Synaptic elimination and the complement system in Alzheimer's disease

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ABSTRACT

The mechanisms behind Alzheimer's disease (AD) are largely unknown. The disease is to a large extent hereditary, and the best pathophysiological correlate to the severity of the symptoms is loss of synapses.

The general aim of this thesis was to examine the hypothesis that AD is primarily a synaptic disease – with an emphasis on complement-mediated elimination of synapses.

Animal models of AD have shown that long-term potentiation (LTP) of synapses is inhibited by beta amyloid (Aβ). LTP is considered to be a physiological correlate to learning and memory, and Aβ is a peptide that constitute the extracellular plaques that characterise AD. The Aβ induced inhibition of LTP has been shown to be dependent on the receptor for advanced glycation end products (RAGE). In the present thesis I present results that suggest an association of a functional single nucleotide polymorphism (SNP) in the gene encoding RAGE with diagnosis of AD. Thus linking the synapse related pathophysiology observed in animal models, to human patients with AD.

During development, synapses in the retinogeniculate system and the sensorimotor cortex of mice are eliminated in a complement mediated manner. Since AD pathology primarily affects the hippocampus, we sought to investigate whether the complement system mediates elimination of hippocampal synapses as well. Indeed, by use of complement component 3

(C3) deficient mice, electrophysiological, histological, molecular and behavioural methods, we obtained results that suggest this for a fact.

Considering the importance of synapse loss in AD we decided to measure the levels of the complement proteins C3, complement component 4 (C4), complement factor B (CFB) and complement receptor 1 (CR1) in cerebrospinal fluid (CSF) from patients with various degrees of AD. The results showed a trend towards increased complement levels in AD patients. This association, however, was too weak to be of diagnostic value. Nevertheless it supports the notion of complement involvement in AD.

Next we hypothesised that genetic variation in genes encoding complement proteins could potentially be associated with diagnosis of AD. Therefore, we investigated SNPs in the complement genes *CR1, C3, CFB,* and the second complement component (*C2*). Although no such associations were found, we did, however, find an association of *C2/CFB* SNPs with measures of cognitive function (MMSE) and neuronal damage (tau) in AD patients, thus lending further support for the hypothesis of complement mediated synaptic elimination in AD.

I conclude that several lines of evidence suggest that AD might very well be the result of aberrant complement regulation, with improper synaptic elimination as a consequence. Precise knowledge about the mechanisms underlying AD is of great value to research into accurate diagnostic methods and treatments, thus, further research on the subject of synapse elimination in AD is warranted.

Keywords: Alzheimer’s disease, Synapse, Complement, RAGE, Biomarker, Genetics, SNP.

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SAMMANFATTNING PÅ SVENSKA

Mekanismerna bakom Alzheimers sjukdom är i stort sett okända, men det som bäst förklarar symptomens svårighetsgrad är antalet synapser. Med avstamp i detta förhållande är den grundläggande frågeställningen i denna avhandling huruvida Alzheimers sjukdom primärt är en synapssjukdom.

Djurförsök har visat att långtidspotentiering (LTP) av synapser hämmas av amyloid β (Aβ), det protein som till största delen utgör de amyloida plack som karaktäriserar sjukdomen patologiskt. Detta är mycket intressant eftersom LTP anses vara det cellulära substratet för minne och inlärning. Hämningen har visats vara beroende av proteinet RAGE; därför undersökte vi huruvida en variant av genen för detta protein var associerad med Alzheimers sjukdom. En sådan association fanns och detta tolkar vi som att de observationer av RAGE-beroende LTP-hämning som gjorts i djurmodeller av Alzheimers sjukdom skulle kunna vara relevanta för sjukdomens utveckling även hos människor.

Eftersom förlust av synapser karaktäriserar Alzheimers sjukdom undersökte vi fenomenet synapseliminering i hippocampus (den del av hjärnan som först drabbas av Alzheimerspatologi). Här kunde vi med olika elektrofysiologiska metoder visa att möss som saknar genen för komplementkomponent 3 (C3) har en störd synapseliminering, vilket innebär att komplementsystemet skulle kunna vara involverat i Alzheimers sjukdom. Därför mätte vi halterna av komplementproteinerna C3, C4 och CR1 i ryggmärgsvätska från personer med olika grad av Alzheimers sjukdom och resultaten visade på signifikanta skillnader i halterna av dessa.

För att ytterligare stärka upp hypotesen undersökte vi varianter av komplementgenerna *C2*, *C3*, *CFB* och *CR1* hos Alzheimerspatienter och friska kontrollpersoner. Trots betydande stöd i litteraturen för att dessa gener skulle vara associerade med Alzheimers sjukdom fann vi inga associationer mellan dessa genvarianter och Alzheimersdiagnos. Vi fann däremot att en variant av *C2* och *CFB* var associerad med Alzheimerspatienternas kognitiva förmåga och mängden hyperfosforylerat tau (ett protein som är kopplat till Alzhemers sjukdom och hjärnskada).

Sammanfattningsvis presenterar jag i denna avhandling ett visst stöd för hypotesen att den grundläggande problematiken vid Alzheimers sjukdom utgörs av synapspatologi och att den kan vara associerad med förändringar i RAGE- och komplementsystemet.

LIST OF PAPERS

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

I. [*]Daborg J., [*]von Otter M., Sjölander A., Nilsson S., Minthon L., Gustafson DR., Skoog I., Blennow K., Zetterberg H. Association of the RAGE G82S polymorphism with Alzheimer's disease.
J Neural Transm 2010 Jul; 117(7): 861-7.

II. [*]Daborg, J., [*]Perez-Alcazar, M., Stokowska, A., Wasling, P., Björefeldt, A., Beyer, N., Atkins, A.L., Zetterberg, H., Carlström, K., Dragunow, M., Clementson Ekdahl, C., Hanse, E., Pekna, M. Impaired synaptic elimination and compensatory homeostatic plasticity in the hippocampus of mice lacking C3.
In manuscript

III. Daborg J., Andreasson U., Pekna M., Lautner R., Hanse E., Minthon L., Blennow K., Hansson O., Zetterberg H. Cerebrospinal fluid levels of complement proteins C3, C4 and CR1 in Alzheimer's disease.
J Neural Transm 2012 Jul; 119(7): 789-97.

IV. Daborg J., Holmgren S., Abramsson A., Andreasson U., Zetterberg M., Nilsson S., Minthon L., Skoog I., Blennow K., Pekna M., Hanse E., Zetterberg H. Association of complement gene single nucleotide polymorphisms with Alzheimer's disease.
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[*] These authors contributed equally.

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ABBREVIATIONS

$A\beta_{42}$	Amyloid beta (42 amino acid variant)
AD	Alzheimer's disease
AGER	Advanced glycation end-products receptor
AMD	Age-related macular degeneration
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AUC	Area under the curve
C1q	Complement component 1 q
C2	Complement component 2
C3	Complement component 3
C4	Complement component 4
CFB	Complement factor B
CFH	Complement factor H
CNS	Central nervous system
CR1	Complement receptor 1
CSF	Cerebrospinal fluid
EPSP	Excitatory postsynaptic potential
FAD	Familiar Alzheimer's disease
LTP	Long-term potentiation
MCI	Mild cognitive impairment

MMSE	Mini mental state examination
MRI	Magnetic resonance imaging
n	Number of synapses or release sites
NMDAR	N-methyl-D-aspartate receptor
NP	Neuronal pentraxine
NPR	Neuronal pentraxine receptor
OR	Odds ratio
p	Release probability
PPR	Paired pulse ratio
q	Quantal content
RAGE	Receptor for advanced glycation end-products
ROC	Receiver operating characteristic
SAD	Sporadic Alzheimer's disease
SNP	Single nucleotide polymorphism
TACE	Tumor necrosis factor-alpha converting enzyme
VIP	Variable in the projection

1 INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD) was first described by Alois Alzheimer in 1906 (Maurer et al. 1997). It is the most common form of dementia, and it is characterized by a progressive decline in cognitive ability. This can be attributed to the progressive loss of synapses and neurons seen in patients. At least two forms of AD are recognized; early onset familial AD (FAD), where a single mutation in a key AD gene causes AD in an autosomal dominant, fully penetrant manner, and sporadic AD (SAD) where several risk factors, both genetic and environmental, are thought to contribute to the disease.

1.1.1 Diagnosis

Clinically, the disease is foremost characterized by amnesia - beginning as anterograde amnesia, and as the disease progresses this is followed by a retrograde amnesia (Salmon and Bondi 2009). Other psychiatric symptoms are common, and include emotional and attention deficits (Salmon and Bondi 2009). Several tests are used to assess cognitive function when trying to determine diagnosis. One of the simplest and most frequently used is mini mental state examination (MMSE) (Tombaugh and McIntyre 1992; Folstein et al. 1975).

