et al.*, 2017, Ziegler-Waldkirch *et al.*, 2018). Additionally, the amyloid seeding model provides the unique opportunity to study plaque formation within a very defined and early time window. Furthermore, Aβ seeding activity persists for at least six months in the brain, even in the absence of template replication from host-derived Aβ (Ye *et al.*, 2015a). The longevity of Aβ seeds in vivo suggests that they may act as resilient prion-like protein, which may provide novel insights into changes in brain activity preceding the onset of dementia in AD.

The amyloid seeding model not only necessitates a donor extract containing Aβ seeds but also a host that can generate seeding-capable Aβ plaques. For example, injecting brain extracts from non-transgenic mice or non-AD control donors that lacked Aβ deposition failed to seed plaque formation (Meyer-Luehmann *et al.*, 2006, Kane *et al.*, 2000). Growing evidence supports the existence of variant structural strains of Aβ seeds, like tau (Falcon *et al.*, 2015, Goedert, 2015) and PrP (Aguzzi *et al.*, 2007, Collinge and Clarke, 2007, Prusiner, 2013). Injecting Aβ-rich brain extracts, which included Aβ monomers, oligomers, and larger multimeric Aβ species, from aged APP transgenic donor of the same strain as the host mice displayed a strain-specific seeding pattern (Meyer-Luehmann *et al.*, 2006, Watts *et al.*, 2011, Heilbronner *et al.*, 2013). Moreover, different molecular Aβ conformations linked to their functionality have been detected in vivo. This was first observed immunohistochemically, which showed morphological changes in the Aβ staining pattern depending on the characteristics of both the APP-transgenic host and donor seeding extract (Meyer-Luehmann *et al.*, 2006). Additionally, Aβ conformation-specific oligothiophene dyes confirmed that the molecular attributes of Aβ aggregates show intersubject variability among FAD and sporadic AD patients (Rasmussen *et al.*, 2017, Condello *et al.*, 2018).

The extent of Aβ seeding is directly proportional to concentration of the injected brain extract (Meyer-Luehmann *et al.*, 2006, Fritschi *et al.*, 2014b). Additionally, the characteristics and rate of induced Aβ pathology further depend on the brain area injected, with strongest deposition in the entorhinal cortex and the hippocampal molecular layer, granular cell layer as well as the subgranular cell layer in the dentate gyrus (Walker *et al.*, 2002, Meyer-Luehmann *et al.*, 2006, Eisele *et al.*, 2009). Ziegler-Waldkirch and colleagues were the first to show substantial neuronal death following Aβ deposition in vivo (Ziegler-Waldkirch *et al.*, 2018). Using the amyloid seeding model, the authors found a dramatic decrease in proliferation and neurogenesis in the granular cell layer of dentate gyrus in two APP transgenic mouse models. These regions are also severely affected in aging transgenic mice under normal conditions (Radde *et al.*, 2006). The selective vulnerability of these brain regions indicates that the starting point of the seeding cascade is crucial for the route or spread of pathology (Eisele *et al.*, 2009, Meyer-Luehmann *et al.*, 2006, Ziegler-Waldkirch *et al.*, 2018). Furthermore, after injecting the seeding-competent brain extract into the hippocampus, Aβ deposits begin to appear in other brain regions throughout the hippocampal formation (Eisele *et al.*, 2009) and limbic connectome over time (Ye *et al.*, 2015b). As the limbic connectome is a major source of neocortical communication with the hippocampal formation and entorhinal cortex (Suh *et al.*,

2011), these studies suggest that seeded Aβ aggregates traffic along neuronal pathways to spread pathology through the brain, similar to the cell-to-cell transmission model suggested in prion diseases (Lee *et al.*, 2010). However, whether this occurs by random diffusion or by active, cell-specific uptake and transport is yet to be determined in vivo.

Seeding most likely occurs due to the nature of Aβ deposits rather than additional factors that may indirectly stimulate Aβ aggregation, e.g. a virus or an immune response to the foreign brain extracts. To date, the seeding factors remain unknown; however several studies indicate that Aβ itself may drive the mechanism to a large extent (Heilbronner *et al.*, 2013, Langer *et al.*, 2011, Fritschi *et al.*, 2014a, Fritschi *et al.*, 2014b). Disrupting the amyloid-inducing activity of the injected brain extract by heating, reduced the Aβ induction (Meyer-Luehmann *et al.*, 2006). Additionally, denaturation of brain extracts by formic acid treatment or co-inoculation of anti-amyloid antibody abolished the seeding effect (Meyer-Luehmann *et al.*, 2006, Duran-Aniotz *et al.*, 2014). These findings support the direct role of Aβ as the seeding agent. Preparation of synthetic $A\beta_{40}$, $A\beta_{42}$ or a mixture of both failed to induce detectable Aβ deposition (Meyer-Luehmann *et al.*, 2006) unless the concentration was increased 10-fold with a longer incubation period (Stohr *et al.*, 2012). Of note, the seeding potency of $A\beta_{38}$ or $A\beta_{43}$ found in AD patients has not been reported. Furthermore, amyloid seeding has been attempted in an ex vivo hippocampal slice culture model, however it appears to be sustainable only upon frequent exposure to high levels of synthetic Aβ (Novotny *et al.*, 2016), similar to PrP seeding potency (Miller *et al.*, 2013, Wang *et al.*, 2010). Moreover, ASC-deficient APP/PS1deltaE9 display reduced seeding and spreading of Aβ, suggesting that the secreting adaptor protein ASC specks found in the core of amyloid plaques may play an active role in initiating cross-seeding of Aβ plaques (Venegas *et al.*, 2017).

The seeding model has influenced other research fields, such as the aggregation and spreading of tau (Guo *et al.*, 2016, Clavaguera *et al.*, 2013), α-synuclein (Volpicelli-Daley *et al.*, 2011, Luk *et al.*, 2012) or TAR DNA-binding 43 protein found in frontotemporal lobar degeneration cases (Porta *et al.*, 2018). Additionally, it has been used as an in vivo model for cross-seeding studies to investigate the interactions between amyloid and other proteopathic co-pathology such α-synuclein, or tau (Gotz *et al.*, 2001, Bachhuber *et al.*, 2015, Guo *et al.*, 2013, He *et al.*, 2018, Venegas *et al.*, 2017, Bolmont *et al.*, 2007, Rasmussen *et al.*, 2017). The seeding model has also been used to test treatment options such as immunization (Meyer-Luehmann *et al.*, 2006) or environmental enrichment as a non-pharmacological approach (Ziegler-Waldkirch *et al.*, 2018). Finally, recent studies suggest that microglia may contribute to the transport and processing of seeds in a disease stage-dependent manner (Asai *et al.*, 2015, Venegas *et al.*, 2017).

