The role of microglia in Alzheimer's disease

Investigating mechanisms regulating amyloid-β clearance

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Gothenburg 2016

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ISBN 978-91-628-9858-8

Printed in Gothenburg, Sweden 2016
Ineko, Gothenburg

Till mina underbara tjejer

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia today. The disease is characterized by the presence of amyloid plaques and neurofibrillary tangles in the brain, accompanied by a progressive neurodegeneration leading to memory loss. The underlying cause of the disease is largely unknown, but most evidence suggests that altered amyloid-β (Aβ) production is the putative cause of the disease. AD is also characterized by an inflammatory status in the CNS, although it is unclear whether the inflammation is the cause or the consequence of the disease. The aim of this thesis was to examine the role of microglia, the main immune cell in the brain, with regard to AD pathogenesis. This was carried out by experimental studies using the zebrafish as a model system in a combination with clinical analyses investigating biochemical differences and genetic variation in AD patients compared to controls. In paper I, we developed a novel zebrafish larvae model to study Aβ toxicity. The results indicated that injection with Aβ in animals leads to increased neurodegeneration and memory impairments, results similar to the pathological picture of AD. We also observed that oligomerization of the Aβ peptides was crucial for the neurotoxicity and that Aβ-induced neurodegeneration and memory impairments were mediated by separate pathways. In paper II, we used the newly developed zebrafish model to investigate how microglia interacts with injected Aβ. Confocal imaging revealed that microglia engulfs Aβ rapidly and that microglia is protective against Aβ-induced toxicity. Animals with reduced expression of microglia showed elevated levels of Aβ in the brain together with an increased neurodegeneration compared to controls. We also investigated the role of the p2y12 receptor and found that it plays a key role

in the microglia-mediated clearance of Aβ in the brain. In paper III, we investigated genetic variation in the purinergic *P2Y12* gene in a case-control study and found a haplotype to be associated with increased risk of AD. In paper IV, we performed a genetic analysis of *SORT1*, encoding sortilin 1, a receptor expressed on microglia, and identified a genetic variant strongly associated with a reduced risk of AD. In paper V, we identified two markers, chitotriosidase and YKL-40, both representing inflammation, to be upregulated in cerebrospinal fluid (CSF) of AD patients. In summary, we developed a new zebrafish model that can be used to study Aβ-induced pathology, where p2y12 was found to be protective against Aβ toxicity. Our data also suggest that AD patients have increased inflammation in the CNS and that *P2Y12* together with *SORT1* are possible risk genes for AD. This indicates that the zebrafish and humans share molecular mechanisms of neuroinflammation and our new model can in the future be used to explore new target genes for AD diagnosis and development of therapeutics.

Keywords: microglia, neuroinflammation, amyloid-beta, Alzheimer's disease, genetic association, biomarker, cerebrospinal fluid, zebrafish, clearance, in vivo imaging

ISBN: 978-91-628-9858-8

SAMMANFATTNING PÅ SVENSKA

Alzheimers sjukdom (AD) är den mest förekommande typen av demens och karakteriseras av ansamlingar av senil plack och neurofibriller i hjärnan. Sjukdomen kännetecknas även av en progressiv nervcellsdegeneration som med tiden leder till minnesproblem. Den underliggande orsaken till sjukdomen är fortfarande okänd, men mycket tyder på att produktionen av amyloid-β (Aβ), den huvudsakliga komponenten i placken, kan ligga till grund för sjukdomsutvecklingen. Inflammation är också karaktäristiskt för AD, där ökad aktivering av immunceller kan ses i hjärnan hos AD patienter. Syftet med denna avhandling var att studera hur inflammation är kopplat till AD, med primärt fokus på den huvudsakliga immuncellen i hjärnan, mikroglia. I den första artikeln utvecklade vi en ny zebrafiskmodell för att studera Aβ-toxicitet. Resultaten visade att fiskar som injicerats med Aβ hade en ökad celldöd i hjärnan och minnesproblem som överensstämmer med den patologiska profilen hos AD-patienter. Studien visade även att minnesproblemen och neurodegenerationen som orsakades av Aβ sker via olika mekanismer. I den andra artikeln visade våra resultat att mikroglia skyddar mot Aβ-toxicitet i zebrafiskmodellen. Vi såg även att microgliareceptorn p2y12 är inblandad i bortrensningen av Aβ. I den tredje artikeln utvärderade vi vårt tidigare fynd i ett patientmaterial och fann en haplotyp i *P2Y12* genen som var associerad med ökad risk för AD. I den fjärde artikeln fortsatte vi att studera ytterligare en receptor uttryckt på microglia och identifierade en genetisk variation i *SORT1* som var associerad med en minskad risk för sjukdomen. I den femte artikeln fastställde vi att två markörer som representerar aktivering av immunceller, chitotriosidase och YKL-40, mätt i ryggvätska (CSF), var uppreglerade hos patienter som lider av AD. Vi har i denna avhandling utvecklat en ny zebrafiskmodell för att studera Aβ-patologi som sedan följts upp i humanstudier. I denna modell observerade vi även att mikroglia är skyddande mot Aβ-toxicitet, och att p2y12-receptorn är inblandad i bortrensandet av Aβ. Våra analyser i patientmaterial tyder även på att AD-patienter har en ökad grad av inflammation i hjärnan jämfört med kontroller. Humanstudierna påvisade även genetisk variation i receptorerna *SORT1* och *P2Y12* mellan AD patienter och kontroller, vilket kan ligga till grund för den inflammatoriska profil dessa patienter uppvisar. Våra komparativa studier påvisar även att zebrafisken och människan har liknande underliggande mekanismer för mikrogliafunktion vilket betyder att zebrafiskmodellen i framtiden kan fungera som en plattform för att studera och utveckla mediciner som förebygger AD.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Kettunen, P, **Andersson, C-H,** Sundell, J, Offenbartl, M I, Ahmadniaye, G, Olsson, M, Abramsson, A and Zetterberg, H. *Amyloid-β disrupts sensitization of the touch-evoked startle response in larval zebrafish.* Submitted 2016.

II. **Andersson, C-H,** Strand, S and Kettunen, P. *p2y12 mediates amyloid-β clearance in zebrafish larvae*. Manuscript

III. **Andersson, C-H,** Hansson, O, Wallin, A, Zetterberg, H, Blennow, K, Skoog, I, Andreasen, N, Nilsson, S and Kettunen, P. *A haplotype of the purinergic P2Y12 gene is associated with increased risk of Alzheimer's disease*. Submitted 2016.

IV. **Andersson, C-H,** Hansson, O, Wallin, A, Zetterberg, H, Blennow, K, Skoog, I, Andreasen, N, Nilsson, S and Kettunen, P. *A genetic variant of the sortilin 1 gene is associated with reduced risk of Alzheimer's disease*. Accepted for publication in Journal of Alzheimer's disease 2016.

V. Rosén, C, **Andersson, C-H,** Andreasson, U, Molinuevo, JL, Bjerke, M, Rami, L, Lladó, A, Blennow, K and Zetterberg, H. *Increased levels of chitotriosidase and YKL-40 in cerebrospinal fluid from patients with Alzheimer's disease.* Dementia and Geriatric Cognitive Disorders EXTRA. 4(2):297-304 2014.

CONTENT

ABBREVIATIONS

Aβ	Amyloid-β
AD	Alzheimer's disease
ADP	Adenosine diphosphate
APOE	Apolipoprotein E
APP	Amyloid precursor protein
CNS	The central nervous system
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DPF	Days post fertilization
DPI	Days post injection
ELISA	Enzyme-linked immunosorbent assay
fAD	Familial Alzheimer's disease
GWAS	Genome-wide association study
LD	Linkage disequilibrium
MCI	Mild cognitive impairment
MCP-1	Monocyte chemoattractant protein-1
MMSE	Mini mental state examination
MO	Morpholino antisense oligo

MS222	Tricaine methanesulfonate
NFT	Neurofibrillar tangles
P-tau	Phosphorylated tau
P2Y12	G_i-coupled purinergic $P2y_{12}$ receptor
PSEN1	Presenilin-1
PSEN2	Presenilin-2
PTU	1-phenyl 2-thiourea
sAD	Sporadic Alzheimer's disease
SNP	Single nucleotide polymorphism
SORT1	Sortilin 1
T-tau	Total tau
TREM2	Triggering receptor on myeloid cells 2

1 INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD) was first reported at a meeting in Tübingen, Germany, in 1906 where the German psychiatrist Alois Alzheimer described the clinical features and the neuropathology of a demented patient with progressive memory loss, as summarized in a case report one year later (Alzheimer et al., 1995). After a *post mortem* examination of the diseased patient's brain, Alois Alzheimer defined the neuropathological findings as accumulation of plaques and tangles, later identified as aggregates of amyloid-β (Aβ) and protein tau, accompanied by extensive atrophy of the brain (Alzheimer et al., 1995). The disease would not become known as AD until 1910, when Kraepelin named it so in the chapter "Presenile and Senile Dementia" in the 8th edition of his Handbook of Psychiatry.

AD is today the most common form of dementia with a prevalence of over 10% in the elderly population (Blennow et al., 2006). Dementia can be described as an acquired syndrome of impaired cognition. The measurements of cognition are traditionally performed by a comparison of the patient to a norm, where the norm is a matched healthy population with the same age, education and gender (Tarawneh and Holtzman, 2012). The heterogeneity of the norm, where several healthy individuals suffer from age-related memory impairments (Sliwinski et al., 1996), makes the detection of cognitive decline hard. With the increase in expected lifespan and the lack of effective preventive treatments, the prevalence and socioeconomic burden of AD is believed to only increase with time.

