Studies on the Involvement of the Immune system in Alzheimer's disease

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LIST OF PUBLICATIONS

Andrea Marcello, Oliver Wirths, Thomas Schneider-Axmann, Malin Degerman-Gunnarsson, Lars Lannfelt, Thomas A. Bayer

Reduced levels of IgM autoantibodies against N-truncated pyroglutamate Aβ in plasma of patients with Alzheimer's disease

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Pyroglutamate Abeta pathology in APP/PS1KI mice, sporadic and familial Alzheimer's disease cases

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Breyhan H, Wirths O, Duan K, Marcello A, Rettig J, Bayer TA.

APP/PS1KI bigenic mice develop early synaptic deficits and hippocampus atrophy.

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Andrea Marcello, Oliver Wirths, Thomas Schneider-Axmann, Malin Degerman-Gunnarsson, Lars Lannfelt, Thomas A. Bayer

Circulating immune complexes of Aβ and IgM in plasma of patients with Alzheimer's disease

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Inflammatory changes are tightly associated with neurodegeneration in the brain and spinal cord of the APP/PS1KI mouse model of Alzheimer's disease.

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PhD THESIS

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ABSTRACT

The main subject of this doctoral work is the role of the immune system in Alzheimer's disease (AD), and how its understanding may help to develop novel diagnostic and therapeutic options for the disease.

This study is structured in 2 parts, the first centred on mouse models of AD and in particular on the relationship between pathological features and inflammation in the brain of APPPS1/KI mice, while in the second part the focus is shifted on immune system involvement in human *in vivo*.

The APP/PS1KI mouse model for AD has been previously shown to harbour severe pathological alterations, including behavioural deficits, axonal degeneration and hippocampal neuron loss starting at the age of 6 months. We observed early activation of the inflammatory marker GFAP already at 2 months, followed at 6 months by reduced levels of pre- and post-synaptic markers. Inflammatory processes are considered to play an important role in the progression of neurodegenerative changes in AD, and these data add further evidence to their association with axonal degeneration and neuron loss.

It has previously been shown that immune complexes (IC) of a given biomarker with class M immunoglobulins (IgM) provide better performances compared to the unbound biomarker in a number of cancer entities. In the present work, we investigated IC of IgM-Aβ as a potential biomarker for Alzheimer's disease (AD). Aβ–IgM concentration has been measured in 75 plasma samples from patients with AD, individuals with mild cognitive impairment (MCI), and healthy age- and sex-matched controls (HC). Using an ELISA assay detecting Aβ–IgM complexes, we observed that high levels of Aβ–IgMs were detectable in HC and MCI patients; however, there was no significant difference to the AD group.

To overcome the impossibility to discriminate IC of specific Aβ forms, we measured in the same plasma samples the level of IgM autoantibodies directed against different Aβ epitopes as potential diagnostic biomarkers for Alzheimer's disease (AD). The mean level of anti-$Aβ_{3(pE)}$-IgM was significantly decreased in AD patients compared to HC. In the group of MCI patients there was a significant positive correlation between anti-$Aβ_{3(pE)}$-IgM and cognitive decline expressed as MMSE (rho = 0.58, df = 13, p = 0.022). These observations indicate that the level of IgM autoantibodies against $Aβ_{3(pE)}$ represents a

promising plasma biomarker for AD and correlates with the cognitive status of individuals at risk of developing AD.

These data are in line with the idea of a possible pivotal role of $A\beta_{3(pE)}$ in AD development, as shown by a novel mouse model expressing only truncated $A\beta_{3(pE)-42}$ demonstrating high neurotoxicity of this peptide. To better analyze pyroglutamate-positive plaque load in the brain, a novel monoclonal antibody highly specific for $A\beta_{3(pE)}$ has been developed.

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LIST OF ABBREVIATIONS

APP: amyloid precursor protein

ACh**:** acetylcholine

AChE: acetylcholinesterase

AD: Alzheimer's disease

ADAM: A Disintegrin And Metalloprotease

ANOVA: one-way analysis of variance

ApoE: apolipoprotein E

Aβ: amyloid-β

$A\beta_{3(pE)}$: amyloid-β protein starting at position 3 with pyroglutamate

BACE: beta-site amyloid precursor protein cleaving enzyme

BBB: blood brain barrier

CNS: central nervous system

CSF: cerebro-spinal fluid

DAB: 3,3-diaminobenzidine

EC: entorhinal cortex

F: female

FAD: familial Alzheimer's disease

FCS: fetal calb serum

FTD: fronto-temporal dementia

GSK: glycogen synthase kinase

hAPP: human APP

HC: healthy controls

HRP: horseradish-peroxidase

IC: immune complex

i.e.: id est

IHC: Immunohistochemistry

IgG: class G immunoglobulin

IgM: class M immunoglobulin

LTP: long term potentiation

M: male

MAPK: mitogen-activated protein kinase

MCI: mild cognitive impairment

MRI: Magnetic Resonance Imaging

NFT: neurofibrillary tangles

NAbs: natural antibodies

NMDA: N-Methyl-D-Aspartate

NSAID: non-steroidal anti-inflammatory drug

OxLDL: oxidized LDL

P2: synaptosome fraction

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PET: Positron emission tomography

pGlu: pyroglutamate

PHF: paired helical filaments

PS: presenilin

PS1KI: PS1 knock-in

QC: glutaminyl cyclase

RT: room temperature

RT-PCR: reverse transcription polymerase chain reaction

S2: whole brain fraction

SDS: sodium dodecyl sulfate

TBA: truncated beta amyloid

WT: wildtype

Chapter 1

Introduction

1.1 ALZHEIMER`S DISEASE

Alzheimer's disease (AD) represents nowadays the most common form of senile dementia. AD is characterized by proceeding memory deficits and ongoing loss of daily-life abilities. First signs of the early disease state are short-term memory loss and visual-spatial confusion, often accompanied by aphasia, disorientation or lack of inhibition. Many patients show changes in the way they approach their social surrounding with previously unknown violent outbursts or distinct passivity. Close relatives are normally the first to notice such changes in the character and behaviour of the person affected by AD and who is showing the first signs of cognitive impairment. As the disease progresses, together with proceeding cognitive decline, patients show also deterioration of musculature and a loss of mobility. Patients in the final stage are unable to perform even the simplest tasks, like walking or eating without assistance, suffer from incontinence of the bladder and/or the bowel, speak often in an utterly disorganized way and in the terminal phases require permanent supervision. This care is mostly achieved by close relatives of the demented persons; therefore, besides the aforementioned effects of AD on patients, it is in general the entire familiar environment which suffers the impact of AD, often pushing one family member to sign out of its job in order to assist the relative. Furthermore, most family members taking care of demented persons are themselves already in an advanced age and are therefore, due to their health conditions, not best suited to fulfil this task.
Currently, an estimated 5.3 million Americans of all ages have Alzheimer's disease, corresponding to one in eight persons aged 65 and older (13%). It has been estimated that the economic value of the care provided by family and other unpaid caregivers of people with Alzheimer's and other dementias was $94 billion, which is the results of the average wage per hour of a caregiver. One study found that 87% of caregivers of people with Alzheimer's and other dementias were taking care of a relative, and the remaining 13% were taking care of a non-relative, including a friend or neighbour (association 2009).

1.2 CURRENT TREATMENTS FOR AD

Despite the social benefit, but also the unquestioned economic potential that a treatment for AD would generate, there are to date no effective therapies on the market and likely

none is going to appear in the next few years. To date, only few medicaments are available for the treatment of AD; unfortunately, these drugs do not target the underlying disease mechanism but deliver only limited symptomatic benefits, with no overall slowdown in the progress of the disease. They are acetylcholinesterase (AChE) inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. Briefly, since a reduced activity of cholinergic neurons has been observed in AD patients, which has been proposed to have some impact on the characteristically memory loss, drugs like *Donepezil*, *Rivastigmine* and *Galantamine* aim to stabilize levels of the neurotransmitter acetylcholine (ACh) in the brains by inhibiting AChE, the enzyme responsible for its degradation; however, these drugs only delay the first symptoms by some months. NMDA receptor antagonists like *Memantine* are based on the finding that in AD sustained activation of NMDA receptors may finally lead to the degeneration of cholinergic neurons. NMDA- receptor antagonists block the excessive glutamate-induced activation of the receptor that occurs under pathological conditions in AD (Hull et al. 2006).

Epidemiological studies also suggested potential for non-steroidal anti-inflammatory drugs (NSAIDs), estrogens, HMG-CoA reductase inhibitors (statins) or tocopherol (vitamin E) to prevent AD. However, prospective, randomised studies have not convincingly been able to demonstrate clinical efficacy (Hull et al. 2006). Besides these therapeutic approaches a number of products has been suggested having beneficial properties against AD, including red wine for its content of flavonoids with antioxidant activity, omega-3-fatty acids, Gingko Biloba extracts, vitamins B and others, but none of these results has been confirmed (Morris 2009). However, what all these therapies and supplements lack is that they do not address the underlying disease pathomechanism .

Given the substantial failure of previous drugs, a new generation of pharmacological treatments is being developed. They have the potential to halt the progression of AD because they are aimed to directly lower the neurotoxic effects of the amyloid-β (Aβ, see below) peptide rather than just targeting the symptoms of the disease by restoring the activity of altered neurotransmitter function. So far, several approaches to modulate Aβ metabolism have been tried, with varying success. The most renown, and initially most promising, was the Aβ active vaccination trial AN-1792 in 80 people with AD; however, this trial was stopped early after 6% of the participants developed meningoencephalitis. Although there was no overall improvement in clinical profile in patients who received this active immunization, clearance of amyloid plaques in the brain did occur and, despite the effect being independent of antibody response, this trial provided an important proof of

concept (Holmes et al. 2008). Passive immunization with humanised monoclonal antibodies with specificity to Aβ peptides might potentially reduce side-effects, but on the other hand it presents issues relative to its multiple dosing, including lower patients compliance and potential overdose risks. However, immunotherapy is a promising strategy and several treatments are currently under clinical development, including antibodies with different specificities and of different classes.

Another category of second generation medicaments for AD aims to the modification of the amyloid metabolic pathway. PBT2 is a metal-protein-attenuating compound that affects copper-mediated and zinc-mediated toxic oligomerization of Aβ. In a randomised placebo-controlled study of 78 patients with PBT2 (Lannfelt et al. 2008), no serious adverse events were seen; compared to the placebo group, the treated group showed a significant dose-dependent decrease in cerebro-spinal fluid (CSF) Aβ coupled to an improvement in cognition in two tests of executive function. A trial with another anti-Aβ drug, *tarenflurbil* (flurbiprofen), showed a decrease in Aβ production with a good tolerance, and evidences of a dose-related effect on measures of daily activities and global function in 210 people with mild AD, however without any effect on people with moderate AD (Wilcock et al. 2008).

Dimebon, a non-selective antihistamine, has shown positive effects on both cognition and activities of daily living, with improvements at 12 months and a low incidence of side-effects in a study of 183 people with AD (Doody et al. 2008).

In any case, in order for these drugs to be successful, they should be given at the earliest stages of the disease—possibly before symptoms appear. Thus, the facility to identify people at risk or to diagnose AD at an early stage is essential. It is, in fact, becoming more and more evident that rather than therapy, the best approach in dealing with AD is prevention.

1.3 AD PREVENTION

Prevention in AD acquires great importance if we consider the extremely long asymptomatic latent period of this disease. Even an intervention that delayed disease onset by just a few years could dramatically reduce the burden of this disease on society and public health-care systems (Brookmeyer et al. 1998). To this end, there has been recently great focus on the identification of potential preventive factors for dementia, and

epidemiologic research has suggested various candidates, including modifiable lifestyle factors, such as social contacts, leisure activities, physical exercise, and diet, as well as some pharmacologic strategies, such as hormone replacement therapy, NSAIDs, and Ginkgo biloba. In addition, the treatment of vascular risk factors could be important. Some of these factors have been targeted by interventions tested in randomized controlled trials, but many of the results obtained are in conflict with those obtained in observational studies (Hull et al. 2006).

A preventive strategy based on the use of a pharmacologic treatment would seem to be a relatively simple method of preventing AD. Since an extremely high number of people would be exposed to the treatment without ever developing AD, a good risk-benefit ratio would be necessary. Until such a treatment will be discovered, lifestyle factors (diet, social engagement, cognitive stimulation, physical exercise) seem the most reasonable candidates for prevention trials at the current time, in particular because of their safety. Because of the difficulties in conducting multi-domain intervention, randomized controlled trials would not represent an cost-effective and thus economically-appealing approach, and large public health interventions at the population level could be required. However, such interventions would have to be feasible, cost effective, and easily transferable in order to have a real public health impact (Coley et al. 2008). In addition, the best results from an effective prevention strategy would be obtained by having at disposal a reliable method for early diagnosis. tToday many CSF biomarkers with a good diagnostic value exist to predict the onset of AD; unfortunately, CSF measurement is a painful and invasive exam, inappropriate to be used as mass-screening method. For this reason, other more accessible biological fluids are being examined for the presence of new potential biomarkers, but the results have been so far contradictory and not satisfying. More sophisticated diagnostic criteria need to be established that take into account the potential of current diagnostic biomarkers, whether they are in the blood, cerebrospinal fluid, or visualised by Positron Emission Tomography (PET) brain imaging (Dubois et al. 2007).

1.4 FAMILIAL AND SPORADIC TYPE OF AD

There are 2 different forms of AD: familial or early-onset AD (FAD) and sporadic or late-onset-AD, the latter representing the vast majority of the total cases. 90-95% of AD cases are, in fact, sporadic with proceeding age as the major identifiable risk factor, while only 5-

10% of the disease cases are genetically based (Tanzi 1999). FAD is caused by Mutations associated with the genes encoding the amyloid precursor protein (APP, chromosome 21), Presenilin-1 (PS1, chromosome 14), and Presenilin-2 (PS2, chromosome 1) (Fig. 1). These mutations foster abnormal processing of APP and therefore lead to overproduction of the most common Aβ peptides $A\beta_{1-40}$ and $A\beta_{1-42}$. Additionally, the ε4 allele of apolipoproteinE (ApoE) has also been shown to be associated with an increased risk for late-onset AD (St George-Hyslop 1999; Lambert et al. 2009).

However, the causes of sporadic AD, besides ageing, remain uncertain, though extensive research efforts are being made which address this issue. Widely discussed is the influence of environmental agents (e.g., heavy metals), intrinsic factors (e.g., cytokines), and dietary factors (e.g., cholesterol) (Lahiri et al. 2007).

A)

Codon	Mutation	Phenotype
665	Gln Asp	Late onset AD - no segregation
670/671	Lys-Met Asn-Leu	FAD; increased A production
673	Ala Thr	No disease phenotype
692	Ala Gly	FAD and cerebral hemorrhage; increased A
693	Glu Gly	Late onset AD - no segregation
	Glu Gln	HCHWA-D
713	Ala Val	Schizophrenia - no segregation
	Ala Thr	AD - no segregation
716	Ile Val	FAD
717	Val Ile	FAD; increased long A isoforms
	Val Phe	FAD
	Val Gly	FAD

B)

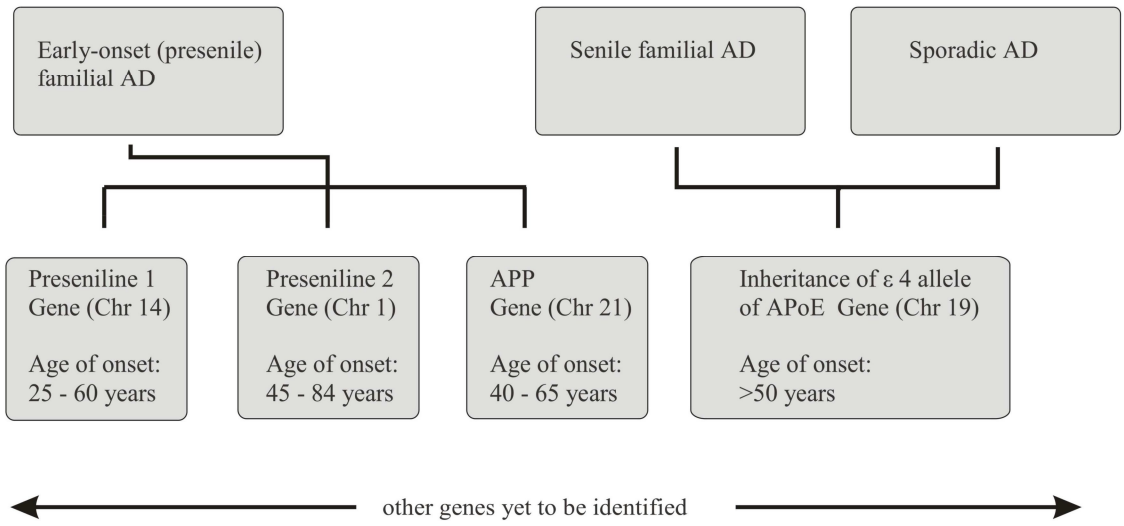


Figure 1 (A) Missense mutations in the APP gene (HCHWA-D: amyloidosis Dutch type; FAD: familiar Alzheimer disease). Data taken from (St George-Hyslop 2000) (B) Genetic mutations implicated in familiar or sporadic onset of Alzheimer's disease (according to (St George-Hyslop 1999)).

1.5 NEUROPATHOLOGICAL HALLMARKS OF ALZHEIMER`S DISEASE IN HUMAN

Neuropathologically, brains of AD patients are characterized traditionally by the presence of senile plaques consisting of Aβ and neurofibrillary tangles of hyper-phosphorylated tau-protein. Functionally, the brain presents extensive neuron loss, synaptic dysfunction, vascular deficits and inflammation.

1.5.1. Aβ PRODUCTION

Aβ is produced in the brain by enzymatic cleavage of the transmembrane protein APP (Gandy et al. 2000). APP mRNA alternative splicing produces at least 8 distinct isoforms, namely APP677, APP695, APP696, APP714, APP733, APP751, APP752 and APP770, APP695 being the most common isoform in the brain (Golde et al. 1990; Sandbrink et al. 1997).

APP processing leads to the production of different fragments, depending on distinct secretases acting on it. In the non-amyloidogenic, non-pathogenic pathway APP is cleaved first by the enzyme α-secretase, leading to the release of sAPPα, and subsequently by γ-secretase, which produces the N-terminally truncated Aβ fragment “p3”(Carter et al. 2001). α-secretase cleaves APP within the Aβ sequence at position 17, therefore p3 represents the fragment $Aβ_{17-40/42}$. As a consequence, α-cleavage of APP prevents the formation of $Aβ_{1-40/42}$ which are the most prominent Aβ-species in AD affected brains. The identity of α-secretase and its mechanism of regulation remains unclear; main candidates are the ADAM family of proteases (A Disintegrin And Metalloprotease) members ADAM9, 10 and 17 (Hiraoka et al. 2007).

In the amyloidogenic pathway, APP is initially cleaved by β-secretase and the sAPPβ fragment is released; the remaining transmembrane peptide C99 is then processed by γ-secretase leading to the generation of Aβ (Van Broeck et al. 2007). β-Secretase has been reported to be a type I transmembrane glycosylated aspartyl protease, while γ-secretase is a high-molecular-weight protein complex comprising at least the four proteins Aph-1, Pen-2, nicastrin and PS1/2, with the latter containing the active site (Guntert et al. 2006; Van Broeck et al. 2007). Though the exact intracellular cleaving sites of γ-secretase and the nature of this enzyme are still not fully understood, it is commonly accepted that γ-

secretase cleavage of C99 produces peptides of different lengths, the most prominent of which are $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ (Carter et al. 2001) (Fig. 2).

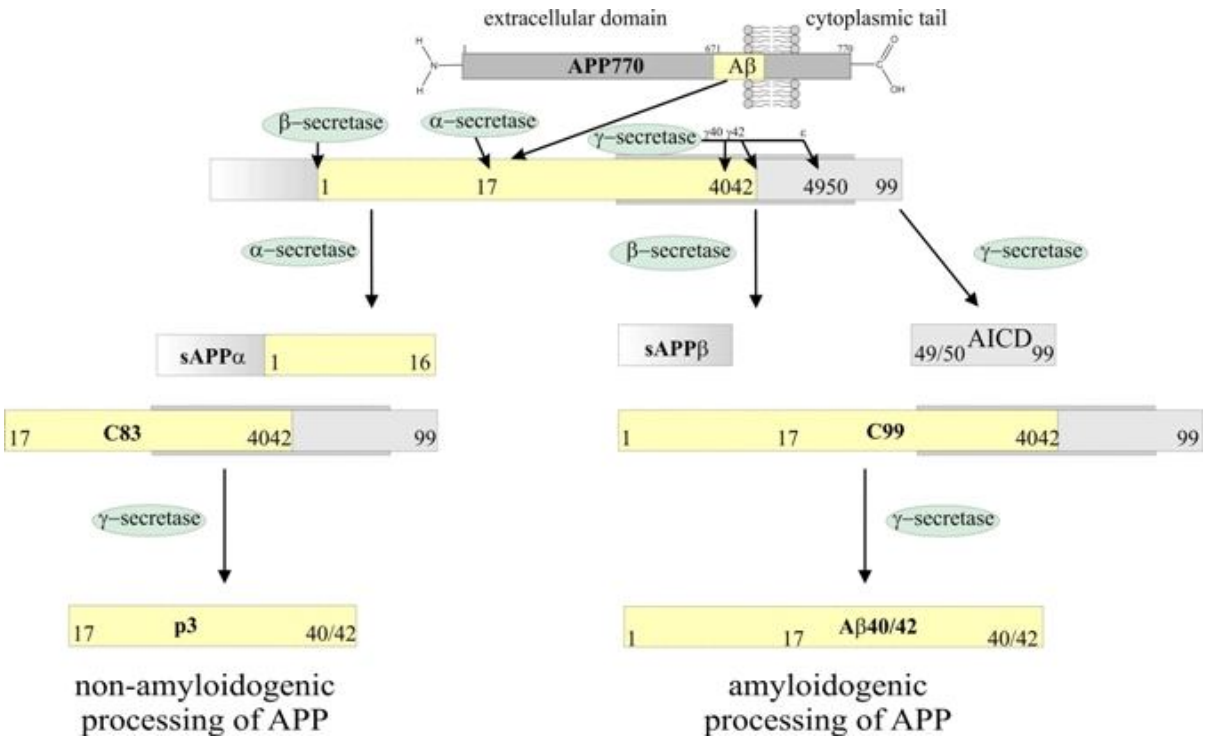


Figure 2 Non-amyloidogenic and amyloidogenic pathway of APP processing by subsequent proteolytic cleavage by **α**-/**γ**-secretase (non-amyloidogenic pathway) or **β**-/**γ**-secretase (amyloidogenic pathway) leading to the generation of $A\boldsymbol{\beta}_{1\text{-}40/42}$.

The cleavage sites of the enzymes involved in APP processing are all near to or at those positions in the APP sequence where FAD mutations have been found (Fig. 3); as a consequence, all FAD mutations in the genes for APP, PS1 and PS2 result in increased production of Aβ. Notably, mutations in PS1 and PS2 have been shown to enhance the production of $A\beta_{1\text{-}42}$ *in vitro* and *in vivo* (Xia 2000; Takeda et al. 2004). While $A\beta_{1\text{-}40}$ in human AD patients is the far most abundant Aβ species with an estimated 1000-fold excess compared to $A\beta_{1\text{-}42}$, the latter one has been shown to be much more prone to aggregate and to have highly neurotoxic properties (Selkoe 2001).

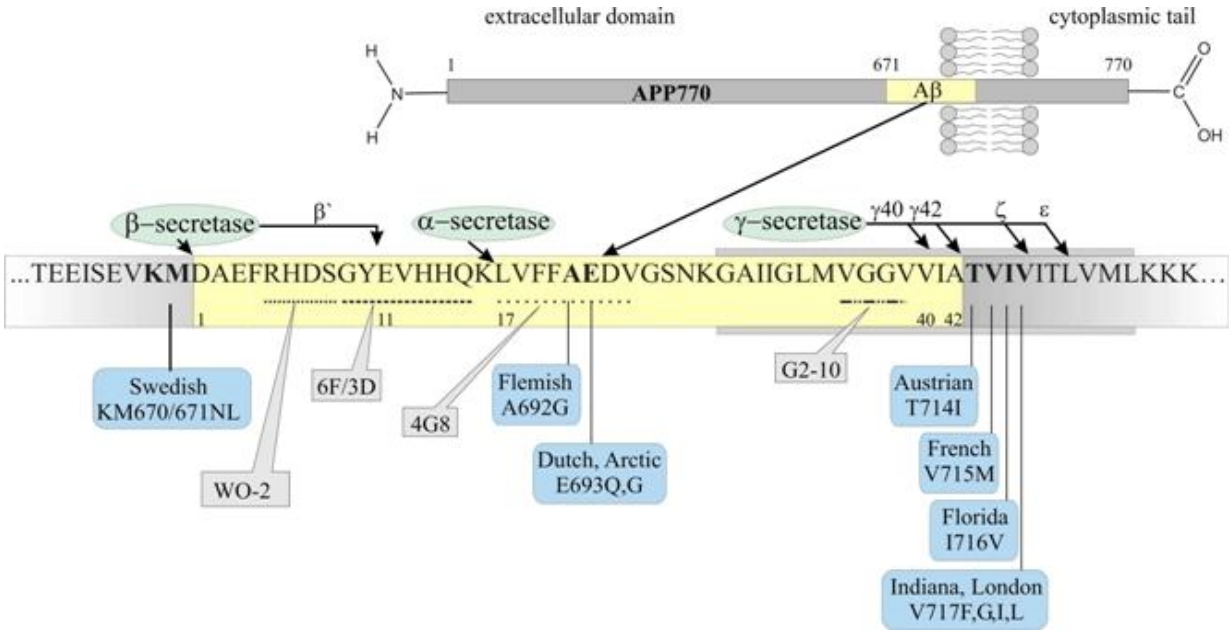


Figure 3 Schematic representation of the APP protein, including the A**β** sequence, cleavage sites of **α**-, **β**-, and **γ**-secretase, important mutations in the APP protein related to familial AD and binding sites of important antibodies.