Traditionally, AD has been a *post mortem* diagnosis. Aside from progressive cognitive deterioration, the patient also has to show some specific pathological changes. These hallmarks of the disease were described as plaques and tangles by Alois Alzheimer over a century ago. In the eighties the plaques were shown to consist mainly of the 42 amino acid long β-amyloid peptide ($A\beta_{42}$) (Glenner and Wong 1984; Masters et al. 1985; Wong et al. 1985), whereas the tangles were shown to be made up of hyper-phosphorylated tau protein (Grundke-Iqbal et al. 1986). These two molecules now constitute the core cerebrospinal fluid (CSF) biomarkers for AD (Blennow et al. 2010). Diagnosis is chiefly based on clinical assessments, but in recently revised diagnostic criteria biomarkers have entered as important adjuncts (http://www.alzheimersanddementia.org/content/ncg). Other methods utilised include different brain imaging techniques, such as magnetic resonance imaging (MRI).

In the present thesis AD subjects fulfilled the DSM-IIIR criteria of dementia (Spitzer et al. 1987) and were either pathologically confirmed or fulfilled the

criteria of probable AD defined by NINCDS-ADRDA (McKhann et al. 1984) which was further supported by CSF biomarkers.

MCI is defined as not normal but not demented. Cognitive impairment can be reported by the patient him- or herself, but is preferably also confirmed by an informant, and in objective neuropsychological tests. An alternative criterion is evidence of cognitive decline over time. Otherwise the patient should be able to lead an ordinary life with only minimal impairment in complex instrumental functions (Winblad et al. 2004). Around 50% of MCI patients eventually develop AD, whereas the remaining 50% have benign cognitive impairment or suffer from other neurodegenerative conditions.

Although there is no cure for AD, a correct diagnosis is important to exclude other diagnoses for which treatment might be available. It is also important in order to provide the patient and relatives with correct information and a reasonable prognosis so they know what to expect of the future, enabling them to make arrangements and plans in good time. Lastly, efforts in making accurate early diagnoses will hopefully pay off when treatments become available.

1.1.2 Epidemiology

In 2011 AD had a prevalence of about 34 million cases worldwide (Barnes and Yaffe 2011). The incidence is approximately 1% and increases exponentially with age (Reitz et al. 2011). Due to increased life expectancies, the prevalence has been estimated to triple over the next 40 years (Barnes and Yaffe 2011). The suffering and economic impact cannot be overstated.

Age is the major risk factor for AD. FAD has a marked earlier onset than SAD; typically it starts in the late 40's or early 50's and has an absolute genetic aetiology. Although the present thesis only concerns the more common SAD, it is important to note that FAD and SAD are clinically and neuropathologically similar, and since all known FAD mutations are located in genes involved in Aβ processing, a common conclusion is that Aβ should play a central role in AD.

SAD, in contrast to FAD, has a later onset, and a more complicated aetiology, where several risk factors are thought to contribute. Twin studies have shown that as much as 60-80% of the risk is of hereditary origin (Gatz et al. 2006). The only confirmed susceptibility gene is the ε4 allele of the apolipoprotein E gene (*APOE* ε4) (Corder et al. 1993; Kurz et al. 1996; Poirier et al. 1993). Although *APOE* ε4 has been suggested to account for 20-30% of the risk (Reitz et al. 2011), it is neither necessary nor sufficient for

disease (Myers et al. 1996). This leaves a lot to explain, and probably several small effect susceptibility genes work together in a complex manner to cause the disease. Other risk factors are female sex, and vascular disease and its associated risk factors (Reitz et al. 2011; Bendlin et al. 2010).

1.2 Synapses

The brain is the organ responsible for our mental experiences and abilities. It achieves this through the workings of a complex network of neurons and glial cells. The human brain houses approximately 100 billion neurons, these are interconnected via approximately 100 trillion synapses (Squire 2008).

1.2.1 Synaptic transmission

Synapses transfer, process and store information; accordingly, the synapse should be regarded as the ultimate functional unit in the central nervous system (CNS). The vast majority of synapses in the brain are glutamatergic (Megias et al. 2001), meaning that the transmitter substance released from the presynaptic bouton in order to convey a signal from the presynaptic cell to the postsynaptic cell, is glutamate. There also exist GABAergic synapses, these are typically inhibitory; further there is a class of modulatory synapses which utilize a vast range of transmitters, modulating neural activity via volume transmission, the lack of specificity is why these structures should not be considered as synapses.

The basis for neural transmission is excitability. When a neuron is excited enough, it fires an action potential along its axon, thereby increasing the probability of exciting the neurons it is connected to. Excitability is regulated in part by the neuron itself, this is called intrinsic excitability, and in part by the sum of synaptic input it receives from other neurons, extrinsic excitability. Extrinsic excitability is positively regulated by excitatory synaptic input, and negatively regulated by inhibitory synaptic input – the balance between excitation and inhibition is of utmost importance in neuronal networks.

When a great enough number of excitatory synapses are activated, either spatially or temporally, onto a given neuron, it will fire an action potential that will spread along its axon, reaching all of the presynaptic terminals where it will give rise to a Ca^{2+} transient, and through this momentarily increase the probability of transmitter release, if transmitter is released, the transmitter substance will diffuse over the synaptic cleft and bind to receptor molecules in the postsynaptic membrane. These receptors are coupled to ion

channels, and in the case of an excitatory synapse, this opening will lead to a transient depolarisation of the postsynaptic membrane, known as an excitatory postsynaptic potential (EPSP). If this EPSP is accompanied by several other EPSPs in the postsynaptic neuron, this neuron will in turn convey the signal to the neurons it is connected to. However, this is not a linear process; it depends on the efficacy of each synapse, if the signal will be transferred to the postsynaptic neuron.

Synaptic efficacy is determined by three parameters, $n*p*q$, where n is the number of synapses or release sites, p is the release probability of a single synaptic vesicle, and q is the quantal size, which is the magnitude of the postsynaptic response to release of a single synaptic vesicle (Korn and Faber 1991).

1.2.2 Synaptic plasticity

Plasticity is a fundamental aspect of neural networks - neither intrinsic excitability nor synaptic efficacy is fixed. These parameters are constantly changing in an activity-dependent manner. During development these phenomena ensure that the neuronal network is efficiently wired; in the more mature brain, however, they provide the brain with the necessary means to adapt to a changing environment, and to store information, thus enabling anticipation of the future (Kandel and O'Dell 1992).

One of the more thoroughly investigated forms of plasticity is long-term potentiation (LTP) of the glutamate synapse in the hippocampus (Kerchner and Nicoll 2008). LTP is commonly regarded as the neurophysiological substrate for learning and memory. Although there are good reasons to believe that this is actually true, it has not been shown that LTP is neither necessary nor sufficient for learning and memory (Martin et al. 2000). The concept was invented by Donald Hebb in the monumental book *The organisation of behaviour* (Hebb 1949). Briefly, he suggested that the connections between neurons that are active simultaneously, should be strengthened, thus providing a physiological substrate for lasting association. More than two decades later this hypothetical phenomenon was indeed observed by use of extracellular field recordings in rabbits (Bliss and Lomo 1973), and spurred new interest in the search for the engram. A puzzling question that was left unanswered was how two neurons could "know" that they were active simultaneously. The putative coincidence detector was finally shown to be the N-methyl-D-aspartate receptor (NMDAR) by Wigström and Gustafsson in 1986 (Wigstrom and Gustafsson 1986). The NMDAR is both voltage- and ligand-gated, meaning that in order for the

channel to open, it requires glutamate, which is released from the presynaptic terminal, in conjunction with postsynaptic depolarisation which is needed to relieve the channel from of a magnesium ion that blocks the channel at resting membrane potential. Hence, if the NMDAR is located in the postsynaptic membrane, the channel only opens when the two neurons that are connected via the synapse, are simultaneously active. Moreover, the NMDAR is permeable to calcium ions, and these work as intracellular second messengers, signalling that the synapse should be reinforced. There has been some debate as to whether the strengthening is of a pre- or postsynaptic locus, and the answer seems to be both, mainly depending on which synapse the investigator is examining. In the hippocampal CA3/CA1 synapse, most researchers agree that LTP is expressed as an increased number of glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) type in the postsynaptic membrane.

LTP is balanced by the process of long-term depression (LTD). The function and mechanisms of LTD is less well understood, but it appears as if at least some forms of LTD are dependent on the NMDAR. This is a puzzling feature, but it is generally believed that LTP requires a robust and temporally precise activation of the NMDARs, whereas an intermediate or scanty activation leads to LTD (Malenka and Bear 2004).

Other kinds of synaptic plasticity include various forms of short-term plasticity and homeostatic plasticity. Short-term plasticity is usually of a presynaptic nature, affecting release probability in various ways (Zucker and Regehr 2002). Probably these phenomena aid in the processing of information, rather than storage of it. Paired pulse plasticity is perhaps the most familiar form of short-term plasticity; it is frequently used to investigate release probability, and the common conception is that residual calcium after the first pulse increases the release probability of the second pulse and therefore giving rise to a greater postsynaptic response. This occurs in synapses with a low release probability and is called facilitation; when the second pulse generates a smaller postsynaptic response, the phenomenon is called depression, and this occurs in synapses with a high initial release probability.