AD commonly affects people that are 65 years or older, but the pathology is thought to begin decades before the emergence of cognitive symptoms (Price and Morris, 1999). Prior to the clinical manifestation of AD, individuals often display signs of mild cognitive impairment (MCI), where the term "MCI due to AD" refers to the pre-dementia phase of AD (Albert et al., 2011). MCI is a risk factor for AD, and the criterion for MCI is a slight but significant deviation from the standard neuropsychological performance results from age-matched controls. An MCI diagnosis can be reversible, seen in patients suffering from temporary depression or medically-induced impairment of

cognitive functions. While many individuals with MCI later progress to AD, some remain stable or develop other types of dementia (Winblad et al., 2004).

The progression is slow in the early phase of AD, which results in difficulties to find early signs of the disease, which also leads to patients seeking medical evaluation quite late. Early signs of AD include impairments in the short-term memory, whereas the working memory and long-term memory are affected to a less degree (Tarawneh and Holtzman, 2012). An early stage of AD may also be characterized by changes in the personality, language impairments and depressive symptoms (Tarawneh and Holtzman, 2012, Zubenko et al., 2003). Moderate to severe AD is marked by a progressive decline in cognitive functions accompanied by inability in executive functions and logical reasoning (Tarawneh and Holtzman, 2012). The behavioral symptoms are also often more severe at these stages and may include hallucinations (Lauter, 1968), delusions (Reisberg et al., 1996), aggression and anxiety (Devanand et al., 1997). AD patients during the final stages of the disease are completely dependent on comprehensive nursing and show greatly reduced cognitive function, language skills and basic motor functions. The cause of death is often due to complications in aspiration or infections (Tarawneh and Holtzman, 2012).

The disease is also manifested by a progressive neurodegeneration, ultimately leading to a fatal outcome (Blennow et al., 2006). The underlying molecular causes of the disease are poorly understood. A genetic background can explain the incidence of so-called early-onset familial AD (fAD), where genes involved in the amyloidogenic processing of amyloid-β, contain single mutations which are inherited in an autosomal dominant fashion (Goate et al., 1991, Sherrington et al., 1995, Levy-Lahad et al., 1995). The familial form of the disease is rare, with an occurrence below 0.1% (Harvey et al., 2003). For the remaining AD cases, so-called sporadic AD (sAD), the underlying causes are less well established, although several contributing risk factors have been identified, including age (Ferri et al., 2005) genetic variation in the gene *APOE* (Corder et al., 1993, Poirier et al., 1993) and *TREM2* (Guerreiro et al., 2013, Jonsson et al., 2013), family history (Mayeux et al., 1991), gender (Baum, 2005) and cardiovascular disease (CVD) (Stampfer, 2006).

1.1.1 Diagnosis

Clinically, the AD diagnosis is set according to the fulfillment of the criteria for dementia according to DSM-V, as well as the criteria for possible or

probable dementia caused by AD according to the National Institute of Neurological and Communicative Disorders – Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). The diagnosis of possible or probable AD dementia can be strengthened by biomarkers reflecting AD pathology (McKhann et al., 2011). Since the NINCDS-ADRDA criteria used for diagnosis of AD are traditionally mainly based on exclusion of other dementias, the clinical manifestation of the disease is usually accompanied by other clinical and psychiatric examinations. The International Working Group (IWG) has contributed largely with new research criteria for the diagnosis of AD. In 2007, the group proposed new ways to diagnose AD with the use of positron emission tomography (PET) and cerebrospinal fluid (CSF) biomarkers reflecting amyloid and tau pathology (Dubois et al., 2007). The IWG-2 criteria allow for a positive diagnosis of AD with either typical or atypical AD symptoms, prior to *post mortem* examination. Diagnosis according to the IWG-2 criteria also enable fulfillment of AD criteria in a pre-demented state, when patients nowadays often seek medical advice (Dubois et al., 2014). A mini mental state examination (MMSE) is commonly used to assess cognitive function of the patients (Folstein et al., 1975). Structural neuroimaging, such as computed tomography (CT) or magnetic resonance imaging (MRI), serves to exclude other causes leading to dementia, such as brain tumors, cerebral infarcts, white-matter lesions (WMLs), among others.

1.1.2 Neuropathology

Characteristic neuropathological hallmarks for AD are neurofibrillary tangles (NFT), composed of intracellular aggregates of hyperphosphorylated tau and senile plaques of Aβ (Braak and Braak, 1991). Moreover, inflammatory recruitment of microglia and astrocytes are contributing to the pathological profile and are found in close proximity to senile plaques (Perlmutter et al., 1990, Wisniewski et al., 1992, Wisniewski and Wegiel, 1991).

Tau

The tau protein is normally situated on microtubules in neurons where it functions as a stabilizing structure. In AD however, the tau protein is misfolded, hyperphosphorylated and dislocated from the microtubules (Iqbal et al., 2005). The tau pathology is seen early in the disease progress, although it is unknown whether the pathology is a cause or a consequence of the disease. The spreading of the neurofibrillary degeneration follows a rather stereotypical pattern in AD patients, where the NFTs are first observed in the

allocortex of the medial temporal lobe and spreads to the associative isocortex (Braak et al., 2006). The severity and amount of the NFT distribution correlate well with the progression of the dementia (Arriagada et al., 1992, Bierer et al., 1995, Gomez-Isla et al., 1997), as well as the affected neuronal areas involved in the neuropsychological impairments shown in AD patients (Arnold et al., 1991, Braak and Braak, 1991).

Amyloid-β

The presence of extracellular Aβ plaques is the second hallmark seen in the brain of AD patients. The Aβ peptide is generated from the amyloidogenic processing of the amyloid precursor protein (APP), cleaved by β-secretase and γ-secretase (Zhang et al., 2011). The plaque formation is a result of Aβ peptides undergoing a conformational change which generates oligomeric β-sheet structure that further aggregates into insoluble fibrils (Blennow et al., 2006). The fibril structure of Aβ was initially thought to cause the neurotoxicity seen in AD, but more recent evidence indicates that the oligomeric form might be the dangerous component (Walsh et al., 2002, Shankar et al., 2008). Aβ plaques can be divided into two categories, diffuse and dense-core plaques. The dense-core plaques are mainly of β-sheet conformation and are associated with neuronal loss, dystrophic neurites, synaptic loss and reactive astrocytes and microglia (Itagaki et al., 1989, Knowles et al., 1999, Pike et al., 1995, Urbanc et al., 2002). These plaques are also more often found in patients suffering from AD, whereas the diffuse plaques are present in healthy elderly (Masliah et al., 1990). Aβ plaques mainly accumulate in the isocortex where the 42 amino acid long peptide, $A\beta_{1\text{-}42}$, and N-terminally truncated forms are the main components of the plaques (Masters et al., 1985). The structure of $A\beta_{1\text{-}42}$ is believed to be the underlying cause for accumulation seen in plaques, as the hydrophobic properties makes the peptide more prone to aggregate. A high degree of AD patients also show cerebral amyloid angiopathy, where the more soluble $A\beta_{1\text{-}40}$ peptide is commonly situated in vessel walls which affects cortical capillaries, small arterioles and various arteries (Serrano-Pozo et al., 2011).

During normal conditions, the Aβ peptide is cleared away from the brain through either enzymatic degradation (Carson and Turner, 2002), secretion across the blood-brain barrier (Shibata et al., 2000) or microglia phagocytosis (Lee and Landreth, 2010). In AD however, the aggregation of Aβ in the senile plaques is thought to be the result of a skewed production / clearance ratio of the peptide. The Aβ cascade hypothesis (Fig. 1) (Hardy and Higgins, 1992) suggests that Aβ is the putative cause for the pathology seen in AD, which might be the results of increased levels of Aβ forming plaques. The underlying causes of fAD also support this theory, as all mutations causing the disease are involved in amyloidogenic processing of APP.

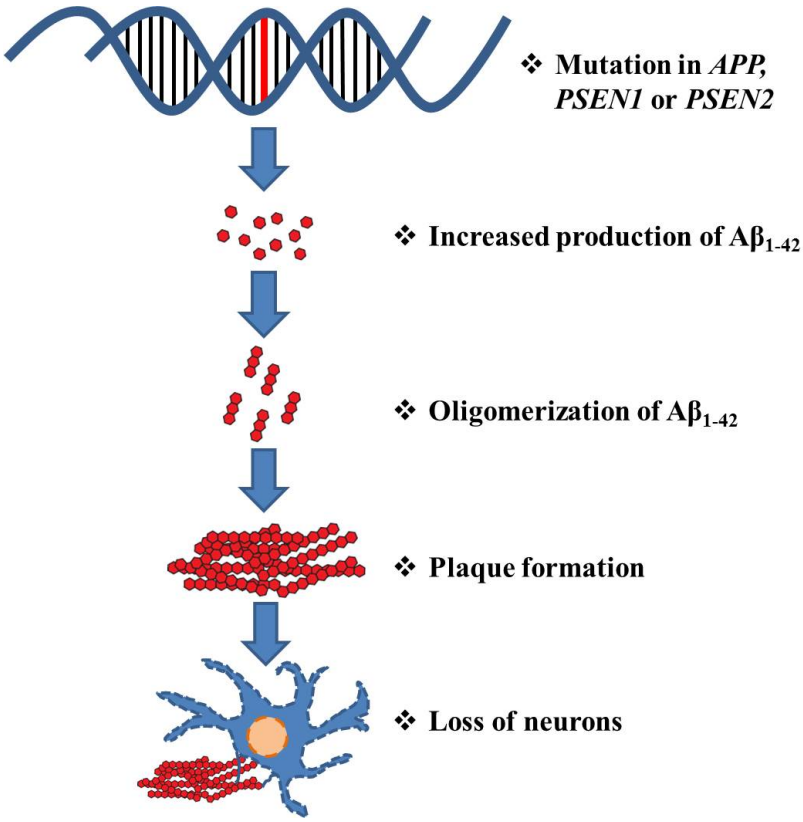

Figure 1. The Aβ cascade hypothesis proposes an explanation of the series of events leading to AD pathology. Mutations and/or other factors disrupt the Aβ production/clearance homeostasis, which in turn generates neurotoxic effects from the accumulation and aggregation of Aβ.