1.5.2. Aβ PLAQUE DEPOSITION

The formation of senile plaques consisting mainly of Aβ peptides is the most evident hallmark of AD brains. It is widely accepted that production and aggregation of Aβ is a key event in the pathology of AD (Van Broeck et al. 2007).

The molecular mechanism with which Aβ initiates neurodegeneration remains so far unknown. Until recently, it was common knowledge that extracellular insoluble Aβ plaques were directly responsible for neuron loss and AD development, but recent evidences point to intracellular Aβ as being the actual main toxic agent (see (Tseng et al. 2004; Wirths et al. 2004) for review). This revised version of the amyloid hypothesis is based on the finding that Aβ accumulation initiates in the intraneuronal space while extracellular formation of plaques is a subsequent event (Masters et al. 1985; Wirths et al. 2004) (Fig. 4).

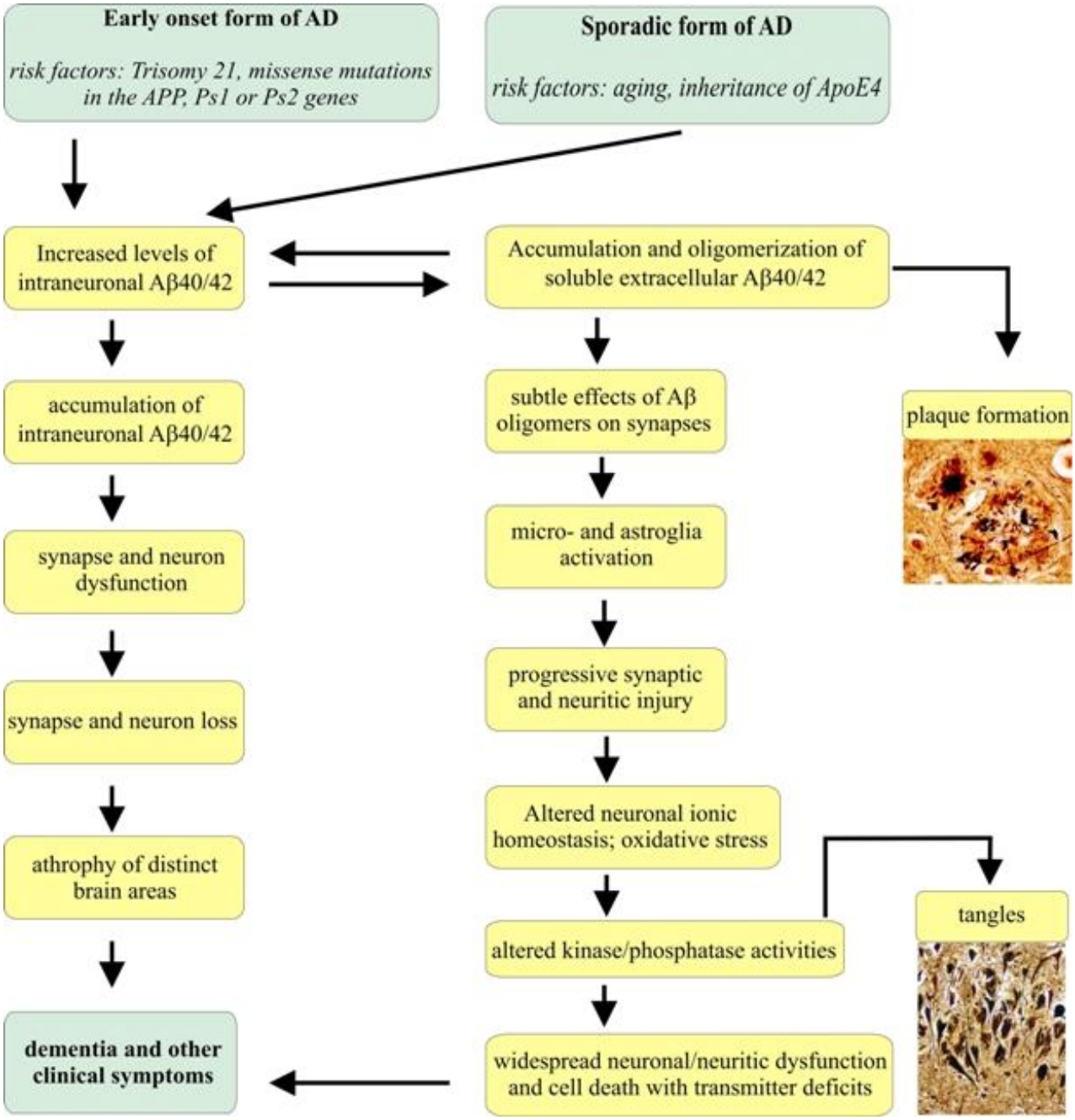


Figure 4 Two versions of the Amyloid hypothesis: Sequence of pathogenic events leading to AD pathology is shown in the classical understanding according to Hardy et al. (right part of figure) and in a revised version according to Wirths et al. (left part of figure) that focuses on the contribution of intraneuronal accumulation of Aβ42 to the development of AD pathology. Both vicious cascades may interfere and contribute in parallel as well to the cognitive decline symptomatic for AD affected patients, as suggested by horizontal arrows.

The rationale for this hypothesis was the observation of deficits in behaviour, synaptic transmission and long-term potentiation well before first signs of plaque pathology in several transgenic mouse models of AD (Holcomb et al. 1998; Hsia et al. 1999; Moechars et al. 1999). The hypothesized pathological role of intracellular Aβ was supported by the finding, among others, that this pool of Aβ represents an early and integral component of

the pathogenesis of the human muscle disorder IBM (inclusion body myositis) (Mendell et al. 1991; Askanas et al. 1993). Additionally, immunohistochemical analysis of human postmortem brain and APP/PS1 transgenic mouse brains clearly showed an age-dependent increase of $A\beta_{1-42}$ -species within neurons (Gouras et al. 2000; D'Andrea et al. 2001; Busciglio et al. 2002; Tabira et al. 2002; Wirths et al. 2004). The importance of intracellular Aβ has been supported by several recent studies ((Glabe 2001; Gouras et al. 2005) for a review). Most convincing, in an APP/PS1KI mouse model of AD a strong correlation between early accumulation of intraneuronal Aβ in CA1/2 region of the hippocampus starting already at 2 months of age and extensive neuron loss (~50%) in this area of the hippocampal pyramidal cell layer was reported (Casas et al. 2004).

Furthermore, it is now generally accepted that Aβ is most toxic in a soluble, oligomeric aggregation state (Fig. 5), rather than aggregated β-sheet-containing amyloid fibrils (Selkoe 2001; Klein 2002). It has been demonstrated that soluble oligomeric $A\beta_{42}$, and not amyloid plaques, correlates best with cognitive dysfunction in AD (McLean et al. 1999; Naslund et al. 2000), and oligomers are formed preferentially within neuronal processes and synapses rather than extracellularly (Walsh et al. 2000; Takahashi et al. 2004). Recently, our group also reported that transient intraneuronal Aβ rather than extracellular plaque pathology correlates with neuron loss in the frontal cortex of APP/PS1KI mice (Christensen et al. 2008).

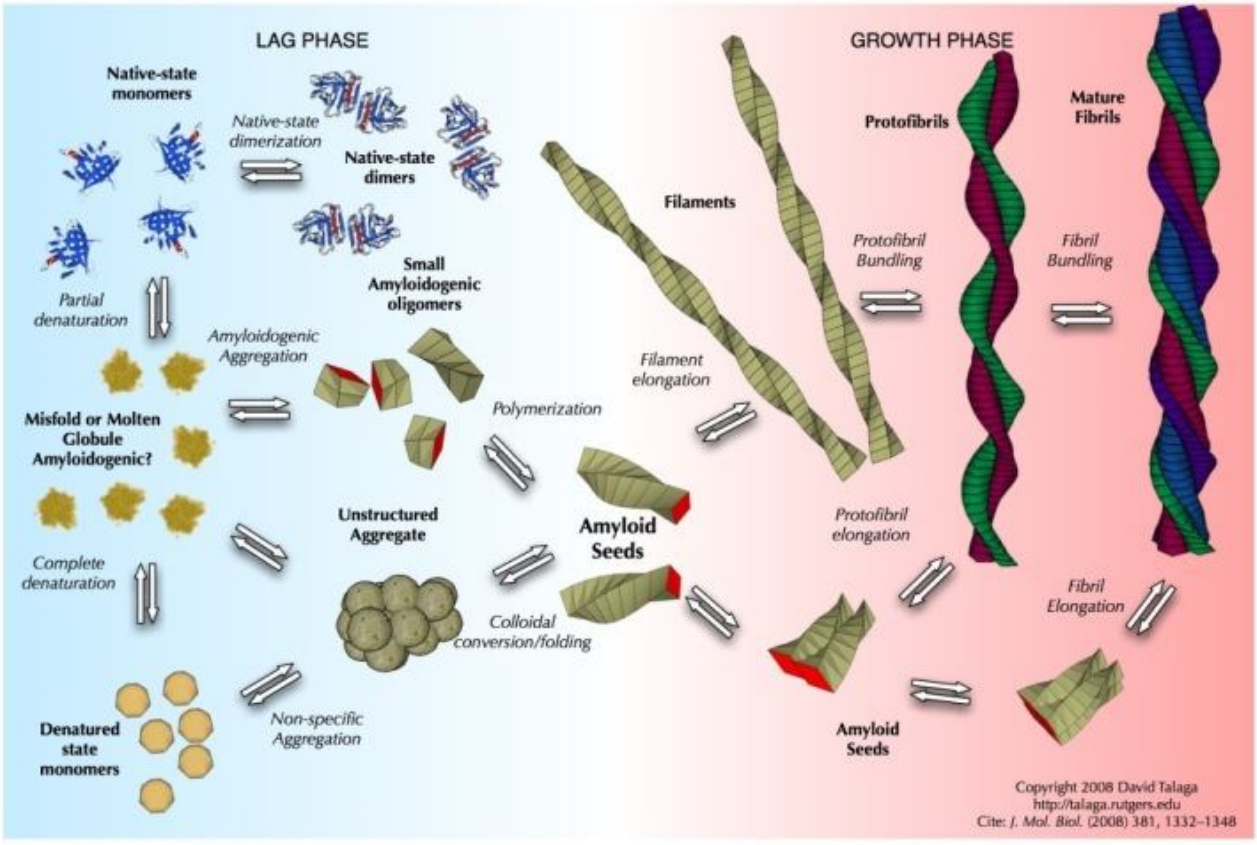


Figure 5 Possible pathways for plaque formation highlighting the different phases and aggregation states. Although mature fibrils, which eventually aggregate into plaques, are the endpoint of the process, it is not clear yet which Aβ aggregation state is best correlated to the course of the disease image taken from http://talaga.rutgers.edu/research/amyloid.php.

1.5.3. NEUROFIBRILLARY TANGLES

Brain neurofibrillary tangles (NFT) represent, together with amyloid plaques, the two characterizing hallmarks of AD. NFTs appear as bundles of abnormal filaments called paired helical filaments (PHF), composed of highly phosphorylated forms of the ~55 kDa microtubule-associated protein tau. It is commonly accepted that the high degree of phosphorylation of PHF-tau leads to microtubule disorganization and the generation of neurofibrillary lesions (Boutajangout et al. 2002). Six tau isoforms generated by alternative splicing of tau RNA are known in the human brain (Wagner et al. 1996). Tau protein binds and stabilizes microtubules and promotes microtubule assembly *in vitro* (Goedert et al. 1990); it is therefore believed to have an active role in the formation and maintenance of axons. In this regard, it has been shown that antisense oligonucleotides down-regulating tau expression in primary cerebellar neurons impair the generation of new axons (Wagner

et al. 1996). The phosphorylation process of tau takes place at serine/threonine residues preceding a proline and is mediated by a number of proline-kinases including e.g. members of the mitogen-activated protein kinase (MAPK) family, glycogen synthase kinase 3α (GSK3α) and 3β (GSK3β) or Cyclin-dependent kinase 5 (CDK5). At a cellular level, hyperphosphorylated tau is mostly found in the somatodendritic compartment of the neurons (Zheng et al. 2002) (Fig. 6).

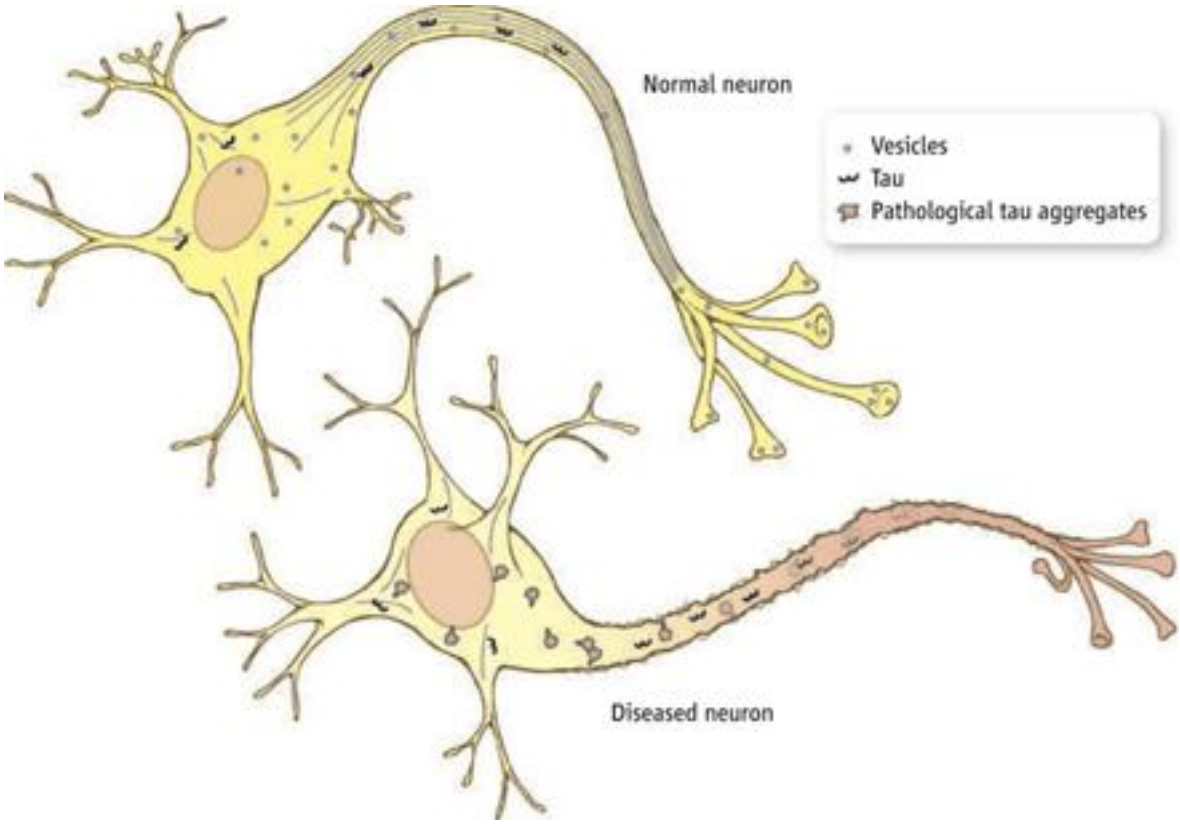


Figure 6 Tau malfunction in AD: In a healthy neuron (top), tau stabilizes the microtubules (blue lines) that transport materials to the nerve terminals. In AD, tau is unable to bind to the microtubules and forms abnormal aggregates, the so called paired helical filaments (PHF-tau). This process fosters degeneration of microtubules and induces impaired neuronal functioning (graphics taken from Marx 2007).

The precise pathophysiology of tau is still a matter of discussion. Rapoport et al. reported *in vitro* Aβ-induced neurotoxicity initiated by tau: incubation of fibrillar Aβ with cultured hippocampal neurons expressing either mouse or human tau showed clear signs of neurodegeneration, while no such effect was observed in tau-knockout mice under the same conditions (Rapoport et al. 2002). On the other hand, Zheng et al. demonstrated *in vitro*, that Aβ can induce tau hyperphosphorylation in rat primary septal cultures (Zheng et al. 2002). Microtubular destabilization provoked by hyperphosphorylated tau aggregates

has been speculated to lead to impairment of cellular transport, (Mandelkow et al. 2003), cellular geometry, and neuronal viability (Cummings et al. 1998).

1.5.4. HIPPOCAMPAL SHRINKAGE, NEURON LOSS AND SYNAPTIC DEFICITS

In AD patients, synaptic density decline in the brain appears to be the pathological hallmark that best correlates with the extent of cognitive loss during the disease development, if compared to plaque pathology, NFTs, neuron loss, and neurotransmitters deficits (Coleman et al. 2003).

Two independent studies revealed a correlation coefficient of 0.7 between synapse density and cognitive status of AD patients (Coleman et al. 2003). In well accordance with the event of synapses loss, Reddy et al. analyzed levels of synaptic proteins in brains of AD patients and reported a loss of pre-synaptic proteins (synaptotagmin, synaptophysin, and Rab3A), synaptic membrane proteins (Gap43 and synaptobrevin) and post-synaptic proteins (neurogranin and synaptopodin) in AD patients when compared to healthy controls (HC) (Reddy et al. 2005). Synaptic decline appeared to be more severe in the frontal cortex compared to the parietal cortex of AD patients (Reddy et al. 2005).

Neuron loss in AD represents a further marker of cognitive decline. Shepherd et al. reported a neuron loss in frontal superior cortex of 36% in early onset AD cases and of 22% in sporadic AD cases compared to HC (Shepherd et al. 2007). In patients with mild AD a 32% neuron loss has been observed in the entorhinal cortex (EC), an area of the cortex believed to have a crucial role as a gateway connecting the neocortex and the hippocampal formation. The EC receives afferents from widespread association cortex and limbic areas, projects to the dentate gyrus of the hippocampal formation, receives afferents from the hippocampus, and sends afferents back to association neocortex. The EC is a region highly vulnerable in AD and layers II and IV of the EC have been shown to be among the first regions affected with tangles in Down syndrome and in normal aging. In severe cases of AD, a decrease in the neuron number of up to 90% was measured in layer II and of up to 70% in layer IV when compared with HC, whose neurons number remained constant between 60 and 90 years of age (Gomez-Isla et al. 1996).

The hippocampal formation of the brain is the region that shows the most prominent atrophic changes caused by AD. In an MRI analysis of AD patients brain Bobinski et al. found a decline of hippocampus volume of about 30% compared to HC (Bobinski et al.

2000). Several MRI-based studies indicate hippocampal atrophy as a relatively specific early stage marker of AD (Bobinski et al. 2000; Jack et al. 2002; Silbert et al. 2003), and a strong correlation between hippocampal atrophy and cognitive decline has also been reported (Petersen et al. 2000; Jack et al. 2002; Chetelat et al. 2003). On the other hand, other studies suggest MRI-assessment of EC volume as a more suitable marker for diagnosis of early stage AD compared to hippocampal volume, allowing also better differentiation between mild cognitive impairment (MCI) and early stage of AD (Pennanen et al. 2004).

It is well established that APP undergoes fast axonal transport (Koo et al. 1990) and plays an important role in axonal and synaptic processes physiology. Abnormal focal axonal swellings, that correspond to pathological accumulation of axonal cargoes and transport proteins, are evident in AD brains (Stokin et al. 2005). Recently, axonal deficits and degeneration have been described in AD (Dai et al. 2002) and in different APP transgenic mouse models overexpressing human APP in brain and spinal cord (Stokin et al. 2005; Wirths et al. 2006) including APP/PS1KI mice (Wirths et al. 2006). These axonal alterations are visible as varicosities and spheroids, containing abnormal accumulations of mitochondria and other organelles together with axonally-transported material like neurofilaments, synaptic proteins and APP. Stokin et al. have pointed out that inhibition of axonal transport leads to increased intraneuronal Aβ accumulation (Stokin et al. 2005). Interestingly, intraneuronal Aβ accumulation precedes axonal degeneration in APP/PS1 models (Wirths et al. 2006), where aberrant intraneuronal Aβ accumulation coincided with impaired axonal integrity (see APP/PS1KI chapter).

1.5.5. BRAIN INFLAMMATION

Neuropathological and neuroradiological studies have demonstrated that inflammatory processes, like microglia activation and astrocyte reactivity, occur early during the course of the disease (reviewed in (Eikelenboom et al. 2006). These processes are widely thought to be triggered by plaques and tangles formation (Arnaud et al. 2006). For example, reactive microglia surrounding senile plaque cores have been consistently reported in AD brains (Rogers et al. 1988; McGeer et al. 2001) (Fig. 7).

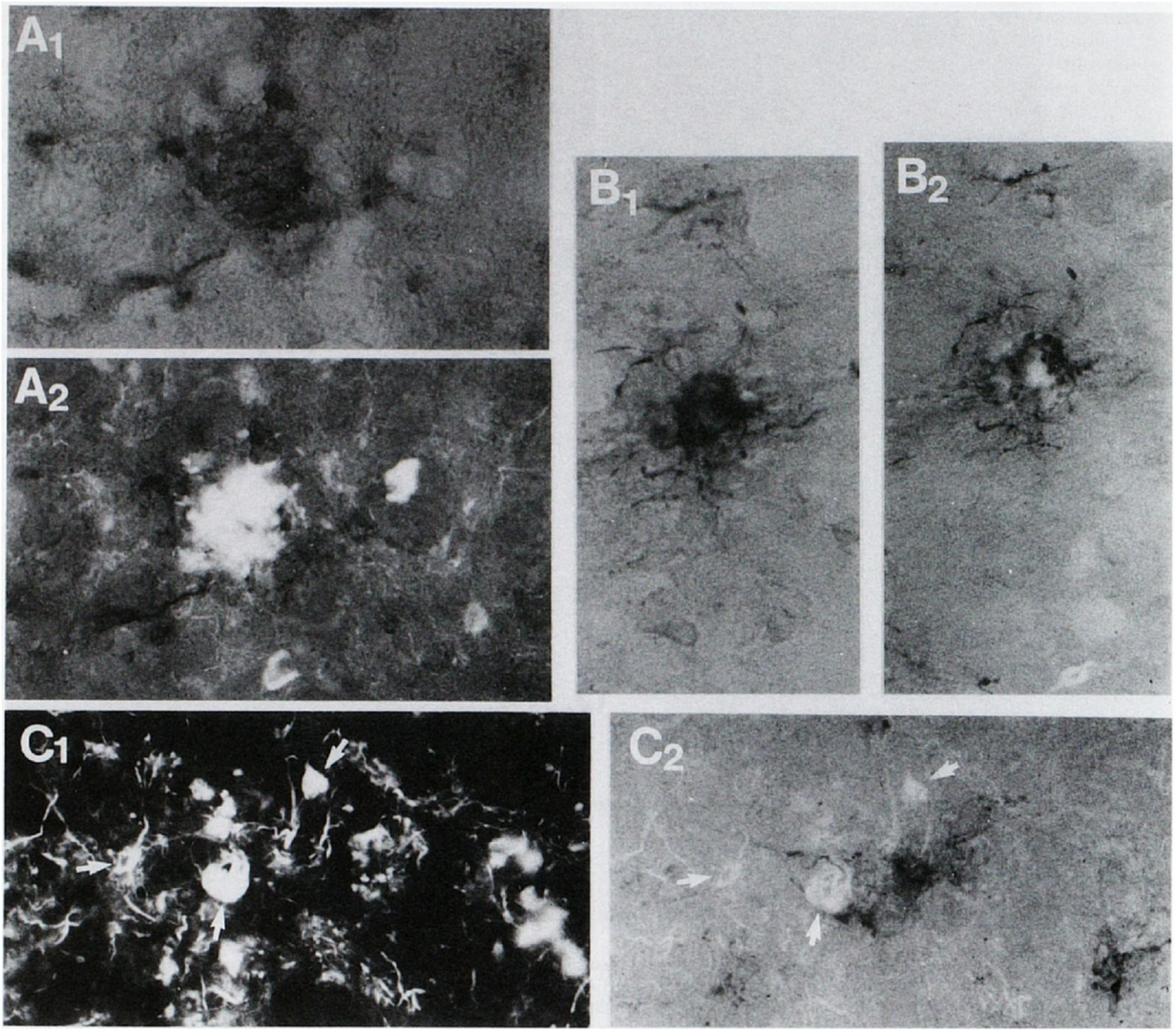


Figure 7 Clusters of HLA-DR immunoreactivity in gray matter of AD cortex are highly correlated with AD pathology. **A1,B1** HLA-DR positive clusters, bright field optics (200 x). **A2,B2** The same fields with dim bright field and thioflavin S fluorescence showing plaque cores. **C1,C2** Unlike plaques, which co-localize almost universally with HLA-DR positive clusters, neurons filled with neurofibrillary tangles (arrowheads) sometimes co-localize with HLA-DR clusters and cells, but sometimes do not. Large, unravelling tangles appear most vulnerable. Panel C1 is fluorescence only. Panel C2 adds dim bright field (200x) (Rogers et al. 1988)

While the event of inflammation in the brain was formerly believed to be a peripheral phenomenon of the disease, the hypothesis of neuroinflammation as one of the potential triggers for neurodegeneration and other pathological hallmarks of AD today is gaining in acceptance (Streit et al. 2004; Arnaud et al. 2006); however, the putative associations and causative links between these AD hallmarks have not been fully elucidated.