The aforementioned forms of synaptic plasticity affects specific synapses to enable processing and storage of information. Homeostatic plasticity on the other hand, is thought to globally affect synaptic transmission, thereby keeping the relative differences in synaptic efficacy intact (Turrigiano 2008; Vitureira et al. 2011).

1.2.3 Synaptogenesis and synaptic elimination

The generation of functional neuronal networks during brain development is based on an extensive iteration of synaptogenesis (the formation of new synapses) and synaptic elimination (Hua and Smith 2004). Since generation and elimination of synapses is relatively fast, occurring on a scale of minutes to hours, and extensive generation/elimination of synapses is thought to continue up until puberty (Hua and Smith 2004; Bourgeois et al. 1994; Bourgeois and Rakic 1993; Zecevic and Rakic 1991), the evolving neuronal networks are expected to have the possibility of testing the functionality of a vast number of combinations. In this scenario, a disturbance of synaptic elimination is expected to decrease the functionality of the synaptic networks, since it would force the brain to keep the synaptic connections that form, regardless of functionality. Accordingly, faulty regulation of synapse numbers has been implicated in the aetiology of several psychiatric disorders (Penzes et al. 2011). Schizophrenia and autism spectrum disorders probably have aetiologies of a developmental origin, whereas AD could be the result of a faulty reactivated developmental synaptic elimination (Penzes et al. 2011; Wasling et al. 2009).

Synaptogenesis, which would be the positive regulation of n, has been quite extensively studied (Christopherson et al. 2005; Eroglu et al. 2009; Kucukdereli et al. 2011; Terauchi et al. 2010). However, as in most biological systems, the parameter n is also under negative regulation. Although this process is far less well understood, it has been shown that synaptogenesis is limited by proteins in the Nogo receptor family (Wills et al. 2012), and recent efforts have begun to unravel the mechanisms of synaptic elimination in the cerebrum.

There are good reasons to believe that LTP and LTD are involved in synaptic elimination. Since the functional synapses are those that participate in synchronous firing, and this circumstance also leads to potentiation of them, it should be expected that LTP protects from elimination, perhaps indirectly, by reducing the probability of LTD (Peineau et al. 2007) – which on the other hand, is often the consequence of asynchronous firing, thus, affecting synapses with little value in the network. In accordance, it has been shown that LTD can induce elimination of synapses (Nagerl et al. 2004; Shinoda et al. 2005; Kamikubo et al. 2006; Bastrikova et al. 2008; Becker et al. 2008).

Interestingly, it was recently shown that freezing behaviour after fear conditioning correlated with spine elimination in the frontal association cortex of mice (Lai et al. 2012). Excitatory synapses are often localized to

small dendritic protrusions called spines, and the Lai et al. (2012) study suggests that elimination of synapses are directly involved in learning; alternatively that impaired elimination leads to an inefficient neuronal network, or perhaps most likely, both. Either way, the study strongly implicates the process of synaptic elimination in the formation of memories.

The details of the elimination process are largely unknown, but it has been shown that the complement system is somehow involved (Stevens et al. 2007; Paolicelli et al. 2011; Chu et al. 2010; Schafer et al. 2012).

The complement system is probably better known for its role in the innate immune response. It consists of more than thirty proteins, mainly zymogens, which when activated, aggregate and catalyse the formation of active complement components, forming a cascade that ultimately works to eliminate foreign objects such as pathogens (Sarma and Ward 2011). This is done either by opsonisation and attraction of macrophages, or by formation of the membrane attack complex which forms a pore in the infected cell membrane and thereby induces direct cell lysis. Exactly how complement proteins work to eliminate synapses is still not known. However, elimination is most likely initiated by complement component 1q (C1q) (Stevens et al. 2007), the starting point of the classical complement cascade.

Studies of transgenic mice, lacking the genes encoding C1q and complement component 3 (C3) revealed that the complement system is involved in eliminating retinogeniculate connections (Stevens et al. 2007) and synapses in the sensorimotor cortex (Chu et al. 2010). A role for microglia has been implicated by showing how inhibition of microglial motility resulted in defective synapse elimination in the hippocampus (Paolicelli et al. 2011). In addition, Paolicelli and colleagues (2011) also reported that they found the postsynaptic density protein PSD95 in microglia, suggesting that these cells engulf synapses. A recent study corroborates this by presenting evidence for that retinogeniculate synapses are actually engulfed by microglia, at least the presynaptic portion (Schafer et al. 2012). It was further shown that this was dependent on the complement component 3 receptor (C3R) and C3 signalling (Schafer et al. 2012).

Aside from the complement system the proteins semaphorin 3A/F and 5B, and ephrin have also been implicated in synapse elimination (Bagri et al. 2003; O'Connor et al. 2009; Tada and Sheng 2006; Fu et al. 2007). Another group of proteins that have been implicated are the neuronal pentraxins (Bjartmar et al. 2006). This is interesting since some of these molecules are situated in synapses (Gerrow and El-Husseini 2007) and can bind C1q

(Sarma and Ward 2011), thus providing a potential platform for the classical complement cascade.

1.3 Synaptic pathophysiology in AD

The major clinical hallmark of AD is amnesia, and as already mentioned, Aβ seems to play a central role in AD pathogenesis. Interestingly, the pathology usually debuts in the hippocampal formation in the temporal lobe of the brain; an anatomic structure that is intimately associated with learning and memory (Squire and Wixted 2011). Since the cellular substrate for learning and memory is thought to be LTP, Aβ might in some way inhibit LTP. Indeed, quite a few animal studies have shown this for a fact (Shankar et al. 2008; Townsend et al. 2007; Walsh et al. 2002; Klyubin et al. 2008; Knobloch et al. 2007; Kamenetz et al. 2003; Chang et al. 2006; Chapman et al. 1999). The mechanism by which Aβ inhibits LTP is not clear however, but a number of pathways have been implicated through various experimental studies. The phosphatases: calcineurin (Chen et al. 2002), PP1 (Knobloch et al. 2007), and striatal enriched phosphatase (Chin et al. 2004); the kinases: p38 MAPK (Wang et al. 2004), cdk5 (Wang et al. 2004), Erk/MAPK (Townsend et al. 2007), Akt/PKB (Townsend et al. 2007), GSK3β (Koh et al. 2008; Takashima et al. 1996), PKA/CREB (Vitolo et al. 2002), and CAMKII (Townsend et al. 2007; Zhao et al. 2004); the insulin receptor (Townsend et al. 2007), the receptor for advanced glycation end products (RAGE) (Arancio et al. 2004; Origlia et al. 2009; Origlia et al. 2008), glutamate uptake (Li et al. 2009) and the prion protein (Kessels et al. 2010; Barry et al. 2011) have all been shown to be implicated in the Aβ-induced LTP inhibition.

There are also some studies suggesting that the threshold for LTD is lower in animal models of AD (Hsieh et al. 2006; Li et al. 2009; Cheng et al. 2009). This is very interesting since it has also been shown that LTD can lead to synapse elimination (Nagerl et al. 2004; Shinoda et al. 2005; Kamikubo et al. 2006; Bastrikova et al. 2008; Becker et al. 2008) and loss of synapses is the pathophysiological feature of AD brains that best correlates with the severity of the symptoms (Scheff and Price 2003; DeKosky and Scheff 1990; Terry et al. 1991).

Other studies have shown that application of Aβ to a slice of hippocampal tissue induces synapse elimination in the slice (Qu et al. 2011; Shankar et al. 2007; Shrestha et al. 2006). Knockout mice, lacking APP have been shown to have more synapses than wild type (WT) mice (Priller et al. 2006); conversely, transgenic mice overexpressing human APP have fewer synapses than WT mice (Koffie et al. 2009; Smith et al. 2009; Qu et al. 2011).

Recent animal studies have shown that synaptic elimination is dependent on the complement system. Interestingly, knockout of C1q in transgenic mice expressing human APP with FAD mutations produces a minor neuropathological phenotype (Fonseca et al. 2004). In line with this mechanism of elimination, investigation of *post mortem* brain tissue from AD patients have shown increased levels of complement mRNA (Yasojima et al. 1999), this finding has been further supported by studies showing increased levels of complement proteins in the CSF from AD patients (Finehout et al. 2005; Wang et al. 2011). In addition, it has been shown that the genes encoding complement receptor 1 (CR1) and complement factor H (CFH) are associated with AD diagnosis (Harold et al. 2009; Lambert et al. 2009; Naj et al. 2011; Seshadri et al. 2010; Zetterberg et al. 2008).

In conclusion, these findings warrant a closer investigation of the involvement of the complement system and synaptic elimination in AD.

2 AIM

2.1 The general aim

The general aim of this thesis was to examine the hypothesis that AD is primarily a synaptic disease – with an emphasis on complement-mediated elimination of synapses.

2.2 The specific aims

I. To examine if genetic variation in the gene encoding the RAGE receptor is associated with AD.

II. To investigate if the complement system is involved in elimination of synapses in the hippocampus of mice.

III. To measure the levels of C3, C4, and CR1 in CSF from patients with MCI, probable AD, MCI patients who later developed probable AD, and compare them with the levels in healthy controls, with the purpose of evaluating these proteins as potential biomarkers for AD, and to gain evidence for complement involvement in AD.