1.1.3 Cerebrospinal fluid biomarkers for AD

CSF is a transparent liquid, produced by ependymal cells and fills the ventricles of the brain and surrounds the brain and spinal cord. CSF analysis can be used as a measurement of changes in the brain after neuronal injuries or in neurological disorders. This is especially useful since the CSF measurements could be used to find neuronal changes prior to the emergence of dementia. In AD, CSF measurements are used to strengthen an AD diagnosis as well as to distinguish the clinical features from other neurological disorders or other dementias. The levels of $A\beta_{1-42}$ are decreased in CSF of AD patients, which is thought to represent an accumulation of the peptide in plaques and leading to a reduced diffusion into the CSF. For tau, the levels of total tau (T-tau) and phosphorylated tau (P-tau) are elevated in the CSF in AD patients, probably due to an increase in neuronal degeneration leading to leakage of these proteins to the CSF (Blennow et al., 2001).

1.2 Microglia

The concept of microglia was first introduced by Pio Del Rio-Hortega in a series of articles published between 1919 and 1927 (Kettenmann et al., 2011). Rio-Hortega was able to describe the features of the cells in a very precise manner, where he concluded that microglia are of mesodermal origin, entering the brain during early development in an amoeboid form, transforming into a resting state characterized by a branched morphology in the mature brain and having the capability to migrate and proliferate.

1.2.1 The role of microglia in the CNS

Microglia are the main innate immune cells of the CNS. They show both different origin and characteristics compared to other monocytes and astrocytes. During the development of CNS, microglia are involved in synaptic maintenance (Schafer and Stevens, 2015) and the clearance of apoptotic cells (Kettenmann et al., 2011). In the mature brain, microglia exist as a stable population surveying the microenvironment for injuries or invading pathogens. Since the blood-brain barrier allows very little infiltration of immune cells into the CNS, microglia cells need to be very plastic (Kettenmann et al., 2011). During normal conditions, the cell maintains a resting state, characterized by a branched morphology (Nimmerjahn et al., 2005). Upon injury or exposure to foreign pathogens, microglia enter an activated state. This state is characterized by a withdrawal

of its processes and a change into an amoeboid morphology, accompanied by an increased motility and shift in surface receptors (Colton and Wilcock, 2010, Rock et al., 2004). Activation can also promote a local proliferation of the microglia population (Kettenmann et al., 2011). The activation of microglia has shown to be of a heterogenic nature rather than "on" or "off". The initial response to an activating stimulus is a pro-inflammatory phenotype, characterized by secretion of a plethora of factors promoting inflammation and neurotoxicity (Laurenzi et al., 2001, Liu et al., 2005, Suk et al., 2001, Kreutzberg, 1996, Liu et al., 2002). Upon inflammatory resolution, microglia converts into an anti-inflammatory state which dampens the inflammation and stimulates cellular repair (Fig. 2) (Cherry et al., 2014).

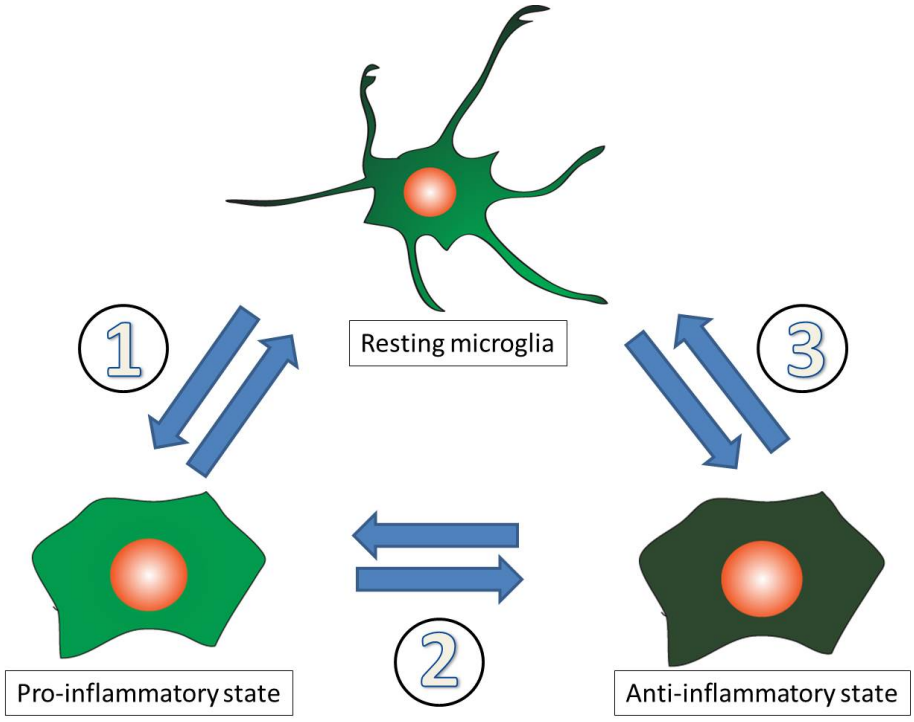

Figure 2. Microglia are characterized by a ramified morphology during normal conditions in the CNS. Activation leads to a change in morphology and gene expression, often to a pro-inflammatory state (1). Upon resolution of the inflammation, microglia reduce the inflammatory response by promoting cellular repair (2) until normal conditions are reached. Microglia then enter a quiescent state (3).

1.2.2 Microglia and AD

AD is characterized by a low-grade neuronal inflammation, primarily driven by microglia (Fig. 3) (Heppner et al., 2015). Most evidence indicates that the Aβ production exceeds the clearance ratio in AD, consequently generating abnormal amounts of Aβ. In that regard, a decreased clearance capacity of microglia could be the explanation for the Aβ plaque buildup seen in AD patients, a theory strengthened by several studies. Firstly, accumulations of activated microglia are found in close proximity to Aβ plaques (McGeer et al., 1987, Itagaki et al., 1989). Brain imaging (Stefaniak and O'Brien, 2016) and measurements of markers representing activation of microglia (Griffin et al., 1989, Hye et al., 2006), also suggest a neuroinflammatory status in AD patients. The amount of activated microglia correlates with cognitive ability in AD patients, which indicates a pathological activation of the cells (Edison et al., 2008). Animal and cell studies also indicate that the properties of microglia change over time, where alterations in morphology, migration and phagocytic property (Caldeira et al., 2014) are observed, ultimately shifting microglia towards a more pro-inflammatory phenotype (Hickman et al., 2008).

Two of the main genetic risk factors for sAD, the *ϵ4* allele of apolipoprotein E (*APOE)* and missense mutation in triggering receptor on myeloid cells 2 (*TREM2*), are genes expressed on microglia and both involved in immune activation in the brain. ApoE plays a crucial role in the degradation of Aβ and the carriers of the *ϵ4* allele have a 3-4 fold increase in the risk of developing AD (Corder et al., 1993, Poirier et al., 1993). Activation of TREM2 promotes phagocytic pathways in microglia (Takahashi et al., 2007, Frank et al., 2008, Piccio et al., 2007) and is accompanied by anti-inflammatory signaling (Piccio et al., 2007). The loss of a functional TREM2 leads to an increased Aβ burden together with less clearance of toxic debris (Guerreiro et al., 2013, Neumann and Takahashi, 2007, Wang et al., 2015, Jonsson et al., 2013).

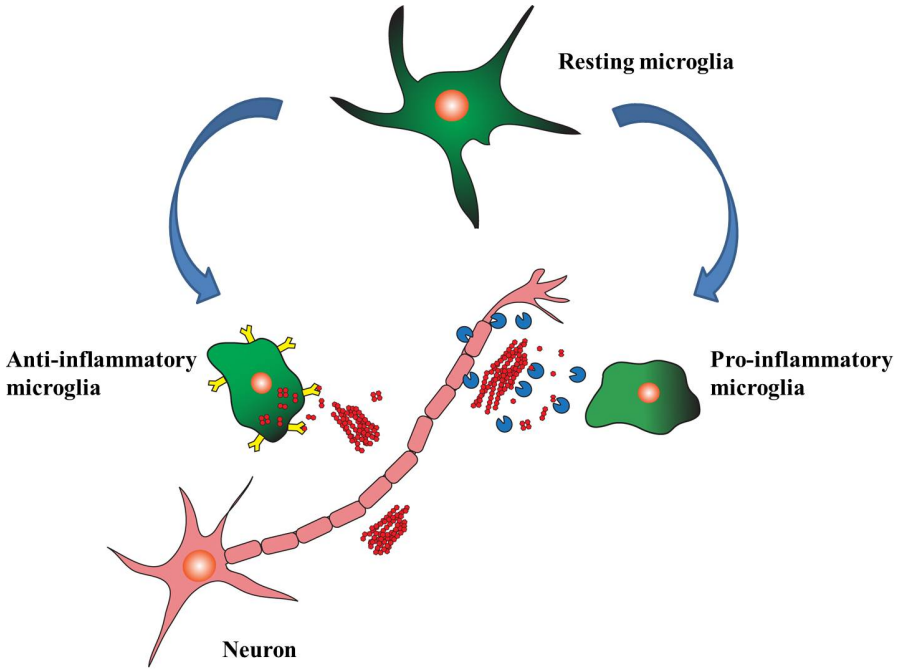

Figure 3. Microglia degradation of Aβ (red dots) can be mediated by phagocytosis or by enzymatic degradation. The anti-inflammatory state promotes phagocytic properties of microglia whereas the pro-inflammatory state is characterized by the release of factors that can be toxic for neurons.