2. Microglia and AD

Microglia constitute approximately 10% of the total glial population in the healthy adult brain. Together with meningeal, choroid plexus, and perivascular macrophages, microglia compromise the brain myeloid cell population that plays a pivotal role in the CNS immune response and homeostasis. As the main innate immune cells of the CNS, microglia represent the first line of immunological defence by constantly surveying their microenvironment for signs of damage or debris allowing them to respond rapidly to focal injury or invading

pathogens (Davalos *et al.*, 2005, Mazaheri *et al.*, 2017). Microglia have several functions in the developing CNS, including (1) axon growth and synaptogenesis, (2) synaptic pruning, (3) angiogenesis, (4) support of neurogenesis, (5) recognising and responding to abnormal events including focal injury or neuropathological insults such as Aβ plaques, and (6) apoptosis and phagocytosis (Ransohoff, 2016). These functions are central to disease progression and modulation because chronic glial activation is a prominent feature of aging and often goes hand-in-hand with pathological protein accumulation as well as cell death, both of which constitute central characteristics of neurodegeneration. Although microglia's response to injury is usually beneficial, it may also be detrimental, especially in chronic inflammation observed in AD (Hickman *et al.*, 2008, Lucin and Wyss-Coray, 2009).

2.1 Disease-associated microglia

Depending on their microenvironment, microglia were considered to be selective to two states: resting or activated. Anti-inflammatory ramified microglia were described as resting, or M2, often observed during healthy physiological conditions and morphologically characterised by long processes and a small cell body. In contrast, the M1 activated amoeboid microglia were illustrated as hypertrophied cell bodies with short processes, and characterised by production of pro-inflammatory cytokines and nitric oxide (Wyss-Coray and Rogers, 2012). Ramified microglia were found throughout plaque-free brain regions, whereas the amoeboid microglia were typically associated with senile plaques (Brawek *et al.*, 2014). However, recent advances in transcriptomics (Butovsky *et al.*, 2014, Hammond *et al.*, 2018, Keren-Shaul *et al.*, 2017, Mazaheri *et al.*, 2017, Krasemann *et al.*, 2017) indicate that the M1/M2 dichotomy do not truly reflect the heterogeneous microglial populations present in disease-associated inflammation.

Comprehensive single-cell RNA sequencing analysis of AD, ALS and aging mouse models show that microglia are present in a continuum of distinct phenotypes that are highly dependent on their spatiotemporal context (Kang *et al.*, 2018, Hammond *et al.*, 2018, Keren-Shaul *et al.*, 2017). For example, microglia heavily cluster around Aβ plaques in both mice and humans (Figure 1.3) (Meyer-Luehmann *et al.*, 2008, Serrano-Pozo *et al.*, 2013, Parhizkar *et al.*, 2019) and modulate Aβ pathogenesis (further discussed in 3.3.1). These subset of microglia are called disease-associated microglia (DAM) (Keren-Shaul *et al.*, 2017) or microglia of neurodegenerative phenotype (MGnD) (Krasemann *et al.*, 2017). While DAM markers such as Clec7a, Cd68 and Cst3 are upregulated during disease conditions, homeostatic microglial markers such as P2ry12, P2ry13, and Cx3cr1 are downregulated (Keren-Shaul *et al.*, 2017, Masuda *et al.*, 2019). DAM typically display upregulation of genes involved in pathways triggering phagocytosis, migration and chemotaxis, as well as lysosomal and lipid metabolism such as ApoE, Lpl, Dap12, and Trem2 (Kang *et al.*, 2018, Keren-Shaul *et al.*, 2017, Krasemann *et al.*, 2017, Mazaheri *et al.*, 2017, Hammond *et al.*, 2018, Masuda *et al.*, 2019), all of which are well-known AD risk factors (Lambert *et al.*, 2013).

3. Triggering Receptor Expressed on Myeloid Cells 2

TREM2 belongs to a family of innate immune receptors referred to as triggering receptors expressed on myeloid cells (Bouchon *et al.*, 2000). In humans, the *TREM2* gene is located on chromosome 6p21.1 or 17C in mouse and encodes a 230 amino acid glycoprotein. Many of the TREM and TREM-like (*TREML*) genes such as *TREM1*, *TREML1*, *TREM2*, *TREML2*,

TREML3, and *TREML4* are conserved in mice and humans with only *Trem3* and *Trem6* unique to mice and *TREML3* to humans. TREM2 is a type 1 cell surface transmembrane glycoprotein, and consists of an extracellular V-type immunoglobulin (Ig)-like domain as well as a short cytoplasmic tail (Figure 1.6) (Bouchon *et al.*, 2000). It is selectively expressed on microglia in the brain (Sessa *et al.*, 2004, Neumann and Takahashi, 2007), but also abundantly found in the periphery in a subgroup of myeloid cells, including bone osteoclasts, dendritic cells, alveolar and peritoneal macrophages, granulocytes and Kuppfer cells (Bouchon *et al.*, 2001, Cella *et al.*, 2003, Paloneva *et al.*, 2003, Colonna and Wang, 2016). Trem2 expression levels increase with aging (Jay *et al.*, 2015, Guerreiro and Hardy, 2013, Jiang *et al.*, 2014, Brendel *et al.*, 2017) and vary depending on the particular region of the CNS, with a higher expression in the white matter and hippocampus in the brain (Chertoff *et al.*, 2013, Forabosco *et al.*, 2013). High levels of Trem2 expression were reported in CD45-expressing cells (Jay *et al.*, 2015, Savage *et al.*, 2015), suggesting that Trem2-expressing cells could be of a peripheral origin and may be found in the brain due to infiltrating monocytes. However, these findings were later disputed using parabiosis experiments in AD mouse model reporting that Trem2-expressing cells were mainly resident microglia rather than infiltrating monocytes (Wang *et al.*, 2016). DAM often display elevated Trem2 expression in AD, ALS, and aging mouse models (Keren-Shaul *et al.*, 2017, Krasemann *et al.*, 2017). Keren-Shaul and colleagues revealed that DAM activation occurs in a Trem2-dependent manner during which microglia acquire a phagocytic phenotype associated with synthesis and degradation of lipids (Keren-Shaul *et al.*, 2017). Moreover, microglia appear to be locked in a homeostatic state with impaired chemotaxis and reduced responses to neuronal injury in Trem2-deficient mice (Mazaheri *et al.*, 2017).