The importance of inflammation in the pathogenesis of AD was indirectly confirmed by epidemiological investigations that revealed a decreased incidence of AD in subjects using anti-inflammatory drugs, especially NSAIDs (Wyss-Coray 2006). However, clinical trials designed to inhibit inflammation have failed in the treatment of AD patients suggesting that

anti-inflammatory agents have more protective than therapeutic effect. Despite ongoing research, the extent to which neuroinflammation contributes to disease pathogenesis is still not fully understood. Moreover it is also not clear whether inflammation in AD brains represents a protective reaction to neurodegeneration or if it is rather a destructive process that contributes to further loss of brain function (Akiyama et al. 2000).

1.6. MODELLING OF HUMAN AD-LIKE FEATURES IN TRANSGENIC MICE

1.6.1. OVERVIEW OF AD TRANSGENIC MOUSE MODELS

Transgenic mouse models are valuable tools to study the aetiology of human disorders, including AD; they have greatly contributed to our understanding of its pathogenesis. Whereas the majority of all AD cases occurs sporadically, a small percentage of familial early-onset AD cases develops due to mutations of APP or Presenilin (reviewed in (Bayer et al. 2001)); transgenic mouse models generally are based on these mutations. Many models have been described so far that display to a higher or lower extent the neuropathological features typically seen in AD cases: early pathological changes, such as deficits in synaptic transmission (Hsia et al. 1999), changes in behaviour, differential glutamate responses, learning deficits and deficits in long-term potentiation were all reported in several mouse models for AD overexpressing APP (Holcomb et al. 1998; Moechars et al. 1999; Puolivali et al. 2002; Billings et al. 2005; Oakley et al. 2006). However, Aβ deposition did not correlate with the behavioural phenotype (Holcomb et al. 1999); moreover, none of them exhibits all AD-typical hallmarks alone.

Typically, mouse models overexpressing mutant human APP (hAPP) show increased production of Aβ and reveal a more or less severe pattern of diffuse or compact amyloid plaques in immunohistochemical staining. These mice display also synaptic transmission deficits detectable well before the build-up of massive plaque pathology. Some of these APP-based models also display features like neurodegeneration or cognitive deficits. Early pathological alterations before onset of plaque deposition that might be related to intraneuronal Aβ accumulation have also previously been reported in other mouse models. These include deficits in synaptic transmission (Hsia et al. 1999) or changes in behaviour and deficits in long-term potentiation (LTP) (Moechars et al. 1999).

Other mice models contain a human mutant tau transgene as can be found in familial forms of fronto-temporal dementia (FTD), where extensive tangle formation is a dominant feature. Oddo et al. developed a triple-transgenic mouse model, harbouring mutant transgenes for tauP301L, APPK670N,M671L and PS1M146V. This model shows plaque pathology, tangles and also displays synaptic transmission deficits (Oddo et al. 2003).

Until recently, however, modelling axonal degeneration and neuronal loss remained elusive. APP23 transgenic mice presented a small loss of pyramidal neurons of the hippocampal formation (Calhoun et al. 1998), which did not reflect the dramatic reduction in AD patients (Gomez-Isla et al. 1996). Recently, mouse models carrying both APP and PS1/2 transgenes have been shown to further increase Aβ production and to significantly accelerate the development of AD-like phenotype and pathology. Mild neuron loss has been documented around amyloid plaques in PSAPP mice (Urbanc et al. 2002). Moreover, 2 different mouse models carrying multiple mutations in the PS1 and APP genes have been described that present significant neuron loss in the hippocampus (Casas et al. 2004; Schmitz et al. 2004).

Schmitz et al. crossed human APP751 transgenic mice harbouring the Swedish and London mutations (APP751SL) with the human mutant PS1 (M146L) transgenic line. The hAPP gene was under control of the THY1 promoter, which drives gene expression specifically in neurons. In 17 month-old APP/PS1 M146L mice a 25% reduction of CA1-3 neurons compared to age-matched control mice has been observed, a reduction larger than what could be explained only by amyloid plaque pathology (Schmitz et al. 2004).

Casas et al. used the same APP751SL mice, but crossed them with PS1 knock-in (PS1KI) mice harbouring two human PS1 mutations (M233T/L235P) in the endogenous mouse PS1 gene; thus, expression of mutated PS1 takes place under control of the endogenous promoter. These mutations were specifically selected because of their link to very early onset FAD at 29 (L235P) and 35 (M233T) years of age. This model, named APP/PS1KI, displays at 10 months a neuron loss of over 50% in the CA1 region, and the histopathological onset of AD-like features is evident already at 2 months of age with massive accumulation of intraneuronal Aβ (Casas et al. 2004).

In the next paragraph, the focus will be on this latter model, which is the main model used in this lab and the model used in this PhD work.

1.6.2. THE APP/PS1KI MOUSE MODEL

Due to the London mutation, which increases the affinity for gamma-cleavage at position 42, the predominant species of Aβ in these mice is $Aβ_{42}$. However, after measurement of total Aβ, a relative share of $Aβ_{42}$ of 85% was determined at 4 months, while 4 months old APPSL littermates revealed only a 30% on the total Aβ plaque composition of this form. This indicates that the presence of FAD-linked PS1KI mutations in APP/PS1KI mice contributes significantly to the formation of $Aβ_{42}$. APP/PS1KI mice display widespread and numerous round compact Aβ deposits within the subiculum, cortical, hippocampal, and thalamic areas, starting at 2.5 months, unlike APPSL mice, which show only very few deposits restricted to the areas of the subiculum and deeper cortical neuronal layers with a delayed onset at 6 months of age.

Detailed analysis of the hippocampal CA1-3 subfield and of the dentate gyrus showed that APP/PS1KI mice display a significant reduction of the hippocampal pyramidal cell layer thickness, this being particularly prominent in the CA1 region in 6 months old females, which showed a neuron loss of 33%. Neuron loss was detected to take place in the CA1/2 pyramidal cell layer of the hippocampus to an extent of 50% at 10 months of age with a macroscopically evident loss already at six months of age (Casas et al. 2004) (Fig. 8).

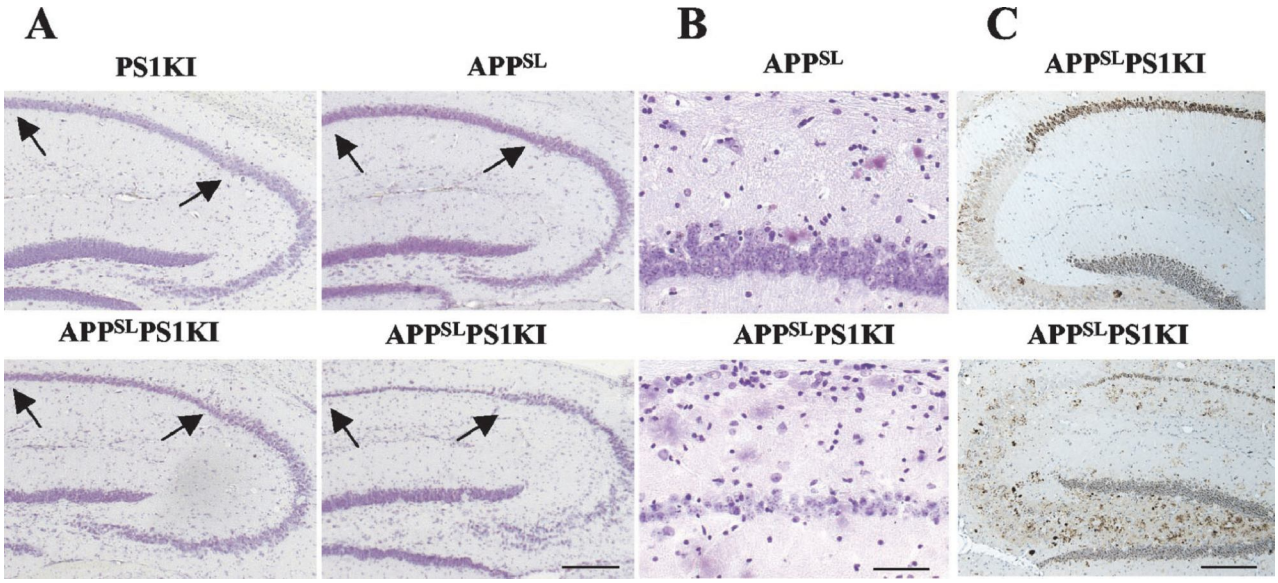


Figure 8 APPSLPS1KI transgenic mice develop massive neuronal loss in the hippocampus. **A** Representative photomicrographs of cresyl violet-stained sagittal brain sections of 10-month-old PS1KI, APPSL, and two APPSLPS1KI mice at low magnification. Note the deeply reduced thickness of the CA1/2 pyramidal cell layer indicated between arrows in the APPSLPS1KI brain (bottom). **B** Higher magnification views of the cresyl violet-stained CA1/2 subfield of a representative APPSL (top) and APPSLPS1KI (bottom) mouse are shown. **C** APP immunostaining of the hippocampal formation in 2 (top)- and 10 (bottom)-month-old APPSLPS1KI mice. APP staining reveals a very strong APP expression in CA1/2 subfield with a faint labelling in CA3 where no neuronal loss was detected. Note again

the reduced thickness of CA1/2 subfield with the APP neuronal immunostaining. Scale bars: 150 _m (A); 50 _m (B); 100 _m (C). Figure from (Casas et al. 2004).

APP/PS1KI mice showed also prominent axonopathy: immunohistochemical and ultrastructural analysis by electron microscopy in APP/PS1KI mice revealed age-dependent characteristic axonal swellings, spheroids, axonal demyelination and ovoids, which are myelin remnants of degenerated nerve fibers (Wirths et al. 2007) (Fig. 9).

Generally, plaque pathology in APP/PS1KI mice is preceded by the accumulation of oligomeric and fibrillar Aβ deposits in brain and spinal cord motor neurons in young mice; many of these peptides present various N-terminal modifications like truncations and N-terminal glutamate cyclization (Wirths et al. 2007).

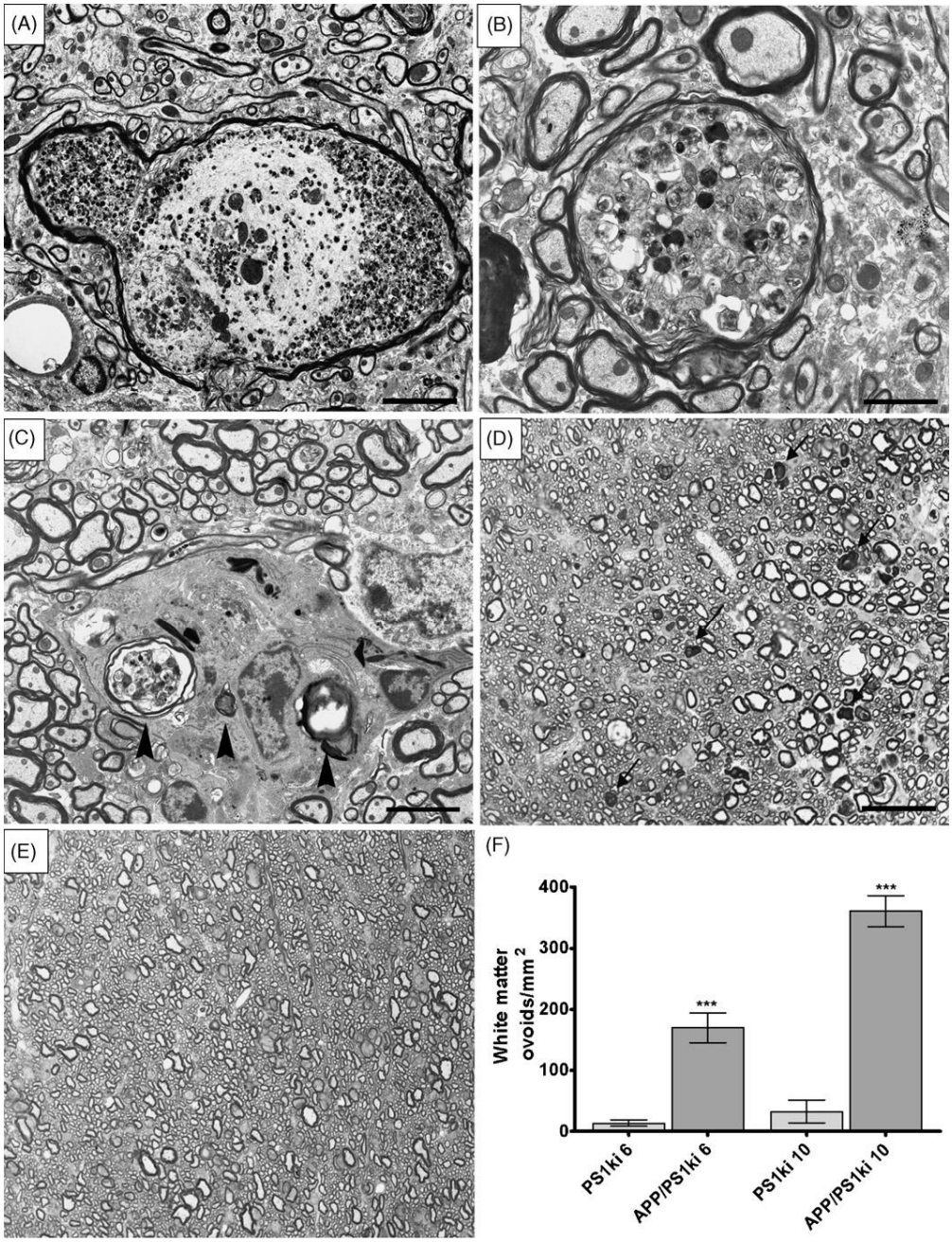


Figure 9 Electron micrographs and semithin spinal cord white matter sections of 10-month-old APP/PS1KI mice. Dilated axons demonstrating an accumulation of mitochondria and various dense vesicles (A and B). Macrophage containing phagocytosed axonal material (arrowheads), representing myelin ovoids (C). Toluidine blue stained sections of the cervical spinal cord with numerous white matter ovoids in a 10-month-old APP/PS1ki mouse (D), whereas PS1KI mice were almost devoid of myelin ovoid pathology (E). Quantification revealed a significant increased density of profiles of white matter ovoids in APP/PS1KI mice compared to PS1ki control mice (F). Error bars, S.E.M.; ***P < 0.001. Scale bars: (A) 7.5µm; (B) 2.5 µm; (C) 5.5 µm; (D and E) 33 µm (Wirths et al. 2007)

1.6.2.1. RELATED SIDE PROJECT: AD-LIKE RELEVANT FEATURES IN TRANSGENIC MOUSE MODELS

The APP/PS1KI mouse model has been shown to harbour deficits in cognitive and motor performance already at the age of 6 months (Wirths et al. 2008), with extensive loss of

CA1 pyramidal neurons quantifiable in 50% at 10 months of age (Casas et al. 2004), accompanied by severe axonal degeneration (Wirths et al. 2007). This is the only mouse model so far, developing abundant hippocampal neuron loss within the age of 1 year (Casas et al. 2004).

Successively to intracellular accumulation of Aβ, six-months-old APP/PS1KI mice show abundant extracellular plaques harbouring a broad variety of N-truncated Aβ, dystrophic neurites and astrogliosis (Casas et al. 2004). APP/PS1KI mice have a good resemblance of pathological and behavioural hallmarks with the actual disease, therefore they represent a suitable model for studying human-like AD-pathological processes.

Since no neuron loss and no behavioural phenotype was detected in these mice at 2 months of age compared to age matched PS1KI control mice a systematic analysis of various inflammatory and synaptic markers has been performed in pre-symptomatic (2-month-old) and affected APP/PS1KI mice (6-month-old).

Inflammation

Though the occurrence of neuroinflammatory events represents a phenomenon well described in AD, the extent to which and the way how inflammatory processes are involved with the pathology of the disease are still a matter of debate. It is often suggested that Aβ plaques, tangles and the event of neurodegeneration with the exposition of intracellular DNA and neurofilaments to the extracellular environment are the main triggers for the activation of several generic inflammatory pathways in human brains affected by AD.

Inflammatory changes have been previously described in a variety of different APP transgenic mouse models (Benzing et al. 1999; Stalder et al. 1999; Mehlhorn et al. 2000; Apelt et al. 2001; Bornemann et al. 2001; Qiao et al. 2001; Richards et al. 2003; Heneka et al. 2005). Additionally, this lab performed an extensive and detailed study on APP/PS1KI mice which included a broad range of inflammation markers (Fig. 10) like cytokines and cytokine receptors, toll-like receptors, transcription factors and acute phase proteins, as well as proteins involved in metal homeostasis and oxidative stress defence like the metallothioneins family (Wirths et al. 2008).

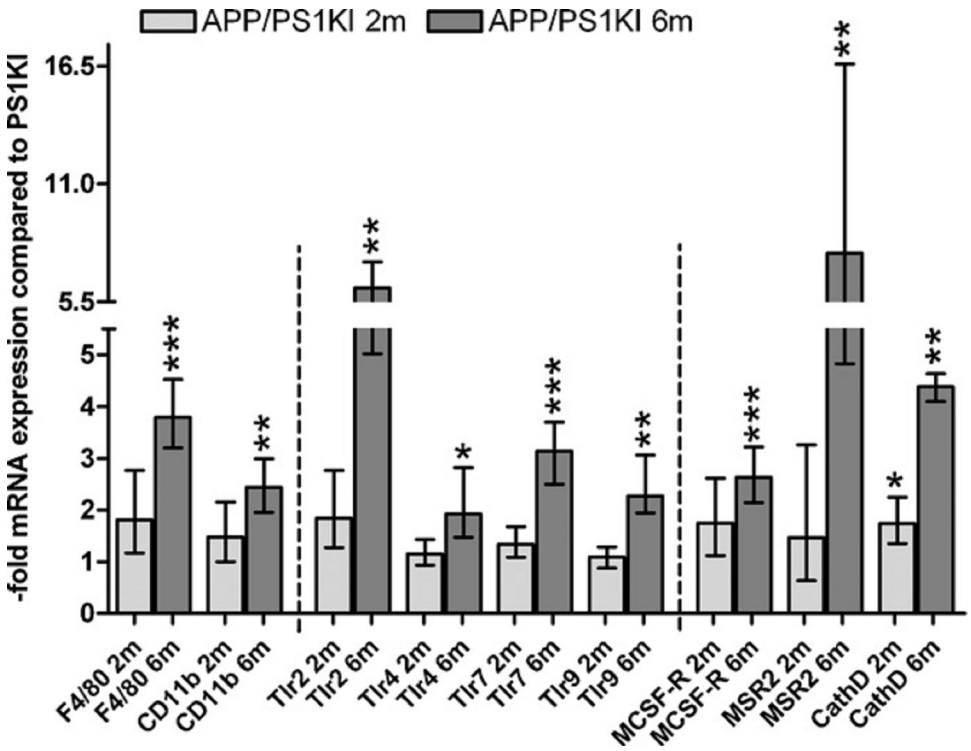


Figure 10 Quantitative real-time PCR shows consistent activation of microglia markers F4/80 and CD11b, members of the Toll-like receptor family (Tlr2, Tlr4, Tlr7, Tlr9) representing innate immune receptors, as well as for MCSFR and MSR2 at the age of 6 months on APP/PS1KI mice compared to their PS1KI littermates. In addition, Cathepsin D levels were significantly increased at both 2 and 6 months of age, indicating early microglial activation. Figure from (Wirths et al. 2008).

The scope of this side project was to relate specific phases of inflammation to specific phases of the pathological processes connected to AD by measuring the expression of an inflammation protein marker, GFAP, in young, asymptomatic -month-old mice and diseased 6-month-old mice. Since the causality relationship between AD and inflammation is still unclear, a closer look to the temporal order by which changes in the expression of inflammatory genes take place in relation to the uprising of typical AD pathological characteristics is of interest. Such information may help to spread light over the matter of whether inflammation of the brain is a side effect of AD pathology or whether inflammatory processes may contribute to the progress of cognitive decline in AD patients.

Synaptic deficits

It is well established that AD patients suffer from working memory deficits (Baddeley et al. 1991). However, motor performance deficits have also been described to occur in AD patients e.g. gait disturbances, disturbed activity level and balance, as well as general motor signs (Alexander et al. 1995; O'Keeffe et al. 1996; Pettersson et al. 2002; Scarmeas et al. 2004).

APP/PS1KI mice develop severe age-dependent axonal degeneration (Wirths et al. 2006) (Fig. 9), as well as loss of hippocampal CA1/2 neurons starting at the age of 6 months (Casas et al. 2004). In addition, deficits in induction of long-term synaptic changes have also been observed (Breyhan et al. 2009).

Recently, it is becoming widely accepted that the soluble oligomers and the β-sheet containing amyloid fibrils are the toxic forms of Aβ (Selkoe 2001; Klein 2002; Harmeier et al. 2009). Considering that oligomers are formed preferentially within neuronal processes and synapses rather than extracellularly (Walsh et al. 2000; Takahashi et al. 2004), the aim of the present study was to investigate and quantify how some synaptic markers are affected by the overproduction of Aβ in these mice.

1.7. PYROGLUTAMATE-Aβ

One of the most coherent arguments pointing against the Amyloid hypothesis is the finding of Aβ plaques in the brains of elderly subjects without any sign of cognitive decline. It has been speculated that the difference between AD patients and plaques-bearing HC is to be searched in the plaques composition, rather than in the sheer presence of plaques (Piccini et al. 2005). Differences in the Aβ species composing plaques have been described repeatedly over the years. It is an established fact that amyloid plaques do not consist only of full length, unmodified $Aβ_{1-40}$ and $Aβ_{1-42}$, but rather of a mix of Aβ peptides of different lengths, often bearing post-translational modifications.

In fact, besides Aβ peptides starting with an aspartate at position 1, a variety of different N-truncated Aβ peptides have been identified in AD brains. Truncated peptides in majority beginning with phenylalanine at position 4 of Aβ have been reported as early as 1985 by Masters et al. (Masters et al. 1985). In contrast, no N-terminal sequence could be obtained from cores purified in a sodium dodecyl sulfate-containing buffer, which led to the assumption that the N-terminus could be blocked by a post-translational modification (Gorevic et al. 1986; Selkoe et al. 1986).

Initial difficulties in sequencing Aß peptides purified from human brain tissue were in part explained by the presence of N-terminal truncated Aβ starting with pyroglutamate ($Aβ_{3(pE)}$) shown using mass spectrometry of purified Aβ peptides (Mori et al. 1992). Saido and co-workers (Saido et al. 1995) showed then that $Aβ_{3(pE)}$ represents a dominant fraction of Aβ peptides in senile plaques of AD brains and hypothesized a mechanism in which $Aβ_{3(pE)}$

aggregation precedes and works as a seed for the aggregation of the other Aβ species, due to its higher hydrophobicity (Saido et al. 1996). This was later confirmed by other reports investigating AD and Down's syndrome post-mortem brain tissue; in particular, Iwatsubo et al. reported massive aggregation of $Aβ_{N1(L\text{-}isoAsp)}$, $Aβ_{N1(D\text{-}Asp)}$, and of $Aβ_{3(pE)}$. Additionally, weak staining patterns were detected for pyroglutamate $Aβ_{N11\text{-}42(pE)}$ and for $Aβ_{N17(Leu)}$ (Iwatsubo et al. 1996). Lemere et al. reported in 29-year-old Down's Syndrome patients a stronger immunoreactivity for $Aβ_{3(pE)}$ than for $Aβ_{N1\text{-}42}$; $Aβ_{3(pE)}$ was also the species showing the most consistent age-dependent increase (Lemere et al. 1996). Harigaya et al. quantified the contribution of $Aβ_{3(pE)}$ to the total plaque load in AD patients to be of up to 25% (Harigaya et al. 2000). Subsequently, also Piccini et al. showed that soluble Aβ aggregates found in AD are characterized by a predominance of N-truncated variants of Aβ over the full-length protein $Aβ_{1\text{-}42}$; in particular, $Aβ_{3(pE)}$ was the prevailing N-truncated Aβ species (Piccini et al. 2005).

In *in vitro* experiments Schilling et al. have shown that cyclization of glutamate at position 3 can be driven enzymatically by glutaminyl cyclase (QC) (Fig. 11c) (Schilling et al. 2004). The same enzyme has a many-fold increased affinity for the Glutamine residue. In addition, it has been shown that QC inhibition significantly reduced $Aβ_{3p(E)}$ formation *in vitro* and *in vivo*, emphasizing the importance of QC activity during cellular maturation of pyroglutamate-containing peptides (Cynis et al. 2006; Schilling et al. 2008).