Several cytokines and chemokines secreted by microglia have been found to have altered expression levels in AD patients. The factors interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, all contributing to a pro-inflammatory phenotype of microglia, are upregulated in AD patients (Sastre et al., 2006) and the respective genes have been associated to an increased risk of the disease (Nicoll et al., 2000, Capurso et al., 2004, McCusker et al., 2001).

Secreted APP and Aβ are also capable of activating microglia, shown by several studies (Frackowiak et al., 1992, Perlmutter et al., 1990, Barger and Harmon, 1997). The mechanisms underlying the Aβ-induced activation of microglia can be direct or indirect. The direct activation involves a physical

interaction between Aβ and innate receptors on microglia, including the pattern recognition receptors (PRRS) (Salminen et al., 2009), toll-like receptors (TLRs) (Lehnardt et al., 2003, Walter et al., 2007) and scavenger receptors (SR) (Paresce et al., 1996). The interaction can promote phagocytosis, production of cytokines and chemokines, or proliferation, depending on the receptor activated (Solito and Sastre, 2012). The neurotoxic features of Aβ aggregation initiate the indirect activation of microglia. Cell death, a hallmark of AD, is accompanied by release of factors which initiates microglia migration towards the site of injury, followed by an activation of the microglia cell (Haynes et al., 2006). Although CNS inflammation is seen in patients suffering from AD, it is unknown whether microglia is driving the progression of the disease, or if microglia activation is a result of the disease. It is possible that microglia, activated by Aβ or other AD-related pathological factors, stall in a pro-inflammatory state, consequently promoting a chronic inflammation in the brain (Heppner et al., 2015).

1.2.3 The P2Y12 receptor

The P2Y12 receptor was first identified on platelets, where it regulates their activation during the clotting process (Foster et al., 2001, Hollopeter et al., 2001, Andre et al., 2003, Dorsam and Kunapuli, 2004). The receptor belongs to the metabotropic purinergic receptor family and is a common clinical target for treatments used to prevent thrombosis in cerebrovascular and cardiovascular diseases (Foster et al., 2001, Hollopeter et al., 2001). P2Y12 mediates the activation of platelets mainly by binding extracellular ADP, secreted from other activated platelets or damaged blood cells (Hollopeter et al., 2001). In the CNS, P2Y12 is exclusively expressed on microglia cells (Butovsky et al., 2014). The role of the P2Y12 receptor on microglia is similar to the function on platelets, where it guides microglia towards sites of injuries by binding ADP molecules (Haynes et al., 2006, Sieger et al., 2012). It also mediates closure of the blood-brain barrier in the case of leakage (Lou et al., 2016). P2Y12 is supposedly most crucial for an immediate response to injury in the CNS, as the expression of the gene is high during the resting state and decreased upon activation (Haynes et al., 2006).

1.2.4 The sortilin 1 receptor

Sortilin is a sorting receptor encoded by the *SORT1* gene. The protein was first identified in a screen for endocytic receptors (Petersen et al., 1997), it belongs to the VPS10P domain receptor family (Willnow et al., 2008) and is

expressed on a variety of cells, including microglia (Nykjaer and Willnow, 2012). Sortilin is synthesized as a precursor protein and localized in the *trans*Golgi network in an inactive form (Nielsen et al., 2001). Upon activation, sortilin can be redirected to the cell surface, where it either acts as a transmembrane surface binding site, or undergoes cleaving which results in a release of the ligand binding site in a soluble form into the extracellular space. Both pathways involve binding of unrelated ligands, which often leads to endocytosis. The activation of sortilin can also relocate the protein to other cellular compartments such as the endosome, where it mediates transport of bound ligands to lysosomes for degradation (Nykjaer and Willnow, 2012).

Genetic variation in sortilin has mainly been associated with CVD (Takeuchi et al., 2012, Jones et al., 2013, Lee et al., 2013, Arvind et al., 2014, Angelakopoulou et al., 2012, Qi et al., 2011) and is strongly correlated with serum levels of cholesterol (Arvind et al., 2014, Kathiresan et al., 2008, Willer et al., 2008, Shirts et al., 2011, Keebler et al., 2009, Gupta et al., 2010, Walia et al., 2014). Some evidence suggests an involvement of sortilin in AD pathology, as it has been shown to promote APP cleavage (Finan et al., 2011, Tan and Evin, 2012) and deliver extracellular Aβ to lysosomes for degradation (Carlo et al., 2013). Patients suffering from AD have also revealed elevated levels of sortilin in their brains (Saadipour et al., 2013).

1.3 Genetic variation

The human population is approximately 99.9% genetically identical, leaving the remaining 0.1% of the genome together with environmental factors as the contributors for individual differences (Feuk et al., 2006, Kidd et al., 2008). Genetic variation comes in many forms, where DNA can be inserted or deleted from the genome (insertions/deletions), but also show variations in copy number (CNVs), where genomic regions have been duplicated. The most common forms of genetic variation are single nucleotide polymorphisms (SNPs) which are the result of introduction of stable mutations in the genome (Fig. 4). A SNP is the genetic variation at a specific base pair in the genome, where the type of nucleotide varies among individuals. A rare variant at a certain locus is referred to as a polymorphism, granted it is present in ≥1% of the population. The variation in a SNP usually consists of two possible nucleotides, where the alternatives are called alleles. Since humans have two copies of each chromosome, the two alleles accounts for three possible outcomes. Carriers of two copies of the same allele are

called homozygous, whereas carriers of one of each are called heterozygous. The allelic combination makes up for the genotype for a certain loci.

The effect of a SNP varies, depending where it is located. A mutation in a coding region, i.e. exon, can cause a change of the amino acid sequence (non-synonymous), introduce a pre-mature stop codon (nonsense) or have no effect on the transcribed codon (synonymous). The vast majority of SNPs are located in so-called non-coding regions of the genome, i.e. introns, where the function is less known. Even though intronic regions are usually not transcribed, SNPs in these regions could affect the transcription levels of genes located nearby.

During meiosis, the maternal and paternal genetic material is mixed to generate a greater genetic diversity in the offspring. The DNA is exchanged between homologous chromosomes in a recombination event, where SNPs located in close vicinity to each other are more likely to be inherited together, a phenomena termed linkage disequilibrium (LD). This information is essential in genetic studies, as the variation in one SNP (tag SNP), can impute the genetic variation in a larger genomic region.

1.3.1 Genetic association studies

The aim of genetic association studies is to detect associations between one or more polymorphisms and a trait. The trait could be a quantitative measurement, or a discrete attribute, such as a disease. The design of a genetic association study can be a so-called case-control study, where the study aims to identify rare genetic variants only present in either of the group. The case group is usually patients suffering from a certain disease, whereas the control group is healthy individuals representing the general population. There are also family-based approaches, where the heritability of genetic variation is investigated with regard to a trait present in a family.

The genetic variation is generally investigated with the use of tag SNPs, where genotyping of a few SNPs reflects the genetic variation in a whole gene. The tag SNP does not always represent a site of biological importance, but it might however reflect the variance in another site that has an important function. The selection of tag SNPs are done through collective databases, such as the International HapMap Project, which summarizes all genetic variance, i.e. SNPs across the whole genome. The LD pattern of the SNPs in a gene determines which and how many tag SNPs that should be selected for

the specific region. The combination of two or more SNPs, referred to as a haplotype, can also be used to investigate the variation in a population.

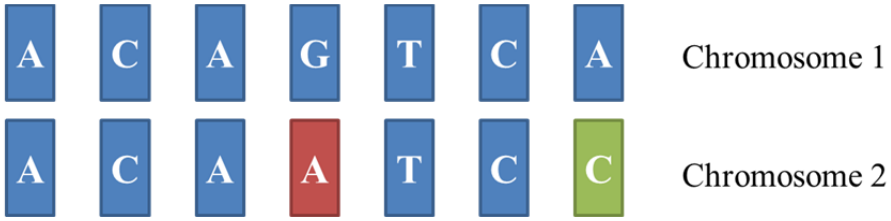

Figure 4. Chromosome 1 and 2 represent the two variants of a chromosome pair. SNPs are the variation in a single nucleotide at a specific position in the genome, marked by red and green in this illustration. A haplotype is the combination of two or more SNPs at certain positions, in this case the combination of the red and the green nucleotide.

A genetic association can be investigated through a candidate gene approach or via a genome-wide association studies (GWAS). Candidate gene approaches are hypothesized driven, where prior knowledge of a gene leads to the suspicion of a possible biological contribution of the gene to the disease or trait. GWAS are on the other hand used as an unbiased screen of the whole genome for SNPs associated to a disease or trait. One common concern with GWAS is that the findings often lack a known biological relevance (McClellan and King, 2010).

1.3.2 Genetic contribution to AD

The genetic background of fAD is characterized by mutations causing the disease in an autosomal dominant manner. The first identified risk factor for the familial form of the disease was a mutation in the *APP* gene (Goate et al., 1991). Following, investigation of families with AD revealed several new mutations in the APP gene as the putative cause for the disease (Mullan et al., 1992, Hardy, 1992). Amyloidogenic processing of APP generates Aβ peptides, a pathway involving the cleavage of APP by β-secretase and γ-secretase. Two proteins, PSEN1 and PSEN2, are both a part of the γ-secretase complex and their genes have been shown to contain mutations in the

majority of fAD cases (Sherrington et al., 1995, Levy-Lahad et al., 1995). These mutations are thought to make γ-secretase more prone to cleave APP at a specific site which leads to a longer form of Aβ, i.e. $A\beta_{1-42}$. This longer form of Aβ is more hydrophobic than the shorter forms and the most common Aβ specie seen in the Aβ plaques in AD patients.