TREM2 binds to multiple ligands including microbial products, phospholipids, as well as zwitterionic and anionic lipids released during neuronal and glial damage to activate TREM2 signalling (Figure 1.6). TREM2 mutations appear to blunt the signalling activity, suggesting that the positively charged arginine residue may be critical for initiating TREM2 signalling. Other ligands include nucleic acids, proteoglycans as well as heat shock protein 60 (Kober *et al.*, 2016), which play an active role in phagocytosis (Stefano *et al.*, 2009). Additionally, specific lipid species initiate TREM2 signalling, either as a lipoprotein complex or components of a cellular membrane (Cannon *et al.*, 2012, Poliani *et al.*, 2015, Song *et al.*, 2016, Wang *et al.*, 2015). For example, TREM2 binds to high-density lipoproteins (HDL), low-density lipoproteins (LDL) as well as apolipoproteins (Apo), such as ApoA1, ApoA2, ApoB, ApoE and ApoJ (also known as clusterin) in vitro (Yeh *et al.*, 2016, Atagi *et al.*, 2015, Bailey *et al.*, 2015, Song *et al.*, 2016). ApoE lipidation does not appear to be essential for ApoE-TREM2 binding to occur (Atagi *et al.*, 2015, Bailey *et al.*, 2015, Jendresen *et al.*, 2017), although Yeh and colleagues reported that ApoE lipidation augmented this interaction (Yeh *et al.*, 2016). Moreover, TREM2 appears to bind to all ApoE isoforms with similar affinities (Atagi *et al.*, 2015, Bailey *et al.*, 2015).

TREM2 is shuttled to and from the plasma membrane via the secretory pathway, that is, the trans-Golgi network (Prada *et al.*, 2006, Varnum *et al.*, 2017), endosomes (Raha *et al.*, 2017) and exocytic vesicles (Prada *et al.*, 2006). TREM2 undergoes maturation by N-linked glycosylation during this transport process (Park *et al.*, 2015), however the biological relevance of these modifications remains unknown. Full-length membrane bound TREM2 undergoes

proteolytic processing by a disintegrin and metalloproteinases (ADAM) α-secretases, ADAM10 or ADAM17, which results in ectodomain shedding between H157 and S158 residues generating a soluble form of TREM2 (sTREM2) (Figure 1.6) (Feuerbach *et al.*, 2017, Kleinberger *et al.*, 2014, Schlepckow *et al.*, 2017, Thornton *et al.*, 2017, Wunderlich *et al.*, 2013). This cleavage terminates TREM2 signalling and its downstream effector functions. Interestingly, p.H157Y was recently identified as an AD risk variant (Jiang *et al.*, 2016), and shows increased sTREM2 levels, reduced cell surface TREM2 as well as impaired phagocytosis in vitro (Schlepckow *et al.*, 2017).