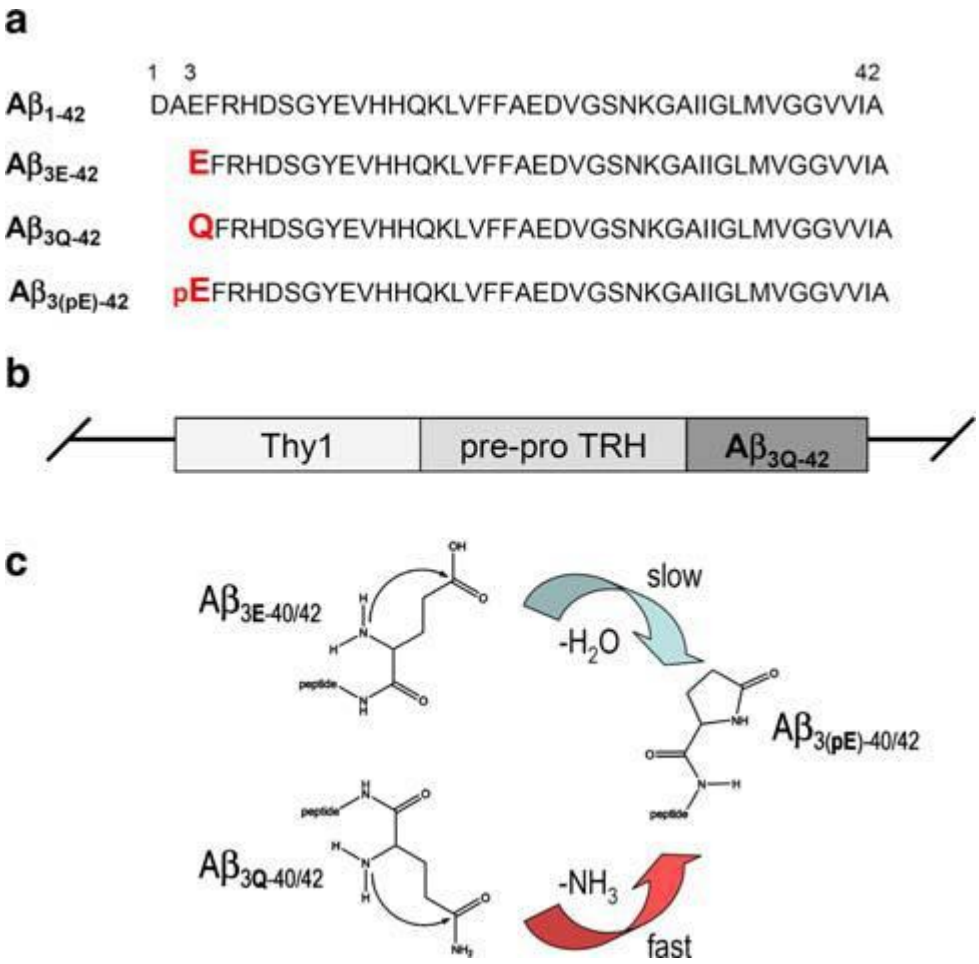


Figure 11 Sequence of Aβ $_{1–42}$ and Aβ$_{3(pE)}$. **a** Aβ $_{1–42}$ starts at position 1 with aspartate (D), Aβ$_{3E--42}$ at position 3 with glutamate (E), and Aβ$_{3Q–42}$ with glutamine (Q). Both N-truncated Aβ$_{3E--42}$ and Aβ$_{3Q–42}$ peptides can be converted into pyroglutamate- Aβ$_{3(pE)}$. **b** Schematic drawing of the transgenic vector. TBA2 transgenic mice express Aβ$_{3Q–42}$ under the control of the Thy1 promoter fused to the signal peptide of the pre-prothyrotropin- releasing hormone. **c** Aβ$_{3(pE)}$ or Aβ$_{3Q}$ serves as substrates for generation of Ab3(pE). The conversion of pyroglutamate from N-terminal glutamate (E) is slow, in contrast to fast pyroglutamate (pE) formation from glutamine (Q) (Wirths et al. 2009).

N-terminal deletions enhance aggregation (Masters et al. 1985; Pike et al. 1995; He et al. 1999; Schilling et al. 2006) and stability (Kuo et al. 1998) of Aβ peptides *in vitro*; this probably leads as well to an increased toxicity compared to full-length Aβ (Russo et al. 2002). Russo et al. showed that neuron and astrocyte cultures are affected to a significant higher extent by exposition to Aβ$_{3(pE)-\ 40/42}$ rather than by the corresponding full-length species and that Aβ$_{3(pE)}$ shows a higher resistance to degradation by astrocytes. However, other studies reported that the toxicity of Aβ$_{3(pE)-40/42}$ is similar to that of Aβ$_{1-40}$ and Aβ$_{1-40}$ (Tekirian et al. 1999), and that Aβ$_{3(pE)}$ is not the major variant in AD brain (Lemere et al. 1996).

To verify Aβ$_{3(pE)-x}$ toxicity *in vivo,* this group has recently generated a new transgenic mouse model expressing Aβ starting at position 3 with glutamine instead of glutamate and ending at position 42 (TBA2 mouse line, Fig 11a,b) to increase the conversion rate to

pyroglutamate (Wirths et al. 2009). This model showed for the first time that intraneuronal $A\beta_{3(pE)–42}$ accumulation is sufficient for triggering neuron death and inducing an associated neurological phenotype in a transgenic mouse model. The severity of the neurological phenotype observed in TBA2 mice, accompanied by Purkinje cell loss and premature mortality reflects the *in vivo* toxicity of $A\beta_{3(pE)-42}$. Aβ staining in the cerebellum was completely restricted to the intraneuronal compartment further supporting the notion that intraneuronal pathology is instrumental in neuron loss and that extracellular plaque deposition has no drastic effect on cell survival (Fig. 12).

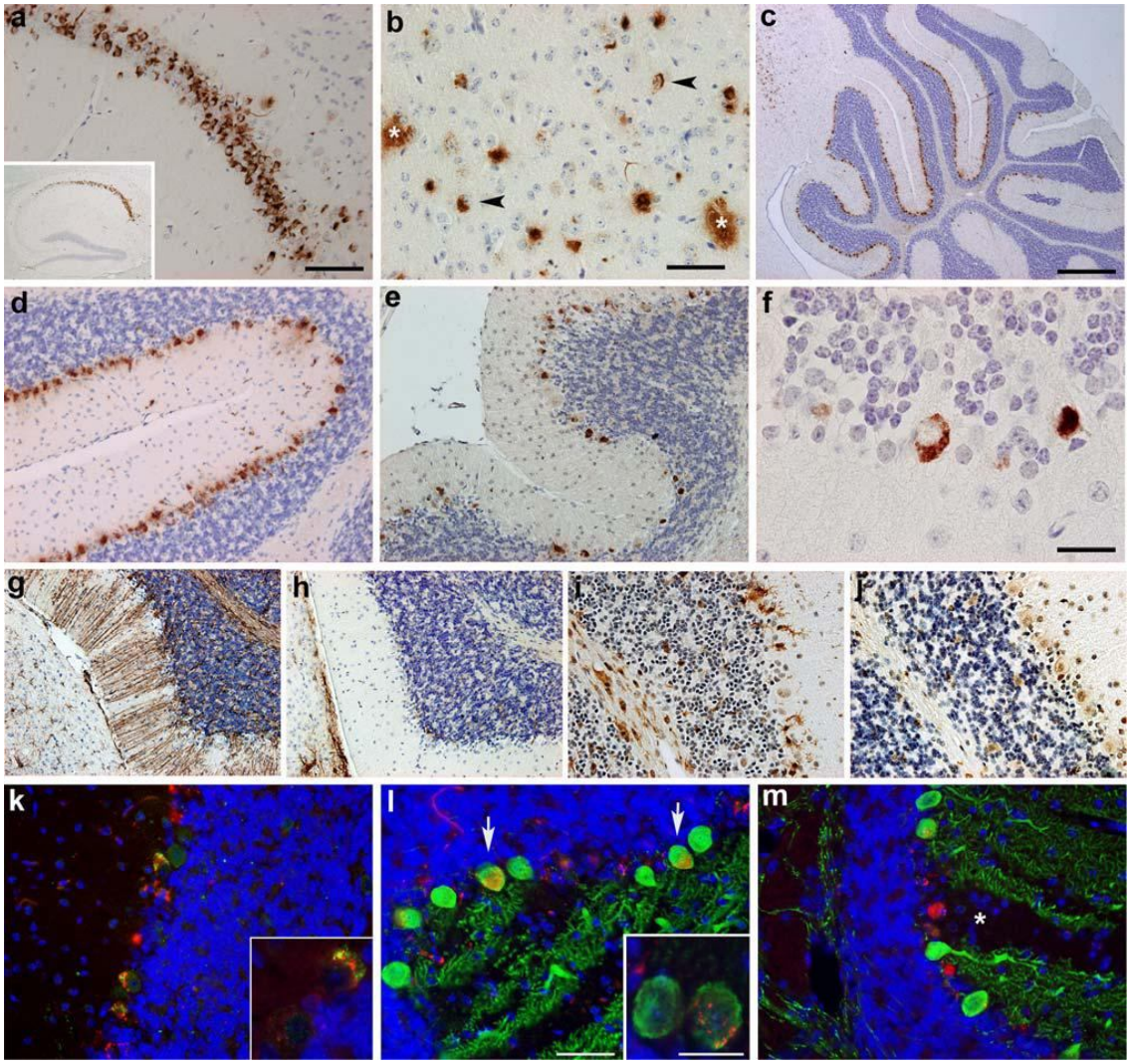


Figure 12 Immunohistochemical staining of TBA2 mouse brain (2-month-old). **a** Immunostaining with 4G8 revealed strong Aβ accumulation in the CA1 pyramidal layer of the hippocampus (inset shows a hippocampus overview at low magnification). **b** Intra- (arrowhead) and extracellular Aβ (asterisk) in the thalamus shown by 4G8 staining. **c, d** Aβ staining (4G8) in the cerebellum is almost completely restricted to the Purkinje cell layer. **e, f** Most Purkinje cells accumulated $A\beta_{3(pE)}$ as shown by an antibody against $A\beta_{3(pE)}$. g GFAP staining of a TBA2 mouse revealed prominent Bergmann glia immunoreactivity, whereas wildtype animals (**h**) were consistently negative. The microglia marker Iba1 revealed microglia clusters surrounding Purkinje cells and in white matter tracts in TBA2 mice (**i**), but not in wildtype littermates (**j**). k Immunostaining of Purkinje cells with 4G8 (red) and anti-ubiquitin (green) antibodies showing abundant ubiquitin immunoreactivity in 4G8-positive Purkinje cells. **l, m** Staining of Purkinje cells using antibodies against calbindin (green) and 4G8 (inset shows high magnification of a 4G8- and calbindin-positive Purkinje cell). Note absent calbindin (asterisk) and extracellular Aβ staining indicating Purkinje cell loss. Only 4G8- positive remnants can be seen. Scale bars a, d, e, g–j 100 lm; b, k–m 50 lm; c 500 lm; f inset k, l 20 lm (Wirths et al. 2009).

Kuo et al (Kuo et al. 2001) have used an integrated chemical and morphological comparison of the Aβ peptides and amyloid plaques present in the brains of APP23 transgenic mice and human AD patients. The lack of post-translational modifications such as terminal degradation, isomerization, racemization, and pyroglutamyl formation of Aβ from APP23 mice provides an explanation for the differences in solubility of Aß from human AD and transgenic mouse plaques. The same group also demonstrated that in another transgenic mouse model (Tg2576), Aβ peptides showed a pronounced N-terminal degradation, post-translational modifications, and crosslinkages that were frequently observed in the compact Aβ peptide deposits found in AD brain. Thus, under *in vivo* conditions, truncated Aβ molecules appeared to be generated by hydrolysis at multiple sites rather than by post-mortem N-terminal degradation (Kalback et al. 2002).

1.7.1. RELATED PROJECT: DEVELOPMENT OF A NOVEL MONOCLONAL ANTI-$A\beta_{3(pE)-X}$-ANTIBODY

A striking feature of the APP/PS1KI mouse model is the progressive development of a complex pattern of N-truncated Aβ variants that well parallels the composition of Aβ species observed in human AD brains. In detail, human Aβ isovariants 2/3-42, 4/5-42, 8/9/10/11-42, 12/13/14-42 and most notably $A\beta_{3(pE)-x}$ were determined by proteomic analysis of brain lysates in a two-dimensional western-blot (Casas et al. 2004). Notably, the APP/PS1KI mouse model was the first to show accumulation of $A\beta_{3(pE)}$.

This group showed recently in the same mice that intraneuronal aggregation of N-terminal modified Aβ variants corresponds to robust learning deficits at the age of 6 months, age-dependent axonopathy, neuron loss in the CA1 region of the hippocampus, synaptic deficits, and hippocampus atrophy (Wirths et al. 2007; Wirths et al. 2008; Breyhan et al. 2009). Recently, a new mouse model has been generated which expresses only N-truncated $A\beta_{3(pE)}$ in neurons, and demonstrated for the first time that this peptide is neurotoxic *in vivo* inducing neuron loss and an associated neurological phenotype (Wirths et al. 2009).

Given the increasing interest in $A\beta_{3(pE)}$, we have generated two novel monoclonal antibodies which we have characterized as highly specific for $A\beta_{3(pE)}$ peptides and herein used to analyze plaque deposition in APP/PS1KI mice and compared its pattern with that present in brain tissue from sporadic and familial AD cases.

1.8. AD BIOMARKERS

The earliest pathological events leading to Alzheimer's disease (AD) are estimated to occur 10–20 years before the appearance of the first cognitive changes in affected patients (Price et al. 1999; Yamaguchi et al. 2001). Therefore, identification of biomarkers predictive of AD might allow screening for individuals who may benefit from preventive therapies like treatment with acetylcholine esterase inhibitors before the development of cognitive impairment.

As of today, few biomarkers are known which are able to give information about disease advancement.

1.8.1. CHOLESTEROL

Recently, Mielke et al. reported in a population-based 70-year-old birth cohort followed for 18 years that increasing levels of plasma total cholesterol at ages of 70, 75, and 79 are related to a reduced risk of dementia between ages 79 and 88 (Mielke et al. 2005). However, the association of high levels of plasma cholesterol with a decreased risk of AD is in contrast to other reports that suggest high total plasma cholesterol to be a risk factor for later dementia (Kuusisto et al. 1997) .

However, Brain cholesterol is assumed to represent a permanent pool of exclusively locally synthesized cholesterol that is protected from exchange with the periphery by its inability to cross the blood-brain-barrier (Bjorkhem et al. 2004). The main elimination reaction of cholesterol from the brain occurs by its conversion to 24(S)-hydroxycholesterol by enzymatic activity of CYP46, which is exclusively expressed in human brain, (Dietschy et al. 2001; Lutjohann et al. 2002). It has been shown that 24(S)-hydroxycholesterol in contrast to cholesterol is capable of crossing the blood-brain-barrier and therefore can be detected in easily accessible body fluids like plasma or serum (Bjorkhem et al. 2001). In AD patients the levels of 24(S)-hydroxycholesterol have been shown to be significantly elevated in early stages of the disease, potentially due to disruptions in the blood-brain-barrier. The higher efflux of 24(S)-hydroxycholesterol has been hypothesized to be a result of neuron loss or an altered cholesterol metabolism in AD (Locatelli et al. 2002). Therefore, elevated 24(S)-hydroxycholesterol plasma levels have been discussed as an easily accessible biomarker for the diagnosis of AD in early stages of the disease, but up to now

there is no clear evidence for its suitability as an early-onset marker for AD (Schonknecht et al. 2002; Teunissen et al. 2003; Irizarry 2004) .

1.8.2. CSF BIOMARKERS

Aβ peptide and tau protein are supposed to have a central role in the pathogenesis of AD (Hardy et al. 2002). This considered, a large series of studies in blood (Zhang et al. 2004) and cerebrospinal fluid (CSF) (Hampel et al. 2004) have been conducted in order to evaluate the possibility to use their measurement as a predictive biomarker for the diagnosis of AD.

As a matter of fact, most of these studies report about decreased $A\beta_{1-42}$ and increased tau and phospho-tau in CSF of AD affected patients compared to non-demented persons (Blennow et al. 2003; Sunderland et al. 2005); today, measurement of CSF level of these peptides is considered a reliable diagnostic procedure with 75–95% sensitivity and specificity (Jensen et al. 1999; Blennow 2004; Lewczuk et al. 2004). However, broad appliance of CSF scans as a routine test for early AD diagnosis is not possible due to the fact that lumbar puncture, especially if repeated over time, represents an invasive procedure which is not well tolerated by most of the patients. Contrastingly, blood is an easily accessible body fluid and a blood diagnostic test would be highly preferable.

1.8.3. BLOOD BIOMARKERS

Unfortunately, studies on plasma Aβ levels have shown contradictory results: while some groups reported a correlation between CSF and blood levels of Aβ in HC but not in AD and MCI patients (Giedraitis et al. 2007), others found no correlation at all, neither in AD patients (Mehta et al. 2001) nor in HC (Mehta et al. 2005).

However, the fact that around 500 mL of CSF are absorbed into the blood every day let us speculate that suitable AD biomarkers can be found also by specific plasma analysis (Hye et al. 2006).

So far, measurement of Aβ levels in plasma yielded highly contrasting results, ranging from increased Aβ40 and decreased Aβ42 in AD patients compared to HC (Xu et al. 2008), no differences (Vanderstichele et al. 2000; Fukumoto et al. 2003) or increased

Aβ42 in AD patients (Matsubara et al. 1999). Although the significance of Aβ for diagnosing Alzheimer's disease is controversial, many studies agree on the idea that high plasma concentrations of Aβ40 and low plasma concentrations of Aβ42 indicate an increased risk of dementia (reviewed in (Kawarabayashi et al. 2008)).

The different findings in these studies may be due to differences in study designs, including variations in age and disease severity of included subjects. A longitudinal study showed that plasma Aβ40 and Aβ42 levels measured at the age of 70 years were not significantly associated with incident AD (Sundelof et al. 2008). All of the studies, though, have shown substantial overlap in plasma Aβ40 and Aβ42 levels between patients and controls, thus limiting the diagnostic value of plasma Aβ assessment.

Plasma Aβ levels are commonly quantified with enzyme-linked immunoabsorbent assays (ELISA) using antibodies against a variety of Aβ epitopes (for example (Suzuki et al. 1994; Mehta et al. 2000)). Quantification of Aβ in blood is technically difficult and limited by the fact that most of it is bound to blood components, including lipids serum albumin, α2-macroglobulin, immunoglobulins, apolipoprotein J, transthyritin, apoferritin, as well as complement components C1q and C3 (Biere et al. 1996; Kuo et al. 2000).

1.8.4. IMMUNOLOGICAL BIOMARKERS

Besides the detection of biochemical AD markers, autoimmune responses to molecules involved in AD pathology have become a current research focus (Nath et al. 2003; Gruden et al. 2004). There seems to be a reduction in the magnitude of lymphocyte stimulation by APP in AD patients (Trieb et al. 1996), and anti-Aβ natural antibodies (NAbs) seem to be present in sera and CSF of AD patients and HC (Du et al. 2001). However, whether autoimmunity plays a direct role in the pathogenesis of AD, or whether NAbs can be used as biomarkers for AD, is still open to debate (Singh 1997; McGeer et al. 2001; Tarkowski et al. 2003).

1.8.4.1. RELATED PROJECT: IMMUNE COMPLEXES OF Aβ AND IgM IN THE BLOOD OF AD, MCI PATIENTS AND HC

Recently, it has been shown for many forms of cancer that Immune complexes (ICs) of a peptide used as biomarker with immunoglobulins of class M (IgM) have better performances as a diagnostic tool than the equivalent unbound peptide (Beneduce et al. 2005; Castaldi et al. 2005; Beneduce et al. 2007). These biomarkers are physiological self-proteins which are overexpressed in cancer due to the unnatural overgrowth of the cancer cells producing them.

Since AD is also characterized by excessive accumulation of self peptides, in the present study the level of circulating immune complexes (ICs) between Aβ and IgM has been investigated.

1.8.4.2. RELATED PROJECT: ANTI- $A\beta_{3(pE)}$-IgM IN AD

As mentioned above, $A\beta_{3(pE)}$ is suggested to play a crucial role in the development of AD, since deposition of it occurs early in AD and $A\beta_{3(pE)}$ exhibited pronounced toxicity in neuronal and glial cell cultures (Hosoda et al. 1998; Russo et al. 2002). This is probably due to the fact that pyroglutamate modification confers proteolytic resistance (Saido 1998; Russo et al. 2002) and an initial rate of aggregation up to 250-fold higher when compared to normal Aβ (He et al. 1999; Schilling et al. 2006). The drastically increased proteolytic stability of $A\beta_{3(pE)}$ might also be the reason for the correlation of the $A\beta_{3(pE)}$ content with severity of the sporadic AD, as suggested in a previous study (Guntert et al. 2006). In vitro studies showed that $A\beta_{3(pE)}$ probably serves as a nidus for and promoter of aggregation of full length Aβ (Schilling et al. 2006).

Therefore, it can be hypothesized that the prevention of $A\beta_{3(pE)}$ formation might represent a new strategy for the causal treatment of AD as well as other pyroglutamate-related amyloidosis; it is consistent with this idea that a higher natural immunoreactivity specific for $A\beta_{3(pE)}$ also participate in delaying the onset of the disease. In fact, reduction or inhibition of accumulation of $A\beta_{3(pE)}$ could, in turn, prevent Aβ aggregation by clearance of a major nucleation factor and enhance neuronal survival.

In this study, a new assay aimed to measure the level of NAbs against N-terminus of $A\beta_{3(pE)}$ as well as other specific Aβ epitopes has been developed. The purpose was to

investigate whether and which natural IgM immunoglobulins are present in the blood of AD patients, MCI individuals and HC, and to search for possible differences between groups. In details, the levels of anti-Aβ-, anti-$Aβ_{x-42}$-, anti-$Aβ_{x-40}$-, and anti-N-terminus of $Aβ_{3(pE)}$-IgMs have been measured. The findings may also point to the role that NAbs play in the pathogenesis of the disease.

1.9 Focus of the present work

Aim 1: Expression analysis of proteins in APP/PS1KI mice

The first part deals with the Western blot characterization of whole brain and synaptosomal fractions of APP/PS1KI mice in order to support data from mRNA studies and electrophysiological recordings showing synaptic deficits and inflammation processes developing between 2 and 6 months of age, corresponding to the time span when cognitive impairment and neuron loss have been shown to establish.

Aim 2: Characterization of novel monoclonal anti-pyroglutamate Abeta antibodies.

The second part deals with the characterization of 2 monoclonal antibodies directed against $Aβ_{3(pE)-x}$ generated by Synaptic Systems and the validation by dot blot, ELISA and immunohistochemistry.

Aim 3: Establishing of an ELISA test for Abeta-IgM immune complexes and validation in human plasma samples

The third part deals with the validation and measurement of the presence of immune complexes of Aβ and IgM in the plasma of AD and MCI patients and HC to evaluate whether the level of such immune complexes is correlated with the disease state of the individual. In order to perform these measurement a novel ELISA test has been developed.

Aim: 4 Establishing of an ELISA test for autoantibodies against pyroglutamate Abeta-IgM and validation in human plasma samples

The fourth part deals with development of a novel ELISA test for the measurement of anti-$Aβ_{3(pE)-x}$-IgM autoantibodies in the human plasma. The presence of the autoantibodies has been validated by HPLC purification and western blot. The test was validated using 30 AD,

15 MCI and 30 HC plasma samples to evaluate differences in the mean level of autoantibodies in the 3 studied groups.

Chapter 2

Material and methods

2.1 STATISTICAL ANALYSIS

For synaptic and inflammatory markers, and for blood biomarkers, differences between groups were tested with either one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni's multiple comparison tests, paired t-test for pair-wise comparisons or unpaired t-tests as indicated in the figure legends. All data were given as means ± standard deviation. The diagnostic powers were determined by ROC curve analysis. Chi-square test on independence was used to analyze, if the gender distribution was significantly different between the three groups and if there is a difference between the two patient groups in relation to the number of family members with dementia. All tests were two-tailed.

Significance levels were given as follows: ***P < 0.001; **P < 0.01; *P < 0.05. All calculations were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA).

2.2 APP/PS1KI MICE

The generation of APP/PS1KI mice has been described in details in Casas et al. 2004 (Casas et al. 2004). In brief, human mutant APP751, harbouring the Swedish and London mutations (APP751SL) is expressed under the control of the murine Thy-1 promoter, whereas murine PS1 with two FAD-linked mutations (PS1 M233T and PS1 L235P) is expressed under the control of the endogenous mouse PS1 promoter (“knock-in”). All mice named as PS1KI were homozygous for PS1 knock-in mutations; the APP/PS1ki mice harboured one additional hemizygous APP751SL transgene. All animals were handled according to German guidelines for animal care. All mice were kept on standard mouse diet and all experiments were carried out according to German animal protection guidelines. In total 6 APP/PS1KI and 6 PS1KI mice have been used in the current study (generous gift of Dr. Laurent Pradier, Sanofi-Aventis, France).

2.3 TBA2 MICE

The generation of TBA2 mice has been described in Wirths et al 2009 (Wirths et al. 2009). These mice have been generated by inserting the cDNA of a murine thyrotropin-releasing hormone-Aβ fusion protein mTRH-Aβ3Q–42 into the vector pUC18 containing the murine Thy-1 sequence applying standard molecular biology techniques and verified by sequencing. The transgenic mice were generated by male pronuclear injection of fertilized C57Bl/6J oocytes (PNI, generated by genOway, Lyon, France). The resulting offspring were further characterized for transgene integration by PCR analysis and after crossing to C57Bl/6J wildtype mice for transgene expression by RT-PCR. The line with the highest transgene mRNA expression was selected for further breeding (named TBA2).

2.4 PROTEIN EXTRACTION FROM MOUSE BRAIN

For western blot, mouse brain hemispheres were homogenized in 10 volumes of lysis buffer (0.32M Sucrose, 5mM HEPES, pH 7.5) including complete protease inhibitor cocktail (Roche), and centrifuged at 12,000*g* for 20 minutes at 4 °C. The supernatants were immediately frozen and stored at -80 °C until further analysis.