The most common genetic risk factor for sporadic AD cases, which accounts for over 99% of all AD cases, is the gene *APOE*. In 1993, two groups independently reported an association between the *ϵ4* allele of the gene and the risk of AD (Corder et al., 1993, Poirier et al., 1993). There are three common polymorphisms in *APOE* seen in humans, coined the *ϵ2*, *ϵ3* and *ϵ4* alleles, where the *ϵ2* allele is present in 1-5%, *ϵ3* in 50-90% and *ϵ4* in 5-35% of the population (Mahley and Rall, 2000). Heterozygous carriers of the *ϵ4* allele has a 3-4 fold increase in the risk of developing AD with a 10 year decreased time of onset. For homozygous carriers, the time of onset is lowered by 20 years combined with an 8-10 fold increase in the risk of developing the disease (Corder et al., 1993, Farrer et al., 1997, Poirier et al., 1993, Meyer et al., 1998).

More recently, a rare missense mutation in *TREM2* was identified in AD patients by two different groups (Guerreiro et al., 2013, Jonsson et al., 2013). Although the rare polymorphism in *TREM2* occurs with less frequency than the *ϵ4* allele of *APOE,* the rare variant shows a similar effect size of the risk of developing AD and confers the risk independently of *APOE* genotype (Jonsson et al., 2013). The receptor is expressed on microglia cells and the mutation is thought to decrease the phagocytic properties, which in turn leads to an increase in a pro-inflammatory status (Takahashi et al., 2005).

2 AIM

2.1 The general aim

The aim of this thesis was to investigate the role of microglia in the pathogenesis of AD, with special emphasis on the microglia receptors P2Y12 and sortilin 1.

2.2 Aim of the individual papers

1. To establish an *in vivo* model for Aβ-mediated neurotoxicity using the zebrafish (*Danio rerio)* as a model organism with the help of behavioral experiments and brain imaging.

2. To evaluate the role of microglia with regard to injected Aβ in zebrafish larvae, with primary focus on the p2y12 receptor and clearance capacity of microglia cells.

3. To examine weather genetic variation in the *P2Y12* gene is associated with the risk of AD, cognitive function and characteristic CSF AD biomarkers in a case-control population study.

4. To investigate if common genetic variation in the gene encoding the sortilin 1 receptor is associated with the risk of AD, cognitive function and CSF biomarkers present in AD patients.

5. To study if inflammation is present in the brain of AD patients by measuring and comparing levels of CSF biomarkers representing inflammation in AD patients and controls.

3 METHODS

3.1 Ethics

The human population studies (paper III and IV) included in this thesis contained material from Swedish cohorts. Samples were collected from Lund, Gothenburg/Mölndal and Piteå and the participants or their close relatives gave their consent for collection of samples and the participation in the studies. The studies were conducted in accordance with the Helsinki Declaration and the procedures were approved by regional ethical committees in Lund, Gothenburg and Umeå. For paper V, the cohort was recruited from the Alzheimer's Disease and Other Cognitive Disorders Unit, Hospital Clinic, Barcelona, Spain and approved by the Hospitals Clinic's ethics committee.

For the zebrafish studies (paper I and II), all experimental protocols were approved by the animal ethics committee in Gothenburg and followed the guidelines of the Swedish Board of Agriculture.

3.2 Subjects

3.2.1 Patients

The case-control studies (paper III and IV) consisted of 620 patients diagnosed with AD and 1107 controls. The samples were recruited from different cohorts, where AD patients were recruited from Piteå (n = 182) and Malmö (n = 438), and controls from Gothenburg/Mölndal (n = 674) and Malmö (n = 433). This patient material was used in both paper III and IV and detailed information regarding the clinical examination of the patients and controls is found in the corresponding paper. In paper V, the patients (n = 25) and controls (n = 25) were recruited from the Alzheimer's Disease and Other Cognitive Disorders Unit, Hospital Clinic, Barcelona, Spain and detailed information regarding clinical diagnosis and characteristics of the material is given in paper V.

3.2.2 Zebrafish

The zebrafish (*Danio rerio)* used in the animal experiments were wildtype of the AB strain (paper I and II) and the Tg(*apoE:GFP)* line (paper II) which

was generously provided by Francesca Peri (Fig. 5) (Peri and Nusslein-Volhard, 2008). The animals were kept at the Institute for Neuroscience and Physiology at the University of Gothenburg, at a constant temperature of 28°C and a 14:10 hour light-dark cycle. The embryos were raised at 28.5°C in embryo media (5.03 mM NaCl; 0.17 mM KCl; 0.33 mM CaCl2 · $2H_2O$; 0.33 mM $MgCl_2 \cdot 6H_2O$).

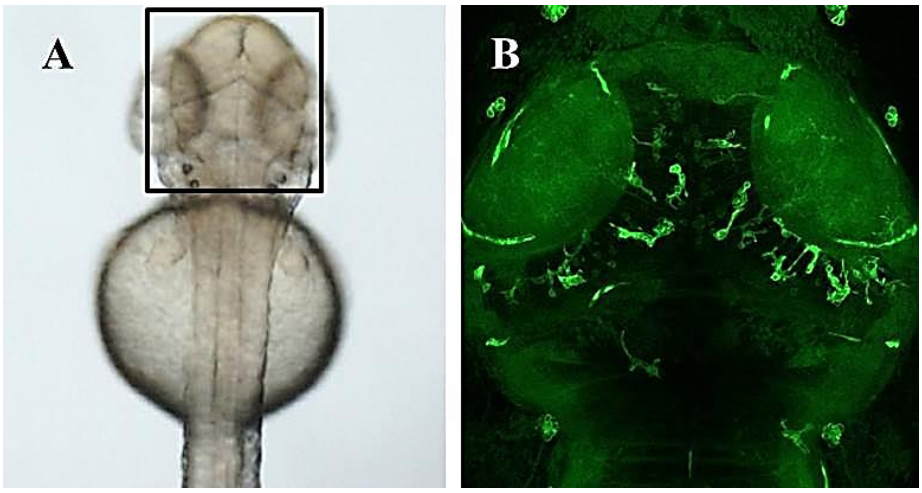

Figure 5. (A) Image of Tg(apoE:GFP) zebrafish at 2 dpf. (B) Confocal image of the area represented by the square in (A) at 4 dpf. The green microglia cells are expressing GFP.

3.3 Genotyping

3.3.1 SORT1

The sequence of the gene encoding sortilin 1, *SORT1*, (Gene ID: 6272) was obtained from the University of California, Santa Cruz (UCSC) Genome Browser using the assembly GRCh38/hg38 (Kent et al., 2002). The selected SNPs in *SORT1* were genotyped by LGC Genomics (http://www.lgcgroup.com/) using KASP™ genotyping assay technology. In short, the KASP genotyping assay contains two allele-specific forward primers and one common reverse primer for the SNP sequence of interest.

The forward primers have different tail sequences which enables identification of homozygosity or heterozygosity depending on which primer(s) that binds to the sequence.

3.3.2 P2Y12

The sequence of *P2Y12* (Gene ID: 64805) was acquired from the UCSC Genome Browser using the GRCh38/hg38 assembly (Kent et al., 2002). The genotyping was performed according to the description for *SORT1* genotyping.

3.4 Zebrafish

3.4.1 Morpholino injections

Morpholino antisense oligos (MO) are designed to bind to complementary RNA which leads to a knock-down or alteration of a gene expression of interest. The binding of a MO does not degrade the RNA, but rather acts as a physical blockage which inhibits translation of the gene.

Fertilized eggs were injected with MO into the yolk at the one-cell stage to ensure maximum penetrance of the MO (Fig. 6) (Gene Tools, LLC, Philomath, OR, USA; http://www.gene-tools.com). In paper I, animals were injected with MO against *tp53* with the sequence of 5'-GCGCCATTGCTTTGCAAGAATTG-3', or with a control MO, with the sequence 5'-CCTCTTACCTCAGTTACAATTTATA-3'. In paper II, animals were injected with control MO, *tp53* MO as well as a MO against *pu.1/spi-1* with the sequence 5'-GATATACTGATACTCCATTGGTGGT-'3 (Rhodes et al., 2005) and *p2y12* 5'-AGCTGCGTTGTTTGCTCCATTGAT-'3 (Sieger et al., 2012).

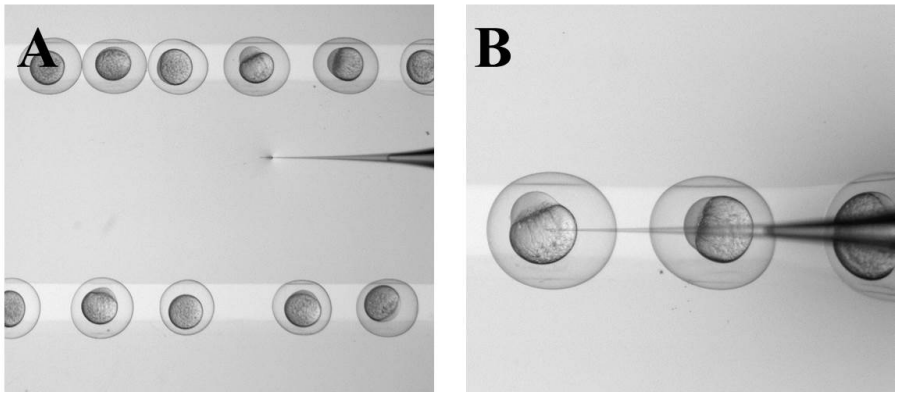

Figure 6. (A) Fertilized zebrafish eggs are collected and placed in wells directly after they are laid. (B) The eggs are then injected with MO solution into the yolk at the one-cell stage to ensure maximum penetration of the MO.