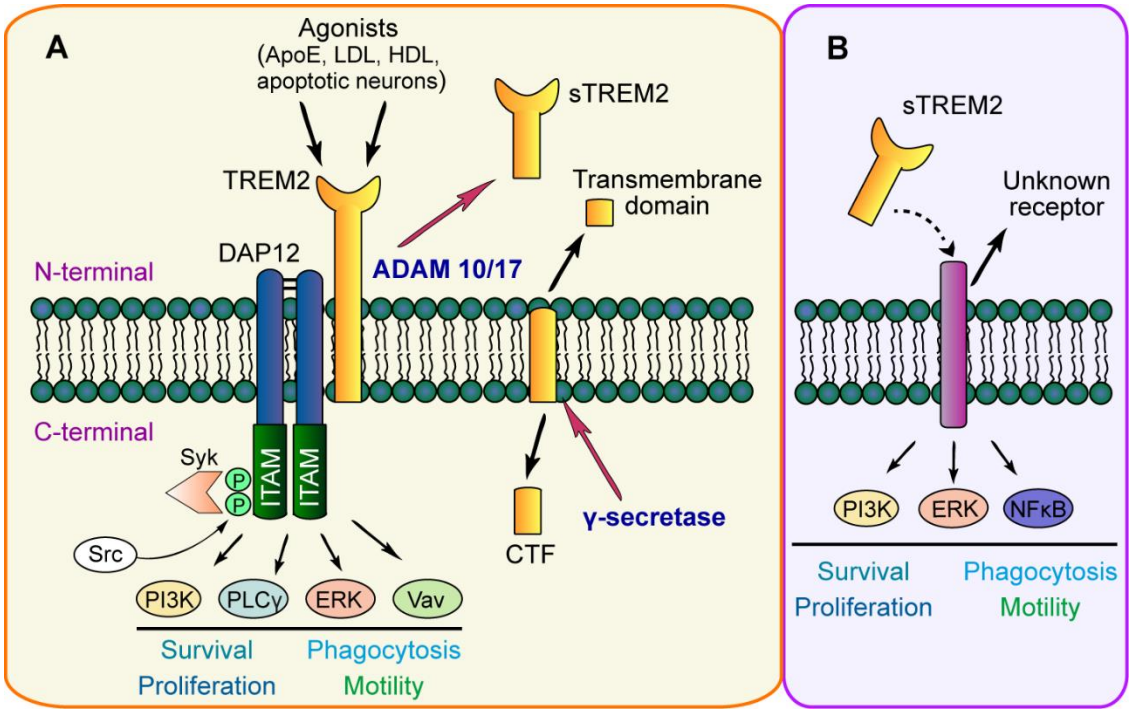

Figure 1.6 TREM2/DAP12 signalling. (A) TREM2 binds to several ligands, and signals through its Ig-like V-type domain. The positively charged conserved lysine residue on TREM2 transmembrane region (amino acid 186) electrostatically interacts with negatively charged aspartic acid residue in DNAX-activating protein of 12 kDa (DAP12). Src kinase phosphorylates the DAP12 immunoreceptor tyrosine-based activation motifs (ITAM). This phosphorylation recruits and activates spleen tyrosine kinase (Syk), which triggers downstream effectors including extracellular signal-regulated kinases (ERK), phosphoinositide 3-kinase (PI3K), phospholipase C-γ (PLCγ) and Vav. Subsequent intracellular Ca^{2+} efflux and actin cytoskeletal reorganisation promotes microglial functions such as motility and phagocytosis. TREM2 undergoes sequential proteolytic processing by ADAM 10 or 17 resulting in ectodomain shedding of sTREM2. The remaining TREM2 transmembrane domain is subsequently cleaved by γ-secretase, releasing a C-terminal fragment (CTF) (Glebov *et al.*, 2016, Wunderlich *et al.*, 2013). TREM2 cleavage by ADAMs terminates the downstream signalling cascade. (B) The receptor for sTREM2 is unknown. The ectodomain shedding appears to activate PI3K, ERK and nuclear factor (NF)-κB pathways and enhances several microglial functions related to phagocytosis. (Adapted from (Konishi and Kiyama, 2018)).

sTREM2 is readily found in in biological fluids such as serum and CSF in both mice and humans (Henjum *et al.*, 2016, Kleinberger *et al.*, 2017, Kleinberger *et al.*, 2014, Wunderlich *et al.*, 2013). CSF sTREM2 levels are thought to reflect the degree of microglial activation in the brain during disease progression (Heslegrave *et al.*, 2016, Piccio *et al.*, 2016) as well as the severity of brain atrophy due to its association with neuronal injury markers (Suarez-Calvet *et*

al., 2016b). Interestingly, CSF sTREM2 levels peak specifically during the early symptomatic stages of AD (Suarez-Calvet *et al.*, 2016a), suggesting that sTREM2 may be a biomarker for not only disease severity but also cognitive decline that manifests in the earlier stages of AD (Jiang *et al.*, 2014).

3.1 TREM2/DAP12 and Nasu Hakola disease

DAP12 belongs to the type 1 transmembrane adapter protein family (Wilson *et al.*, 2000). It is abundantly expressed on immune cells such as natural killer cells, myeloid cells and neutrophils, and forms an ITAM-containing disulphide-linked homodimer (Figure 1.6). DAP12 itself is thought to have no ligand-binding capability due to its short extracellular domain (Lanier, 2009). To initiate an intracellular signalling cascade, TREM2 interacts with DAP12 within the transmembrane region through an electrostatic association (Bouchon *et al.*, 2000), which triggers a downstream signalling event regulating cell migration and survival, phagocytosis or cytokines release (Figure 1.6) (Colonna *et al.*, 2007). Homozygous loss-of-function mutation in *TREM2* or *DAP12* leads to a rare neurodegenerative disease associated with leukodystrophy, osteoporosis and a presenile form of frontal lobe dementia called Nasu Hakola disease (NHD, also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy, PLOSL) (Klunemann *et al.*, 2005, Paloneva *et al.*, 2001, Paloneva *et al.*, 2002). To date, 11 *TREM2* mutations have been reported to cause NHD, some of which include p.W78X, p.W44X, p.K186N, and p.D134G (Paloneva *et al.*, 2002). Additionally, amyloid PET imaging revealed extensive Aβ pathology in a patient carrying the p.Q33X TREM2 variant (Ghezzi *et al.*, 2017), which is more commonly found in FTD patients (Guerreiro *et al.*, 2013b). Moreover, NHD patients develop bone cysts as early as the second decade of life, with dementia manifesting due to atrophy of the corpus callosum and frontal lobe regions. Other pathological changes include loss of myelin, gliosis, and enlarged brain ventricles, which also affect motor functions. As a result, NHD patients perish as early as the late fourth decade of life. Currently there are no NHD mouse models available, however Trem2- or Dap12-deficient mice display similar phenotypes including impaired myelination, reduced amyloid plaque-associated microglia and increased accumulation of diffuse Aβ plaques (Mazaheri *et al.*, 2017, Wang *et al.*, 2015, Keren-Shaul *et al.*, 2017, Wang *et al.*, 2016, Yuan *et al.*, 2016, Parhizkar *et al.*, 2019, Cantoni *et al.*, 2015, Piccio *et al.*, 2007, Kaifu *et al.*, 2003, Nataf *et al.*, 2005). Moreover, TREM2 expression appears to be severely downregulated in dendritic cells from NHD patients, suggesting that disrupted TREM2 expression or signalling may be responsible for NHD symptoms.

3.2 TREM2 and FTD

Heterozygous or homozygous *TREM2* coding variants such as p.T66M (Guerreiro *et al.*, 2013b, Le Ber *et al.*, 2014), p.W198X (Giraldo *et al.*, 2013), p.Q33X and p.Y38C (Guerreiro *et al.*, 2013b) are associated with FTD. Although the p.T66M and p.Y38C heterozygous variants are found in AD patients, they are more commonly associated with FTD (Guerreiro *et al.*, 2013b, Jonsson and Stefansson, 2013). Other AD-associated TREM2 variants such as p.T96K, p.L211P and p.R47H also increase the risk of developing FTD (Thelen *et al.*, 2014, Rayaprolu *et al.*, 2013). The p.T66M and p.Y38C mutations that reside in the Ig-like domain are misfolded in the ER (Kober *et al.*, 2016, Park *et al.*, 2015) and show impaired cell surface

trafficking (Kleinberger *et al.*, 2014, Song *et al.*, 2017), which may explain how TREM2 loss-of-function affects FTD-like syndrome (Guerreiro *et al.*, 2013b). Additionally, the glycosylation pattern of these mutations is also affected indicating impairment in maturation that could affect TREM2 function (Park *et al.*, 2015).