For synaptosome enrichment, brain hemispheres were homogenized in 10 volumes of 0.32 M sucrose, 5 mM HEPES, pH 7.5 including complete protease inhibitor (Roche) using a glass-teflon homogenizer. Homogenates were centrifuged at 10,000g for 10 min and the supernatant was centrifuged at 12,000g for additional 20 min. The resulting pellet contains the crude synaptosomal fraction.

2.5 PHOTOMETRIC MEASUREMENT OF PROTEIN CONCENTRATION

Protein concentration was measured by use of the commercially available Roti-Quant universal solution (Carl Roth GmbH, Karlsruhe, Germany), based on the biuret reaction. Absorbance was measured by use of a photometer at 595 nm. Concentrations of proteins were calculated by comparison with a calibration curve derived from BSA samples with a range in concentration from 0 – 20 mg/mL. All samples were measured in a transparent

96-well culture plate. In each well 2 µL of sample were diluted with 200 µL of detection solution Roti-Quant universal and incubated for 30 min at 37 °C before measurement.

2.6 WESTERN BLOT

For inflammatory and synaptic markers, and Aβ, brain homogenates containing equal amount of proteins were used for SDS-PAGE electrophoresis. For IC and IgM localization, fractions corresponding to 280 nm absorbance peaks after HPLC purification were tested. Electrophoresis was performed using 4–12% sodium dodecylsulfate–polyacrylamide gels (Vario-Gel, Anamed, Germany). Proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, USA) by semi-dry blotting and then blocked in 10% non-fat dry milk in TBS containing 0.05% Tween (TBS-T) for 1 h at room temperature. For Aβ detection, the membrane was heated in PBS in a microwave for 5 minutes right after transfer. Blots were then incubated with the primary antibody directed against the protein of interest overnight at 4 °C followed by secondary peroxidase-conjugated antibodies for 2 h at room temperature, or alternatively, with peroxidase-conjugated goat anti-human-IgM (Sigma-Aldrich) at 1:1,000 in TBS-T for 2 h. Protein bands were revealed using chemiluminescence solution and peroxide hydrogen as substrates on a Curix60 developing machine (Agfa-Gevaert N.V., Mortsel, Belgium).
Quantification was done by densitometry of Western blot bands using ImageJ software (NIH, http://rsb.info.nih.gov/ij). A mouse monoclonal antibody against Tubulin (Chemicon, California, USA) or actin was used to ensure equal loading and measure relative quantity of markers.

2.7 GENERATION OF MONOCLONAL ANTI-$A\beta_{3(pE)}$ ANTIBODIES

The first phases of the antibodies development have been carried out at Synaptic System (Goettingen, Germany). Briefly:

Phase I / Immunization:

3 balb/c mice were immunized with 90 to 150 µg of a KLH coupled pyroglutamate-Aβ-peptide with the sequence pEFRHD-C. Animals are immunized over a period of 17 days

every second day and one day before fusion. As immune stimulating agent incomplete Freund's adjuvant combined with CpG oligo-nucleotides has been used.

Phase II / Fusion:

After immunization cells from knee lymph knots and spleen of all three animals are prepared and fused with the P3-X63-Ag8 myeloma cell line in the presence of PEG 1500. After fusion cells are plated on 24-well plates resulting in 360 oligo clones each consisting of 5-10 clones. Cells are cultivated for 10 days in selective and conditioned medium selecting for fused cells only.

Phase III / Screening and subcloning:

All 360 oligo clone supernatants are screened by 3 different ELISA using as capture molecules $A\beta_{3(pE)-7}$, $A\beta_{1-7}$ and Fetal calb serum (FCS). The clones showing the highest $A\beta_{3(pE)-7}$/ $A\beta_{1-7}$ and $A\beta_{3(pE)-7}$/FCS signals ratio were selected and further analyzed.

2.8 DOT BLOT

1 µL of each studied Aβ peptides (see Table 2) was spotted on a nitrocellulose membrane in serial dilutions with a starting concentration of 50ug/mL in PBS and air dried. The membrane has been blocked with 10% non-fat dry milk in TBS-T for 1 h at 4°C. The blot was then incubated overnight at 4°C with the selected supernatant diluted 1:25 in TBS-T 5% non-fat dry milk. After 1 h incubation at room temperature with a peroxidase conjugated goat anti-mouse-IgG antibody (Sigma Aldrich), dots were developed using enhanced chemiluminescence.

Peptide	**sequence**
$A\beta_{3(pE)-7}$	pEFRHD-C
$A\beta_{3(pE)-42}$	pEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
$A\beta_{1-42}$	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
$A\beta_{3-7}$	EFRHD-C
$A\beta_{1-4}$	DAEF-C

Table 1 peptides used for the dot blot analysis

2.9 IMMUNOHISTOCHEMISTRY ON PARAFFIN SECTIONS

Mice were anaesthetized and transcardially perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brain samples were carefully dissected and postfixed in 4% phosphate-buffered formalin at 4 °C.

Human brain samples from frontal cortex were obtained from the Netherlands Brain Bank (NBB), Amsterdam, Holland.

Immunohistochemistry was performed on 4 µm paraffin sections according to the protocol showed below. Supernatants from oligoclones cultures were used as primary antibodies in 1:10, 1:100 and 1:1000 dilution in PBS with 10% FCS (fetal calf serum) and detected with biotinylated secondary anti-mouse-IgG antibodies (Dako Denmark A/S, Glostrup, Denmark).

1. Deparaffinize:
    a. 2x5 min xylol
    b. 10 min 100% ethanol
    c. 5 min 95% ethanol
    d. 1 min 70% ethanol
    e. 1 min distilled water
2. Peroxidase Block
    a. 30 min in 200 mL PBS with 2 mL 30% H2O2
3. microwave treatment
    a. 10 min in 0.01M citrate buffer pH 6.0 (citric acid monohydrate)
    b. 15 minutes cool down
4. wash 1min in distilled water.
5. wash 15 min PBS + 0,1% Triton-X100
6. wash 1 min with PBS
7. formic acid treatment
    a. 3 min incubation in 88% formic acid
    b. 2x 1 min wash in PBS
8. unspecific block for 1 h in PBS +10% FCS (fetal calf serum) +4% dry milk (40mg/ml)
9. Discard blocking solution
10. overnight incubation with primary antibody in PBS+10% FCS room temperature

11. wash 15 min with PBS+ 0,1% Triton-X100
12. 1 h incubation with secondary antibody 1:200 in PBS including 10% FCS for 1 h at 37 °C
13. wash 15 min with PBS
14. 1,5 h incubation with ABC-Vectastain-Complex solution (1:100 solution A + 1:100 Solution B in PBS +10% FCS) at 37 °C
15. wash 15 min with PBS
16. stain with DAB (3,3-diaminobenzidine-tetrahydrochloride, Sigma) 25 mg/mL in 50 mM Tris/HCl pH 7.5
17. wash in PBS
18. Hematoxylin staining
    a. 40 seconds in Hematoxylin (Mayers hemalaun solution)
    b. 5 min tap water
19. ascending ethanol-series,
    a. 1 min 70% ethanol
    b. 5 min 95% ethanol
    c. 10 min 100% ethanol
    d. 2x10 min Xylol
20. 7. embedding with a quick hardening mounting medium

2.10 PATIENTS AND SAMPLES

Plasma samples (stored at -70 °C) from 30 out-patients with mild to moderate AD, 15 patients with mild cognitive impairment (MCI) and 30 healthy controls (HC) were analyzed. The patients were recruited at the Memory Clinic at the Department of Geriatrics, Uppsala University Hospital. All AD patients were diagnosed according to DSM IV (association 2000) and NINCDSADRDA (McKhann, Drachman et al. 1984). The MCI patients met the Petersen criteria (Winblad, Palmer et al. 2004) for MCI. All plasma samples were a generous gift from Prof. Lars Lannfelt, Uppsala University Hospital.

2.11 GELFILTRATION

Plasma samples from 5 AD patients and 5 HC showing high reactivity to Aβ-IgM immune complexes or anti-Aβ autoantibodies in a preliminary assay were grouped and analyzed by gel filtration HPLC. Five hundred microliters of pooled samples were analyzed using a gel-filtration column Superdex 200 10/300 GL (GE Healthcare, Freiburg, Germany) on an ACTA Basic system (GE Healthcare, Freiburg, Germany). The elution was carried out in PBS at a flow rate of 1 ml per minute, and sample absorbance was monitored at 280 nm. Fractions were collected every 30 seconds, immunoreactivity was tested by ELISA and the fractions corresponding to the highest immunoreactivity were pooled and used as reference standard. Before running the samples, a calibration run was carried out according to the manufacturer's instructions.

2.12 ELISA ASSAYS

Three different ELISA assay were newly designed for the detection of Aβ, Aβ-IgM immune complexes (IC) and anti-Aβ autoantibodies.

2.12.1 Aβ ELISA

The Aβ level in gel-filtration fractions was determined as follows: 96-well ELISA plates were coated with 50 µL per well of 1 µg/mL of mouse anti-human Aβ antibody directed against residues 17-24 (4G8, Signet Laboratories, Dedham, MA, USA) in PBS at 4 °C overnight and then blocked for 2 h with 3% BSA in PBS at 37 °C. After blocking, 50 µL of gel-filtration fractions in PBS containing 1% BSA and 0.05% Tween 20 (PBS-B-T) were incubated for 1.5 h at 37 °C. The wells were then washed with PBS containing 0.05% Tween 20 and incubated with 50 µL of rabbit polyclonal anti-Aβ 692 (generous gift from Gerd Multhaup, Free University of Berlin) at a 1:1,000 dilution in PBS-B-T for 1.5 h at 37 °C. Aβ was revealed using peroxidase-conjugated goat anti-rabbit-IgG (Dako, Glostrup, Denmark) in a 1:1,000 dilution in PBS-B-T and developed with TMB (Pierce manufacturing, Appleton, IL, USA) and hydrogen peroxide as substrate. The results of Aβ determination in the gel-filtration fractions were reported in optical density.

2.12.2 Aβ–IgM IC ELISA

96-well ELISA plates were coated with 50 µL per well of 1 µg/mL of 4G8 antibody (Signet Laboratories, Dedham, MA, USA) in PBS at 4 °C overnight and then blocked for 2 h with 3% BSA in PBS. After blocking, 50 µL of serially diluted reference standard or samples in PBS-B-T were incubated for 1.5 h at 37 °C. The Aβ–IgM complex was revealed using peroxidase-conjugated anti-human-IgM (Sigma-Aldrich, Munich, Germany) in PBS-B-T and developed with TMB and hydrogen peroxide as the substrate. Aβ–IgM IC levels in the plasma were expressed in arbitrary units/mL (AU/mL) by using gelfiltration-purified Aβ–IgM reference standards to design a calibration curve, where the undiluted standard was assigned 1,000 AU/ml; concentration was determined by interpolation of samples absorbance on the calibration curve. The linear range of the assay was between 3.9 and 62.5 AU/ml. The samples with values above 62.5 AU/ml were further diluted and re-measured. The assay was standardized and its analytical performance evaluated: inter- and intra-assay coefficient of variation (CV) was less than 10%. Aβ–IgM cut-off was selected as the best combination of specificity and sensitivity according to the receiver-operating characteristic (ROC) curve.

2.12.3 ANTI-Aβ-IgM ELISA

96-well ELISA plates were coated with 50 µL per well of the following antigens: 1 µg/mL of a synthetic fragment of human Aβ starting at position 3 with a modified pyroglutamate and ending at position 7 (pEFRHD); 1 µg/mL of synthetic full length human Aβ 1-42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA); 0.1 µg/mL of synthetic human Aβ38-42 (N-GVVIA); 0.1 µg/mL of synthetic human Aβ37-40 (N-GGVV) (all peptides from Synaptic Systems GmbH, Goettingen, Germany) in PBS at 4°C overnight and then blocked for 2 hours with 3% BSA in PBS. After blocking, 50 µL of fractions, serially diluted reference standards or samples in PBS-B-T were incubated for 1.5 h at 37°C. The IgM autoantibodies were revealed using peroxidase-conjugated anti-human-IgM (Sigma, Saint Louis, MI, USA) in PBS-B-T and developed with TMB (Pierce manufacturing, Appleton, IL, USA) and Hydrogen peroxide as the substrate. The amount of IgM in the plasma was expressed in arbitrary units/mL (AU/mL) by using Gel-filtration purified Aβ-IgM reference standards to design a calibration curve; concentration was

determined by interpolation of samples absorbance on the calibration curve. The samples with values above 32 AU/mL (matching the upper limit of the calibration curve) were further diluted and re-measured. The assay was standardized and its analytical performance evaluated: coefficient of variation (CV) inter- and intra-assay was less than 10%. Range of linearity of the assay was between 0.9 and 32 AU/mL.

2.13 ANTIBODIES USED IN THE PROJECT

ANTIBODY	SOURCE	MANUFACTURER	WORKING DILUTION
Primary antibodies			
PSD95	Rabbit	Cell Signalling Technology, Inc, Danvers, MA, USA	1:1000
Clathrin light chain	Mouse	Synaptic System, Goettingen, Germany	1:2000
SNAP25	rabbit	Sigma, Saint Louis, MI, USA	1:5000
GFAP	mouse	Chemicon International, Temecula, California, USA	1:3000
WO-2 (human $A\beta_{5-8}$)	mouse	Genetic company, Schlieren, Switzerland	WB 1:5000 ELISA 1:500
4G8 (human $A\beta_{17-24}$)	mouse	Signet Laboratories, Dedham, MA, USA	1:1000
692	rabbit	Generous gift from Prof. Gerd Multhaup, FU Berlin	1:2000
β-Actin	mouse	Sigma, Saint Louis, MI, USA	1:2000
tubulin	rabbit	Chemicon International, Temecula, California, USA	1:3000
HRP-conjugated Secondary antibodies			
Anti-mouse-IgG	goat	Dako, Glostrup, Denmark	1:1000
Anti-rabbit-IgG	Swine	Dako, Glostrup, Denmark	WB 1:3000 ELISA 1:5000
Anti-human-IgM	goat	Sigma, Saint Louis, MI, USA	IC ELISA 1:3000 Anti-Aβ-IgM ELISA 1:20000
Biotinylated secondary antibodies			
Anti-mouse-IgG	rabbit	Dako, Glostrup, Denmark	1:200

Table 2 working dilution, source and manufacturer of the antibodies used in the PhD project

Chapter 3

Results

3.1 AD-LIKE RELEVANT FEATURES IN TRANSGENIC MOUSE MODELS

Inflammation

2 and 6 months-old APP/PS1KI and PS1KI mice were tested by western blot to measure the level of GFAP expression in the brain. Western blotting verified the up-regulation of GFAP in 2- ($P < 0.01$) and 6-month-old APP/PS1KI mice ($P < 0.05$) on the protein level (Fig. 12) compared to PS1KI mice. This result was corroborated by immunohistochemical staining against GFAP and S100A6, showing strong astrogliosis in the hippocampus of 6-month-old APP/PS1KI mice, whereas age-matched PS1KI were almost devoid of any astroglial reaction (Wirths et al. 2008).

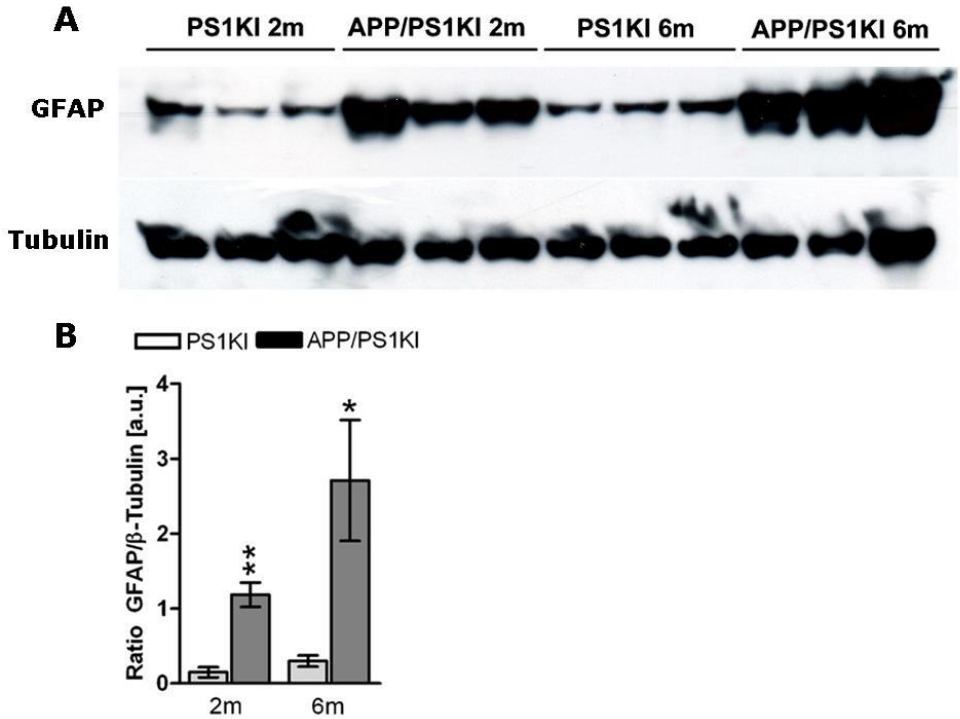


Figure 13 A Protein levels of GFAP are significantly increased in both 2- and 6-month-old APP/PS1KI mice,as shown in a representative Western blot **B**. Figure from (Wirths et al. 2008).

Synaptic deficits

2 and 6 months old APP/PS1KI and PS1KI brains were homogenized and synaptosome-enriched fractions (P2) separated from the whole-brain fractions (S2). Western blot analysis confirmed the enrichment of P2 fractions with synaptic specific marker PSD95, while tubulin was enriched in the S2 fraction, as expected (Fig. 13). Interestingly, western blot analyses evidenced a high enrichment of Aβ in the synaptosome fraction, the

enrichment being more marked in 6 months-old mice, while APP level was constant for P2 and S2 fractions for both the considered time points. These finding clearly indicate a strong association of Aβ with the synaptic area.

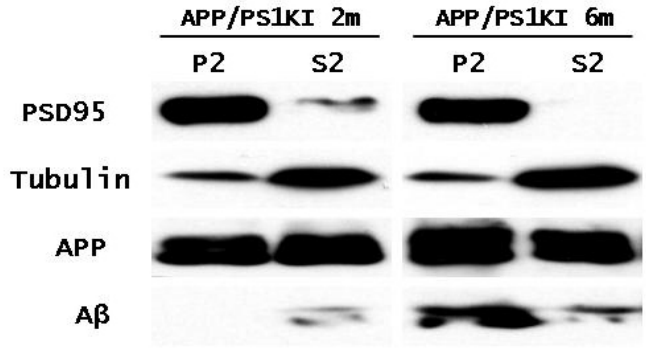


Figure 14 Western blot of P2 and S2 fractions of 2 and 6 months old APP/PS1KI mice for synaptosome marker PSD95, Tubulin, APP and Aβ. The image confirms the purification of the synaptosome fraction, and an enrichment of Aβ in it.

The levels of pre- and post-synaptic proteins were quantified by Western blot in whole brain extracts and synaptosome enriched fractions of 2 and 6 month-old mice. The proteins analyzed were: Synaptosome-associated protein of 25 kDa (SNAP25), a highly conserved protein anchored to the cytosolic face of membranes required for exocytosis of presynaptic vesicles; neuron-specific Clathrin light chains, which is enriched in synaptic nerve terminals participating in presynaptic vesicle endocytosis; PSD-95, a major protein at the postsynaptic density which has an important function in regulating the surface expression of glutamate receptors, thereby representing a critical factor regulating synaptic plasticity.

At 2 months, there was no difference between APP/PS1KI and PS1KI control mice. In whole brain Western blots only PSD-95 levels were significantly reduced at the age of 6 months in APP/PS1KI mice (Fig. 14). Of interest, the levels of all three markers were significantly decreased in synaptosome-enriched fractions in 6 month old mice (Fig. 14).

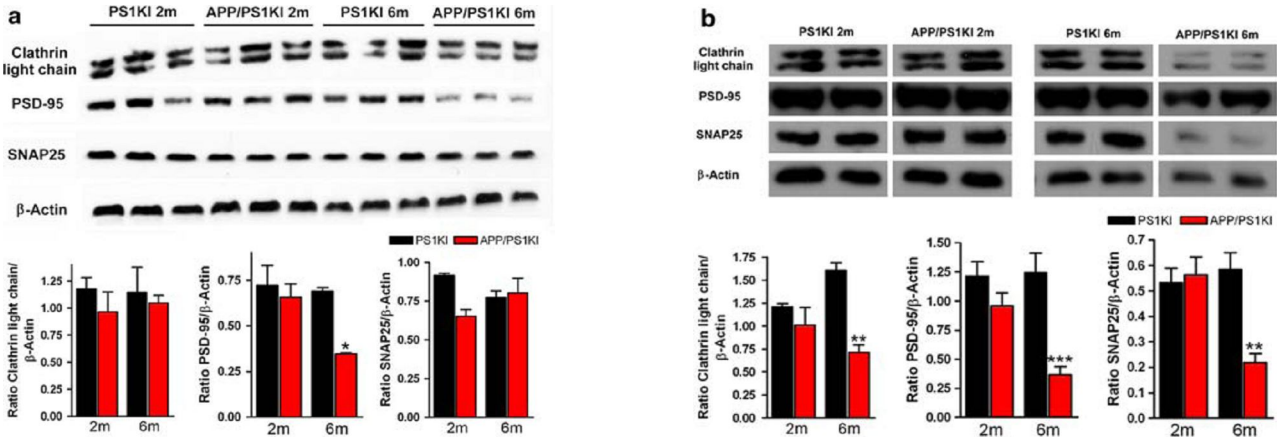


Figure 15 Western blot analysis of pre- (clathrin light chain; SNAP25) and post-synaptic markers (PSD-95). **A** While in whole brain lysates, only the levels of PSD-95 declined significantly, **B** the levels of all synaptic markers were decreased in synaptosome enriched fractions in 6-month-old APP/PS1KI mice. At 2 months, there was no difference between APP/PS1KI and PS1KI control mice. Values are given as mean values ± SEM, ***P\0.001; **P\0.01, *P\0.05. Figure from (Breyhan et al. 2009).

3.2 DEVELOPMENT OF NOVEL MONOCLONAL ANTI-Aβ$_{3(pE)}$-ANTIBODIES

After hybridoma cells have been created from both long- and short-term immunization protocols, they are plated to obtain oligoclones each consisting of 5-10 clones. By screening each oligoclone's supernatant by ELISA those showing highest ratio of reactivity against Aβ$_{3(pE)-7}$ over reactivity against Aβ$_{3-7}$ and the FCS blank control have been selected and further characterized for their specificity and affinity by IHC analysis (Tab. 4 and 5).

Clone-Nr.	ELISA OD$_{492nm}$ 07Synap31 (pEFRHD-C)	ELISA OD$_{492nm}$ 07Synap32 (DAEFRHD-C) =control peptide	ELISA OD$_{492nm}$ FCS	Subclasses
7	1,689	0,360	0,175	1, 2a, M
48	1,170	0,339	0,339	2a, M
50	0,888	0,214	0,090	2b, M
72	1,199	0,389	0.090	1, 2a, M
101	1,507	0,322	0,121	2a, 3, M
157	2,205	0,398	0,297	2b, M
192	1,327	0,246	0,123	2a
223	1,164	0,386	0,303	1, 2b, M
234	0,750	0,219	0,176	2a, M
239	1,200	0,339	0,106	1, 2a, M
245	2,206	0,283	0.093	M
248	1,596	0,365	0,040	1, 2a, 3, M
314	2,008	0,221	0,185	2a, M
357	1,771	0,255	0,144	2a, M

Table 3 ELISA of first oligoclones series from long-term immunization protocol. The selected oligoclones are evidenced in grey (courtesy of Synaptic System GmbH, Goettingen)

Clone-Nr.	ELISA OD_{492nm} 07Synap31 (pEFRHD-C)	ELISA OD_{492nm} 07Synap32 (DAEFRHD-C) =control peptide	ELISA OD_{492nm} FCS	Subclasses
70	1,2	0,09	0,1	1/2a/2b
73	0,13		0,03	1
93	1,4	0,15	0,24	1/2b/M
44	1,9	0,05	0,04	2b/M
106	1,6	0,05	0,05	2a/M
117	1,3	0,06	0,12	1/2a/M
141	2,0	0,17	0,11	2a/M
165	1,2	0,18	0,07	1/2a/M
121	1,4	0,15	0,1	1/2a/M
136	2,0	0,15	0,12	M

Table 4 ELISA of first oligoclones series from short-term immunization protocol. The selected oligoclones are evidenced in grey (courtesy of Synaptic System GmbH, Goettingen)

Supernatants from oligoclones 72, 157, 192, 245, 248 and 357 of the long-term immunization set, and from oligoclones 70 106 141 and 136 of the short-term immunization set were used to stain by IHC brain sections from APP/PS1KI and TBA2 mice and evaluate their capacity to stain $A\beta_{3(pE)}$-positive plaques (Tab. 6).