3.4.2 Aβ injections

In paper I and II, Aβ-species were delivered into the brain of zebrafish larvae through injections at 2-6 days post fertilization (dpf). The animals were first anaesthetized in tricaine methanesulfonate (MS222) and embedded in 1.5% low melting temperature agarose (Sigma-Aldrich) prior to brain injection, performed using a microinjector (Eppendorf FemtoJet®). For detailed information regarding concentrations, oligomerization protocols and type of Aβ species used, see individual paper for specifics.

3.4.3 Confocal microscopy

All imaging experiments in paper I and II were performed using a LSM 710 confocal microscope (Zeiss) at the Centre for Cellular Imaging, the Sahlgrenska Academy. Brain images were collected from collapsed z-stacks, taken at 1-2 µm intervals using Zen Zeiss software. Images were analyzed in ImageJ (Schneider et al., 2012).

Prior to imaging, animals were treated with 0.003% 1-phenyl 2-thiourea (PTU) 24 hours post fertilization (hpf) to prevent pigmentation. The zebrafish larvae were embedded in low melting point agarose and anaesthetized in MS222 just before the experiments. For cell death experiments, AB wildtype

animals were used and incubated for 45 min in 5µg/ml acridine orange that stains for dead cells. For TAMRA-Aβ_{1-42} clearance and TAMRA-Aβ_{1-42} accumulation in microglia analysis, AB wildtype and Tg(*apoE:GFP)* animals were used. Acridine orange and GFP was excited by a 488 nm argon laser and TAMRA-Aβ_{1-42} by a 561 nm diode pumped solid state laser.

3.4.4 Behavioral tests

In paper I, animals underwent behavioral sensitization, a form of non-associative learning, to test whether learning impairments are present in animals injected with Aβ species. The test was performed by giving electrical pulses on the skin as stimuli to zebrafish larvae, which evoked a startle response, before and after a training event. The training consisted of an electrical stimulus, and the difference in responsiveness before and after training gave a measurement of how the larvae sensitized. For detailed information regarding the sensitization protocol, see paper I.

3.5 CSF analyses

CSF samples from patients and controls were collected through lumbar puncture in the L3/L4 or L4/L5 interspace according to standardized procedures (Blennow et al., 2010). The samples were centrifuged after collection and then stored in -80°C until they were analyzed.

In paper III, IV and V, CSF levels of Aβ_{1-42} were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) specific for Aβ_{1-42} (Innotest™ β-amyloid (1-42), Fujirebio, Ghent, Belgium) and CSF levels of P-tau and T-tau were measured using a sandwich ELISA (Innotest™ hTAU-Ag, Fujirebio, Ghent, Belgium). The levels of CSF biomarkers representing inflammation in paper V were measured using various techniques. The Meso Scale Discovery technique (MSD Human MCP-1; Meso Scale Discovery, Gaithersburg, Md., USA) was used to measure CSF levels of monocyte chemoattractant protein-1 (MCP-1). An ELISA (R&D Systems, Minneapolis, Minn., USA) was used to determine levels of YKL-40 and the activity of chitotriosidase was determined through an enzyme assay previously described (Hollak et al., 1994, Mattsson et al., 2011). All biochemical measurements were performed by board-certified laboratory technicians who were blinded to clinical data. The analyses were performed in one round of experiments using one batch of reagents, and intra-assay coefficients of variation were below 10%.

3.6 Statistical analyses

3.6.1 Zebrafish experiments

All statistical analyses in paper I and II were performed in Microsoft Excel and IBM SPSS Statistics version 20, (New York, NY, USA), and the graphical representations were plotted in GraphPad Prism 5 and 6 (GraphPad Software Inc., La Jolla, Calif., USA). Statistical comparisons between groups were performed using unpaired t-test whereas paired t-test was used for intragroup comparisons. All plotted data are presented as mean±SEM unless otherwise stated. P-values < 0.05 were considered statistically significant.

3.6.2 Gene association analyses

In paper III and IV, an unpaired t-test was performed for continuous parameters and Pearson chi-square test was used for categorical parameters when patients and controls were compared. Single SNP association with diagnosis was performed using logistic regression with an additive genetic model. Identified covariates included in the statistical model were sex and number of *APOE ϵ4* alleles for SNP association to disease. Single SNP associations to continuous parameters were only performed in the patient group with a linear regression model and sex, age and number of *APOE ϵ4* alleles as cofactors. Haplotype analyses for association with disease or continuous parameters were performed using various window sizes, stated in detail in the individual papers. Correction for multiple testing was carried out by a permutation test, set to 10 000 permutations. All statistical analyses were carried out in IBM SPSS Statistics version 20, (New York, NY, USA) and Plink v1.07 (REF). P-values < 0.05 were considered statistically significant.

3.6.3 CSF biomarker analyses

In paper V, comparisons of CSF biomarker levels and activity between controls and AD patients were performed using the Mann-Whitney U test. Correlations were analyzed using Spearman rank correlation coefficient test and for the differentiation of patients and controls, a receiver operating characteristic (ROC) curve was contracted for each individual marker. All statistical analyses were carried out using the software SAS version 9.3 (SAS Institute Inc., Cary, N.C., USA) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, Calif., USA).

4 RESULTS AND DISCUSSION

4.1 Paper I

To study AD pathology in animal models has been shown to be challenging. Most research investigating Aβ toxicity is based on rodent models, where mutations in specific genes are required for the animals to develop AD-like pathologies. The transgenic animals express mutations in genes responsible for the emergence of fAD in humans and the observed pathology in the form of Aβ plaque burden and memory impairment vary extensively (Elder et al., 2010). Another concern is the fact that most cases of AD are sporadic, where patients show no variation in the genes involved in familial AD. Therefore, it is of great importance to develop new complementary techniques to study Aβ toxicity.

In this study, we developed a new zebrafish *in vivo* model to study Aβ-caused neurotoxicity. The ability to easily manipulate gene expression, the high genetic homology to humans, the fast external embryonic development and transparent nature of the embryo are all favorable features for *in vivo* experiments in neurodegenerative disorders such as AD. Therefore, we decided to deliver Aβ into the brain of the fish rather than using transgenic methods, to overcome impaired and uncontrolled Aβ production. This technique also allows for investigation whether different species of Aβ affect neurons differently.

As AD is characterized by a progressive neuronal degeneration accompanied by memory loss in humans, we wanted to validate that Aβ, the main toxic component found in the brain of AD patients, show similar effects in zebrafish larvae. To this end, we developed a novel behavioral preparation for zebrafish larvae to study a form of non-associative learning called sensitization. We showed that during normal conditions, larvae were able to sensitize to an electrical stimuli which elicits the startle response. Remarkably, when animals were injected with oligomerized Aβ, they were no longer able to sensitize, which supports the theory of Aβ oligomerization as a putative cause for the observed memory impairments (Walsh et al., 2002, Barker et al., 2002).

Next, we evaluated the neurodegenerative properties of the Aβ injection. Animals injected with oligomerized Aβ showed significant increase of dead cells in the brain compared to controls. The cell death was widespread in the brain and not located exclusively in the vicinity of the regions responsible for the startle response, which suggests that the learning impairments are not caused by direct toxicity to these cells. To ensure that the Aβ was responsible for the observed neurodegeneration, fluorescent Aβ was injected and was shown to co-localize well with dead cells in the brain and was absent at the regions responsible for the startle response.

Patients suffering from AD are today generally treated with symptomatic medication, as there is no cure for AD. Here, we wanted to evaluate if the most commonly used treatments for AD, i.e. the partial NMDA receptor antagonist memantine and the acetylcholinesterase inhibitor donepezil, had an effect on the Aβ-caused phenotype seen in the zebrafish larvae. Indeed, treatment of zebrafish larvae with memantine and donepezil prevented the memory impairments caused by Aβ. This indicates that the mechanisms underlying Aβ toxicity, more exactly memory dysfunction, are conserved between human and the zebrafish. The results also provide insight that the zebrafish can function as a model to screen for AD therapeutic treatments.

Our results from the zebrafish fits well to the data from rodent models using Aβ injections causing block of long-term potentiation (LTP) (Walsh et al., 2002) and long-term memory (Balducci et al., 2010) that can be protected by memantine (Klyubin et al., 2011). However, our zebrafish model serves a versatile alternative to the rodent models available today, including transgenic mice strains. Firstly, the memory impairments and neurodegeneration appears much faster than the commonly used transgenic mouse models that require up to 6 -12 months before cognitive symptoms appear (Spires-Jones and Knafo, 2012). Secondly, the size and permeability of the zebrafish larvae allows for quick and uncomplicated drug delivery which permits future screening of novel compounds that can serve as AD therapies, as the delivery of drugs does not require injections. Additionally, the molecular pathways of Aβ toxicity can be probed pharmacologically and with genetic modifications including MOs and novel techniques such as CRISPR mutations in a way that is not possible to do with other, higher vertebrate models.

Lastly, as the model stands today it can unfortunately not contribute with knowledge on the causes of the elevated Aβ levels in the brains of AD patients as the Aβ peptides in our model were delivered to the brain. Although the zebrafish has two copies of the *APP* gene (Musa et al., 2001) and expresses the enzymes processing APP to Aβ (Groth et al., 2002, Leimer et al., 1999, Moussavi Nik et al., 2012), no Aβ has yet been detected in zebrafish brain tissue, endogenously or in genetic models. This indicates that transgenic zebrafish models overexpressing Aβ similarly to mouse transgenic models are not feasible. However, as we and others hypothesize that AD is caused by an imbalance of the production /clearance ration, the latter can be regulated in our model as seen in paper II.