IV. To evaluate the complement genes *CR1*, *C3*, *C2* and *CFB* as possible susceptibility genes for AD.

3 METHODOLOGICAL CONSIDERATIONS

3.1 Subjects

The subjects of the different studies were chosen to best provide an answer to the questions posed in the specific aims. In general I have preferred to work with human subjects for both scientific and ethical reasons - mice do not become demented nor can they provide consent.

3.1.1 Patients

For details on diagnostic procedures and other information on the patients please refer to the different papers. The patients in our studies were diagnosed with MCI, AD or other neurodegenerative conditions using standardised clinical methods and test batteries performed by psychiatrists and psychologists at specialised memory clinics. Controls were cognitively normal according to these tests. A clinical diagnosis of AD is not perfect and it has been estimated that 10-16% misdiagnoses occur in relation to neuropathology (Brunnstrom and Englund 2009; Galasko et al. 1994; Victoroff et al. 1995), which often is considered the gold standard diagnostic method. Biomarkers for AD neuropathology, e.g., CSF levels of Aβ42 and tau proteins, may help to increase the diagnostic accuracy compared with clinical criteria (Blennow et al. 2010), but studies showing this for a fact are still lacking. Clinical AD diagnoses in papers I and IV were confirmed either by a typical CSF tau and Aβ biomarker profile or at autopsy according to the neuropathological CERAD criteria for definitive AD (Mirra et al. 1991).

3.1.2 Mice

To study the role of the complement system in elimination of synapses in the hippocampus, mice deficient in C3 were used (Pekna et al. 1998). These mice do not lack the whole gene, but the critical exon 24, which renders the gene product incomplete and thus non-functioning (Pekna et al. 1998). To ensure almost identical genetic backgrounds between WT controls (C57Bl/6) and C3 knockout mice (C3 KO), the C3 KO mice were backcrossed for 13 generations.

3.2 Genotyping

Genotyping of single nucleotide polymorphisms (SNPs) was made by use of TaqMan allelic discrimination, a polymerase chain reaction (PCR)-based assay. PCR is a method of DNA amplification, based on the molecular mechanisms of DNA replication (Mullis and Faloona 1987). A sample of DNA is heated to approximately 95ºC to make the two DNA strands separate, subsequently the temperature is lowered to 50-60ºC in order to enable hybridisation of two oligonucleotides (predesigned DNA molecules that bind to a specific region of the genome) that flank the desired sequence of the sample DNA and work as primers for the DNA polymerase. Then the temperature is raised again, to 72ºC, and the heat-stable DNA polymerase, Taq polymerase, will commence with the work of replicating the DNA sequence singled out by the primers. Thus, the sequence of interest is doubled for each cycle.

The TaqMan allelic discrimination is accomplished by cleavage of a fluorescent dye from the TaqMan probe by the exonuclease activity of the Taq polymerase as it replicates the SNP-containing sequence. Two probes are used in the reaction, one for each allele, thus enabling identification of heterozygotes and both kinds of homozygotes (Livak et al. 1995).

PCR is easy in theory but difficult to optimize in practice. In the present thesis all genotyping experiments were performed by certified lab technicians specialised in molecular genetics, using commercially available kits.

TaqMan allelic discrimination is an excellent method for genotyping a limited number of SNPs since no post-PCR handling is required, thus, minimizing the risk of contamination.

3.3 Electrophysiology

Electrophysiology was chosen as the primary method for quantifying synapses for the reason that the assessment only concerns functional synapses.

3.3.1 Hippocampal slice preparation

The hippocampus (fig. 1 in paper II) has been extensively studied in the past 40 years. It is a brain formation of fundamental importance for learning and memory, it is easily recognised, and the cell layers are arranged in a laminar way which makes the structure very suitable to use when recording

extracellular field potentials (Andersen 2007). In addition, the hippocampus is the site in the brain where AD pathology usually first appears, making it highly relevant to study considering the aims of this thesis.

The acute hippocampal slice preparation was chosen for most of the electrophysiological studies because it leaves much of the circuitry intact while still allowing for pharmacological manipulation.

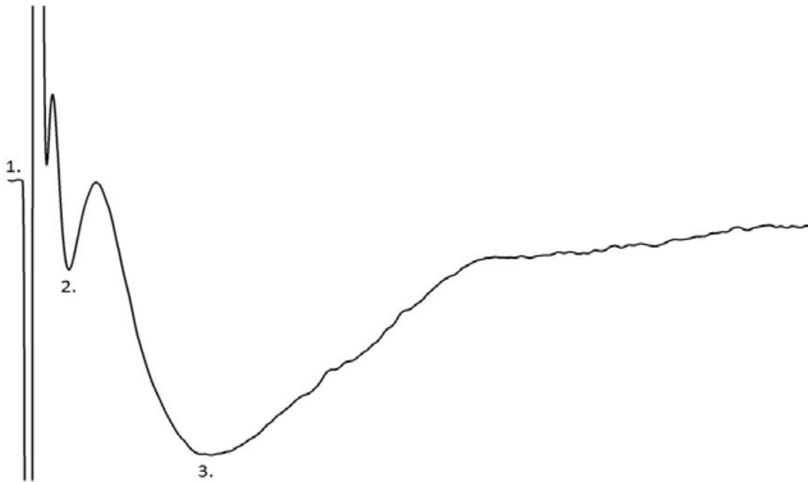

Figure 1. A typical fEPSP. The first transient deflection seen in this picture (1.) is the shock artefact generated by the electrical stimulation. The second, smaller deflection (2.) is the fibre volley which corresponds to the action potentials evoked by the stimulation. The action potentials will subsequently promote release of transmitter in the presynaptic terminals, leading to the third larger deflection (3.) which is the postsynaptic potential.

3.3.2 Extracellular field recordings

The field EPSP (fEPSP) recorded in a hippocampal slice is generated by the actions of hundreds of neurones, and subsequently tens of thousands of synapses. The pyramidal neurons in CA1 are organised in one layer with their apical dendrites projecting perpendicular from that layer. The currents generated by synaptic activity in these dendrites will flow intracellularly in the same general direction towards the soma where they will exit and flow back along the apical dendrites in the extracellular space, thereby generating an electrical field. Experimentally, such field potentials can easily be evoked

by electrical stimulation of the axons of the pyramidal cells in CA3, called the Shaffer collaterals. The recorded fEPSP will reflect the events of its making (fig. 1) and constitutes a great sample of synapses.

The strength in this approach is also its weakness; while many synapses are sampled and thus provide a robust measurement, it is impossible to differentiate between different types of synapses. The relevance of such a differentiation in the present thesis is, however, very limited.

Input/output measurements

Input/output measurements are an excellent and very straight forward way to measure synaptic efficacy. The size of the fibre volley corresponds to the number of axons stimulated (Andersen et al. 1978), whereas the fEPSP is the response from the activated population of synapses. By testing different stimulation intensities and plotting the magnitude of the fEPSP against that of the volley a measure of synaptic efficacy per axon can be obtained.

The strength of this method is its simplicity; however, this is also its weakness, since we cannot know which quantal parameters (i.e. n, p or q) are changing.

Paired pulse recordings

This type of stimulation paradigm, where two synaptic responses are elicited in close succession, is commonly used to detect changes in the quantal parameter p (Branco and Staras 2009). The size of the second pulse in relation to the first pulse is described by the paired pulse ratio (PPR), and calculated as the second pulse divided by the first pulse. When the PPR is larger than 1, the plasticity is called facilitation and taken as a sign of a low initial p (Zucker and Regehr 2002).

Interpretation of PPR data should be made with caution, however; changes in release probability as a consequence of changes in vesicle pool size, results in no or very modest changes in PPR (Hanse and Gustafsson 2001; Abrahamsson et al. 2005). Moreover, if postsynaptic changes (i.e. changes in q) occur in a subpopulation of either high- or low-release probability synapses, the PPR would change, thus falsely hint on a change in p. Nevertheless, paired pulse recordings are simple, and an excellent way to probe for changes in release probability.

MK- 801 experiments

MK-801 is an irreversible open channel blocker, specific for the NMDAR. This can be utilised to estimate release probability in an almost direct manner

since the rate of decay of the NMDAR EPSP magnitude is dependent on the presynaptic release probability (Hessler et al. 1993; Wasling et al. 2004). When a synaptic vesicle is released, the opposing NMDARs open; the resulting EPSP is recorded, but since MK-801 is present, these NMDARs are irreversibly blocked, which means that the synaptic NMDARs are turned off in a release-dependent way. A limitation of this method is that the decay also depends on the open probability of the individual NMDARs. In this study we have no information on this, and cannot exclude that there is a difference between the studied groups. There is, however, no reason to believe that knockout of exon 24 in *C3* should affect the open probability of the NMDARs.

Burst recordings

Single pulses are very common in electrophysiological experiments; however, in the hippocampus *in vivo*, neurons often fire bursts of action potentials. This is why it can be of importance to investigate how some physiological phenomena respond to burst stimulation. A problem is that the EPSPs become distorted by back propagating action potentials, to a large extent this problem can be overcome by measuring the initial slope of the EPSP, instead of measuring the amplitude or perhaps the area under the whole burst response.