Knocking in p.T66M missense mutation in mice showed a gene dose-dependent reduction in sTrem2 in the brain, serum, and CSF due to ER retention of mutant immature sTrem2 (Kleinberger *et al.*, 2017). Decreased cell surface Trem2 resulted in loss of Trem2 function as observed by a decline in phagocytic ability of bone marrow-derived macrophages as well as reduction in TSPO and fluorodeoxyglucose (FDG) PET, which are indicative of decreased inflammation and impaired brain glucose metabolism or cerebral blood flow respectively. Interestingly, the p.T66M knock-in model also revealed evidence of poor removal of myelin debris with aging as observed by increased microglial nodules throughout the brain (Kleinberger *et al.*, 2017). Although no bone-related pathology was observed in these mice, the model may help address several important questions such as how cerebral blood flow affects TREM2 maturation and whether sTREM2 or full length TREM2 may influence hypometabolism and neuronal activity in the brain in a cell-autonomous manner. Moreover, it may serve as an essential loss of Trem2 function model, which may be more suitable for comparing the clinical situation than manipulating gene expression, as is currently typical.

3.3 TREM2 and AD

Although TREM2 variants are rare with allele frequencies below 1%, several heterozygous TREM2 variants are found to confer higher AD risk including p.R47H, p.R62H, p.D87N, p.H157Y, p.T96K, p.W191X, p.L211P and R136Q (Bonham *et al.*, 2017, Guerreiro *et al.*, 2013a, Jiang *et al.*, 2016, Jin *et al.*, 2014, Jin *et al.*, 2015, Jonsson *et al.*, 2013). The TREM2 variants include early stop sites and ectodomain mutations, which consequently produce partially or non-functional protein (Giraldo *et al.*, 2013). The p.R47H variant found more commonly in European and North American populations increases the risk of LOAD by approximately 3-fold, with similar odds ratio to single *APOE* ε4 allele (Guerreiro and Hardy, 2013, Lill *et al.*, 2015). Additionally, it is associated with higher total tau and phosphorylated tau in CSF (Cruchaga *et al.*, 2013). The p.R47H variant has since then been confirmed to be associated with both early onset AD and LOAD in various case populations independent of the *APOE* ε4 risk variant (Jin *et al.*, 2014, Pottier *et al.*, 2013, Ruiz *et al.*, 2014, Sims *et al.*, 2017). LOAD patients with the p.R47H variant present an earlier onset of disease symptoms including faster cognitive decline (Slattery *et al.*, 2014, Del-Aguila *et al.*, 2018).

While NHD-associated TREM2 variants impact protein expression, stability, folding and transport, the AD-associated TREM2 variants seem to impact the efficiency of TREM2 signalling (Kleinberger *et al.*, 2014, Ma *et al.*, 2016, Song *et al.*, 2016, Kober *et al.*, 2016). Moreover, p.R47H variant AD patients show impaired microglial clustering around plaques (Yuan *et al.*, 2016, Parhizkar *et al.*, 2019), resulting in larger, more diffuse plaques (Yuan *et al.*, 2016). Reduced plaque-surrounding microglia was also recently reported in 5xFAD mice overexpressing human p.R47H TREM2 but lacking endogenous expression of murine Trem2 (Song *et al.*, 2018). The microglial pathology the p.R47H mice were comparable to Trem2-deficient 5xFAD mice, suggesting that the mutation causes a loss-of-function (Song *et al.*,

2018). Murine p.R47H Trem2 knock-in also confirmed loss-of-function effects as a result of reduced Trem2 mRNA and protein production (Cheng-Hathaway *et al.*, 2018). However, Trem2 haploinsufficieny appeared to be an artefact introduced by CRISPR resulting in atypical splicing of exon 1 and 2 in the p.R47H murine mutation, which was otherwise not observed in humans carrying the p.R47H coding variant (Xiang *et al.*, 2018). These findings highlight the need to determine how different TREM2 variants influence alternative splicing and their functional consequences.

3.3.1 TREM2, microglia function and Aβ pathology

Microglia play an active role in regulating amyloid pathology by phagocytosis. However, the inconsistent results from microglia depletion studies using APP transgenic mice raise a critical question: At which disease stage does microglia most efficiently restrain amyloidogeneisis? Microglia depletion in vivo has shown that the timing and duration of microglia interaction with amyloid pathology plays an important role in its phagocytic abilities. Herpes simplex virus thymidine kinase (HSTK) is also known as a suicide gene as it converts ganciclovir (GCV) into toxic DNA replication inhibitors that results in cell death (Czako and Marton, 1994). Thus, expressing HSTK on a CD11b promoter, which is expressed in macrophages and microglia, results in microglia depletion upon GCV treatment in APPPS1 mice crossed with HSTK-expressing mice (Grathwohl *et al.*, 2009). Similarly, treating 5xFAD mice with macrophage colony stimulating factor 1 receptor (CSFR1) inhibitor results in microglia depletion (Spangenberg *et al.*, 2016). Both microglia depletion systems do not affect plaque burden after onset of Aβ pathology (Spangenberg *et al.*, 2016, Grathwohl *et al.*, 2009). However, depleting microglia by CSFR1 inhibition prior to Aβ deposition dramatically reduced plaque pathology in 5xFAD mice (Sosna *et al.*, 2018), suggesting that microglia may be involved in the initial seeding stage of plaque formation. Thus by eliminating newly seeded plaques, Trem2 may have protective functions at least early during amyloidogenesis (Parhizkar *et al.*, 2019).

A protective function of TREM2 is also supported by disease-associated TREM2 variants causing a loss-of-function (Abud *et al.*, 2017, Kleinberger *et al.*, 2017, Kleinberger *et al.*, 2014, Song *et al.*, 2018, Yeh *et al.*, 2016). Trem2 haploinsufficiency or total genetic ablation reduces plaque-associated microglia (Jay *et al.*, 2015, Wang *et al.*, 2015, Ulrich *et al.*, 2014), suggesting that Trem2 signalling is required to activate and recruit microglia to the plaques. Additionally, transcriptomic analyses of plaque-surrounding microglia show major changes in their molecular markers related to chemotaxis, proliferation, response to wounding and locomotion, compared to microglia that retain a homeostatic profile when no plaques are present (Krasemann *et al.*, 2017, Mazaheri *et al.*, 2017). Thus, Trem2-expressing microglia appear to be essential for promoting microglial association to plaques and constitutes a major population of DAM in Aβ-depositing mouse models (Keren-Shaul *et al.*, 2017, Krasemann *et al.*, 2017). Impaired microglial clustering around plaques have also been reported in AD cases with different TREM2 variants (Parhizkar *et al.*, 2019, Yuan *et al.*, 2016). Additionally, loss of Trem2 function shows increased seeding of early plaque deposits, most likely due to impaired Aβ clearance by microglia (Parhizkar *et al.*, 2019). The inability of microglia to cluster around Aβ results in enlarged and more diffused plaques, which appear as star-shaped fibrils (Yuan *et al.*, 2016, Wang *et al.*, 2016). Such changes in plaque morphology as well as neuritic plaques