4.2 Paper II

Our previous study (paper I) showed that Aβ injections in zebrafish larvae induce cell death and memory impairments. However, as our experiments indicated degradation of the Aβ peptide over time, we decided to investigate the role of microglia cells, the innate immune cells of the brain, with regard to Aβ interaction and clearance. Microglia are highly motile and adaptive cells and serve as the initial immunological response to pathogens and injuries in the brain. Microglia has also been suggested to be involved in AD pathology, although the contribution of the cell remains unclear. Even though microglia are capable of phagocytose and degrade Aβ, studies also indicate that the cells are promoting a constant inflammation in AD patients which is harmful for the brain, possibly accounting for the progressive neurodegeneration.

We started by injecting fluorescent Aβ into the brain of zebrafish larvae expressing GFP in microglia cells. Confocal imaging revealed an interaction between the microglia cells and Aβ. The cells were shown to not only interact with the Aβ peptide, but also to engulf it, seen through accumulation of internalized peptide in the microglia cells.

As we had previously seen that the Aβ dispersion varied over time, we wanted to investigate this further. An initial observation was that the amount of peptide decreased in the course of time, seen through continuous measurements of Aβ injected animals at several time points. The Aβ was spread extensively around the brain directly after injection, whereas a more condensed state was displayed at later time points in which the peptide was

concentrated in small clusters. We hypothesized that these clusters were accumulations of Aβ inside of microglia cells, and that the decreased amounts of peptide was due to internal degradation mechanism. To test this, we knocked-down the expression of microglia cells in the animals and witnessed a great reduction in Aβ clearance capacity, as injected animals no longer showed any difference in peptide amounts at different time points. These animals also revealed a great increase in cell death compared to Aβ-injected controls, which suggests that a failure in clearance potentiates the Aβ-caused toxicity. Taken together, these results demonstrate a protective role of microglia with regard to Aβ toxicity in the zebrafish larvae.

Additionally, we were interested in exploring the underlying mechanisms of how microglia find and clear away Aβ from the brain. We aimed at evaluating the role of the purinergic receptor P2Y12, as a potential candidate for Aβ clearance. P2Y12 is mainly expressed on platelet cells where it regulates activation and aggregation of platelets in the presence of injury. The receptor is also expressed in the brain, exclusively on microglia, where it mediates microglia migration towards sites of injuries by binding ADP molecules. Subsequently, we hypothesized that Aβ caused neurodegeneration leads to secretion of ADP molecules from dead cells, which in turn activates the p2p12 receptor, promoting microglia migration towards sites of Aβ. To test this theory, we knocked down the expression of the zebrafish p2y12 receptor prior to injection on Aβ and detected differences in processing of Aβ in fish with and without p2y12 receptors. To our knowledge, p2y12 has not previously been associated with Aβ processing *in vivo*.

In this project we have observed the interaction between microglia and externally applied Aβ and not endogenously overexpressed Aβ. However, we expect that the molecular mechanisms investigated will be relevant for disease progression of AD and possibly other neurodegenerative diseases.

With the publication of the first $CX3CR1^{GFP/WT}$ mouse in 2000 it was possible to follow microglia activity *in vivo* in vertebrates (Jung et al., 2000, Nimmerjahn et al., 2005). However, few studies have so far focused on *in vivo* imaging of microglia clearance of Aβ. The comprehensive *in vivo* imaging studies by Condello *et al.* and Jung and coauthors (Condello et al., 2015, Jung et al., 2015) have reported microglia accumulation around Aβ plaques, as previously seen in histological analyses of human tissue and in transgenic mice models. Although *in vivo* time-lapse imaging of microglia

activity has been done in transgenic mice with microglia marked (Liu et al., 2010), many of these studies are lacking the high resolution in time that we can access in the zebrafish, that enable mechanistic analysis of the events underlying Aβ clearance.

4.3 Paper III

Our previous studies provided results which indicated that the purinergic receptor P2Y12 was involved in microglia-dependent clearance of Aβ in zebrafish larvae. Animal models do however mimic traits present in humans, and the underlying mechanisms responsible for the observed phenotypes might not be the same in animals as in humans. Therefore, in an attempt to validate our zebrafish model and also confirm our result which suggests P2Y12 to play a role in AD pathology, we conducted a gene association study in a Swedish AD patient material consisting of 620 AD patients and 1107 controls.

P2Y12 is solely expressed on microglia cells in the CNS (Guerreiro et al., 2013), where it guides the microglia towards sites of injury by binding ADP molecules (Sieger et al., 2012, Haynes et al., 2006). We hypothesized that the P2Y12 receptor might aid microglia to sites of neurodegeneration and Aβ plaques by sensing cues of ADP molecules, a theory strengthened by our previous zebrafish experiments. To our knowledge, P2Y12 has so far not been associated with AD in clinical studies.

In this study, we selected SNPs that covered the *P2Y12* gene, and investigated weather genetic variation in the gene was associated with the risk of AD. The genetic variation was also analyzed with regard to cognitive function in AD patients, a so-called MMSE score, as well as CSF biomarkers characteristic for AD, i.e. CSF levels of $A\beta_{1-42}$, T-tau and P-tau.

Out of the genotyped SNPs in the *P2Y12* gene, we found a nominal association between one gene variant of *P2Y12* and a reduced risk of AD. Next, we investigated whether haplotype variants were associated with the risk of AD and we identified several haplotypes to be strongly associated to disease status.

Lastly, we conducted an analysis to investigate if genetic variation in *P2Y12* influenced cognitive function or levels of characteristic CSF biomarkers for

AD in the patient group. Here, we found a number of SNPs that were nominally associated with altered levels of CSF $A\beta_{1-42}$ in AD patients. Clinical studies indicate that patients suffering from AD display decreased CSF levels of $A\beta_{1-42}$, as a result from $A\beta_{1-42}$ accumulation in plaques in the brain and less peptide in the CSF (Blennow et al., 2001). From our results, we hypothesize that the association between *P2Y12* and the altered $A\beta_{1-42}$ levels might be due to a decreased clearing capacity of microglia, hence less $A\beta_{1-42}$ in the CSF. Even though we found associations between SNPs in *P2Y12* and CSF levels of $A\beta_{1-42}$, no results remained significant after correcting for multiple testing. No associations were observed for SNP association to MMSE score or CSF levels of P-tau or T-tau.

In conclusion, the underlying mechanisms behind the association between the *P2Y12* haplotype and an increased risk of AD remain unclear. It is possible that the haplotype influences P2Y12-mediated microglia guidance towards sites of injury, as both SNPs in the haplotype are located in a region suggested to regulate the expression of *P2Y12* (Timur et al., 2012). Decreased expression of the receptor could thereby obstruct microglia migration in response to Aβ-caused neurodegeneration, where P2Y12 binds ADP molecules released from dead cells to a lesser extent. On the other hand, cardiovascular implications are commonly linked to the presence of many types of dementia, including AD. As P2Y12 is expressed by platelets and functions as a key regulator of platelet activation, it is fascinating to speculate that the haplotype could increase the platelet aggregation at injured sites in the brain. The neurodegeneration present in AD patients leads to secretion of ADP molecules from dead cells, which in turn recruits and activates platelets. Abnormal platelet aggregation can progress into events of pathological thrombosis, which ultimately leads to ischemic events, further inducing the neurodegeneration. It is therefore of great importance to replicate these findings, and to evaluate the risk for AD in combination with markers representing cardiovascular implication to better understand the putative link between cardiovascular implications and the risk of development of dementia.

4.4 Paper IV

Sortilin 1, a member of the VPS10P domain receptor family (Cattaneo et al., 2003), is a transmembrane receptor encoded by the *SORT1* gene. The receptor mediates intracellular trafficking of ligands and is highly expressed

in the CNS, where it plays an important role in neuronal viability and function (Nykjaer and Willnow, 2012). Sortilin 1 has previously been proposed to be involved in the pathogenesis of AD based on both *in vivo* and *in vitro* studies. Firstly, AD and MCI patients had increased cortical protein levels of sortilin 1 (Mufson et al., 2010) where the levels correlate well with the severity of the pathology. Secondly, experiments in mice revealed that sortilin acts as a receptor for apoE in neurons and that animals lacking sortilin 1 display increased Aβ plaque burden (Carlo et al., 2013). A member of the VPS10P domain receptor family, *SORL1*, has previously been suggested as a candidate gene for AD (Lee et al., 2008, Rogaeva et al., 2007). SNPs located in *SORT1* have been shown to influence plasma levels of cholesterol and increase the risk of myocardial infarcts (Carlo et al., 2014), but so far has no genetic variation in *SORT1* been correlated with the risk of AD.

In this study, we performed a genetic association analysis investigating association between variation in *SORT1* and the risk of AD in a Swedish case-control cohort. We selected both novel SNPs as well as SNPs that has previously been associated with other diseases. All SNPs were analyzed with regard to disease status, along with cognitive function and CSF biomarkers characteristic for AD. We identified one SNP that was strongly associated with a reduced risk of AD, which also survived correction for multiple testing. We also found two SNPs to be nominally associated with altered CSF levels of $A\beta_{1-42}$.

As previously mentioned, sortilin 1 can act as a receptor for apoE ligands. Knock-down of sortilin 1 has revealed an impaired neuronal uptake of apoE/Aβ complexes and increased Aβ plaque pathology in mice (Carlo et al., 2013, Takamura et al., 2012). Hence, we hypothesize that the SNP associated with decreased risk of AD might influence the uptake of apoE/Aβ complexes, thus promoting the clearance of Aβ. The nominal association between two SNPs and altered CSF levels of $A\beta_{1-42}$ also supports this theory, as it is an indication of Aβ pathology present in AD patients. It is also possible that the SNP identified in our study to reduce the risk of AD has an effect on plasma lipid levels, a theory based on numerous previous studies identifying strong associations between *SORT1* and altered plasma levels of lipids in humans (Arvind et al., 2014, Willer et al., 2008, Walia et al., 2014, Shirts et al., 2011, Keebler et al., 2009, Kathiresan et al., 2008, Gupta et al., 2010). In other words, genetic variation in *SORT1* leading to altered lipid levels might explain the underlying mechanism underlying the relation between CVD and

lipid levels as known risk factors for AD. Therefore, it is of great importance to include measurements and markers for lipid levels and CVD when future genetic association studies on *SORT1* are performed with regard to AD.