3.3.3 Whole cell patch clamp recordings

The patch-clamp technique was invented by Sakmann and Neher in 1976 (Neher and Sakmann 1976). Briefly, the technique is based on establishing a high resistance seal between a glass pipette containing an electrode, and a cell, thus enabling control of the cell membrane voltage or current. By setting the voltage over the membrane, or clamping the membrane potential as it is normally put, currents flowing over the cell membrane can be measured. In the present work, this has been utilised in the simplest possible way by measuring miniature synaptic currents. The major advantage of whole cell recordings is that they enable a dissection of the quantal parameters.

In the present investigations, field recordings were done when possible, but to estimate quantal size, patch clamp whole cell recordings were necessary. Aside from being more technically demanding, whole cell experiments also carry the disadvantage of being further invasive by disrupting the intracellular milieu; this have been shown to greatly affect the possibility of inducing LTP (Malinow and Tsien 1990), but it has most likely not affected the results presented here.

3.4 Behavioural testing

To investigate if the C3 KO mice displayed an abnormal learning ability, we assessed their aptitude for contextual and cued fear conditioning. Two paradigms of fear conditioning (brief delay and trace), were tested.

Training was carried out in two different settings (differing in visual appearance and smell), one setting was safe (no shocks delivered) and one setting was unsafe (shocks delivered). The shocks were paired with tones. In the brief delay paradigm the tone and shock were temporally close, whereas they were more separated in the trace paradigm.

The basic concept is that contextual fear conditioning (using the cues that are present in the conditioning box throughout the entire conditioning session) tests hippocampal-dependent learning and memory, while cued fear conditioning (using the cue that is explicitly paired with the shock, the tone in this case) tests hippocampal-independent/amygdala-dependent learning and memory. Animals with hippocampal lesions can still learn cued fear conditioning, but are impaired on contextual fear conditioning (Otto and Poon 2006). Cued fear conditioning becomes hippocampal-dependent when there is a longer delay between the end of the tone and the beginning of the shock. Hence, memory of the tone-shock association using the brief delay procedure reflects hippocampal-independent memory, while memory of the contextual cue-shock association in either procedure and memory of the tone-shock association in the trace delay procedure reflect hippocampal-dependent memory.

3.5 Antibody- based assays

The biggest issue with antibody-based assays is the specificity of the antibodies used. In the present work, all antibodies used were commercially available and specificity was assured by the manufacturers. No additional validation was performed.

3.5.1 Immunohistochemistry

In order to corroborate the electrophysiological findings, immunohistochemical detection of synapses was performed. Thin sections of hippocampal tissue were made. These were incubated with an antibody specific for the presynaptic protein VGLUT1, the primary antibody. This was followed by incubation with a fluorescently labelled antibody against the primary antibody, thus providing amplification of the signal, while

maintaining the specificity of it, and enabling visual detection of presynaptic terminals. Images were then taken by use of confocal microscopy.

Because of the small size and vast amount of synaptic puncta, an automated high content analysis of the images was performed (Dragunow 2008) by an investigator blind to the genotype of the sections.

3.5.2 Western blot

Western blot is a commonly used method to detect a specific protein in a sample. Relative difference in amounts of the protein can also be estimated.

Briefly, proteins were extracted from freshly dissected hippocampal tissue, and total protein concentrations were measured. Subsequently, equal amounts of protein were added to a gel, and under the influence of an electric field the proteins run down the gel, distributed according to size. Then the proteins were transferred to a membrane that was incubated with a primary antibody, followed by incubation with an enzyme linked secondary antibody which catalyses a reaction producing a chemiluminescent signal which is quantified and taken as a measure of the protein.

The drawback with western blot is that it does not provide any absolute concentrations, but when the question posed only regards relative differences, western blot is a sufficient method.

3.5.3 ELISA

ELISA is an abbreviation for enzyme-linked immunosorbent assay, and that is exactly what it is. An antibody specific for the protein of interest is attached to a surface (e.g. well, plate, or beads), the sample containing the protein to be analysed is added to the surface, and as a consequence of the specific antibodies attached to this, the protein will be adsorbed to the surface (immunosorbed). This is followed by washing, thus leaving only the protein of interest on the surface, then an enzyme linked or biotinylated antibody is added. In the case of a biotin-conjugated antibody, a secondary streptavidin-conjugated enzyme is added. Thus the protein to be analysed is sandwiched between two specific antibodies and each such complex is linked to an enzyme. In the final step, a substrate for the enzyme is added, and the reaction is quantified using a set of standard samples containing known concentrations of the analyte.

An ELISA can be more sensitive than a western blot, and allows for measurement of absolute concentrations. This is necessary for analysis of all

chemical biomarkers where concentrations are indicative as opposed to mere presence.

3.6 qPCR

Real time quantitative PCR (qPCR) is a PCR-based technique for quantifying nucleic acids. In the present thesis it was combined with reverse transcription PCR, where mRNA is transcribed to its corresponding complementary DNA (cDNA). The cDNA is then amplified by PCR. Instead of quantifying the PCR product after the reaction, the PCR product is measured during the reaction in real time. Here this was accomplished by the presence of a molecule that fluoresce when it binds to double stranded DNA (SYBR GREEN), thus when the single-stranded DNA recombines in the PCR cycle. Quantification is based on comparison with a standard curve, and to correct for variation between different samples, the signal from the gene of interest is normalised to that of a stably expressed reference gene.

3.7 Statistics

Most science is based on comparisons, e.g. different experimental settings, study groups or changes over time. The common way of dealing with the analysis is to evaluate two hypotheses – the null hypothesis, according to which the difference between the groups is zero, and the alternative hypothesis, according to which there is a difference in the material. Normal variation in combination with chance can result in seemingly substantial differences between different samples; therefore it is important to estimate the element of chance. This is usually done by calculating a p-value which is the probability of the observation occurring while the null hypothesis is true. Differences are denoted as significant if the p-value fall under a stipulated cut-off level, usually set to 0.05. Thus, if the p-value is lower than 0.05, the null hypothesis should be refuted, and the alternative hypothesis accepted.

If the null hypothesis is rejected though no true difference actually exists, a Type I error has been made (the probability for this to occur is the stipulated significance level). Conversely, if the null hypothesis is accepted while a true difference actually exists, a Type II error has been made. To avoid Type II errors it is necessary to have a large enough sample size in respect to the variability in the variable investigated. The necessary sample size can be estimated by a power calculation. Statistical power is the probability of detecting a true difference, if it exists, hence of not making a Type II error.

Hypothesis testing can be made by use of a vast number of more or less complicated statistical methods. Statistical software enables non-statisticians to perform the tests without knowledge about the actual calculations. However, this comes with the risk of performing wrong tests and making faulty conclusions. This possible limitation has been restricted by seeking advice from relevant experts in the field.

Since falsely significant differences are identified by chance according to the chosen significance level, and this applies to each test being made, it is often advisable to correct the p-value for multiple testing when several tests are made. The easiest way to do this is by use of the Bonferroni method, where the p-values are simply multiplied by the number of tests that were performed. However, these methods are often very conservative, as a consequence real differences might be discarded.

Alleles in a randomly mating population without any evolutionary selection pressures will occur in stably fixed proportions. This is called Hardy-Weinberg equilibrium. In the gene association studies, Hardy-Weinberg equilibrium was assessed by comparing theoretical genotype distributions, calculated on the basis of observed allele frequencies, with observed genotype frequencies using χ_2-test. This ensures detection of genotyping errors that could otherwise lead to false associations. However, Hardy-Weinberg disequilibrium might also reflect natural selection as a consequence of an actual association.

In gene association studies, risk estimations for carriers of the susceptibility gene are relevant, not only to estimate the risk, but also as a measure of the effect size of the studied gene. Since most diseases are uncommon, a randomly selected cohort or a selection of carriers and non-carriers, are very impractical because of the large sample size needed in such approaches. The common way is instead to use a case-control design. However, this means that a proper risk ratio cannot be calculated, since the study population was not randomly selected from the whole population. Instead an odds ratio (OR) has to be used. The OR represents the risk of an outcome and is interpreted in relation to 1 which means that the subject under study has no effect on risk. An OR of more than 1 means an increased risk, whereas an OR of a number less than 1 should be interpreted as protective.

4 SUMMARY OF RESULTS

4.1 Association of RAGE with AD diagnosis

In an attempt to provide a link between recent animal studies showing that the RAGE receptor mediates the LTP inhibiting effect of Aβ, and actual human AD patients, we investigated the genetic variability in the gene encoding RAGE (somewhat confusingly the gene is named advanced glycation end-products receptor (*AGER*)), and it's relation to AD.

While working on this project, a study (Li et al. 2010a) on the very same subject was published. This group showed an association of the functional 82S SNP with AD in a Chinese cohort. In the field of genetics this is actually a good thing, since independent replications in separate populations are needed. In contrast to the study by Li et al. (2010) all AD cases in our material were neurochemically confirmed and of European origin (further demographics are given in Table 1, paper I).