Clone-Nr.	TBA2 (undiluted)	APP/PS1KI (undiluted)	APP/PS1KI serial dilutions
72	+ Purkinje cells		
157	+++ Purkinje cells, some Cortex and Hippocampus neurons	Plaques and intracellular in CA1 / background	1:10 / background 1:100 +++ 1:1000 +++
192	+++ Pyramidal cells 1:100 no staining	Plaques and intracellular in CA1 / no background	1:10 ++ 1:100 negative 1:1000 negative
245	+ Purkinje cells		
248	+++ 1:1000	Plaques and intracellular in CA1 / no background	1:10 +++ 1:100 +++ 1:1000 ++
357	++ Pyramidal cells		
70	(+)		
106	(+)		
141	+++ Purkinjecells, negative in CA1	+++ in Plaques and CA1	1:10 +++ 1:100 +++ 1:1000 ++
136	Astrocytes	Astrocytes	

Table 5 IHC on brain slices of transgenic AD mouse models stained with oligoclones of anti-$A\beta_{3(pE)}$ monoclonal antibodies. Grey-highlighted samples are those showing intense specific staining for Aβ plaques and low background. The arbitrary intensity of the staining is indicated with 0 to 3 + crosses.

Dot Blot analysis

Supernatants from oligoclones 72, 157, 192, 245, 248, 357 and 141 showed good affinity for Aβ plaques and very low unspecific background and have therefore been selected for further analysis. Their specificity for the pyroglutamate modification and their sensitivity threshold have been evaluated with a dot blot experiment against N-terminal fragment and full-length $Aβ_{3p(E)-42}$, full-length $Aβ_{1-42}$ and N-truncated native $Aβ_{3-7}$ (Tab. 7).

Clone-Nr.	$Aβ_{3(pE)-7}$	$Aβ_{3-7}$	$Aβ_{3(pE)-42}$	$Aβ_{1-42}$
72	5 ng	negative	0,5 ng	negative
141	5 ng	Negative	50 ng	negative
157	5 ng	Negative	50 ng	negative
192	5 ng	Negative	5 ng	negative
245	0,5 ng	Negative	0,5 ng	negative
248	5 ng	Negative	5 ng	negative
357	0,5 ng	Negative	0,5 ng	negative

Table 6 Dot blot against 4 Aβ-derived peptides of selected anti-$Aβ_{3p(E)}$ oligoclones. All samples showed no cross-reaction with unmodified peptides, and a sensitivity threshold of at least 50 ng

Immunohistochemistry on AD brain

Brain slices obtained from post mortem AD patients at braak stage 4 are stained with the selected supernatants to verify the recognition of Aβ plaques in human (Tab. 8).

Clone-Nr.	1:10	1:100
72	-	-
141	+++ some background	+++
157	+++	+++
192	+ 1:10	++ 1:10
245	-	-
248	+ 1:10	++ 1:10
357	+	+

Table 7 IHC on post-mortem brain slides from AD patients

1ST SUBCLONING

Selected oligoclones have been subcloned in order to obtain purer composition of supernatant in terms of Immunoglobulin classes. Of the 7 clones selected, clones 141, 157, 248 and 357 produced new colonies. The subclones have been tested in IHC experiments on mouse brain to assess whether there had been improvement in specificity and sensitivity and further select the best candidates (Tab. 9).

141	APP/PS1KI serial dilutions
A8	1:10 +++ 1:100 +++ 1:1000 ++
C6	1:10 +++ some background 1:100 +++ 1:1000 +++
E2	1:10 +++ some background 1:100 +++ some background 1:1000 +++
F8	1:10 +++ 1:100 ++ 1:1000 -
G3	1:10 ++++ some background 1:100 +++ 1:1000 +++
H5	1:10 +++ some background 1:100 +++ 1:1000 ++

157	APP/PS1KI serial dilutions
B7	1:10 +++ 1:100 +++ 1:1000 ++
B9	1:10 +++ 1:100 +++ 1:1000 +++
B10	1:10 +++ 1:100 ++ 1:1000 ++
C3	1:10 ++ 1:100 ++ 1:1000 +
C9	1:10 +++ 1:100 +++ 1:1000 ++
C12	1:10 +++ 1:100 +++ 1:1000 +++
D1	1:10 +++ 1:100 +++ 1:1000 +++

248	APP/PS1KI serial dilutions
B5	1:10 +++ 1:100 +++ 1:1000 +++
C2	1:10 +++ 1:100 +++ 1:1000 +++
C3	1:10 +++ 1:100 ++ 1:1000 ++
E2	1:10 +++ 1:100 +++ 1:1000 +++
F2	1:10 ++++ 1:100 +++ 1:1000 +++
G6	1:10 +++ 1:100 ++ 1:1000 -

357	APP/PS1KI serial dilutions
G7	1:10 + 1:100 + 1:1000 -
H1	1:10 + 1:100 + 1:1000 -
H3	1:10 ++ 1:100 ++ 1:1000 +
H4	1:10 + 1:100 - 1:1000 -
H6	1:10 + 1:100 + 1:1000 -
H7	1:10 + 1:100 + 1:1000 -

Table 8 IHC on slides from APP/PS1KI mice with the supernatant obtained from the first subclones. Each supernatant has been tested in 3 dilutions (1:10, 1:100, 1:1000) and the intensity of the signal evaluated visually assigning a score either negative (-) or positive (+s). In grey are highlighted the best candidates.

2ND Subcloning

Colonies producing the supernatants displaying the best staining properties have been further subcloned to obtain monoclonal colonies. The supernatants obtained have been used in an IHC experiment (Tab. 10) to determine the 2 clones having the highest sensitivity and specificity characteristics.

141H5	APP/PS1KI serial dilutions
C5	1:100 - 1:1000 -
E4	1:100 + 1:1000 -
E8	1:100 - 1:1000 -
F1	1:100 + 1:1000 -
H1	1:100 - 1:1000 -

157B10	APP/PS1KI serial dilutions
C2	1:100 +++ 1:1000 +++
C4	1:100 + 1:1000 +
D2	1:100 ++ 1:1000 -
E1	1:100 + 1:1000 -
F6	1:100 ++ 1:1000 -
H9	1:100 +++ 1:1000 +++

248 B5	APP/PS1KI serial dilutions
A11	1:100 + 1:1000 -
B1	1:100 +++ 1:1000 +++
E2	1:100 + 1:1000 -
E5	1:100 +++ 1:1000 +++
F1	1:100 - 1:1000 -
G4	1:100 + 1:1000 -
H1	1:100 +++ 1:1000 +++

Table 9 IHC on brain slices from APP/PS1KI mice with supernatants from second subcloning to determine the 2 clones having the highest sensitivity and specificity characteristics.

Supernatants 248b5b1 (re-named 2-48, subclass IgG1) and 157b10H9 (re-named 1-57, subclass IgG2b) have finally been selected as showing the best results for staining of $A\beta_{3p(E)}$-positive plaques. Figures 16, 17 and 18 show the comparison of sensitivity and specificity in IHC and dot blot of the oligoclones, the first subclones and the second and final subclones.

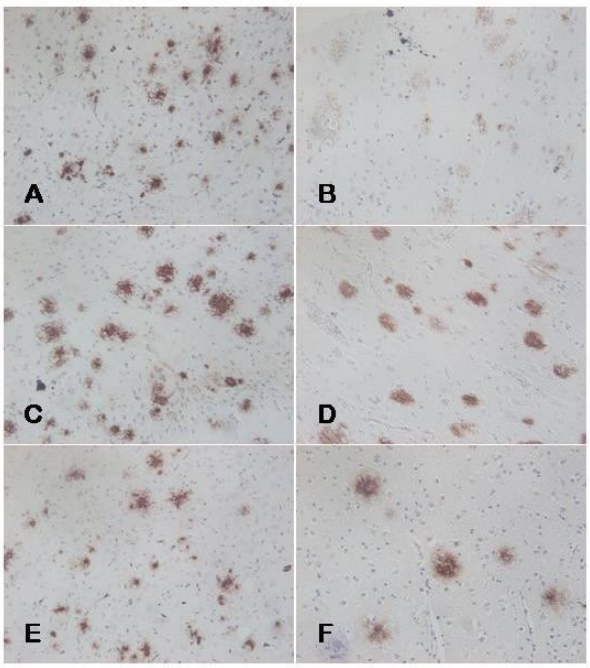


Figure 16 Immunohistochemical staining of brain sections from APP/PS1KI mice (A, C, E) and AD patients (B, D, F) with successive subclones of the 2-48 antibody: 248 (A, B), 248B2 (C, D), and the definitive 248B2B1 (E, F)

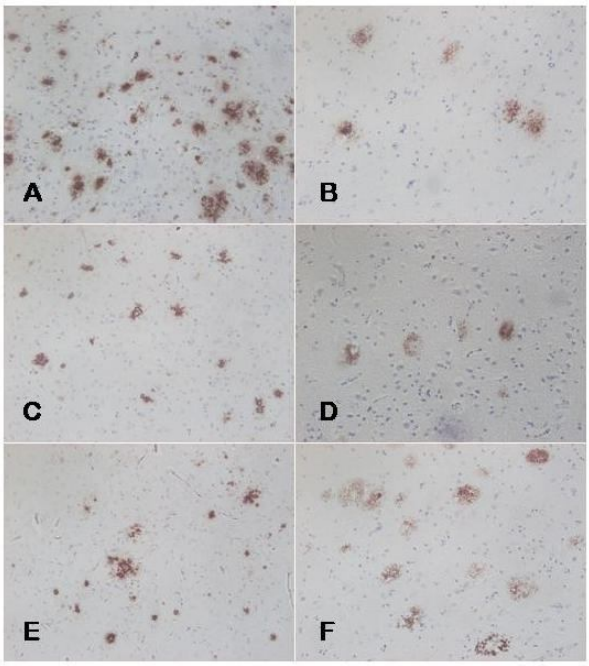


Figure 17 Immunohistochemical staining of brain sections from APP/PS1KI mice (A, C, E) and AD patients (B, D, F) with successive subclones of the 1-57 antibody: 157 (A, B), 157B10 (C, D), and the definitive 157B10H9 (E,F).

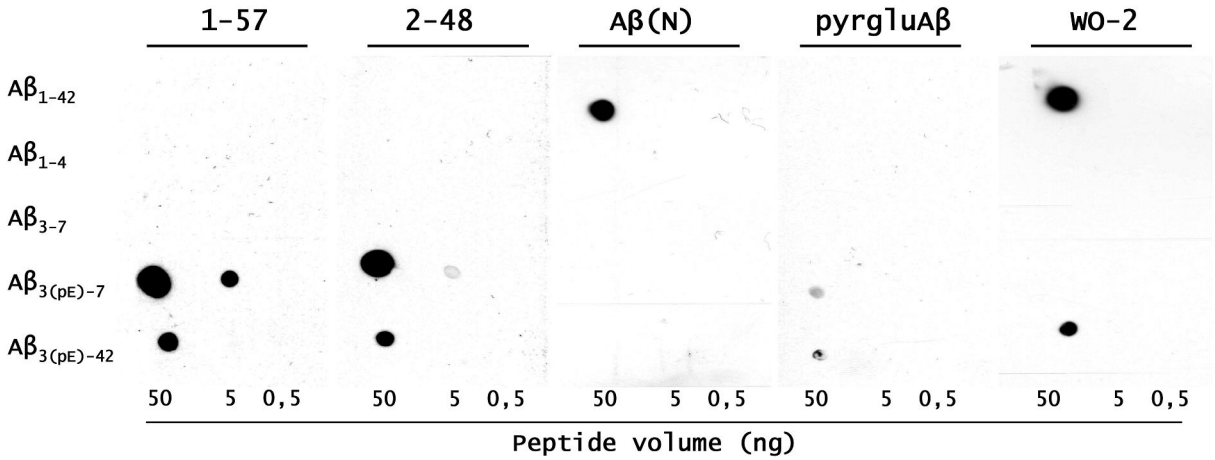


Figure 18 Comparison of Dot Blot performance for the newly developed 1-57 and 2-48 monoclonal antibodies and 3 commercially available antibodies. The peptides used for recognition are $A\beta_{1-42}$, $A\beta_{1-4}$, $A\beta_{3-7}$, $A\beta_{3(pE)-7}$ and $A\beta_{3(pE)-42}$. AβPyrGlu is a commercially available monoclonal antibody recognizing specifically Aβ starting at position 3 with a pyroglutamate, Aβ(N) recognize only Aβ starting at position 1 and WO-2 is directed against residues 4 to 10

3.3 IMMUNE COMPLEXES OF Aβ AND IgM IN THE BLOOD OF AD, MCI PATIENTS AND HC

There were no significant age differences between AD patients, MCI patients and HC ($F = 0.3$, $df = 2, 72$; $P = 0.74$). Patients with MCI diagnosis were not significantly older at onset of the disease than AD patients ($F = 2.3$, $df = 1, 42$; $P = 0.13$). As expected, the mean

MMSE score was higher in MCI compared to AD patients (F = 44.3, df = 1, 40; P\0.0005). Gender distribution was not significantly different between the three groups (Chi2 = 1.2, df = 2; P = 0.55) and the proportion of family members with dementia did not differ significantly between the two patients groups (Chi2 = 0.8, df = 1; P = 0.38) (Table 11).

Demographic data of the studied groups.

	AD		MCI		HC	Chi^2	d.f.	*p*
Gender (male; female)	m:12; f: 18		m: 8; f: 7		m:11; f: 19	1.2	2	0.55
Dementia in family	yes: 16; no: 12		yes: 6; no: 8			0.8	1	0.38
	n	m ± SD	*n*	m ± SD	*n*	m ± SD	*F*	d.f.	*p*
Age (years)	30	72.3 ± 6.7	15	73.7 ± 4.3	30	72.5 ± 5.2	0.3	2.72	0.74
Age at onset (years)	30	67.9 ± 7.9	14	71.4 ± 4.9			2.3	1.42	0.13
MMSE (at screening)	27	17.3 ± 6.1	15	27.9 ± 1.3			44.3	1.40	<0.0005

AO: Alzheimer's disease, MCI: mild cognitive impaired individuals, HC: healthy controls, m: mean, SD: standard deviation, Chi^2: Chi-square statistic, *F*: *F*-slatistic, d.f.: degrees of freedom, *p*: probability, m: male, f: female.

Table 10 Demographic data of the studied groups**.**

In order to identify circulating Aβ–IgM complexes, plasma samples from ten individuals were pooled and analyzed by gel filtration. Fractions collected from the column were tested for the presence of generic Aβ and Aβ–IgM complexes using dedicated ELISA assays (Fig. 19A). A strong immunoreactivity against Aβ was observed in the fraction eluting at high molecular weight (>500 kDa) (Fig. 19A).

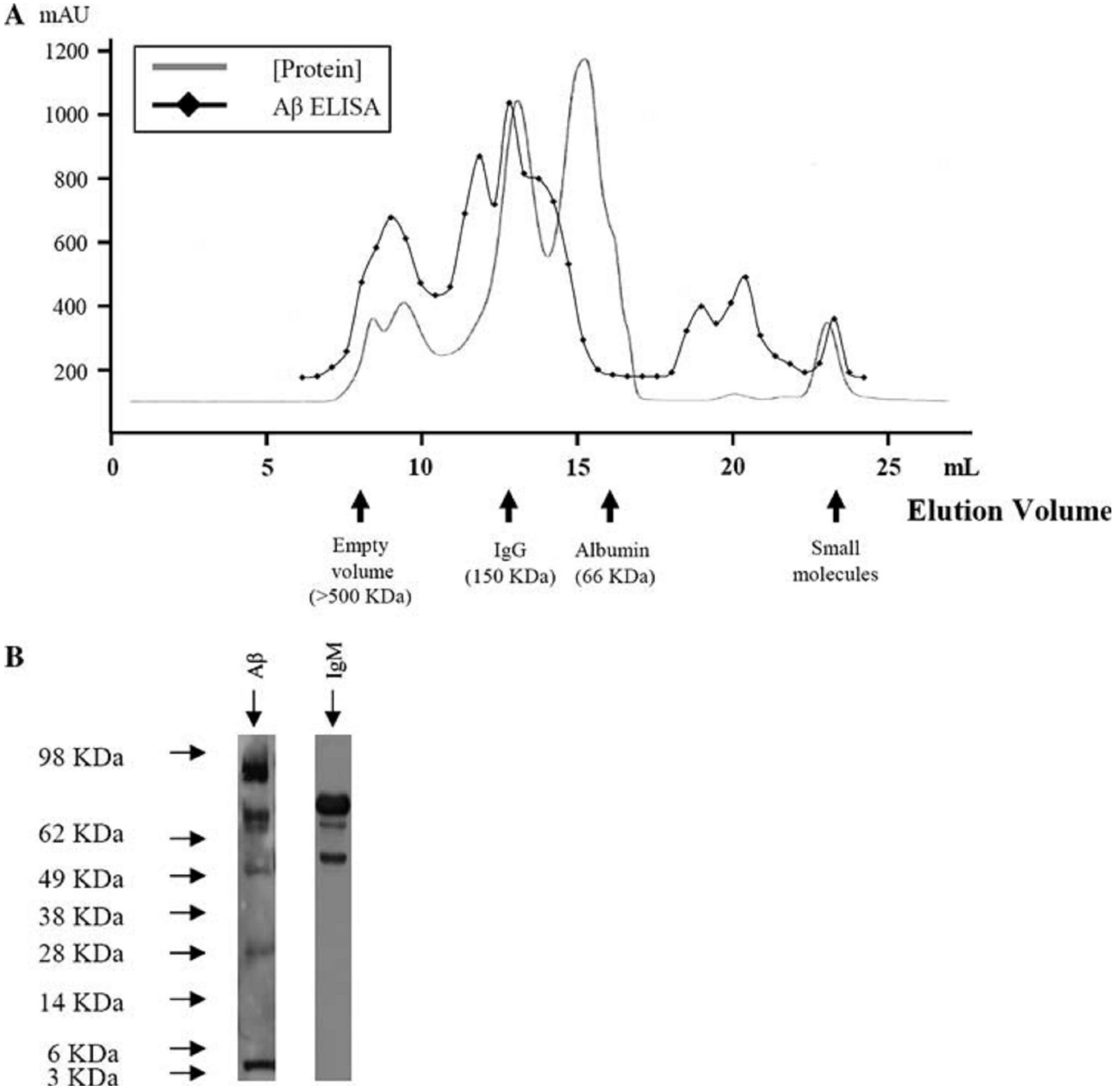


Figure 19 (**A**) Gel-filtration HPLC of pooled plasma samples. Proteins are separated according to their molecular weight, with the larger proteins eluting first. Total protein concentration of each fraction was measured at 280 nm (gray line). The characteristic peaks were as follows: empty volume with a MW[500 kDa (IgMs), IgGs with a MW of *150 kDa, and the albumin peak at *66 kDa. A direct ELISA performed on each fraction to measure the quantity of generic Aβ (diamonds) shows that most of Aβ elutes in the empty volume and IgG fractions suggesting that Aβ(Kuo et al. 2000) in the blood is strongly associated with antibodies. (**B**) Western blot against Aβ in the empty volume (IgM) fraction and IgM showing the presence of Aβ in the IgM fraction.

In the same fraction high reactivity for complexes of Aβ and IgM was also found, thus providing the first evidence of the occurrence of circulating Aβ–IgM immunecomplexes (Fig. 20C). Reactivity for generic Aβ was also found in the fractions eluting at 150 kDa (IgG fraction) and at low molecular weight (Fig. 19A), confirming previous observations (Kuo et al. 2000). The high molecular weights fraction contained components migrating at the

expected molecular weight for reduced IgM that were stained by antihuman-IgM antibodies (Fig. 19B).

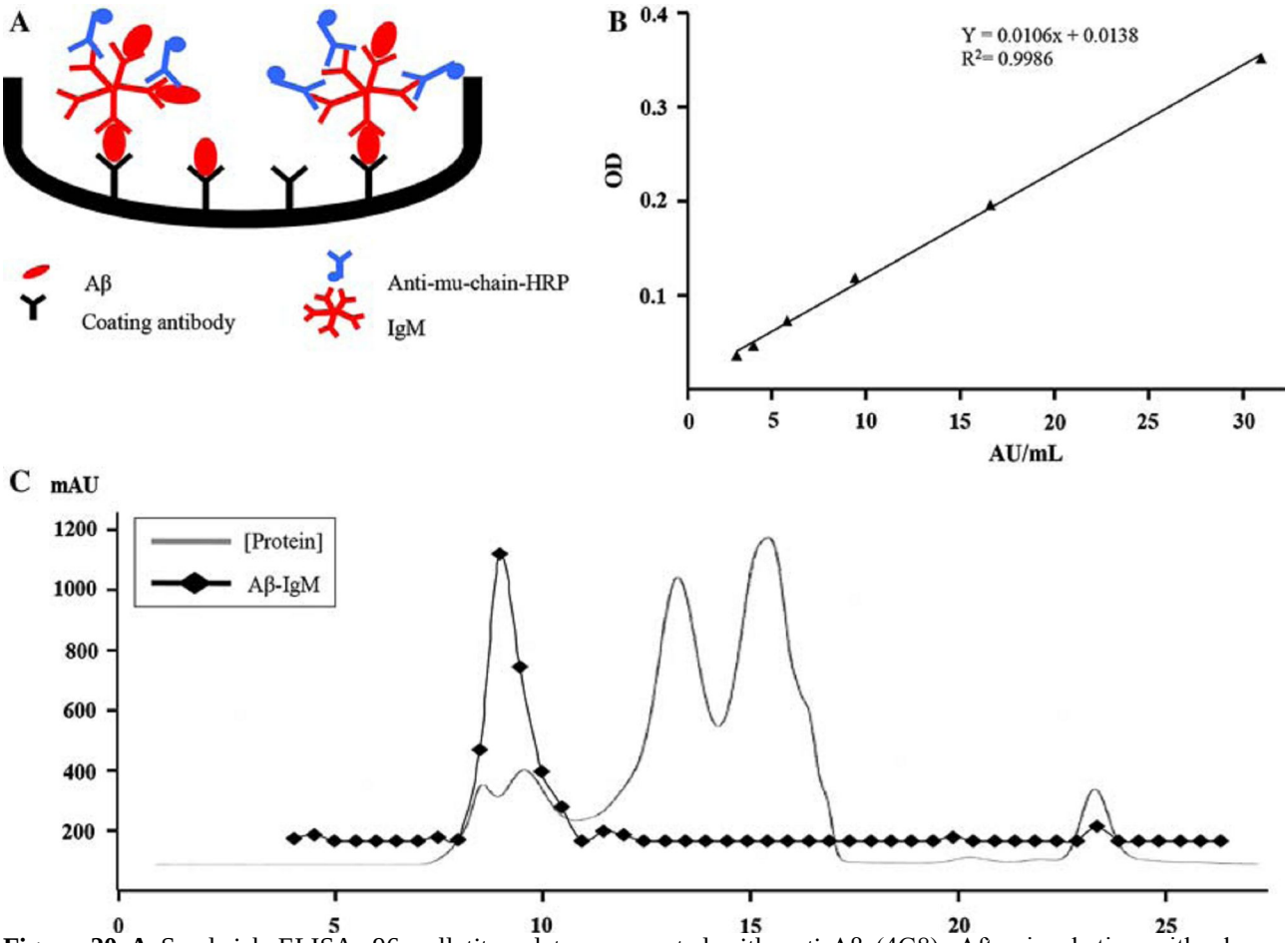
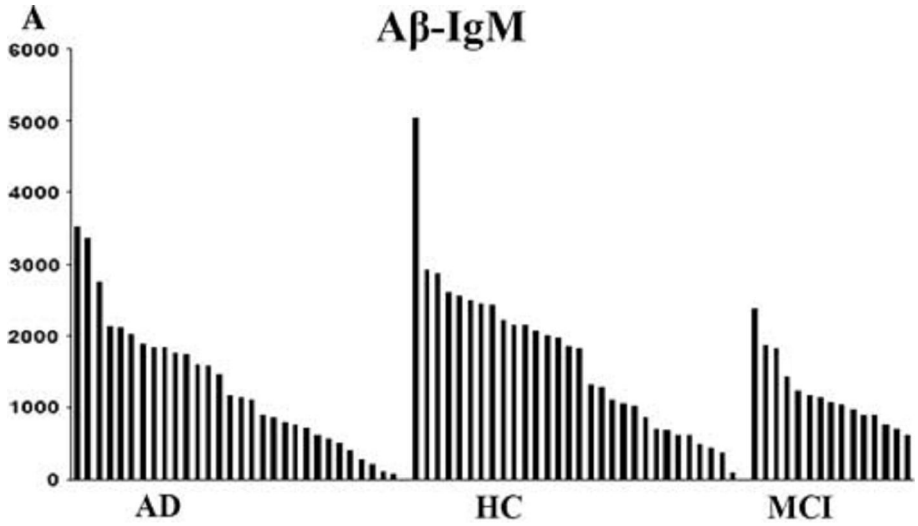
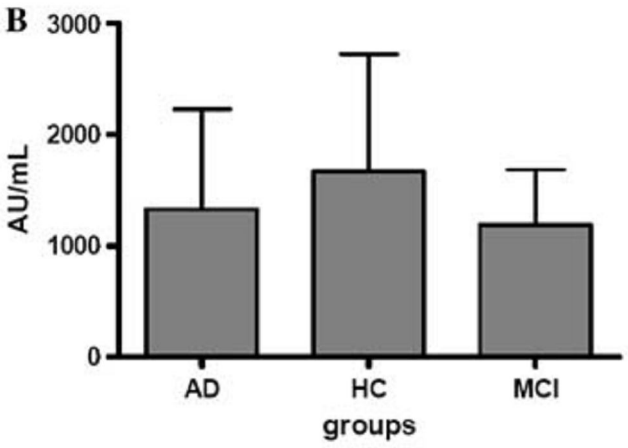


Figure 20 A Sandwich ELISA. 96-well titer plates are coated with anti-Aβ (4G8). After incubation with plasma samples, the wells are washed and incubated with an anti-IgM heavy chain antibody conjugated with HRP. Signal of bound molecules is therefore only produced by complexes of both IgM and antigen. **B** Calibration curve obtained by serial dilutions of the IgM fraction. **C** Aβ-IgM (diamonds) immune complexes in the fractions derived from gel-filtration HPLC compared to total protein concentration (solid line).