4.5 Paper V

The pathological hallmarks present in AD, i.e. the extracellular aggregations of Aβ together with intracellular fibrils of phosphorylated tau protein can be visualized indirectly in CSF samples. Numerous studies have shown that AD patients display elevated CSF levels of total T-tau and abnormal levels of phosphorylated P-tau, while the levels of $A\beta_{1-42}$ are decreased compared to healthy controls (Blennow et al., 2010, Blennow et al., 2001). The measurements of CSF biomarker levels are used as a complement to the clinical examination, but are not sufficient to set a diagnosis. One major limitation with the AD diagnosis today is the inability to predict incipient AD in cognitively healthy individuals. Here, CSF biomarkers representing early neuronal changes prior to the emergence of cognitive impairments could serve to detect the risk of AD, as well as to provide a better understanding of the underlying mechanisms responsible for the emergence of AD.

Lately, studies have shown that AD is accompanied by an activation of the immune system, also contributing to the pathogenesis of the disease (Heppner et al., 2015, Zhang et al., 2013). Also, the main genetic risk factor for AD, *APOE* (Poirier et al., 1993, Corder et al., 1993), together with the recent identification of the genes *TREM2* (Guerreiro et al., 2013, Jonsson et al., 2013) and *CD33* (Bradshaw et al., 2013) as candidate genes for AD, has further strengthened this theory as all these genes are linked to immune cell activity. We believe that measurements of markers representing the state of activation in the immunological activity could serve as an excellent tool to evaluate AD pathology and to find new insights in the pathogenesis of the disease.

In this study, we investigated the association between three CSF biomarkers representing inflammation in a cohort consisting of AD patients and healthy controls. The AD patients were selected based on a clinical examination together with a positive profile for the characteristic AD biomarkers. The controls were non-demented individuals with a negative CSF profile for AD biomarkers. The selected biomarkers were chitotriosidase, an enzyme secreted by activated macrophages, YKL-40/chitinase 3 like 1 (CHI3L1), a

glycoprotein expressed on astrocytes and microglia, and MCP-1, a protein involved in the recruitment of monocytes produced by microglia.

We showed that the CSF levels of chitotriosidase and YKL-40 were significantly increased in the AD patients compared to controls, results in agreement with previous reports (Mattsson et al., 2011, Watabe-Rudolph et al., 2012, Craig-Schapiro et al., 2010, Perrin et al., 2011). No significant difference was observed for MCP-1. The levels of YKL-40 has also been proposed to serve as a prognostic marker for progression of AD with high diagnostic utility (Perrin et al., 2011, Craig-Schapiro et al., 2010), which was also observed in our analysis (area under the ROC (receiver operating characteristic) curve (AURUC) = 0.88). An interesting result was that the inflammatory biomarkers had no correlation to the core AD biomarkers, $A\beta_{1-42}$ T-tau or P-tau in the patients. This finding suggests that inflammatory status might affect the risk of AD independently of Aβ or tau pathology. Since CSF levels of YKL-40 are altered in very early stages of the disease, YKL-40 measurements could be a valuable diagnostic tool to find early sings of AD in cognitive healthy individuals. The results of paper V resonate well with recent studies showing increased CSF concentration of secreted TREM2 in dementia and pre-dementia stages of AD (Henjum et al., 2016, Suarez-Calvet et al., 2016, Piccio et al., 2016, Heslegrave et al., 2016).

5 CONCLUSION

Apart from accumulation of senile plaques and neurofibrils, AD is also characterized by low-grade CNS inflammation. It is however unclear whether activation of the immune system fuels or antagonizes the progression of the disease, indicated by the contradictive results from both animal and clinical studies. In this thesis, we aimed at developing new tools to study mechanisms, including inflammatory processes, related to Aβ toxicity that can be used to validate clinical data or to discover new molecular mechanisms to be further analyzed in humans.

The results presented in this thesis provide new insight in how the zebrafish can be used as a model to study AD pathology. We showed that the toxic effects of Aβ injections in zebrafish were similar to the characteristics of AD pathology and that commonly used AD symptomatic treatments protect against the learning problems seen in our model. Also, the microglia receptor p2y12 was shown to play an important role in the microglia-mediated clearance of Aβ. This finding was confirmed in a human population study where a haplotype in *P2Y12* was found to be associated with an increased risk of AD. Taken together, these results indicate that the zebrafish and human share the underlying mechanisms of Aβ-caused toxicity and that the zebrafish is a suitable animal model to study and develop drugs to prevent or treat AD.

In addition to the zebrafish experiments showing the importance of microglia in protection against Aβ toxicity, the results from the human population studies included in this thesis also suggest an involvement of inflammation in AD. CSF analysis revealed that two biomarkers, chitotriosidase and YKL-40, were both increased in AD patients compared to controls. Both markers represent activation of the immune system and could be suitable for diagnostic purposes together with the core AD biomarkers. We also investigated genetic variation in *SORT1*, the gene coding a receptor expressed on microglia, and found associations to decreased risk of AD.

Taken together, we have shown that microglia play an important role in AD, seen through zebrafish experiments and in associations studies of AD risk and variation in microglia receptor genes and inflammatory CSF biomarkers. We also developed a new zebrafish model to study AD-related pathology, such as learning impairment, neurodegeneration and inflammation that could serve as a tool to develop new therapeutics for treatment of the disease.

6 FUTURE PERSPECTIVES

AD is today the leading cause of dementia in the world. With a growing population and an increased expected lifespan, the need for effective treatments is of great importance. One major issue with the development of effective drugs is the heterogeneity of the disease, as the pathology varies and most cases are sporadic, lacking clear genetic components. Another concern is the inconclusive results from animal models, where the findings are not supported by clinical studies. It is therefore important to develop new models to study pathology related to AD. The results should thereafter be evaluated in clinical studies, to confirm or contradict if the underlying mechanism is present and is a contributing factor to the development of the disease. Findings of factors contributing to AD without knowledge of the underlying biological function could also be explored in animal models.

Historically, most research of AD pathology has focused on the histopathological hallmarks of AD, Aβ and tau, as the putative cause for the disease. It is widely accepted that Aβ pathology is present long before the emergence of cognitive impairments in AD patients, which suggests that targeting Aβ for treatment purposes might not be optimal. Growing evidence suggests that other features, such as CVD and inflammation, are strongly involved in AD and might contribute to the early development of the disease. Therefore, to fully investigate the mechanisms responsible for AD, one should also evaluate the role of secondary features such as inflammatory status and CVD-related markers in the brain of AD patients.

Epidemilogical studies have shown that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD. However, clinical trials have failed to show positive effects from NSAID treatment in AD patients. The conflicting results are most likely due to that inflammation precede the neurodegeneration features of AD. Dampening general inflammation is therefore only protective prior to AD, not after the disease is manifested. It is also important to further investigate the mechanisms underlying AD related inflammation, to be able to develop more suitable drugs. Reducing general inflammation might not be the correct way, but rather find pathways responsible for a protective activation of the immune system, which could be used as treatment for manifested AD.

ACKNOWLEDGEMENT

I would like to sincerely thank everyone who was involved in the work of this thesis, especially:

Petronella Kettunen, my main supervisor, for your enthusiasm, your hard work, your sense of humor and hospitality! I am very thankful for everything you have taught me and for giving me the opportunity to work with you. I could not have asked for a better supervisor!

Henrik Zetterberg, my co-supervisor, for your enthusiasm, your will to always help out and for sharing your scientific knowledge with me.

Alexandra Abramsson, my co-supervisor, for all the help in the lab and discussions about running, and also for your help with MO design!

Staffan Nilsson, for your sense of humor, your endless patience in teaching me how statistics work and all work with the genetic studies.

Sabine Strand and all other students, for your excellent work in the lab during your Master's and your contribution to the zebrafish articles.

Anna Zettergren, for all discussions, all help and assistance you provided for the genetic studies.

Anders Wallin, for your hospitality, deep knowledge of AD and related disorders and help with all writing!

Mona Seibt Palmér, for all the help with the patient material and your endless pipetting!

Rakesh Banote, for being a great friend, co-worker and for introducing me to Indian beer!

Pradeep Kumar, for being an awesome office-mate and for all cookies you have given me!

Maria Olsson, my office-mate for all these years, for all the discussions about everything and everyone, for your help with all my work and great personality!

Anna Staffas, my former office-mate, for all discussions about music, food and everything in-between!

Johan Stenberg, for all great discussions and assistance during the final hours of the thesis writing!

Maja Offenbartl, for all nice discussions, wine drinking and lab work!

All group colleagues; **Malin Edling, Maria Holmström, Tugce Munise Satir, Faisal Nazir, Petra Bergström, Lotta Agholme, Elisa Alexandersson and Linda Werme.**

All colleagues at the Department of Clinical Chemistry!

The staff at Centre for Cellular Imaging (CCI), especially **Julia Fernandez-Rodriguez** and **Maria Smedh**.

The people at "Farmen" for the great board game evenings and parties; **Jakob Näslund, Erik Studer, Jesper Sundell, Daniel Eckernäs, Daniel Hovey, Lina Jonsson, Sara Karlsson and Lars Westberg**

My parents, **Lennart** and **Ingela Andersson** and my brothers **Niklas Andersson** and **Markus Trojer**.

And especially, **Emilia Möller Rydberg**, for your endless patience and support you have showed during this time. I could not have done this without you! And our little one, my daughter **Olivia Rydberg** for making me think of things more relevant than science.

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