Our study investigated some additional SNPs in *AGER* (Table 2, paper I), although none of these were associated with AD, the 82S allele was associated with an increased risk of AD (Pc=0.04, OR=2.0, 95% CI 1.2–3.4) (Table 3, paper I). There was no genetic interaction between *AGER* 82S and *APOE* ε4 in producing increased risk of AD (p=0.21) (Table 4, paper I), and none of the *AGER* SNPs showed association with Aβ42, T-tau, Ptau181 or MMSE scores.

RAGE may affect both production and accumulation of Aβ in the brain (Chaney et al. 2005; Cho et al. 2009; Deane et al. 2003). Since the 82S variant of RAGE has an increased ligand-binding affinity (Hofmann et al. 2002; Osawa et al. 2007) this could lead to increased signalling, which in turn, would accelerate APP-processing through BACE1 (since BACE1 has been shown to be positively regulated by RAGE (Cho et al. 2009)), and thereby increase Aβ production. An increased transport of peripheral Aβ into the brain would be expected as well, since RAGE has been shown to transport Aβ across the blood-brain barrier into the brain (Deane et al. 2003). However, we found no association between G82S genotype and Aβ levels. Another possibility is that the 82S variant may be more effective in mediating the LTP-inhibiting effect of Aβ (Arancio et al. 2004; Origlia et al. 2009; Origlia et al. 2008), a hypothesis that remains to be tested.

In conclusion, the present results, together with those from Li et al. (2010), suggest that *AGER* is a susceptibility gene for AD.

4.2 Complement mediated synapse elimination in the hippocampus

The complement system has been shown to be involved in elimination of retino geniculate synapses (Stevens et al. 2007) and synapses in the sensorimotor cortex (Chu et al. 2010) of mice.

Considering that the first brain region to be affected in AD is the hippocampus, and that loss of synapses is perhaps the most relevant feature of the disease; investigation of the mechanisms of synapse elimination in the hippocampus is highly warranted.

Thus we decided to investigate if the number of synapses is altered in mice lacking C3. The hypothesis was that these mice should have an increased number of synapses as a result of impaired synaptic elimination.

An increased number of synapses should lead to an increased synaptic efficacy, when measured as the average synaptic response to a given number of stimulated axons. Surprisingly, the input/output measurements showed no difference between C3 KO and WT animals (Fig. 1, paper II). However, there was an increased PPR, indicating lower release probability in the C3 KO mice (Fig. 2, paper II). According to the formula for synaptic efficacy, $n*p*q$, a decreased release probability could potentially mask an increased synapse number. In order to ascertain the decreased release probability in the C3 KO mice, we recorded NMDAR EPSPs in the presence of MK-801. The resulting MK-curves showed that the release probability was indeed lowered (Fig. 3, paper II), thus supporting the original hypothesis that C3 KO mice have an increased number of synapses.

This conclusion assumes that the quantal size remain unchanged. To test this, we made whole cell patch clamp recordings of miniature EPSCs. Since no difference in the size of the AMPAR EPSCs was observed (Fig. 4, paper II), we conclude that quantal size is not changed in C3 KO mice. In conjunction with a decreased release probability and unaltered input/output, the only sensible conclusion is that the C3-deficient mice must have an increased number of synapses.

This conclusion gained further support when we analysed the result of the burst stimulations. C3 KO mice displayed an increased synaptic response to burst-stimulations (Fig. 5, paper II), probably due to the normalising effect on release probability of the short-term plasticity induced by the burst.

To corroborate the electrophysiological findings, we made immunohistochemical stainings of VGLUT1 in thin sections of hippocampal tissue from WT and C3 KO mice. Again surprised, we found that C3 KO mice had a lower density and smaller puncta of VGUT1 staining (Fig. 6, paper II), thus implying fewer synapses. An explanation for this result that would still fit together with the previous results, is, that in the C3 KO mice, synapses are more numerous, but smaller, so small that they creep under the detection level for immunohistochemistry. This rational comes with the prediction that the VGLUT1 protein levels in C3 KO mice should not be lower than in WT mice. Indeed, western blot and qPCR experiments showed that VGLUT1 expression was not different between C3 KO and WT mice, thus supporting the notion of more numerous but smaller synapses. In further support of this notion, it has been shown that mice lacking C1q, a protein found upstream of C3 in the classical complement cascade, in fact, have smaller synaptic boutons (Chu et al. 2010).

Considering the importance of proper synapse elimination during development, C3 KO mice should display some learning disabilities. We confirmed this prediction by subjecting the animals to fear conditioning, showing that the C3 KO mice were blessed with an impaired memory for the foot shocks when the learning paradigm was hippocampus-dependent (Fig. 7, paper II). Interestingly, this opens for the possibility that synaptic elimination in the amygdala is not impaired by the lack of C3.

In the last experiment of this study, we explored the possibility of an increased propensity for epileptiform activity in the brains of the C3 KO animals. Such an effect was seen in the previous study of C1q deficient mice (Chu et al. 2010) and could possibly be explained by the increased burst response reported in the present study. This notion is further supported by the fact that inhibitory GABAergic synapses do not seem to be eliminated in a complement-dependent manner (Chu et al. 2010). Therefore we recorded spontaneous activity *in vivo* in C3 KO and WT mice. We did, however, not observe any spontaneous seizures in the C3 KO mice (Fig. 8, paper II).

In conclusion, these results implicate the complement system in the elimination of glutamate synapses in the hippocampus. They also suggest that the increased neuronal activity, an expected consequence of deficient

elimination and too many glutamate synapses, is partially compensated by reduced release probability, possibly via homeostatic plasticity.

4.3 Complement levels in AD CSF

Previous reports have shown that *post mortem* brain tissue from AD patients have increased levels of complement mRNA (Yasojima et al. 1999), and that AD CSF has increased levels of a certain isoform of C4b (Finehout et al. 2005), C3 and factor H (Wang et al. 2011). Since the complement system seems to be involved in AD we evaluated the complement proteins, C3, C4 and CR1 as potential CSF biomarkers for AD.

C3 and C4 CSF levels were significantly higher in AD patients as compared to stable MCI patients (Table 1, and Fig. 1, paper III). Since the core biomarker patterns were similar between the control and stable MCI groups, and the MCI-AD and AD groups (Table 1, paper III), these were merged into a control/stable MCI group and an MCI-AD/AD group (Fig. 2, paper III). Statistical comparison of these two groups showed that the CR1 levels were significantly increased in the MCI-AD/AD group. C3 and C4 levels were not significantly different between the groups, but there was a trend towards higher C3 levels in the MCI-AD/ AD group ($p = 0.068$).

To evaluate if the levels of C3, C4, or CR1 could be of diagnostic utility, receiver operating characteristic (ROC) curves were created for the different analytes. The core biomarkers T-tau, P-tau and Aβ42 could differentiate AD from controls with an area under the curve (AUC) of 0.96. When we added the complement proteins to this model, the AUC did not improve (Fig. 3, paper III). The variable importance in the projection (VIP) plots show the contributions of the different analytes to the model (Fig. 3, paper III). Although C3 and CR1 contributed to some extent, the magnitude was too small to be of clinical use.

In addition to the primary analyses described above, we also investigated potential correlations between the different variables in the two merged groups (Table 2, paper III). The results showed that the levels of the complement proteins correlated with age and albumin ratio, an indicator of the blood-brain barrier function. Correlations were also seen between age, MMSE and albumin ratio.

The correlation between complement levels and age has been reported in a previous study (Wang et al. 2011) and is somewhat unsettling considering the significant differences in age seen between the diagnostic groups in the

present study. However, the correlations of complement factors with age were not straight-forward. When examining correlations in the four subgroups separately, the correlations remained in controls and stable MCI patients, but disappeared in the AD subgroups, in spite of a large age span in those groups as well (Table 1, paper III).

As expected, T-tau and P-tau levels were correlated in both groups. T-tau and P-tau were also correlated with age, Aβ42 and CR1 in the non-demented subjects. In AD patients, on the other hand, we observed a weak correlation between MMSE and Aβ42, MMSE and C4.

In conclusion, the results of this study speak for complement involvement in the disease process occurring in patients with AD. None of the complement proteins C3, C4 and CR1 are, however, suitable as CSF biomarkers for clinical use.

4.4 Complement gene SNPs in AD

Clearly the genes of the complement system are interesting as candidate susceptibility genes for AD. *CR1* and *CFH* have been implicated by previous studies (Harold et al. 2009; Lambert et al. 2009; Naj et al. 2011; Seshadri et al. 2010; Zetterberg et al. 2008; Ferrari et al. 2012; Hu et al. 2011). For the present gene association study, we selected genes that had previously been found associated with the neurodegenerative disease age-related macular degeneration (AMD) which in turn is epidemiologically linked to AD (Kaarniranta et al. 2011; Kirby et al. 2010). These genes were: *C2*, *C3*, and the gene encoding complement factor B (*CFB*). We also included *CR1*, since this gene has been associated with AD in several genome wide association studies (Harold et al. 2009; Lambert et al. 2009; Naj et al. 2011; Seshadri et al. 2010; Ferrari et al. 2012; Hu et al. 2011).