were also observed in AD patients carrying the p.R47H variant, similar to that observed in Trem2-deficient mice (Yuan *et al.*, 2016). Earlier studies reported inconsistent results about the effect of TREM2 on amyloid plaque pathology (Jay *et al.*, 2015, Ulrich *et al.*, 2014, Wang *et al.*, 2015), although it was later identified to be an age- and disease progression-dependent effect (Jay *et al.*, 2017, Parhizkar *et al.*, 2019). Moreover, Trem2 upregulation increases the phagocytic ability of plaque-surrounding microglia as indicated by plaque-derived material found in the DAM population (Keren-Shaul *et al.*, 2017, Yuan *et al.*, 2016, Parhizkar *et al.*, 2019).

Recently, Zhong and colleagues reported a protective function of sTREM2 in Aβ-depositing mice (Zhong *et al.*, 2019). They demonstrated that similarly to full-length Trem2, sTREM2 reduced plaque deposition and associated dystrophic neurites by enhancing microglial proliferation, migration and clustering around plaques as well as lysosomal degradation of Aβ. Additionally, sTREM2 rescued long-term potentiation and memory deficits, which are otherwise impaired by Aβ plaque deposition in 5xFAD mice. All effects were attenuated following microglia depletion, suggesting that sTREM2 mediates its protective effects primarily through microglia and that microglia are central to modifying synaptic plasticity and amyloidogenesis. Thus, the CSF sTREM2 increase observed at the prodromal mild cognitive impairment stage of AD patients (Suarez-Calvet *et al.*, 2016b) may represent a protective response to the initial threat caused by Aβ plaques that likely drive the pathogenic cascades of AD (Hardy and Higgins, 1992, Hardy and Selkoe, 2002). The functional similarities between sTrem2 and Trem2 may pose a challenge for therapeutic modulation of TREM2. This is because while blocking ADAM-mediated cleavage may enhance the protective functions of TREM2 due to increase signalling, reducing sTrem2 may present detrimental side effects that would be difficult to predict, especially since a receptor for sTrem2 has not yet been identified.

4. ApoE – Structure and function

ApoE is highly induced in DAM similarly to Trem2 (Keren-Shaul *et al.*, 2017, Krasemann *et al.*, 2017). Under healthy physiological conditions, the 299 amino acid glycoprotein is highly expressed in the liver, and in the brain, primarily by astrocytes (Zhang *et al.*, 2014, Zhang *et al.*, 2016), as well as microglia and damaged neurons to a lesser extent (Xu *et al.*, 2006). ApoE is mainly known as a major cholesterol carrier in the brain that maintains the structure of specific lipoproteins (Kim *et al.*, 2009a). Native ApoE is primarily lipidated by Adenosine triphosphate-binding cassette transporter A1 (ABCA1) in the brain. Additionally, ApoE facilitates transport of lipids among different cell types by serving as a ligand for the LDL receptor (LDLR) family and lipoprotein receptor-related protein (LRP) (Kim *et al.*, 2009a) (Figure 1.7).

Compared to humans that express three major isoforms of ApoE, with ApoE3 being the most predominant isoform, mice express only one type of ApoE protein (Kim *et al.*, 2009a). The human ApoE include two separate N- and C-terminal domains joined by a flexible hinge. The N-terminus includes the receptor binding regions, which differs in the ApoE isoform residues (ApoE4, arginine 112; ApoE3 and ApoE2, cysteine 112). The C-terminal domain contains the lipid-binding region (residues 244-272) (Bu, 2009). The cysteine residue at 112 found in both ApoE2 and ApoE3 may form disulphide-linked dimers, whereas the arginine residue in ApoE4

significantly impedes lipid binding (Weisgraber, 1990, Weisgraber and Shinto, 1991). Additionally, while ApoE3 and ApoE4 that have arginine at residue 158 display normal receptor binding activity, ApoE2 with cysteine shows impaired receptor binding abilities and is associated with the recessive form of type III hyperlipoproteinemia (Ahn *et al.*, 2009, Weisgraber *et al.*, 1990, Weisgraber *et al.*, 1994, Weisgraber and Shinto, 1991). This suggests that the differences in one or two amino acids in the N- and C-terminal domains significantly alter their interaction and preference to bind lipid and receptor proteins involved in cholesterol uptake (Mahley and Huang, 2012). To date, it is not clear how the function of ApoE as a lipid redistributor is mechanistically related to several diseases including AD, Down's syndrome-associated dementia and CAA. However, the ε4 allele disease risk association suggests that ApoE might contribute to these diseases by influencing synaptic plasticity, tau hyperphosphorylation, impairments in BBB integrity, neuroinflammation, as well as Aβ aggregation and clearance (Holtzman and Fagan, 1998, Kim *et al.*, 2009a, Mahley and Huang, 2012).