To assess the relevance of these findings, plasma samples from 75 patients with AD, MCI, and from healthy donors were analyzed for the presence of Aβ–IgM ICs. Serial dilutions of the gel filtration-purified IC fraction have been used to draw a reference calibration curve. By interpolation with the standard curve, Aβ–IgM IC concentration in the plasma samples has been expressed in arbitrary units (AU)/ml. According to ANOVA test, no significant differences among the three diagnostic groups were observed (Student t test P value AD vs. HC = 0.18, AD vs. MCI = 0.61, MCI vs. HC = 0.10) (Fig. 21). ROC curve analysis showed that specificity was only 46.67% when sensitivity was set at 80%, and by setting specificity above 80%, sensitivity was 26.67% (Table 12).





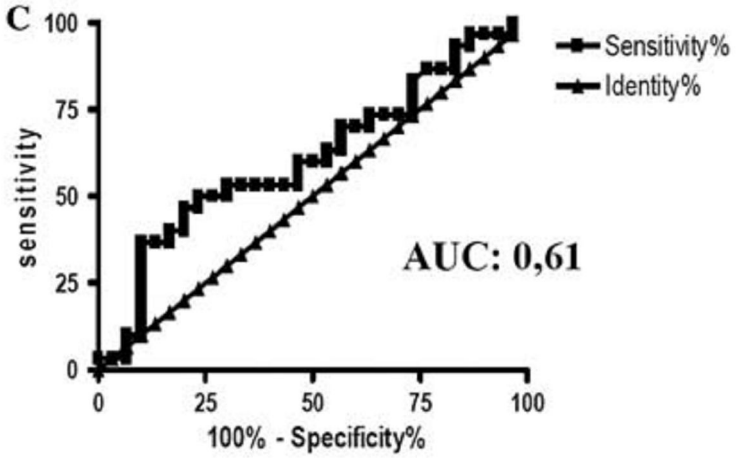


Figure 21 Analysis of Aβ–IgM immune complexes. a Levels of Aβ–IgM immune complexes in the different diagnostic groups. b The comparison of the mean values do not show any significant difference. c ROC curve analysis shows a certain difference in the distribution of the immune complexes plasma concentration between AD and HC groups, which however was not significant

Biomarker	Cut-off (AU/mL)	Sensitivity	Specificity	PPV	NPV
Aβ-IgM	<1926	80% (24/30)	46.67% (16/30)	60%	70%
	<648.5	26.67% (8/30)	80% (6/30)	57%	52%

Table 11 Comparison of specificity, sensitivity, positive predictive value (PPV = true positive (TP)/TP ? false positive), negative predictive value (NPV = true negative (TN)/TN ? false negative) of Aβ-IgM comparing AD patients with the healthy control (HC) group

3.4 ANTI-$A\beta_{3(pE)}$-IGM in AD

The general procedure for the screening of naturally occurring autoantibodies (Nabs) in the studied groups is summarized in Fig 22

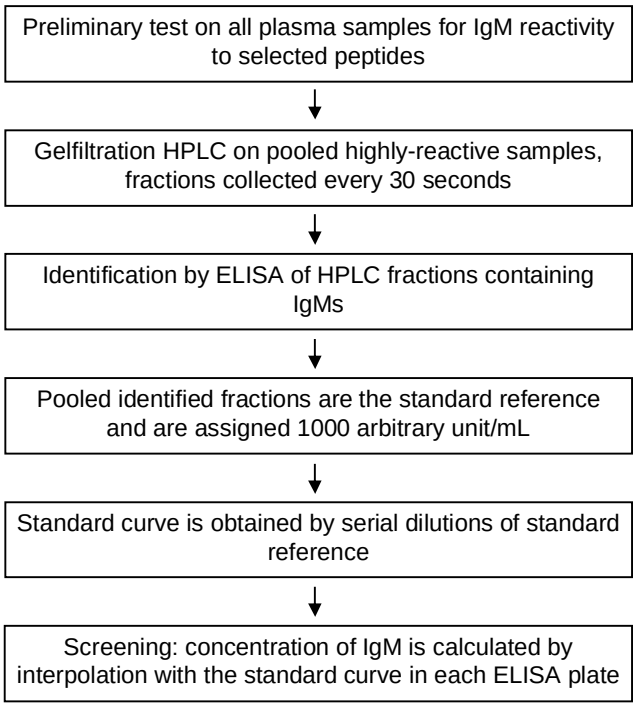


Figure 22 Successive steps for the selection and purification of the reference standard for the screening of NAbs plasma level.

The three groups studied for immune complex presence were used to measure the level of specific NAbs directed against some relevant Aβ epitopes: plasma samples from 10 individuals were pooled and analyzed by gel filtration. Fractions collected from the column were tested for the presence of anti- $A\beta_{3(pE)}$-IgM using ELISA assays (Fig. 23). A strong immunoreactivity against anti- $A\beta_{3(pE)}$-IgM was observed in the fractions eluting at high

molecular weight (> 500 kDa) (Figure 23A). The fractions contained components migrating at the expected molecular weight for reduced IgM that were stained using an anti-human IgM antibody (Figure 23B).

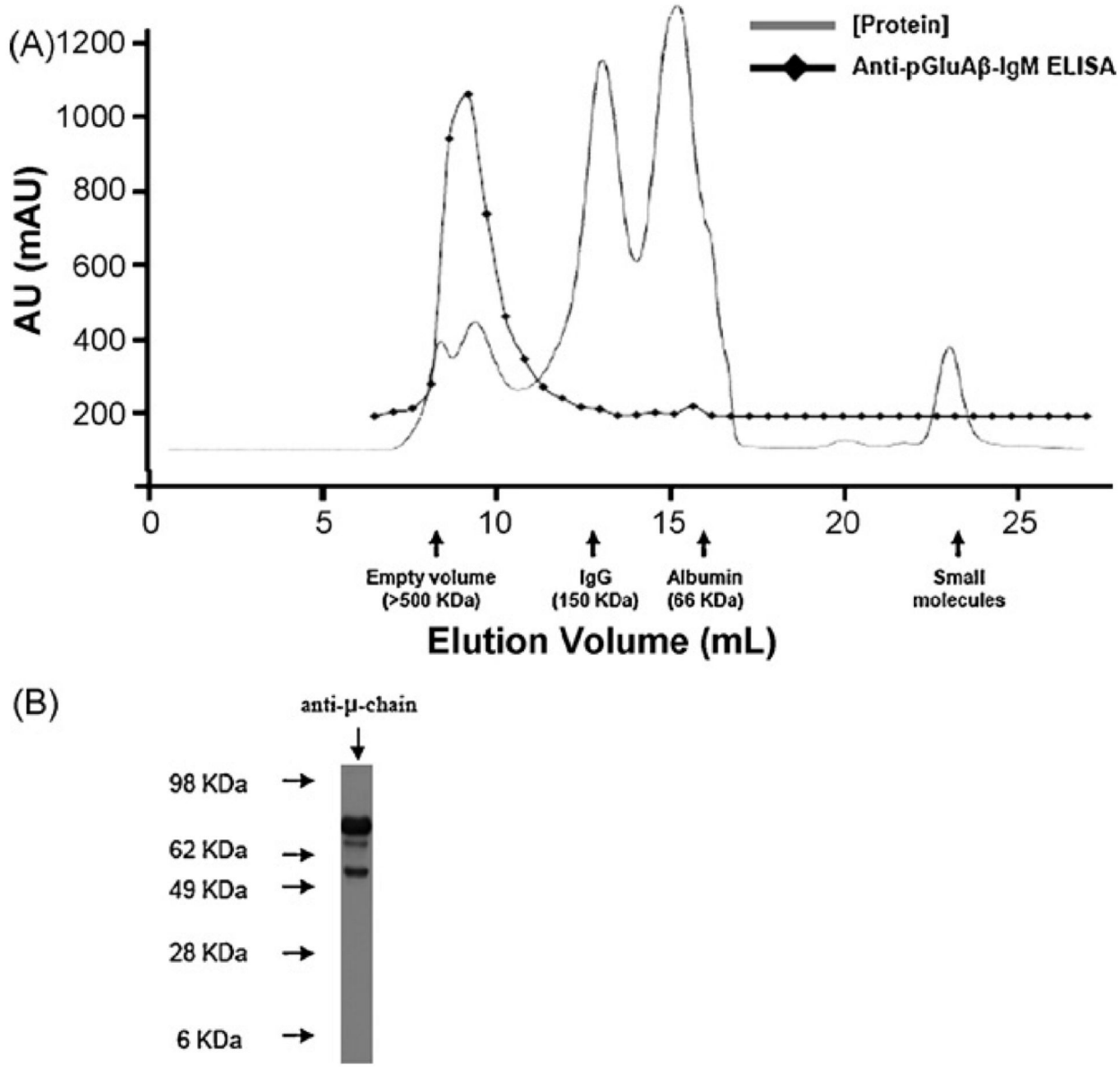


Figure 23 (A) Gel-filtration HPLC of pooled plasma samples. Proteins are separated according to their molecular weight, with the larger proteins eluting first. Total protein concentration of each fraction was measured at 280 nm (grey line). The characteristic peaks were as follows: empty volume with a MW>500 kDa (larger proteins and aggregates), IgGs with a MW of ~150 kDa, and the albumin peak at~ 66 kDa. A direct ELISA performed on each fraction to measure the quantity of anti-Aβ3(pE)-Aβ-IgM (squares) confirms that IgMs elute in the empty volume fractions. (B) Western blot against IgM heavy chain in the empty volume fraction.

To assess the relevance of these findings, plasma samples from 75 patients with AD, MCI, and from healthy controls were analyzed for the presence of natural IgM autoantibodies

against different Aβ epitopes. Serial dilutions of the gel filtration-purified IgM fractions have been used to draw a reference calibration curve (Figure 24B).

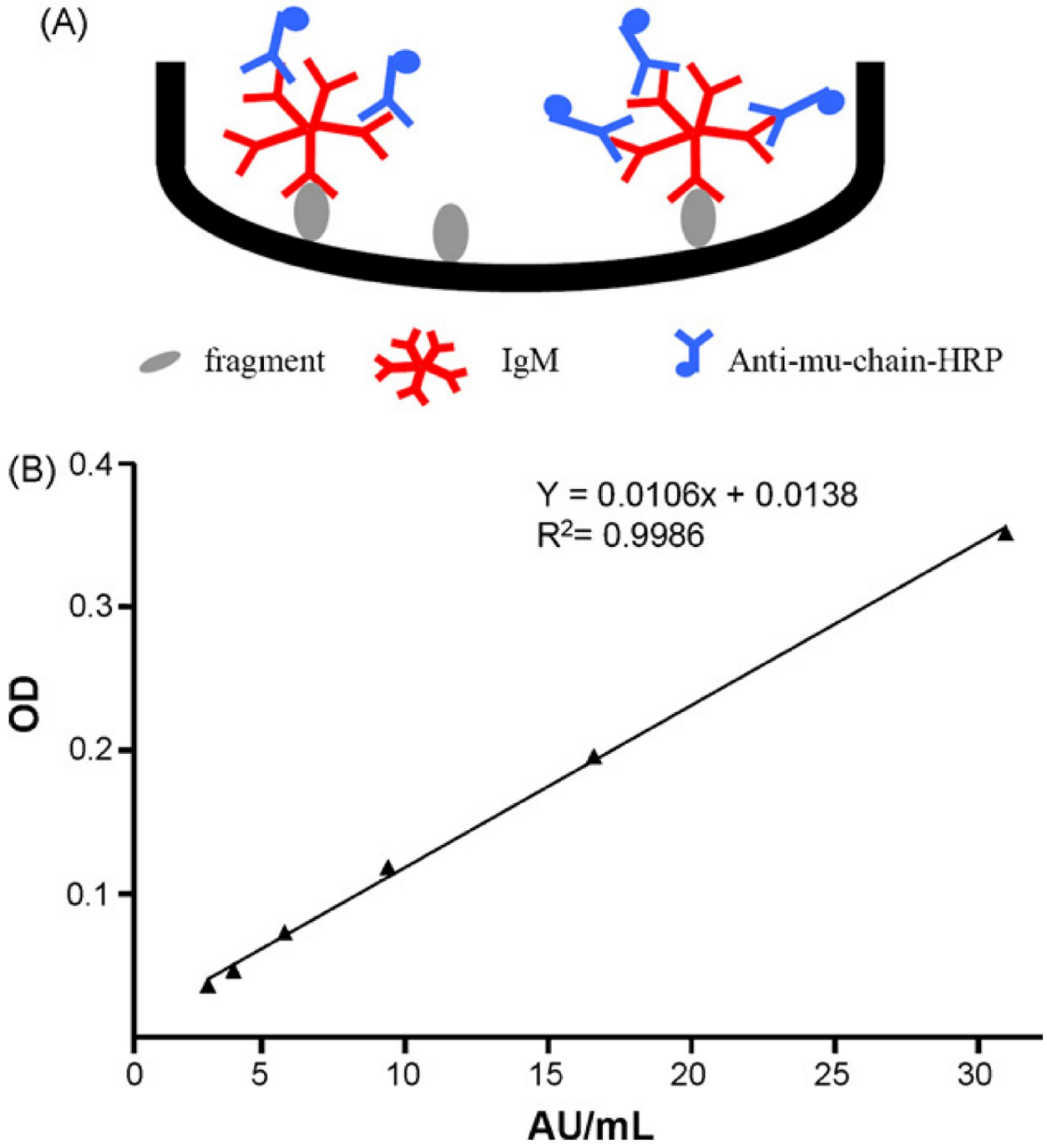


Figure 24 (A) Sandwich ELISA: 96-well titer plates are coated with different Aβ fragments (grey). After incubation with plasma samples, the wells are washed and incubated with an anti-IgM-heavy chain antibody conjugated with HRP (Blue). Signal of bound molecules is therefore only produced by complexes IgMs (red) recognizing that specific fragment. (B) Calibration curve obtained by serial dilutions of the IgM fraction.

By interpolation with the standard curve, anti-Aβ-IgM concentration in the plasma samples has been expressed in arbitrary units per mL (AU/mL). The titer of IgM autoantibodies against Aβ1-42, Aβx-40 and Aβx-42 was not significantly different between AD patients and HC (data not shown). The mean level of anti-$A\beta_{3(pE)}$-IgM was significantly decreased in AD patients as compared to healthy controls (3-group ANOVA: F = 3.2, df = 2, 72, p = 0.045; AD vs. HC: - 29%, p = 0.021). In addition, there was a trend for anti-Aβ3(pE)-IgM reduction in MCI patients versus healthy controls (-28%, p = 0.071), while there was no significant difference between the two patient groups (AD vs. MCI: -2%, p = 0.92) (Figure 25 and table 13).

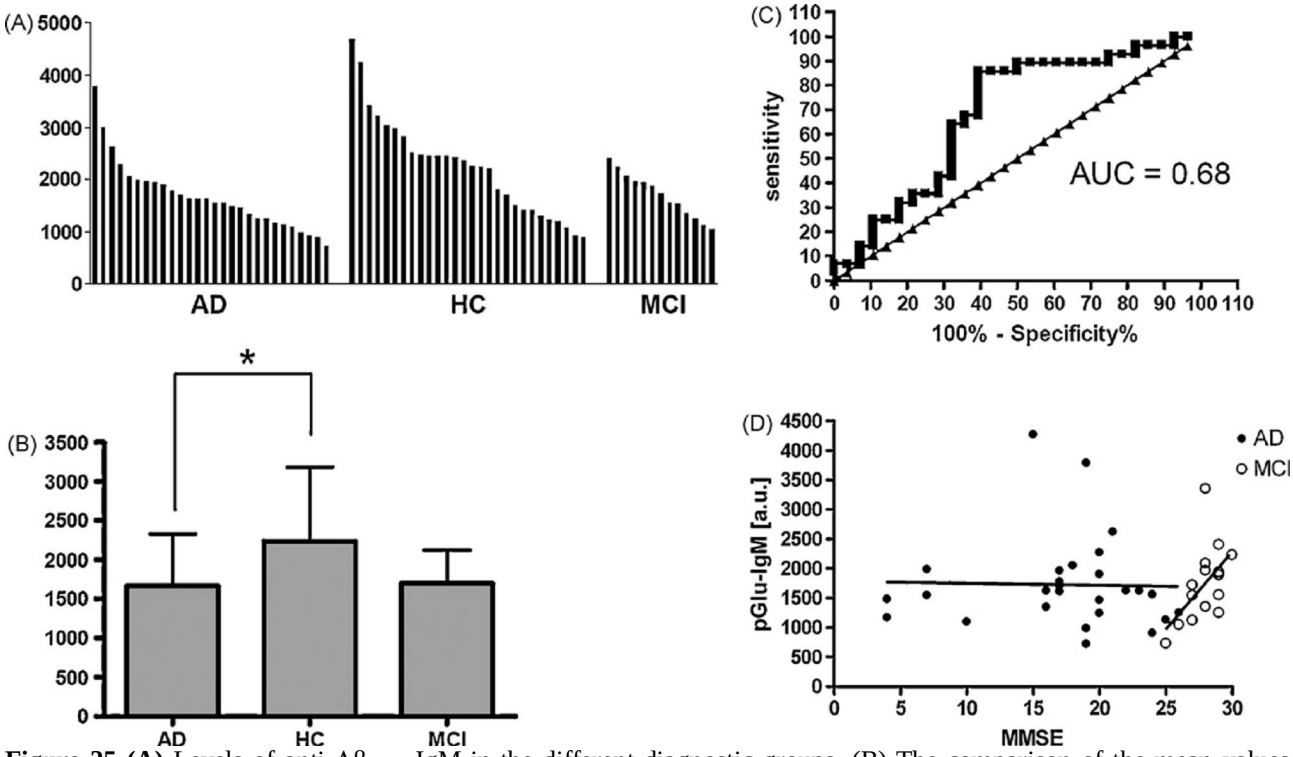


Figure 25 (A) Levels of anti-$A\beta_{3(pE)}$-IgM in the different diagnostic groups. (B) The comparison of the mean values shows a significant reduction in AD group compared to HC. (C) ROC curve analysis shows a discrete Area Under the Curve (AUC) of 0.68 in discriminating AD and HC samples. By setting sensitivity at a value higher than 80% corresponding specificity was 60%. (D) While the anti-$A\beta_{3(pE)}$-IgM levels correlated significantly with MMSE scores in MCI patients (rho = 0.58, df = 13, p = 0.022), no correlation was observed in the group of AD patients.

	AD		MCI		HC		ANOVA			Subgroup comparison		
	n	m ± SD	*n*	m ± SD	*n*	m ± SD	3-Group comparison			AD vs. HC (*p*)	MCI vs. HC (*p*)	MCI vs. AD (*p*)
							F	d.f.	*p*			
Anti-pGlu-Aβ IgM	30	1706 ± 338	15	1741 ± 648	30	2405 ± 1532	3.2	2.72	0.045	0.021	0.071	0.92

AD: Alzheimer disease, MCI: mild cognitive impairment, HC: healthy controls, ANOVA: analysis of variance, m: mean, SD: standard deviation, *F*: *F*-statistic, d.f.: degrees of freedom, *p*: probability.

Table 12 Comparison of anti-$A\beta_{3(pE)}$-IgM between the diagnostic groups.

ROC curve analysis of the anti-Aβ3$_{(pE)}$-IgM showed that specificity was 60% when sensitivity was set to 80%, and sensitivity was 32% when specificity was set to 80%, comparing AD patients with HC, with an area under the curve (AUC) of 0.68 (Figure 25C, Table 14).

Interestingly, in the group of AD patients there was no significant correlation between anti-Aβ3$_{(pE)}$-IgM and cognitive decline analyzed by MMSE (rho = -0.1, df = 25, p = 0.65). These two variables correlated however significantly positive in MCI patients (rho = 0.58, df = 13, p = 0.022; Figure 25D).

Biomarker	Cut-off (AU/mL)	Sensitivity	Specificity	PPV	NFV
Anti-pGlu-Aβ IgM	<2016	80% (24/30)	60% (12/30)	67%	75%
	<1277	33% (10/30)	80% (6/30)	63%	55%

Table 13 Comparison of specificity, sensitivity, Positive Predictive Value (PPV = true positive (TP)/TP+false positive), Negative Predictive Value (NPV = true negative (TN)/TN+false negative) of anti- Aβ3(pE)-IgM assay when setting alternatively sensitivity and specificity to 80%, according to ROC curve analysis.

Chapter 4

Discussion

4.1 AD-LIKE RELEVANT FEATURES IN TRANSGENIC MOUSE MODELS

Inflammation

Beside the two classic hallmarks, amyloid plaques and NFT, AD development is also characterized by diffuse Inflammation of the brain, as previously demonstrated in the brain of human AD patients showing astrocyte activation (Dickson 1997), as well as microglial responses (Rogers et al. 1988; Griffin et al. 1995).

Considering that in 6 month-old APP/PS1KI mice a profound hippocampal neuron loss, accompanied by impairment in cognitive and motor tasks, is detectable, (Casas et al. 2004; Wirths et al. 2008), aim of this work was to investigate GFAP expression in young pre-symptomatic (2 months) and adult (6 months) APP/PS1KI and PS1KI control mice.

Western blot analysis of protein level in young APP/PS1KI mice revealed an up-regulation of GFAP already at 2 months compared to PS1KI controls, and the increase appeared to be significantly higher in 6 months-old APP/PS1KI mice.

This result was in line with quantitative real-time PCR and immunohistochemical staining against GFAP and S100A6 in the same mice, showing strong astrogliosis in the hippocampus of 6-month-old mice, whereas age-matched PS1KI mice were almost devoid of any astroglial reaction (Wirths et al. 2008). These data are consistent with previous results obtained in other APP-transgenic mouse models where astro- and microgliosis has been consistently reported (Frautschy et al. 1998; Benzing et al. 1999; Bornemann et al. 2001; Gordon et al. 2002; Richards et al. 2003). Although the relationship between extracellular amyloid plaques and astrocytes is not completely clear, recent data suggest that astrocytes were able to internalize Aβ peptides, thereby supporting the role of astrocytes as active Aβ clearing cells in the central nervous system (Pihlaja et al. 2008).

In support of a role for inflammation in AD, inflammatory changes have been linked to axonal degeneration in a number of other neurodegenerative conditions. In fact, in murine experimental autoimmune encephalomyelitis (EAE), an overlap between the earliest manifestations of axonal damage and astrocytic responses has been reported (Wang et al. 2005). Furthermore, in experimental spinal cord injury (SCI) an early up-regulation of genes related to inflammation (Ahn et al. 2006), as well as infiltration of blood-derived monocytic cells and their morphologic transformation into microglia in zones of acute, anterograde (Wallerian) axonal degeneration induced by entorhinal cortex lesion (ECL) was reported (Bechmann et al. 2005).

In conclusion, this analysis of inflammatory changes in APP/PS1KI mice, coupled with the preceding findings in the same mice illustrated in the introduction, indicates early brain astroglial activation, which comes along with the first deposition of extracellular plaques already at the age of 2 months. Subsequently, when other pathological alterations like axonal degeneration, hippocampal neuron loss and behavioural deficits are also evident, GFAP expression is further increased, in agreement with the expression of the other components of the immune system previously analyzed.

Synaptic deficits

An emerging role for intracellular Aβ accumulation has been previously shown in human AD (Gouras et al. 2000; D'Andrea et al. 2002). It has been observed that Aβ localizes predominantly to abnormal endosomes (Cataldo et al. 2004), multivesicular bodies and within pre- and postsynaptic compartments (Takahashi et al. 2002; Langui et al. 2004). Takahashi et al. (Takahashi et al. 2004) demonstrated that $A\beta_{42}$ aggregates into oligomers within endosomal vesicles and along microtubules of neuronal processes, both in cultured Tg2576 neurons, as well as in Tg2576 and human AD brain. Moreover, several other APP transgenic mouse models showed impairment of basal synaptic transmission (Hsia et al. 1999; Moechars et al. 1999; Fitzjohn et al. 2001).

In this project, strongly reduced levels of pre- and postsynaptic markers in the synaptosome fraction have been detected in the same time span where LTP deficits had been previously observed (see introduction). Interestingly, only the synaptosome fraction displayed such reduction, while the whole brain fraction showed no differences, except for post-synaptic density protein PSD-95, which might indicate a stronger pathological effect at the post-synaptic site. Notably, Aβ has also been found for the first time to be highly enriched in the synaptosome fraction; this finding might indicate a direct link between Aβ accumulation and synaptic deficits.

This group previously observed that different Aβ peptides strongly accumulate within CA1 neurons in the critical period between 2 and 6 months of age (Wirths et al. 2004); from the observation of LTP deficits (see introduction), it has been speculated that there is a narrow period for the onset of the synaptotoxic effect, since 4-months-old APP/PS1KI mice and 6-months-old APP/PS1KI hemizygous mice with only one PS1 knock-in allele show no synaptic deficits.