No significant associations between the investigated SNPs and risk of AD were detected in our material (Table 3, paper IV). *C2/CFB*, (these two genes are tightly linked, and therefore inherited together) however, was associated with MMSE and T-tau in AD subjects; since the ORs are in the same direction as in previous studies on AMD (Sun et al. 2012) and the overlaps of the confidence intervals are substantial, it is quite possible that we failed to detect an actual association in this study. The same rational goes for the *CR1* replication. Meta-analysis of the SNP investigated here ends up with an OR=1.17 (www.alzgene.org) and is well within our estimated CI=(0.84 – 1.37), thus our result supports rather than refutes the previously found associations.

In conclusion, the study failed to detect any associations of the selected complement SNPs and AD diagnosis. An association of the *C2/CFB* SNPs with T-tau levels and MMSE was found. These two parameters reflect the seriousness of the symptoms, and the association lends some support to the hypothesis of aberrant complement regulation in AD.

5 DISCUSSION

In the present thesis I have explored some predictions of the hypothesis that AD is primarily a synaptic disorder. I have shown that a variant of the gene encoding the RAGE protein, which has been linked to synaptic dysfunction in animal models of AD, is associated with AD diagnosis. This study was followed by animal experiments in which the mechanisms of synaptic elimination in the hippocampus were investigated. Consistent with the results from previous studies in other brain areas, we found that synapses were eliminated in a complement mediated manner. This finding led us to explore certain neurochemical and genetic aspects of the complement system in patients with AD. The investigations showed a trend towards increased complement levels in AD CSF, and an association of *C2/CFB* variants with MMSE and tau levels in AD patients.

In the following section I will present a general discussion on the pathogenesis and pathophysiology of AD. I will present a possible sequence of events that lead to AD, and discuss the results presented in my thesis in this hypothetical context. Finally, I will give my view on the ultimate reasons as to why people develop AD.

5.1 The events leading to AD – a proposed model

The common conception is that in AD, Aβ kills neurons. This is in principal correct, but several crucial steps in the process have been overlooked. Without doubt Aβ as well as tau are involved in AD. However, it is the number of synapses that best predict the severity of the dementia (DeKosky and Scheff 1990; Scheff and Price 2003; Terry et al. 1991). This comes as no surprise since synapses constitute the physical locus of memory storage. Lastly, AD is associated with a loss of neurons; this is, however, occurring in the later stages of the disease.

5.1.1 It all starts with Aβ

In FAD, the disease causing mutations all affect Aβ processing, either by favouring the more toxic 42 amino acid long variant, or by simply promoting production, with increased amounts as a consequence. In SAD, however, no such linear relationship exists. A multitude of risk factors, foremost genetic ones, are thought to cause the disease. Aside from ageing, APOE ε4 is the most prominent risk factor, and is known to be involved in Aβ clearance.

Perhaps compromised Aβ clearance is an initial culprit in SAD, followed by positive feedback loops, including RAGE, which will increase the production of Aβ when the scales tip over.

5.1.2 Altered synaptic plasticity by Aβ

Aβ inhibits LTP (Chang et al. 2006; Chapman et al. 1999; Kamenetz et al. 2003; Klyubin et al. 2008; Knobloch et al. 2007; Shankar et al. 2008; Townsend et al. 2007; Walsh et al. 2002) and promotes LTD (Cheng et al. 2009; Hsieh et al. 2006; Li et al. 2009). Several mechanisms have been proposed (Pozueta et al. 2012); of particular interest is a study showing that Aβ blocks glutamate uptake, thereby altering the induction thresholds for LTP and LTD, such that LTD is promoted on the expense of LTP (Li et al. 2009).

"What about tau?" some would ask. Tau is most certainly involved in AD pathogenesis (Hardy et al. 1998). No FAD-mutations are found in the gene encoding tau, however. Mutations do exist in *tau*, and causes dementing disorders such as frontotemporal dementia with Parkinsonism, this is, however, not associated with any Aβ pathology (Hardy and Selkoe 2002). Thus, it seems that in AD, Aβ is the causative agent, and that tau follows. Nevertheless, tau has been shown to be necessary for Aβ to inhibit LTP (Shipton et al. 2011) suggesting that Aβ is sufficient to initiate the pathologic cascade, but also that tau is necessary for its continuing rampage.

A possible reason for the shift towards LTD could be an increased tonic NMDAR signalling since it has been shown that increasing the extracellular concentration of glutamate by blocking the glutamate uptake promotes LTD (Li et al. 2009; Yang et al. 2005). Additional findings pointing towards dysregulation of NMDARs in AD are those that have implicated the prion protein in Aβ pathophysiology (Stys et al. 2012; You et al. 2012; Parkin et al. 2007; Griffiths et al. 2011). The prion protein regulates NMDAR desensitisation, and it has been shown that Aβ perturb this function of the prion protein, thus increasing the tonic activity of these receptors. This could potentially explain the findings of Aβ-induced excitotoxicity as well as the shift towards LTD and eventually apoptosis.

5.1.3 Synapses are marked for destruction

A possible link between LTD and elimination of synapses is a family of proteins called neuronal pentraxins (NP). These have been shown to be necessary for LTD in the hippocampus (Cho et al. 2008) and serve as binding sites for C1q (Perry and O'Connor 2008). Moreover, the levels of certain NPs

are increased in FAD CSF (Ringman et al. 2012). NP1 and NP2 (a.k.a. NARP) are secreted molecules that bind to, and cluster AMPARs. NP receptor (NPR) is a transmembrane protein that binds to AMPAR and NP1 and NP2, thereby potentially anchoring AMPARs in the synaptic cleft. Stimulation of LTD (which would be increased by Aβ) also stimulates the enzyme tumor necrosis factor-alpha converting enzyme (TACE) to cleave the transmembrane domain of NPR, thus releasing it from the membrane (Cho et al. 2008). This event is associated with endocytosis of AMPAR and LTD (Cho et al. 2008). One can speculate that the released AMPAR-NP complex, act as an initiation point for the complement cascade when it moves outside the synaptic cleft. The complement proteins that would then bind to the AMPAR-NP complex could be of various origins. The main source of complement proteins in the body is the liver. A compromised blood-brain barrier can give peripheral complement access to the brain. This could be of pathological relevance, and it is interesting to note that Aβ-RAGE interaction can disrupt the blood-brain barrier (Kook et al. 2012). This pathway is not necessary, however, since complement is also produced in the brain. In the brain, complement components are secreted by both glia and neurons, mainly during development, but also in the adult brain in response to disease or trauma (Veerhuis et al. 2011; Stephan et al. 2012). Interestingly, Aβ can modulate complement activation (Wang et al. 2009), and knockout of C1q in a mouse model of AD reduced the decrease in synapse markers and activation of glia, otherwise observed in this model (Fonseca et al. 2004).

5.1.4 Engulfment of synapses by microglia

Several scenarios could follow once complement has marked a synapse. The complement cascade might reach its final stage in forming the membrane attack complex with subsequent lysis of the synapse as a consequence. Another possibility would be that the complement molecules act as a phagocytosis signal, attracting microglia that would respond by engulfing the synapse. Microglia have been implicated in the events of synaptic elimination by impairing their motility, and the observation of synaptic proteins in microglial endosomes (Paolicelli et al. 2011). Since hindering microglial motility impairs synaptic elimination, their role is conceivably beyond merely engulfing debris from cell-autonomous elimination. Therefore it seems more likely that complement marks synapses for elimination, and that this process is carried out in a most direct manner by phagocytosing microglia.

5.1.5 Neuronal death

An interesting consequence of fewer synapses is that it means less LTP, which aside from impaired learning abilities, also would lead to a decrease of

trophic factors to the cell (Hardingham and Bading 2010). Moreover, LTD is dependent on the apoptosis protein caspase-3 (Li et al. 2010b). It is conceivable that excessive LTD in the absence of LTP drives the neuron to apoptosis. In this respect it is interesting to note that overexpression of NP1 increases the levels of activated caspase-3, the number of apoptotic cells in neuronal cultures (Abad et al. 2006) and that caspase-3 is increased in synaptosomes from AD brains compared to controls (Louneva et al. 2008). In addition, NP1 knockdown rescues these cells from Aβ induced toxicity (Abad et al. 2006). Moreover, *post-mortem* analyses of AD brains have shown that NP1 is associated with senile plaques and the synaptic protein SNAP-25 (Abad et al. 2006).

5.1.6 Summary of the model

To sum up, Aβ inhibits LTP and promotes LTD. This leads to cleavage of NPR, which enables AMPARs with associated NPs to escape the synaptic cleft, as they get overrun by complement binding to the NPs they attract microglia that engulf the synapse. The declining numbers of non-plastic synapses manifest as anterograde amnesia in the patient; decreasing synapse numbers and extensive LTD ultimately leads to accumulating apoptosis promoting factors and the subsequent death of neurons, manifesting as retrograde amnesia, and in the end, death of the patient. The model should be regarded as a working hypothesis, and is in need of rigorous testing. Outlined below is a discussion on how the results presented in this thesis render some support for some of the steps in the model.