4.1 ApoE and Aβ pathology

ApoE was found to be a constituent of amyloid plaques (Corder *et al.*, 1993, Strittmatter *et al.*, 1993b) with a positive correlation between copies of APOE ε4 alleles and senile plaque density (Rebeck *et al.*, 1993, Schmechel *et al.*, 1993). The APOE ε4 gene dosage has also been closely linked to fibrillar Aβ and low $A\beta_{42}$ levels in CSF (Morris *et al.*, 2010, Sunderland *et al.*, 2004). Earlier studies on ApoE often produced inconsistent findings as it was later found that the affinity for ApoE to bind to Aβ highly depended on the lipidation state of ApoE, the Aβ species, as well as the pH levels of the in vitro model system (LaDu *et al.*, 1994, Sanan *et al.*, 1994, Strittmatter *et al.*, 1993a). The isoform-dependent effects on Aβ aggregation was better understood using transgenic mice expressing human ApoE alleles crossed with APP transgenic models. For example, while Aβ burden was significantly reduced in ApoE-null PDAPP mice (Bales *et al.*, 1999), mice lacking endogenous ApoE but expressing ε3 or ε4 isoform displayed less Aβ deposition compared to mice expressing endogenous ApoE. These findings suggest that human ApoE influences Aβ clearance (Holtzman *et al.*, 2000). It is not yet known how exactly ApoE interacts with Aβ and influences its clearance, though several mechanisms have been proposed including, enzymatic degradation (Jiang *et al.*, 2008), cellular uptake (Thal *et al.*, 2000, Yeh *et al.*, 2016, Ulrich *et al.*, 2018), interaction with receptors and transporters on cell surface (Verghese *et al.*, 2013), transport across the BBB (Cirrito *et al.*, 2005), and ISF-CSF flow (Castellano *et al.*, 2011, Cirrito *et al.*, 2003). ApoE4-expressing PDAPP mice display higher ISF Aβ levels with a slower Aβ clearance rate compared to ApoE3 mice. In comparison, ApoE2-expressing mice show lowest ISF Aβ and fastest Aβ clearance (Cirrito *et al.*, 2003). Additionally, immunotherapy studies using anti-ApoE antibody in amyloid plaque-bearing mouse models have shown to reduce Aβ plaque burden in an antibody dose-dependent manner (Liao *et al.*, 2014, Kim *et al.*, 2012). Targeting ApoE with anti-sense oligonucleotides from birth also appeared to reduce Aβ plaque pathology; however, the effect was lost if the treatment started after onset of plaque formation (Huynh *et al.*, 2017).

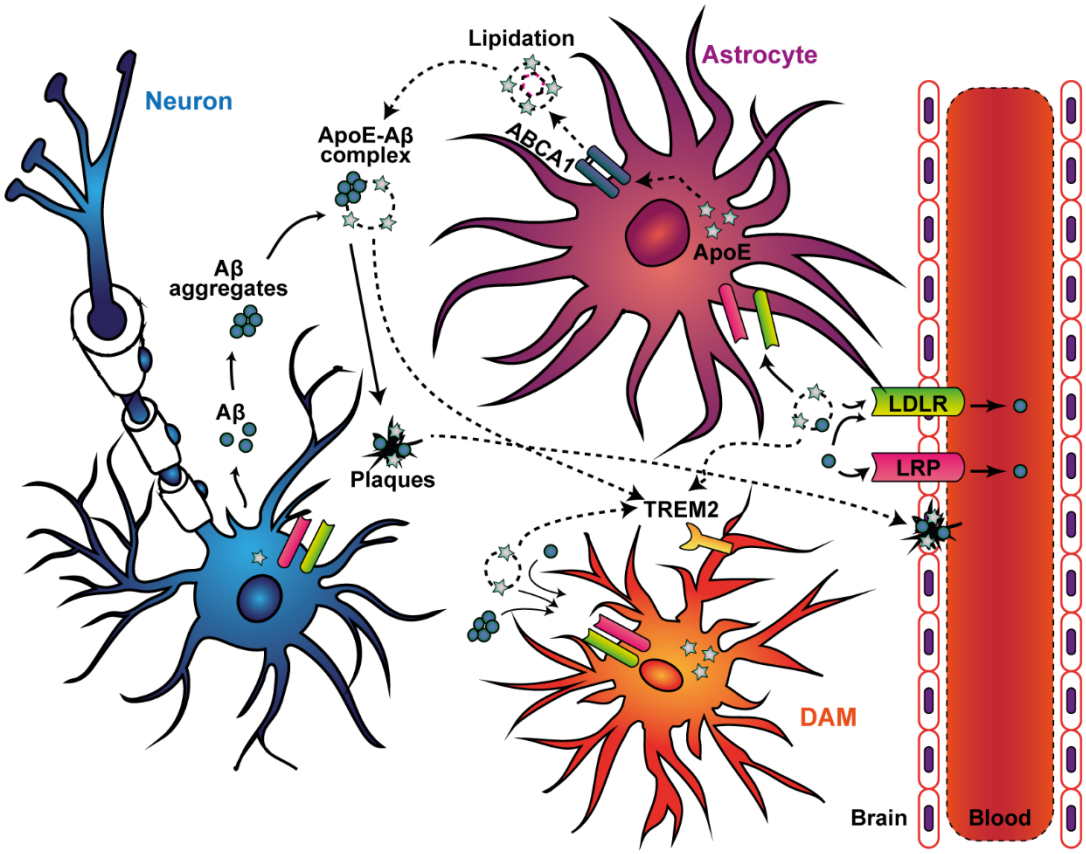

Figure 1.7 ApoE production, lipidation and its interaction with Aβ. Under stress or inflammation, ApoE is produced by astrocytes and DAM, and to a lesser extent in neurons. ApoE is lipidated in the astrocytes by ABCA1 and secreted into the interstitial fluid. The process begins once sufficient phospholipids and cholesterol bind to ABCA1, which triggers a conformational change leading to its dimerisation. The lipidated dimers immobilise actin filaments on the plasma membrane until lipid-free ApoE directly binds to the ABCA1 dimer. Upon binding, ApoE is lipidated by ABCA1 and leaves the dimer, which once again dissociates into a monomer to begin the process again. ApoE-containing lipoprotein particles are endocytosed into other cells via LDLR and LRP, the major metabolic receptors for ApoE. Amyloidogenic APP processing leads to Aβ production and release from neurons into the interstitial fluid (ISF). ApoE may bind to monomeric and smaller oligomeric soluble Aβ species and facilitate its aggregation into Aβ plaques in an isoform-dependent manner. However, ApoE could also bind to Aβ to facilitate its uptake and clearance through microglia in a TREM2-dependent manner or across the BBB. Accordingly, ApoE may bind to TREM2 to promote a DAM profile and enhance phagocytic uptake of Aβ.