This coincided with hippocampus atrophy, beginning CA1 neuron loss and synaptic failure, thereby linking Aβ accumulation with neurodegenerative mechanisms. These observations

also provide additional evidence that Aβ is able to trigger typical neurodegenerative mechanisms in AD independent of neurofibrillary tangle formation (Breyhan et al. 2009).
In conclusion, the data presented here provide evidence that intraneuronal accumulation of different Aβ peptides correlates well with a robust dysfunction in synaptic functionality/alteration of synaptic functionality in the APP/PS1KI mouse model for AD.

4.2 $A\beta_{3(pE)-x}$ IN AD

Considering the increasing importance research is giving to pyroglutamate-modified Aβ and the absence of an effective and specific antibody for it, we developed a novel monoclonal antibody directed against the N-terminal pyroglutamate modification at position 3 of Aβ ($A\beta_{3(pE)}$). Wild type mice have been immunized with an Aβ fragment constituted of residues 3 to 7 starting with pyroglutamate to generate antibody-producing hybridoma cells. After 2 successive subcloning steps, aimed to purify colonies producing class G immunoglobulins (IgGs) of a single subclass, the 2 subclones showing the best characteristics of sensitivity and specificity in dot-blot and IHC experiments have been selected. The antibodies showed the capacity to stain large numbers of plaques positive for $A\beta_{3(pE)}$ patients with sporadic and familial AD including those carrying mutations in APP (arctic and Swedish). The specificity was confirmed by dot-blot experiments comparing 1-57 and 2-48 with a generic anti-Aβ-antibody (WO-2, residues 5-8), an antibody directed against the native N-terminus of Aβ and a commercially available anti-$A\beta_{3(pE)-x}$. The dot blot experiment showed no reactivity to both full-length native Aβ and unmodified Aβ starting at residues 3. Antibodies 1-57 and 2-48 have been contemporarily further characterized in other experiments. Avidity for each antibody was calculated by BIAcore (GE Healthcare, Uppsala, Sweden), obtaining KD-values of 17.7 nM for 1-57 and 9.1 nM for 2-48, which confirmed a very high affinity for both antibodies (Wirths et al. 2009). In an SDS-PAGE experiment (Wirths et al. 2009) under denaturing conditions the two antibodies specifically detected $A\beta_{3(pE)-42}$ without any visible cross reactivity against other Aβ epitopes and not even against N-truncated Aβ starting with a pyroglutamate modification at residue 11. Finally, by immunohistochemical staining of human brain slices, it has been showed that large numbers of plaques positive for $A\beta_{3(pE)}$ are present both in patients with sporadic AD and with familial AD. The amount of plaques positive for $A\beta_{1-x}$ is less abundant in the control cases. Interestingly, in APP/PS1KI there is a continuous increase in $A\beta_{3(pE)}$ plaque load with increasing age, while the density for $A\beta_{1-x}$ plaques declined with aging. It is

therefore possible to assume that in particular the peptides starting with position 1 of Aβ are N-truncated as disease progresses (Wirths et al. 2009).

N-truncated $Aβ_{3(pE)}$ peptides have been identified by several groups from AD brains (Mori et al. 1992; Saido et al. 1995; Iwatsubo et al. 1996; Saido et al. 1996; Kuo et al. 1997; Russo et al. 1997; Hosoda et al. 1998; Tekirian et al. 1998; Harigaya et al. 2000; Kuo et al. 2001; Miravalle et al. 2005; Piccini et al. 2005; Guntert et al. 2006; Piccini et al. 2007) and they have been reported to be the dominant Aβ species in AD brain (Saido et al. 1995). In addition, other N-terminal truncated peptides have been identified such as $Aβ_{5–40/42}$ (Takeda et al. 2004), $Aβ_{11–40/42}$ (Lee et al. 2003; Liu et al. 2006), and Flemish and Dutch N-terminally truncated Aβ peptides (Demeester et al. 2001).

Although the precise formation process and the regulation of $Aβ_{3(pE)}$ are at the moment unknown, Saido et al. suggested that the removal of Aβ N-terminal amino acids 1 and 2 could be carried out by a hypothetical peptidase (Saido et al. 1995); in this regard it has been very recently shown that aminopeptidase-A may be responsible for the N-terminal truncation of the aspartate at position 1 of full-length Aβ (Sevalle et al. 2009).

In *in vitro* experiments Schilling et al. have shown that cyclization of glutamate at position 3 can be driven enzymatically by glutaminyl cyclase (QC) (Schilling et al. 2004). In addition, it has been shown that QC inhibition significantly reduced $Aβ_{3p(E)}$ formation *in vitro* and *in vivo*, emphasizing the importance of QC activity during cellular maturation of pyroglutamate-containing peptides (Cynis et al. 2006; Schilling et al. 2008). It has been postulated that pyroglutamate may protect the peptide from exopeptidase degradation and make it more prone to accumulate, thus explaining the observed 250-fold increased acceleration of the initial formation of Aβ aggregates (Schilling et al. 2006) and increased toxicity compared to full-length Aβ (He et al. 1999; Russo et al. 2002).

To further verify this *in vivo*, this group has recently generated transgenic mice expressing Aβ starting at position 3 with glutamine and ending at position 42 (TBA2 mouse line) (Wirths et al. 2009). This model showed for the first time that intraneuronal $Aβ_{3(pE)–42}$ accumulation is sufficient for triggering neuron death and inducing an associated neurological phenotype in a transgenic mouse model. The severity of the neurological phenotype observed in TBA2 mice, accompanied by Purkinje cell loss and premature mortality reflects the *in vivo* toxicity of $Aβ_{3(pE)-42}$. Aβ staining in the cerebellum was completely restricted to the intraneuronal compartment further supporting the notion that intraneuronal pathology is instrumental in neuron loss and that extracellular plaque deposition has no drastic effect on cell survival.

4.3 AD BIOMARKERS

4.3.1PREVENTION AND DIAGNOSIS

Today's few available options for AD have the only effect of slowing down the progress of the disease for a few months, as can be expected considering the neurodegenerative nature of the disease. Moreover, even though the clinical symptoms of AD are detectable at an advanced age, the molecular processes leading to AD start up to 20 years before the onset of the first symptoms. For these reasons AD treatment must necessarily act on early, asymptomatic phases of the disease; it would therefore be more useful to develop preventive, rather then therapeutic, treatment.

The most obvious way to deal with AD prevention is to act on modifiable risk factors. Not so long ago, the traditional risk factors for AD (advanced age, familial aggregation and the APOE ε4 allele) kept prevention somewhere at the bottom of the list of priorities in AD research and in clinical practice. However, during the recent years, evidence has accumulated from long-term population-based studies that several modifiable risk factors (especially vascular and lifestyle-related), besides their customary association with cardiovascular disease, are also important for AD. The genetic constitution of an individual is currently non-modifiable, but interactions between genetic susceptibility and environmental factors create opportunities for preventing diseases of multifactorial origin, such as AD.

Like for many other chronic diseases, efficient AD prevention can only be achieved by taking a life-long view of the disease. High blood pressure, especially at midlife, has been associated with an increased AD risk later in life (Launer et al. 2000; Kivipelto et al. 2002). It has been reported that high serum total cholesterol values at midlife increase the risk of late life AD (Kivipelto et al. 2002; Whitmer et al. 2005). A diet rich in saturated fats and cholesterol may also increase the risk of AD, whereas polyunsaturated fatty acids and fish seem to be protective. However, contradictory findings exist as well (Luchsinger et al. 2004).

Another lifestyle-related factor which has been suggested to be connected with AD is physical activity. Some shorter term longitudinal studies indicated an inverse association between regular and high intensity leisure time physical activity, or some specific form of physical activity (e.g. dancing, walking) and dementia/AD risk, whereas other found no association (Fratiglioni et al. 2004). Social and mental activities have also been suggested

to protect against AD (Fratiglioni et al. 2004); however, none of these findings has been consistently proven.

4.3.2 CURRENT SEROLOGICAL BIOMARKERS FOR AD

Clearly, any prevention strategy could be more easily targeted if coupled to an efficient tool for early diagnosis of AD. At present, only CSF Aβ and Tau level are well established AD biomarkers with high sensitivity and specificity (Galasko 1998; Kanai et al. 1998; Tapiola et al. 2000; Andreasen et al. 2001; Lewczuk et al. 2004). Although much better for clinical practise, blood Aβ measurement is controversially discussed as a useful biomarker for AD. Unfortunately, there seems to be no correlation between CSF and blood Aβ level, as shown by several groups (Mehta et al. 2000; Vanderstichele et al. 2000; Mehta et al. 2001; Matsumoto et al. 2007). Studies have shown a decreased blood level of Aβ in AD subjects (Pesaresi et al. 2006; Xu et al. 2008), while others showed an increase (Kosaka et al. 1997; Matsubara et al. 1999), or no statistical differences (Tamaoka et al. 1996; Fukumoto et al. 2003).

The same inconsistency emerged from a series of prospective studies, in which a higher risk for developing AD has been associated both with higher baseline plasma $A\beta_{42}$ concentration (Pomara et al. 2005) or higher $A\beta_{40}$ with no effect on $A\beta_{42}$ (van Oijen et al. 2006). Finally, Graff-Radford et al. (Graff-Radford et al. 2007) linked a high risk for developing AD to a lower $A\beta42/A\beta_{40}$ ratio.

4.3.3IMMUNE COMPLEXES

Recently, ICs of known cancer biomarkers with IgM have attracted investigators' attention because they have been proven to correlate better than unbound biomarkers with pathology in different cancer types (Beneduce et al. 2005; Castaldi et al. 2005; Beneduce et al. 2007; Beneduce et al. 2008), characterized by an over- or mis-production of an endogenous protein.

It is well known that IgM complexes are in equilibrium in blood with the unbound respective antigen; it is reasonable to think that the same equilibrium holds true for any IgM complex in blood. Already established ICs as biomarkers include: PSA–IgM for prostate cancer (Beneduce et al. 2007), carcinoembryonic antigen–IgM for colorectal cancer (Castaldi et

al. 2005) alpha fetoprotein–IgM and squamous cell carcinoma antigen-IgM for liver cancer (Beneduce et al. 2004; Beneduce et al. 2005). Moreover, IgM ICs have also been shown to be uncorrelated with basal IgM levels (Beneduce et al. 2008). Nydegger (Nydegger 2007) discussed in a review article biochemical properties of immune complexes in general, their formation and their equilibrium state, stating that immune complex IgM-antigen interaction is extremely high compared to other biochemical interactions.

Goals of this study were to verify the occurrence of ICs of Aβ and IgM in the plasma of AD and MCI patients and HC, in order to evaluate a possible correlation between the IC level and AD.

Plasma samples from 30 AD patients, 15 MCI patients, and 30 age- and sex-matched HC have been analyzed to determine the levels of circulating Aβ–IgM ICs. After proteins have been separated and purified by size-exclusion gelfiltration, Aβ was primarily found to be associated with the IgM and the IgG fractions using a dedicated ELISA assay. This is the first characterization of circulating Aβ–IgM complexes in AD. The Aβ–IgM assay did not show any significant difference among the three groups, in contrast with previous findings (Gruden et al. 2007), proving that although there is great interest around autoimmunity in AD, IC levels cannot be used as a diagnostic marker. It should be noted, however, that variability of IC level was high within the groups. This high variance leading to an overlap between AD patients and HC might be partly due to biological variability.

Consensus criteria for a suitable biomarker (Trojanowski et al. 1998) for AD were not met. On the other hand, the presence of circulating ICs against neuronal antigens supports the notion that autoimmune- mediated processes may be involved in neurodegenerative disorders. Further investigations are needed to elucidate the pathophysiologic role of these complexes; it will be interesting in particular to evaluate in future studies possible relationships of Aβ-IC levels with the clinical development of MCI patients, and with AD patients' response to immune therapies.

4.3.4 ANTI-$A\beta_{3(pE)}$-IgM NATURAL ANTIBODIES

4.3.4.1 PREVIOUS STUDIES ON NATURAL ANTIBODIES IN AD

Naturally occurring anti-Aβ antibodies were first isolated from human B cells (Gaskin et al. 1993); since then several epidemiologic studies have confirmed the presence of anti-Aβ NAbs in the blood of both AD patients and HC, although with contradictory results.

To evaluate a possible use as biomarkers for AD, levels of NAbs against $A\beta_{1-40}$ and $A\beta_{1-42}$ have been measured both in mice ((Nath et al. 2003; Li et al. 2007; Sohn et al. 2007) and humans (Gaskin et al. 1993; Hyman et al. 2001; Nath et al. 2003; Henkel et al. 2007; Gustaw et al. 2008; Szabo et al. 2008) but as of today there is no agreement whether there is a difference between AD patients and HC. Depending on the study, titer of anti-$A\beta_{42}$ autoantibodies was reported to be lower in AD patients compared to healthy individuals (Du et al. 2001; Weksler et al. 2002), higher (Nath et al. 2003); another study reported no correlation between autoantibodies titers and plasma $A\beta_{40}$ or $A\beta_{42}$ levels (Hyman et al. 2001).

More recent studies focusing on autoantibodies against oligomeric forms of Aβ have also given contradictory results, with some studies reporting a lower immunoreactivity in AD patients compared to HC (Moir et al. 2005), while others showed a higher titer (Gruden et al. 2007) or no significant differences (Britschgi et al. 2009).

4.3.4.2 ANTI- $A\beta_{3(pE)}$-IgM LEVELS

The goal of the present study was to characterize the occurrence of IgM autoantibodies against Aβ in the same samples used for the IC project, and to evaluate a possible correlation between the immunological profiles of individuals with AD.

The same plasma samples used for the IC project have been analyzed to determine the levels of circulating Aβ-IgM. After gel-filtration and size fractionation, high reactivity against the $A\beta_{3(pE)-7}$ peptide was found in the IgM fractions using a dedicated novel ELISA assay. A significant decrease in the level of autoantibodies against $A\beta_{3(pE)-x}$ was observed in AD patients as compared to HC. To note, the MCI group showed a mean level of anti-$A\beta_{3(pE)-x}$-IgM in between that of the AD group and of the HC, even though it didn't differ significantly from them, reflecting the heterogeneous and intermediate nature of the group; interestingly, in the MCI group there was a significant positive correlation between anti-$A\beta_{3(pE)-x}$-IgM and cognitive status (MMSE score). None of the other IgM autoantibody levels against Aβ showed any significant difference between the groups. These results indicate that circulating $A\beta_{3(pE)-x}$-IgMs result from autoimmune mediated processes involved in AD. However, whether this immune activity is defensive or toxic is still a matter of debate (Woulfe et al. 2002; Nath et al. 2003; Geylis et al. 2005); in any case,

autoimmunity driven homeostasis of plasma protein levels is a natural process and its understanding may be useful in both diagnosing and treating AD.

The fact that only anti-$A\beta_{3(pE)-x}$-IgMs show difference between the groups gives further support to the pathological role proposed for the pyroglutamate-modified peptide. As it has recently been shown by this group, $A\beta_{3(pE)}$-positive plaques are abundant in sporadic and familial AD cases, and at least in the APP/PS1KI mouse model, the $A\beta_{3(pE)-x}$ plaque load increases in an age-dependent manner, which is not the case for total Aβ and $A\beta_{1-x}$ (Wirths et al. 2009).

The exact role of NAbs in AD is still not clear; a possibility includes that they serve as a ‘‘buffering system’’ to keep free potential toxic endogenous peptides and proteins under homeostatic control and lead to their clearance (Gruden et al. 2007); according to this idea, a lower clearance activity by NAbs would facilitate Aβ, and pyroglutamate-modified-Aβ in particular, accumulation and aggregation. In support to this homeostatic role, there are experimental evidences of anti-Aβ-IgM NAbs able to catalyze hydrolysis of Aβ peptide (Taguchi et al. 2008), and to promote clearance of Aβ plaques (Dodel et al. 2003), as shown in neuronal cell cultures (Du et al. 2003) and animal models of amyloid pathology (Wilcock et al. 2003).

4.3.4.3 FUNCTION OF IGM NABS IN ATHEROSCLEROSIS

The results presented here, together with the positive results from immunotherapy studies, allow speculation on a possible role as housekeeping agents for NAbs in AD. On this subject, it may be interesting to look at NAbs in atherosclerosis, whose role is better-known, and speculate on a possible analogy with AD.

Like AD, atherosclerosis is also a slow, continuously developing disease, evolving from intimal thickening to increasingly complex lesions, consisting of cells derived from the circulation, wall cells, but also of extracellular matrix material and lipoproteins (LDL). Oxidized-LDL (OxLDL) are recognized and bound by natural anti-OxLDL-IgGs; successive excess uptake by macrophages results in foam cell formation within atherosclerotic plaques which lead to pathological inflammation. Tsimikas et al found a lower anti-OxLDL-IgM level and a higher IgG level in the blood of patients at risk for coronary stenosis (Tsimikas et al. 2007), while Su et al found a direct correlation of anti-OxLDL-IgM with decreased risk of carotid atherosclerosis in hypertensive patients (Su et al. 2006).

As a further evidence, mice immunized with heat-killed phosphorylcholine-containing pneumococci have a high titer of anti-OxLDL IgM and show reduced formation of atherosclerotic lesions (Binder et al. 2003). Similarly, passive immunization with the same antibodies lead to reduced lesion formation (Faria-Neto et al. 2006). In both cases, IgM seem to carry out this atheroprotective effect by binding OxLDL and thus neutralizing its pro-inflammatory effects and inhibiting its uptake by macrophages (Horkko et al. 1999; Chang et al. 2004).

Considering the toxic nature of self peptides also in AD, and the related inflammatory events, we suggest a similar protective role for IgM. Moreover, the opposite roles of IgMs and IgGs in atherosclerosis may also explain the contradictory results in previous studies on anti-Aβ-NAbs in AD, which were mostly based on IgGs only or total Igs titer measurement ((Binder et al. 2005) for a review).

Recently, a very detailed study on autoantibodies directed against many different Aβ forms has been published (Britschgi et al. 2009) which also shows an abundant titer of NAbs directed in particular against post-translationally-modified Aβ. The authors found a decreasing titer of NAbs with age, although no differences between AD patients and HC have been found. However, it is to note that the analysis of plasma autoantibodies has been limited to IgGs. The latter also show to be protective *in vitro* on cultured neurons; clearly, pro-inflammatory effects could not be evaluated in such an experiment.

4.3.4.4 ANTI-Aβ ANTIBODIES IN THERAPY

APP transgenic mice injected intravenously with monoclonal anti-Aβ antibody showed a rapid and massive increase of CNS-derived Aβ in the plasma, further suggesting that antibodies may participate in Aβ clearance from the brain (DeMattos et al. 2001).

The mechanism proposed for this immune-mediated elimination of Aβ is that natural antibodies bind Aβ in the blood, thus reducing its effective free concentration. This leads to increase the concentration gradient between brain and blood, enhancing efflux mechanisms' effectiveness in clearing Aβ out of the brain.

This idea has been recently challenged with a surprising result from Yamada et al. After intraperitoneal injection of anti-Aβ or control anti-α-synuclein murine antibodies, the authors microinjected radioactive $A\beta_{1\text{-}40}$ into the brain of mice and by monitoring the

radioactivity they showed that clearance of Aβ from the brain was slower in mice injected with anti-Aβ when compared to the controls (Yamada et al. 2009).

There are evidences for a therapeutic use of anti-Aβ antibodies in human, as it is being shown by current experimental immunotherapeutic approaches. Hock et al. showed that active immunization against aggregated $A\beta_{42}$ resulted in a slower cognitive decline in a dose-dependent fashion. Patients generating a higher titer of anti-Aβ-antibodies performed better in cognitive tests than patients who did not respond to the immunization protocol (Hock et al. 2003). In this study, the increase in antibody titer did not correspond to a decrease in Aβ blood levels; in contrast, significant changes were observed in serum and CSF of patients with AD after treatment with intravenous anti-Aβ-IgGs (Dodel et al. 2002).

The levels of Aβ autoantibodies of the IgG class for monitoring the effect of passive or active immunization in AD have already been evaluated by recent studies. It is speculative to discuss a possible role of Aβ–IgM levels as a predictive indicator of immunotherapy efficacy.

4.3.4.5 NAbs REGULATION

It is important to evaluate whether and how IgM production is regulated, in order to understand whether NAbs can be considered as a biomarker or rather as a risk factor. IgM is a protein constitutively produced by the B-1 subset of B-cells. There is a number of factors which have been found to influence the production of IgMs. TLR9 induces a signal cascade that ultimately activates NF-kB to promote the transcription of several molecules including CD38 and IgM in B-cells (Peng 2005). In primary biliary cirrhosis, hyperproduction of IgM from B-cells is induced by stimulation of TLR9 by bacterial CpG oligodeoxynucleotides (Kikuchi et al. 2005), indicating that IgM production can be induced as a reaction to a noxious challenge. IgM production can also be altered in vivo by chemical substances such as ursodeoxycholic acid (UDCA). UDCA reduces IgM production possibly by reducing cell activation and down-regulating the NF-kB intracellular signalling pathway (Kikuchi et al. 2009).

Normal antigens binding on surface IgMs do not trigger B-1 cells antibody responses; however, certain antigenic stimuli, like phosphorylcholine in the context of an appropriate carrier can induce antibody response, although it does not lead to the generation of memory cells.

B-1 cells have been found to be stimulated in their maturation and IgM secretion by interleukin-5 (IL-5), which mediates also the expansion of type T15 and EO6 natural IgM in mice (Takatsu 1998; Moon et al. 2004). Additionally, several Th2 cytokines, such as IL-9 and IL-10 have also been reported to participate in B-1 cells development and function (Nisitani et al. 1995; Vink et al. 1999). Mutations that disrupt positive regulators of BCR signalling, such as CD19, PI-3K p85, vav, BTK, or CR1/CR2, result in decreased B-1 cell numbers, while mutations in inhibitory regulators such as SHP-1, CD22, CD72 or Lyn, can lead to increased B-1 cell numbers (Berland et al. 2002).

In murine models of autoimmune diseases, it has been found that genetic factors contribute to determine B-1 cells compartment size, and a series of loci has been identified which are linked to B-1 cells expansion (Hayakawa et al. 1986; Hamano et al. 1998; Pers et al. 2002; Li et al. 2004). It is possible to speculate that like a lowered activation threshold against physiological self-antigens leads to autoimmune disease, so a similar mechanism against pathological self-antigens may lead to increased protection. It's possible to imagine a genetic background with a lowered activation threshold for B-1 cells, inducing proliferation and NAbs secretion in response to normally sub-threshold activation signals; oppositely, an increased activation threshold would decrease NAbs production and their protective function.

Chapter 5

Summary and conclusions

Synaptic and inflammatory markers

Quantitative western blot of different synaptic and inflammatory markers on APP/PS1KI mice evidenced how the pathology in these mice reflects for many characteristics the pathology in human patients. Synaptic dysfunction seems to be initiated between 2 and 6 months, the same period where neuron loss starts; however, the inflammatory marker GFAP has been found over-expressed already at 2 months. These finding may suggest that inflammatory events precede and possibly contribute to neuron loss. This is consistent with the observation that Aβ is enriched in the synaptosome fraction, giving a further indication that Aβ carries out its pathological effect by acting directly at the synaptic level.

Monoclonal anti-pyroglutamate antibodies

The two antibodies developed in this work showed a striking specificity and a good sensitivity, especially when compared in a dot blot experiment to a commercially available antibody directed against the same epitope. There is no cross-reaction detected whatsoever with other unmodified Aβ fragments; hence they represent a valuable tool for the study of $Aβ_{3(pE)-x}$.

AD blood biomarkers

The failure to obtain the same results seen in cancer with the measurement of IC in AD can be due to at least two main reasons. First, it was not possible to measure IC of specific Aβ subpopulations, including $Aβ_{x-42}$ or $Aβ_{3(pE)-x}$, since the epitopes are masked by the bound IgM. Therefore, only measurement of total Aβ in complex with IgMs was possible, this excludes the possibility of revealing a specific recognition by IgM of those Aβ forms that in sporadic AD appear to be more relevant in causing neuronal dysfunction. Secondly, ICs level in cancer is probably higher as a consequence of the fact that the number of antigen-producing cells is increased. In AD, increase in Aβ production seems to account for only a small minority of cases, specifically those bearing inherited mutations in APP or PS1/2; this may suggest that many AD cases are caused by a faulty clearance of Aβ, rather than by its overproduction. Proteins involved in Aβ clearance are, among others, apolipoprotein E (whose allele E4 is less effective in clearing Aβ), proteases like neprilysin and insulin-degrading enzyme and NAbs against Aβ ((Selkoe 2001) for review). Measuring specific IgM NAbs, rather than ICs, made it possible to bypass these issues.

The finding that anti-$Aβ_{3(pE)}$-NAbs level is lower in AD patients, while ICs are not changed, may indicate that a low level of this autoantibody could be a situation preceding the clinical

symptoms of AD, rather than being a consequence of AD development; in this case it would acquire a predictive value for AD. It can be speculated that less efficacy in clearance of $A\beta_{3(pE)}$ by IgM concurs in favouring its accumulation both intracellularly and in the amyloid plaques.

It is not clear, however, whether and how natural IgMs levels are regulated, in particular in regard to the development of the disease. A better understanding of its regulation will also help to determine whether anti-$A\beta_{3(pE)}$-IgM NAbs are a biomarker or a risk factor for AD. In this respect, it will be interesting to evaluate the levels of anti-$A\beta_{3(pE)}$-IgM level in a prospective study on AD and MCI patients and HC. (2009)

Measurement of anti-$A\beta_{3(pE)}$-IgM has proven to be a valuable tool for the study of AD *in vivo* in humans, with potential applications ranging from diagnosis, therapy and monitoring of the disease; the findings of this work have also the potential to shed light on the involvement of the immune system and on the role of pyroglutamate in AD development.

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