5.2 The present results in relation to the model

RAGE has been implicated in the production of Aβ (Cho et al. 2009), as well as the transport of Aβ from the periphery to the brain over the blood brain barrier (Deane et al. 2003). Thus, the 82S variant of the RAGE receptor, which has an increased ligand binding affinity (Hofmann et al. 2002; Osawa et al. 2007), could lead to increased levels of Aβ. Although we did not observe any associations of *AGER* genotypes with Aβ levels, it is possible that such an association is visible only in the early stages of the disease, since high affinity would be most relevant in a context of low ligand concentration. Moreover, RAGE has been shown to mediate the Aβ-inhibition of LTP (Arancio et al. 2004; Origlia et al. 2009; Origlia et al. 2008), and to activate microglia (Bianchi et al. 2010). Aβ levels, LTP-inhibition, and activation of microglia are all important features of the proposed model, and by linking the

82S allele of *AGER* to AD, we have provided circumstantial evidence in support of the model.

Complement mediated synaptic elimination is a key feature of the proposed model. Our results suggest that synapses in the hippocampus are eliminated through the actions of the complement system. This is a basic requirement for the model to hold up, since AD pathology primarily affects the hippocampus. These results were obtained in animal experiments; mice, do not become demented, however, and these results are in need of confirmation in humans. If true, however, a prediction that follows is that CSF complement levels would be altered in AD as a result of on-going synapse elimination in the hippocampus. Therefore, we investigated the levels of complement proteins in CSF from AD patients. Our results revealed a trend towards higher levels of complement in CSF from AD subjects, thus, supporting the model by suggesting increased complement activation in patients diagnosed with AD.

In light of the previous findings linking aberrant complement regulation to AD, we hypothesised that some SNPs in genes encoding complement proteins could be associated with AD diagnosis. The selected SNPs did, however, not show any associations with diagnosis of AD. An association would have had to be regarded as support for the model, but the fact that we did not find any associations do not really speak against it. Moreover, the results were likely a consequence of an overestimated effect size on our behalf, to detect the differences with a satisfactory precision, a larger sample size would have been needed. We did, however, find an association of *C2/CFB* genotype with MMSE and t-tau in AD patients. This is interesting since CFB is a part in the alternative pathway of the complement cascade; this pathway mainly functions to amplify the cascade and would thus not initiate it. This could mean that AD patients that carry the minor *CFB* allele have a more intense complement cascade, and thereby more adverse symptoms of the disease (i.e. lower MMSE and higher t-tau). This reasoning also lends some support to the model, in which complement activation is one of the key events.

A wide range of methods has been used during the work on this thesis, thus approaching a holistic view on the subject. The downside of this, however, is that it limits the depth of the investigation. My primary interest has been disease mechanisms and pathogenesis, a subject that is often researched by use of animal models. I have mostly studied human subjects, however. This is of major importance since only humans develop AD. Regrettably, our material was somewhat heterogeneous with respect to age between the

diagnostic groups; on the other hand we have used a well characterised material so the diagnoses are quite certain

5.3 Final speculations on AD pathogenesis

The proposed sequence of events leading to AD could explain how people get sick, but not why. The initial piece of the puzzle, the ultimate explanation, is still missing in the proposed model.

A common conception is that given enough time everyone will develop AD. Although not proven for a fact, this notion has something to it. The question should perhaps not be if a person will develop AD; rather, the right question would be when. Aging is the major risk factor for AD. I will argue that genetics set the stage, and that over time the brain will do the rest. The neural network of the brain is a complex system. As such it potentially ends up in an attractor. Perhaps dementia is an inevitable point-attractor, and our brains have evolved to handle just so many iterations, i.e. the passing of information through the system. The system constantly changes in response to the input it receives, with synapses growing stronger and weaker, but perhaps, given enough time, many synapses will have become so strong that they are no longer significantly weakened, with a non-plastic network as a result. This notion is supported by findings showing that senescent rats have larger unitary EPSPs, as well as a lowered threshold for LTD and a partially impaired LTP (Burke and Barnes 2010). Aβ is released in a stimulus dependent manner (Cirrito et al. 2005), and it might be that very strong synapses release more Aβ, thus dooming the surrounding synapses, and thereby eventually dooming themselves. In this context it is interesting to note that APOE ε4 transgenic mice show a loss of synapses with aging, and that this is accompanied with larger synapses (Cambon et al. 2000).

Genetic background would predispose different individuals to different ages of onset. APOE genotype, for instance, is associated with age of onset (Corder et al. 1993; Kurz et al. 1996; Poirier et al. 1993). A simple life without intellectual challenges would also be a risk factor, since exposing the network to the same kind of input over and over again, with little variation would bring it to a non-plastic state much faster. Indeed, participation in cognitively stimulating activities is associated with decreased risk of AD (Wilson et al. 2002). In terms of the "point-attractor hypothesis" sketched above, genetic background and types of input would determine the number of iterations the neuronal network of an individual can withstand.

Probably the setup of genes of a person, should also be regarded as a complex system, meaning that the relation between susceptibility genes and risk is not linear, and that clinical symptoms should be regarded as emergent properties of this complex system (Khachaturian 2000). Perhaps more or less specific combinations of genes work in synergy, though in different ways, nevertheless producing the same or at least similar outcome. In genetic studies of AD we look for alleles that increase or decrease the risk for the disease by comparing AD subjects with normal controls. Perhaps this is not an optimal approach. AD is a relatively common disease, and some say that eventually everyone will get it. Could it be that we fail to detect the SAD causing genes because they are too common, and thus get lost in a sort of genetic noise? This is a conceivable scenario if these genes do indeed work in synergy, by themselves posing a very small risk, but in specific clusters causing a great risk. This highlights the level on which future genetic association studies should be made, and also the importance of control subjects. Some people grow very old without developing dementia. Although it seems as if Aβ is sufficient to initiate the events leading to AD, non-demented elderly people can have substantial plaque pathology (Bennett et al. 2006; White 2009; O'Brien et al. 2009; Iacono et al. 2009). Something is clearly missing in our understanding of AD - perhaps these extraordinary non-demented people should be more extensively studied?

6 CONCLUSIONS

I. In paper I we identified *AGER*, the gene encoding RAGE, as a probable susceptibility gene for SAD.

II. In paper II we show that the key complement protein C3 is involved in elimination of synapses in the hippocampus of mice, and that the subsequent increase of synapses that follow deletion of C3 is partially compensated by a reduced release probability in glutamate synapses.

III. In paper III we concluded that C3, C4 and CR1 are not suitable as CSF biomarkers for AD. There was, however, a trend towards increased levels of these proteins in AD, and we interpret this trend as support for the hypothesis of complement involvement in AD pathogenesis.

IV. In the final paper of this thesis we concluded that there was no association of *C2*/*CFB*, *C3* or *CR1* with AD in the investigated population. This does not mean that no such association exists; it should rather be interpreted as if the association exists, the effect size is very small. Finally, we concluded that there was an association of *C2/CFB* with MMSE and high tau levels in AD patients.

Thus, in the present thesis I have presented evidence in favour of viewing AD as a disease primarily affecting synapses.

7 FUTURE PERSPECTIVES

Most scientific work increases our knowledge about the world, but perhaps even more it makes us realise the limitations of our knowledge. The work presented here is not different as it awakes at least as many questions as it provides answers for. Below I will suggest some potential future directions for those with interest and recourses.

In paper I we identify an increased risk for AD in subjects with the 82S variant of RAGE. We speculate that the 82S variant of RAGE might be more effective in mediating the LTP-inhibiting action of Aβ. This could be tested by constructing transgenic mice that carry this variant only and evaluate how they respond to Aβ. Additional gene association studies could also be made to further tie the LTP-inhibiting effect of Aβ seen in animal studies to actual AD patients. Interesting candidate genes would be those encoding key LTP proteins, e.g. the NMDAR, protein kinase C, CAMK II.

In paper II we show that C3 is involved in elimination of synapses in the hippocampus of mice. The natural course of direction here, would be to directly investigate the involvement of the complement system in AD pathophysiology, e.g. if C3 KO mice are protected from Aβ-induced synapse elimination. Constructing a double transgenic mouse, deficient in C3, and carrying human APP with a FAD mutation would be a promising approach.

Although we concluded that none of the investigated complement proteins were suitable CSF biomarkers for AD in paper III, the CSF levels of several complement proteins remain unknown. A thorough investigation of complement levels, including the activation products, is definitely warranted.

In paper IV we failed to detect any associations of the investigated complement genes, most likely due to the small effect size of most susceptibility genes for SAD. An interesting question is how many risk alleles a person can have, but not develop AD. Are the risks additive or do they work in synergy? Perhaps different forms of AD can be distinguished by specific sets of susceptibility genes. To test this holistic hypothesis a very large material would be necessary, since most susceptibility genes are quite uncommon. A way to reduce the sample size, however, would perhaps be to only include patients with known family history.

Be that as it may, the answer to the puzzle of AD will most likely be centred on synaptic function, and include the terms age and genetics.

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