More recent studies suggest that ApoE interacts with Aβ to affect its fibrillogenesis (Liu *et al.*, 2017) in an isoform dependent manner (Castellano *et al.*, 2011, Fitz *et al.*, 2012). While ApoE is largely lipidated in the brain, altering the ApoE lipidation state has a profound impact on Aβ fibrillization and inflammation. For example, LDLR-deficient 5xFAD mice displayed increased thioflavine S-positive fibrillar plaque burden compared to 5xFAD controls, with reduced microgliosis and astrogliosis (Katsouri and Georgopoulos, 2011). Similar results have been reported in Tg2576 transgenic mice lacking LDLR expression (Cao *et al.*, 2006). In support of these findings, overexpression of LDLR in APPPS1 mice reduced endogenous ApoE levels and inhibited Aβ deposition, as well as increased Aβ clearance (Kim *et al.*, 2009b). Furthermore, the intronic microRNA-33 supresses ABCA1 expression through translational repression or by inducing mRNA decay (Koldamova *et al.*, 2005, Rayner *et al.*, 2010). Thus,

ablating microRNA-33 expression significantly increased ABCA1 levels and ApoE lipidation, which in turn reduced Aβ levels in the brain (Kim *et al.*, 2015). Similarly, overexpression of ABCA1 has shown to increase ApoE lipidation with a corresponding decrease in Aβ deposition in PDAPP mice (Wahrle *et al.*, 2008). Whereas ABCA1 deficiency reduces ApoE levels in the brain and consequently elevates Aβ deposition in APP23 mice (Koldamova *et al.*, 2005).

4.1.1 TREM2-APOE modulation of Aβ pathology

The effects of APOE on immune activation have largely focused on astroglia due to their significant contribution to modulation of synaptic activity, inflammation and regulation of the BBB. As nascent ApoE is primarily lipidated by ABCA1, which is heavily expressed in astrocytes in mice (Zhang *et al.*, 2014, Zhang *et al.*, 2016), studies on loss of ApoE function in the brain parenchyma or the BBB were mainly focused on astroglia (Funato *et al.*, 1998, Thal *et al.*, 2000, Koistinaho *et al.*, 2004). Earlier studies indicated that ApoE affects receptor-mediated phagocytosis by microglia (Paresce *et al.*, 1997, Paresce *et al.*, 1996, Uchihara *et al.*, 1995). However, most of these studies were performed in vitro, which may have heavily influenced the microglial signature, and therefore function (Butovsky and Weiner, 2018). Until recently, not much was known about the impact of loss of ApoE function on microgliosis. Interestingly, similar to Trem2 knockout mice (Wang *et al.*, 2016, Yuan *et al.*, 2016), ApoE-deficient APPPS1-21 or APP/PS1de9 displayed impaired microglial clustering around Aβ plaques, which appeared enlarged with star-shaped fibrils (Ulrich *et al.*, 2018). Furthermore, ApoE isoforms appear to have differential effects on microglial clustering around plaques, with ApoE4 displaying increased number of microglia around plaques compared to ApoE3 in mice (Rodriguez *et al.*, 2014) but not in AD patients (Serrano-Pozo *et al.*, 2013). Additionally, loss of Trem2 function mice display reduced ApoE/Aβ colocalisation with significantly decreased microglial ApoE compared to astroglial ApoE levels (Parhizkar *et al.*, 2019). With recent developments in transcriptomic analysis, we now know that microglia also express genes required for ApoE production or lipidation, such as ABCA1 (Zhang *et al.*, 2014, Zhang *et al.*, 2016). This suggests that along with ApoE secreted by astroglia, ApoE of microglial origin may have a profound effect on its phagocytic ability to modulate plaque pathology by influencing its aggregation and/or clearance. Thus, TREM2 and APOE may share similar mechanisms involving LOAD pathogenesis as a result of changes in microglial pathology (Krasemann *et al.*, 2017). For example, TREM2 may bind to ApoE accumulated on the surfaces of damaged or dead neurons, which may enhance opsonisation and promote neuronal phagocytosis by microglia. However, the exact mechanism through which the interaction occurs, or whether they are upstream or downstream of each other remains to be determined in vivo.

II. Aim of the study

Microglia are considered one of the key players in maintaining brain homeostasis through phagocytic removal of harmful pathogens such as Aβ plaques. In fact, microgliosis is one of the key pathological hallmarks of AD and other neurodegenerative diseases, as loss of microglial function is believed to be crucial in determining the outcome and progression of LOAD. This is supported by the recent GWAS reporting that more than half of AD risk genes are implicated in microglial and innate immune cell function, including the two top risk genes *APOE* and *TREM2*. The *APOE* ε4 allele is long known to be the strongest genetic risk factor for AD due to its pro-aggregating effects on Aβ as well as impaired plaque clearance. Both APOE and TREM2 mRNA expression are upregulated in microglia clustering around plaques and TREM2 deficiency is consistently reported to reduce the number of microglia around plaques. However, the functional role of TREM2 in amyloid metabolism remains controversial due to conflicting results from different transgenic model systems studied at different stages of Aβ pathology. Furthermore, it remains unclear whether these results from mouse models translate to human AD pathology. As Aβ accumulation and deposition in the brain likely drives the pathogenic cascade of AD, the major goal of my PhD thesis was to provide a critical link between Aβ metabolism and TREM2-dependent microglial response in the pathogenesis of AD.

Do disease-associated *TREM2* variants confer a loss-of-function? How does the FTD-associated p.T66M variant impact microglial function in vivo?

When I started my PhD, all functional studies on TREM2 were published using Trem2 knockout mice, however very little was reported on the functional effects of disease-associated *TREM2* variants. Therefore, I collaborated with Gernot Kleinberger and colleagues to characterise a mouse model expressing the FTD-like syndrome-associated Trem2 p.T66M mutation at an endogenous level. This variant has not been linked to AD, such as the p.R47H variant. However, in comparison, the p.T66M mutation displays stronger TREM2 loss-of-function effects on TREM2 maturation, shedding and phagocytic uptake in vitro. Thus, to understand its functional consequences on microglia in vivo, I collected brain, cerebrospinal fluid and serum samples from WT, heterozygous and homozygous p.T66M-expressing mice of different ages.

What is the factor connecting amyloidogenesis and microglial phagocytosis? How does the TREM2-Aβ interaction fit in the amyloid cascade hypothesis?

To investigate a potential interaction between TREM2 and Aβ pathology, I used the amyloid seeding paradigm and analysed Trem2-dependent effects on early Aβ plaque formation as well as associated microgliosis in APPPS1 mice. For this, I included both APPPS1/Trem2$^{-/-}$ and APPPS1/Trem2$^{p.T66M}$ mice as loss of Trem2 function models. I extended the analyses to study changes in amyloidogenesis and microglial activity with aging in APPPS1 mice, which were not exogenously seeded. Lastly, I examined whether loss of Trem2 immunomodulatory functions can be linked to Aβ metabolism through changes in APOE and whether these pathological events can be confirmed in AD patients with *TREM2* variants.

III. References

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